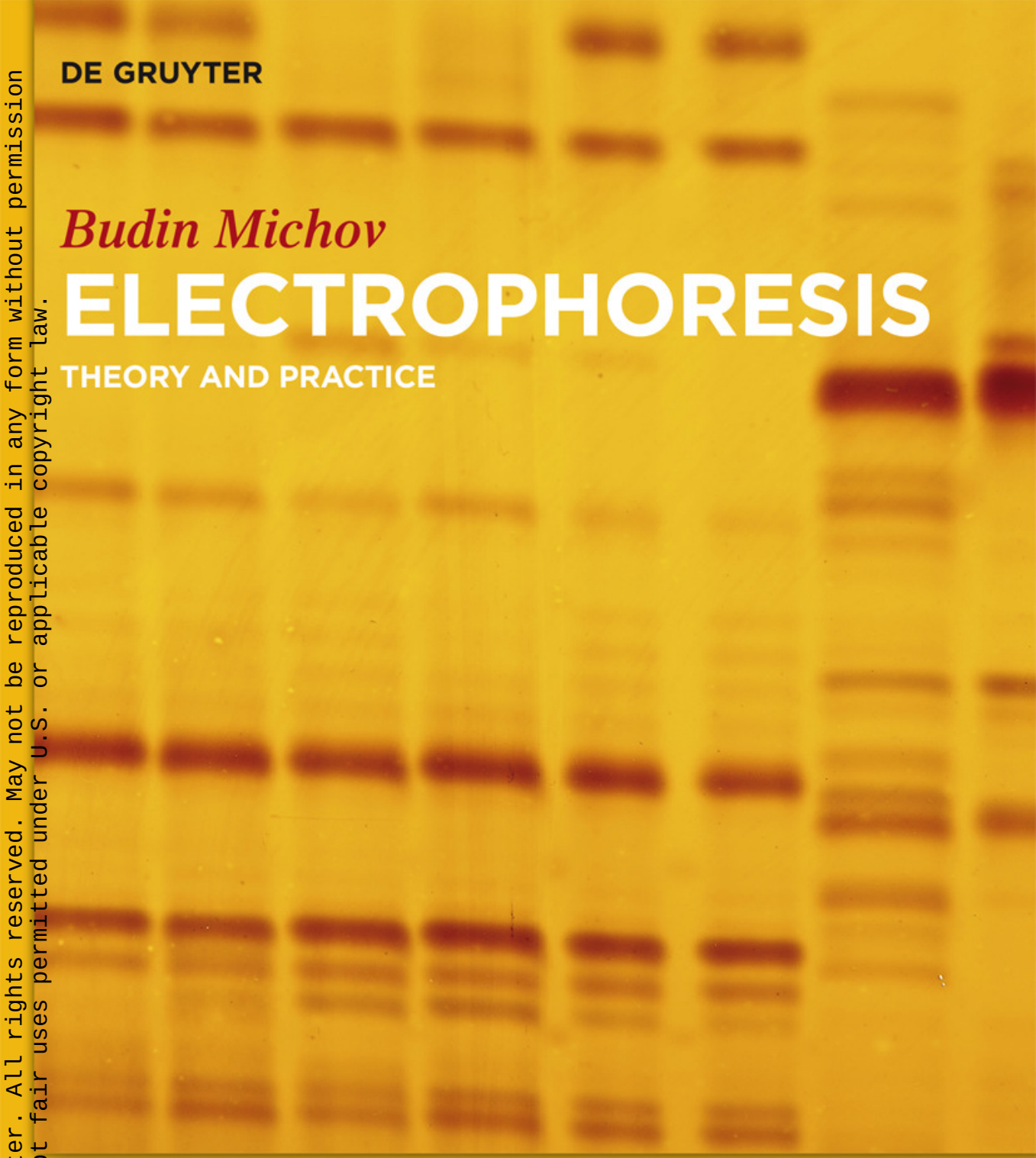

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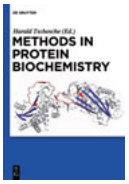

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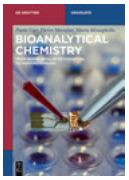

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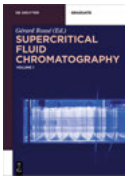

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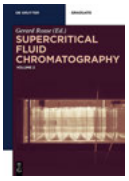

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Budin Michov

Electrophoresis – Theory and Practice

In Medicine and Biochemistry

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To those scientists who have worked lifelong hard in patience and modesty using knowledge and techniques to research molecules and ions of our organism.

About the Author

Prof. Budin M. Michov, MD, PhD, DSc studied medicine and earned his PhD at the Medical University of Sofia. He worked as biochemist and chemist at Technical University in Munich, and as a scientific adviser at SERVA Electrophoresis in Heidelberg, Germany. On the basis of his patented method, he founded a company in Germany for electrophoretic methods and products. Later, he was the chairman of the Department of Biochemistry at the Medical Faculty of Sofia University. Prof. Budin Michov published articles on a new complex compound in TRIS-borate buffers, mobility calculation of composed ions, calculating the TRIS-borate ion mobility, ionic mobility parameter, simplifying the Henry function, electrophoresis in one buffer at two pH values, electrophoresis in expanded stationary boundary, TRIS-formate-taurinate buffer system for SDS electrophoresis in homogeneous polyacrylamide gels, electrophoresis of CSF proteins in agarose gels, geometric and electrokinetic radii and electric potentials of charged and dissolved particles, and more. He invented a new device for concentration gradient system, and buffer recirculator for electrophoresis. Prof. Budin Michov published several books, for example: *Electrophoresis*, Walter de Gruyter, Berlin/New York (1996), in German; *Molecular Mechanisms of Inflammation and Allergy* (1977); *Chemistry in Medicine* (2001); and *Encyclopedia of Molecular Medicine* (2018).

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Preface

It is my pleasure to present this book on electrophoresis.

Electrophoresis is referred to as moving of charged dissolved particles in an electric field, which causes their resolution as a result of their velocities and interaction with the separation medium.

Nowadays, numerous electrophoresis techniques are known. They are used for resolving proteins and nucleic acids, chromosomes, viruses, proteins of cell membranes (plasma, lysosomal, nuclear, and other), cell organelles (mitochondria, ribosomes), cells (red cells, tissue cells, and parasites), and so on. The resolving is performed by charge, size, or binding affinity.

The electrophoresis methods are used in biochemistry, proteomic and genomic studies, microbiology, forensics, and molecular biology. By using electrophoresis almost all separations of blood proteins and DNA are carried out.

It was a challenge and a hard work for me to write this book after the great progress in protein and nucleate electrophoresis in the last years. Nevertheless, I will be happy if the book will help the readers to understand and use the theoretical basics and diverse methods of electrophoresis.

My thanks to Dr. Bernhard Seitner who supported me in word and deed during my efforts on the book.

Nuremberg, Germany
Budin Michov

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Contents

Abbreviations

η	Dynamic viscosity
ζ	Electrokinetic potential of an ion, in V
α	Ionization degree of an electrolyte, dimensionless
$\mu_{\infty i}$	Absolute mobility of ion *i*, in $m^2/(s\ V)$
μ_i	Mobility of ion *i*, in $m^2/(s\ V)$. [No difference exists between the terms ionic mobility and electrophoretic mobility.]
μ_i'	Effective mobility of ion *i*, in $m^2/(s\ V)$
[B]	Equilibrium concentration of substance B, in mol/dm^3 (mol/l) or $kmol/m^3$
$[H^+]$	Equilibrium concentration of the proton in mol/dm^3 (mol/l) or $kmol/m^3$
$\mathbf{[H^+]}$	Dimensionless proton concentration. $\mathbf{[H^+]} = [H^+]/[H^+]_0$, where $[H^+]_0$ is the standard proton concentration of 1 mol/dm^3
$[OH^-]$	Equilibrium concentration of the hydroxide ion, in mol/dm^3 (mol/l) or $kmol/m^3$
$[\mathbf{OH}^-]$	Dimensionless concentration of the hydroxide ion. $[OH^-] = [OH^-]/[OH^-]_0$, where $[OH^-]_0$ is the standard hydroxide concentration of 1 mol/dm^3
A	Adenine
ACES	N-(2-**ac**etamido)-2-amino**e**thane**s**ulfonic acid
ADP^{2-}	**A**denosine **di**phosphate
$a_{i(pi)}$	Electrokinetic radius of ion *i* (polyion *pi*)
ALP	**Al**kaline **p**hosphatase
Ammediol	2-**Am**ino-2-**me**thyl-1,3-propane**diol**
APS	**A**mmonium **p**eroxydi**s**ulphate
ATP^{3-}	**A**denosine **t**ri**p**hosphate
B^-	**B**orate ion
BAC	N,N-**b**is(**a**cryloyl)**c**ystamine
BBS	**B**ES-**b**uffered **s**olution
BCIP	5-**B**romo-4-**c**hloro-3-**i**ndolyl **p**hosphate
BES	N,N-**b**is(2-hydroxyethyl)-2-amino-**e**thane**s**ulfonic acid
BICINE	N,N-**bi**s(2-hydroxyethyl)-gly**cine**
BIS	N,N'-methylene**bis**acrylamide, N, N'-methylenediacrylamide
BISTRIS	**Bis**(2-hydroxyethyl)-amino-**tris**-(hydroxymethyl)-methane
bp	Base pairs
BPB	**B**romo**p**henol **b**lue
BSA	**B**ovine **s**erum **a**lbumin
C	Cytosine
C	Crosslinking
CA	**C**ellulose **a**cetate
CAPS	N-**c**yclohexyl-3-**a**mino**p**ropane**s**ulfonic acid
CBB	**C**oomassie **b**rilliant **b**lue
CBV	**C**oomassie **b**rilliant **v**iolet
CHAPS	3-[(3-**ch**olamidopropyl)dimethyl**a**mmonio]-**p**ropane**s**ulfonate
CHES	2-(**c**yclo**h**exylamino)**e**thane**s**ulfonic acid
c_i	Volume concentration of ion *i*, in mol/dm^3 (mol/l) or $kmol/m^3$
CTAB	N-**c**etyl-N,N,N-**t**rimethyl**a**mmonium **b**romide
dATP	**D**eoxy**a**denosine **t**ri**p**hosphate
ddNTP	**Di**deoxy**n**ucleoside **t**ri**p**hosphate
DEAE	**Di**ethyl**a**mino**e**thyl
DIPSO	3-N, N-bis(2-hydroxyethylamino)-2-hydroxypropanesulfonic acid

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Disc	**Disc**ontinuous
DMS	**Dim**ethyl **s**ulfate
DMSO	**Dim**ethyl **s**ulf**o**xide
DNA	**D**eoxyribo**n**ucleic **a**cid
dNTP	**D**eoxy**n**ucleoside **t**ri**p**hosphate
DTE	1,4-**Di**thio**e**rythritol
DTT	1,4-**Di**thio**t**hreitol
2D	Two-dimensional
EBT	**E**riochrome **b**lack **T**
EDTA	**E**thylene**d**initrilo**t**etra**a**cetate (**e**thylene**d**iamine**t**etra**a**cetate)
Eq.	Equation(s)
G	**G**uanine
G^-	**G**lycinate ion
GABA	**γ-A**mino**b**utyric **a**cid
GlyGly	**Gly**cyl**gly**cine
HB	Boric acid
HDL	**H**igh **d**ensity **l**ipoproteins
HEPES	4-(2-**h**ydroxy**e**thyl)**p**iperazine-1-**e**thane**s**ulfonic acid
HEPPS	4-(2-**h**ydroxy**e**thyl)**p**iperazine-1-**p**ropane**s**ulfonic acid
HEPPSO	4-(2-**h**ydroxy**e**thyl)**p**iperazine-1-(2-hydroxy**p**ropane**s**ulf**o**nic acid)
HG	Glycine
HRP	**H**orse**r**adish **p**eroxidase
I	Ionic strength, in mol/kg or mol/dm^3 (mol/l)
$\boldsymbol{I}$	Dimensionless ionic strength. $\boldsymbol{I} = I/I_o$, where I_o is the standard ionic strength of 1 mol/dm^3
I_c	Volume ionic strength in mol/dm^3 (mol/l)
IEF	**I**so**e**lectric **f**ocusing
Ig	**I**mmuno**g**lobulin
I_m	Mass ionic strength in mol/kg
IPG	**I**mmobilized **p**H **g**radient
IPG-Dalt	2D electrophoresis: IPG / SDS gel electrophoresis
Iso-Dalt	2D Electrophoresis: IEF / SDS electrophoresis
K	Thermodynamic equilibrium constant; different dimensions
$\boldsymbol{K}$	Dimensionless thermodynamic equilibrium constant. $\boldsymbol{K} = K/K_O$, where K_O is the standard thermodynamic equilibrium constant, which has the dimensions of K
K_c	Concentration equilibrium constant; different dimensions
kb	**K**ilo**b**ases
kbp	**K**ilo**b**ases **p**airs
$\boldsymbol{K_c}$	Dimensionless concentration equilibrium constant; $\boldsymbol{K_c} = K_c/K_O$, where K_O is the concentration equilibrium constant. Dimensions of K_c
K_R	Retardation coefficient
K_w	Ionic product of the water, in $(\text{mol/dm}^3)^2$, $(\text{mol/l})^2$
LDL	**L**ow **d**ensity **l**ipoproteins
LSB	**L**ow-**s**alt **b**uffer
Mbp	**M**ega**b**ases **p**airs
ME	2-**M**ercapto**e**thanol
MES	2-(N-**m**orpholino)**e**thane**s**ulfonic acid
m_i	Mass concentration (molality) of ion i, in mol/kg
MOPS	3-**Mo**rpholino-**p**ropane**s**ulfonic acid

MOPSO	3-(N-**mo**rpholino)-2-hydroxy-**p**ropane**s**ulf**o**nic acid
M_r	Relative molecular mass (ionic, polyionic mass). It represents the ratio between the mass of a particle, and 1/12th of the mass of carbon isotope ^{12}C, dimensionless
NaBPB	**B**romo**p**henol **b**lue sodium salt
NAP	**N**ucleic **a**cid **p**urifier
NBT	**N**itro **b**lue **t**etrazolium
Nonidet	**Non**-**i**onic **det**ergent
OD	**O**ptical **d**ensity
PAGE	**P**oly**a**crylamide **g**el **e**lectrophoresis
PAS	**P**eriodic **a**cid-**S**chiff reagent
PGM	**P**hospho**g**luco**m**utase
pH	Hydrogen exponent (pondus, potentia hydrogenii) – the negative logarithm of $[H^+]$, dimensionless
pH(I), pI	Isoelectric point (pondus isoelectricus), dimensionless
p*K*	Negative logarithm of *K*, dimensionless
p*K'*	Negative logarithm of *K'*, dimensionless
pK_c	Negative logarithm of K_c, dimensionless
PBS	**P**hosphate-**b**uffered **s**aline
PCR	**P**olymerase **c**hain **r**eaction
PMSF	**P**henyl**m**ethane**s**ulfonyl **f**luoride
PVC	**P**oly**v**inyl **c**hloride
PVDF	**P**oly**v**inylidene **dif**luoride
r	Molecular radius
R_μ	Relative mobility
R_f	**R**atio to **f**ront
$r_{i(pi)}$	Geometric **r**adius of **i**on *i* (**p**oly**i**on *pi*)
RN	**R**ibo**n**ucleate
RNA	**R**ibo**n**ucleic **a**cid(s)
s	Number of ionic species in the solution, dimensionless
SA	**S**odium **a**cetate
SDS	**S**odium **d**odecyl **s**ulfate
SDS-PAGE	**SDS p**oly**a**crylamide **g**el **e**lectrophoresis
SSC	**S**aline-**s**odium **c**itrate
STE	**S**odium chloride-**T**RIS-**E**DTA
T	Polyacrylamide concentration, total monomeric concentration
T	**T**hymine
TAE	***T**RIS-**a**cetate-**E**DTA*
TAPS	N-[**t**ris(hydroxymethyl)methyl]-3-**a**mino**p**ropane**s**ulfonic acid
TAPSO	N-[**t**ris(hydroxymethyl)methyl]-3-**a**mino-2-hydroxy**p**ropane**s**ulf**o**nic acid
TBE	**T**RIS-**b**orate-**E**DTA
TBS	**T**RIS-**b**uffered **s**aline
TBST	**T**RIS-**b**uffered **s**aline with **T**ween 20
TCA	**T**ri**c**hloro**a**cetic acid
TE	**T**RIS-**E**DTA
TEA	**T**ri**e**thanol**a**mine
TES	N-[**t**ris(hydroxymethyl)methyl]-2-amino**e**thane**s**ulfonic acid
TMEDA (TEMED)	N,N,N',N'-tetra**m**ethyl**e**thylene**d**i**a**mine, 1,2-bis(dimethylamino)ethane
TMPTMA	1,1,1-**Tr**i**m**ethylol**p**ropane **tr**i**m**eth**a**crylate

TNE	**T**RIS-**N**aCl-**E**DTA
TRICINE	N-[**tri**s(hydroxymethyl)methyl]-gly**cine**
TRIS	**Tri**s(hydroxymethyl)-aminomethane
TTBS	**T**ween 20-**TBS**
TX	**T**riton **X**100
UV	**U**ltrav**i**olet light
VLDL	**V**ery **l**ow **d**ensity **l**ipoproteins
z_i	Charge number (electrovalence) of ion *i*, dimensionless

NB: To derive logarithmic values of ionization constants, use dimensionless ionization constants. They can be considered as ratios between the ionization constants and a standard constant of 1 mol/dm^3 or 1 mol/l. Dimensionless concentrations of H^+ (or other ions) can also be defined as ratios between the concentrations of H^+ (or other ions) and a standard concentration of H^+ (or other ions), which should also be 1 mol/dm^3 or 1 mol/l. The same is valid for ionic strengths.

1 Fundamentals of electrophoresis

Electrokinetic phenomena refer to a variety of effects resulting from the electric double layer. An example is the electrophoresis; other examples include electroosmosis, sedimentation potential, and streaming potential [1].

Today, the term *electrophoresis* means moving of charged dissolved particles in an electric field, which causes their resolution depending on their velocities and interaction with the separation medium [2,3,4]. In its current form, the electrophoresis appears in the 1930s after the studies of Tiselius [5].

The electrophoresis of positively charged particles (cations) is called *cataphoresis*, while the electrophoresis of negatively charged particles (anions) is called *anaphoresis*.

Electrophoresis is the basis of numerous resolving techniques for proteins and nucleic acids, chromosomes, viruses, cell membranes (plasma, lysosomal, nuclear, and more), cell organelles (mitochondria and ribosomes), cells (red cells, tissue cells, and parasites), and so on. The separation takes place by charge, size, or binding affinity, and is used in medicine, biochemistry, proteomic and genomic studies, forensics, molecular biology, and microbiology.

Using electrophoresis, more than half of all separations and almost all separations of blood proteins and DNA are carried out.

1.01 Overview on electrophoresis

Proteins and nucleic acids form polyions. In an electric field, the positively charged polyions move toward the negative pole, while the negatively charged polyions move toward the positive pole.

Usually, the polyions to be resolved are applied onto a separation medium, which on its turn is placed into an electrophoresis cell connected to a power supply. When voltage is applied, larger and less charged polyions move slower through the medium, while smaller and more charged polyions move faster (Figure 1-1).

Mostly, electrophoresis is carried out in agarose or polyacrylamide gels, soaked with buffers. Other separation media are starch gel, cellulose acetate, and paper; however, they have lost their actuality. Electrophoresis can also be carried out in

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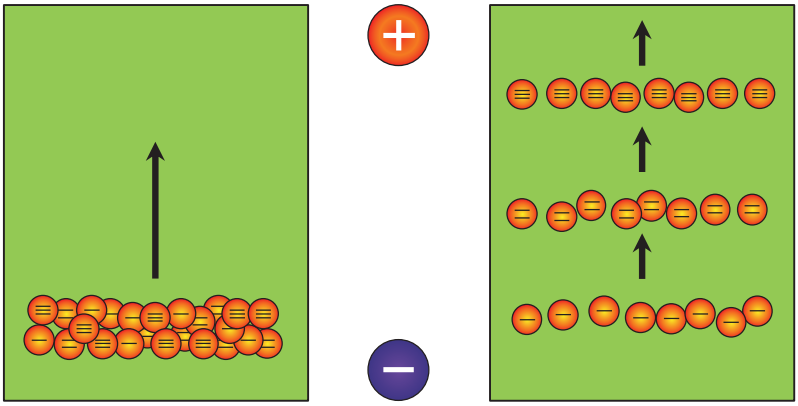

Figure 1-1: Electrophoresis of unipolar particles with different electric charges.
Left – start of electrophoresis; *Right* – end of electrophoresis

buffers only, or in gradient polyacrylamide gels. The buffers are electrolyte solutions, which create constant pH value, for example TRIS-borate buffer, TRIS-acetate-EDTA buffer, TRIS-histidinate buffer, and Good's buffers.

Since proteins and nucleic acids are mostly colorless, their movement through the gel cannot be followed during electrophoresis. Therefore, tracking dyes are usually included in the sample buffer. During electrophoresis of negatively charged proteins, Bromophenol blue, xylene cyanol (it runs slower than Bromophenol blue), or orange G is used; during electrophoresis of positively charged proteins, Bromocresol green or Methylene blue is used.

The anion of **B**romo**p**henol **b**lue (BPB, 3′,3″,5′,5″-tetrabromophenolsulfonphthalein) is colored blue at alkali and neutral pH values. It moves toward the anode before the proteins and nucleic acids. As it has reached the anodic end of the separation medium, the electrophoresis must be stopped.

The proteins can be separated also in pH gradients, where they stop at their isoelectric points (pI). This electrophoresis, called *isoelectric focusing*, can be carried out in pH gradients made by carrier ampholytes, or immobilines.

The electrophoresis takes place in horizontal or vertical separation cells. The electrodes can be placed on the gel or in separate electrode tanks.

Except electrophoresis cells and power supplies, thermostats for reserving the resolving medium temperature, and densitometers or scanners for analyzing the *pherograms* (the medium with the resolved polyions) are used. Semiautomated electrophoresis devices are also offered on the market.

The electrophoretic velocity v of a polyion is proportional to its effective mobility μ' and the electric field strength E. In its turn, the effective mobility depends on the total electric charge of the polyion and is inversely proportional to the viscosity of the separation medium. The total electric charge is determined by the buffer pH

value. The viscosity of the resolving medium depends on the medium structure and the temperature.

The electric field strength is equal to the ratio between the voltage and the distance between the two electrodes. Since the distance remains constant during electrophoresis, the polyion velocity depends only on the voltage.

The Joule heating that is produced during electrophoresis should be replaced from the electrophoresis cell. The most electrophoretic techniques take place in simple cells; however, some of techniques require circulating cooling.

In gel electrophoresis, the polyions interact with the resolving medium and, as a result, separate each from other. Some of the charged particles, such as serum, cerebrospinal fluid, and urinary proteins, as well as deoxynucleates or ribonucleates, can be separated without any structural changes. The undiluted molecules, however, must be converted with the help of detergents in soluble molecules prior to electrophoresis. For example, the cell membrane lipoproteins must be transformed in soluble micelles.

The detection of the resolved polyions is carried out directly or indirectly. The direct detection takes place in the resolving medium by nonspecific or specific staining methods, enzyme-substrate reactions, immune precipitation, autoradiography, and fluorography. The indirect detection is performed by immune printing or blotting.

The proteins are stained with Amido black 10B, Coomassie brilliant blue, or silver; and DNA are visualized using ethidium bromide which intercalates into DNA and fluoresces under ultraviolet light. If the polyions in the gel contain radioactive atoms, autoradiograms can be obtained. After the electrophoresis, the gels with the results can be saved in a dry form, or recorded with a camera. The intensity of the bands or spots can also be measured and compared against markers in the same gel, using specialized software.

After electrophoresis, *blotting* of proteins and nucleic acids can take place. With this technique, the resolved bands can be immobilized onto blot membranes and treated afterward. The blotting methods are simple and usually contain four steps: electrophoresis of proteins or nucleic acids; transfer and immobilization of the bands on blot membranes; binding of analytical probes to the blotted substances; and their visualization.

After being transferred onto a blot membrane, the immobilized proteins or nucleic acids are localized by probes, such as antibodies or DNA, that specifically bind to the polyions of interest. Then, the probe position is visualized usually by autoradiography.

The main blotting techniques used nowadays are Southern, Northern, and Western blotting.

1.01.1 Separation media

Electrophoresis can be performed in solutions, but the diffusion there hinders from good resolution of the components. In order to limit the diffusion, solid

media are used. The earliest solid medium was cellulose contained in filter paper sheets – paper electrophoresis, which was studied 1951 by Kunkel and Tiselius [6]. The introducing of cellulose acetate membranes was the next step of the cellulose electrophoresis. They have large pores that do not retain proteins either chemically or physically.

Today the most common is the gel electrophoresis. Depending on the gel, agarose and polyacrylamide gel electrophoresis are mostly used.

Electro(end)osmosis, or **e**lectro**o**smotic **f**low (EOF), of the gels has a great effect on electrophoresis. It appears when a solid medium (a porous matrix, capillary tube, membrane, microchannel, or other materials) possesses ionized chemical groups on its internal surface. Under these circumstances, the liquid phase moves over the solid surface in order to maintain the thermodynamic equilibrium. The electroosmosis is related to the ζ potential of the solid surface and the (di)electric constant of the electrolyte solution. Its value is pH dependent. The electroosmosis leads to diffusion of the resolved bands in the gel electrophoresis but helps in the capillary electrophoresis.

1.01.2 Electrophoresis resolution and sharpness

The most important characteristics of the electrophoretic methods are their resolution and sharpness.

The *resolution* is referred to the ability of an electrophoresis method to separate two sample components from each other, and depends on the Gaussian profiles of the bands. It is calculated by dividing the distance between the centers of adjacent bands by their average bandwidths.

The *sharpness* is equal to the reciprocal value of the bandwidth; narrower the bands, the higher is the sharpness.

The resolution and sharpness depend on the separation medium, the properties of the polyions to be separated, and the temperature. At higher temperature, the diffusion is increased. To reduce diffusion, the electrophoresis is carried out at relatively low temperatures, mainly at 10–15 °C.

1.01.3 Detection of resolved bands

The majority of polyion bands are not visible to the naked eye, with a few exceptions. For example, the separated hemoglobins (which are red colored) are seen on a nitrocellulose membrane. A couple of methods are created for detection, localization and quantitation of separated bands and spots.

Proteins can be stained in the gels. Common dye is Coomassie brilliant blue which can detect 0.3 μg of protein in a spot. DNA is usually detected using the fluorescent intercalating of ethidium bromide. Both proteins and DNA can react with

silver ions to form black bands. This method is 100 times more sensitive than the other staining methods and can detect 2 ng of protein in a spot.

The process of staining is followed by destaining the gel background to remove unbound dye. In some cases, placing the gel on an **u**ltra**v**iolet (UV) lightbox or under a UV lamp can be revealed UV absorbing bands, or UV fluorescence bands.

Sometimes the resolved bands ought to be extracted from the gel. For this purpose, a blotting technique is applied to transfer the polyions onto membranes. The membranes are fabricated usually from nitrocellulose, nylon, or **p**oly**v**inylidene **d**i-**f**luoride (PVDF).

The first blotting procedure was described by Ed Southern in 1975 (Southern blotting). In this method (capillary blotting), diffusion of DNA from an after-electrophoresis gel to a membrane was achieved by pressure applied to a sandwich of gel, a wetted membrane, and a stack of blotting paper. The process takes up to 24 h but can be speeded up by using a vacuum through the gel. The transfer in an electric field (electroblotting) is faster than the capillary one.

The blotting of RNA bands is called Northern blotting, and the blotting of proteins is called Western blotting.

The Western blotting with immunodetection is performed as follows: The resolved proteins are transferred onto a membrane (nitrocellulose or PVDF), which has been soaked in an appropriate solution to block its nonspecific binding sites. Then, the membrane is incubated in a solution with an enzyme-linked second antibody against the first antibody to probe the immobilized proteins. The enzyme is usually a peroxidase or alkaline phosphatase, which transform appropriate substances into visual products.

When filter papers are used to soak the gel buffer and together with it the bands onto a membrane, the method is called semidry blotting. Once the polyions have been fixed on the membrane, they can be probed to be detected.

If prior to electrophoresis radioactive atoms have been incorporated in the polyions, their position in a gel or on a membrane can be determined by autoradiography. For this purpose, the gel or the membrane is placed on a photographic film or overlaid with it and is let to expose in the dark and cold to show the positions of the radioactive bands. After this, the bands in the gel can be cut out to be eluted and scintillated.

The modern methods for both detection and characterization of proteins also involve mass spectrometry.

1.02 Comparison between electrophoresis and other separation methods

The electrophoresis has important advantages against the most competing methods: ultracentrifugation and chromatography. In electrophoresis, enzymatic activities

or immunological identities of proteins can be detected directly in the gel, or indirectly on a blot membrane. Similar possibilities do not exist in any of the competing methods.

In addition, no expensive devices for detecting the resolved polyions are needed after electrophoresis, as is the case with the chromatography. Also, evaluation devices (densitometer and computer) are not blocked during electrophoresis. Such option is available only in the thin layer chromatography.

However, electrophoresis has also disadvantages: its separation medium, with exception of the capillary and free-flow electrophoresis, can be used only once; on the contrary, a chromatography column can be used many times.

1.02.1 Ultracentrifugation and chromatography

In *ultracentrifugation*, as in electrophoresis, a stationary phase is present. However, the separation of sample components is carried out in a gravity field and depends only on their masses.

The *chromatography* (from Greek *chroma* "color" and *graphein* "to write") is a set of techniques for separating of mixtures. The mixtures are dissolved in a fluid (*mobile phase*), which moves through another material (*stationary phase*). The mixture components travel at different speeds, for the sake of differential partitioning between the both phases.

The chromatography was employed in Russia by Mikhail Tsvet [7,8,9]. He separated plant pigments such as chlorophyll, carotenes, and xanthophylls using calcium carbonate as stationary phase, and petrol ether/ethanol mixtures as mobile phase. Since these components have different colors (green, orange, and yellow, respectively), he gave the method the current name.

The chromatography may be analytical or preparative. The analytical chromatography is carried out with smaller volumes of mixture. Preparative chromatography is used to purify the components of a mixture.

There are numerous chromatographic techniques. They can be divided in the following groups: techniques by chromatographic bed shape, techniques by physical state of mobile phase, and techniques by separation mechanisms. To the techniques by chromatographic bed shape belong the following: column chromatography, planar chromatography, paper chromatography, thin layer chromatography, and displacement chromatography.

The *column chromatography* is a technique in which the particles of the stationary phase are situated within a tube, through which the mobile phase flows.

In 1978, Clark Still [10,11] introduced the *flash column chromatography* (flash). Here, the solvent is driven through a column by applying pressure. This allows most separations to be performed in less than 20 min. The columns for flash column chromatography may be connected with detector and fraction collector.

The *planar chromatography* is characterized with a plane stationary phase. The support for the stationary phase can be a paper (paper chromatography) or a glass plate (thin layer chromatography). Different compounds in the sample mixture travel different distances according to their interactions with the stationary phase. The identification of unknown substances is made by comparing their **r**etention **f**actors (R_f) to a known R_f.

In the *paper chromatography*, the sample is placed on chromatography paper end as a dot or line, and the paper is set in a container with a solvent. As the solvent rises through the paper, it crosses through the sample mixture. Because the paper consists of cellulose, the nonpolar compounds in the mixture travel farther, whereas more polar substances bind to the cellulose and travel slower.

The ***t**hin **l**ayer **c**hromatography* (TLC) is similar to the paper chromatography. Instead of paper stationary phase, it uses a stationary phase of a thin adsorbent layer like silica gel, alumina, or cellulose on a glass plate. It is faster than the paper chromatography and offers better separations.

In the *displacement chromatography*, molecules with higher affinity to the chromatography matrix (the displacer) compete effectively for binding sites, and displace molecules with fewer affinities. As a result, different biomolecules move with different velocities.

Chromatography methods that use the physical state of the mobile phase are: gas chromatography, liquid chromatography, and affinity chromatography.

The ***g**as **c**hromatography* (GC) (**g**as-**l**iquid **c**hromatography, GLC) is based on the equilibrium between the positions of the analytes to be resolved in a solid (or viscous liquid) stationary phase, and a mobile gas (usually helium). The stationary phase is adhered to a solid matrix inside a larger metal tube (a packed column) or to a small-diameter (0.53–0.18 mm inside diameter) glass or fused-silica tube (a capillary column). Capillary columns give superior resolution and are therefore widely used. However, the high temperatures used in the gas chromatography make it unsuitable for high molecular mass biopolymers or proteins; but it is well suited in the environmental monitoring and industrial chemistry.

The ***l**iquid **c**hromatography* (LC) is a separation technique in which the mobile phase is a liquid. In the ***h**igh **p**erformance **l**iquid **c**hromatography* (HPLC), the sample is forced by a liquid (the mobile phase) at high pressure through a column that is packed with a porous monolithic layer of particles or a porous membrane (the stationary phase).

The ***a**ffinity **c**hromatography* (AC) is based on noncovalent interactions between analytes to be resolved and specific molecules. It is often used in biochemistry to purify proteins labeled with compounds such as His-tags, biotin, or antigens, which bind specifically to the stationary phase. After purification, these tags are removed to obtain pure proteins.

To the separation techniques belong also ion exchange chromatography, gel filtration chromatography, **e**xpanded **b**ed **a**dsorption (EBA) chromatography, and reversed-phase chromatography.

The *ion exchange chromatography* (ion chromatography) uses ion exchange mechanisms to separate charged compounds as anions, cations, amino acids, peptides, and proteins. It usually takes place in columns with charged stationary phase presented by an ion exchange resin whose charged groups retain oppositely charged compounds.

The *gel filtration chromatography* is used to separate molecules according to their hydrodynamic volume. Smaller molecules are trapped in the pores of the gel and are removed by the mobile phase flow. Molecules that are greater than the pores are not retained and therefore pass faster through the gel.

In the ***e**xpanded **b**ed **a**dsorption* (EBA) chromatography, the target proteins are captured from the crude flow when it passes through a column with adsorbent beads. So the crude flow can be treated directly, without the traditional clarification and pretreatment steps.

In the ***r**eversed-**p**hase **c**hromatography* (RPC), the mobile phase is more polar than the stationary phase. As a result, hydrophilic molecules in the mobile phase are eluted first, while hydrophobic molecules tend to be adsorbed by the hydrophobic stationary phase. The proteins are resolved by decreasing the salt concentrations, increasing the concentrations of detergent (which disrupts hydrophobic interactions), or changing the pH value.

Other types of chromatography are the two-dimensional and chiral chromatography.

If a given solvent system cannot separate some analytes, they can be additionally resolved in a new solvent system with different physicochemical properties. This method is named *two-dimensional chromatography*. The sample is spotted on one corner of a square plate, then resolved in the first solvent system, and air dried. After this, the square plate is rotated by 90° and the sample is resolved in the second solvent system.

The *chiral chromatography* is used to separate enantiomers, which have no chemical or physical differences. To take place, either the mobile phase or the stationary phase must be chiral.

To analyze nucleic acids, genomes, and proteins, computational simulation, named *Monte Carlo methods* (*experiments*), is used [12]. The Monte Carlo methods are a broad class of computational tests, which can give information, if a chemical reaction can happen. With them, different experiments can be conducted: breaking bonds, introducing impurities at specific sites, or changing the molecule structure.

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1.1 Ions and polyions

All electrophoretic methods, including isoelectric focusing, are performed in buffers. Before we describe the buffers as an indispensable part of electrophoresis, we will briefly report on electrolytes.

1.1.1 Electrolytes

Electrolytes are compounds which build ions in a solution. Such compounds are, for example, the bases and acids, often called protolytes [1,2].

According to Brønsted theory [3], a base is an electrolyte, which binds a proton coming from an acid; and an acid is an electrolyte, which gives a proton to a base. Hence, acetate ion (CH_3COO^-) and ammonia (NH_3) are bases, while acetic acid (CH_3COOH) and ammonium ion (NH_4^+) are acids.

There is a difference between the total and the net charge of a polyion. The total charge is the sum of all the electric charges of the polyion in an infinitely dilute solution. The net charge is less (in absolute units) than the total charge. Responsible for this are the counterions in the buffer that are bound to the polyionic surface.

1.1.1.1 Concentration dissociation constant and ionic strength

The magnitude for the affinity of a base and an acid to a proton is the dissociation (ionization) constant, defined in mol/kg, or mol/dm^3 (mol/l). The acid HA which dissociates according to the chemical balance

$$HA \leftrightarrow H^+ + A^-$$

is characterized by the concentration dissociation constant

$$K_c = \frac{[H^+][A^-]}{[HA]} \tag{1.1-1}$$

where $[H^+]$, $[A^-]$, and [HA] are the equilibrium concentrations, in mol/dm^3, or mol/l, of the proton, base A, and acid HA, respectively. This equation can also be wrote in a logarithm form, namely as

$$\mathrm{pH} = \mathrm{p}K_c + \log\frac{[\mathrm{A}^-]}{[\mathrm{HA}]} \tag{1.1-2}$$

The concentration dissociation constant depends on the temperature and ionic strength of the solution.

The temperature exerts counterinfluence on the concentration dissociation constant. On the one hand, it labilizes the chemical bond between the proton and the rest of the molecule and, therefore, supports the dissociation process; on the other hand, it partially restores the hydration shell around the protolyte molecule and, therefore, suppresses the dissociation process.

The ionic strength influences on the dissociation constant because it represents the ionic concentrations in the solution and the charge numbers of the ions. Two types of ionic strengths are used: I_c (volume concentration ionic strength) and I_m (mass concentration ionic strength). They are defined by the equations:

$$I_c = \frac{1}{2}\sum_{B=1}^{s} z_B{}^2 c_B \tag{1.1-3}$$

and

$$I_m = \frac{1}{2}\sum_{B=1}^{s} z_B{}^2 m_B \tag{1.1-4}$$

where s is the number of ionic species formed by the compound B, z_B is the number of proton charges (the electrovalence) of ion B, c_B is the volume concentration of ion B in mol/dm^3, or mol/l, and m_B is the molality of ion B, in mol/kg. The ionic strength I_m is a more precise magnitude; however, the ionic strength I_c (abbreviated as I) is more commonly used because of the complicated calculation of I_m.

If $I = 0$, the dissociation constant is known as *thermodynamic dissociation constant K*. The thermodynamic dissociation constant depends only on the temperature of the solution.

The relationship between K_c and K is given by the following equation:

$$\mathrm{p}K = \mathrm{p}K_c - 0.5\left(z_{HA}{}^2 - z_{H^+}{}^2 - z_{A^-}{}^2\right)\frac{I^{1/2}}{1 + I^{1/2}} \tag{1.1-5}$$

where pK and pK_c are the negative logarithms of K and K_c, respectively, and I (a dimensionless value) is the ratio between the ionic strength and the standard ionic strength, equal to 1 mol/l. When an electrolyte, such as CH_3COOH, dissociates giving only two unipolar ions, eq. (1.1-5) is simplified to give

$$pK = pK_c + \frac{I^{1/2}}{1 + I^{1/2}} \tag{1.1-6}$$

If the expression in the parentheses in eq. (1.1-5) has a negative value, then $pK > pK_c$. If a same charge is present on both sides of the equilibrium process, such as in the dissociation of NH_4^+ in a proton and an ammonia molecule, then $pK = pK_c$.

The pK values of protolytes, which are important for the electrophoresis, are listed in Table 1.1-1. From the pK values, with the aid of eqs. (1.1-5) and (1.1-6), the pK_c values at different ionic strengths can be calculated. These calculations can be used for defining the composition of buffer solutions.

Table 1.1-1: Structures and pK values of protolytes, which are important for electrophoresis.

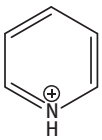
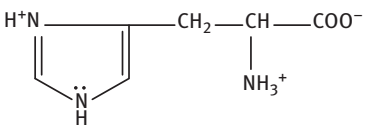
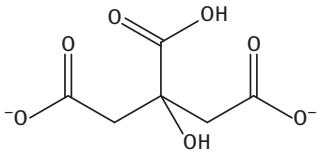
Protolytes	Chemical formulas	M_r	p$K_{0°C}$	p$K_{25°C}$
Hydroxonium ion	H_3O^+	19.02		−1.74
Phosphoric acid	H_3PO_4	98.00	2.06	2.15
Glycinium ion	$^+H_3NCH_2COOH$	76.07	2.44	2.35
Alaninium ion	$^+H_3NCH(CH_3)COOH$	90.09	2.51	2.43
β-alaninium ion	$^+H_3NCH_2CH_2COOH$	90.09	3.65	3.55
Formic acid	HCOOH	46.03	3.78	3.75
Lactic acid	$CH_3CHOHCOOH$	90.08	3.88	3.84
4-aminobutyricium ion (GABA-Ion)	$^+H_3NCH_2CH_2CH_2COOH$	104.12	4.09	4.03
Acetic acid	CH_3COOH	60.05	4.78	4.76
Propionic acid	CH_3CH_2COOH	74.08	4.90	4.87
Pyridinium ion		80.10	5.50	5.21
Histidinium ion		156.16	6.35	6.04
Hydrogen citrate		190.12		6.40

Table 1.1-1 (continued)

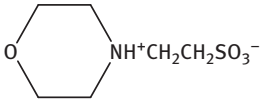
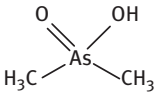
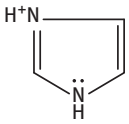
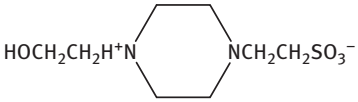
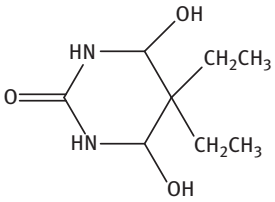
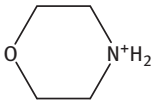
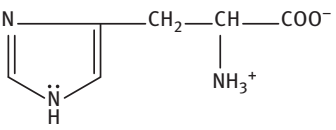
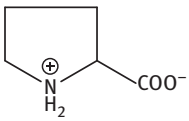
Protolytes	Chemical formulas	M_r	p$K_{0°C}$	p$K_{25°C}$
MES		195.20	6.45	6.16
Cocadylic acid		138.00	6.61	6.27
BISTRIS ion	$(HOCH_2CH_2)_2NH^+C(CH_2OH)_3$	210.24	6.88	6.50
ACES	$H_2NCOCH_2NH_2{}^+CH_2CH_2SO_3{}^-$	182.21	7.38	6.84
Imidazolium ion		69.08	7.46	6.99
Dihydrogen phosphate ion	$H_2PO_4{}^-$	97.00	7.32	7.20
TES	$(HOCH_2)_3NH_2{}^+CH_2CH_2SO_3{}^-$	229.24	8.00	7.48
HEPES		238.30	7.89	7.52
Triethanolaminium ion	$(HOCH_2CH_2)_3NH^+$	150.19	8.35	7.80
Barbital (Veronal)		184.20	8.40	7.98
TRIS ion	$(HOCH_2)_3CNH_3{}^+$	122.14	8.84	8.07
TRICINE	$(HOCH_2)_3CNH_2{}^+CH_2COO^-$	179.17	8.64	8.09
Glycylglycine	$^+H_3NCH_2CONHCH_2COO^-$	132.12	8.98	8.25
BICINE	$(HOCH_2CH_2)_2NH^+CH_2COO^-$	163.17	8.78	8.34
Morpholinium ion		88.12	8.85	8.60

Table 1.1-1 (continued)

Protolytes	Chemical formulas	M_r	p$K_{0°C}$	p$K_{25°C}$
Ammediolium ion	$HOCH_2(^+H_3N)C(CH_3)CH_2OH$	106.14	9.56	8.83
Asparagine	$H_2NCOCH_2CH(NH_3^+)COO^-$	132.12	9.04	8.86
Histidine		155.16		8.97
Taurine	$^+H_3NCH_2CH_2SO_3^-$	125.14	9.74	9.06
Boric acid	H_3BO_3	61.83	9.50	9.24
Ammonium ion	NH_4^+	18.03	10.12	9.29
Ethanolaminium ion	$^+H_3NCH_2CH_2OH$	62.08	10.35	9.54
Alanine	$^+H_3NCH(CH_3)COO^-$	89.09		9.69
Glycine	$^+H_3NCH_2COO^-$	75.07	10.50	9.78
β-alanine	$^+H_3NCH_2CH_2COO^-$	89.09		10.19
4-Aminobutyric acid (GABA)	$^+H_3NCH_2CH_2CH_2COO^-$	103.12	11.37	10.56
Proline		115.13	11.33	10.64
Water	H_2O	18.02	16.63	15.74

1.1.2 Electric double layer of a charged particle

In a solution, the charged particles are in electrostatic interactions with oppositely charged ions (counterions). The counterions form ionic atmospheres. The surface of a charged and solvated (hydrated) ion or polyion and its ionic atmosphere build an electric double layer [4,5]. The electric field strength inside the electric double layer can be from 0 to over 10^9 V/m.

There are several models of the electric double layer. Most common are the models of Helmholtz, Gouy–Chapman, Stern, Grahame, and Bockris–Devanathan–Müller.

1.1.2.1 Model of Helmholtz

The first model of the electric double layer, known as the Helmholtz model, was developed by Helmholtz [6] and Perrin [7]. According to it, the counterions are at a certain distance from the charged surface. As a result, similarly to a capacitor electric double layer, the electric potential decreases linearly by the distance from the charged surface (Figure 1.1-1).

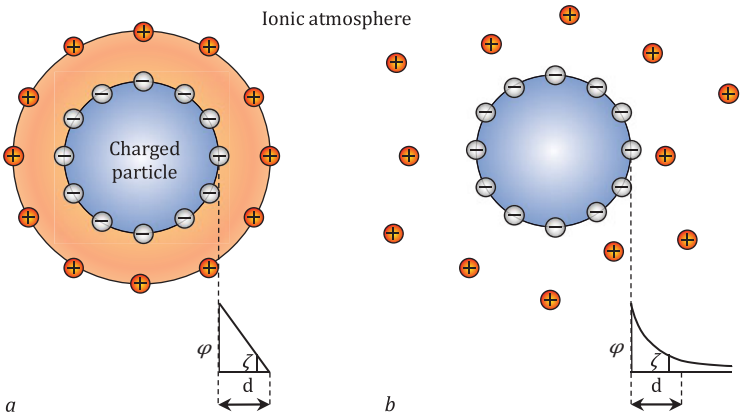


Figure 1.1-1: Models of the electric double layer according to Helmholtz (*a*) and Gouy–Chapman (*b*).

According to this model, a charged particle can be accepted as a ball capacitor, which consists of two concentric spheres. The electric potential on the surface of the charged particle (the inner electric potential of Helmholtz) is

$$\varphi_{in} = \frac{Q}{4\pi\varepsilon a} \tag{1.1-7}$$

where Q is the electric charge of the particle, in C; ε is the (di)electric permittivity of the solvent (water), which is equal to the product of the relative (di)electric permittivity ε_r and the (di)electric-constant ε_0 (8.854 187 817 • 10^{-12} F/m); and a is the radius of the particle in m. The electric potential of the ionic atmosphere of the particle (the outer electric potential of Helmholtz) is

$$\varphi_{ex} = \frac{Q}{4\pi\varepsilon(a+d)} \tag{1.1-8}$$

where d is the thickness of the electric double layer. It follows from eqs. (1.1-7) and (1.1-8) that the electrokinetic potential ζ of a charged particle and its ionic atmosphere may be described by the expression

$$\zeta = \frac{Q}{4\pi\varepsilon a} - \frac{Q}{4\pi\varepsilon(a+d)} = \frac{Qd}{4\pi\varepsilon a(a+d)} = \frac{Q}{4\pi\varepsilon(1+a/d)} \tag{1.1-9}$$

The Helmholtz model does not take into account the diffusion of ions in solution, the possibility of adsorption of ions onto the charged surface, and the interaction between the charged surface and the dipoles of the solvent.

1.1.2.2 Model of Gouy–Chapman

Gouy [8], and Chapman [9] have shown that the fixed arrangement of the counterions in the model of Helmholtz does not exist, because they are moving to and way from the charged particle. According to the model of Gouy–Chapman, the number of counterions, and thereby the electric potential of the double layer, decreases exponentially with the distance from the charged surface (Figure 1.1-1).

The counterions are not fixed on the charged surface, but diffuse in the solvent. The relationship between the counterion concentration at the charged surface, c, and the counterion concentration in the solution, c_0, is

$$c = c_0 e^{-\left(\frac{ze\varphi}{kT}\right)} \tag{1.1-10}$$

where z is the number of elementary charges on the counterion, e is the elementary charge, k is the Boltzmann constant, and φ is the potential of the charged surface.

The model of Gouy–Chapman assumes the ions as points. Besides, it is not applicable for highly charged electric double layers. An improvement of this theory, known as the modified Gouy–Chapman model, includes the size of the ions [10].

Theory of Debye–Hückel

Later Debye and Hückel [11] developed a similar theory of the electric double layer. According to their theory, the thickness of the ionic atmosphere

$$\frac{1}{\kappa} = \frac{1}{F}\left(\frac{\varepsilon RT}{2I}\right)^{1/2} \tag{1.1-11}$$

where κ is the Debye–Hückel parameter in m^{-1}, F is the Faraday constant (96,485.332 12 C/mol), R is the molar gas constant [8,314.46 J/(kmol K)], T is the thermodynamic temperature in K, and I is the ionic strength of the solution in mol/dm^3 (mol/l).

It follows from eq. (1.1-11) that the thickness of the ionic atmosphere depends exclusively on the ionic strength, that is, on the concentration and the electrovalence of all ions in the solution. Moreover, the electric double layer, according to Gouy–Chapman, can be considered as a spherical capacitor, if d in the Helmholtz equation (1.1-9) is replaced by the magnitude $1/\kappa$. Then the following equation is obtained:

$$\zeta = \frac{Q}{4\pi\varepsilon a(1+\kappa a)} \tag{1.1-12}$$

1.1.2.3 Model of Stern

In 1924, Stern [12] suggested a combination of the Helmholtz model and the Gouy–Chapman model. According to his model, the ionic atmosphere consists of a

fixed (adsorption, inner) part, known as adsorption layer, and a diffuse (free, outer) part, known as diffuse layer. The thickness of the fixed layer is equal to Δ, and the thickness of the diffused layer is equal to $1/\kappa$. The fixed atmosphere is composed of hydrated counterions, which are adsorbed on the hydrated charged surface. The diffuse layer is composed of the remaining counterions, which are also hydrated. The border between the fixed and diffuse parts is named *slipping plane*. The ions of the diffuse layer are moving freely and its concentration decreases exponentially with the distance from the charged surface (Figure 1.1-2).

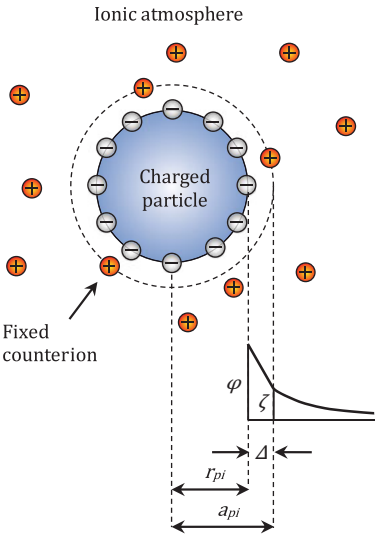


Figure 1.1-2: Spherical protein polyion of radius r_{pi} possessing a negative total charge of $-12e$ is covered with the fixed part of its ionic atmosphere of Δ thickness. The fixed part consists of three counterions with a positive net charge of $3e$. The diffuse part, which has a thickness of $(1/\kappa - \Delta)$, contains the remaining counterions, which have a positive total charge of $9e$.

The fixed part of the ionic atmosphere is moving in an electric field together with the charged particles, while the counterions of the diffuse part separate from the fixed part and are moving to the opposite pole. As a result, the electric potential of the charged particle decreases at the beginning linearly up to the boundary between the fixed and the diffuse part of the ionic atmosphere, then decreases exponentially [13,14,15].

The model of Stern explains best the properties of a polyion. It is known that the pK_c values of some chemical groups of the proteins differ by up to 1.5 pH units from the pK_c values of the same groups in the corresponding amino acids. This can be explained so: If a protein polyion has a positive total charge, its protons are repelled and hydroxide ions from the solution are attracted. As a result, the pK_c values of the acidic groups reduce. When a protein polyion has a negative total charge, its hydroxide ions are repelled and protons from the solution are attracted. In this case, the pK_c values of the alkaline groups grow up.

When the positive and the negative charges of a polyion are equal, the polyion is located in its *isoelectric point*. Then the ζ-potential of the polyion is equal to 0. The isoelectric point depends on the pH value of the solution, which influences the ionization of the polyion [16,17].

The ζ-potential cannot be measured directly but can be calculated using theoretical models and the ionic (polyionic) electrophoretic mobility. The widely used theory for calculating the ζ-potential is that of Smoluchowski [18]. Smoluchowski's theory is valid for dispersed particles of any shape and concentration. However, it is valid only for a thin double layer, when the Debye length, $1/\kappa$, is much smaller than the particle radius a, namely

$$\kappa a \gg 1 \tag{1.1-13}$$

When the Debye length is much larger (e.g., in some nanocolloids), the following equation is obtained:

$$\kappa a < 1 \tag{1.1-14}$$

The Stern model has numerous limitations: the ions are treated as point charges, all interactions in the diffuse layer are assumed as Coulombic, the (di)electric permittivity is accepted as a constant throughout the double layer, and the fluid viscosity is assumed as unchangeable above the slipping plane.

1.1.2.4 Model of Grahame

Grahame [19] proposed that some ionic or uncharged species can penetrate the Stern layer. This could occur, if ions lose their solvation shell as they approach the charged surface. This model proposes the existence of three regions. The **i**nner **H**elmholtz **p**lane (IHP) passes through the centers of the adsorbed ions; the **o**uter **H**elmholtz **p**lane (OHP) passes through the centers of the solvated ions at a distance of their closest approach to the electrode; and the diffuse layer is the region beyond the OHP.

1.1.2.5 Model of Bockris–Devanathan–Müller

In 1963, Bockris, Devanathan, and Müller [20] suggested that attached molecules of the solvent, such as water, have a fixed alignment on the electrode surface. The inner Helmholtz plane passes through the centers of these molecules, and the outer Helmholtz plane passes through the centers of the solvated ions. The diffuse layer is the region beyond the outer Helmholtz plane (Figure 1.1-3).

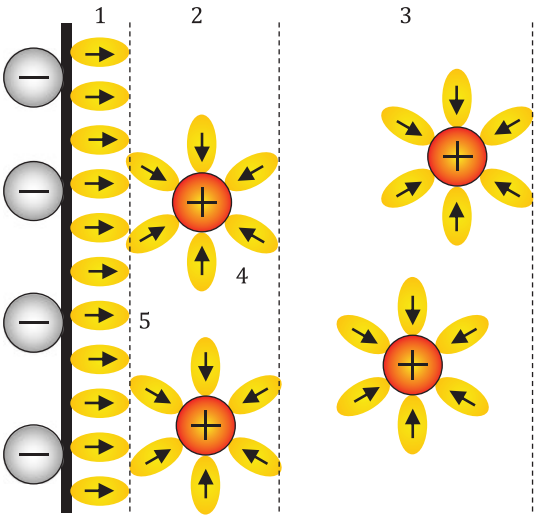


Figure 1.1-3: Schematic presentation of the Bockris–Devanathan–Müller model.
1. Inner Helmholtz plane; 2. Outer Helmholtz plane; 3. Diffuse layer; 4. Solvated ion; 5. Solvent molecules

1.1.3 Two radii and two electric potentials of a charged particle

Recently, we have proposed that every charged and dissolved particle has two radii and two electric potentials [21].

We assume that the counterion is in continuous movement toward and from the central ion due to the electrostatic forces of attraction and repulsion forces of diffusion. Therefore, it can be suggested that in every infinite small interval of time a part of the counterion is linked with the central ion, forming the **ad**sorption layer (*ad*) of the ionic atmosphere, while the remaining part of the counterion forms the **di**ffusion layer (*di*) of the ionic atmosphere.

In line with these considerations, we assume that the radius of the central ion, which we will call *geometric radius R*, increases in the presence of ionic atmosphere to another radius, which we will call *electrokinetic radius A*. These radii characterize two different charged particles that should be called *geometric* and *electrokinetic ion*, the electrokinetic ion being, of course, the larger particle. Hence, the electrokinetic radius is not constant, but varies to $A + dA$, as $R \leq A \leq R + r$, where r is the geometric radius of the counterion, and $A - R = \Delta$ (the thickness of the adsorption layer).

In addition, the geometric ion has a specific electric potential, which we will call *geometric* (thermodynamic) *potential*, and the electrokinetic ion is characterized by another electric potential, which we will call *electrokinetic potential* (Figure 1.1-4). Besides, the electrokinetic potential, φ is a function not only of the distance from it, but also of the ionic strength of the solution.

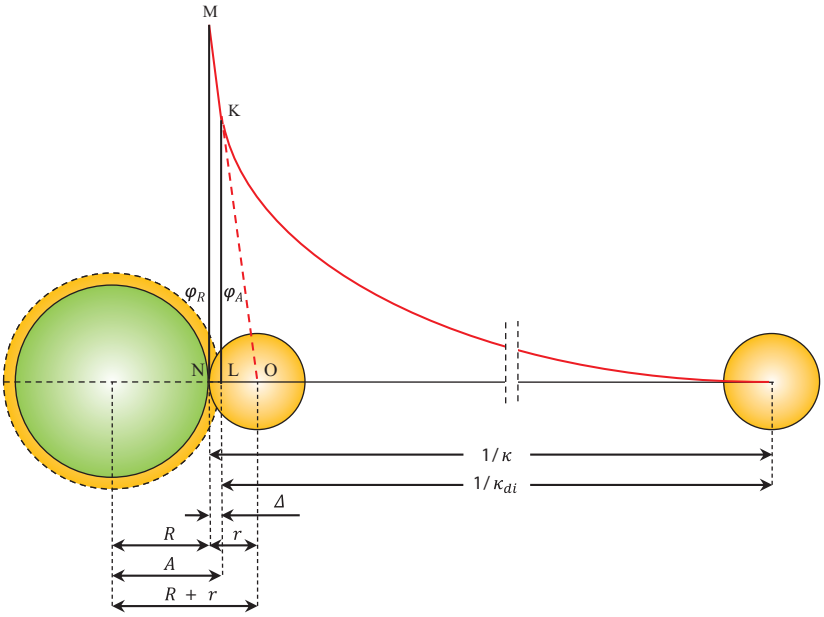


Figure 1.1-4: Electric potentials and radii of the central ion and its ionic atmosphere. φ_R – electric potential of the geometric ion; φ_A – electric potential of the electrokinetic ion; R and r – geometric radii of the central ion and of its counterion, respectively; A and $R + r$ – electrokinetic and the maximum electrokinetic radius of the central ion; Δ – thickness of the adsorption layer; $1/\kappa$ – thickness of the ionic atmosphere; $1/\kappa_{di}$ – thickness of the diffuse layer of the ionic atmosphere

On the surface of the electrokinetic ion, the electric potential of the electrokinetic ion and its ionic atmosphere that is known as ζ-potential is

$$\varphi_A = \varphi_R \frac{1 - \kappa(A - R)}{1 + \kappa R} \tag{1.1-15}$$

where φ_R is the electric potential of the geometric ion and its ionic atmosphere. The electric field of the ionic atmosphere (the counterions) is located around the central ion, whose electric charges create an internal electric field. Therefore, the electric potential on the surface of the geometric ion and its ionic atmosphere is

$$\varphi_R = \varphi_{R_0} + \varphi_{1/\kappa} \tag{1.1-16}$$

where φ_{R_0} is the electric potential of the geometric ion.

When $1/\kappa = \Delta$, that is, on the surface of the electrokinetic ion, the electric potential of the electrokinetic ion and its ionic atmosphere (ζ-potential) is

$$\varphi_A = \varphi_R(1 - \kappa r) \tag{1.1-17}$$

Using the above equations, we proved that

$$A = R + \kappa r^2 \tag{1.1-18}$$

where $r \le \kappa^{-1} \le \infty$, that is, $0 \le \kappa r \le 1$

The last equation shows that the electrokinetic radius A is a function of the parameter κ, namely of the ionic strength of the solution I, at a slope of r^2. According to it, the thickness of the adsorption layer is

$$\Delta = A - R = \kappa r^2 \tag{1.1-19}$$

Assuming that $R = nr$, it follows that

$$A = nr + \kappa r^2 = r(n + \kappa r) \tag{1.1-20}$$

If $n = 1$, the last equation is simplified to give

$$a = r(1 + \kappa r) \tag{1.1-21}$$

where a is the electrokinetic radius of the central ion and r is the geometric radius of the central ion and its counterion.

Having in mind the existence of two radii for each charged particle, we [22] integrated the differential equations of Henry's function [23,24] in the interval $r, 2r$, and obtained the new function

$$f(\kappa r) = \frac{1 + \kappa a}{1 + \kappa r} = \frac{1 + \kappa r + (\kappa r)^2}{1 + \kappa r} \tag{1.1-22}$$

which helps us to obtain the electrokinetic potential as

$$\zeta = \frac{1.5\mu\eta}{\varepsilon} \frac{1 + \kappa r}{1 + \kappa a} \tag{1.1-23}$$

The deduced equations show that the electrokinetic radius A of a central ion is a function of the parameter of the Debye–Hückel κ, hence a function of the ionic strength of a solution I, while the geometric radius R of the central ion is constant. The slope of the curve $dA/d\kappa$ of the displayed equations is equal to r^2, that is, to the square of the geometric radius r of the counterion. When the thickness of the ionic atmosphere $1/\kappa \to \infty$, that is, when $\kappa \to 0$, then $A \to R$; and when $1/\kappa \to 0$, that is, when $\kappa \to 1/r$, then $A \to R + r$.

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1.2 Proteins and nucleic acids form polyions in a solution

The electrophoresis is used for separation of proteins and nucleic acids. These compounds split protons in a solution (the acidic proteins and nucleic acids) or bind protons (the alkaline proteins). As a result, they convert themselves into charged particles (polyions). The proteins and nucleic acids can be resolved electrophoretically in native or denatured state.

1.2.1 Structure and conformation of proteins

The proteins form the backbone of every cell or organism. In addition, they catalyze, as enzymes, all biochemical reactions as well as the release of energy, and are transporters, hormones, signal transmitters, and so on.

Proteins are made up of amino acid residues and can contain in addition non–amino acid compounds. To the first group belong globular proteins (soluble proteins with oval-shaped molecules, such as albumins and globulins) and fibril proteins, also called scleroproteins (water-insoluble proteins with long molecules such as collagens and keratins). The second group includes protein complexes that are composed of a protein part and an exterior compound. According to the exterior compound, the complex proteins are subdivided into glycoproteins, lipoproteins, phosphoproteins, metalloproteins, and nucleoproteins.

Four levels of the protein structure are known.

The *primary structure* of a protein is referred to as the position of amino acid residues (the amino acid sequence) in the polypeptide chain. The polypeptide chain is formed by condensation reactions between adjacent amino acids, resulting in formation of peptide groups (Figure 1.2-1*a*). So, proteins can be determined as polyamides.

The *secondary structure* of a protein is referred to as the spatial folding of the polypeptide chain. It is strengthened mostly by hydrogen bonds. A hydrogen bond is formed between an oxygen atom of a keto group (=C=O) of a peptide group, and a proton of an imino group (=NH) of another peptide group, located at a distance of 0.28 nm.

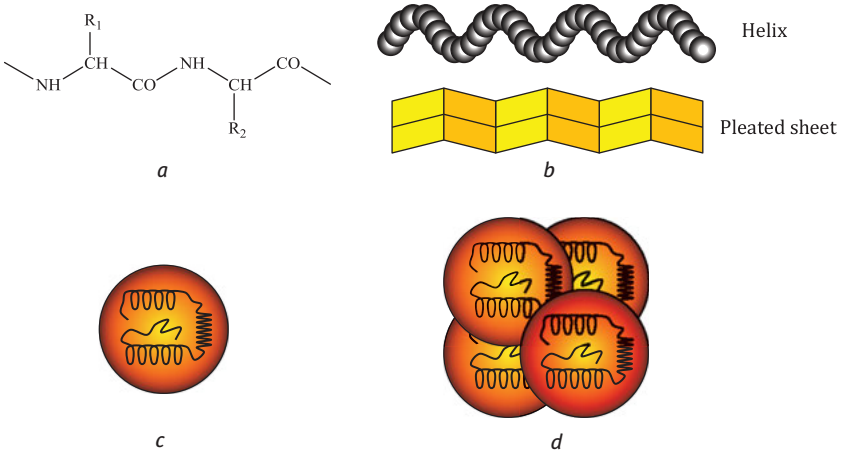


Figure 1.2-1: Structure of proteins.
(*a*) Primary structure; (*b*) Secondary structure; (*c*) Tertiary structure; (*d*) Quaternary structure

The hydrogen bonds are located within a polypeptide chain or between adjacent polypeptide chains. In the first case, they screw the polypeptide chain forming an alpha helix; in the second case, they fold it in a beta sheet (Figure 1.2-1*b*).

The *alpha helix* (α-helix) consists of 3.6 amino acid residues per turn, and is right- or left handed. Its side groups face outward and react with adjacent groups and solvent molecules.

The *beta sheets* (β-sheets), generally twisted, consist of β-strands connected laterally by backbone hydrogen bonds. In an extended β-strand, side chains point straight up, then straight down, then straight up, and so on. The β-strands in a β-sheet are aligned so that their α-C atoms are adjacent and their side chains point in the same direction.

The *tertiary structure* of a protein is referred to as the spatial folding of the axis of its polypeptide chain. It is caused mainly by proline, because it, as an imino acid, bends the axis of the polypeptide chain. Only globular proteins have tertiary structure (Figure 1.2-1*c*).

The tertiary structure is stabilized by disulfide bonds within the polypeptide chain. They are formed by dehydrogenation between cysteine –SH groups. Hydrophobic bonds, hydrogen bonds, and ionic bonds are also of importance for the building of the tertiary structure.

Hydrophobic bonds inside a protein molecule are formed between the hydrocarbonic groups of valine, leucine, isoleucine, and phenylalanine residues. Hydrogen bonds exist between peptide groups or between peptide groups and polar groups of amino acid residues (–OH, $–CONH_2$, *etc.*). Ionic bonds are formed between positively charged groups of lysine, arginine, and histidine residues and negatively charged groups of glutamic and aspartic acid residues.

As a rule, the internal of a globular protein is hydrophobic, whereas its surface is coated with charged groups. As a result, every globular protein is surrounded on the outside by aqueous dipoles, which form a hydrate envelope.

A protein with tertiary structure is myoglobin (M_r = 17,000). It has dimensions of 2.5 × 3.5 × 4.5 nm, and a predominantly α-helical structure. The whale myoglobin is built of a polypeptide chain of 154 amino acid residues that are arranged in eight α-helix sections. It contains also a heme, which is embedded between the α-helical sections.

Lysozyme is another protein with tertiary structure. It has mixed structure: half of the molecule has a pleated region and a small piece of an α-helix, and the other half contains three α-helical regions.

In contrast to globular proteins, the proteins of biological membranes carry hydrophobic groups on the outside, which form hydrophobic bonds to membrane lipids. As a result, the membrane proteins are insoluble in water (Figure 1.2-2).

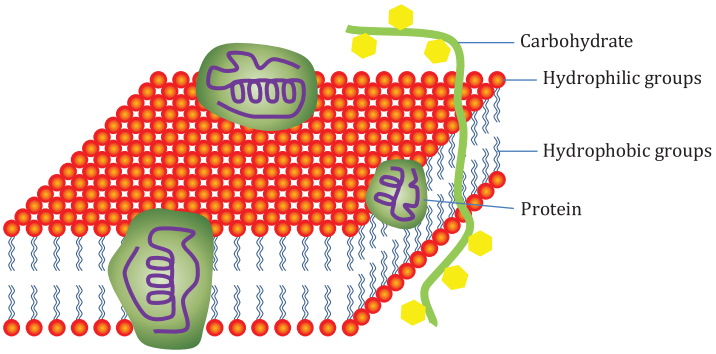


Figure 1.2-2: Structure of a biological membrane.

The *quaternary structure* of a protein is referred to as a structure of several protein molecules (polypeptide chains) named *subunits*. The subunits may be identical or different. Their spatial arrangement in the protein molecule is held together by disulfide, hydrogen, and ionic bonds (Figure 1.2-1*d*).

1.2.1.1 Masses and charges of proteins

The electrophoretic mobility of proteins is determined by their masses and electric charges. The masses depend on the number of amino acid residues. The amino acid number in most proteins extends from less than a hundred to many thousands. Since the relative molecular mass M_r of an amino acid residue is approximately 100, the relative molecular mass of the proteins is approximately 10,000 to several 100,000.

The magnitude of a total electric charge is given by the equation

$$Q_i = z_i e \tag{1.2-1}$$

where z_i is the total number of elementary charges (electrovalence) of the particle i and the fixed part of its ionic atmosphere, and e is the electric charge of the proton, equal to 1.602 1892•10^{-19} C. Since z_i has a positive or negative value, the value of Q_i can have different signs.

A difference exists between the total charge and the net charge of a polyion. The total charge is the sum of all the electric charges, which the polyion carries: the charges of the amino acid residues, metal ions, cofactors, and so on. The net charge is less (in absolute value) than the total charge due to the counterions which are bound to the polyion surface, according to Stern model. The higher the ionic strength of the solution, the smaller the net charge is.

The proteins are amphoteric polyelectrolytes that form in an aqueous solution charged particles (polyions) with both positive and negative electric charges. The charges are carried by ionizable groups in the amino acid residues. The negatively charged proteins are known as *proteinates*, and the positively charged proteins as *protein polycations*.

The proteins contain different ionizable groups. At 25 °C and an ionic strength of I = 0.1 mol/l, the groups can be arranged according to their pK_c values in the following series [1,2]: terminal α-carboxyl group α-COOH (pK_c = 3.0 to 4.3), β-COOH of the aspartic acid residue (pK_c = 3.0 to 5.4), γ-COOH of the glutamic acid residue (pK_c = 3.9 to 6.4), imidazole group $-C_3N_2H_3$ of the histidine residue (pK_c = 5.6 to 7.0), terminal α-amino group α-NH_2 (pK_c = 7.6 to 8.5), the thiol group of the cysteine residue –SH (pK_c = 9.1 to 10.8), ε-NH_2 of the lysine residue (pK_c = 9.4 to 10.9), phenol group of the tyrosine residue $-C_6H_4OH$ (pK_c = 9.8 to 11.7), and the amidino group $-C(NH)NH_2$ of the arginine residue (pK_c = 11.2 to 12.6) (Table 1.2-1).

In a neutral aqueous solution, the carboxyl groups of the aspartic and glutamic acid residues split protons and obtain negative charges. On the contrary, the imidazole group of the histidine residue, the amino group of the lysine residue, and the amidino group of the arginine residue (its imino group) bind protons and become positively charged.

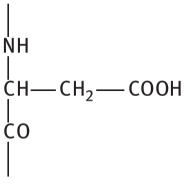
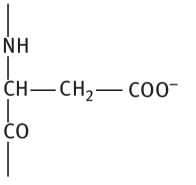
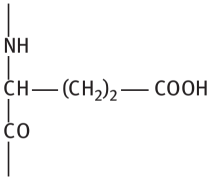
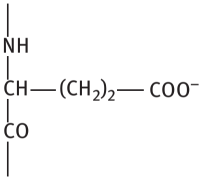
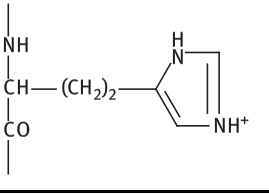
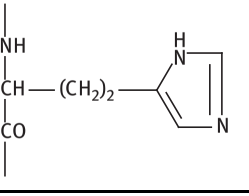
The splitting or binding of a proton depends on the pH value of the solution: at a high pH value the first case is predominating, at a low pH value the second case is prevailing. The intracellular pH is equal to 6.5 to 7.0; the extracellular pH, for example in the blood plasma, is equal to 7.4. Therefore, the amino acid residues are mainly in an ionized state.

The total charge (the number of elementary charges) of a protein polyion is calculated as a sum of the negative and positive charges. For example, if a proteate has 200 negative and 180 positive charges, its total number of elementary charges is

$$z = -200 + 180 = -20$$

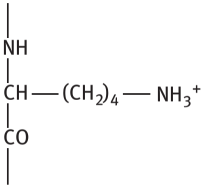
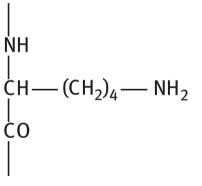
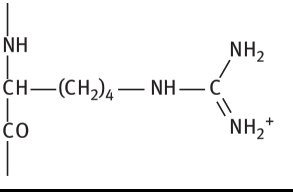
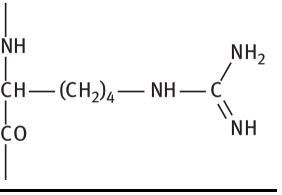
In proteids, for example glycoproteins and phosphoproteins, the total charge depends also on the additional acidic groups of the sugar residues, and on the additional dihydrogen phosphate, respectively, which carry negative charges.

Table 1.2-1: Electric charges of the amino acid residues of proteins at neutral pH value.

Residue of	2	3	4	5	6	7	8	9	10	11	12	pH
Aspartic acid pK_c = 3.0 to 5.4												
Glutamic acid pK_c = 3.9 to 6.4												
Histidine pK_c = 5.6 to 7.0												

(continued)

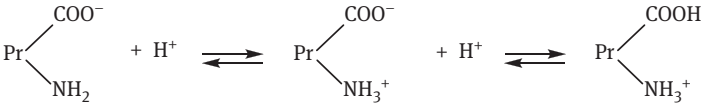
Table 1.2-1 (continued)

Residue of	2	3	4	5	6	7	8	9	10	11	12	pH
Lysine pK_c = 9.4 to 10.9												
Arginine pK_c = 11.2 to 12.6												

The pH ranges, suitable for calculating the charges of a protein polyion and close to the neutral pH range, are 8.5 to 9.0 and 5.0 to 5.5. At pH = 8.5 to 9.0, the groups α-COOH, β-COOH, and γ-COOH are deprotonated, and ε-NH_2 and $-C(NH)NH_2$ are protonated. The groups $-C_3N_2H_3$, α-NH_2, –SH, and $-C_6H_4OH$ are not ionized. This pH value is most commonly used for electrophoresis of acidic proteins.

1.2.1.2 Isoelectric points of proteins

Proteins are amphoteric molecules. That is why they can be considered as zwitterions and denoted by the formula $H_3^+N–Pr–COO^-$. Their charges vary when the proton concentration in the solution is changed:



where the carboxyl groups at low pH values and the amino, imidazole, and amidino groups at high pH values are neutral. The pH value at which the negative charges of an amphoteric polyion neutralize its positive charges and its total charge becomes equal to zero, is defined as *isoelectric point*. Typically, the isoelectric point is noticed as pI; however, this term should be replaced by the term "pH(I) point," because the isoelectric point is a pH value [3].

If the total charge of a protein polyion is set as a function of the pH value, a curve is obtained, which is intersected by the abscissa axis at the isoelectric point of the protein (Figure 1.2-3).

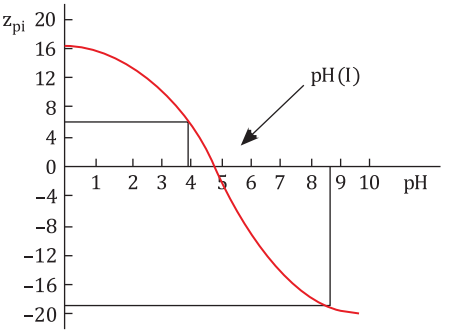


Figure 1.2-3: The total charge of a protein polyion depends on the pH value of the buffer. This protein has 19 negative charges at pH = 8.7, but 6 positive charges at pH = 3.9.

There is a difference between the terms "isoelectric point" and "**p**oint of **z**ero **c**harge" (PZC). The isoelectric point is the pH value at which the particle does not move in an ionic solution located in an electric field. Hence, the isoelectric point depends on the ionic strength of the buffer. The higher the ionic strength, the more are the adsorbed

counterions. The point of zero charge is the pH value at which the particle has zero total charge in endless diluted solution, hence, in absence of adsorbed ions on its surface [4,5]. The isoelectric point is simpler to measure than the point of zero charge; therefore, it is used predominantly.

The values of the isoelectric point decrease while increasing the temperature [6]. For example, at 25 °C they can be by 0.6 pH units lower than at 4 °C [7]. This difference is larger at basic pH ranges than at acidic pH ranges, since the amino groups are more strongly affected by temperature than the carboxyl groups.

The absence of an electric charge in the isoelectric point destroys the electric double layer of a protein and makes it defenseless against precipitants. Then, methanol and ethanol can bind water dipoles and disrupt the hydration shell of the protein polyion and, as a result, precipitate it. Precipitation can also be caused by salts, which also destroy the hydration shell.

The pH(I) values of 95% of the known proteins are located in the pH range 3 to 10 [8]. The acidic glycoprotein of chimpanzee, whose pH(I) = 1.8, seems to have the lowest isoelectric point; and the lysozyme from human placenta, whose pH(I) = 11.7, seems to have the highest isoelectric point.

1.2.1.3 Native and denatured proteins

The secondary, tertiary, and quaternary structure of proteins determine their biological activity (enzyme and hormone reactions, antigen-antibody reactions, oxygen transport, *etc.*). This is their native state. The change in the quaternary, tertiary, and secondary structure of proteins is referred as *denaturation*. It is usually accompanied by loss of biological activity.

The denaturation of proteins is caused by different factors:

- Dehydration by organic solvents (alcohols)
- pH decreasing (acid denaturation)
- High temperature (heat denaturation)
- Ionic or nonionic detergents
- Urea in higher concentration
- High-energy radiation (UV, X-rays, and β-rays).

When denaturing is carried out, the interactions, which stabilize the native protein structure, are broken. The polypeptide chains are unfolded and new interactions take place between them. The hydrophobic groups, previously hidden in the protein internal, come outside and the protein becomes insoluble.

The mild denaturation is referred as the quaternary structure of a protein disintegrates but its tertiary structure remains intact. It is caused by urea, guanidine, or phenol in higher concentration, which destroy only the hydrogen bonds.

The nativity and denaturation of a polyion play a crucial role in electrophoresis. In certain separations, the protein nativity (e.g., of enzymes) should be maintained;

in other separations, the denaturation provides additional information about the polyion structure.

1.2.2 Structure and conformation of nucleic acids

Nucleic acids are the largest macromolecules in the biosphere. They consist of numerous mononucleotide residues which are connected to each other by phosphodiester bonds in polynucleotide chains. The phosphodiester bond represents a phosphoric acid residue, which connects a 3′ hydroxyl group of a mononucleotide residue with the 5′-hydroxyl group of an adjacent mononucleotide residue.

A mononucleotide residue consists of a base, a sugar (deoxyribose or ribose), and a phosphate. The bases contain purine or pyrimidine. Purine bases are adenine and guanine, pyrimidine bases are cytosine, uracil, and thymine. Uracil is not present in the deoxyribonucleic acids (DNA); thymine is not present in the ribonucleic acids (RNA).

The base sequence of the mononucleotide residues, attached by covalent bonds each with other, in a nucleic acid single strand is referred to as *primary structure* of a nucleic acid. The *secondary structure* of a nucleic acid is referred to as the spatial folding of the single strand, stabilized by noncovalent base pairing. The base pairing occurs between two single strands or inside a single strand, that is, it can be inter- or intramolecular. DNA contains usually intermolecular, and RNA contains usually intramolecular base pairing.

The base pairs are formed by hydrogen bonds (hydrogen bonding) between partially negatively charged oxygen or nitrogen atoms, and partially positively charged hydrogen atoms of covalently bonded purine and pyrimidine bases. There are two hydrogen bonds between adenine and thymine (uracil) and three hydrogen bonds between guanine and cytosine.

According to the DNA model of Watson–Crick [9], most DNA molecules resemble a double screw (double helix). The double helix has a diameter of about 2 nm and winds containing 10 base pairs each. The sugar rings and the phosphodiester bridges are located on its surface; the base pairs are perpendicular to its axis (Figure 1.2-4).

The DNA double helix is also held together by hydrophobic interactions, formed between the superposed (stacked) bases (base stacking). The two polynucleotide chains are of opposite polarity, each ending with a dihydrogen phosphate residue, which esterifies the 5′-hydroxyl group of a deoxyribose residue.

In the model of Watson–Crick, both DNA polynucleotide chains form right-wound helix. Another model with a left-wound helix is called *Z*-conformation. The *Z*-conformation makes up less than 1% of the chromosome structure in the eukaryotes. The transition from the right-wound double helix to the *Z*-conformation is carried out *in vitro* at high salt concentration.

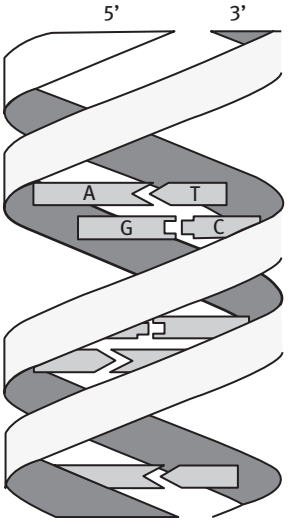


Figure 1.2-4: Double helix of DNA.
A – adenine; T – thymine; G – guanine; C – cytosine

There are three different classes of ribonucleic acids: messenger RNA, ribosomal RNA, and transfer RNA. The messenger ribonucleic acids (mRNA) have relative molecular masses of hundred thousand to several million. It is synthesized as a much larger molecule in the nucleus, but is reduced later enzymatically in the cytoplasm. The ribosomal ribonucleic acids (rRNA) are located in the ribosomes of higher organisms. The transfer ribonucleic acids (tRNA) are relatively small molecules containing 75–85 mononucleotide residues and rare bases.

DNA has linear or circular molecules, which are double stranded or single stranded. The chromosomes of eukaryotes contain double-stranded linear DNA. RNA may also be linear or circular. mRNA, rRNA, and tRNA are single-stranded linear RNA.

The nucleic acids are biopolymers with very long molecules, especially DNA. For example, DNA of the human genome contains three billion base pairs, spread between 23 chromosomes. Because a base pair is $0.34 \bullet 10^{-9}$ m long, DNA of a chromosome is a few centimeter long. In nonhelical state, the total DNA of a human cell would be 2 m long.

The conformation of the DNA molecule effects the DNA movement. Supercoiled DNA migrates faster than relaxed DNA because the supercoiled DNA is more compact. The velocity of DNA movement changes under various electrophoresis conditions [10].

Nucleic acids contain alleles and loci [11]. *Alleles* are variant forms of the genes. Each person has two alleles at a same locus of DNA, one allele contributed by the biological mother, and the other allele contributed by the biological father. *Loci*

(singular *locus*) are the fixed positions on a chromosome, like the position of a gene or a genetic marker.

1.2.2.1 Masses and charges of nucleic acids

The relative molecular mass M_r of a mononucleotide residue is approximately 330. This means that three mononucleotide residues in DNA or RNA have M_r of about 1,000.

Very often, the DNA mass is indicated with the number of the DNA bases, *b* (in the double helical DNA with the number of base pairs, *bp*), whereby a series of 1,000 bases is called kilobases, *kb* (a series of 1,000 base pairs is called kilobase pairs, *kbp*). A *kb* has a relative molecular mass of about 330,000, three kilobases of approximately 10^6.

Each mononucleotide residue has a negative charge in neutral or alkaline solutions, and the number of elementary charges can be calculated when the relative nucleate mass is divided by −330. Hence, in a solution the nucleic acids are polyanions (nucleates), which migrate in an electric field in the anodic direction. In contrast to the proteins, the number of elementary charges of the nucleic acids does not change in neutral or alkaline buffers. Consequently, the ratio of the electric charge to the mass of all DNA and RNA polyions is constant and therefore their mobilities are the same [12].

Nucleate mobilities depend only on the ionic strength and the nativity of the polyions. For example, the mobility of the native DNA from calf thymus is $15.1 \bullet 10^{-9}$ $m^2/(sV)$ at an ionic strength of 0.1 mol/l, and $21.7 \bullet 10^{-9}$ $m^2/(sV)$ at an ionic strength of 0.01 mol/l (for the denatured DNA from calf thymus, these data are $13.3 \bullet 10^{-9}$ and $19.0 \bullet 10^{-9}$ $m^2/(sV)$, respectively). The increased temperature also diminishes the nucleate mobilities because the denaturation increases the nucleate volume.

Polyions of this length cannot be analyzed by electrophoresis. They must be broken into smaller pieces, which can be performed in two ways: with restriction endonucleases, and by multiplication of DNA or RNA in the ***p**olymerase **c**hain **r**eaction* (PCR). The PCR products can be resolved with the aid of disc-electrophoresis and detected by silver staining.

Polynucleotide chains, which have complementary base sequences, can unite. This phenomenon is known as *hybrid formation*. Hybrids may be formed between two DNA polynucleotide chains or between a DNA and an RNA polynucleotide chain.

1.2.2.2 Native and denatured nucleic acids

The nucleic acids, as the proteins, can be in a native or a denatured state (Figure 1.2-5). The native nucleic acids contain base pairs; the denatured nucleic acids, however, contain no base pairs. This means that double-stranded nucleic acids exist only under

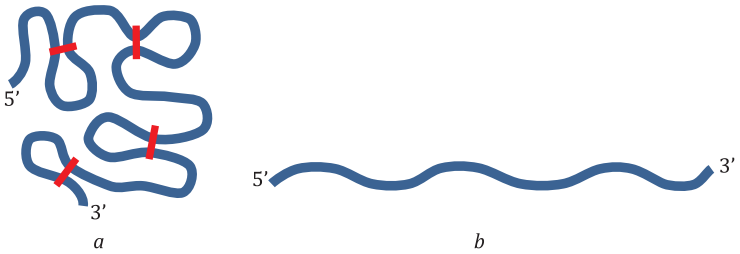


Figure 1.2-5: Native (*a*) and denatured (*b*) nucleic acid (rRNA). The base pairs are shown in red.

native conditions, whereas single-stranded nucleic acids can occur under native or denaturing conditions.

The nativity of a double-stranded nucleic acid or a hybrid depends on the number of hydrogen bonds between the two strands and the repulsion between the negatively charged phosphate groups of the polynucleotide chains. If the strength of hydrogen bonds is greater than the strength of repulsion between the negatively charged phosphate groups of nucleic acids, they remain natural; if the binding forces of base pairing are not sufficient, the double helix denatures. If the repulsion between the negative charges of the polynucleotide chains is reduced (e.g., by increasing the ionic strength), the double helix becomes stable.

The native DNA molecules may be present in globular or linear conformation. The globular molecules are either supercoiled or circled.

Many factors are known which disrupt the inter- or intramolecular base pairing and cause denaturing of nucleic acids: high temperature, alkaline pH values, methylmercury hydroxide, glyoxal or formaldehyde, and urea and formamide.

Temperature. The building of base pairs releases energy. Therefore, a supply of thermal energy allows the paired regions to separate from each other – a process named *thermal denaturing*. The thermal denaturing of DNA can be compared with the melting of the crystal lattice of DNA, and therefore is called DNA melting. The DNA melting occurs at 70 to 90 °C in a saline solution. It destroys the DNA double helix into single strands. As a result, the physical properties of DNA (viscosity, light absorption, and optical rotation) change.

The melting of nucleic acids depends on the base pairing: GC-rich DNA have a higher melting point, as three hydrogen bonds connect guanine and cytosine; in contrast, TA-rich DNA have a lower melting point, as two hydrogen bonds connect adenine and thymine.

To be destroyed, approximately 2 °C for each AT- (AU-) base pair and 4 °C for each GC-pair are necessary. So, a 20-bp-long DNA, containing 10 AT-pairs and 10 GC-pairs, should melt at 10•2 °C + 10•4 °C = 60 °C.

Alkaline pH values. Another possibility to break hydrogen bonds in nucleic acids is to increase the pH value. Increasing their concentration, the negative charged

hydroxide ions (OH^-) interact with the protons in the hydrogen bonds and form with them own hydrogen bonds, which denature the nucleates into single strands. This method is used to study DNA single strands exceeding 1,000 nucleotides, in alkaline agarose gels.

The denaturing at high pH values is, however, unsuitable for RNA nucleates of more than 1,000 mononucleotides. The reason is the fact that RNA has an additional OH-group on the second position of ribose, which hydrolyzes RNA. Therefore, RNA is usually denatured by methylmercury hydroxide, glyoxal or formaldehyde, and is separated in denaturing agarose gels.

Methylmercury hydroxide, glyoxal, or formaldehyde. These chemicals are volatile and toxic, especially methylmercury hydroxide, so they must be carefully handled. Methylmercury hydroxide, glyoxal, or formaldehyde denature nucleates because they react with the nitrogen atoms in the purine and pyrimidine rings, which are involved in the base pairing. Methylmercury hydroxide ($H_3C–Hg^+OH^-$), as well as glyoxal (OHC–CHO) or formaldehyde (H–CHO), are used in denaturing agarose gels, but cannot be used in denaturing polyacrylamide gels, as they react with the catalysts of the polymerization process.

Urea and formamide. Highly concentrated urea ($H_2C–CO–NH_2$), alone or in combination with formamide ($OHC–NH_2$), forms hydrogen bonds with the nucleic acid bases and, as a result, destroys the base pairs. So it breaks the secondary structure of the nucleic acids, namely the inter- and intramolecular base pairing in DNA or RNA. These chemicals are used for determining the base sequence in nucleic acids.

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1.3 Electrophoresis is running in buffers

Electrophoresis is carried out in aqueous solutions. In acidic solutions, the concentration of hydrogen ions is very high. They move to the cathode where gaseous hydrogen is freed:

$$2H^+ + 2e^- \rightarrow H_2\uparrow$$

In alkaline solutions, the concentration of hydroxide ions is very high. They move to the anode where gaseous oxygen is freed:

$$4OH^- \rightarrow 2H_2O + O_2\uparrow + 4e^-$$

To keep the concentration of H^+ and OH^-, hence, the pH values constant, all electrophoretic methods, with the exception of isoelectric focusing, are carried out in buffers.

1.3.1 Buffers

According to the concept of Brønsted [1], buffers are systems of weak bases and their conjugate acids. Their titration curves show a plateau region (buffer zone) which includes up to 1.5 pH units (Figure 1.3-1). In the buffer zone, the buffer pH value changes insignificantly when base or acid is added or the buffer solution is diluted.

The buffers obey the equation of Henderson–Hasselbalch

$$pH = pK_c + \log\frac{[Base]}{[Acid]} \tag{1.3-1}$$

With the aid of this equation, the buffer pH values can be calculated, if the pK_c of the buffering acid and the equilibrium concentrations of the base and its conjugated acid, for example $[NH_3]$ and $[NH_4^+]$ or $[CH_3COO^-]$ and $[CH_3COOH]$, are known. When pH = pK_c, the concentrations of the base and the acid are equal, i.e. half of the protolyte is ionized.

To explain the buffer function, let us present the equilibrium process of acid HA:

$$HA \leftrightarrow H^+ + A^-$$

When a strong acid is added, the process

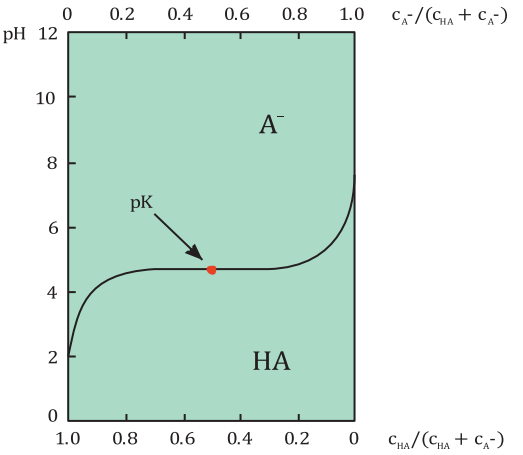


Figure 1.3-1: Titration of acetic acid (HA).

$$A^- + H^+ \leftrightarrow HA$$

occurs, which can be described by the equation

$$pH = pK_c + \log \frac{[A^-] - [H^+]}{[HA] + [H^+]} \tag{1.3-2}$$

When a strong base is added, the process

$$HA + OH^- \leftrightarrow A^- + H_2O$$

occurs, which can be described by the equation

$$pH = pK_c + \log \frac{[A^-] + [H^+]}{[HA] - [H^+]} \tag{1.3-3}$$

It follows from eqs. (1.3-2) and (1.3-3) that the buffer pH value does not significantly change when the concentration of the added base or acid is lower than the concentrations of the buffering base $[A^-]$ and buffering acid [HA]. If the buffer solution is diluted, the concentrations of the base and acid decrease but not the ratio $[A^-]/[HA]$. Therefore, the pH value does not change significantly, too.

1.3.1.1 Buffer capacity

Each buffer has a specific buffer capacity β. The buffer capacity is equal to the changed concentration of a buffer base or an acid, causing a change of the buffer pH value by one pH unit. Mathematically

$$\beta = \frac{dc_A}{d\mathrm{pH}} \tag{1.3-4}$$

where c_A is the base concentration.

Let us derive the expression of buffer capacity. For the equilibrium reaction

$$HA \leftrightarrow H^+ + A^-$$

the total concentration of the base in the buffer solution is

$$c_A = [A^-] + [OH^-] - [H^+] \tag{1.3-5}$$

where $[OH^-]$ is the equilibrium concentration of hydroxide ion. The equilibrium concentration of the buffer anion $[A^-]$ can be calculated from the equation

$$K_c = \frac{[H^+][A^-]}{[HA]} \tag{1.3-6}$$

Since the total concentration of the acid and base of the buffering electrolyte is

$$c = [HA] + [A^-] \tag{1.3-7}$$

it follows from eq. (1.3-6) that

$$[A^-] = \frac{cK_c}{K_c + [H^+]} \tag{1.3-8}$$

It is known that

$$[OH^-] = \frac{K_w}{[H^+]} \tag{1.3-9}$$

where K_w is the ionic product of water, equal to about 10^{-14} $(mol/l)^2$ at 25 °C. Hence, it follows from eqs. (1.3-7), (1.3-8), and (1.3-9) that

$$\beta = \frac{d\left(c\frac{K_c}{K_c + [H^+]} + \frac{K_w}{[H^+]} - [H^+]\right)}{d(-\log[H^+])} = \ln 10\left(c\frac{K_c[H^+]}{(K_c + [H^+])^2} + \frac{K_w}{[H^+]} + [H^+]\right) \tag{1.3-10}$$

In neutral, weakly acidic or weakly basic buffers, the concentrations of the proton and hydroxide ion are relatively low. Therefore, their values can be neglected and equation (1.3-10) can be simplified to give

$$\beta = \ln 10\ c\frac{K_c[H^+]}{(K_c + [H^+])^2} = \ln 10\ c\frac{10^{-pK_c - pH}}{\left(10^{-pK_c} + 10^{-pH}\right)^2} \tag{1.3-11}$$

According to eqs. (1.3-10) and (1.3-11), the buffer capacity depends on the concentration and the pH value of the buffer. When pH = pK_c, it follows from eq. (1.3-10) that

$$\beta = \ln 10\left(\frac{c}{4} + \frac{K_w}{[H^+]} + [H^+]\right) \tag{1.3-12}$$

and from eq. (1.3-11) that

$$\beta = \ln 10 \frac{c}{4} \tag{1.3-13}$$

For a mixture of buffer electrolytes, eqs. (1.3-10) and (1.3-11) can be converted into

$$\beta = \ln 10 \left\{ \Sigma c \frac{K_c[\mathrm{H}^+]}{(K_c + [\mathrm{H}^+])^2} + \frac{K_w}{[\mathrm{H}^+]} + [\mathrm{H}^+] \right\} \tag{1.3-14}$$

and

$$\beta = \ln 10 \ \Sigma c \frac{K_c[\mathrm{H}^+]}{(K_c + [\mathrm{H}^+])^2} = \ln 10 \Sigma c \frac{10^{-\mathrm{p}K_c - \mathrm{pH}}}{\left(10^{-\mathrm{p}K_c} + 10^{-\mathrm{pH}}\right)^2} \tag{1.3-15}$$

respectively, where c is the total concentration of each electrolyte.

Equations (1.3-14) and (1.3-15) can be transformed, if the magnitude of K_c is replaced by the equilibrium concentrations of ions and molecules from eq. (1.3-6), into

$$\beta = \ln 10 \frac{[\mathrm{A}^-][\mathrm{HA}]}{[\mathrm{A}^-] + [\mathrm{HA}]} \tag{1.3-16}$$

and

$$\beta = \ln 10 \Sigma \frac{[\mathrm{A}^-][\mathrm{HA}]}{[\mathrm{A}^-] + [\mathrm{HA}]} \tag{1.3-17}$$

Because $[\mathrm{A}^-] = \alpha c$, and $[\mathrm{HA}] = c(1-\alpha)$, where α represents the dissociation degree, the last two equations can be simplified to give

$$\beta = \ln 10 \alpha(1-\alpha)c \tag{1.3-18}$$

and

$$\beta = \ln 10 \Sigma \alpha(1-\alpha)c \tag{1.3-19}$$

The dependence of the buffer capacity of the acetate buffer on the pH value and on the total concentration of buffering electrolyte is shown in Figure 1.3-2.

Let us estimate how a buffer reacts when pH is changed. Let us assume that a buffer is created after neutralization of a weak acid of concentration c with a strong base of concentration x. Because all basic molecules react stoichiometrically with the weak acid, the Henderson–Hasselbalch equation (eq. 1.3-1) can be presented as

$$\mathrm{pH} = \mathrm{p}K_\mathrm{c} + \log \frac{x}{c-x} \tag{1.3-20}$$

To calculate the pH sensitivity of a buffer, the first derivative of eq. (1.3-20) should be derived. Using the relationship between the natural and decimal logarithms

$$\log \mathrm{n} = \frac{\ln \mathrm{n}}{\ln 10} \tag{1.3-21}$$

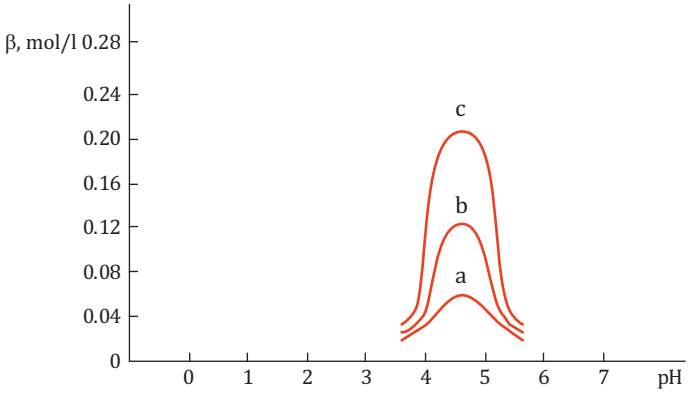


Figure 1.3-2: Dependence of the buffer capacity β, in mol/l, of the acetate buffer (CH_3COO^-/CH_3COOH) on the pH value and the total buffer concentration c, in mol/l.
(a) c = 0.1 mol/l; (b) c = 0.2 mol/l; (c) c = 0.4 mol/l

eq. (1.3-20) is transformed to give

$$\frac{d\mathrm{pH}}{dx} = \frac{1}{\ln 10}\left[\frac{1}{x} - \frac{1}{c-x}(-1)\right] = \frac{c}{\ln 10x(c-x)} \tag{1.3-22}$$

From the last expression, it can be determined that a buffer is more effective, if its first derivative is in minimum, that is, when its reciprocal value, named buffer capacity (s. above), has a maximum value.

To find when the quotient $d\mathrm{pH}/dx$ has its minimum, the second derivative of eq. (1.3-20) must be derived:

$$\frac{d^2\mathrm{pH}}{dx^2} = \frac{c(2x-c)}{\ln 10x^2(c-x)^2} \tag{1.3-23}$$

According to the last equation, the second derivative is equal to 0 when $x = c/2$; hence, when the added base neutralizes the half of the weak acid. If this value is substituted in the Henderson–Hasselbalch equation, then

$$\mathrm{pH} = \mathrm{p}K_c + \frac{1}{\ln 10}\ln\frac{2c}{2c} = \mathrm{p}K_c \tag{1.3-24}$$

The last equation indicates that a buffer is most effective when its pH value is equal to the pK_c value of its buffering acid. The closer the pK_c values to the pH value, and the higher the buffer concentration, the greater is the buffer capacity. The buffer capacity falls to 33% of the maximum value at pH = pK_a ± 1, and to 10% at pH = pK_a ± 1.5. Therefore, the difference between the values of pH and pK_c should not exceed 0.75, that is, $|\mathrm{pH} - \mathrm{p}K_c| \leq 0.75$. For example, when a buffer must have a pH

value of 4.3, no acetate buffer should be used, but a benzoate buffer, because $pK_{C_6H_5COOH} = 4.20$, while pK_{CH_3COOH} is equal to 4.76.

1.3.1.2 Calculating buffer pH values

According to the chemical equilibrium

$$HA \leftrightarrow A^- + H^+ \tag{1.3-25}$$

an acid dissociates equal amounts of hydrogen ion and anion. The equilibrium concentrations of the three components can be calculated in a **R**eaction, **I**nitial, **C**hange, **E**quilibrium (RICE) table (Table 1.3-1).

Table 1.3-1: RICE table for a monoprotic acid.

R	[HA]	[A^-]	[H^+]
I	c_0	0	y
C	$-x$	$+x$	$+x$
E	$c_0 - x$	x	$x + y$

The first row, labeled *I*, lists the initial conditions: the concentration of the acid is c_0, so the concentration of A^- is zero; y is the initial concentration of added strong acid. If strong alkali is added, y will have a negative sign because alkali removes hydrogen ions from the solution. The second row, labeled *C*, specifies the changes that occur when the acid dissociates. The acid concentration decreases by the amount $-x$, and the concentration of A^- increases by the amount $+x$. The third row, labeled *E*, joins the above two rows to show the equilibrium concentrations in the buffer.

To find x, the equilibrium constant

$$K_a = \frac{[H^+][A^-]}{[HA]} \tag{1.3-26}$$

is used. Substituting the concentrations with the values in the last row of the RICE table, eq. (1.3-26) is transformed into

$$K_a = \frac{x(x+y)}{c_0 - x} \tag{1.3-27}$$

which gives

$$x^2 + (K_a + y)x - K_a c_0 = 0 \tag{1.3-28}$$

Changing the values of c_0, $K_{a.}$ and y, the last equation can be solved for x. Assuming that $\mathrm{pH} = -\log[\mathrm{H}^+]$, it follows that

$$\mathrm{pH} = -\log(x + y) \tag{1.3-29}$$

1.3.2 Buffers used in electrophoresis

In electrophoresis, acidic, neutral, or alkaline buffers are used (Table 1.3-2). The alkaline buffers are most widespread because the most proteins and the nucleic acids are acidic.

Table 1.3-2: Buffers used in electrophoresis.

Buffer and pH	Concentrations	Preparation
Acidic buffers		
Acetate buffer pH = 5.3, 10x	0.60 mol/l sodium acetate CH_3COOH	Adjust 49.22 g anhydrous sodium acetate with 1 mol/l acetic acid to pH = 5.3. Add deionized water to 1,000.0 ml.
TRIS-chloride buffer pH = 6.8, 4x	0.50 mol/l TRIS HCl	Titrate 60.57 g TRIS with HCl in 800 ml deionized water to pH = 6.8. Add deionized water to 1,000.0 ml.
Neutral buffers		
TRIS-citrate-EDTA buffer pH = 7.2, 10x	0.25 mol/l TRIS Citric acid 0.01 mol/l Na_2EDTA	Adjust 30.29 g TRIS with 2 mol/l citric acid to pH = 7.2 and add 3.72 g $Na_2EDTA.H_2O$. Add deionized water to 1,000.0 ml.
Hydrogen phosphate buffer pH = 7.3, 10x	0.0034 mol/l KH_2PO_4 0.0071 mol/l Na_2HPO_4	Mix 1.36 g/l KH_2PO_4 and 1.78 g/l Na_2HPO_4 in ratio 435:565 (V/V).
Alkaline buffers		
TRIS-hydrogen phosphate-EDTA buffer pH = 7.7, 10x	0.35 mol/l TRIS 0.35 mol/l NaH_2PO_4 0.01 mol/l Na_2EDTA	42.40 g TRIS 49.69 g Na_2HPO_4 3.72 g $Na_2EDTA \cdot H_2O$ Add deionized water to 1,000.0 ml.
TRIS-formate buffer pH = 7.8, 10x	1.29 mol/l TRIS 1.00 mol/l formic acid	156.27 g TRIS 38.36 ml formic acid Add deionized water to 1,000.0 ml.

Table 1.3-2 (continued)

Buffer and pH	Concentrations	Preparation
TRIS-hydrogen phosphate-EDTA buffer pH = 8.0, 10x	0.80 mol/l TRIS H_3PO_4 0.02 mol/l Na_2EDTA	Dissolve 96.91 g TRIS in 800 ml deionized water and adjust with 85% phosphoric acid (about 15.5 ml) to pH = 8.0. 7.44 g $Na_2EDTA \cdot H_2O$ Add deionized water to 1,000.0 ml.
TRIS-acetate-EDTA buffer pH = 8.3, 50x	2.00 mol/l TRIS CH_3COOH 0.05 mol/l Na_2EDTA	Dissolve 242.28 g TRIS in 800 ml deionized water Adjust with acetic acid to pH = 7.2 18.61 g $Na_2EDTA \cdot H_2O$ Add deionized water to 1,000.0 ml.
TRIS-borate-EDTA buffer pH = 8.3, 10x	0.89 mol/l TRIS 0.89 mol/l boric acid 0.02 mol/l Na_2EDTA	107.81 g TRIS 55.03 g boric acid 7.44 g $Na_2EDTA \cdot H_2O$ Add deionized water to 1,000.0 ml.
TRIS-taurinate-EDTA buffer pH = 8.5, 2x	0.564 mol/l TRIS 0.782 mol/l taurine 0.002 mol/l Na_2EDTA	68.32 g TRIS 97.86 g taurine 0.74 g $Na_2EDTA.H_2O$ Add deionized water to 1,000.0 ml.
TRIS-TRICINEate buffer pH = 8.6	0.324 mol/l TRIS 0.096 mol/l TRICINE	39.25 g TRIS 17.20 g TRICINE Add deionized water to 1,000.0 ml.
Barbitalate buffer pH = 8.6	0.05 mol/l sodium barbitalate 0.01 mol/l barbital	10.31 g sodium barbitalate 1.84 g barbital Add deionized water to 1,000.0 ml.
TRIS-chloride buffer pH = 8.8, 4x	1.5 mol/l TRIS HCl	Dissolve 181.71 g TRIS in 800 ml deionized water and titrate with HCl to pH = 8.8. Add deionized water to 1,000.0 ml.
TRIS-borate-EDTA buffer pH = 8.8, 10x	0.58 mol/l TRIS 0.29 mol/l boric acid 0.02 mol/l Na_2EDTA	70.26 g TRIS 17.93 g boric acid 7.44 g $Na_2EDTA \cdot H_2O$ Add deionized water to 1,000.0 ml.
TRIS-taurinate buffer pH = 9.0, 4x	1.96 mol/l TRIS 0.54 mol/l taurine	237.43 g TRIS 67.58 g taurine Add deionized water to 1,000.0 ml.
Glycinate buffer pH = 10.5, 10x	0.20 mol/l glycine NaOH	Adjust 15.01 g glycine with 0.1 mol/l NaOH to pH = 10.5. Add deionized water to 1,000.0 ml.

The most common buffers for nucleic acids are **T**RIS-**a**cetate-**E**DTA (TAE) and **T**RIS-**b**orate-**E**DTA (TBE) buffers. TRIS-acetate-EDTA buffer has lower buffer capacity than TRIS-borate-EDTA buffer but provides better resolution for larger DNA.

TRIS-borate-EDTA buffer was introduced by Peacock and Dingman [2]. Its pH value is usually 8.3 at TRIS concentration of 50–100 mmol/l (Table 1.3-2).

We proved that the TRIS-borate buffer contains a complex compound formed by a condensation reaction between boric acid and TRIS [3]. The compound, which was referred to as *TRIS-boric acid*, has a zwitterionic structure. It dissociates a hydrogen ion and a *TRIS-borate* ion whose mobility was calculated [4]. The existence of TRIS-boric acid in the TRIS-borate buffer was reproved 30 years later in a vast article by a large scientific group in Paris [5].

The formation of TRIS-boric acid and the dissociation of its protonated amino group take place according to following scheme (Figure 1.3-3):

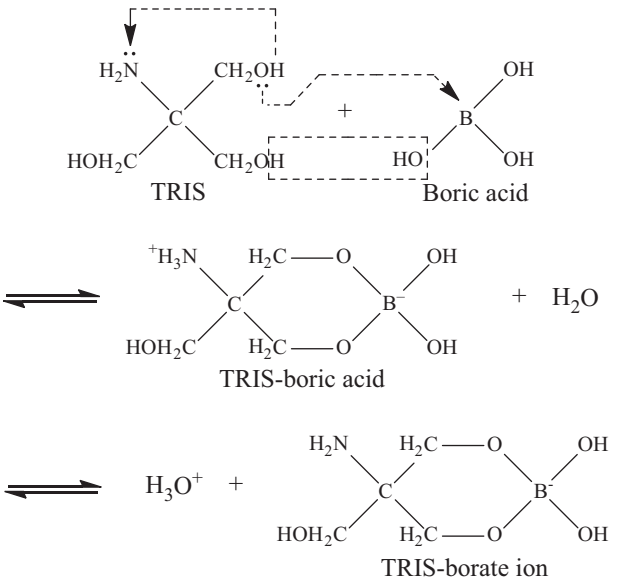


Figure 1.3-3: Formation and dissociation of TRIS-boric acid.

It should not be forgotten that borate ion interacts with *cis* diols, such as those found in RNA, forming complex compounds.

Many other buffers have been proposed, for example, containing **l**ithium **b**orate (LB), histidine, and so on. LB is ineffective in resolving DNA fragments larger than 5,000 bp. However, because of its low conductivity, much higher voltage could be used (up to 35 V/cm), which shortens the electrophoresis time. In 3 g/dl agarose gel containing 1 mol/l lithium borate, nucleates of one base pair difference can be resolved.

1.3.3 Biological buffers

The biological buffers are subdivided into intracellular and extracellular buffers.

The major *intracellular buffer* is the hydrogen phosphate buffer (HPO_4^{2-}/$H_2PO_4^-$), often incorrectly referred to as phosphate buffer. Its maximum buffer capacity is at pH = 6.7. In addition to the hydrogen phosphate buffer, the cell contains other buffers of limited importance, for example the glucose-6-phosphate and adenosine triphosphate buffer.

The most important *extracellular buffer* is the hydrogen carbonate buffer (HCO_3^-/H_2CO_3), known also as bicarbonate buffer. Its carbonic acid is in equilibrium with the dissolved carbon dioxide $CO_{2(dis)}$, which in turn is in equilibrium with the atmospheric carbon dioxide $CO_{2(atm)}$. These processes and the dissociation of carbonic acid can be expressed as:

$$CO_{2(atm)} \leftrightarrow CO_{2(dis)} + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

The pK_c value of carbonic acid is equal to 3.8, but the highest buffer capacity of the hydrogen carbonate buffer is at pH = 6.3. This "missing" match between theory and practice can be explained as follows: the balance between carbonic acid and the dissolved carbon dioxide is strongly shifted toward carbon dioxide – only 0.0026 of the dissolved carbon dioxide exists as carbonic acid. As a result, the pH value of a hydrogen carbonate buffer is determined not only by the ratio HCO_3^-/H_2CO_3, but also by the equilibrium $CO_{2(dis)} \leftrightarrow H_2CO_3$. The p$K$ value of the total reaction is equal to 6.3.

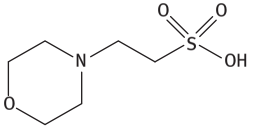
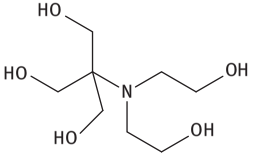
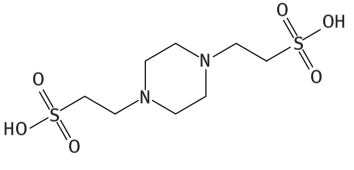
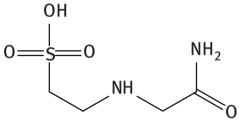
The equilibrium between the dissolved carbon dioxide and carbonic acid explains the influence of the atmospheric carbon dioxide on the pH value of the hydrogen carbonate buffer: According to the Henry's law, the concentration of a dissolved gas depends on its partial pressure. Thus, if the partial pressure of carbon dioxide is increased in the atmosphere, the pH value of the buffer decreases, and vice versa.

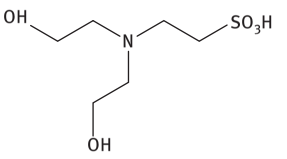
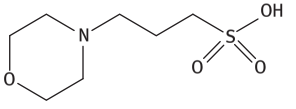
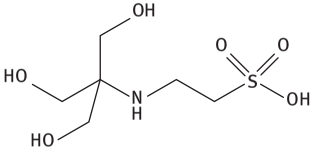
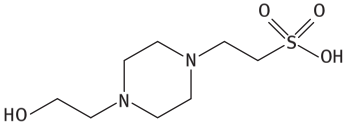
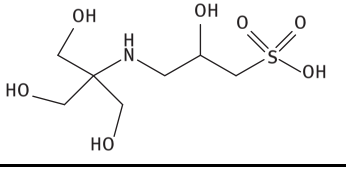
Fluids in and surrounding cells of the living organisms are kept at a constant pH. To study biological processes in the laboratory, scientists use *biological buffers*, which cover the pH range of 2 to 11. Many of them were described by Norman Good and colleagues [6,7,8]. Most of Good's buffers are solutions of new zwitterionic compounds, for example: MES, ADA, PIPES, ACES, MOPSO, MOPS, BES, TES, HEPES, DIPSO, TAPSO, POPSO, HEPPSO, PEPPS, TRICINE, BICINE, TAPS, and more.

The specifications of the biological buffers used in the electrophoresis techniques are given in Table 1.3-3. Their pK_a values are temperature and concentration dependent.

The most of Good's buffers, except MES, MOPS, and PIPES, form complexes with metals. Piperazine-containing buffers (PIPES, HEPES, POPSO, and EPPS) can form radicals and should be avoided in studies of redox processes in biochemistry [9,10]. TRICINE is photo-oxidized by flavins, and therefore reduces the activity of flavone enzymes at daylight. ADA, POPSO, and PIPES are poorly soluble in water,

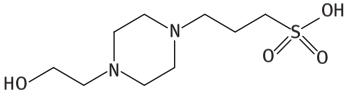
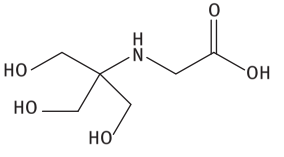
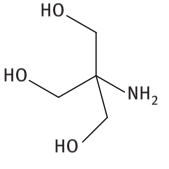
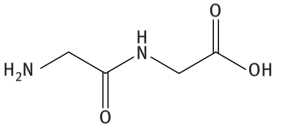
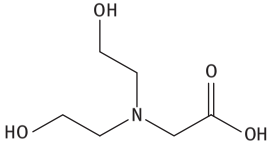
Table 1.3-3: Biological buffers used in electrophoresis.

Buffer	Full compound name	Chemical formulas	Mol. mass	pK_a at 25 °C	Buffering range
MES	2-(*N*-morpholino)-ethanesulfonic acid		195.24	6.10	5.4–6.8
BISTRIS	2,2-Bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol		209.24	6.50	5.8–7.2
PIPES	1,4-Piperazinediethane-sulfonic acid		302.37	6.76	6.1–7.5
ACES	2-(carbamoylmethylamino)-ethanesulfonic acid		182.20	6.78	6.1–7.5

BES	N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid		213.25	7.09	6.4–7.8
MOPS	3-Morpholinopropane-1-sulfonic acid		209.26	7.20	6.5–7.9
TES	2-{[Tris(hydroxymethyl)-methyl]amino} ethanesulfonic acid		229.25	7.40	6.7–8.1
HEPES	2-[4-(2-hydroxyethyl)-piperazin-1-yl] ethanesulfonic acid		238.30	7.48	6.8–8.2
TAPSO	3-[[1,3-dihydroxy-2-(hydroxymethyl) propan-2-yl]amino]-2-hydroxypropane-1-sulfonic acid		259.28	7.60	6.9–8.3

(continued)

Table 1.3-3 (continued)

Buffer	Full compound name	Chemical formulas	Mol. mass	pK_a at 25 °C	Buffering range
HEPPS	3-[4-(2-Hydroxyethyl)-piperazin-1-yl] propane-1-sulfonic acid		252.33	8.00	7.3–8.7
TRICINE	N-(2-Hydroxy-1,1-bis-(hydroxymethyl)ethyl) glycine		179.17	8.05	7.4–8.8
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol		121.14	8.06	7.4–8.8
Gly-Gly	2-[(2-Aminoacetyl)amino]-acetic acid		132.12	8.20	7.5–8.9
BICINE	2-(Bis(2-hydroxyethyl)-amino)acetic acid		163.17	8.26	7.6–9.0

TAPS	3-[[1,3-dihydroxy-2-(hydroxymethyl) propan-2-yl]-amino]propane-1-sulfonic acid		243.28	8.40	7.7–9.1
CHES	2-(Cyclohexylamino)-ethanesulfonic acid		207.29	9.49	8.8–10.2

but their monosodium salts are good soluble. ADA absorbs UV light below 260 nm, and ACES absorbs it at 230 nm and below.

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1.4 The polyions move in electric field

The mobilities play a very important role in the electrophoresis of proteins and nucleic acids. Therefore, they must be well described and characterized.

1.4.1 Ionic and polyionic mobility

In an electric field, a charged particle (ion or polyion) is transported by the electrophoretic force F_e (in N) to the counter-pole. This force is directed toward the frictional force F_f (in N). It is caused by the dipole molecules of the solvent (water) which are on the path of the moving particle. If the charged particle moves with a constant speed, the two forces are equal to each other:

$$F_e = F_f \tag{1.4-1}$$

It is known that

$$F_e = QE = z_\infty eE \tag{1.4-2}$$

where Q is the electric charge of the particle, in C, which is equal to the product of the number of its elementary charges z_∞ in an infinitely diluted solution and the elementary charge e; and E is the strength of the electric field, in V/m. Simultaneously, it is known that

$$F_f = f v_\infty \tag{1.4-3}$$

where f is the friction coefficient (in Js/m^2), and v_∞ is the velocity of a charged particle (in m/s) in an infinitely dilute solution. For spherical particles, according to Stokes' law,

$$f = 6\pi\eta r \tag{1.4-4}$$

where η is the dynamic viscosity of the solvent (in Pa s), and r is the radius of the charged particle (in m). From eq. (1.4-1)–(1.4-4), the equation

$$z_\infty e = 6\pi\eta r \frac{v_\infty}{E} \tag{1.4-5}$$

is obtained.

It is known also that

$$\frac{v_\infty}{E} = \mu_\infty \tag{1.4-6}$$

where μ_∞ is the absolute mobility of the particle, in $m^2/(sV)$, that is, in an infinitely dilute solution. Then, in accordance with eq. (1.4-5) and (1.4-6), it follows that

$$\mu_\infty = \frac{z_\infty e}{6\pi\eta r} \tag{1.4-7}$$

In reality, a charged particle moves together with a part of its ionic atmosphere. Therefore, an electrokinetic potential ζ is measured on the charged particle that is less than its absolute potential φ_0. According to most scientists, ζ-potential is measured on the slipping plane between the particle (together with its adsorbed counterions), and the diffuse part of its ionic atmosphere. As a result, the mobility of the charged particle decreases, when increasing the ionic strength:

$$\mu = \frac{v}{E} = \frac{ze}{6\pi\eta r} \tag{1.4-8}$$

At its isoelectric point, the electric charge of a protein is zero and its mobility is zero, too.

The ionic mobility also depends on the temperature of the solution. It is established that for a 10 °C temperature increase, the mobility rises by approximately with 20%. However, a too-elevated temperature will slow down the mobility of proteins and nucleates since they denature and their volumes increase.

Regardless of their masses, nucleates have the same mobility because their charge density is equal. However, if an electrophoretic separation takes place in a gel, the larger nucleates meet greater resistance, which decreases their mobility.

1.4.2 Effective mobility

In contrast to strong electrolyte solutions, effective mobility μ' is measured in weak electrolyte solutions. This is due to the fact that not all molecules are dissociated, but only a part of them, namely αc, where α is the dissociation degree (dimensionless) and c the total electrolyte concentration, in mol/dm^3 [1,2]:

$$\mu' = \alpha\mu \tag{1.4-9}$$

Consequently, the effective mobility of an ion in a solution of weak acid or base depends on the pH value of the solution.

1.4.3 Equations of polyionic mobility

In the scientific literature, a few equations for polyionic mobility are known: equation of Smoluchowski, Hückel, Onsager, Robinson–Stokes, parametric equation, and quadratic equation.

1.4.3.1 Equation of Smoluchowski

Smoluchowski [3,4] found that the mobility μ of a charged particle can be expressed by the following equation

$$\mu = \frac{\zeta\varepsilon}{\eta} \tag{1.4-10}$$

where ε is the (di)electric permittivity of the solvent (water), which equals the product of the relative (di)electric permittivity ε_r and the (di)electric constant ε_0 (8.854 187 817•10^{-12} F/m).

1.4.3.2 Equation of Hückel

Hückel [5] showed that the ionic atmosphere reduces the absolute mobility $(1+\kappa a)$ times, according to the equation

$$\mu = \frac{\mu_\infty}{1+\kappa a} = \frac{\zeta\varepsilon}{1.5\eta} \tag{1.4-11}$$

where κ is the reciprocal value of the ionic atmosphere thickness (1/κ), and a is the radius of a charged particle. When the concentration of a solution (buffer) increases, the thickness of the ionic atmosphere decreases, that is, the sum $(1+\kappa a)$ increases. This means that the ζ-potential depends on both the charge of the particle and the concentration of the solution (buffer).

1.4.3.3 Henry's function

Comparing the equations of Smoluchowski (1.4-10) and Hückel (1.4-11), we can establish that the mobility, according to the first equation, is 1.5 times higher than the mobility, according to the second equation. In order to unite both equations, Henry [6,7] added to the Hückel equation his complex function $f(\kappa a)$ and received

$$\mu = \frac{\zeta\varepsilon}{1.5\eta} f(\kappa a) \tag{1.4-12}$$

where η is the dynamic viscosity of the solution (in Pa s), and

$$f(\kappa a) = 1 + \frac{(\kappa r)^2}{16} - \frac{5(\kappa r)^3}{48} - \frac{(\kappa r)^4}{96} + \frac{(\kappa r)^5}{96} - \frac{11}{96} e^{\kappa r} \int_{\infty}^{\kappa r} \frac{e^{-t}}{t} dt \tag{1.4-13}$$

is the function of Henry.

According to Henry's function, the mobility of an ion or polyion depends not only on the ionic strength of the solution, which is present in the parameter κ, but also on the radius of the charged particle a. When $\kappa a \to \infty$, then $f(\kappa a) \to 1.5$, and eq. (1.4-12) is transformed into the equation of Smoluchowski (1.4-10); when $\kappa a \to 0$, then $f(\kappa a) \to 1.0$, and eq. (1.4-12) gives the Hückel equation (1.4-11).

1.4.3.3.1 New expression of Henry's function

We proved that each ion (polyion) has two radii: a geometric r and an electrokinetic a [8]. Besides we showed that the radius a is a function of the Debye–Hückel parameter, according to the equation

$$a = r(1 + \kappa r) \tag{1.4-14}$$

where $0 \le \kappa r \le 1$. In addition, we established that every ion and its ionic atmosphere are characterized by the following potentials: φ_0, φ_r, ζ, and φ_a. According to our concept and the theories of Gouy [9], Chapman [10], and Debye–Hückel, the geometric potential

$$\varphi_o = \frac{ze}{4\eta\varepsilon r} \tag{1.4-15}$$

the potential of the geometric ion and its ionic atmosphere

$$\varphi_r = \frac{ze}{4\eta\varepsilon a} = \frac{ze}{4\eta\varepsilon r(1 + \kappa r)} = \frac{\varphi_o}{1 + \kappa r} \tag{1.4-16}$$

the electrokinetic potential

$$\zeta = \frac{ze}{4\eta\varepsilon r(1 + \kappa a)} = \frac{\varphi_o}{1 + \kappa a} = \varphi_r \frac{1 + \kappa r}{1 + \kappa a} \tag{1.4-17}$$

and the potential of the electrokinetic ion and its ionic atmosphere

$$\varphi_a = \frac{zeexp[-\kappa(a - r)]}{4\pi\varepsilon a(1 + \kappa r)} = \frac{\varphi_r}{1 + \kappa a} = \frac{\zeta}{1 + \kappa r} = \zeta \frac{r}{a} \tag{1.4-18}$$

If we introduce the two ionic radii and the four potentials in the Henry's expressions, we obtain a new function, which we refer to as $f(\kappa r)$ [11]:

$$f(\kappa r) = \frac{1+\kappa a}{1+\kappa r} = \frac{1+\kappa r+(\kappa r)^2}{1+\kappa r} \tag{1.4-19}$$

Now eq. (1.4-12) is converted into

$$\mu = \frac{\zeta\varepsilon}{1.5\eta} f(\kappa r) = \frac{\zeta\varepsilon}{1.5\eta}\frac{1+\kappa a}{1+\kappa r} \tag{1.4-20}$$

When $\kappa r \to 0$, then $f(\kappa r) \to 1$, and

$$\mu = \frac{\zeta\varepsilon}{1.5\eta} \tag{1.4-21}$$

that is, the equation of Hückel occurs; and when $\kappa r \to 1$, then $f(\kappa r) \to 1.5$, and

$$\mu = \frac{\zeta\varepsilon}{\eta} \tag{1.4-22}$$

that is, the equation of Smoluchowski occurs.

In order to explain better the derived equations, we show on Figure 1.4-1 the graphics of $f(\kappa r)$ and $2/3\, f(\kappa r)$ as functions of $-\log[-\log(\kappa r_i)]$. It can be seen that there is no difference between the two functions: $2/3\, f(\kappa r)$ must be multiplied by 1.5 to obtain the derived function $f(\kappa r)$, which is identical to the Henry's function $f(\kappa a)$.

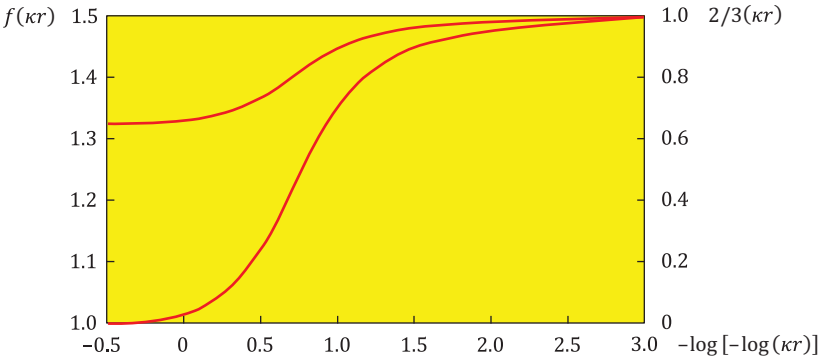


Figure 1.4-1: Dependence of functions $f(\kappa r)$ and $2/3(\kappa r)$ on $-\log[-\log(\kappa r)]$. The function $f(\kappa r)$ is presented on the left ordinate axis, whereas the function $2/3(\kappa r)$ is presented on the right ordinate axis.

On Figure 1.4-2 are given the graphics of $f(\kappa r)$ as a function of κr and of its logarithmic values.

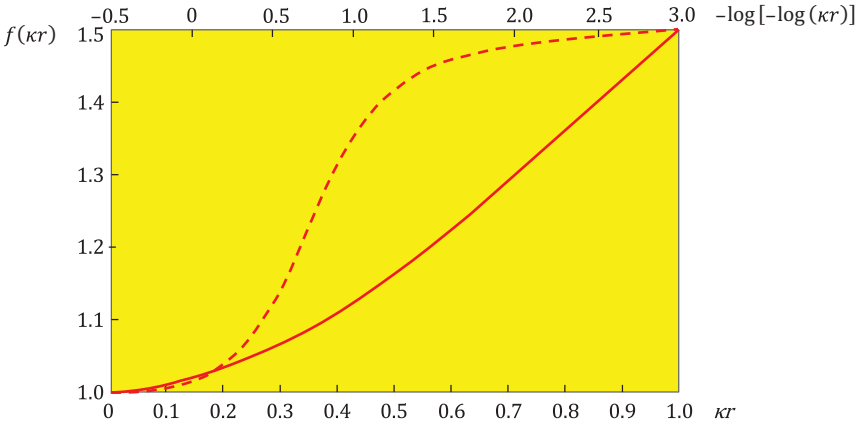


Figure 1.4-2: The dependence of function $f(\kappa r)$ on κr (the solid line) and on its logarithmic values $-\log[-\log(\kappa r)]$ (the dashed line).

1.4.3.4 Equation of Onsager

Onsager [12,13] proposed that the polyion mobility depends on the phenomena cataphoresis and relaxation. *Cataphoresis* causes additional friction force, which reduces the polyionic mobility in an electric field. It occurs because the diffuse part of its ionic atmosphere is moving towards the polyion. *Relaxation* is referred to the restructuring of the complex polyion – ionic atmosphere in an electric field: both the polyion and its ionic atmosphere move in opposite directions in an electric field, which results in polarization of the complex and additional reduction of the polyionic mobility (Figure 1.4-3).

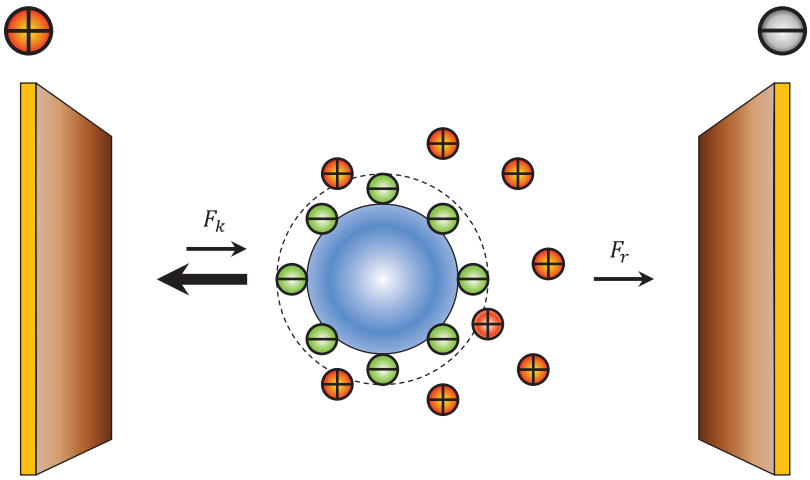


Figure 1.4-3: Influence of the cataphoretic strength (F_k) and relaxation strength (F_r) on the polyionic mobility.

The effect of cataphoresis and relaxation on the particle mobility is described by the Onsager equation

$$\mu = \mu_\infty - \left(\mu_{cat} + \mu_{rel}\right) = \mu_\infty - zsI^{1/2} \tag{1.4-23}$$

where μ_∞ is the absolute mobility of the polyion; μ_{cat} and μ_{rel} are its cataphoresis and relaxation mobility, respectively; z is the number of its elementary charges (the electrovalence); s is the slope of the Onsager calibration line; and I is the ionic strength of the solution (buffer).

1.4.3.5 Equation of Robinson–Stokes

The Onsager equation can be used only for solutions with low ionic strengths. At higher ionic strength (up to 0.1 mol/l), the equation of Robinson–Stokes [14] can be applied in a good agreement with the experiment:

$$\mu = \mu_\infty - \frac{sI^{1/2}}{1 + \kappa a} \tag{1.4-24}$$

According to this equation, the ions and polyions are not considered as point charges, as in Onsager theory, but as charged particles. Therefore, $I^{1/2}$ is divided by the sum $(1 + \kappa a)$, where the radius a of the charged particle can have different values, depending on the experimental conditions.

1.4.3.6 Parametric equation

The ionic (polyionic) mobility can be calculated also according to the linear parametric equation

$$\mu = \mu_\infty - p \tag{1.4-25}$$

where p is the parameter of the ionic (polyionic) mobility [15] expressed as

$$p = \frac{ze\kappa}{6\pi\eta} \tag{1.4-26}$$

For weak electrolytes, the above equation is transformed into

$$\mu' = \alpha(\mu_\infty - p) \tag{1.4-27}$$

1.4.3.7 Quadratic equation

If eq. (1.4-14), which represents our concept of existing of two radii of a charged particle (a geometric and electrokinetic radius), is inserted in the equation of Robinson–Stokes (1.4-24) and the resulting expression is simplified, the following quadratic equation is obtained:

$$\mu = \mu_\infty - \frac{sI^{1/2}}{1 + \kappa r + \kappa^2 r^2} \tag{1.4-28}$$

The experimental values of the ionic mobility can be well expressed by the parametric and the quadratic equation as well as by the equation of Robinson–Stokes. However, while in the Robinson–Stokes equation the context-dependent value of a is used, the quadratic equation contains the well-defined geometric radius r of the ion. The experimental data lie closest to the results obtained by the quadratic equation.

If the ionic mobility is multiplied by its electrovalence and the Faraday constant F, the ionic equivalent conductivity Λ_i, in $Sm^2/kmol$, is obtained. If the equivalent conductivities of all ions are summarized, the molar electroconductivity of the electrolyte solution Λ_m is obtained, also in $Sm^2/kmol$:

$$\Lambda_m = \Sigma\Lambda_i = F\Sigma z_i \mu_i \tag{1.4-29}$$

The molar electroconductivity is equal to the ratio between the specific conductivity γ of the electrolyte solution and the molar concentration of the electrolyte c:

$$\Lambda_m = \frac{\gamma}{c} \tag{1.4-30}$$

From the last two equations, the specific electroconductivity of an electrolyte solution can be calculated:

$$\gamma = \Sigma\gamma_i = F\Sigma c_i z_i \mu_i \tag{1.4-31}$$

where γ_i is the specific ionic electroconductivity, measured also in S/m.

The electroconductivity values can be calculated using Onsager equation, Robinson–Stokes equation, or the parametric equation. The values, calculated according to the Onsager equation, are far from the experimental values when $I > 0.01$ mol/dm^3 (Figure 1.4-4).

1.4.4 Mobilities of ions used in electrophoretic methods

In Table 1.4-1, the absolute mobilities of ions are listed [16,17,18], which are used in electrophoretic methods.

1.4.4.1 Calculating the mobilities of composed ions

We propose equations for calculating the mobilities of ions that could be considered as composed of ions with known mobilities [19].

Different ions have different shapes but in order to simplify the equations all ions could be considered as spherical. The spherical shapes are recommended because Stokes law concerns the ions as spheres.

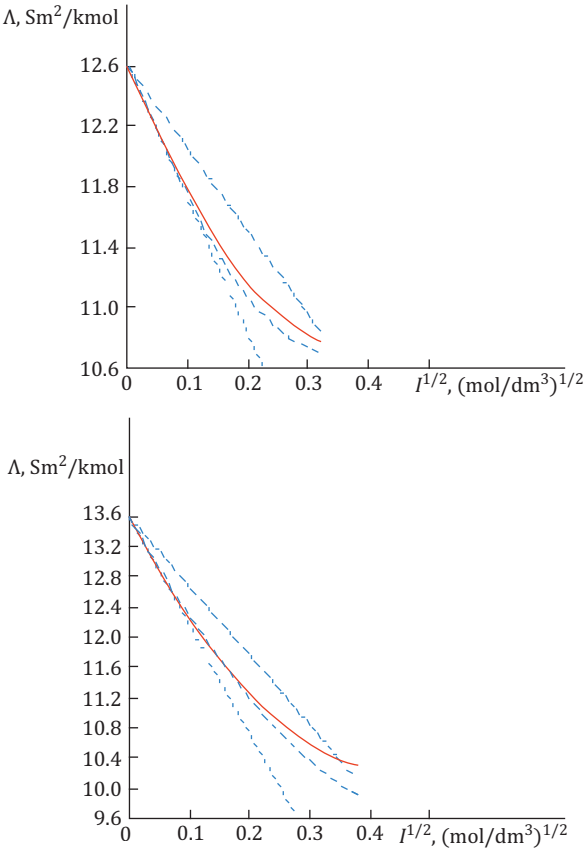


Figure 1.4-4: The electroconductivities (Λ) of solutions of NaCl (up) and $CaCl_2$ (down) at 25 °C, according to Onsager equation (dotted line); according to Robinson–Stokes equation when a is equal to $4 \bullet 10^{-10}$ m for 1-1, and $4.31 \bullet 10^{-10}$ m for 1-2 electrolytes (dashed line); and according to the parametric equation (the dotted-dashed line). It is supposed that $\mu_{\infty Na^+} = 51.93 \bullet 10^{-9}$, $\mu_{\infty Cl^-} = -79.13 \bullet 10^{-9}$, and $1/2\ \mu_{\infty Ca^{2+}} = 30.84 \bullet 10^{-9}$ $m^2/(s\ V)$. The experimental results are presented as solid red curves.

Let us accept that ion x is composed of ions a and b (all ions under discussion are meant to be hydrated) after splitting off small ions or molecules such as H^+, HO^-, H_2O, H_2, and so on. Let us also admit that the volume of ion x is approximately equal to the sum of the volumes of forming ions. If we express the volumes of all ion spheres with the formula $V = \frac{4}{3}\pi r^3$ and simplifying the equation, the following expression is obtained:

$$r_x^3 = r_a^3 + r_b^3 \tag{1.4-32}$$

where r_x, r_a, and r_b are the radii of the composed and forming ions, respectively.

Table 1.4-1: Absolute ionic mobilities, in $m^2/(sV)$, at two temperatures.

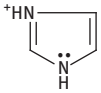
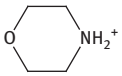
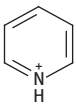
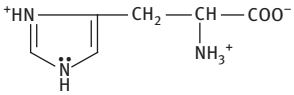
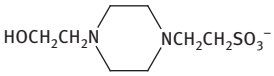
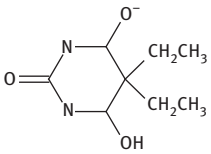
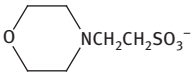
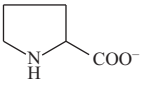
Ions	Chemical formulas	$\mu_\infty \bullet 10^9$ (0 °C)	$\mu_\infty \bullet 10^9$ (25 °C)
Hydronium ion	H_3O^+	248.74	362.39
Ammonium ion	NH_4^+	40.32	76.26
Potassium ion	K^+	39.57	76.18
Sodium ion	Na^+	27.40	51.95
Ethanol ammonium ion	$^+H_3NCH_2CH_2OH$	23.92	47.59
Imidazolium ion		26.65	43.74
Morpholinium ion		20.69	40.37
Pyridinium ion		23.92	38.55
Glycinium ion	$^+H_3NCH_2COOH$	15.73	36.52
4-aminobutyricum ion (GABA ion)	$^+H_3NCH_2CH_2CH_2COOH$	18.46	33.63
Alaninium ion	$^+H_3NCH(CH_3)COOH$	17.46	32.67
1/2 Calcium ion	Ca^{2+}	15.55	30.84
Triethylammonium ion	$(HOCH_2CH_2)_3NH^+$	16.22	30.26
β-Alaninium ion	$^+H_3NCH_2CH_2COOH$	17.22	29.30
Ammediolium ion	$HOCH_2(^+H_3N)C(CH_3)CH_2OH$	13.37	27.86
TRIS ion	$(HOCH_2)_3CNH_3^+$	12.75	27.86
1/2 Magnesium ion	Mg^{2+}	16.07	27.49
Histidinium ion		14.73	24.01
BISTRIS ion	$(HOCH_2CH_2)_2NH^+C(CH_2OH)_3$	12.00	24.01
HEPESate ion		−9.52	−22.85
TRICINEate ion	$(HOCH_2)_3CNHCH_2COO^-$	−10.51	−24.01
Barbitalate (Veronalate) ion		−13.99	−24.97
TESate ion	$(HOCH_2)_3NHCH_2CH_2SO_3^-$	−12.75	−26.90
ACESate ion	$H_2NCOCH_2NHCH_2CH_2SO_3^-$	−14.24	−27.86

Table 1.4-1 (continued)

Ions	Chemical formulas	$\mu_\infty \bullet 10^9$ (0 °C)	$\mu_\infty \bullet 10^9$ (25 °C)
MESate ion		−13.49	−28.19
1/2 Hydrogen phosphate ion	HPO_4^{2-}	−14.15	−29.71
Asparaginate ion	$H_2NCOCH_2CH(NH_2)COO^-$	−14.98	−29.78
Glycylglycinate ion	$H_2NCH_2CONHCH_2COO^-$	−16.97	−30.26
4-Aminobutirate ion (GABAate ion)	$H_2NCH_2CH_2CH_2COO^-$	−16.22	−32.19
Borate ion	$B(OH)_4^-$	−18.21	−33.63
Taurinate ion	$H_2NCH_2CH_2SO_3^-$	−17.71	−34.11
Dihydrogen phosphate ion	$H_2PO_4^-$	−17.46	−34.28
BICINEate ion	$(HOCH_2CH_2)_2NCH_2COO^-$	−19.70	−36.04
Propionate ion	$CH_3CH_2COO^-$	−18.21	−37.10
Glycinate ion	$H_2NCH_2COO^-$	−20.94	−38.45
Prolinate ion		−17.22	−39.89
Lactate ion	$CH_3CHOHCOO^-$	−18.95	−40.22
1/2 Sulfate ion	SO_4^{2-}	−21.25	−41.47
Acetate ion	CH_3COO^-	−22.18	−42.36
Hydrogen sulfate ion	HSO_4^-	−25.40	−52.00
Formate ion	$HCOO^-$	−30.88	−56.60
Chloride ion	Cl^-	−42.95	−79.09
Hydroxide ion	OH^-	−108.83	−204.92

It is known that the electric field acts on a moving ion with a force which is in equilibrium with the friction force defined by Stokes equation [20]. Transforming this equilibrium we obtain the equation

$$r = \frac{ze}{6\pi\eta\mu_\infty} \ [\mathrm{m}] \tag{1.4-33}$$

where z is the ionic electrovalency, e the electron charge ($1.6021892 \bullet 10^{-19}$ C), η the dynamic viscosity of the water, and μ_∞ the absolute ionic mobility, that is, the mobility in an extremely diluted solution. Hence, eq. (1.4-32) can be represented as

$$\left(\frac{z_x}{\mu_{\infty x}}\right)^3 = \left(\frac{z_a}{\mu_{\infty a}}\right)^3 + \left(\frac{z_b}{\mu_{\infty b}}\right)^3 \tag{1.4-34}$$

which gives

$$\mu_{\infty x} = z_x \left[\left(\frac{z_a}{\mu_{\infty a}} \right)^3 + \left(\frac{z_b}{\mu_{\infty b}} \right)^3 \right]^{-1/3} \quad [\mathrm{m^2/(sV)}] \tag{1.4-35}$$

If ion x is formed by two equal ions with absolute mobility $\mu_{\infty a}$ and if the composed ion and the forming ions have the same electrovalency, eq. (1.4-35) is transformed in

$$\mu_{\infty x} = 2^{-1/3} \mu_{\infty a} \, [\mathrm{m^2/(sV)}] \tag{1.4-36}$$

Equations (1.4-35) and (1.4-36) can be applied for extremely diluted solutions. However, every ion is surrounded by counterionic atmosphere whose radius

$$\kappa^{-1} = 1.9885 \bullet 10^{-12} \, (\varepsilon_r T/I)^{1/2} \, [\mathrm{m}] \tag{1.4-37}$$

where ε_r is the relative electric permittivity, T the thermodynamic temperature, and I the ionic strength of the solution [21]. This atmosphere diminishes the absolute mobility of an ion $(1 + \kappa r)$ times, that is,

$$\mu = \frac{\mu_\infty}{1 + \kappa r} \quad [\mathrm{m^2/(sV)}] \tag{1.4-38}$$

From eqs. (1.4-33) and (1.4-38) follows that

$$\mu_\infty = \frac{\mu}{2} \pm \left[\frac{\mu}{2} \left(\frac{\mu}{2} + \frac{ze\kappa}{3\pi\eta} \right) \right]^{1/2} \quad [\mathrm{m^2/(sV)}] \tag{1.4-39}$$

The last equation can be shortened, if the square root is expanded into a power series and if we take only the first correction term. Then eq. (1.4-39) is transformed into

$$\mu_\infty = \mu + \frac{ze\kappa}{6\pi\eta} \quad [\mathrm{m^2/(sV)}] \tag{1.4-40}$$

We gave evidence for the existence of a complex ion in TRIS-borate buffers which we called TRIS-borate ion [22]. TRIS-borate ion $[(HO)_2B^-(OCH_2)_2C(CH_2OH)NH_2, TB^-]$ can be considered as composed of a TRIS ion $[(HOCH_2)_3CN^+H_3, HT^+]$ and a borate ion $[B(OH)_4^-, B^-]$ when a proton and two water molecules are split off.

Let us calculate the mobility of TRIS-borate ion at different temperatures and same ionic strength – for example, at 0 and 25 °C, if I = 0.01 mol/l [23]. It is known that at 0 °C (273.15 K) and I = 0.01 mol/l $\mu_{HT^+} = 10.18 \bullet 10^{-9}$ and $\mu_{B^-} = -15.65 \bullet 10^{-9}$ m^2/(sV) [24]. Having in mind that $z_{HT^+(B^-)} = (\pm)1$ and that at the same temperature ε_r = 88.00 and $\eta = 1.787 \bullet 10^{-3}$ Pa s, we can calculate that zeκ/(6πη) = ± $1.54 \bullet 10^{-9}$ m^2/(sV). Hence, from eq. (1.4-40) follows that

$$\mu_{\infty HT^+} = 10.18 \bullet 10^{-9} + 1.54 \bullet 10^{-9} = 11.72 \bullet 10^{-9}\,\mathrm{m^2/(sV)}$$

and

$$\mu_{\infty B^-} = -15.65 \bullet 10^{-9} - 1.54 \bullet 10^{-9} = -17.19 \bullet 10^{-9}\,\mathrm{m^2/(sV)}$$

Then eq. (1.4-35) can be transformed to give

$$\mu_{\infty TB^-} = -\left[\left(\frac{1}{11.72 \bullet 10^{-9}}\right)^3 + \left(\frac{-1}{-17.19 \bullet 10^{-9}}\right)^3\right]^{-1/3} = -10.69 \bullet 10^{-9}\,\mathrm{m^2/(sV)}$$

When I = 0.01 mol/l, according also to eq. (1.4-35),

$$\mu_{TB^-} = -10.69 \bullet 10^{-9} + 1.54 \bullet 10^{-9} = -9.15 \bullet 10^{-9}\ \mathrm{m^2/(sV)}$$

At 25 °C (298.15 K) and I = 0.01 mol/l μ_{HT^+} = 24.06•10⁻⁹ and μ_{B^-} = −29.84•10⁻⁹ m²/(sV) [4]. Taking into account that at this temperature ε_r = 78.54 and η = 0.8904•10⁻³ Pa s, it can be calculated that $ze\kappa/(6\pi\eta)$ = (±)3.14•10⁻⁹ m²/(sV). Hence, it follows from eq. (1.4-40) and (1.4-35) that at 25 °C $\mu_{\infty HT^+}$ = 27.20•10⁻⁹ m²/(sV), $\mu_{\infty B^-}$ = −32.98•10⁻⁹ m²/(sV), and $\mu_{\infty HB^-}$ = −23.45•10⁻⁹ m²/(sV), and when I = 0.01 mol/l

$$\mu_{TB^-} = -23.45 \bullet 10^{-9} + 3.14 \bullet 10^{-9} = -20.31 \bullet 10^{-9}\ \mathrm{m^2/(sV)}$$

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1.5 Electrophoresis is carried out in different solid media

The electrophoresis is carried out in buffers or solid media containing buffers in their pores. Usually, the solid media act as sieves and separate the polyions according to their volumes.

Different solid media are used for electrophoresis. The paper, thin layers of aluminum oxide, cellulose powder, and so on on glass or plastic plates have historical significance. Nowadays, cellulose acetate, starch (quite rarely), and, most of all, polyacrylamide and agarose gels are used.

Agarose gels are used for proteins and DNA fragments of usually 50,000–200,000 bp in size; the resolution of over 6 Mb DNA is carried out by pulsed-field electrophoresis [1]. Polyacrylamide gels are usually used for proteins and for small fragments of DNA (5–500 bp). The agarose gels are run horizontally; the polyacrylamide gels are run in horizontal as well in vertical position.

1.5.1 Cellulose acetate

Cellulose **a**cetate (CA) is composed of glucose residues that are esterified by acetic acid. Each glucose residue is β-glycosidically connected with the hydroxyl group at the C-4 atom of the next glucose residue. In this way, long fibril molecules are formed, which are linked each with other by hydrogen bonds. Cellulose acetate possesses large pores which have no sieving effect on the migrating polyions; therefore, the electrophoretic separation is fulfilled only according to the electric charges of proteins.

The CA films used for electrophoresis do not counteract the diffusion, as a result of which the resolution of CA electrophoresis is of low grade. The films offered on the market (Sartorius, Göttingen) have dimensions of 140 × 57 mm, 145 × 57 mm, or 145 × 70 mm. They are widely used in the clinical routine.

A similar product is Cellogel. It represents cellulose acetate gel, fixed on a film (Chemetron, Milan). Cellogel is stored in methanol solution to prevent drying out. Prior to electrophoresis, the Cellogel membranes are cut into strips with a length of 145 mm, and a width of 25 or 75 mm, and are used for running distance of approximately 90 mm.

1.5.2 Starch gel

The starch gel – the first gel for electrophoresis – was introduced in 1955 by Smithies [2,3]. It was derived from hydrolyzed potato in concentrations of 5–10 g/dl [4,5]. The starch gel has sieving effect on proteins [6,7], since its pores are close to the volume of proteins. However, it is slightly opaque. The proteins can be visualized using Amido black staining.

The starch gel was supplanted by agarose and polyacrylamide gels because of its lack of reproducibility. Today it is performed only for separation of some enzymes in the genetics [8,9].

1.5.3 Agarose gel

Agarose is one of the two main components of agar, which is extracted from red seaweed [10]. It consists of linear polysaccharide chains, which are built of approximately 400 residues of agarobiose. Agarobiose contains an α-(1→3)-linked β-D-galactopyranose and a β-(1→4)-linked 3,6-anhydro-L-α-galactopyranose [11]. The 3,6-anhydro-L-α-galactopyranose represents L-galactose that possess an anhydro-bridge between the third and sixth positions. Some D- and L-galactose units can be methylated; ionizable groups are also found in small quantities [12] (Figure 1.5-1).

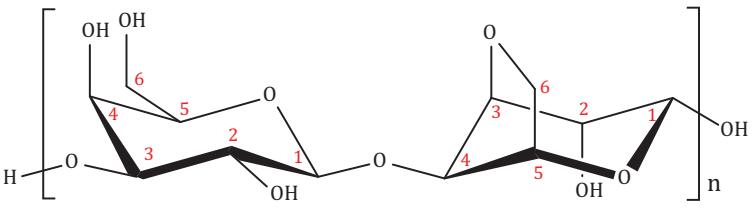


Figure 1.5-1: Structure of the residue of an agarobiose monomer.

Each polysaccharide chain contains about 800 galactose residues, and has M_r between 100,000 and 200,000 [13]. Many chains form helical fibers which build

supercoiled structures of a radius of 20–30 nm [14]. The fibers are quasi-rigid and have a different length that depends on the agarose concentration [15]. They form pores of diameter from 50 to 200 nm, whereas higher agarose concentrations form lower pore diameters. This structure is held together by hydrogen bonds and can be disrupted by heating that melts the gel to a liquid.

Agarose is available as a white powder which can be dissolved in boiling buffers, such as TAE or TBE [16]. Then the agarose solution is cooled in molds to produce slabs. A comb is often placed in the solution to create wells for applying the samples. The agarose gel represents a nontoxic and chemically inert medium, whose preparation does not require catalysts. Its structure is stable in the pH range of 4 to 9.

The agarose concentration in the gel is usually 0.7–2.0 g/dl. Low-concentration gels (0.1–0.2 g/dl) are fragile and are not easy to handle; high-concentration gels are brittle. In a 0.16 g/dl agarose gel, the pores have a diameter of about 500 nm; and in a 0.075 g/dl agarose gel, they have a diameter of about 800 nm [17]. For protein and DNA separations, 1 g/dl agarose gel is usually used, whose pores have a diameter of about 150 nm.

The agarose gels are optimal for electrophoresis of proteins with M_r larger than 200,000 [18]. Gels with a concentration of 0.8–1.0 g/dl are suitable for electrophoresis of 5–10 kb DNA fragments; gels with a concentration of 2 g/dl gel are suitable for electrophoresis of 0.2–1 kb DNA fragments.

The agarose gels are used for electrophoresis of proteins of different masses, as well of nucleates, especially DNA, chromosomes, and viruses. Agarose gels are also used for immunodiffusion and immunoelectrophoresis, because their fibers function as an anchor for immunocomplexes. Pulsed-field and field-inversion electrophoreses can be run on agarose gels, too.

The agarose gels are usually cast on support films. The support films are made of 0.18–0.20 mm polyester film that is covalently bound to a thin layer of agarose gel. The layer binds to the melt agarose when cast. The supported gels can be cut in different size, are stable at temperatures up to 110 °C, and are transparent to UV light above 310 nm.

The gelling and melting temperatures of agarose gels vary depending on the agarose type. Agaroses derived from *Gelidium* have a gelling temperature of 34–38 °C and a melting temperature of 90–95 °C, while agaroses derived from *Gracilaria* have a gelling temperature of 40–52 °C and a melting temperature of 85–90 °C. The gelling temperature is a function of the concentration of the methyl group in agarose: increasing methylation lowers the gelling temperature [19].

Low-melting agarose. The standard agarose melts at 80–90 °C, when DNA denatures. To hinder this, low-melting agarose was invented, which melts at 30–35–40 °C. Low-melting agarose resembles chemically modified agarose that possess hydroxyethyl groups in its polysaccharide chains and fewer sulfate groups [20]. However, it has a lower resolution and lower mechanical strength than the standard agarose [21]. Low-melting agarose allows gel slices with DNA to be melt after

electrophoresis and placed at disposal to polymerases, restriction endonucleases, and ligases.

High-strength agarose. If very large molecules have to be resolved, large pores in the agarose gel are needed, which are characteristic for low-concentrated gels. However, when the agarose concentration falls to 0.4 g/dl, gels degrade easily. The mechanical strength of an agarose gel is expressed in g/cm^2, that is, it is equal to the mass, which can be carried by 1 cm^2 agarose gel. The standard gels have mechanical strength of about 1,000 to 2,000 g/cm^2, the low-melting gels of about 200 g/cm^2. To overcome this disadvantage, high-strength agarose was discovered that has higher mechanical strength at low concentration – up to 6,000 g/cm^2. This agarose is used for pulsed-field electrophoresis of chromosomes.

Small-pore agarose. Compared to polyacrylamide gel, the agarose gel separates badly nucleates with length less than 1,000 bp, because of its relatively large pores. To overcome this, a small-pore agarose was produced.

Charge free agarose. As mentioned, the agarose gels contain charged groups, which cause electroosmosis. To prevent this, chemically modified agarose was invented that has no acidic groups [22,23].

Electroosmosis. Electroosmosis is referred to as movement of the solvent (water) when located in an electric field [24]. It is caused in the following way: Residual chemical groups of sulfuric, pyruvic, or carbonic acid on agarose molecule split hydrogen ions in neutral and alkaline buffers, and transform themselves into negatively charged sulphate, pyruvate, or carboxylate ions. The negatively charged agarose gel does not migrate to the anode (stationary phase); however, its positively charged counterions migrate, together with their own hydration envelopes, to the cathode (the mobile phase). As a result, the cathode water increases (hydrates the pole), while the anodic gel pole loses water (dehydrates).

The electroosmosis is a disturbing factor for electrophoresis. To avoid it, agarose types with less acidic groups were prepared – uncharged agaroses [25]. The reduction of electroosmosis results in a better resolution of proteins. Simultaneously, it is a useful phenomenon for the electrophoresis of nucleic acids, as it retards the DNA movement.

Agarose is used also in the chromatography, because it does not absorb biomolecules, has good flow properties, and tolerates extremes of pH and ionic strength as well as high concentration of denaturants [26]. Sepharose (cross-linked beaded agarose), Superose (highly cross-linked beaded agarose), Superdex (covalently linked dextran to agarose), and more are different agarose-based matrices for gel filtration chromatography.

1.5.4 Polyacrylamide gel

The **p**oly**a**cryl**a**mide (PAA, poly(2-propenamide)) gel, introduced in 1959 by Raymond and Weintraub [27], is a copolymer of the monomer acrylamide and the comonomer

BIS. It is the best medium for electrophoretic separations [28,29]. Polyacrylamide gel is relatively nontoxic, because it contains minute residual amounts of acrylamide after its production [30].

Contrary to other gels, the polyacrylamide gel has some important advantages:

1. It is hydrophilic and electrically neutral.
2. It does not show electroosmosis, since it has almost no electric charges.
3. It is transparent to light of wavelengths above 250 nm, chemically inert, and thermostable.
4. It can be prepared with desired pore size [31], changing its concentration. So, sieving effect can exist, which increases the resolution of the electrophoresis. By altering the concentrations of two monomers in the gel and the ratio between them, the pore size of a polyacrylamide gel can be altered in a reproducible manner.
5. It does not interact with polyions to be resolved or with dyes.

1.5.4.1 Acrylamide

Acrylamide (prop-2-enamide, M_r = 71.08) (Figure 1.5-6) crystallizes in white crystals, which are soluble in water, methanol, glycerol, acetone, trichloromethane, and other solvents, and has a boiling point at 84.5 ± 0.3 °C. It is highly neurotoxic and accumulates in the body. Acrylamide polymerizes spontaneously during prolonged storage.

Acrylamide purification. The purification of acrylamide is carried out by recrystallization in the following steps:

- 100 g of acrylamide are dissolved at 60 °C in 200 ml chloroform and filtered;
- The filtered solution is cooled to 22 °C, when acrylamide crystallizes;
- The solvent is removed through a Buchner funnel with the aid of a vacuum pump;
- The purified acrylamide is stored in a dark container.

1.5.4.2 Bisacrylamide

Bisacrylamide (BIS, N,N'-methylene-bis-acrylamide, N,N'-methylene-diacrylamide, M_r = 154.17) (Figure 1.5-6) is a white less toxic cross-linker, also soluble in water. It melts at 185 °C and polymerizes spontaneously during prolonged storage.

1.5.4.3 The magnitudes *T* and *C*

The acrylamide (the monomer) and BIS (the comonomer) are often referred to as *monomers* and their solution as *monomer solution*. Hjerten [32] improved formulas for the *total monomeric concentration T* and the *cross-linking degree C* of a gel, which we have transformed into:

$$T = a + b \quad (\mathrm{g/dl}) \tag{1.5-1}$$

and

$$C = \frac{b}{a+b} \quad \text{(dimensionless)} \tag{1.5-2}$$

where a and b are the concentrations of acrylamide and BIS, respectively, in g/dl.

The total monomer concentration T and the cross-linking C determine the pore size. Any increase or decrease away from $C = 0.05$ makes the pore size larger, as the pore size is a parabolic function with a vertex at $C = 0.05$.

1.5.4.4 Alternative cross-linkers

The BIS cross-linked PAA gels have high optical transparency and good mechanical properties at low concentrations, are electric neutral, and can have variable porosities. However, their relatively low stability in alkaline solutions and their reduced optical transparency at certain concentrations limit their application. Therefore, besides BIS, alternative cross-linkers: DATD [33], BAP (PDA) [34], AcrylAide (*FMC*, Rockland, Me.), and more are developed (Table 1.5-1).

Some of the cross-linkers are used for liquefaction of polyacrylamide gel to obtain the separated polyions after electrophoresis. If the gel contains DHEBA [35], the liquefaction is carried out by oxidation with periodic acid; if the gel contains BAC [36], the BAC disulfide bonds are split with thiols. However, the gel liquefaction denatures the polyions. PDA (BAP) is used to reduce the silver stain background in SDS-PAGE gels.

Irrespective of all that, the alternative cross-linkers have many disadvantages; therefore, BIS is still the most used comonomer.

1.5.4.5 Initiator-catalyst systems

The copolymerization of acrylamide and BIS requires free radicals, which are generated by initiator-catalyst systems [37]. Among them, the APS-TMEDA system is optimal (Figure 1.5-2).

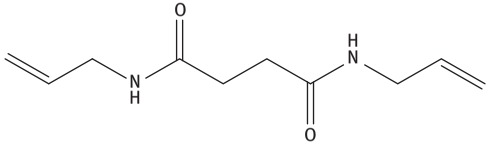
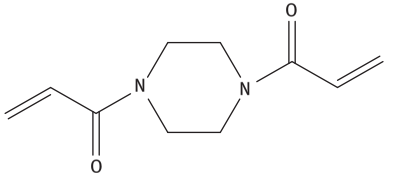
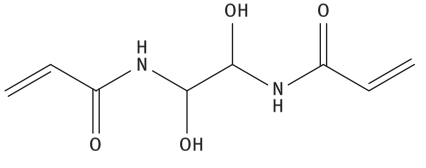
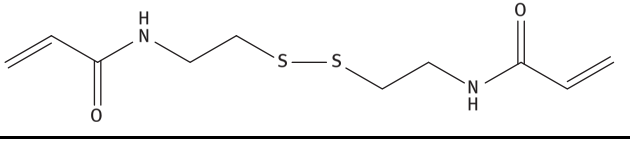
The *initiator* APS (**a**mmonium **p**eroxydi**s**ulfate, ammonium persulfate, $M_r = 228.18$) dissociates in solution giving two ammonium cations and a peroxydisulfate anion. Then peroxydisulfate anion forms two sulfate free radicals [38] (Figure 1.5-3):

APS is used in concentrations of 1–4 mmol/l (0.02–0.10 g/dl). Compared to other oxidizing agents, it is preferred because it releases no molecular oxygen, which inhibits the copolymerization.

The velocity of copolymerization process increases with the increase of concentrations of the monomers and APS. Therefore, we propose that TMEDA concentration should remain constant, while the APS concentration should be changed, according to the formula:

$$c_{APS} = 0.32(\text{g/dl})^2/T \ \ [\text{g/dl}] \tag{1.5-3}$$

Table 1.5-1: Some alternative cross-linkers.

Cross-linkers	Chemical formulas	M_r
DATD (N,N'-**Dia**llyl**t**artar**d**iamide)		228.25
BAP (1,4-**b**is-**a**cryloyl-**p**iperazine), or PDA (**P**iperazine-**d**iacryl**a**mide)		194.23
DHEBA (N,N'-(1,2-**Dih**ydroxy**e**thylen)-**b**is-**a**crylamide)		200.20
BAC (N,N'-**b**is-**a**cryloyl**c**ystamine)		260.38

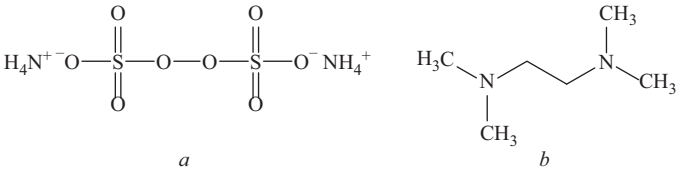


Figure 1.5-2: Chemical formulas of APS (*a*) and TMEDA (*b*).

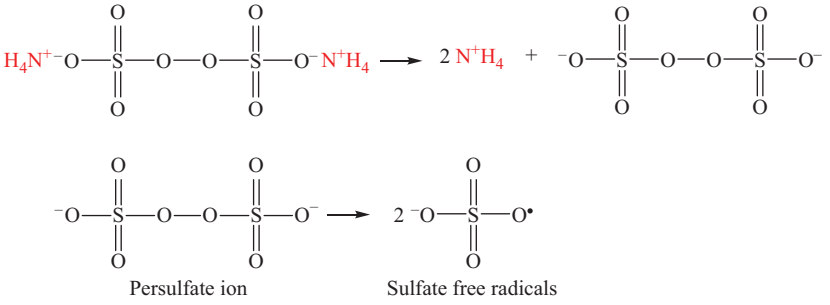


Figure 1.5-3: Building of sulfate free radicals from APS.

This means that at T = 5 g/dl c_{APS} should be 0.06 g/dl, at T = 11 g/dl it should be 0.03 g/dl, and at T = 16 g/dl it should be 0.02 g/dl.

The tertiary amine TMEDA [N,N,N',N'-**t**etra**m**ethyl**e**thylene**dia**mine, TEMED, 1,2-bis(dimethylamino)-ethane, M_r = 116.21] is the most commonly used *catalyst*, in concentrations of 2–4 mmol/l (0.02–0.06 ml/dl). It accelerates the decomposition of peroxydisulfate ions into sulfate free radicals.

TMEDA is a hygroscopic compound, which accumulates water. The air oxygen in water oxidizes it in yellow-colored products. They do not have the TMEDA activity; therefore, the copolymerization process needs higher TMEDA concentration after a while.

The increase of the concentration of free radicals (the concentrations of ammonium peroxydisulfate and TMEDA) results in a decrease of the average polymeric chain length, an increase in the gel turbidity, and a decrease in the gel elasticity. The decrease of the concentration of free radicals leads to reverse effects.

APS and TMEDA are used at approximately equimolar concentrations in the range of 1 to 10 mmol/l.

Riboflavin (riboflavin-5′-phosphate) generates also free radicals in a photochemical reaction, often in combination with TMEDA. When irradiated with blue to ultraviolet light (UV) in the presence of oxygen, it converts into a leuco form, which initiates the copolymerization (Figure 1.5-4). This is referred to as a *photochemical copolymerization*.

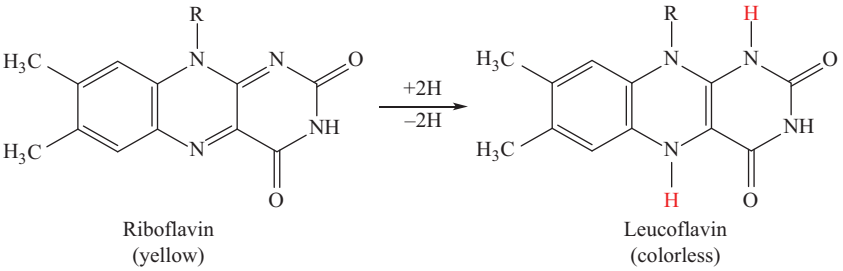


Figure 1.5-4: Riboflavin and its leuco form.

1.5.4.6 Copolymerization of acrylamide and BIS

The copolymerization of acrylamide and BIS proceeds in two steps. At first, the sulfate radicals add to alkene (the vinyl groups of acrylamide) molecules forming sulfate ester radicals, which take part in the polymerization producing long polymeric chains [39] (Figure 1.5-5).

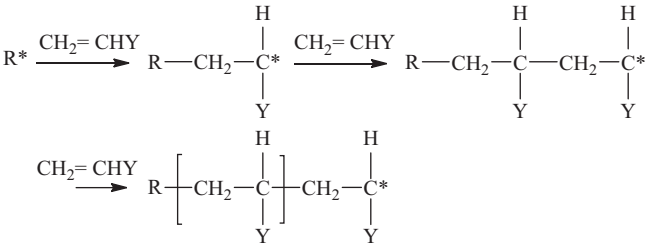


Figure 1.5-5: Polymerization of an alkene (CH_2 = CHY) with the help of a sulfate radical (R*).

Then the polymeric chains are cross-linked by BIS molecules. So, the spatial structure of the polyacrylamide gel is formed, which can be compared to a sponge [40,41] (Figure 1.5-6).

The copolymerization is most efficient at alkaline pH, but diminishes rapidly at pH values below pH = 6. Therefore, for low-pH gels, riboflavin and TMEDA are used.

The viscosity, elasticity, and strength of a polyacrylamide gel depend on the ratio between the length of polyacrylamide chains and the frequency of the cross-linkages, that is, on the ratio between the molar concentrations of acrylamide and BIS. If the ratio between the molar concentrations of acrylamide and BIS is 200:1 (between the mass concentrations 100:1), the polyacrylamide gel is elastic, soft, and transparent, because it has long chains. If the ratio between the molar concentrations of acrylamide and BIS is less than 20:1 (between the mass concentrations 10:1), the gel is fragile, brittle, and dull, since it is made of short polyacrylamide chains.

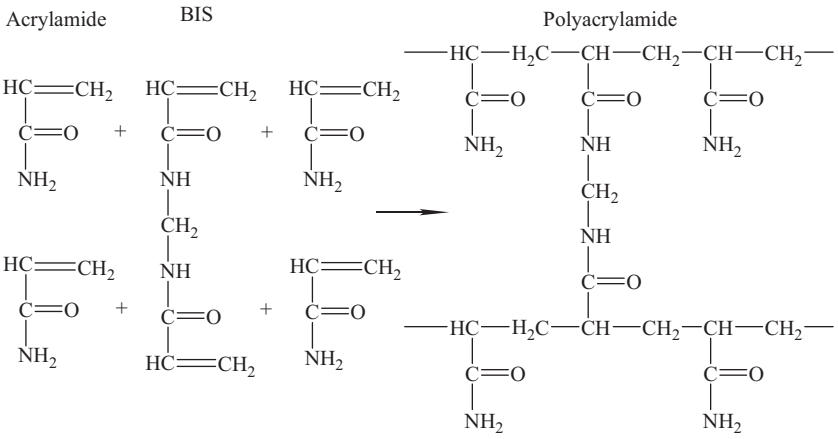


Figure 1.5-6: The copolymerization of acrylamide and BIS gives polyacrylamide gel. A little part of the neutral carboxamide groups in the polyacrylamide gel can hydrolyze receiving negatively charged carboxylic groups.

When T is increased at fixed C, the number of polyacrylamide chains increases and the pore size decreases in a nearly linear relationship. Higher-concentration gels (with higher T) have smaller pores. The relationship of C to the pore size is more complex. When T is held constant and C is increased, the pore size decreases to a minimum at about $C = 0.05$. When C is increased above 0.05, the pore size is increased, presumably because of formation of shorter polymer chains. Gels with low T (e.g., 7.5 g/dl) are used for separation of large proteins, while gels with high T (e.g., 15 g/dl) are used for small proteins (Table 1.5-2).

Table 1.5-2: Acrylamide concentration and pore size of polyacrylamide gels suitable for separation of different proteins. $C = 0.05$.

Polyacrylamide, g/dl	Pore diameter, nm	Protein mass range, $M_r \cdot 10^{-3}$
5.0	3.6	35–300
7.5	3.0	25–250
10.0	2.6	20–200
12.5	2.3	15–150
15.0	2.0	10–100
20.0	1.8	7–70

It is recommended that for the most denaturing DNA and RNA gels the value of C is 0.05 (19:1 acrylamide/BIS) and for the most native DNA and RNA gels the value of C is 0.033 (29:1). For most protein and SDS-protein gels, the standard C value is 0.0026 (37.5:1) (Table 1.5-3).

Table 1.5-3: Composition of polyacrylamide gels for various applications.

T, g/dl	C	Proteins, $M_r \bullet 10^{-3}$	Native DNA and RNA, bp	Denatured DNA and RNA, bp
4	19:1	100–200	100–1,500	70–500
6	19:1	40–150	60–600	40–400
8	19:1	20–100	40–500	20–100
10	19:1	15–70	30–300	15–70
12	19:1	8–60	20–150	8–60
5	29:1	>150	200–2,000	>150
6	29:1	50–200	80–800	50–200
8	29:1	30–125	60–400	30–125
10	29:1	20–100	50–300	20–100
12	29:1	10–70	40–200	10–70
20	29:1	>30	<40	<30
6	37.5:1	60–200		60–200
8	37.5:1	50–150		50–150
10	37.5:1	25–100		25–100
12	37.5:1	15–80		15–80

The gel of T = 4 g/dl and C = 0.03 has no sieving properties; therefore, native proteins migrate through it according to their free mobility.

Usually the monomers are dissolved in concentrated stock solutions with T = 30–40 g/dl. Before usage, the monomer stock solutions should be deaerated under moderate vacuum for about 15 min. The initiator-catalyst system is added finally, before the solution is cast.

The APS and TMEDA concentrations of 0.05 g/dl each are sufficient for the copolymerization of resolving gels. Under these conditions, the visible copolymerization process happens in 15–20 min, but the gel should be used in 90 min [42]. If stacking gels are needed, they polymerize more rapidly, in 8–10 min, with 0.05 g/dl APS and 0.1 g/dl TMEDA. It is important that total acrylamide is polymerized. If this is not the case, the nonpolymerized acrylamide binds covalently to the polyions during electrophoresis [43].

Besides the initiator-catalyst system, the copolymerization between acrylamide and BIS depends on the pH value, temperature, purity of the chemicals, and inhibitors.

The hydroxide ions accelerate the copolymerization process. Therefore, it should take place at basic pH values. On the contrary, the rate of the copolymerization process decreases at low pH values.

The temperature accelerates the copolymerization process; at temperatures below 20 °C, it slows down.

The chemicals involved in the monomer copolymerization (acrylamide, BIS, and catalysts) must be extremely pure. Acrylamide and BIS should be stored in dark containers in the refrigerator and recrystallized, if necessary. The most common impurity is the acrylic acid, which is formed during the spontaneous hydrolysis of acrylamide. It binds to the gel during the copolymerization, thus giving the gel negative electric charges. Such gels show electroosmosis during electrophoresis.

Inhibitors of the copolymerization are the oxygen and peroxides, the latter building oxygen at their cleavage. The oxygen slows down the process, because it catches free radicals. Therefore, the copolymerization should be carried out with exclusion of air.

Except in water, the monomers may copolymerize in glycerol, ethylene glycol, sucrose solution, urea solution, or other liquids. As these chemicals have higher viscosity, they accelerate the copolymerization process without changing the gel properties. If necessary, nonionic detergents (Triton X-100, Nonidet P 40) in concentration of 0.5 to 2 ml/dl, or ionic detergents (SDS) in concentration of 0.1 to 0.5 g/dl can be added to the monomer solution.

Similar to the polyacrylamide gel is the gel, which is formed by the monomer **N**-**a**cryloyl-**t**ris(hydroxymethyl)aminomethane (NAT) [44,45].

Two types of polyacrylamide gels are known: homogeneous and gradient gels.

1.5.4.7 Homogeneous polyacrylamide gels

Homogeneous polyacrylamide gels have different thickness. Thin and ultrathin polyacrylamide gels are preferable. They are cast on a support film or support fabric and are used for horizontal electrophoresis. The support film and fabric for polyacrylamide gel are usually prepared from pretreated polyester, which binds chemically to the polyacrylamide gel.

In the following table (Table 1.5-4), recipes for casting homogeneous polyacrylamide gels of different concentrations are given.

Table 1.5-4: Recipes for casting homogeneous polyacrylamide gels of different concentrations.

T, g/dl	4	5	6	7	8	9	10	11	12
Buffer, 4x, ml	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00
Monomer solution $T = 50$ g/dl, $C = 0.03$, ml	8.00	10.00	12.00	14.00	16.00	18.00	20.00	22.00	24.00
10 g/dl TMEDA, ml	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46
10 g/dl APS, ml	1.00	0.80	0.67	0.57	0.5	0.44	0.40	0.36	0.33
Deionized water to, ml	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

T, g/dl	13	14	15	16	17	18	19	20	21
Buffer, 4x, ml	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00
Monomer solution $T = 50$ g/dl, $C = 0.03$, ml	26.00	28.00	30.00	32.00	34.00	36.00	38.00	40.00	42.00
10 g/dl TMEDA, ml	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46
10 g/dl APS, ml	0.31	0.29	0.27	0.25	0.24	0.22	0.21	0.20	0.19
Deionized water to, ml	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Thin and ultrathin polyacrylamide gels

The thin gels are 0.5–1.0 mm thick; the ultrathin gels are thinner than 0.5 mm. Both gels have the following advantages against the vertical gels [46]: the temperature gradient between the gel surfaces is smaller since the heat dissipates easier; the samples applied on the gel surface have smaller volumes; electrode strips, soaked with electrode solutions, can be placed on the gel; the staining and destaining procedures are quickly performed.

The thin and ultrathin horizontal gels on support films [47] can be cut into strips and stored dry. However, they are not suitable for electroblotting. Alternatives for this purpose are the fabric-supported gels [48]. The fabric is commonly made from polyester fibers, which form pores with a diameter of 10–60 μm [49].

1.5.4.8 Gradient polyacrylamide gels

The gradient polyacrylamide gels (pore gradient polyacrylamide gels) were introduced by Margolis and Kenrick [50]. Their concentration varies continuously from one gel end to the other gel end, resulting in a continuous changing of the pore diameters [51,52]. In the linear gradient gels the total concentration T increases linearly at a constant cross-linking degree C; however, the average pore radius increases

exponentially. The maximum pore radius $r_{max} = a - T^{-b}$, where a and b are empirical constants [53]. The T-value of the gradient gels is usually 4–28 g/dl.

During electrophoresis in a gradient polyacrylamide gel, polyions stop where they cannot move away through the located pores. Their molecular masses can be determined, comparing the bands with the bands of polyions with known molecular masses.

There are two types of gradient gels: linear and exponential.

1.5.4.9 Rehydratable polyacrylamide gels

After the polymerization, in the polyacrylamide gels remain monomer rests, APS, and TMEDA. They can disturb the polyions and inactivate the enzymes to be separated. In addition, they, especially the carrier ampholytes, diminish the adhesion of the support film or fabric. All these problems were overcome after rehydratable (clean) gels were introduced [54,55]. They are produced after the cast gels were washed three times for 20 min each in deionized water and rinsed afterward in 2 ml/dl glycerol. Finally, they are dried overnight in the air and stored in the refrigerator.

The rehydratable gels have many advantages over the wet gels: They contain no residual monomers (acrylamide and BIS) and no residual catalysts (APS and TMEDA); can be stored at −20 °C in a dried form for a very long time (in contrast to the wet gels that hydrolyze after a certain time); can be cut in desired dimensions when necessary; and can be rehydrated using solutions containing carrier ampholytes, detergents (Triton X-100, *etc*.), or urea.

However, the rehydratable gels have also some disadvantages: the rehydration requires appropriate solutions; the washing, drying, and rehydration take time; and the rehydrated gels deposit water on their surface.

1.5.4.10 Polyacrylamide gels with high polyol concentration

The electrophoresis, particularly the isoelectric focusing, can be carried out on polyacrylamide gels with high concentrations of polyols, for example 30–60 g/dl glycerol [56]. The polyacrylamide gels with higher polyol concentration offer some advantages: they tolerate increased salt concentrations in the samples, for example 0.3–0.6 mol/l NaCl, which causes less band deformations; eliminate partially the electroosmosis; bear higher field strengths; and the resolution of the electrophoresis and isoelectric focusing increases due to the slower diffusion.

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1.6 General theory of electrophoresis

According to the double-layer theory, a charged particle in a solution is screened by diffuse atmosphere ions, which have the same charge but opposite sign. The electric field exerts on the charged particle moving electrostatic force (F_{el}), which equilibrates the friction force (F_f), appearing while the charged particle is moving through the medium, and the retardation force (F_{ret}), appearing while the diffuse atmosphere ions are migrating opposite to the moving charged particle (Figure 1.6-1). Hence,

$$F_{el} + F_f + F_{ret} = 0 \tag{1.6-1}$$

The most well-known and widely used theory of electrophoresis was developed in 1903 by Smoluchowski [1,2]. According to it,

$$\mu = \frac{\zeta\varepsilon}{\eta} = \frac{\zeta\varepsilon_r\varepsilon_0}{\eta} \tag{1.6-2}$$

where μ (in $m^2/(s\ V)$) is the mobility of the polyion *pi*, ζ (in V) is its zeta potential (i.e., the electrokinetic potential at the slipping plane of the polyionic double electric layer), ε (in F/m) is (di)electric permittivity of the medium, ε_r (dimensionless) is the relative (di)electric permittivity of the medium, ε_0 is the (di)electric constant ($8.854\ 187\ 818 \bullet 10^{-12}$ F/m), and η (in Pa s) is the dynamic viscosity of the medium.

The electrophoretic velocity v (in m/s) of a polyion can be calculated from the equation

$$v = \mu E \tag{1.6-3}$$

Hence, it is a product of the polyionic mobility and the strength (intensity) of the electric field E (in V/m). The last equation shows that the electrophoretic velocity depends on factors, which affect the polyionic mobility and field strength [3].

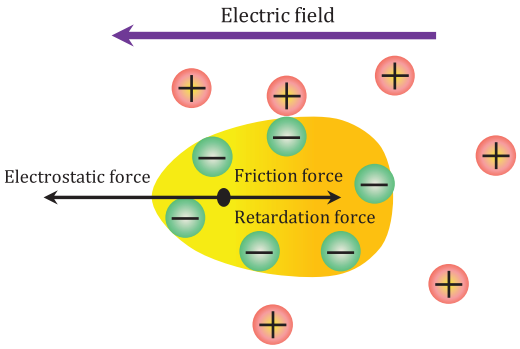

Figure 1.6-1: Scheme of electrophoresis.

1.6.1 What is the polyionic mobility depending on?

According to our ideas [4,5], the relation

$$A = R + \kappa r^2 \tag{1.6-4}$$

exists, where A (in m) is the electrokinetic radius, R (in m) is the geometric radius of a polyion, κ (in m^{-1}) is the parameter of Debye–Hückel [6], and $r \leq \kappa^{-1}$ (in m) is the geometric radius of the counterion.

Besides, we have proved that the polyionic mobility

$$\mu = \frac{\zeta\varepsilon}{1.5\eta} f(\kappa r) \tag{1.6-5}$$

The function $f(\kappa r)$ [7] is described by the equation

$$f(\kappa r) = \frac{1+\kappa a}{1+\kappa r} = \frac{1+\kappa r+(\kappa r)^2}{1+\kappa r} \tag{1.6-6}$$

where a and r are the electrokinetic and the geometric radii of the counterion, respectively. The function $f(\kappa r)$ is similar to the Henry function $f(\kappa a)$ [8,9]; however, in contrast to the Henry function, it has a real and definitive value, unlike the function $f(\kappa a)$.

If we introduce eq. (1.6-6) in eq. (1.6-5), the following expression is obtained:

$$\mu = \frac{\zeta\varepsilon}{1.5\eta}\frac{1+\kappa a}{1+\kappa r} \tag{1.6-7}$$

According to it, when $\kappa r \to 0$, then

$$\mu = \frac{\zeta\varepsilon}{1.5\eta} \tag{1.6-8}$$

that is, the equation of Hückel [10] occurs, and when $\kappa r \rightarrow 1$, then

$$\mu = \frac{\zeta \varepsilon}{\eta} \tag{1.6-9}$$

that is, the equation of Smoluchowski occurs.

The polyionic mobility depends on the polyionic nature, buffer, and medium.

1.6.1.1 Influence of polyionic nature

The polyionic nature determines the geometric potential φ of the polyion. The φ-potential depends on both the electric charge of the polyion and its geometric radius.

1.6.1.2 Influence of buffer

The buffer influences the polyionic mobility mainly by its pH value and its ionic strength, as well as by its (di)electric permittivity and its temperature. These factors influence the polyionic ζ-potential.

Buffer pH. A buffer keeps the pH value of a solution unchanged. This is very important for electrophoresis. For strong electrolytes, which are completely ionized, the pH value, that is, the proton concentration, does not influence the ionic mobility. However, in weak-acid and weak-base buffers, as well as in ampholyte buffers, the ionic mobility is a function of the dissociation degree of the buffer electrolyte, according to the equation

$$\mu_{i(pi)}{}' = \alpha \mu_{i(pi)} \tag{1.6-10}$$

In this equation, $\mu_{i(pi)}{}'$ is the effective mobility of the ion i (polyion pi), in $m^2/(s\ V)$; α is the dissociation degree, and $\mu_{i(pi)}$ is the mobility of the ion (polyion) of the weak protolyte.

Let us consider a macromolecule, which has to be separated, as a weak monobasic acid; let us also ignore its numerous ionizable side groups. If we represent the macromolecule as HPi, from the definition of the dissociation degree and eq. (1.6-10) follows that

$$\mu_{pi^-}{}' = \mu_{pi^-} \frac{[\mathrm{pi}^-]}{[\mathrm{Hpi}] + [\mathrm{pi}^-]} \tag{1.6-11}$$

where $[\mathrm{pi}^-]$ is the equilibrium concentration of the polyanion pi^-, in mol/l, and [Hpi] is the equilibrium concentration of the nondissociated macromolecules, also in mol/l.

The last equation can be transformed according to the mass action law in the expression

$$\mu_{pi^-}{}' = \mu_{pi^-} \frac{K_c}{[H^+] + K_c} \tag{1.6-12}$$

Equation (1.6-12) shows that the effective mobility of a polyion depends on the pH value. If the H^+ concentration is much smaller than the concentration dissociation constant, the ratio in the equation grows to 1, and $\mu_{pi^-}{}' \rightarrow \mu_{pi^-}$. If the H^+ concentration is much greater than the concentration dissociation constant, the ratio in the same equation declines to 0, and $\mu_{pi^-}{}' \rightarrow 0$.

In reality, the above relationships are more complicated, especially for ampholytes, which have many ionizable groups with different pK_c values. To understand the behavior of an ampholyte, for example a protein, we have to know that the mobility is a scalar magnitude as it can have positive or negative value. The mobilities sum of a zwitterion at its pI value is equal to 0; therefore, it cannot move.

The pH value influences the polyionic effective mobility, whereby the dependence looks like the sigmoidal curve of the titration of a weak acid or a weak base (Figure 1.6-2).

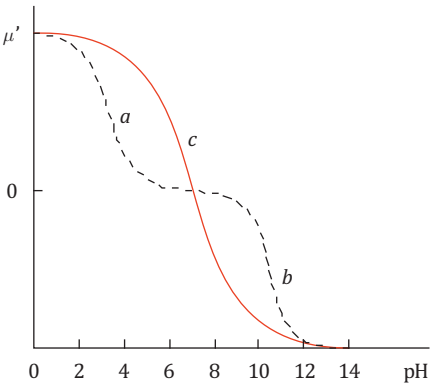

Figure 1.6-2: The effective mobility of the ion of a weak acid (*a*), of the ion of a weak base (*b*), and of an ampholyte (*c*) as a function of the pH value.

Ionic strength of the buffer. The polyionic mobility decreases when the ionic strength of a buffer increases [11]. This can be explained with the concept of the geometric and electrokinetic radii (s. above). When the ionic strength increases, the product κr_{pi} grows, the electrokinetic radius of the polyion, a_{pi}, is enlarged, and the ζ-potential is decreased. The reverse takes place when the ionic strength decreases; then the electrokinetic radius of the polyion decreases and its ζ-potential increases (Figure 1.6-3).

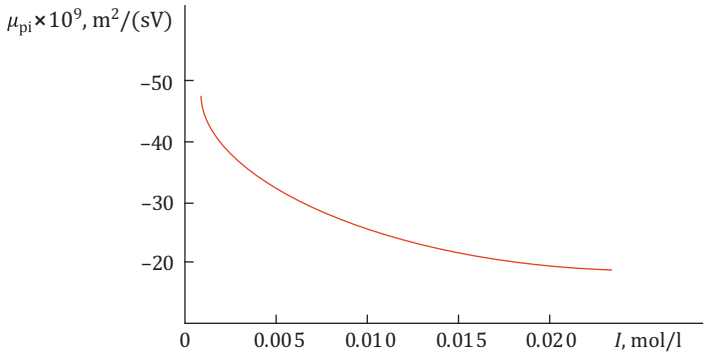

Figure 1.6-3: Dependence of the polyionic mobility on the ionic strength of the buffer.

(Di)electric permittivity of the buffer. The relative (di)electric permittivity ε_r of the buffer solvent (water) remains constant during the electrophoresis. It depends on the temperature: at 0 °C, ε_r is equal to 88.00; and at 25 °C, it is equal to 78.64. Additives, such as detergents, change the relative (di)electric permittivity and, as a result, change the polyionic mobility.

Temperature of the buffer. The temperature decreases the (di)electric permittivity ε of the solvent and its dynamic viscosity η. It has been found that the temperature decreases the dynamic viscosity according to the equation

$$\eta = Ae^{b/T} \tag{1.6-13}$$

where A and b are empirical constants. Studies indicate that, if the temperature increases with 1K, the mobility increases with about 3%.

1.6.1.3 Influence of medium

The medium influences the electrophoresis because of its dynamic viscosity and electroosmosis.

Dynamic viscosity of the medium

In contrast to electrophoresis in a buffer (free electrophoresis), the polyionic mobility in a solid medium is reduced because of an additional dynamic viscosity. The additional dynamic viscosity can be explained by the porous structure of the medium, which causes the charged particles to zigzag through the pores the longer distance s', instead of the shorter distance s. This can be expressed by the equation

$$\mu_t = \mu \left(\frac{s}{s'} \right)^2 \tag{1.6-14}$$

where μ_t is the polyionic mobility in the buffer containing medium and μ is the polyionic mobility only in the buffer.

Electroosmosis

Electroosmosis (electroendosmosis, **e**lectro**o**smotic **f**low, EOF) was first described in 1809 by the Russian scientist Reuss [12]. He showed that water is flowing between particles of silica and other minerals when applying an electric field.

Electroosmosis is referred to as moving of the solvent (water) across a porous material, a capillary tube, a microchannel, or any conduit filled with electrolyte solution, when electric field is applied. It is an essential component of the electrophoresis techniques.

Since the surface charge on a capillary wall is pH-dependent, the electroosmotic flow varies with the changing of the pH value of electrolyte: at a low pH value, it becomes lower; at a high pH value, it becomes higher. The electroosmosis depends also on the temperature and the electrolyte concentration. When the electrolyte concentration is increased, the electroosmosis decreases, and vice versa. It is also decreased also when organic solvents such as methanol are added.

Electroosmosis is an important characteristic of every agarose gel. The agarose gel has acidic groups which carry negative charges in neutral and alkaline buffers. It is the immobile medium (stationary phase); however, its counterions, which are positively charged, move together with their hydration envelopes to the cathode (mobile phase). As a result, the gel cathode binds water (hydrates), whereas the gel anode loses water (dehydrates).

The electroosmosis is a disturbing factor for electrophoresis and isoelectric focusing, as the water flows against the migrating polyions. To avoid this, chemically purified agarose, which contains less acidic groups, is offered on the market. The polyacrylamide gel shows no electroosmosis, as it possesses no dissociating groups.

The electroosmotic flow is an important part of the capillary electrophoresis [13], during which substances are separated according to their electrophoretic mobilities in narrow capillaries, made usually of silica.

1.6.2 What is the electric field strength depending on?

If direct voltage is applied between two electrodes, electric field strength arises:

$$E = \frac{U}{l} \tag{1.6-15}$$

where U is the voltage, in V, and l is the distance between the electrodes, in m. The distance remains unchanged during electrophoresis; therefore, the strength of the electric field depends only on the voltage.

The field strength can also be expressed by the equation

$$E = \frac{J}{\gamma} \tag{1.6-16}$$

where J is the electric current density, in A/m^2, and γ is the specific conductivity of the buffer, in S/m.

1.6.3 Ionic boundaries

Two ionic boundaries in electrophoresis are known: moving and stationary boundary.

1.6.3.1 Moving boundary

The moving boundary [14,15,16] is characterized by the magnitude W, which expresses its volume V swept by the passed electric charge Q [17]:

$$W = \frac{V}{Q} \quad (\mathrm{m}^3/\mathrm{C}) \tag{1.6-17}$$

The relationship between the magnitude W and the effective velocity v' is described by the equation

$$v' = W\frac{I}{S} \tag{1.6-18}$$

where I (in A) is the total electric current and S (in m^2) is the cross section.

During the passage of electric current, the movement of the substance X is indicated by the movement of its boundary. After the passage of the electric charge Q, the boundary creates a new zone 2, which has the volume V_2. The mass balance for the 2→1 boundary may be expressed by the moving boundary equation. It obtains, for the substance L, the following form [18]:

$$\frac{\mu_{L,1}{}'c_{L,1}{}'}{\gamma_1} - \frac{\mu_{L,2}{}'c_{L,2}{}'}{\gamma_2} = W_{2,1}\left(c_{L,1}{}' - c_{L,2}{}'\right) \tag{1.6-19}$$

where 1 and 2 are the subscripts of the effective mobilities and the concentrations of substance L in zones 1 and 2, respectively, and γ (in S/m) is the specific electric conductivity.

During isotachophoresis individual substances form individual zones. To illustrate this, let us consider a simple case of two solutions in a separation channel: one containing the substance XR, and the other containing the substance LR.

During the passage of electric current, the moving boundary 2→1 separates zone 1 of substance L from zone 2 of substance X. When the moving boundary is formed $\left(c_{L,2}{}' = 0, c_{X,1}{}' = 0\right)$, then

$$W_{2,1} = \frac{\mu_{X,2}{}'}{\gamma_2} = \frac{\mu_{L,1}{}'}{\gamma_1} \tag{1.6-20}$$

If X and L are cations and R is an anion, it follows from the above relationships and the electroneutrality law that

$$\frac{c_{X,2}}{c_{L,1}} = \frac{\mu_X}{\mu_X + |\mu_R|} \frac{\mu_L + |\mu_R|}{\mu_L} \frac{z_L}{z_X} \tag{1.6-21}$$

1.6.3.2 Stationary boundary

The stationary ionic boundary [19] does not move in a buffer system. It can be obtained also in a same buffer, if the buffer has different pH values [20].

1.6.4 Regulating function

The regulating function ω was formulated by Kohlrausch [21]. It describes the migration of monovalent ions of strong electrolytes. Later, the Kohlrausch regulating function was adapted also for weak electrolytes.

The Kohlrausch regulating function, valid for strong and weak mono- and multivalent electrolytes, can be expressed by the relationship [22]

$$\omega(x) = \sum_i \frac{c_i{}'(x) z_i}{\mu_i} = const(x) \tag{1.6-22}$$

where $c_i{}'$ is the effective ionic concentration of electrolyte i, present at a given point x along the migration path. The function $\omega(x)$ has a constant value independent on the time of the electric current passage. Hence, the composition of the moving boundary is regulated automatically.

1.6.5 Diffusion

With the time, the resolved band is broadened by diffusion. Assuming that the applied sample forms a thin rectangular concentration body at $t = 0$, then, after a certain time t, Gaussian concentration profile is observed as the result of diffusion. It may be characterized [23] by the standard deviation σ given by the relationship

$$\sigma = \sqrt{2Dt} \ \ [\mathrm{m}] \tag{1.6-23}$$

where D is the *diffusivity* or diffusion coefficient (m^2/s). The diffusivity is proportionality constant between the molar flux and the gradient in the concentration of the species (the driving force for diffusion). It is predicted by the Arrhenius equation:

$$D = D_0 e^{-E_A/(kT)} \ [\mathrm{m}^2/\mathrm{s}] \tag{1.6-24}$$

where D_0 is the maximum diffusivity (at infinite temperature) (m^2/s), E_A is the activation energy for diffusion (J), T is the absolute temperature (K), and k is the Boltzmann constant (1.380 662•10^{-23} J/K).

1.6.6 Joule heating

Another magnitude that is important for the electrophoresis is the Joule heating (the electric power) that is contained in the equation

$$P = IU = I^2R \ \ (\mathrm{W}, \mathrm{watts}) \tag{1.6-25}$$

The heating causes convection currents, diffusional broadening, evaporation, viscosity and pH changes, thermal denaturing of polyions, especially proteins, gel drying, buffer boiling, and even fire. The center of the resolving medium becomes warmer than its outer parts and the temperature difference can reach more than 10 °C, which increases the polyionic mobilites with 2% per 1 °C. These effects can be diminished by external cooling systems, such as circulating cold water around the electrophoresis unit, or using dilute or low conductivity buffers and low voltages (100–500 V).

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1.7 Electrophoresis instrumentation

A modern electrophoresis labor has an electrophoretic cell, a DC power supply, a thermostat, a densitometer with a computer and a printer, casting cassettes, a gradient mixer, a blotter, and other devices (Figure 1.7-1).

The electrophoresis cell should be leveled in order to prevent the buffer to siphon out. The power supply provides a direct electric current and a different voltage. The required voltage and the run time can be set digitally.

Other laboratory devices and equipment that are used for electrophoresis research are: Becher glasses, Erlenmeyer flasks, graduated cylinders, test tubes, glass rods, pipettes, automatic pipettes, a water-jet pump, a heating plate, a magnetic stirrer, magnetic rods, a height-adjustable platforms (laboratory boy), a shaker, a centrifuge, scissors, a spatula, a microwave oven, a dryer or incubator, a UV lamp, a fan, a water pump, a paper-cutting machine, and more.

1.7.1 Electrophoresis cells

The electrophoretic separation is carried out in electrophoretic cells. The electrophoretic cells are prepared by isolate materials such as ceramic, polycarbonate, Plexiglas, PVC, Piacryl, glass, and more. Platinum wires or graphite rods are used as electrodes. For protection, in most cases the electric current is automatically stopped when opening the cell.

The gels have a slab or cylindrical form. The slab gels, either vertical or horizontal, are much more common. They are cast between a pair of glass plates separated by spacer strips. The electrophoretic cells for slab gels are preferable when compared with the electrophoretic cells for cylindrical gels, because the slab gels

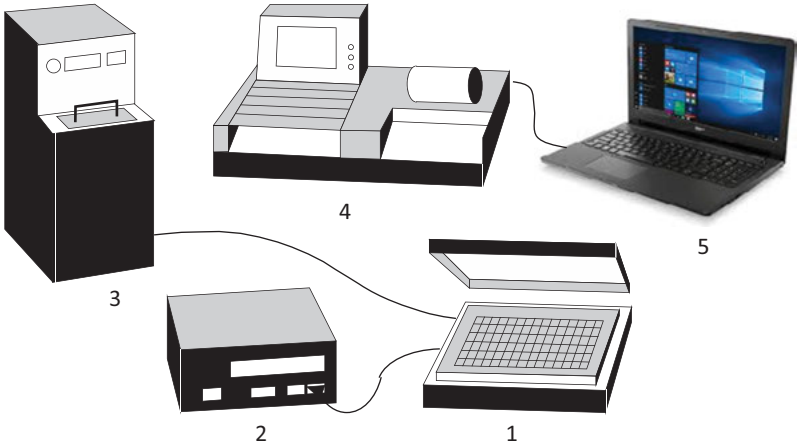

Figure 1.7-1: Apparatuses used for electrophoresis.
1. Electrophoresis cell; 2. Power supply; 3. Thermostat; 4. Densitometer; 5. Computer.

are easier to be analyzed by a densitometer or scanner, and are suitable for immunoelectrophoresis and autoradiography. In addition, the separated polyions are faster stained and the dried gels with the bands can be kept as evidence.

There are two types of electrophoresis cells for slab gels: for horizontal and vertical electrophoresis.

1.7.1.1 Cells for horizontal electrophoresis

The main ingredients of a horizontal electrophoresis cell are the cooling plate and the contact electrodes, which are mounted on a movable electrode holder. They become connected with the slab gel using gels or paper strips on it (Figure 1.7-2).

The cooling plate of a horizontal chamber can be a ceramic plate, a Teflon-coated metal plate, or a glass plate. The Teflon-coated metal plates have the best thermal conductivity; the glass plates offer greater security against a short circuit, however, are fragile. Therefore, the ceramic plates are preferred, because they have the advantages of metal and glass plates. The contact between the gel and the electrodes is direct. The horizontal electrophoresis cell is covered with a lid to prevent evaporation and contamination of the system. The electric power is cut off when the lid is removed.

In a horizontal cell, all species of electrophoresis can be carried out: native and denaturing SDS electrophoresis, analytical and preparative isoelectric focusing, immune and affinity electrophoresis.

1.7.1.2 Cells for vertical electrophoresis

Although the cells for horizontal electrophoresis have many advantages, cells for vertical electrophoresis are still widespread. The reason is that samples of different volumes (2 to 100 µl) can be applied.

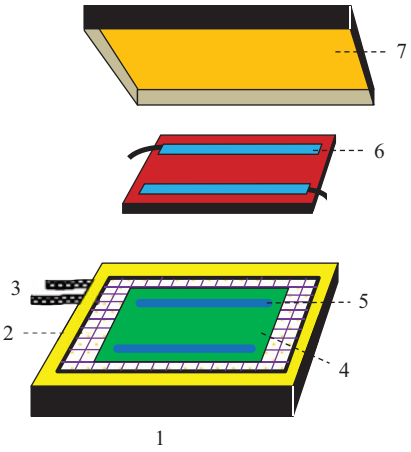

Figure 1.7-2: Electrophoresis cell for horizontal gel electrophoresis.
1. Leveled frame; 2. Cooling plate; 3. Tubings connected to a thermostat; 4. Horizontal gel slab; 5. Electrode strip; 6. Electrode; 7. Safety lid.

In a cell for vertical electrophoresis (Figure 1.7-3), the gels are held vertically between a top and a bottom electrode tank, filled with buffers.

The thickness of the gel in a vertical electrophoresis cell depends on the thickness of the spacers, which can be from 50 µm to 5 mm. Before casting the gel-making

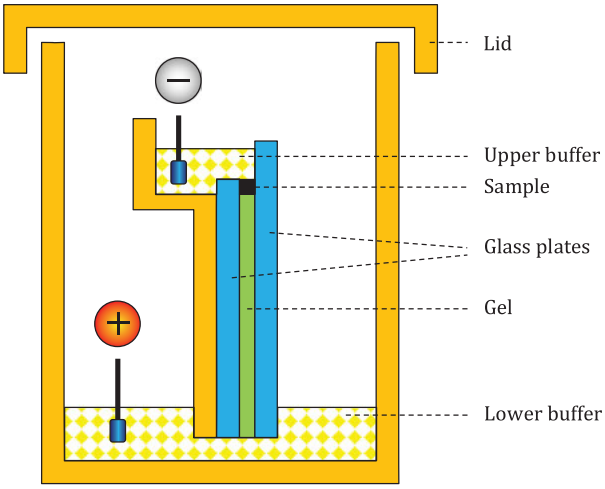

Figure 1.7-3: Construction of a cell for vertical gel electrophoresis. The cell consists of an upper buffer compartment (usually filled with a cathode buffer), which is placed in a lower buffer compartment (usually filled with an anode buffer). The electricity begins to flow after the lid is placed on.

solution, a sample comb is placed in to create sample wells. In order to monitor the progress of the electrophoresis, a marker dye can be added to the sample. When the dye reaches the gel end, the power supply is turned off and the gel is removed from the cassette for staining or blotting.

Vertical separation cells are used also for DNA sequencing. They are characterized by longer separation distances and some of them are equipped with thermostatically controlled cooling plates.

Mini- and midi-cells for rapid analysis are also available on the market. These cells allow the electrophoresis to complete in 35–45 min. The dimensions of the mini-gels are 7 × 8 cm; the dimensions of the midi-gels are 15 × 10 cm.

1.7.2 Power supplies

The electric field is characterized by its field strength E (in V/m), which is directly proportional to the electric voltage U (in V) and inversely proportional to the distance between the electrodes d (in m):

$$E = \frac{U}{d} \tag{1.7-1}$$

Since the distance remains constant during electrophoresis, the field strength can be changed by changing the voltage.

The product of the electric voltage and the electric current I (in A) is referred to as electrical power P (in W):

$$P = UI \tag{1.7-2}$$

There are different types of power supplies:

1. Simple power supplies, which can only be controlled by the voltage.
2. Power supplies, which can generate constant voltage or constant electric current.
3. Power supplies, which can keep constant the voltage, electric current, and electric power.
4. Power supplies, which are connected with a voltage-hour integrator.
5. Programmable power supplies with a microprocessor for desired separation conditions.

A power supply suitable for all types of electrophoresis must produce a voltage of 50 to 5,000 V, and a direct electric current of 5 to 100 mA.

For the pulsed-field electrophoresis, a control unit is connected to the power supply, which alternately drives the electrodes in a north/south and east/west direction.

1.7.3 Thermostats

In electrophoresis, the electric energy is partially converted into heat. If the released heat reaches a high level, it has detrimental effects on the electrophoretic results. Therefore, the electrophoretic cell often has to be cooled.

The cooling of an electrophoretic cell is performed with thermostats. The thermostats control the desired temperature of a liquid and pump it through the electrophoresis cell. Thus, the temperature inside the electrophoretic cell remains constant.

Most electrophoresis types are carried out at temperatures between 5 and 25 °C, usually at 10–15 °C. However, there are electrophoretic methods, which require higher temperatures. The low temperatures are an indispensable condition for SDS gel electrophoresis and isoelectric focusing.

1.7.4 Densitometers

In many cases, it is sufficient to detect the presence or absence of stained bands or spots of the separate polyions with the naked eye. However, it is impossible with the naked eye to determine the exact differences between the intensities (concentrations) of the bands or spots. This can be carried out with the aid of densitometers.

The densitometers offer their results on densitograms, which can be stored and processed in a computer.

1.7.4.1 Optics of a densitometer

The densitometer is a movable photometer, which scans separated polyion bands.

When a light beam (radiant flux) of a certain wavelength and of a certain intensity I_0, in cd, irradiates a substance, a part of it is absorbed, reducing its intensity into I, in cd. According to Beer–Lambert law, the natural logarithm of the ratio of irradiated to transmitted light is referred to as extinction

$$E = ln\frac{I_0}{I} = \varepsilon cd \quad [\text{dimensionless}] \tag{1.7-3}$$

where ε, in m^2/mol, and the layer thickness d, in m, are constants; and c, in mol/l, is the concentration of the substance.

In the analytical chemistry, the term "**o**ptical **d**ensity" (OD, dimensionless) is used. It is different from extinction [1]. OD shows that the absorbance of a solution increases linearly with the concentration of the solution.

The extinction values are calculated from either a reflected or a transmitted radiation, depending on whether the measurement is carried out before or behind the

medium. The densitometers for electrophoretic purposes work usually in the second transmission mode because wet or dried gels, blot membranes, and autoradiograms are examined (Figure 1.7-4).

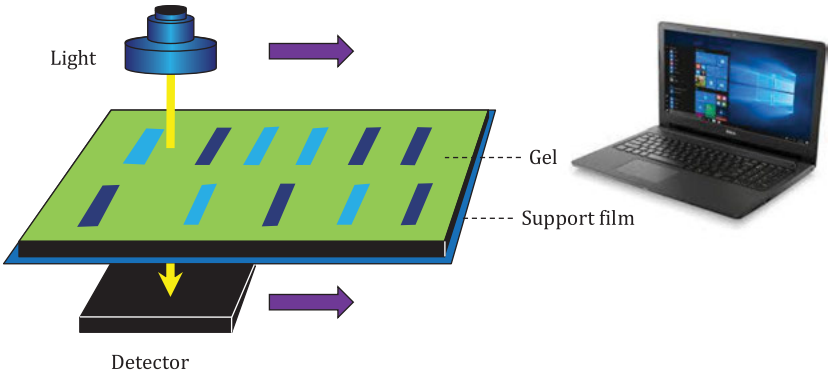

Figure 1.7-4: Principle of a transmission densitometer: a light source and a light detector move along the pherogram, scanning the bands.

The light source is a white light or a laser light. The white light can be adjusted using appropriate filters to a desired wavelength, which increases the sensibility of the method. The laser light has a fixed wavelength.

1.7.5 Gel casting systems

A gel casting system consists of one or more casting cassettes and a gradient mixer. Rarely, gels can be cast in cylindrical glass tubes.

1.7.5.1 Casting cassettes

A casting cassette is constructed by two glass plates and a U-shaped spacer (Figure 1.7-5), which are held together by brackets.

Gel thickness can be varied by spacers inserted into the cassettes prior to the gel formation. They are made of rubber or silicone, and can be glued to one of the glass plates. The distance between the plates can also be determined by Parafilm layers (0.12 mm thick each). There are also casting cassettes with mounted 0.5-mm-thick gaskets (e.g., from Desaga, Heidelberg).

The slab format provides the gels uniformity, so that diverse samples can be compared on a same gel. The slab gels allow good resolution and relatively high voltages without excessive heating, too.

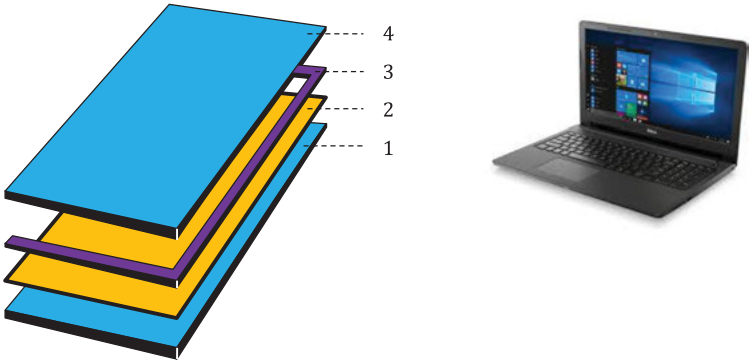

Figure 1.7-5: Assembly of a cassette for casting thin and ultrathin gels.
1. Lower glass plate; 2. Support film; 3. U-shaped spacer; 4. Upper glass plate

1.7.5.2 Gradient mixers

The electrophoresis is often carried out on polyacrylamide gels with increasing concentration or in immobilized pH gradients. To prepare changing concentrations or pH gradients, various gradient mixers are offered.

A simple and widespread gradient mixer consists of two communicating tubes: a mixing chamber and a reservoir, located at the same level (Figure 1.7-6).

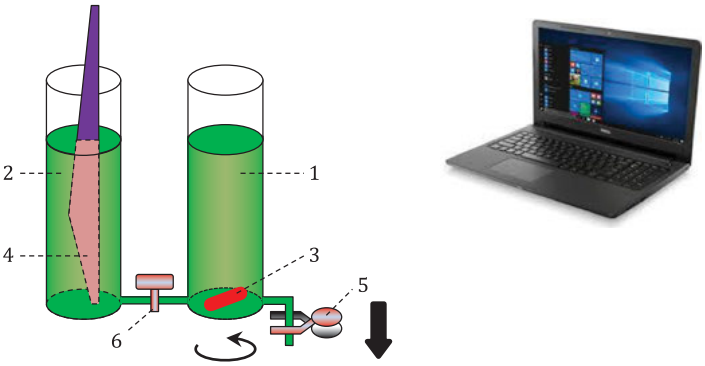

Figure 1.7-6: Gradient mixer.
1. Mixing chamber; 2. Reservoir; 3. Magnetic bar; 4. Compensation bar; 5. Outlet clamp; 6. Pinchcock

The mixing chamber contains a magnetic bar; the reservoir contains a compensation bar. The compensation bar in the reservoir compensates the volume of the magnetic bar in the mixing chamber and the increase of the volume of its solution created during the twisting of the magnetic bar. Thus, the two solutions remain on the same level during the casting, and the solution in the mixing chamber does not flow back into the reservoir. The magnetic bar is driven by a motor.

A simple gradient mixer, constructed by tubes, was described [2]. It is used to generate linear, exponential, and other concentration gradients.

1.7.6 Buffer mixers

During electrophoresis, changes occur in the ionic composition and pH value of the electrode buffers. To avoid this, numerous companies have produced different electrode buffer mixers (recirculators). Most recirculators operate using the same principle: a pump sucks buffer from the one electrode tank and pumps it out into the another electrode tank; after mixing the buffer returns to the first electrode tank, and so on [3,4]. These apparatuses depend on electric power and are too sophisticated.

We propose a water-driven buffer recirculator [5]. Its construction is presented on Figure 1.7-7.

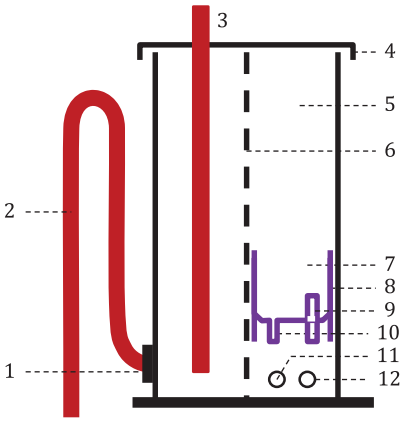

Figure 1.7-7: Scheme of the recirculator.
1. Big aperture; 2. Syphon; 3. Hose; 4. Recirculator lid; 5. Recirculator vessel; 6. Fin; 7. Float; 8. Rib; 9. Inlet tube; 10. Outlet tube; 11. and 12. Small apertures

The recirculator is divided by two vertical fins into two compartments connected each with other (6). The lower part of the one wall has a big aperture (1) connected with a syphon (2); the other wall has two small apertures (11 and 12).

The one half of the recirculator contains a hollow float (7) with leading ribs (8) at its four corners. The bottom of the float possesses two equal tubes: an inlet and an outlet tube. The inlet tube (9) reaches the middle of the float. The tubes are connected with inlet tubing and outlet tubing, respectively. The tubings leave the recirculator vessel through two small apertures (11 and 12). A hose (3) connected with a water source enters the other half of the recirculator through an aperture in the recirculator lid (4). A siphon (2) containing a U-shaped tube is connected to aperture (1).

The water fills the recirculator vessel up through the hose (3) and flows out through the siphon (2). This makes the float move up and down. When the float is in the lower part of the recirculator, it is filled through the inlet tubing with electrode buffer from the electrophoretic device. When it is in the upper part of the recirculator, it feeds the buffer through the outlet tubing into the electrophoretic device.

If the tanks of the electrophoretic device are one above the other, as in the vertical electrophoretic devices, the recirculating buffer mixes with the buffer in the upper tank where it mixes with the electrode buffer, spills over into the lower tank, and then passes into the recirculator float. If the two electrode tanks are on the same level, as in the horizontal electrophoretic devices, the circulating buffer is poured out into one of the tanks, where it mixes with the electrode buffer, and then passes through tubing into the other tank and afterward in the recirculator float.

The quantity of the recirculating buffer and the rate of recirculation can be regulated by changing the lumen of the inlet tubing and the water flow.

1.7.7 Cooling devices

Cooling is often required to maintain the proteins and nucleic acids in their native state. Usually, it is applied to the lower side of vertical gels. As a result, the proteins or nucleic acids that are near to the cooled side migrate slower than the proteins or nucleic acids that are closer to the other side. This causes the bands to slant downward from the cooled to the uncooled side across the gel.

Cooling is not necessary for denatured proteins. In some cases, it is even undesirable. For example, SDS begins to crystallize out of the solution at temperatures below 10 °C, so excessive cooling should harm SDS-PAGE.

1.7.8 Blotters

There are different blotters that are used for various blotting techniques, such as capillary, vacuum, tank, or semidry blotters (s. *Blotting*).

1.7.9 Equipment for semiautomatic electrophoresis

In addition to the classic electrophoresis devices, semiautomatic electrophoresis systems have been developed. The main advantages of a semiautomatic system are the diminished work, the fast and reproducible determination of the results, and their easy documentation.

The first semiautomated electrophoresis system was the PhastSystem [6] (Pharmacia Biotech, Uppsala). It consists of a horizontal separation cell with an integrated thermostat, a power supply, and a staining chamber. The electric current, the temperature in the cell, and the staining can be programmed. The staining chamber can be warmed up to 50 °C and filled with staining solutions using a diaphragm pump. In addition, the system contains a device for rotating the gels in the solutions. For electrophoretic transfer, there is also a blotting unit with graphite electrodes (Figure 1.7-8).

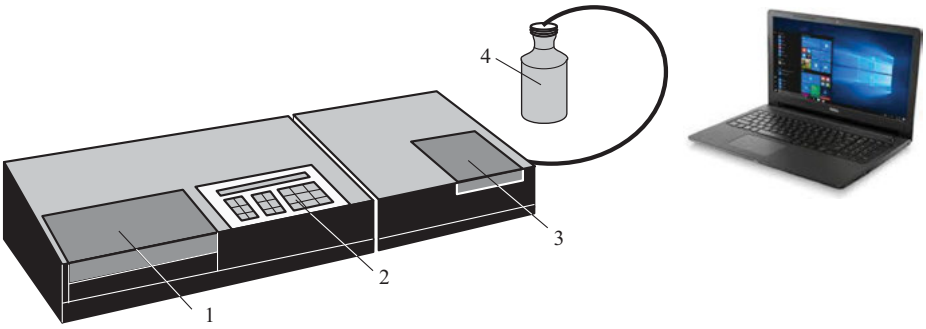

Figure 1.7-8: PhastSystem.
1. Separation cell; 2. Keyboard for programming the separation and next procedures;
3. Staining chamber; 4. Container for solutions

The samples are automatically applied. Film supported precast gels (with dimensions of 50 × 40 × 0.3–0.4 mm) for native electrophoresis, SDS gel electrophoresis, and isoelectric focusing, also Coomassie and silver staining kits are available. The electrophoretic separation and the staining are quickly finished. For example, the SDS electrophoresis in a gradient gel and the additional silver staining take place in ca. 1.5 h.

1.7.10 Equipment for preparative electrophoresis

Devices for preparative electrophoresis have also been developed [7]. Using them, the electrophoretic fractions are contained in separate vessels. Thus, the biomolecules can be separately examined.

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1.8 Classification of electrophoretic methods

Depending on gel position, vertical and horizontal electrophoresis can be distinguished; depending on the volume of the sample to be resolved, analytical and preparative electrophoresis exist.

Proteins and nucleic acids are mostly colorless. Therefore, their movement through the gel cannot be seen. To avoid this, tracking dyes (anionic or cationic) are included in the sample. A known tracking dye is **B**romo**p**henol **b**lue (BPB). It has a negative electric charge and moves toward the anode ahead of proteins and nucleic acids at alkali and neutral pH. When Bromophenol blue has reached the anodic end of the gel, the electrophoresis must be stopped. Other tracking dyes are xylene cyanol, which moves slower, and Orange G, which moves faster than Bromophenol blue.

Vertical and horizontal electrophoresis. The vertical electrophoresis can be performed in slab or cylindrical gels, while the horizontal electrophoresis can be carried out only in slab gels. The horizontal electrophoresis offers numerous advantages over the vertical electrophoresis: easier handling; usage of soaked with buffer electrode strips instead of tank buffers; selection of application points, which is important for native electrophoresis and isoelectric focusing; free access to the gel surface during electrophoresis, and more.

Analytical and preparative electrophoresis. Most electrophoresis methods are analytical. However, there are also methods that allow a preparative separation of polyions. The isoelectric focusing can also be performed analytical or preparative.

The electrophoretic methods can also be classified according to other criteria: the nativity of the polyions to be separated, and the structure of the separation medium. However, the most important characteristic of an electrophoretic system is the buffer used. According to the buffer, all electrophoresis methods can be classified as: zone electrophoresis, isotachophoresis, and isoelectric focusing (Figure 1.8-1). There are also combinations between these methods.

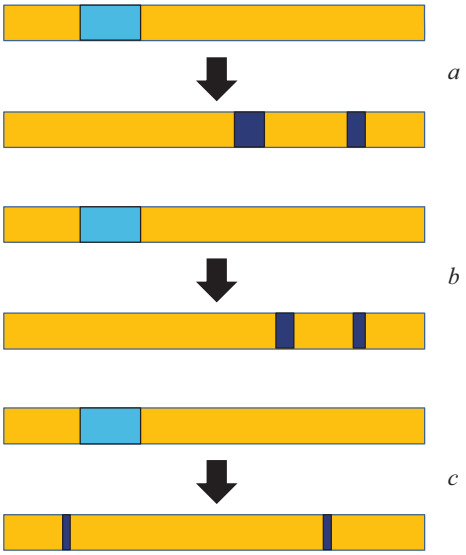

Figure 1.8-1: Scheme of the electrophoretic techniques and their results.
(*a*) zone electrophoresis, (*b*) isotachophoresis, (*c*) isoelectric focusing

Nowadays, a new analytical technique is introduced, namely the dielectrophoresis. It is not a real electrophoretic method, but is closely connected to the electrophoresis.

1.8.1 Zone electrophoresis

The zone electrophoresis is known also as continuous or conventional electrophoresis. It is carried out in one buffer, that is, in an electric field of continuous strength, whose pH value remains constant during the electrophoresis. The zone electrophoresis can take place free (only in a buffer) or in a solid medium soaked with a buffer (Table 1.8-1).

The *free electrophoresis* is a function only of the polyionic charges. However, the polyions diffuse in the buffer. Therefore, the buffer can also contain glycerol, D-sorbitol, sucrose, or other viscous chemicals.

Zone electrophoresis has practical importance in clinical chemistry and biochemistry, where it serves for routine analysis of serum and other proteins. The free zone electrophoresis is used, if larger particles, for example viruses, bacteria, and cells, are to be separated.

Table 1.8-1: Zone electrophoresis methods.

Buffers and separation media	Zone electrophoresis
Buffer as separation medium	Tiselius electrophoresis Capillary electrophoresis Free-flow electrophoresis
Buffer in cellulose acetate membrane	Cellulose acetate electrophoresis
Buffer in agarose gel	Agarose gel electrophoresis Submarine electrophoresis Pulsed-field electrophoresis
Buffer and immunoglobulins in (on) agarose gel	Immunoelectrophoresis Immunofixation
Buffer and ligands in agarose gel	Affinity electrophoresis
Buffer in polyacrylamide gel	Polyacrylamide gel electrophoresis

1.8.1.1 Tiselius electrophoresis

The classic Tiselius electrophoresis [1] is a free zone electrophoresis. The polyions are mixed with a buffer, placed in a U-shaped glass tube and overlaid with the same buffer. When an electric voltage is applied, they separate each from other forming zones (layers) in the two legs of the tube (Figure 1.8-2).

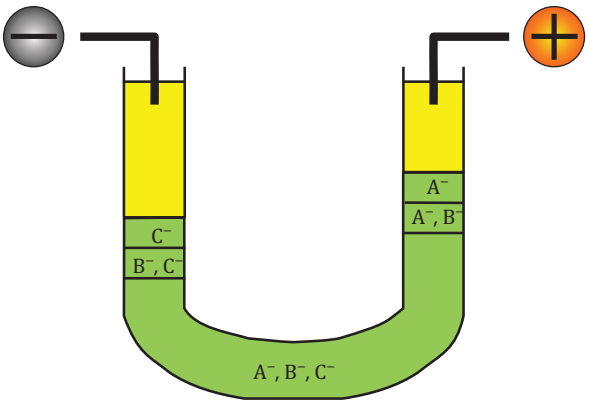

Figure 1.8-2: Tiselius electrophoresis of the anions A^-, B^-, and C^-. After the electrophoresis, four zones can be distinguished in the tube. On its anode end: a pure anion A^- zone and a mixed zone of the anions A^- and B^-; on its cathode end: a pure anion C^- zone and a mixed zone of the anions B^- and C^-.

The separated colorless proteins are detected using a Schlieren-scanning system. It shows the streaks refraction shadows formed by the analytes in the electrophoresis tube, which are focused onto a photographic plate. Today, Tiselius electrophoresis is of little practical usage apart from the preparative electrophoresis and the measuring of ionic mobilities.

1.8.1.2 Capillary electrophoresis

The **c**apillary **e**lectrophoresis (CE), named also **h**igh **p**erformance **c**apillary **e**lectrophoresis (HPCE), is carried out in capillaries or micro- and nanofluidic channels [2,3]. Commonly, CE refers to **c**apillary **z**one **e**lectrophoresis (CZE), but **c**apillary **g**el **e**lectrophoresis (CGE), **c**apillary **i**so**e**lectric **f**ocusing (CIEF), **c**apillary **i**so**t**achophoresis (CIT), and **m**icellar **e**lectro**k**inetic **c**hromatography (MEKC) also belong to it [4]. In the capillary isotachophoresis, the polyions are separated from each other by spacers.

1.8.1.3 Free-flow electrophoresis

In the **f**ree-**f**low **e**lectrophoresis (FFE) [5], a buffer flows perpendicular to an electric field, and the electrophoretic fractions are collected separately. FFE is used for separation of polyions, organelles, and cells. **F**ree-**f**low **i**so**t**achophoresis (FFIT) and **f**ree-**f**low **i**so**e**lectric **f**ocusing (FFIEF) are also known.

1.8.1.4 Solid media electrophoresis

The electrophoresis can be run also in solid separation media that contain buffers in their pores. Diverse media are known: paper, starch, cellulose acetate, agarose, and polyacrylamide. Today, the agarose and polyacrylamide gels are the most widespread electrophoresis media. Their pores can act as a sieve. So, the polyions are separated according to their electric charges, as well as to their volumes. As a result, the electrophoresis resolution increases.

In comparison with the free electrophoresis, the solid media electrophoresis is preferred because the diffusion and convection in the buffer are reduced.

1.8.1.5 Cellulose acetate electrophoresis

The **c**ellulose **a**cetate **e**lectrophoresis (CAE) [6,7] is the simplest electrophoretic method. The cellulose acetate membranes have large pores; therefore, the electrophoretic separation is only charge dependent. Nowadays, it is almost totally replaced by the agarose and polyacrylamide gels electrophoresis.

1.8.1.6 Agarose gel electrophoresis

The **a**garose **g**el **e**lectrophoresis (AGE) [8,9] is widespread. It is used for separation of proteins and nucleates.

Depending on the polyion nativity, two types of agarose gel electrophoresis are known: electrophoresis of native polyions, that is, of polyions with intact structure and properties; and electrophoresis of denatured polyions, that is, of polyions with altered structure and properties. The first electrophoresis is referred to as native agarose electrophoresis, and the second one is referred to as denaturing agarose electrophoresis.

Native agarose gel electrophoresis

The native agarose gel electrophoresis is a zone electrophoresis on agarose gels. It is used for separation of proteins and nucleates.

Submarine electrophoresis

The **s**ubmarine **e**lectrophoresis (SE) is a horizontal agarose gel electrophoresis carried out under a buffer. So, the gel surface does not dry out. It is used as a standard method for separation, identification, and purification of DNA and RNA fragments [10,11].

Denaturing agarose gel electrophoresis

The denaturing agarose gel electrophoresis is a zone electrophoresis carried out in urea containing agarose gels. It is used for separation of denatured nucleic acids.

1.8.1.7 Pulsed-field electrophoresis

The agarose gel electrophoresis is the preferred method for resolving DNA fragments of about 1,000 to 23,000 bp. For larger fragments, for example, chromosomes, the **p**ulsed-**f**ield **g**el **e**lectrophoresis (PFGE) is used [12]. In the pulsed-field gel electrophoresis, DNA fragments greater than 23 kbp are forced by a pulsing field so that nucleates relax and expand alternatively interacting with the gel pores. The separation takes place many hours or even days.

1.8.1.8 Immunoelectrophoresis

The **i**mmuno**e**lectrophoresis (IE) is a combination between a zone electrophoresis in an agarose gel and immune reactions. The immunoglobulins are located in the agarose gel.

1.8.1.9 Immunofixation

The **i**mmuno**f**ixation (IF) is an immunoelectrophoretic method, during which the immunoglobulins are applied on the gel surface after the electrophoresis.

1.8.1.10 Affinity electrophoresis

The **a**ffinity **e**lectrophoresis (AE) is a zone electrophoresis carried out in a ligands containing agarose gel [13,14]. It is based on the interactions between chelating compounds and their ligands.

1.8.1.11 Polyacrylamide gel electrophoresis

The polyacrylamide gel, in contrast to the agarose gel, has no electric charges and, as a result, no electroosmosis. The **p**oly**a**crylamide **g**el **e**lectrophoresis (PAGE) [15,16] is a zone electrophoresis that is performed for separation of native nucleic acids.

In the vertical PAGE systems, samples are poured into wells in the gel. To ensure that they sink to the bottom of the wells, the sample buffer is supplemented with additives that increase its density. Common additives are glycerol or sucrose.

1.8.1.12 Iontophoresis

The **i**onto**p**horesis (IP) is a type of zone electrophoresis, during which ions flow in the human body by applying an electric field. The transport is measured in the units of chemical flux, commonly in $\mu mol/cm^2h$.

Reverse iontophoresis

The reverse iontophoresis is a technique, during which ions are removed from the human body.

1.8.2 Isotachophoresis

The term ***i**so**t**acho**p**horesis* (ITP) originates from the Greek words *isos* (same), *tachos* (speed), and *phorein* (bear). It means migration of ions at the same velocity.

Isotachophoresis takes place in a buffer system consisting of leading and trailing buffers, which form a moving ionic boundary according to the Kohlrausch function [17,18]. The polyions arrange themselves in the moving boundary, according to their effective mobilities [19,20]. In contrast to the continuous electrophoresis, the electric field strength is distributed discontinuously and the pH value changes during the electrophoresis.

If polyanions are to be separated, the anion of the leading electrolyte, the leading anion, should have a higher effective mobility; and the anion of the trailing electrolyte, the trailing anion, should have a lower effective mobility than the effective mobilities of the polyanions. The whole system contains a common counterion.

1.8.2.1 Disc-electrophoresis

The disc-electrophoresis represents a combination between zone electrophoresis and isotachophoresis. It is based on the Ornstein theory [21].

During the disc-electrophoresis, the proteins are first concentrated in a stacking gel according to the principle of the isotachophoresis, and then are resolved from each other in a resolving gel according to the principle of the zone electrophoresis. There are native and denatured disc-electrophoresis methods (Table 1.8-2).

Table 1.8-2: Disc-electrophoresis methods.

Buffers and separation media	Electrophoresis methods
A buffer system in a polyacrylamide gel	Native disc-electrophoresis
A buffer system in a gradient polyacrylamide gel	Gradient gel disc-electrophoresis
A buffer system and SDS in a polyacrylamide gel	SDS disc-electrophoresis
A buffer system and SDS in a gradient gel	Gradient gel SDS disc-electrophoresis
A buffer system and urea in a polyacrylamide gel	Urea disc-electrophoresis

Native disc-electrophoresis

The native disc-electrophoresis is carried out on polyacrylamide gels. It is used for resolving of native polyions, for example serum proteins or nucleates.

The native gels do not contain denaturing agents, so the polyion structure is maintained. Since the proteins remain in native state, they may be visualized by specific enzyme-linked staining.

Native PAGE is used in proteomics and metallomics, and also to scan genes for unknown mutations as in the method single-strand conformation polymorphism.

Gradient native disc-electrophoresis

The native disc-electrophoresis can be carried out also in gradient gels. They act as a molecular sieve, which separates the polyions according to their volume (mass) and conformation [22,23]. Linear and exponential gradient polyacrylamide gels are known.

Denaturing disc-electrophoresis

The denaturing disc-electrophoresis is carried out on gels that contain denaturing agents, for example SDS or urea. SDS is used to lyse lipid membranes of the cells.

The denaturing disc-electrophoresis is run under conditions that disrupt the natural secondary, tertiary, and quaternary structures of proteins, which unfold into linear polypeptide chains. Then the mobilities of the polypeptide chains depend only on their length and their charge-to-mass ratio.

For full denaturation of proteins, reducing agents are also necessary to reduce the disulfide bonds that stabilize the tertiary and quaternary structures. Such agents are β-mercaptoethanol and dithiothreitol.

While the proteins are denatured using SDS, nucleic acids are common denatured using urea in the buffer. Urea, **d**i**m**ethyl **s**ulf**o**xide (DMSO), and glyoxal are the most often used denaturing agents to disrupt RNA and estimate their molecular masses.

The denaturing gel electrophoresis is a component of the methods: **d**enaturing **g**radient **g**el **e**lectrophoresis (DGGE) [24], **t**emperature **g**radient **g**el **e**lectrophoresis (TGGE), and **t**emporal **t**emperature **g**radient **e**lectrophoresis (TTGE) [25] that are used for testing DNA and RNA.

SDS disc-electrophoresis

The SDS disc-electrophoresis resembles a disc-electrophoresis carried out in an SDS-containing gel-buffer system. **S**odium **d**odecyl **s**ulfate (SDS) denatures the proteins and binds to them so that they become strongly negatively charged. Since the ratio of charge/mass is similar for all proteins, the proteins separate from each other only according to their masses. The SDS disc-electrophoresis is one of the most commonly used electrophoresis.

Gradient SDS disc-electrophoresis

As in the native polyacrylamide gel electrophoresis, SDS disc-electrophoresis can be performed in gradient gels. The gradient gels sharpen the protein bands because they separate from each other also according to their volume and conformation.

Urea disc-electrophoresis

The urea disc-electrophoresis is a denaturing disc-electrophoresis on polyacrylamide gels containing urea. It is used for separation of nucleic acids.

1.8.3 Isoelectric focusing

The **i**so**e**lectric **f**ocusing (IEF) is based on the migration of polyions through a pH gradient until they reach their isoelectric points. The pH gradients may be formed by mobile zwitterion ampholytes (carrier ampholytes) or immobile zwitterion ampholytes (immobilines) (Table 1.8-3).

1.8.3.1 Isoelectric focusing with carrier ampholytes

The carrier ampholytes are moving in an electric field until they lose their charges at their isoelectric points and stop. As a result, stationary pH gradients are formed, in which the analyzed substances can be focused into narrow bands. The isoelectric focusing is essential for the protein analysis.

Table 1.8-3: Isoelectric focusing methods.

pH gradient and separation media	IEF
Carrier ampholytes in a polyacrylamide gel	IEF with carrier ampholytes
Carrier ampholytes and urea in a polyacrylamide gel	Denaturing IEF with carrier ampholytes
Carrier ampholytes in an agarose gel	IEF in an agarose gel
Carrier ampholytes and urea in an agarose gel	Denaturing IEF in an agarose gel
Immobilines in a polyacrylamide gel	IEF in immobilized pH gradients
Immobilines and urea in a polyacrylamide gel	Denaturing IEF in immobilized pH gradients

Denaturing isoelectric focusing with carrier ampholytes
This is isoelectric focusing using carrier ampholytes on polyacrylamide gels that contain urea.

Isoelectric focusing on agarose gels
An agarose gel can also be used for isoelectric focusing, because it makes also a solid separation medium with large pores [26,27].

1.8.3.2 Isoelectric focusing in immobilized pH gradients

The immobilines are acrylamide derivatives, which contain buffering groups. They copolymerize into the polyacrylamide gel, so that an **i**mmobilized **p**H **g**radient (IPG) is produced.

Denaturing isoelectric focusing in immobilized pH gradients
This is isoelectric focusing in immobilized pH gradients containing urea.

1.8.3.3 Two-dimensional electrophoresis

The two-dimensional electrophoresis (2D-electrophoresis) represents a combination between isoelectric focusing and SDS disc-electrophoresis, which are carried out sequentially in two mutually perpendicular directions [28,29]. Depending on the pH gradient, there are two types of 2D-electrophoresis: ampholyte 2D-electrophoresis, and immobiline 2D-electrophoresis (Table 1.8-4).

Table 1.8-4: Two dimensional electrophoresis methods.

pH gradient and SDS electrophoresis	2D-electrophoresis
Carrier ampholytes and SDS in a polyacrylamide gel	Ampholyte 2D-electrophoresis
Immobilines and SDS in a polyacrylamide gel	Immobiline 2D-electrophoresis

1.8.4 Dielectrophoresis

Dielectro**p**horesis (DEP) is a method for separation of dielectric particles, when they are placed in a nonuniform electric field [30,31,32]. The particles are not required to be charged. The magnitude of the force exerted on the dielectric particles depends on their electric properties, shape, and volume, and on the medium as well as on the frequency of the electric field.

Although the dielectrophoresis was described far back in the early twentieth century, it was studied and named by Herbert Pohl in the 1950s [33,34]. Later the dielectrophoresis has been used for separation of microparticles [35,36], nanoparticles, and cells.

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Problems 1. Fundamentals of electrophoresis

1.1 Calculate the ionic strength of a solution containing 0.1 mol/l CH_3COONa and 0.2 mol/l NaCl.

1.2 pK value of dihydrogen phosphate ($H_2PO_4^- \leftrightarrow H^+ + HPO_4^{2-}$) is equal to 7.20 at 25 °C. Calculate its pK_c value at I = 0.1 mol/l.

1.3 The total concentration of dissolved CO_2 in deionized water reaches 10^{-4} mol/l, whereby only 0.0026 of it exists as carbonic acid (H_2CO_3). Calculate the pH value of the deionized water, if the dissociation constants of carbonic acid pK_{c1} = 3.8, and pK_{c2} = 10.3.

1.4 A buffer contains 0.1 mol/l CH_3COOH and 0.1 mol/l CH_3COONa at 25 °C. Calculate its pH value, if 0.01 mol/l HCl or 0.01 mol/l NaOH is added. The pK_c value of CH_3COOH is 4.64.

1.5 Calculate the concentrations of CH_3COONa and CH_3COOH (pK_{cCH_3COOH} = 4.64) in an acetate buffer, if its pH value is equal to 5.50 and the total concentration of its electrolytes is equal to 0.2 mol/l.

1.6 Calculate the composition of a carbonate buffer with pH = 10.00 and buffer capacity β = 0.3 mol/l. The concentration dissociation constant of hydrogen carbonate ion p$K_c(HCO_3^-)$ is 10.33.

1.7 Calculate the buffer capacity of a buffer with pH = 5.21 at 25 °C, which contains 0.1 mol/l pyridine and 0.1 mol/l HCl. The pK_c value of pyridinium ion is 5.21.

1.8 Calculate the ratio between the buffer capacities of two buffers at 25 °C, which are composed of CH_3COONa and CH_3COOH. The pH value of the first buffer is equal to p$K_c(CH_3COOH)$ of 4.62; the pH value of the second buffer is equal to 5.50. The total buffer concentration in both solutions is 0.2 mol/ l.

1.9 Calculate the mobility of TRIS ion μ_{HT^+} at 0 °C and at 25 °C, at ionic strengths of 0.01 and 0.07 mol/l. The absolute mobility of TRIS ion is $12.75 \bullet 10^{-9}$ $m^2/(sV)$ at 0 °C, and $27.86 \bullet 10^{-9}$ $m^2/(s\,V)$ at 25 °C. Use the ionic mobility parameter of the linear equation for calculation.

1.10 Using the equation $\mu = \frac{d\,l}{t\,U}$, calculate the mobility of albumin polyion $\mu_{albumin^{n-}}$, if it has run 25 mm (d) on a cellulose strip in 1 h (t), and if the distance (l) between the anode and cathode ends of the strip was 100 mm, and the potential difference was 250 V (U).

1.11 What kind of structure has the water molecule?
A Polar B Amphipathic C Nonpolar D Charged

1.12 Which combination represents a buffer solution?
A A mixture of a weak acid and a strong base
B A mixture of a strong acid and a weak base
C A mixture of a weak acid and its conjugated base
D A mixture of a strong acid and a strong base

1.13 What expresses the equation of Henderson–Hasselbalch?
A Partial pressure of a gas
B pH value of a buffer
C Concentrations of the electrolytes in a given solution
D Osmotic pressure of a solution

1.14 Which is the most important buffer in the cell?
A Hydrogen carbonate buffer
B Hemoglobinate buffer
C Proteinate buffer
D Hydrogen phosphate buffer

1.15 Which is the normal pH value of blood?
A 7.4 B 6.8 C 7.0 D 7.9

1.16 Which buffer in blood has highest buffering capacity?
A Proteinate buffer
B Hemoglobinate buffer
C Hydrogen carbonate buffer
D Hydrogen phosphate buffer

1.17 Which is the pK_b value of ammonia, if the pK value of ammonium ion is 9.25?
A 3.25 B 2.15 C 2.25 D 4.75

1.18 Which buffer is most significant for the blood plasma?
A Proteinate buffer
B Hydrogen carbonate buffer
C Hemoglobinate buffer
D Hydrogen phosphate buffer

1.19 What will be the pH value of a solution, if 10 ml of 0.1 mol/l weak acid (K_a = 10^{-4} mol/l) are mixed with 10 ml of 0.1 mol/l sodium salt of the same acid?
A 5 B 3 C 4 D 6

1.20 What will be the ratio between the concentrations of the charged and uncharged amino groups in a solution with pH = 6.5, if pK value of amino group is equal to 7.5?
A 1:1 B 100:1 C 10:1 D 1:10

1.21 What will be the pH value of a solution obtained, if 10 ml of 0.1 mol/l sodium lactate are added to 10 ml of 0.1 mol/l lactic acid ($pK_a = 3.86$)?
A 3.86 B 4.86 C 2.86 D 1.86

1.22 Blotting techniques are used to identify
A Unique proteins
B Nucleic acid sequences
C Both A and B
D None of the above

1.23 The immobilizing of nucleic acids or proteins on a membrane is called
A Hybridization
B Blotting
C Immobilization
D None of the above

1.24 What is the name of the process of oxidative degradation of polyunsaturated fatty acids in the plasma membrane, resulting in destruction of the cell?
A Saponification
B Hydrolysis.
C Lipid peroxidation.
D Oxide translocation.

2 Electrophoresis of proteins

Proteins are usually analyzed by **SDS p**oly**a**crylamide **g**el **e**lectrophoresis (SDS-PAGE), native gel electrophoresis, **q**uantitative **p**reparative **n**ative **c**ontinuous **p**oly**a**crylamide **g**el **e**lectrophoresis (QPNC-PAGE), or **two d**imensional (2D)-electrophoresis. Most commonly used solid media for protein electrophoresis are: paper, starch gel, cellulose acetate, and above all agarose or polyacrylamide gels.

Paper electrophoresis [1,2] has historical meaning. It was replaced by electrophoresis on cellulose acetate membranes. Glass fiber paper can also be used for electrophoresis; however, it is alkaline and therefore causes a strong electroosmotic flow.

The cell for the paper electrophoresis has two horizontal electrode tanks and is covered with a lid to prevent contact with electricity. Prior to electrophoresis, the paper is soaked with the electrode buffer. The samples are applied on the paper as strokes or spots, the first application being preferred. The resolved polyions are stained as dyes are sprayed over the paper.

Starch gel electrophoresis was introduced in 1955 by Smithies [3]. Starch gels are prepared from partially hydrolyzed potato starch in a concentration of 8–15 g/dl and in form of 5–10 mm layers. The pore size of the starch gels depends on the starch concentration.

The starch gel and electrode tanks usually contain the same buffer. The electrophoretic results are better when the electrode buffer is more concentrated than the gel buffer, hence, the ionic strength of the electrode buffer is higher than the ionic strength of the gel buffer.

Starch gel electrophoresis is completely detached by polyacrylamide gel electrophoresis because of the widely varying starch properties, lack of reproducibility, and inconvenience of handling. Nevertheless, it can be used for separation of human enzymes [4] and in population genetics [5, 6] because the starch gel, as a natural product, is an enzyme-friendly medium.

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2.1 Cellulose acetate electrophoresis of proteins

Cellulose **a**cetate (CA) electrophoresis is widely used for separation of proteins in clinical laboratories.

2.1.1 Theory of cellulose acetate electrophoresis of proteins

Cellulose acetate electrophoresis is a zone electrophoresis that is used mainly for analysis of serum proteins and isoenzymes. It is carried out in simply designed horizontal cells without refrigeration and is characterized by simple handling, small sample volumes, short separation times, and rapid staining and destaining.

The cellulose acetate membranes have very large pores and therefore have no screening effect on proteins to be resolved. The electrophoretic separation depends only on the electric charges of the proteins. The CA membranes counteract the diffusion so that the resolution is modest. The resultant zones are broader than the start zone due to convection.

The theory of cellulose acetate electrophoresis is similar to the theory of zone electrophoresis. The electrophoretic separation depends on the nature of the buffer, the voltage applied, and the distance between the electrodes. For proteins, the total electric charge is given by summing the charges of all ionizable groups at a given pH value. The degree of ionization obeys the Henderson–Hasselbalch equation:

$$\mathrm{pH} = \mathrm{p}K_a + \log\left(\frac{1}{\alpha} - 1\right) \qquad \text{for anions} \tag{2.1-1}$$

and

$$\mathrm{pH} = \mathrm{p}K_a - \log\left(\frac{1}{\alpha} - 1\right) \qquad \text{for cations} \tag{2.1-2}$$

The ionic mobilities also depend on the frictional drag, for example, the viscosity of the buffer. Agents such as **p**oly**e**thylene **g**lycol (PEG) or cellulose derivatives can increase the viscosity of the buffer and, as a result, decrease the mobilities of the proteins.

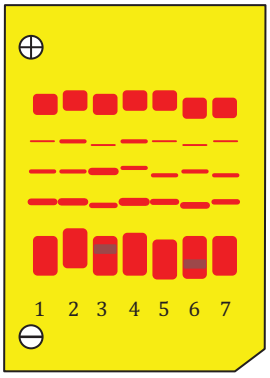
2.1.2 Practice of cellulose acetate electrophoresis of proteins

Prior to electrophoresis, the cellulose acetate membrane is required to be immersed in a buffer for 2–3 min. The excess buffer on the membrane is dried between two filter paper sheets and the CA membrane is spanned on a plastic frame (bridge). The frame is placed into a horizontal electrophoresis cell, where the membrane ends contact the two electrode buffers, and the cell is closed with a lid. Undiluted or twice diluted serum samples are then applied onto the cellulose acetate membrane through holes in the lid. Firstly, the urine and cerebrospinal fluid samples must be concentrated until their protein concentration reaches 2–3 g/dl. Thereafter, the electrophoresis is started.

Generally the electrophoresis is carried out in the 5,5-diethylbarbiturate buffer of Longworth [1] with a pH value of 8.0 to 9.0, known as barbitalate or veronalate buffer. The barbitalate buffer contains a derivative of barbituric acid (an anesthetic drug). Therefore, it should be replaced by a buffer that contains no barbiturate. Such a buffer is the TRIS-taurinate buffer [2].

To obtain clear protein bands after electrophoresis, the background of the CA pherograms must be transparent. This can be achieved when the membrane is soaked in a mixture of dioxane, isobutanol, methanol, or other liquids.

Using CA electrophoresis, five major protein bands (main fractions) of serum, urine, or cerebrospinal fluid can be obtained (Figure 2.1-1).



	Relative concentration
Albumin	60–70%
Alpha-1-globulins	1–4%
Alpha-2-globulins	5–10%
Beta-globulins	8–12%
Gamma-globulins	10–18%

$$\frac{c_{Albumin}}{c_{Globulins}} = 1.5–2.5$$

Figure 2.1-1: Serum proteins separated by cellulose acetate electrophoresis.

2.1.2.1 Cellogel electrophoresis of proteins

Cellogel electrophoresis is similar to cellulose acetate electrophoresis. It is carried out in Cellogel strips. The Cellogel strips also contain cellulose, but are thicker than the CA membranes. Like the cellulose acetate membranes, the Cellogel strips have very large pores and therefore no screening effect exerts on the proteins to be resolved.

The Cellogel sheets are usually manufactured with dimensions of 300 × 110 × 0.2 mm and are stored in methanol solution to stay wet. Prior to electrophoresis, they are cut into smaller strips with a width of 25 or 75 mm and a length of 140 mm, since the running distance is approximately 90 mm. The lower right corner of each strip is cut to distinguish its permeable upper site from its impermeable lower site.

Prior to electrophoresis, the Cellogel strips are dried between filter paper sheets and equilibrated for 5–10 min in the electrophoresis buffer until they begin to sink below the buffer surface. Then they are dried again between filter paper sheets and placed on a plastic bridge (Figure 2.1-2). The bridge is inserted into an appropriate electrophoresis cell, filled with a buffer.

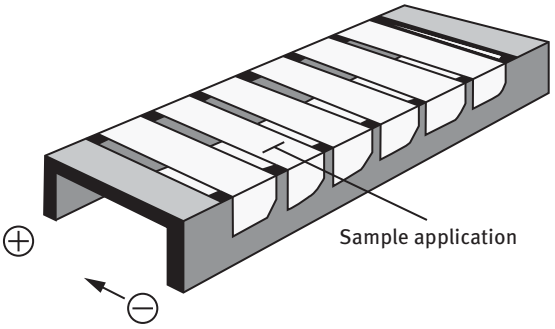


Figure 2.1-2: Bridge for Cellogel strips. When inserted into the electrophoresis cell, the ends of the strips sink into the buffer.

Enzymes, which can be separated and detected by Cellogel electrophoresis, are alkaline phosphatase, lactate dehydrogenase, creatine kinase, leucine amino peptidase, malate dehydrogenase, glucose-6-phosphate dehydrogenase, 3-hydroxyacyl-CoA dehydrogenase, glucose-1-phosphate uridyl transferase, and more [3, 4]. However, nowadays this electrophoresis is displaced by agarose gel electrophoresis.

2.1.3 Protocols

2.1.3.1 Cellulose acetate electrophoresis of serum proteins

Materials and equipment

Barbitalate buffer (pH = 8.6, I = 0.10 mol/l) or
TRIS-taurinate buffer (pH = 9.0, I = 0.05 mol/l) or
TRIS-glycinate buffer (pH = 9.5, I = 0.025 mol/l)
Cellulose **a**cetate (CA) membranes
Ponceau S

Tri**c**hloro**a**cetic acid (TCA)
Acetic acid
Glycerol
Methanol
Dioxane
Isobutanol
Filter paper
Electrophoresis cell
Power supply

Barbitalate buffer

Sodium barbitalate	20.62 g (0.10 mol/l)
Barbital	4.00 g (0.02 mol/l)
Sodium azide	0.65 g (0.01 mol/l)
Deionized water to	1,000.00 ml

Clearing solution

Acetic acid	15.0 ml
87% Glycerol	0.2 ml
Methanol to	100.0 ml
or	
Dioxane	70.0 ml
Isobutanol	30.0 ml

Procedure

- Fill an electrophoresis cell and a Petri dish with an electrophoresis buffer.
- Immerse a CA membrane into the Petri dish for 2–3 min, then dry briefly between filter paper sheets and place onto the electrophoresis cell bridge.
- Put the bridge with the CA membrane into the electrophoresis cell.
- Cover the electrophoresis cell with its lid.
- Apply the samples through the cell lid, using an applicator stamp, onto the cathode side of the membrane.
- Run the electrophoresis at 200–250 V (about 3 mA per membrane) for 25–30 min.
- After electrophoresis, place the CA membrane into the Ponceau S staining solution for 5 min (s. 2.1.3.3).
- Destain the membrane 3 times in 5 ml/dl acetic acid.
- Place the membrane in a Petri with clearing solution for 5 min.
- Place the membrane onto a clean glass plate and roll over with a photo roller until the air bubbles under the membrane are removed.
- Clear the membrane at 70 °C for 5 min.

– Evaluate the red-colored protein bands against a control pherogram, or with a densitometer at 530 nm, or using a scanner. Using a computer program, calculate the relative concentrations of the serum proteins.

2.1.3.2 Cellogel electrophoresis of serum proteins

Materials and equipment
TRIS-barbitalate buffer (pH = 9.2, I = 0.06 mol/l) or
TRIS-taurinate buffer (pH = 9.0, I = 0.05 mol/l) or
TRIS-glycinate buffer (pH = 9.5, I = 0.025 mol/l)
Cellogel membranes
Electrophoresis cell
Power supply

TRIS-barbitalate buffer, pH = 9.2

TRIS	7.20 g (0.06 mol/l)
Sodium barbitalate	10.30 g (0.05 mol/l)
Barbital	1.84 g (0.01 mol/l)
Sodium azide	1.30 g (0.02 mol/l)
Deionized water to	1,000.00 ml

Procedure
– Fill the cell tanks with TRIS-barbitalate buffer or TRIS-taurinate buffer.
– Expose the Cellogel strips for 5 min to a preliminary electrophoresis, which is carried out at field strength of 15 V/cm.
– Apply with a microliter syringe or an applicator 2–5 µl serum on the cathode end of each Cellogel strip. The applicator consists of two parallel wires that capture samples.
– Run electrophoresis at 200 V (about 2.5 mA/strip) for 30–40 min.
– Stain the protein bands after the electrophoresis (s. above).
– Make the Cellogel strips transparent as the CA membranes (s. above).

2.1.3.3 Staining proteins with Ponceau S

Materials and equipment
Ponceau S
Acetic acid
Tri**c**hloroacetic **a**cid (TCA)
CA or Cellogel membranes

Ponceau S staining solution

Ponceau S	0.3 g
Trichloroacetic acid	3.0 g
Deionized water to	100.0 ml

Procedure

- Stain the protein bands in Ponceau S staining solution for 5 min.
- Destain the CA or Cellogel membrane background 3 times in 5 ml/dl acetic acid.
- Clear the membrane in clearing solution for 1 min.
- Place the membrane on a dry glass plate.
- Heat the glass plate at 70 °C for 5 min.
- Scan or densitometer the bands at 525 nm.

A normal pherogram contains 5 bands.

2.1.3.4 Cellogel electrophoresis of urinary and cerebrospinal fluid proteins

Materials and equipment

TRIS-barbitalate buffer (pH = 9.2, I = 0.06 mol/l) or
TRIS-taurinate buffer (pH = 9.0, I = 0.05 mol/l) or
TRIS-glycinate buffer (pH = 9.5, I = 0.025 mol/l)
Coomassie brilliant blue R-250
Methanol
Acetic acid
Cellogel membranes
Electrophoresis cell
Power supply

Procedure

- Apply 150, 50, or 25 µl urine (protein concentration of 0.1–1, 1–3, or 3–6 g/l, respectively), or 150 µl (0.1–1 g/l protein) cerebrospinal fluid.
- Fill the cell tanks with TRIS-barbitalate, TRIS-taurinate or TRIS-glycinate buffer.
- Run electrophoresis at 240 V for 2–3 h.
- Stain in 0.5 g/dl Coomassie brilliant blue R-250 in methanol-acetic acid-water (3:1:6, *V:V:V*) for 10 min.
- Destain the Cellogel background 3 times in methanol-acetic acid-water (3:1:6, *V:V:V*).

2.1.4 Troubleshooting

Problem	Cause	Solution
Prior to electrophoresis		
The surface of the CA membrane or Cellogel strip is spotty.	The drying of cellulose acetate membrane or Cellogel strip has taken too long.	Shorten the drying.
During electrophoresis		
It does not flow or flows too little electric current.	One of the connectors has no or poor contact. There is poor contact between the CA membrane or Cellogel strip, and the electrode buffer.	Check all connections. Check the position of the CA membrane or Cellogel strip.
After electrophoresis		
There are no protein bands on the CA membrane or Cellogel strip.	The proteins have left the CA membrane or Cellogel strip. The proteins have precipitated on the application site.	Monitor the running of the dye front. Let the electrophoresis run in another buffer with a different pH value.
The protein bands are very weak.	The protein concentration was too low. The proteins in the sample were not fully dissolved. The proteins were at their pI values.	Concentrate the sample or apply larger volume. Treat the sample with ultrasound; centrifuge, if opaque; use additives to dissolve the solid components in it. Use a buffer with a different pH value.
Protein bands have built tails.	The protein concentration in the sample was too high. The sample contained solid components.	Apply smaller sample volume or dilute the sample. Prior to applying, centrifuge the sample.
The samples in the adjacent tracks run into one another.	The sample volume was too large.	Concentrate the samples and apply less volume.

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2.2 Agarose gel electrophoresis of proteins

In contrast to the polyacrylamide gels, the agarose gels have large pores. Therefore, they are suitable for separation of native proteins with very high masses [1]. Such a protein is, for example, the complex of pyruvate dehydrogenase [2] that has M_r of $10 \bullet 10^6$. In addition, the agarose gels are simpler in production than the polyacrylamide gels; are nontoxic; and the separation time on them is only 20–30 min. Also, it is possible to produce composite gels of agarose and crosslinked [3,4,5] or linear polyacrylamide gel [6], which can be used for preparative electrophoresis.

The agarose gel electrophoresis is used in research and especially in diagnostics.

2.2.1 Theory of agarose gel electrophoresis of proteins

The theory of agarose gel electrophoresis of proteins [7,8] does not differ from the general theory of agarose gel electrophoresis: In agarose gels, two opposite movements exist: movement of proteins and movement of water (electroosmosis). As a result, α_1-, α_2-, and β-globulins are moving to the anode (in front to the start line), while y-globulins are moving back to the cathode (behind the start line). The electroosmosis helps to separate proteins with small total electric charge.

Agarose gels are used generally in concentrations of 0.7–1.2 g/dl. In 1 g/dl agarose gel, proteins with M_r up to $50 \bullet 10^6$ and with radii of up to 30 nm can be separated. This means that they are suitable for resolving proteins whose radii are equal to 5–10 nm, membrane proteins of high molecular masses [9], and viruses [10].

The most common electrophoresis on agarose gels is the zone electrophoresis, which is run in various buffers [11,12]. Among them, barbitalate buffers (veronalate buffers) are most used. However, the usage of barbituric acid derivatives has been restricted by Medicines Act [13]. Therefore, buffers containing no barbituric acid compounds are preferred, for example, TRIS-taurinate buffer [14].

TRIS-taurinate buffer, pH = 8.5, *I* = 0.10 mol/l	
TRIS	34.16 g
Taurine	48.94 g
NaN_3	0.10 g
Deionized water to	1,000.00 ml

Another suitable buffer for agarose electrophoresis is the TRIS-borate buffer. However, boric acid forms complex compounds with agarose. Therefore, this buffer should not be recommended.

Usually, the agarose gels are cast in the following way: 1.0 g agarose is suspended in 100 ml buffer and boiled until it melts. Then the agarose solution is cast onto a support film, located in a casting cassette or lying on the table. After agarose gels are formed, they can be stored in a damp refrigerator to stabilize their structure.

Horizontal agarose gel electrophoresis is a standard method for separation of proteins in serum, cerebrospinal fluid, urine, and other fluids.

2.2.2 Agarose gel electrophoresis of serum proteins

Blood serum contains many different proteins (Figure. 2.2-1).

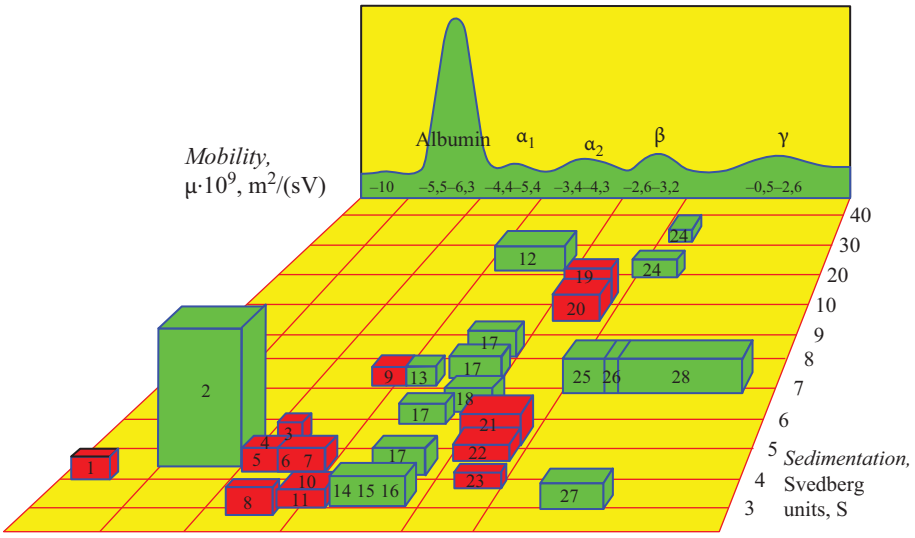


Figure 2.2-1: Normal serum proteins, without lipoproteins, according to Schultze HE and Heremans JF (*Molecular Biology of Human Proteins*. Elsevier Publ. Co., Amsterdam – London – New York, 1966, vol. 1). Modified.
1. Transthyretin (prealbumin); 2. Albumin; 3. 4.6S Postalbumin; 4. Prothrombin; 5. Easy precipitating α_1-glycoprotein; 6. α_{1X}-Glycoprotein; 7. α_1-Antitrypsin; 8. α_1-Acidic glycoprotein; 9. α_1-α_2-Antitrypsin; 10. Tyroxin-binding globulin; 11. α_{GC}-Globulin; 12. α_{2M}-Macroglobulin; 13. Ceruloplasmin; 14. Zn-α_2-Glycoprotein; 15. α_{2HS}-Glycoprotein; 16. α_2-Neuraminoglycoprotein; 17. Haptoglobins; 18. β_{1A}-Globulin; 19. β_{1E}-Globulin; 20. β_{1C}-Globulin; 21. Transferrin; 22. Hemopexin; 23. Plasminogen; 24. Immunoglobulin M; 25. Immunoglobulin A; 26. Immunoglobulin D; 27. β_2-Glycoprotein; 28. Immunoglobulin G

The **s**erum **p**rotein **e**lectro**p**horesis (SPEP) is a method for separating serum proteins. With its help, five main groups (fractions) of serum proteins are obtained: albumin, α_1-globulins, α_2-globulins, β-globulins, and y-globulins (Figure 2.2-2). The ratio between the mass concentrations of albumin and globulins is 1.5–3:1.

After electrophoresis, the separated protein bands can be analyzed visually (comparing them with control pherograms), with the help of scan programs, or densitometrically at 605 nm. Using scan programs or densitometers, the relative and absolute concentrations of the resolved proteins can be calculated.

The high-resolution agarose gel electrophoresis gives twice serum protein fractions as the cellulose acetate electrophoresis (Figure 2.2-3).

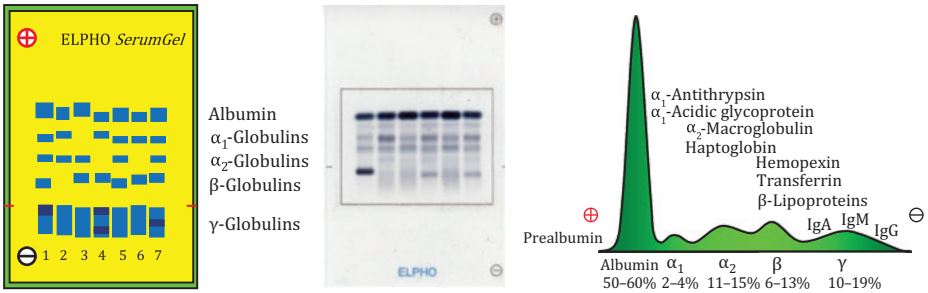


Figure 2.2-2: Pherogram of serum proteins obtained by agarose gel electrophoresis.

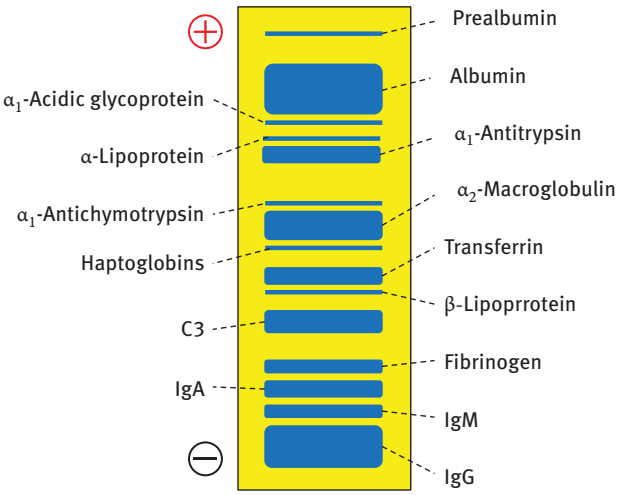


Figure 2.2-3: Pherogram obtained by high-resolution agarose gel electrophoresis.

The serum protein electrophoresis can be used to diagnose different diseases, for example, multiple myeloma, macroglobulinemia, amyloidosis, hypogammaglobulinemia, and more.

2.2.2.1 Albumin

Albumin, along with other 60–70 % of serum proteins, is synthesized in the liver. Its relative molecular mass is approximately 69,000, and its isoelectric point is 4.9.

Albumin has two main functions:

- It determines the osmotic pressure in the blood and as a result regulates the water exchange between the blood and intercellular fluid; and
- It transports different substances, such as free fatty acids, hormones, pigments, and drugs.

Pathological conditions connected with changes in the albumin concentration are: *bisalbuminemia*, characterized with two albumin bands; and *analbuminemia*, characterized with reduction or even absence of the albumin band. Decreased albumin concentration is also observed in nephrotic syndrome (Figure 2.2-4) where albumin passes easily through the damaged glomerular membrane into the urine, due to its low molecular mass. The albumin concentration is low in liver diseases, malnutrition, nutrient absorption disorders, and loss of protein in enteropathy, too. Increased albumin concentration can be observed in acute alcoholism, during pregnancy, and in puberty. Before albumin, *transthyretin* (prealbumin) is moving. It transfers the hormone thyroxine. The concentration of transthyretin is too low, therefore it is usually left undetermined when using common staining methods. Mutations of transthyretin cause familiar amyloidosis.

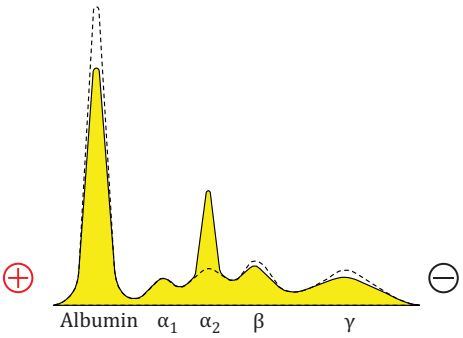


Figure 2.2-4: Nephrotic syndrome. The concentration of α_2-globulins is increased; the concentration of albumin is decreased.

Intermediate zone of albumin-α_1-globulins

The intermediate zone of albumin-α_1-globulins contains α_1-lipoproteins (high density lipoprotein) and α-fetoprotein. The concentration of α_1-lipoproteins is decreased in severe inflammation, acute hepatitis, and liver cirrhosis; the concentration of α-fetoprotein is increased in hepatocellular carcinoma – then a sharp band appears between albumin and α_1-globulins.

2.2.2.2 Alpha-1-globulins

α_1-Globulins constitute the lowest main fraction – only 1–4 % of the serum proteins. α_1-Globulins are: α_1-antitrypsin, α_1-lipoproteins, acidic α_1-glycoprotein, thyroxin-binding globulin, prothrombin, and more.

α_1-Antitrypsin (M_r = 54,000) inhibits trypsin and forms the bulk of α_1-globulins. It has a sulfhydryl group which can bind to thiol compounds. The concentration of α_1-antitrypsin is too low in juvenile pulmonary emphysema and decreases in nephrotic syndrome. Its low concentration in lung emphysema is due to the pulmonary

tissue destruction by neutrophil elastase. Alpha-1-antitrypsin is a protein of the acute phase of inflammation, therefore its concentration increases in acute inflammation.

α_1-Lipoproteins (α-lipoproteins, HDL, M_r = 200,000) transport phospholipids, cholesterol, triacylglycerols, fat soluble vitamins, as A and E, and some hormones.

Acidic α_1-glycoprotein (M_r = 44,100), also called orosomucoid, is an acute phase protein. Orosomucoid and α_1-antitrypsin move together, but orosomucoid is more stained. Its concentration increases in chronic inflammations as well as in some malignancies.

Thyroxin-binding globulin (M_r = 40,000) transfers thyroxine.

Prothrombin (M_r = 68,500) transforms into thrombin in the clotting process.

The concentration of α_1- and α_2-globulins increases in malignant tumors while the concentration of albumin decreases (Figure 2.2-5).

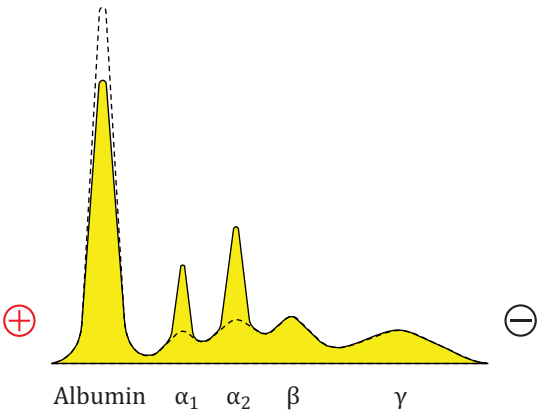


Figure 2.2-5: Increased concentrations of α_1- and α_2-globulins in malignant tumors. The albumin concentration decreases.

Intermediate zone of α_1-α_2-globulins

In the intermediate zone of α_1-α_2-globulins, two pale picks can be seen, which are α_1-antichymotrypsin and vitamin-D-binding protein. α_1-Antichymotrypsin is an acute phase protein and therefore its concentration increases in acute inflammation.

2.2.2.3 Alpha-2-globulins

α_2-Globulins contain 7–12% of total serum proteins. Their most important representatives are: α_2-macroglobulin, haptoglobins, α_2-lipoproteins, and ceruloplasmin.

α_2-Macroglobulin (M_r = 720,000) is an acute phase protein that inhibits plasmin and trypsin and binds to insulin. Its concentration increases in nephrotic syndrome and acute inflammation. The large volume of α_2-macroglobulin molecule does not

allow passing through the glomerular membrane whereas the other proteins pass into the urine. Therefore, its concentration increases significantly (10 times and more) in nephrotic syndrome. It is also slightly elevated at the beginning of diabetic nephropathy.

Haptoglobins (M_r = 100,000) bind to hemoglobins after destroying red blood cells and thus prevent loss of iron. They represent the largest part of the acute phase proteins and lead to an increase in the α_2-globulin concentration in acute inflammation (Figure 2.2-6). On the contrary, their concentration decreases in hemolytic anemia.

α_2-Lipoproteins (prebeta-lipoproteins, VLDL, M_r = 5–20•10^6) transport lipids, mainly triacylglycerols.

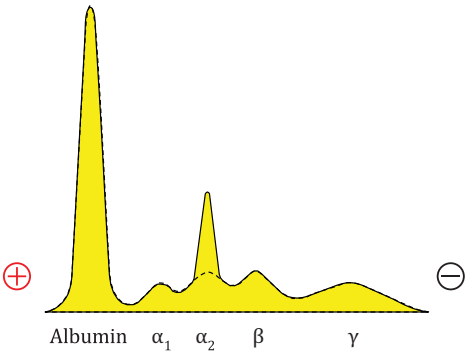


Figure 2.2-6: Increased concentration of α_2-globulins in acute inflammation.

Ceruloplasmin (M_r = 132,000) is an oxidase that takes place in copper metabolism. It is also an acute phase protein.

The concentration of α_2- and α_1-zone increases in patients with malignant tumors and cirrhosis, while the albumin concentration decreases.

Intermediate zone of α_2-β-globulins

This zone contains the prebeta-lipoproteins. Their concentration is increased in type II hypercholesterolemia, hypertriglyceridemia, and nephrotic syndrome. Haptoglobin-hemoglobin complexes are also present. They move in front of haptoglobins.

2.2.2.4 Beta-globulins

The relative concentration of β-globulins is 6–10 % of serum proteins. Most important among them are: hemopexin, transferrin, β-lipoproteins, plasminogen, complement components, and fibrinogen (in plasma).

Hemopexin (M_r = 80,000) binds to heme of hemoglobins.

Transferrin (M_r = 80,000) transfers iron. It is an acute phase protein.

β-Lipoproteins (LDL, M_r = 2.4•10^6) transport lipids, fat-soluble vitamins, and hormones.

When activated, plasminogen (M_r = 143,000) destroys fibrin in the blood clot.

The complement components are glycoproteins.

Fibrinogen (M_r = 341,000) is an important factor of the blood clotting system.

The concentration of β-globulins increases in liver cirrhosis, together with that of y-globulins (β-y-bridge) (Figure 2.2-7).

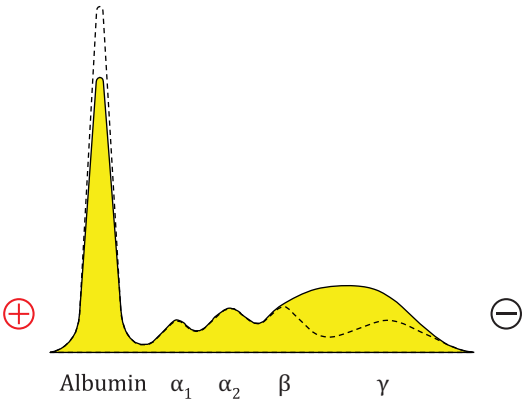


Figure 2.2-7: Beta-gamma bridge in liver cirrhosis.

In more precise agarose gel electrophoresis, β-globulins are separated in two fractions: β_1 and β_2.

Beta-1-globulins

Beta-1-globulins are presented predominantly by transferrin and β-lipoproteins. Increased concentration of transferrin is observed in iron-deficiency anemias, pregnancy, and estrogen therapy. An increased concentration of β-lipoproteins is found in hypercholesterolemia.

Beta-2-globulins

β_2-Globulins are presented predominantly by the **c**omplement component **3** (C3) whose concentration increases during the acute phase of inflammation. On the contrary, their concentration decreases in autoimmune diseases since C3 is bound to immune complexes and removed from blood plasma. Fibrinogen also moves with β_2-globulins, but is absent in the normal serum.

Intermediate zone of β-γ-globulins

In the intermediate zone of β-γ-globulins, C reactive protein (an acute phase protein) is present. When its concentration is high, β-γ-fusion (β-γ-bridge) is monitored.

2.2.2.5 Gamma-globulins

γ-Globulins (immunoglobulins) represent 10–17 % of the total serum protein mass. There are 5 classes of **i**mmuno**g**lobulins (Ig): IgG, IgA, IgM, IgD, and IgE.

IgG (M_r = 160,000) bind specifically to antigens of viral, bacterial or parasite origin, also to bacterial toxins, Rh antibodies, nucleic acids, insulin, and so on.

IgA is located in serum as well in secretions. Serum IgA (M_r = 160,000 to 500,000) are the anti-bacterial agglutinins, anti-nuclear, and anti-insulin antibodies. They move the fastest of all antibodies and therefore are always found in the beginning of gamma zone. When their concentration is increased, IgA can lead to formation of a β-γ-bridge (s. above). Their concentration is high in patients with cirrhosis, respiratory infections, skin disease, or rheumatoid arthritis.

IgM (M_r = 900,000) include antibodies such as ABO isoagglutinines, antibacterial antibodies, Wassermann antibody, anti-thyroglobulin antibodies, and more.

The function of IgD (M_r = 150,000) is still not known.

IgE (M_r = 200,000), known as reagins, play a key role in some allergic diseases, such as hay fever and bronchial asthma.

The concentration of γ-globulins is increased in viral hepatitis (Figure 2.2-8) as well as in multiple sclerosis. A spike-like enlargement of the gamma zone is typical for monoclonal gammopathy; a large increase accompanies the polyclonal gammopathy.

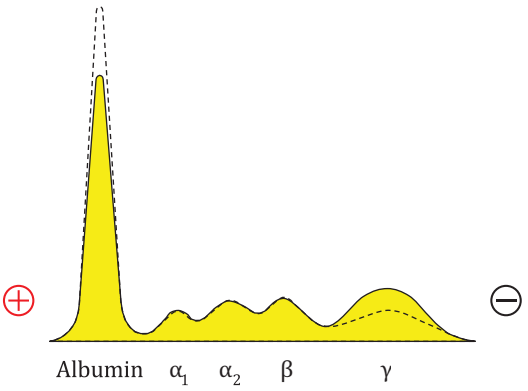


Figure 2.2-8: Increased concentration of γ-globulins and decreased concentration of albumin in virus hepatitis.

The narrow spike in monoclonal gammopathy (Figure 2.2-9) is known as *M spike*. It is malignant or clonal; myeloma is the most common reason of IgA and IgG spikes. Chronic lymphatic leukemia and lymphosarcoma typically occur with an increase of IgM-paraprotein concentration. Nevertheless, there may be up to 8 % of healthy adult patients with monoclonal spikes. Waldenström's macroglobulinemia, amyloidosis, and solitary plasmacytomas are also characterized with M spikes.

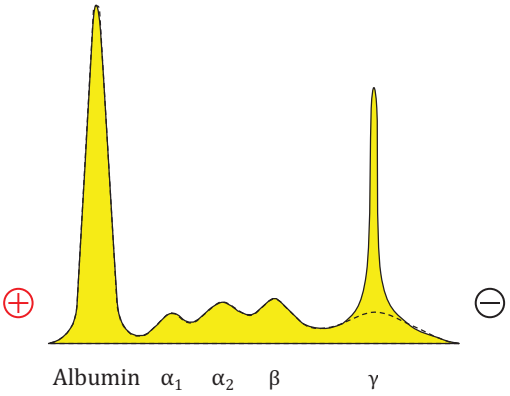


Figure 2.2-9: Increased concentration of immunoglobulins in monoclonal gammopathia.

Polyclonal gammopathy is usually a benign condition. It is typical during severe infections, chronic liver disease, rheumatoid arthritis, systemic lupus, and other connective tissue diseases.

Sometimes lysozyme can be obtained as a narrow band after the gamma-proteins in myelomacytic leukemia.

The reduction of the gamma-zone is called hypogammaglobulinemia. It is normal for infants, but is a symptom for patients with X-linked agammaglobulinemia. In viral hepatitis, the concentration of *y*-globulins is increased, while the concentration of albumin is decreased.

2.2.3 Agarose gel electrophoresis of lipoproteins

Lipoproteins are complexes of lipids and proteins. The lipids are represented by fatty acids, triacylglycerols, free and esterified cholesterol, phospholipids, and sphingolipids. They have exogenous origin, that is, they originate from food, or have endogenous origin, that is, they are synthesized in the body.

The lipoproteins are separated by centrifugation and electrophoresis in 4 density classes: high density lipoproteins, very low density lipoproteins, low density lipoproteins, and chylomicrons (Figure 2.2-10). In addition, intermediate density

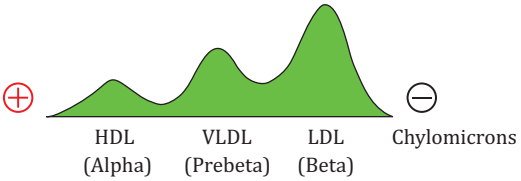


Figure 2.2-10: Normal lipoprotein pherogram.

lipoproteins are distinguished. The proteins of the lipoproteins are named apolipoproteins, and are derived in a few groups – from A to E.

Lipoproteins are water-soluble or fat-soluble. The water-soluble lipoproteins are found in the body fluids (blood, cerebrospinal fluid, and tissue fluid); the fat-soluble lipoproteins are located in the cell membranes (plasma membranes, mitochondrial, lysosomal, and nuclear membranes). The serum lipoproteins are synthesized in liver cells.

The characteristics of lipoproteins are represented in Table 2.2-1.

Table 2.2-1: Characteristics of lipoproteins.

Characteristics	HDL	VLDL	LDL	Chylomicrons
Density (g/ml)	1.210–1.063	1.006	1.063–1.006	1.006
Diameters, nm	7–10	200	10–30	200
Proteins (%)	49	2–13	32	0.5
Triacylglycerols (%)	7	64–80	7	90
Cholesterol (%)	17	8–13	35	6
Phospholipids (%)	27	6–15	25	4

2.2.3.1 High-density lipoproteins

High-**d**ensity **l**ipoproteins (HDL, α-lipoproteins) build the fastest lipoprotein fraction. They contain proteins and phospholipids, both in relatively high concentrations. The synthesis of HDL takes place in the mitochondria and microsomes.

2.2.3.2 Low-density lipoproteins

Low-**d**ensity **l**ipoproteins (LDL, β-lipoproteins) build the largest cholesterol-containing fraction. They move behind the pre-β-lipoproteins. Cholesterol is a widespread steroid in the human body. It is located in the cell membranes. From cholesterol are synthesized bile acids, steroid hormones, and steroid vitamins.

The food seems to be the main source of cholesterol [15,16]. The absorbed cholesterol-esters from the food are transported to the liver, where cholesterol is set free. The free cholesterol is bound to proteins and delivered in the blood as β-lipoproteins.

2.2.3.3 Intermediate-density lipoproteins

Intermediate-**d**ensity **l**ipoproteins (IDL) are formed during the degradation of very low-density lipoproteins. Their name refers to their density being between that of low-density and very low-density lipoproteins. IDL are similar to the low-density lipoproteins and consist of proteins that encircle triacylglycerols and cholesterol esters. Their size is 25 to 35 nm in diameter. IDL bind to receptors in the plasma membrane of liver cells, and with the aid of endocytosis enter the cells where are degraded to form LDL.

IDL, like LDL, can promote the growth of atheroma.

2.2.3.4 Very low-density lipoproteins

Very **l**ow-**d**ensity **l**ipoproteins (VLDL, pre-β-lipoproteins) carry preferably triacylglycerols of endogenous origin. They move after the α-lipoproteins.

2.2.3.5 Chylomicrons

The chylomicrons are composed mainly of exogenous triacylglycerols obtained from the food. They are composed of a lipid droplet surrounded by a thin protein network and phospholipid molecules. The chylomicrons do not move in an electric field.

The triacylglycerols represent glycerol esterified with different fatty acids. The fatty acids of the human body are composed usually by 18 straight-chain carbon atoms, containing one or more double bonds and many single bonds. In the blood, the free fatty acids are bound to albumin and serve as a raw material for the synthesis of endogenous triacylglycerols in the liver [17].

2.2.3.6 Hyperlipoproteinemias

Hyperlipoproteinemias are diseases that are characterized with increased concentration of lipoproteins. They are subdivided in 5 types [18].

Hyperlipoproteinemia type I (hyperchylomicronemia) is characterized by high concentration of chylomicrons. In electrophoresis, a strong chylomicron fraction is observed at the application site, LDL and VLDL are weakly visible, and HDL remain usually unchanged (Figure 2.2-11).

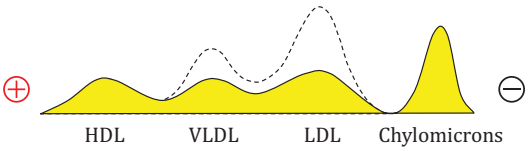


Figure 2.2-11: Hyperlipoproteinemia type I.

In hyperlipoproteinemia type II (hyper-LDL-emia), the concentration of LDL (cholesterol) is increased. The HDL-band is normal. The hyperlipoproteinemia type II is subdivided in two subtypes: type IIa and type IIb. The pherogram of type IIa shows an increased LDL fraction, whereas the VLDL fraction is usually normal. In type IIb, the concentration of LDL as well as of VLDL is increased (Figure 2.2-12).

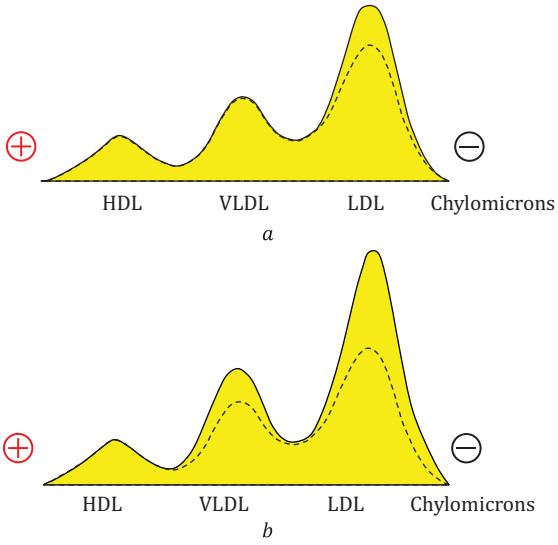


Figure 2.2-12: Hyperlipoproteinemia type IIa (*a*) and type IIb (*b*).

Hyperlipoproteinemia type III (hyper-LDL-hyper-VLDL-emia) is characterized by a broad band over the LDL- and VLDL-fractions (increased cholesterol and triacylglycerols), and a normal α-band fraction (Figure 2.2-13).

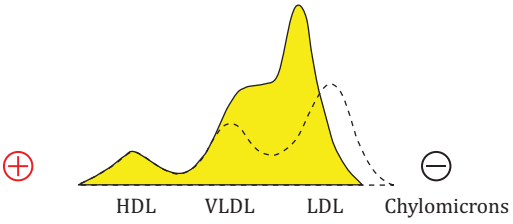


Figure 2.2-13: Hyperlipoproteinemia type III.

The hyperlipoproteinemia type IV (hyper-VLDL-emia) is accompanied by an increased VLDL-fraction. The pherogram shows normal LDL- and HDL-bands (Figure 2.2-14).

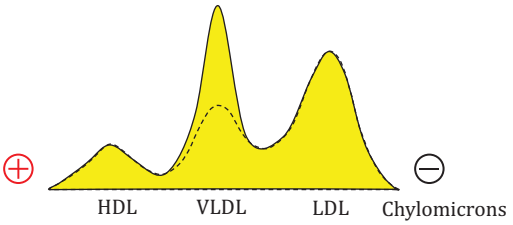


Figure 2.2-14: Hyperlipoproteinemia type IV.

In hyperlipoproteinemia type V (hyper-VLDL-emia and hyperchylomicronemia), a simultaneous increase in the concentration of the exogenous triacylglycerols (chylomicrons) as well as that of the endogenous triacylglycerols (VLDL-fraction) is established (Figure 2.2-15).

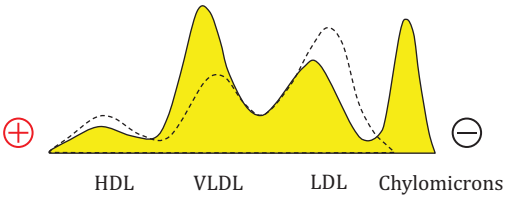


Figure 2.2-15: Hyperlipoproteinemia type V.

2.2.4 Agarose gel electrophoresis of hemoglobins

Electrophoresis, as well as chromatography, gives best information for **h**emoglo**b**ins (Hb). The hemoglobin electrophoresis is carried out in all media, most often in cellulose acetate membranes and agarose gels. Exceptionally good results are obtained using isoelectric focusing on polyacrylamide gel [19,20].

2.2.4.1 Normal hemoglobins

In embryo, the following hemoglobins: Gower 1 ($\zeta_2\varepsilon_2$), Gower 2 ($\alpha_2\varepsilon_2$), Portland I ($\zeta_2\gamma_2$), and Portland II ($\zeta_2\beta_2$) can be established. In fetus, hemoglobin F ($\alpha_2\gamma_2$) can be found [21]. In adults, three types of hemoglobins exist: hemoglobin A (HbA), hemoglobin A_2 (HbA_2), and fetal hemoglobin F (HbF).

Hemoglobin A is a tetramer with M_r = 67,000 that is composed of 4 polypeptide chains (globin chains), each of them with M_r = 17,000, and 4 iron containing prosthetic groups (hemes). The polypeptide chains are designated as α- and β-chains. So, the HbA formula is $\alpha_2\beta_2$. The tertiary structure of every hemoglobin subunit looks like that of myoglobin because they are genetically related.

HbA makes over 95 % of the total hemoglobin in a healthy adult. The rest of 2 % consists of HbA_2 and HbF (Figure 2.2-16). HbF dominates during the fetal

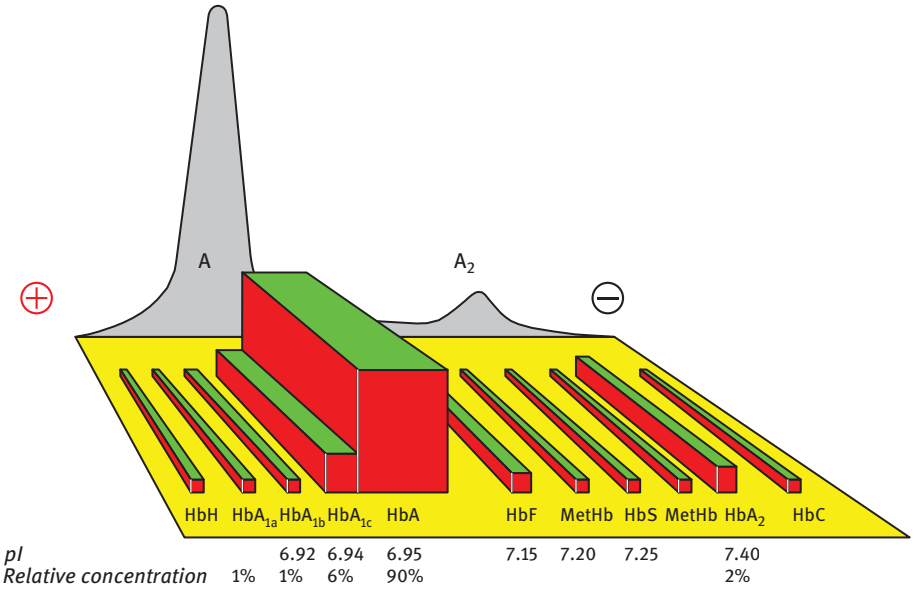


Figure 2.2-16: Hemoglobins.

development, however is replaced later by HbA. The formulas of HbA_2 and HbF are $\alpha_2\delta_2$ and $\alpha_2\gamma_2$, respectively. The synthesis of δ chain of HbA_2 begins in the third trimester. In adults, HbF is restricted in a limited population of red cells called F-cells. The concentration of Hb F is increased in persons with sickle-cell disease and β-thalassemia.

The α-polypeptide chain of all hemoglobins consists of 141 amino acid residues and contains many acidic groups, whereas the non-α-polypeptide chains (β, γ, or δ) consist of 146 amino acid residues and contain few acidic groups. The hemoglobin concentration is 12.8–17.6 g/dl (in average 15 g/dl) in men, and 11.2–16.0 g/dl (in average 14 g/dl) in women.

HbA is partially glycosylated. The glycosylated hemoglobin, called HbA_1, constitutes 8 % of the total hemoglobin mass in the blood. It consists of HbA and glucose that is bound non-enzymatically to the valine residue at the N-end of one of the two polypeptide chains.

There are 3 types of HbA_1: HbA_{1a}, HbA_{1b}, and HbA_{1c}. In healthy people, HbA_{1c} constitutes 6 % of the total hemoglobin, but its concentration is increased several times in patients with diabetes mellitus [22,23]. The concentration of HbA_{1c} reflects the average glucose concentration in the weeks before testing [24,25], whereas direct methods determine the glucose concentration just during the testing time. The concentrations of HbA_{1a} and HbA_{1b} are about 1 g/dl each.

The hem consists of a protoporphyrin residue and a bivalent iron ion (Fe^{2+}).

2.2.4.2 Abnormal and pathological hemoglobins

The globin chains are characterized by their sequence of amino acid residues. If, due to errors in the genetic code, the sequence of the amino acid residues is incorrect, abnormal hemoglobins (hemoglobin variants) are formed. If they do not cause disorders, they are classified as abnormal hemoglobins; if they cause illnesses (hemoglobinopathias), they are called pathological hemoglobins [26].

The most widespread pathological hemoglobins are:

- Hemoglobin D-Punjab ($\alpha_2\beta^{D}{}_2$).
- Hemoglobin H (β_4), formed by a tetramer of β-chains; present in variants of α-thalassemia.
- Hemoglobin Barts (y_4), formed by a tetramer of y-chains; present in variants of α-thalassemia.
- Hemoglobin S ($\alpha_2\beta^{S}{}_2$), a product of a variation in the β-chain gene; found in people with sickle cell disease; causes sickling of red blood cells.
- Hemoglobin C ($\alpha_2\beta^{C}{}_2$), a product of a variation in the β-chain gene; causes a mild chronic hemolytic anemia.
- Hemoglobin E ($\alpha_2\beta^{E}{}_2$), a product of a variation in the β-chain gene; causes a mild chronic hemolytic anemia. It is characterized by an exchange of glutamic acid residue at position 26 of the β-chain with lysine residue. Hemoglobin E is very common among people in Southeast Asia (Myanmar, Cambodia, Laos, and Vietnam) where its prevalence can reach 30 to 40%; and in Northeast India where in certain areas up to 60 % of the population is ill. In Thailand the mutation can reach 50 to 70 %. In Sri Lanka, it can reach up to 40 %. It is found also in Bangladesh and Indonesia. In Europe, families with hemoglobin E were also found, but the European mutation differs from the mutation found in Southeast Asia. This means that the β^{E} mutations have different origin [27,28].
- Hemoglobin SC, a complex heterozygous form with one sickle gene and another encoding HbC gene.
- Hemoglobin Hopkins-2 [29], a variant form of hemoglobin that is sometimes established in combination with HbS in sickle cell disease.

Sickle cell anemia

Sickle cell anemia (sickle cell disease) was first described by Ernest Irons and James Herrick in 1910 [30]. In 1933 Lemuel Diggs and in 1949 J. Neel and E. Beet [31] established its genetic characteristics. In 1949, Linus Pauling [32] invented the unusual hemoglobin S, and explained the illness with an abnormality in its molecule. The actual molecular change in HbS was described in the late 1950s by Vernon Ingram [33]. It has, because of a point mutation, a valine residue instead of glutamic acid residue at position 6 of the β-chain.

HBB gene is responsible for sickle-cell anemia. It is located on the short (p) arm of chromosome 11 at position 15.5. Persons who receive the defective gene from

both father and mother (homozygote persons) develop the disease. Persons who receive one defective and one healthy gene (heterozygote persons) remain healthy. They have normal blood, because they synthesize normal HbA. However, if the oxygen pressure decreases to less than 65 % O_2 saturation, the erythrocytes with HbS deform to give sickle cells.

The homozygote patients with hemoglobin S have a hemoglobin concentration of 6–10 g/dl. Their pherogram shows disturbed synthesis of HbA, compensatory increased synthesis of HbF, and high synthesis of HbS (Figure 2.2-17).

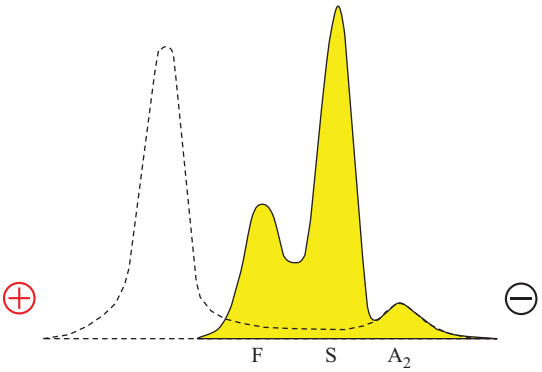


Figure 2.2-17: Sickle cell anemia.

In 1950 another pathological hemoglobin was identified – hemoglobin C [34] (Figure 2.2-16). Its glutamic acid residue at position 6 of the β-chain is exchanged against a lysine residue. This anemia is accompanied by small hematological disturbances. Hemoglobin E was later identified along with hundreds of other hemoglobin variants.

Thalassemias

Thalassemias are diseases in which the synthesis of globin chains of HbA is disturbed. Instead HbA, the body produces the globin chains of HbA_2 or HbF. Thalassemias occur more frequently than the hemoglobinopathies. The heterozygous thalassemia is called *thalassemia minor*. It is characterized by a mild anemia and a light increase of the concentrations of HbA_2 or HbF. The homozygous thalassemia is called *thalassemia major*. It is a serious disorder that is widespread in the Mediterranean. A middle position takes *thalassemia intermedia*.

Alpha-thalassemia. In α-thalassemias, the synthesis of α-chain of HbA is missing or is disturbed. This causes a compensatory excess of β-chains in adults or y-chains in newborns, which leads to formation of tetramers β_4 (HbH) or y_4 (Hb Barts) in blood. These homotetramers are not useful in the body because they, unlike the heterotetramer, have a very high affinity to oxygen and as a result cannot deliver it

to tissues. HbH and Hb Barts move in alkaline electrophoresis faster than HbA (Figure 2.2-18).

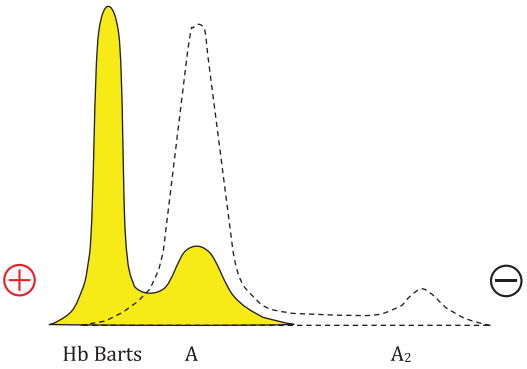


Figure 2.2-18: *α*-Thalassemia.

Beta-thalassemia. In *β*-thalassemias, the *β*-chains of HbA are not synthesized, which leads to increased production of *α*-, *y*-, or δ-chains. As a result, an excess of HbF ($\alpha_2 y_2$) or HbA_2 ($\alpha_2\delta_2$), and of tetramers y_4 or δ_4 is found in the blood.

β-Thalassemia major, called Cooley anemia, if not treated, leads to death in a few years. It is accompanied by increased concentrations of HbA_2 and HbF (Figure 2.2-19).

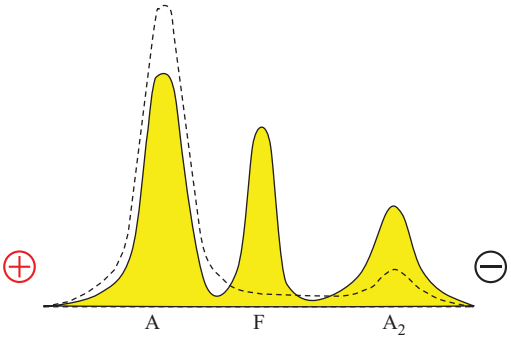


Figure 2.2-19: *β*-Thalassemia.

In addition to *β*-thalassemia major, *β*δ-thalassemia, *β*δ-fusion hemoglobinopathia (with Hb Lepore), and various forms of hereditary persistence of hemoglobin F exist. Hb Lepore is a fusion product between a *β*-chain and a δ-chain (*β*δ-chain). It has the mobility of hemoglobin S. In *β*δ-thalassemia, neither *β*-chains nor δ-chains are synthesized.

Hemoglobin E disease appears when a child inherits the gene for HbE from both parents. In the first months of life, the fetal hemoglobin disappears and the amount

of hemoglobin E increases, leading to mild β-thalassemia. People who are heterozygous for hemoglobin E (one normal allele and one abnormal allele) do not show any symptoms (there is usually no anemia or hemolysis) [35]. Patients who are homozygous for the hemoglobin E allele (have two abnormal alleles) suffer from a mild hemolytic anemia and mild enlargement of the spleen.

People who have hemoglobin E/β-thalassemia possess one gene for hemoglobin E from one parent and one gene for β-thalassemia from another parent. Hemoglobin E/β-thalassemia is a severe disease, and has no cure. It affects more than a million people in the world [36]. The symptoms of hemoglobin E/β-thalassemia are heart failure, enlargement of the liver, problems in the bones, and more.

2.2.4.3 Running hemoglobin electrophoresis

Electrophoretic separation of hemoglobins may be carried out in all separation media. Most used are cellulose acetate membranes and agarose gels because they are easy to handle and provide reliable and reproducible results.

Since the pI points of hemoglobins are at pH = 7.0, the hemoglobin electrophoresis must be performed at higher or lower pH. In alkaline buffers, hemoglobins are negatively charged and migrate toward the anode, whereas in acidic buffers they are positively charged and migrate toward the cathode (Figure 2.2-20).

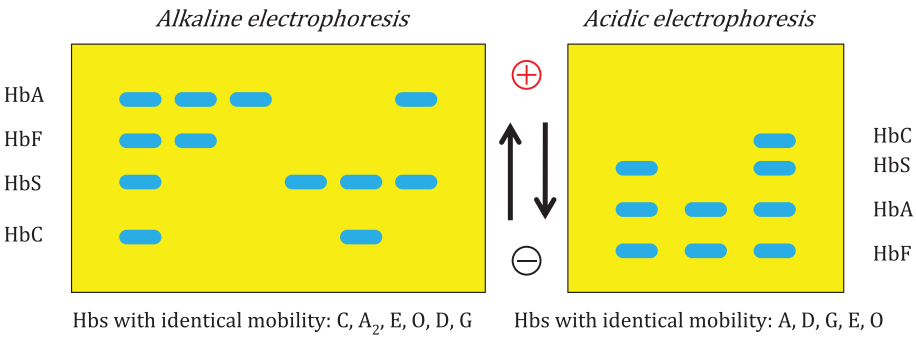


Figure 2.2-20: Hemoglobins obtained by alkaline and acidic electrophoresis.

Alkaline hemoglobin electrophoresis

In the alkaline agarose gel electrophoresis, the hemolysates are applied at the cathode. Routinely, it is carried out in TRIS-barbitalate-EDTA buffer with pH = 8.5. The results in other buffer – TRIS-taurinate-EDTA buffer (also with pH = 8.5) [37] are even better. The electrophoretic separation takes 20–30 min at 80–100 V and room temperature.

After electrophoresis, the Hb bands are fixed in a solution of CCl_3COOH and stained in a solution of Bromophenol blue Na salt in ethanol-acetic acid-water. The

background of the agarose gel (or AC membrane) is destained in ethanol-acetic acid-water.

In the alkaline hemoglobin electrophoresis, hemoglobins A, S, C, and others. can be separated.

Acidic hemoglobin electrophoresis

In the acidic agarose gel electrophoresis, the hemolysates are applied at the anode. The electrophoresis is carried out in a citrate buffer at pH = 6.1. It is used to separate hemoglobins that have equal migration velocities in alkaline electrophoresis. The electrophoresis is carried out at 50–70 V and room temperature for 20–30 min. The fixing and staining are similar to those after alkaline electrophoresis.

In the acidic electrophoresis, HbC is separated from HbE, HbS from HbD or HbG, and HbS from HbC. Using this electrophoresis, also HbF is separated from HbA, which is impossible to be done in the alkaline electrophoresis. In addition, HbA_2 and carboanhydrase are well differed from each other, the band of carboanhydrase being located in neighborhood to the application site.

2.2.5 Agarose gel electrophoresis of cerebrospinal fluid proteins

The protein concentration in the **c**erebro**s**pinal **f**luid (CSF) is about 250 times lower than that in the blood serum. Therefore, CSF must be concentrated prior to electrophoresis. After electrophoresis, the protein bands can be stained with Amido black B10 or Coomassie brilliant blue R-250 or G-250. They can also be stained with silver, if CSF is not diluted.

The CSF is produced by the choroid plexus and meninges. It represents an ultrafiltrate of the serum plasma; therefore, the CSF pherogram is similar to the serum pherogram (Figure 2.2-21). However, there are differences between both pherograms:

- The concentration of prealbumin is too high – it represents 4–5 % of the total CSF proteins.
- The concentration of albumin is not as high as in the blood serum.
- The α_1-globulins contain preferably α_1-antitrypsin and α_1-acidic glycoprotein; the α_2-globulins contain preferably 1–1 haptoglobin type and ceruloplasmin.
- The β-band is split up into two subfractions (β_1 and β_2), whereby β_1-subfraction consists mainly of transferrin and hemopexin; and β_2-subfraction is represented by a specific carbohydrate-arm transferrin called tau-protein (τ-fraction).
- The y-globulins are represented in significantly lower concentration in comparison to the serum y-globulins. They contain trace amounts of IgA and IgM. Often a post-gamma fraction of low concentration, called gamma C, exists.
- The lipoprotein concentration in the cerebrospinal fluid is extremely low.

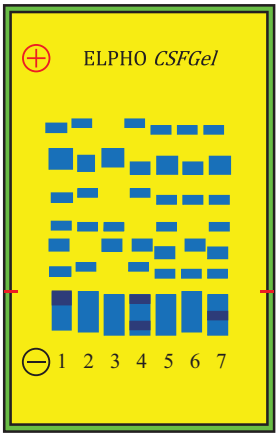


Figure 2.2-21: Electrophoretic bands of CSF proteins.

In pathological conditions, 4 types of CSF pherograms are observed: serum protein type, permeability type, gamma-globulin type, and degenerative type.

Serum protein type. The serum protein type pherogram contains serum proteins in the cerebrospinal fluid. It occurs when the concentration of serum proteins is increased, for example, in multiple myeloma characterized with increased concentrations of IgG and IgA; or in Waldenström's disease characterized with increased concentration of IgM.

Permeability type. The permeability type pherogram exists when the permeability of meningeal and choroidal capillaries is increased, for example, in acute inflammation of the nervous system and meninges – in poliomyelitis, lupus erythematosus, or chronic meningoencephalitis. In these cases, the CSF pherogram shows increased total protein concentration, for example, increased concentration of α- and γ-globulins, and presence of high molecular mass proteins that are normally absent, for example, α_2-macroglobulin, fibrinogen, and IgM.

Gamma-globulin type. Gamma-globulin type pherogram is characterized by increased concentration of CSF γ-globulins, while the concentration of the serum γ-globulins remains normal. This can be observed in chronic and subacute infectious diseases of the central nervous system, for example, in neurosyphilis, neurotuberculosis, meningoencephalitis, trypanosomiasis, chronic lymphocytic meningitis, brain abscesses, and multiple sclerosis.

Degenerative type. Degenerative type pherogram is observed in progressive neurological diseases. In this type, many proteins show different concentrations and altered electrophoretic mobilities. The concentration of β_1-transferrin is low, but the concentration of β_2-transferrin (tau-protein) is high. The altered mobilities result in unclear α-proteins and slow β-proteins.

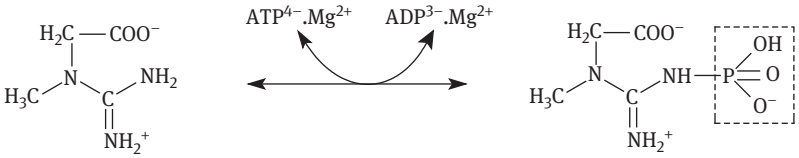
2.2.5.1 Agarose electrophoresis pattern in multiple sclerosis

Multiple **s**clerosis (MS) is probably the most common neurological disease. It is characterized by different symptoms, exacerbations, and remissions. Multiple sclerosis is a demyelinating disease, that is, a disease in which myelin is destroyed. Myelin is composed of 70 % lipids and 30 % protein. The destruction is probably caused by autoantibodies (autoimmunoglobulins).

The agarose gel zone electrophoresis of MS serum gives little diagnostic information. However, the agarose gel zone electrophoresis of cerebrospinal fluid shows an increase of the *y*-globulin concentration, although the *y*-globulin concentration in the CSF is relatively low. In addition, an oligoclonal IgG banding in the gamma aria is seen – up to seven IgG oligoclonal bands but no IgA, IgM, IgD, or IgE. This is the most typical diagnostic criterion for multiple sclerosis [38,39]. It is based on specific immunoglobulins, which are synthesized in corresponding cell clones.

2.2.6 Electrophoresis of creatine kinase

Creatine **k**inase (CK, ATP : creatine N-phosphotransferase, EC 2.7.3.2) catalyzes the reversible phosphorylation of creatine in the mitochondrial membrane, according to the following reaction:



Creatine Phosphocreatine

As a result, ATP formed in the mitochondria by oxidative phosphorylation binds to creatine to form phosphocreatine (creatine phosphate) and ADP. So the concentration of phosphocreatine in muscle cells is fivefold greater than the concentration of ATP. ATP is formed continuously in the reverse reaction. It is the energy source for the muscle contraction.

2.2.6.1 Isoenzymes of creatine kinase and their clinical significance

Creatine kinase is a dimer with M_r = 82,000, which consists of two subunits: B and M. The combination between the subunits gives three isozymes: CK-BB (CK_1), CK-BM (CK_2), and CK-MM (CK_3) [40,41]. The isoenzyme CK-BB moves in agarose or polyacrylamide gel electrophoresis with prealbumin, the isoenzyme CK-BM with the α_1-globulins, and the isoenzyme CK-MM is found in the *y*-globulin region.

The CK isoenzymes (isozymes) form high molecular mass complexes with IgG or IgA. The complex between CK-BB and IgG is a macromolecule with a molecular mass

of more than 250,000. It moves on agarose gel electrophoresis between CK-MM and CK-MB [42].

CK-BB. The isoenzyme CK-BB is contained in the cytosol of brain cells. It dominates over the other CK isoenzymes in bladder, kidney, prostate, uterus, gastrointestinal tract, lungs, placenta, spleen, liver, pancreas, and thyroid gland [43].

CK-BB is not detectable in normal adult serum. It is bound usually to an immunoglobulin forming a complex that moves close to CK-BM. There are some clinical conditions in which the CK-BB activity in the serum is greatly increased. This happens in different cancers (of prostate, breast, stomach, lungs, colon, testes, gall bladder, and in leukemia) [44]. The increased activity in serum reflects the increased concentration of CK-BB in the tumor tissues, inclusive metastases.

CK-BM. CK-BM has its highest activity in the heart muscle. It is a specific indicator of the acute myocardial infarction. The elevated serum activity of CK-BM, in addition to the increased LDH_1/LDH_2 ratio (s. below), the clinical history (previous chest pain), and the abnormal ECG (Q-wave) help diagnose this severe disease (Figure 2.2-22).

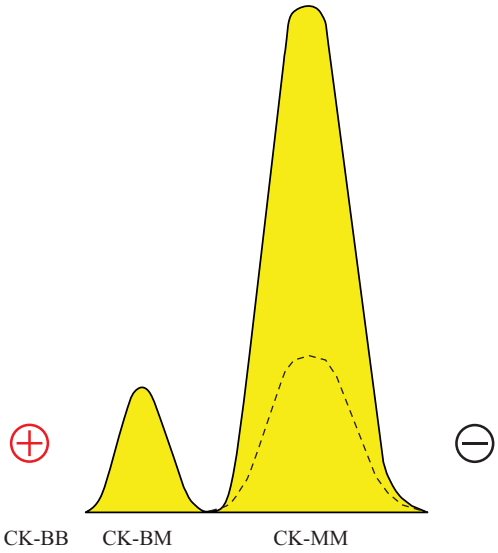


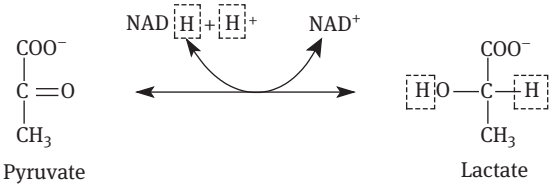
Figure 2.2-22: Increased activity of CK-BM and CK-MM in myocardial infarction.

In acute myocardial infarction, an interruption or reduction of blood supply in the coronary arteries causes anoxia and necrosis of the heart muscle. This damages the plasma membrane of the heart cells, which results in a release of CK-BM into the lymphatic system and then into the peripheral blood. The serum concentration of CK-BM increases in 4 to 8 h after the beginning of infarction, and the high concentration remains up to the 12–18th hour; then it falls down until the 32–40th hour [45].

CK-MM. CK-MM is the most widespread isoenzyme in the skeletal muscle and human serum. It makes more than 50 % of the total CK activity.

2.2.7 Electrophoresis of lactate dehydrogenase

The **l**actate **de**h**y**drogenase (LDH, L-lactate : NAD oxidoreductase, EC 1.1.1.27) is an enzyme, which is located in the cytoplasm. It catalyzes the reduction of pyruvate to lactate according to the following equilibrium, shift to lactate at pH = 7.4:



LDH is a tetramer (M_r = 135,000), which consists of two subunits: H (from ***h****eart*) and M (from ***m****uscle*). Its activity in liver, heart, kidneys, brain, skeletal muscle, and erythrocytes is about 500 times higher than in the normal serum.

2.2.7.1 Isoenzymes of lactate dehydrogenase and their clinical significance

Five LDH isoenzymes exist that can be separated from each other by electrophoresis: LDH_1 (H_4), LDH_2 (H_3M), LDH_3 (H_2M_2), LDH_4 (HM_3), and LDH_5 (M_4). LDH_1, the isoenzyme with the highest negative charge, migrates fastest (its mobility is almost equal to that of serum albumin), while LDH_5 is the slowest isoenzyme (Figure 2.2-23). It remains at cathode in the *y*-globulin zone of serum proteins. LDH_1 predominates in heart, kidney cortex, brain, and erythrocytes, while LDH_5 predominates in skeletal muscle and liver. LDH_2, LDH_3, and LDH_4 are represented in the gall bladder, prostate, and uterus.

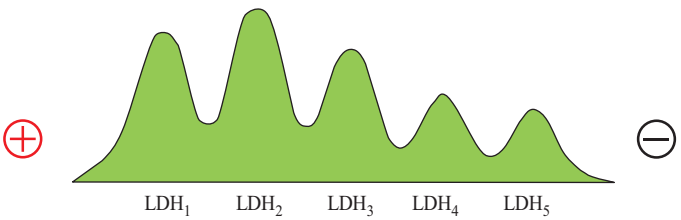


Figure 2.2-23: Normal pherogram of LDH isoenzymes.

The stability of LDH isoenzymes depends on their subunits: LDH_1 has a half-life of about 100 h, LDH_5 of about 10 h.

In the serum of a normal adult, the activity of LDH_2 is higher than that of the LDH_1, the quotient LDH_1/LDH_2 being less than 0.76. In acute myocardial infarction, LDH_1 is released from the myocardium cells into the blood. As a result, the LDH_1/LDH_2 quotient becomes in the first 24–48 h greater than 1 that may continue for days or weeks (Figure 2.2-24).

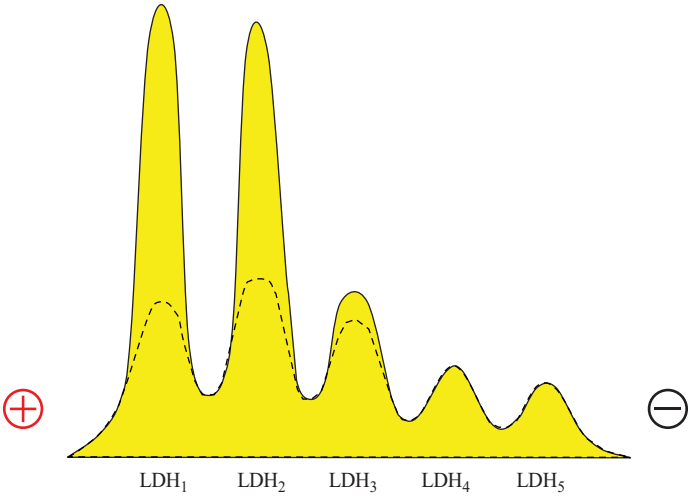


Figure 2.2-24: LDH isoenzymes in myocardial infarction.

There are more diseases in which the concentration of LDH isoenzymes increases: LDH_2 and LDH_3 in pulmonary edema (Figure 2.2-25); LDH_4 and LDH_5 in liver diseases (Figure 2.2-26); and LDH_5 in renal diseases. In malignant neoplasms, all LDH isozymes show increased activity.

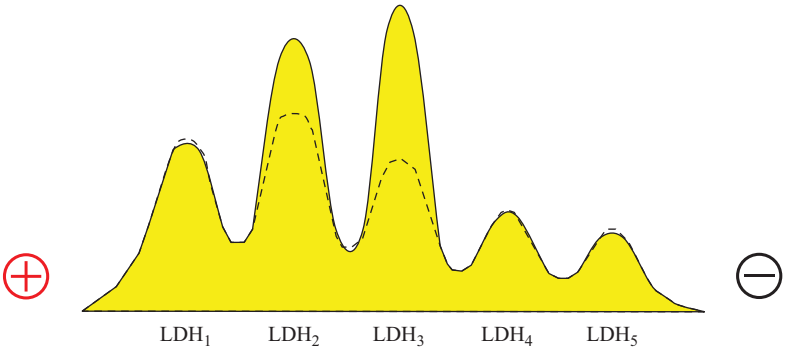


Figure 2.2-25: LDH isoenzymes in pulmonary edema.

The LDH isoenzymes form complexes with IgA and IgG. The LDH-IgA complex consists of two LDH molecules and one IgA molecule. It has a molecular mass of

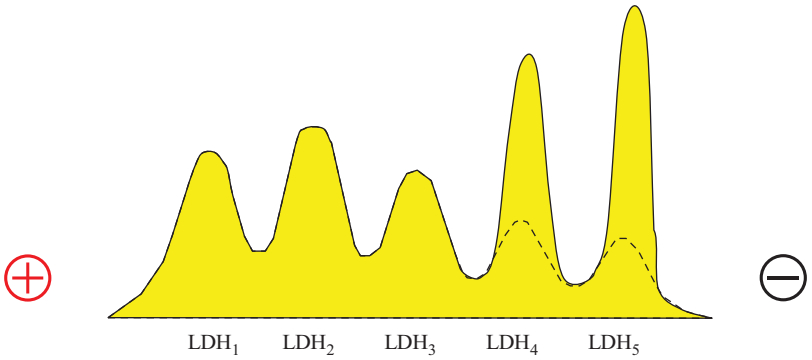


Figure 2.2-26: LDH isoenzymes in toxic hepatitis.

445,000 to 500,000 [46]. When appeared, the pherogram shows a diffuse LDH_3 band in the presence of other LDH fractions. The LDH-IgG complex has a molecular mass of 200,000 to 500,000. It shows a broad band in the LDH_4 or LDH_5 zone, and can appear in many autoimmune diseases, such as rheumatoid arthritis, chronic hepatitis, ulcerative colitis, systematic lupus erythematosus, chronic bronchitis, liver cirrhosis, pericarditis, and more.

2.2.8 Agarose gel electrophoresis of urinary proteins

Normally the urine contains no proteins, but certain diseases (e.g., multiple myeloma) cause a leakage of proteins into the urine. This pathological condition is called *proteinuria*. There are two types of proteinuria: glomerular and tubular (Figure 2.2-27).

In the *glomerular proteinuria*, serum proteins are detected in urine. They have high-molecular mass, which is greater than the albumin mass. The proteins in the glomerular proteinuria are: albumin, α_1-acidic glycoprotein, α_1-antitrypsin, α-lipoproteins, transferrin, haptoglobins, IgA, IgG, and more. β-Lipoproteins and IgM are not found in the urine [47,48].

The *tubular proteinuria* occurs rarely when the tubular absorption fails. The tubular proteins have low molecular mass, which is less than the albumin mass. Compared to the glomerular proteins, they contain much more carbohydrates, which makes them different from the serum proteins. Tubular proteins are: protein hormones [49] (gonadotropins, corticotropin, and erythropoietin), vitamin B_{12}-binding protein, blood group substances [50], retinol-binding protein, and some enzymes: lysozyme, amylase, plasmin, trypsin, pepsin, and LDH [51]. Prior to electrophoresis, these proteins must be concentrated 100-fold to reach a concentration of 2–4 mg/dl. They run before albumin [52,53] and are better separated in SDS disc-electrophoresis.

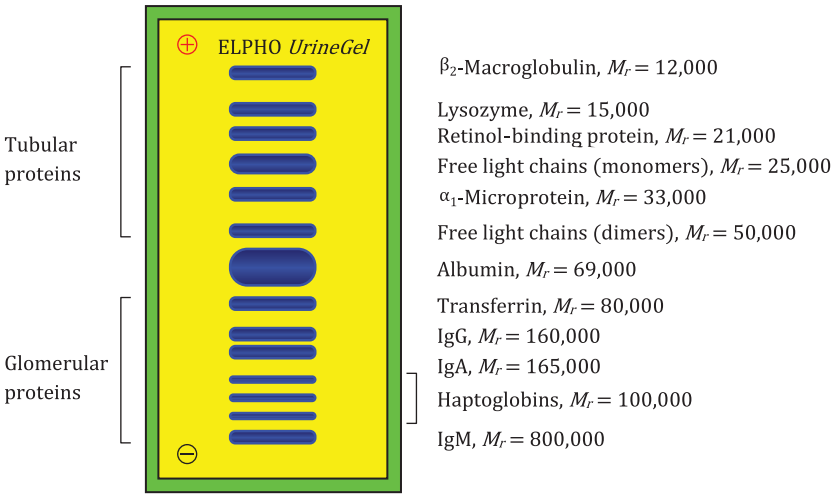


Figure 2.2-27: Pherogram of urinary proteins in patient with mixed proteinuria.

2.2.9 Semi-denaturing detergent agarose gel electrophoresis

Semi-**d**enaturating **d**etergent **a**garose **g**el **e**lectrophoresis (SDD-AGE) was created by Kryndushkin *et al.* [54] in the molecular genetics laboratory of the Russian cardiology research institute. It is used to detect and characterize large mass proteins (with M_r = 200,000–4,000,000 and more), which are stable in 2 g/dl g/dl SDS at room temperature. These proteins cannot enter a polyacrylamide gel, but enter the large pores of agarose gel. The method is useful when studying prions and amyloids [55,56,57].

The original method of SDD-AGE uses a **T**RIS-**a**cetate-**E**DTA (TAE) buffer and incorporates a modified vacuum blotting system (vacuum-assisted capillary transfer) to transfer proteins onto a PVDF membrane. Later Bagriantsev *et al.* [58] have modified this method. They used the traditional wet transfer and a **T**RIS-**g**lycinate-**E**DTA (TGE) buffer. Other methods use semidry transfer or capillary transfer [59].

2.2.10 Protocols

The agarose gels for horizontal electrophoresis (ELPHO, Nuremberg) are located between two polyester films. The support film is connected chemically to the agarose gel, whereas the cover film can be easily removed. The gels have to be stored in a refrigerator at 4 °C. Do not freeze!

2.2.10.1 Agarose gel electrophoresis of serum proteins

Materials and equipment

TRIS
NaON
Formic acid
Taurine
Methylparaben
NaN_3
Amido black 10B (Naphthol blue black)
Ethanol
Acetic acid

TRIS-formate buffer (Gel buffer), pH = 8.8, I = 10 × 0.035 mol/l

TRIS	2.77 g (0.23 mol/l)
NaOH	1.22 g (0.31 mol/l)
HCOOH	1.39 ml (0.37 mol/l)
Taurine	1.53 g (0.12 mol/l)
Methylparaben	0.30 g (0.02 mol/l)
NaN_3	1.95 g (0.30 mol/l)
Deionized water to	100.00 ml

TRIS-taurinate buffer (Electrode buffer), pH = 9.1, I = 10 × 0.06 mol/l

TRIS	1.84 g (0.15 mol/l)
NaOH	2.37 g (0.59 mol/l)
Taurine	12.26 g (0.98 mol/l)
NaN_3	0.65 g (0.10 mol/l)
Deionized water to	100.00 ml

Agarose gel (0.9 g/dl), pH = 8.8, I = 0.035 mol/l

TRIS-formate buffer, 10x	10.00 ml
Agarose ($-m_r$ = 0.16–0.19)	0.90 g
Sucrose (or sorbitol)	2.00 g
Deionized water to	100.00 ml

Bring the mixture to boil in a microwave oven and cast gels.

Electrode buffer, pH = 9.1, I = 0.06 mol/l

TRIS-taurinate buffer, 10x	10.00 ml
Deionized water to	100.00 ml

Staining solution

Amido black 10B	0.20 g
Methanol	30.00 ml
Acetic acid	10.00 ml
Deionized water to	100.00 ml

Destaining solution

Phosphoric acid	0.05 g
Deionized water to	100.00 ml

Procedure

- Unpack an agarose gel and remove its cover film.
- Blot the gel surface with a filter paper strip where the application template will be placed.
- Place a polyester application template on the blotted place according to side marks on the support film.
- Press carefully the template with fingers against the gel to remove air bubbles between the template and gel.
- Dilute serum samples 10 times with deionized water, apply 2–5 µl each in the template slots, and let them diffuse in the gel for 5 min.
- Blot the rest of the samples with a filter paper strip and remove the template together with the paper strip.
- Hang the support film with the gel, gel side down, on the bridge of an electrophoresis cell.
- Insert the bridge, together with the gel, into the electrode tanks of the electrophoresis cell, filled with electrode buffer. So the gel will contact directly with the electrode buffers (Figure 2.2-28).
- Cover the electrophoresis cell with its lid, switch on the power supply, and start the electrophoresis.
- Run electrophoresis at constant voltage of 70 V for 20–25 min.
- Fix the gel after electrophoresis in ethanol – acetic acid – water (5:1:4, *V:V:V*) for 5 min.
- Dry the gel with a hair drier or in a drying oven at room temperature.
- Stain the protein bands in the staining solution for 5 min.
- Destain the gel background several times in the destaining solution.
- Dry the gel for a second time with a hair drier or in a drying oven at room temperature.
- Evaluate the protein bands with a scanner or a densitometer at 600 nm.
  If the Amido black staining is not sufficient, the dried gels can be additionally stained with Coomassie brilliant blue R-250. So, the sensitivity of protein detection is increased 10 times.

Staining of proteins with Coomassie brilliant blue R-250

Materials and equipment

Pherogram

Coomassie **b**rilliant **b**lue **R-250** (CBB R-250)

Methanol
Acetic acid

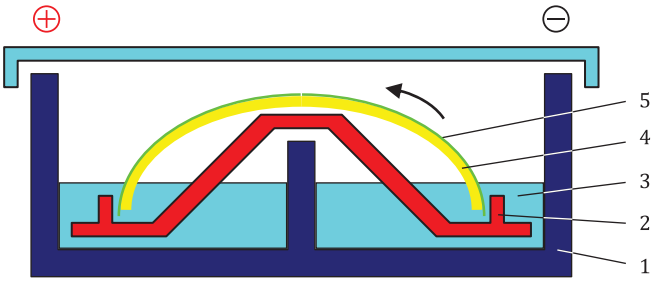


Figure 2.2-28: Direct contact between agarose gel and both electrode buffers.
1. Electrophoresis cell; 2. Bridge; 3. Electrode buffer; 4. Agarose gel; 5. Support film

Coomassie brilliant blue R-250 staining solution

Coomassie brilliant blue R-250	0.3 g
Methanol	30.0 ml
Acetic acid	10.0 ml
Deionized water to	100.0 ml

Filter.

Procedure

- Stain the protein bands in Coomassie brilliant blue R-250 staining solution for 10 min.
- Destain the background 3 times in methanol-acetic acid-water (3:1:6, *V:V:V*).
- Dry the gel or membrane at room temperature.

2.2.10.2 Agarose gel electrophoresis of lipoproteins

The agarose gel electrophoresis of lipoprotein is carried out as the agarose gel electrophoresis of serum proteins. Only the agarose gel is more concentrated (1.5 g/dl) and contains 5 g/dl bovine serum albumin.

Materials and equipment

TRIS
Formic acid
Agarose ($-m_r$ = 0.16–0.19)
Bovine **s**erum **a**lbumin (BSA)
Sucrose or sorbitol
Sudan black B
Ethanol

Agarose gel (1.5 g/dl), pH = 8.8, I = 0.035 mol/l

TRIS-formate buffer, 10x	10.0 ml
Agarose	1.5 g
5 g/dl BSA	10.0 ml
Sucrose (or sorbitol)	2.0 g
Deionized water to	100.0 ml

Bring the mixture to boil in a microwave oven and cast gels.

Electrode buffer, pH = 9.1, I = 0.06 mol/l

TRIS-taurinate buffer, 10x	10.0 ml
Deionized water to	100.0 ml

Staining solution

Sudanblack B	0.2 g
Deionized water to *Filter.*	100.0 ml

Destaining solution

Ethanol	70.0 ml
Deionized water to	100.0 ml

Procedure

- Unpack an agarose gel and remove its cover film.
- Blot the gel surface with a filter paper strip where the application template will be placed on.
- Lay a polyester application template on the blotted place according to the side marks on the support film.
- Press carefully the template with fingers against the gel to remove air bubbles between the template and gel.
- Dilute the serum samples 10 times with deionized water, apply 2–5 µl each into the template slots, and let them diffuse in the gel for 5 min.
- Blot the rest of the samples with a filter paper strip and remove the template together with the paper strip.
- Hang the support film with the gel, gel side down, on the bridge of an electrophoresis cell.
- Fill the electrode tanks of the electrophoresis cell with electrode buffer and place the bridge together with the gel in the cell. As a result, the gel will contact directly the electrode buffers.
- Cover the electrophoresis cell with the lid, switch on the power supply, and start the electrophoresis.
- Run electrophoresis at constant voltage of 70 V for 20–25 min.
- After electrophoresis, fix the gel in ethanol – acetic acid – water (5:1:4, *V:V:V*) for 5 min.
- Dry the gel with a hair drier or in a drying oven at room temperature.

- Mix 5 ml of 1.0 g/dl Sudan black B in ethanol with 45 ml of 60 ml/dl ethanol.
- Stain the lipoproteins in the staining mixture for 10 min. The staining mixture is usable within 24 h.
- Destain the gel in 60 ml/dl ethanol 3 times for 5 min.
- Rinse the gel in deionized water.
- Dry the gel with a hair dryer or in a drying oven.

2.2.10.3 Agarose gel electrophoresis of hemoglobins

The agarose gel electrophoresis of hemoglobins is carried out as the agarose gel electrophoresis of serum proteins. Only the agarose gel is more concentrated (1.5 g/dl). The agarose gel electrophoresis of hemoglobins can be run in alkaline or acidic buffers.

Alkaline agarose gel electrophoresis of hemoglobins

Materials and equipment

TRIS
Formic acid
Sucrose (or sorbitol)

2x Sample buffer, pH = 9.2

1.5 mol/l TRIS-formate buffer, pH = 9.2	4.00 ml
Glycerol	8.00 ml
Bromophenol blue Na salt	0.01 g
Deionized water	28.00 ml

Agarose gel (1.5 g/dl), pH = 8.8, I = 0.035 mol/l

TRIS-formate buffer, 10x	10.0 ml
Agarose ($-m_r$ = 0.16–0.19)	1.5 g
Sucrose (or sorbitol)	2.0 g
Deionized water to	100.0 ml

Bring the mixture to boil in a microwave oven and cast gels.

Electrode buffer, pH = 9.1, I = 0.06 mol/l

TRIS-taurinate buffer, 10x	10.0 ml
Deionized water to	100.0 ml

Procedure

- Wash EDTA containing blood samples in 0.15 mol/l (9.0 g/l) NaCl 3 times.
- Mix 2 volumes of red cells with 2 volumes of deionized water and 1 volume of CCl_4.
- Shake the resulting mixture for 3 min. As a result, the erythrocyte membranes are dissolved (are hemolyzed).

- Centrifuge after 30 min at 3,000 rev/min.
- Dilute the supernatant in a buffer and KCN, until the Hb concentration reaches $7.75 \bullet 10^{-5}$ mol/l (10 g/l), and KCN concentration reaches 0.005 mol/l (0.326 g/l).
- Load on an agarose gel (CA membrane) 10 µl of hemolysate.
- Run electrophoresis as with serum proteins.
- Fix the Hb bands in 0.8 mol/l (130.71 g/l) CCl_3COOH for 15 min.
- Stain in 0.003 mol/l (0.2 g/dl) Bromophenol blue Na salt in ethanol-acetic acid-water (10:1:9, *V:V:V*) for 30 min.
- Destain the background of agarose gel (CA membrane) overnight in ethanol-acetic acid-water (6:1:13, *V:V:V*), or in 0.05 g/dl phosphoric acid.

Acidic agarose gel electrophoresis of hemoglobins

Materials and equipment

Na_3 citrate
Citric acid
D-sorbitol
NaN_3

Solution A (0.2 mol/l Na_3 citrate)

Na_3 citrate	5.88 g
NaN_3	0.07 g
Deionized water to	100.00 ml

Solution B (0.2 mol/l citric acid

Citric acid	4.20 g
NaN_3	0.07 g
Deionized water to	100.00 ml

Natrium citrate buffer, 10x, pH = 6.0, I = 10 × 0.08 mol/l

Solution A	41.5 ml
Solution B	9.5 ml
NaN_3	0.2 g
Deionized water to	100.0 ml

Agarose gel

Natrium citrate buffer	10.0 ml
Agarose	1.0 g
D-sorbitol	10.0 g
Deionized water to	100.0 ml

Bring the agarose mixture to boil in a microwave oven and cast gels.

Procedure

Run electrophoresis as with alkaline hemoglobins. Stain the hemoglobins as the hemoglobins resolved in an alkaline buffer.

2.2.10.4 Agarose gel electrophoresis of cerebrospinal fluid proteins

The agarose gel electrophoresis of cerebrospinal fluid proteins is carried out as the agarose gel electrophoresis of serum proteins.

Materials and equipment

TRIS
Formic acid
Agarose (–m_r = 0.16–0.19)
Sucrose or sorbitol
Amido black 10B (Naphthol blue black)
Ethanol
Acetic acid
Centrifuge concentrators

Agarose gel (0.9 g/dl g/dl), pH = 8.8, I = 0.035 mol/l

TRIS-formate buffer, 10x	10.0 ml
Agarose	0.9 g
Sucrose (or sorbitol)	2.0 g
Deionized water to	100.0 ml

Bring the mixture to boil in a microwave oven and cast gels.

Electrode buffer, pH = 9.1, I = 0.06 mol/l

TRIS-taurinate buffer, 10x	10.0 ml
Deionized water to	100.0 ml

Procedure

- Unpack an agarose gel and remove its cover film.
- Blot the gel surface with a filter paper strip where samples are to be applied.
- Place an application template on the blotted place according to the side marks on the support film.
- Press gently the template against the gel to remove air bubbles between it and the gel.
- Dilute the cerebrospinal samples with deionized water to a protein concentration of 0.20 to 0.40 g/l.
- Concentrate 600 µl of a diluted sample in a centrifuge concentrator rotating it in a 10,000 g centrifuge about 10 min, until the sample reaches a volume of 10 µl. So the concentration of the sample will be about 12 to 24 g/l.

- Apply 2–5 µl of the concentrates each onto the template slots and allow them to diffuse into the gel for 5 min.
- Blot the rest of the samples with a thin filter paper strip and remove the template together with the paper strip.
- Hang the support film with the gel, gel side down, on the bridge of an electrophoresis cell.
- Fill the electrode tanks with electrode buffer and place the bridge with the gel in the cell. So the gel obtains a direct contact with the electrode buffers.
- Close the electrophoresis cell with its lid and turn on the power supply.
- Run the electrophoresis at constant voltage of 70 V for 20–25 min.
- After the electrophoresis, fix the gel in a mixture of ethanol-acetic acid-water (5:1:4, *V:V:V*) for 5 min.
- Dry the gel with a hair dryer or in a drying oven at room temperature.
- Stain the bands in 0.1 g/dl Amido black 10B in the fixing mixture for 5 min.
- Destain the gel background several times in 0.5 g/dl citric acid.
- Dry the gel for a second time with a hair drier or in a drying oven at room temperature.

Silver staining of CSF proteins

Materials and equipment

Preparing solution, 10x

3.00 g/dl (0.12 mol/l) $Na_2S_2O_3$. To be hold in a brown bottle.

Silver stain solution A (Sensitizer)

0.20 g/dl (0.012 mol/l) $AgNO_3$
0.20 g/dl (0.024 mol/l) NH_4NO_3
1.00 g/dl (0.003 mol/l) $H_4[Si(W_3O_{10})_4]$.aq. (silicotungstic acid.aq.)
0.89 ml/dl 37 g/dl HCHO (0.12 mol/l HCHO)

Silver stain solution B (Developer)

8.00 g/dl (0.755 mol/l) Na_2CO_3

Procedure

- Fix a gel in a mixture of ethanol-acetic acid-water (4: 1: 5, *V:V:V*) for 5 min.
- Press the gel: cover it with a thin filter paper, a stack of 4–5 thick filter papers, a glass plate, and a weight of 1–2 kg for 5 min.
- Wash with deionized water for 5 min.
- Press the gel for a second time for 5 min.
- Dry with a hair dryer.
- Prepare the gel in 10-fold diluted preparing solution, 10x for 10 min.
- Rinse twice in deionized water.

- Sensitize the gel in the silver stain solution A for 5 min.
- Add equal volume of the silver stain solution B to develop the protein bands.
- Shake the gel in the mixture until the color of bands reaches desired intensity.
- Stop the chemical reaction in 2 ml/dl acetic acid for 5 min.
- Rinse the gel several times with tap water.
- Dry the gel with a hair dryer at room temperature.
- Clean the back of the support film from metallic silver with a cotton swab.

2.2.10.5 Agarose gel electrophoresis of urinary proteins

Materials and equipment

TRIS
Formic acid
Agarose ($-m_r$ = 0.16–0.19)
Sucrose or sorbitol
Amido black 10B (Naphthol blue black)
Ethanol
Acetic acid

Agarose gel (0.9 g/dl), pH = 8.8, I = 0.035 mol/l

TRIS-formate buffer, 10x	10.0 ml
Agarose ($-m_r$ = 0.16–0.19)	0.9 g
Sucrose (or sorbitol)	2.0 g
Deionized water to	100.0 ml

Bring the mixture to boil in a microwave oven and cast gels.

Electrode buffer, pH = 9.1, I = 0.06 mol/l

TRIS-taurinate buffer, 10x	10.0 ml
Deionized water to	100.0 ml

Procedure

- Unpack an agarose gel and remove its cover film.
- Blot the gel surface with a filter paper strip where samples are to be applied.
- Place an application template on the blotted surface according to the side marks on the support film.
- Press carefully the template with fingers against the gel to remove air bubbles between it and the gel.
- Dialyze and concentrate urine to obtain a protein concentration of 0.2 g/dl.
- Apply 2–5 µl of samples each onto the template slots and let the samples diffuse in the gel for 5 min.
- Blot the rest of the samples with a thin filter paper strip and remove the template together with the paper strip.

- Hang the support film with the gel, gel side down, on the bridge of an electrophoresis cell.
- Fill the electrode tanks of the electrophoresis cell with electrode buffer and place the bridge with the gel in the cell. As a result, the gel obtains a direct contact with the electrode buffers.
- Cover the electrophoresis cell with its lid, switch on the power supply, and start electrophoresis.
- Run electrophoresis at constant voltage of 70–80 V for 20–25 min.
- After the electrophoresis, fix the gel in ethanol-acetic acid-water (5:1:4, *V:V:V*) for 5 min.
- Dry the gel with a hair drier or in a drying oven at room temperature.
- Stain the proteins in Amido black 10B solution for 5 min.
- Destain the gel several times in ethanol-acetic acid-water (5:1:4, *V:V:V*).
- Dry the gel for a second time with a hair drier or in a drying oven at room temperature.

2.2.11 Troubleshooting

Problem	Cause	Solution
Prior to electrophoresis		
The agarose gel is too soft.	The agarose concentration was too low.	Check the recipe. Increase agarose concentration.
	The gelling time was too short.	The gelling time should be at least 60 min at room temperature. It is better, if the agarose gel is stored overnight at 4 °C.
	Urea changed the agarose structure.	Use rehydratable agarose gels.
There are air bubbles in the agarose gel.	The support film or the glass plate of the casting cassette was unclean.	Do not touch the hydrophilic side of the support film with fingers. Wash the glass plate before use.
	The glass plates or the agarose solution were too cold.	Heat the glass plates to 60–70 °C before casting.
The gel separates from the support film and adheres to the glass plate.	An incorrect support film was used.	Do not exchange support films for agarose and polyacrylamide gels.
	The gel was poured on the wrong side of the support film.	Cast the agarose solution on the hydrophilic side of support film; check the sides with water.
	The glass plate was too hydrophilic.	Treat the glass plate with Repel-silane before casting.

(continued)

Problem	Cause	Solution
During electrophoresis		
The electric current does not flow or flows too little during electrophoresis.	Some of the power connections has no or poor contact.	Check all power connections.
The gel “evaporates” (the lid of the separation chamber is covered with water condensation).	The electric power is too high.	Reduce the electric power.
	The cooling is insufficient.	The cooling plate should be made of glass, metal or best of ceramic.
The gel “sweats” (is covered with water drops).	Strong electroosmosis.	Add 20 g/dl glycerol or 10 g/dl sorbitol to the agarose gel.
The gel sparks and burns.	Thin areas in the gel, because the support film was not fixed on the glass plate during gel casting.	Roll strongly the support film on the glass plate of the casting cassette and then cast the agarose solution.
	Poor contact between the gel and the electrode bridges.	Weight the electrodes bridges on the gel with a glass plate.
After electrophoresis		
No protein bands in the gel.	Proteins have left the gel.	Monitor the movement of the dye front.
Protein bands are pale.	The protein concentration or the sample volume was little.	Concentrate the sample, or apply more sample volume.
Protein bands are not sharp or have tails.	Solids in the sample.	Centrifuge the sample before applying them.
	The sample contained too much protein.	Reduce the sample volume or dilute the sample.
	The sample was not dissolved.	Treat the sample with ultrasound.
Adjacent samples run into each other.	The sample volume was too large.	Concentrate the samples and apply less volume.
	The application template did not lie closely on the gel.	Press lightly the application template toward the gel to remove any air bubbles between it and the gel.

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2.3 Immunoelectrophoresis

On agarose gels, immune and affinity electrophoresis can be performed. During the electrophoresis or afterward, proteins bind to specific ligands. Therefore, both techniques are named *ligand electrophoresis*.

Immunoelectrophoresis needs immunoglobulins as ligands. The immunoglobulins recognize exogenous antigens (macromolecules in viruses, bacteria, tissues, and so on). They are produced by plasma cell lines (clones) of the immune system. One clone produces only one class of immunoglobulins. In a healthy organism, there are over 10,000 different clones, therefore the blood serum contains polyclonal immunoglobulins.

2.3.1 Immunoglobulins

Immuno**g**lobulins (Ig, antibodies) are composed of monomers. The Ig-monomer is a glycoprotein. Its protein residue is composed of four polypeptide chains: two **h**eavy (H) and two **l**ight (L) [1]. The polypeptide chains contain **v**ariable (V) and **c**onstant (C) regions. The V-regions form antigen-binding sites (Figure 2.3-1).

The H- and L-polypeptide chains are bound each to other by disulfide bonds: one disulfide bond between an H- and an L-chain, and several disulfide bonds between the H-chains. The Ig-monomer contains also disulfide bonds inside its polypeptide chains that define its tertiary structure.

The heavy chains are 5 types: γ, α, μ, δ, and ε. According to them, 5 classes of immunoglobulins are known: IgG, IgA, IgM, IgD, and IgE, respectively. The light chains exist in two types: κ and λ. Some immunoglobulins contain one monomer (IgG, IgD, IgE), others contain 2–4 (IgA) or 5 monomers (IgM).

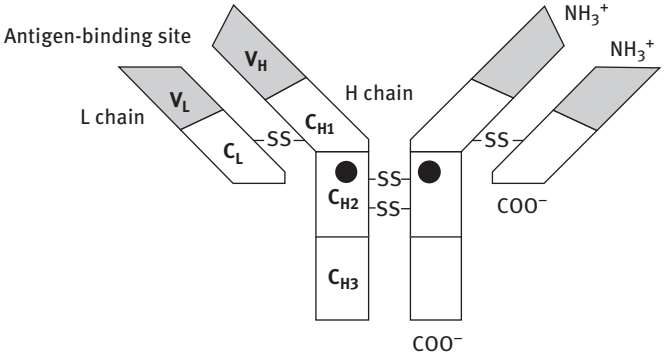

Figure 2.3-1: Structure of immunoglobulin monomer.
V_L and V_H – variable regions of the light and heavy polypeptide chain, respectively; C_L – constant region of the light polypeptide chain; C_{H1}, C_{H2}, and C_{H3} – constant regions of the heavy polypeptide chain; SS – disulfide bond; • – carbohydrate

The function of immunoglobulins is to catch antigens by their antigen binding sites. Thus, they help the complement system to destroy the antigens (e.g., foreign cells).

The electrophoretic separation of immunoglobulins in blood sera is carried out on cellulose acetate membrane [2], or agarose gel [3,4]. The sera are diluted with a buffer, applied onto the slots of a template, separated at usually 80 V for 20–30 min, fixed, stained in a mixture of methanol, acetic acid, and water (3:1:6, *V:V:V*), and finally dried.

Immunoelectrophoresis is a combination between an agarose gel electrophoresis and immune reactions [5,6]. It requires antibodies reacting with resolved proteins. Agarose has been chosen as a gel matrix for this method because it has large pores allowing the proteins to pass. Immunoelectrophoresis is carried out under native conditions, which preserve the conformation and activities of proteins. For this technique, 1 g/dl agarose slabs are usually used, which are 0.5–1 mm thick and contain buffers with pH = 8.6.

At certain antigen-antibody ratios, net-shaped insoluble immune complexes (immune precipitates) are formed (Figure 2.3-2). They remain in the gel, while the non-precipitated proteins can be washed out. Immune precipitates are stained with dyes as Amido black 10B or Coomassie brilliant blue R-250. Nowadays the immunoelectrophoresis is replaced by electroblotting, because it requires less antibodies, and has higher sensitivity.

Immunoelectrophoresis on agarose gels allows additional binding of radioactive antibodies to proteins (antigens). This is used for identification of allergens, which react with IgE.

The following immunoelectrophoretic methods are known: immunoelectrophoresis according to Grabar and Williams, rocket-immunoelectrophoresis according to

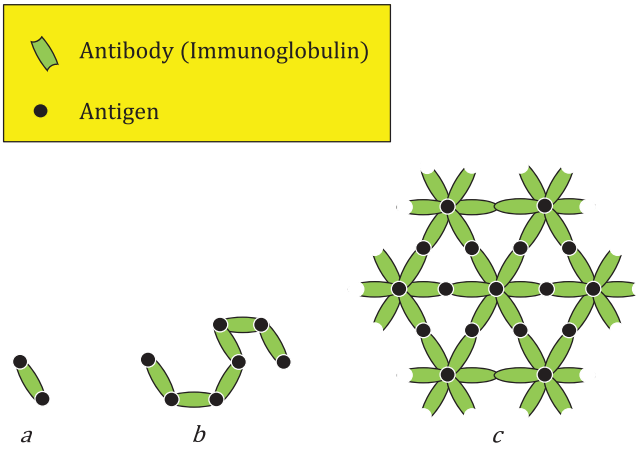

Figure 2.3-2: Hypothetical structure of soluble immune complexes (*a*, *b*) and insoluble immune precipitates (*c*).
The ratio between the molar concentrations of antibodies and antigens is as follows: *a*) 1:2; *b*) 1:1; and *c*) 3:1.

Laurell, counter-current immunoelectrophoresis according to Estella and Heinrichs, crossed immunoelectrophoresis according to Clarke and Freeman, immunofixation, and immunoprinting.

2.3.2 Immunodiffusion electrophoresis according to Grabar and Williams

Immunodiffusion electrophoresis according to Grabar and Williams [7] is a classical immunoelectrophoresis method. It is a combination between agarose gel electrophoresis of proteins (antigens) and diffusion of their antibodies: after electrophoresis, antibodies are applied in channels next to protein bands, which diffuse against the bands forming with them precipitate arcs (Figure 2.3-3). As a result, a great number of serum proteins, and the existence of several immunoglobulin classes are established.

2.3.3 Rocket immunoelectrophoresis according to Laurell

The rocket immunoelectrophoresis was performed by Laurell [8] with some modifications as described by Eriksson *et al.* [9], and Yman *et al.* [10]. It is an electrophoresis of proteins (antigens) on agarose gel containing antibodies. Gel buffer is adjusted to pH = 8.6, so that only the antigens migrate, whereas most antibodies at this pH value cannot move because they are in their isoelectric points.

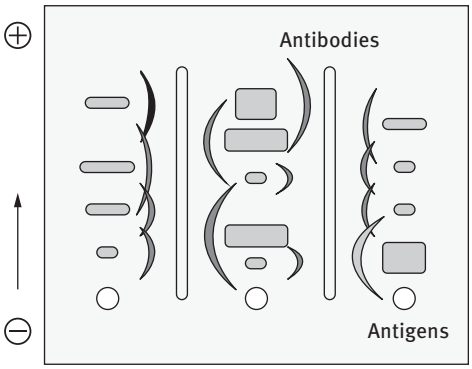

Figure 2.3-3: Immunodiffusion electrophoresis according to Grabar–Williams.

At the beginning of rocket immunoelectrophoresis, the excess antigens result in formation of soluble antigen-antibody complexes. Then, immune precipitates are formed. The immune precipitates are seen as rocket-shaped figures whose surface (height) is proportional to the antigen concentrations (Figure 2.3-4). Therefore, rocket immunoelectrophoresis is a quantitative immunoelectrophoresis. It is used for quantitation of the concentration of human serum proteins.

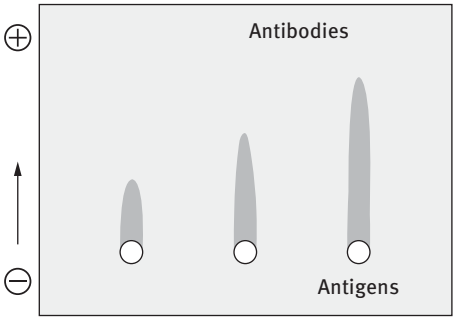

Figure 2.3-4: Rocket immunoelectrophoresis according to Laurell.

2.3.4 Counter-current immunoelectrophoresis according to Estela and Heinrichs

For counter-current immunoelectrophoresis according to Estela–Heinrichs [11], agarose gels with high electroosmosis are used, which contain a buffer with pH = 8.6. At this pH value, the antigens carry negative electric charges, whereas most antibodies are uncharged. The antigens are applied on the anodic end of the gel; their antibodies are applied on the cathodic end of the gel. During electrophoresis, charged antigens run toward the anode, while their antibodies are transported by the electroosmotic flow toward the cathode. When they meet, the antigens and their antibodies precipitate as insoluble immune complexes (Figure 2.3-5).

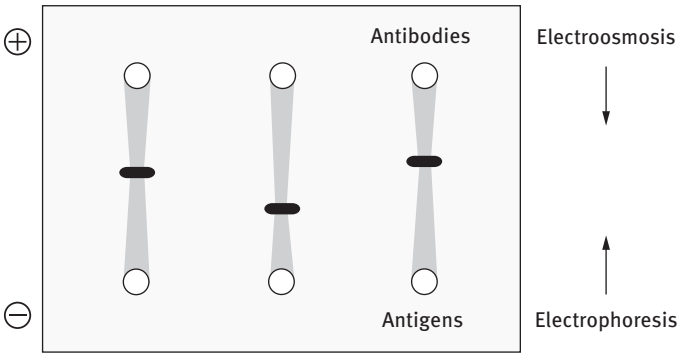

Figure 2.3-5: Counter-current immunoelectrophoresis according to Estela–Heinrichs.

2.3.5 Crossed immunoelectrophoresis according to Clarke and Freeman

Crossed immunoelectrophoresis according to Clarke and Freeman [12] is also called two-dimensional (2D) immunoelectrophoresis. In this method, antigens (proteins) are separated in an agarose gel slab, and then a gel strip containing the bands is cut off and placed on a second agarose gel slab containing antibodies against the antigens. After this, the antigens are separated in a second direction, which is perpendicular to the first one. During the second electrophoresis, immune precipitates are formed whose magnitudes are proportional to the antigen amount. Crossed immunoelectrophoresis is used to study proteins in biological fluids, particularly in human sera (Figure 2.3-6).

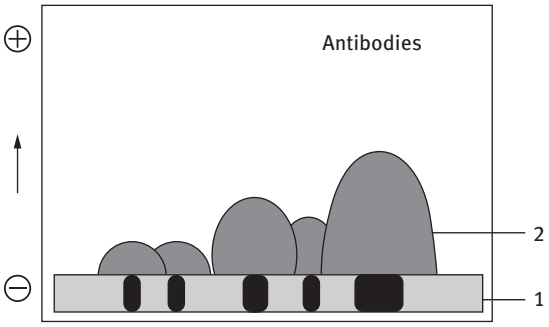

Figure 2.3-6: Crossed immunoelectrophoresis.
1. Agarose gel strip with antigen bands after electrophoresis in the first dimension; 2. Precipitate areas after electrophoresis in the second dimension.

2.3.6 Immunofixation

The immunofixation was described in 1964 [13,14] and used later in modified forms for identification of proteins [15,16,17]. It has few advantages over immunoelectrophoresis. In immunofixation, the proteins are first separated electrophoretically on an agarose gel, and then are coated with corresponding antisera. So, immune complexes are built in the agarose gel [18]. The soluble proteins are washed out with physiological solution, and the immunoprecipitates in the gel are stained (Figure 2.3-7).

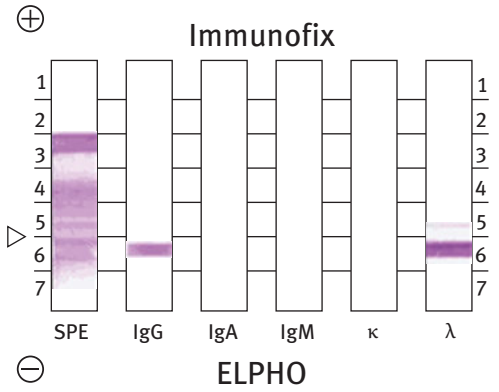

Figure 2.3-7: Immunofixation electrophoresis proving IgG paraproteins of λ-type in the blood serum. SPE – **S**erum **p**rotein **e**lectrophoresis

2.3.7 Immunoprinting

In immunoprinting [19], an agarose gel electrophoresis of antigens (proteins) is carried out. Then the gel is overlaid with an antibody-containing agarose gel or an antibody-containing cellulose acetate membrane. The resolved antigens diffuse into the antibody containing sheet and form immune precipitates.

2.3.8 Protocols

Electrophoresis is carried out in TRIS-glycinate or TRIS-taurinate buffer.

TRIS-glycinate buffer, pH = 8.7

TRIS	1.21 g (0.10 mol/l)
Glycine	3.75 g (0.50 mol/l)
Deionized water to	100.00 ml

TRIS-taurinate buffer, pH = 9.1

TRIS	0.18 g (0.015 mol/l)
NaOH	0.24 g (0.06 mol/l)
Taurine	1.23 g (0.10 mol/l)
Deionized water to	100.00 ml

2.3.8.1 Immunodiffusion electrophoresis according to Grabar and Williams

Materials and equipment

TRIS-glycinate or TRIS-taurinate buffer
Agarose gels
Antibodies

Procedure

- Incise, with the help of a mask, wells in the cathode side of an agarose slab gel for application of samples.
- Excise, using a scalpel, strips between the wells, parallel to the side margins of the gel. Let the gel strips lie in the so formed channels during electrophoresis.
- Add Bromophenol blue Na salt to the samples, and pipette them in the wells.
- Run the electrophoresis at field strength of 10 V/cm and 10 °C for ca. 45 min until Bromophenol blue reaches the opposite gel end.
- After the electrophoresis, remove the gel strips from the channels and fill them with antibody solutions.
- Let the antibodies diffuse in the gel against the sample antigens at room temperature for 15 h to be formed white precipitate arcs.

2.3.8.2 Rocket immunoelectrophoresis according to Laurell

Materials and equipment

TRIS-glycinate or TRIS-taurinate buffer
Agarose gels
Antibody
Polyethylene glycol

Procedure

- Dissolve 1.2 g/dl agarose and 3 g/dl polyethylene glycol in TRIS-glycinate or TRIS-taurinate buffer and heat in a boiling water bath for 7–8 min.
- Allow the solution to cool to 60 °C.
- Add the antibody to the agarose solution.
- Cast the gels on support films placed with their hydrophilic side up on a leveling table, to ensure a uniform gel thickness of 1 mm.
- After gelation, keep the gels in a humidified chamber overnight at 4 °C, in order to allow the gel structure to develop.

- Stamp wells in the gel.
- Pipette the samples into the wells and start the electrophoresis at 10 V/cm and 10 °C for 3 h.

2.3.8.3 Crossed immunoelectrophoresis according to Clarke and Freeman

Materials and equipment
TRIS-glycinate or TRIS-taurinate buffer
Agarose gels
Antibodies

Procedure
- Apply on a gel 0.5 µl serum containing Bromophenol blue Na salt.
- Run electrophoresis in the 1st dimension at 200 V for 60 min.
- After 1D-electrophoresis, cut a gel strip, turn it on 90°, place it on the surface of a new agarose gel containing antibodies, and run electrophoresis in the 2nd dimension at 120 V for 12–15 h.
- After electrophoresis, wash the gel with 0.9 g/dl NaCl for 10 min, press it (s. below) and dry 2 times for 10 min each.
- Stain the bands with Amido back 10B or Coomassie brilliant blue R-250 for 10 min.
- Destain the gel background 2 times in methanol-acetic acid-water (3:1:6, *V:V:V*).
- Dry the gel in a drying oven or at room temperature.

2.3.8.4 Immunofixation of serum proteins

Materials and equipment
TRIS-glycinate or TRIS-taurinate buffer
Agarose gels for immunofixation of serum proteins. They are situated between two films. The lower film is bound firmly to the gel; the upper film can be easily detached from it.
Antisera: Anti-IgG, anti-IgA, anti-IgM, anti-kappa, anti-lambda
Coomassie violet blue R-200 or Amido black 10B
Pipettes

Staining solution

SERVA violet R-200 or Amido black 10B	0.2 g
Methanol	30.0 ml
Acetic acid	10.0 ml
Deionized water to	100.0 ml

Destaining solution

Phosphoric acid	0.05 g
Deionized water to	100.00 ml

Procedure

Preparation and application of sample

- Dilute blood sera with saline at least 20 times to obtain a protein concentration of about 0.35 g/dl. Concentrate the cerebrospinal fluid and urine samples to the same protein concentration. Freshly obtained serum is preferable; however, it may be stored at 2–8 °C for up to 72 hours.
- Blot carefully the agarose gel surface with a thin filter paper where an application template will be placed.
- Place an application template on the blotted gel surface according to the side markers (Figure 2.3-8) and press it gently against the gel surface to remove air bubbles between the gel and template.
- Apply 2–3 µl of samples onto the ELP (**el**ectro**p**horesis) and next slots, and let them diffuse in the gel.
- After 5 min, blot the remaining sample volumes with a filter paper.
- Remove the application template together with the paper strip from the gel.

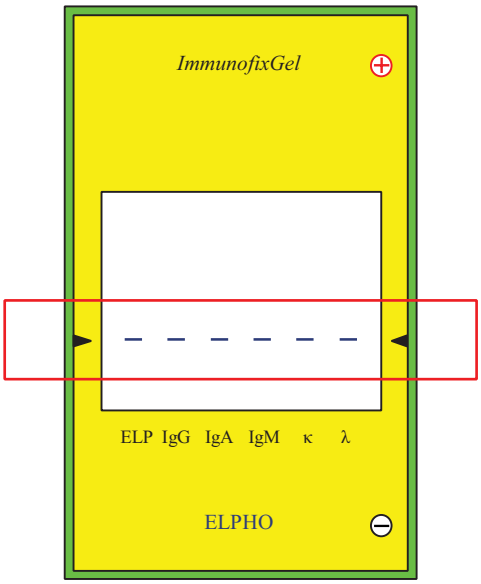

Figure 2.3-8: Application of a template for immunofixation onto an agarose gel.

Electrophoresis

- Fill the tanks of an electrophoresis cell with electrode buffer.
- Hang the support film, with the gel side down, on the bridge of an electrophoresis cell.

- Insert the bridge, together with the gel on it, into the tanks of the electrophoresis cell. So a direct contact occurs between the gel and the electrode buffers (s. *Agarose gel electrophoresis of serum proteins*).
- Cover the electrophoresis cell with its lid, and turn the power supply on.
- Run electrophoresis at constant voltage or electric current (80 V or 20–25 mA, respectively) at room temperature for 20–25 min.

Immunoprecipitation

- After the electrophoresis, dry the agarose gel surface with a filter paper.
- Adjust an antiserum template on the gel, according to the marks on the support film, and press it gently against the gel (Figure 2.3-9).
- Apply with pipettes 50 µl of fixing solution (methanol-acetic acid-water, 3:1:6, *V:V:V*) in the ELP bed and 50 µl of the antisera (G, A, M, κ, and λ) each in the remaining beds.
- Incubate the gel with the antisera on a wet filter paper at room temperature for 30 min.
- Remove the antisera template after the incubation and press the gel dry for 10 min. The dry pressing of the gel is performed in the following way: Place 4–5 filter papers on the gel, a glass plate, and a weight of 1–2 kg (Figure 2.3-10).
- Soak the gel in saline (0.9 g/dl NaCl) for 10 min, and press it dry again.
- Press the gel dry two more times and dry it with a dryer or in a drying oven.

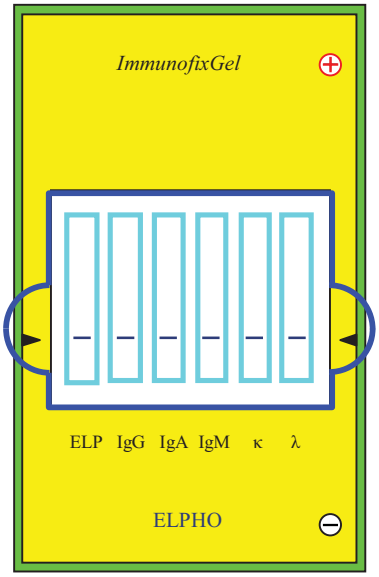

Figure 2.3-9: Adjustment of an antiserum template on agarose gel after electrophoresis.

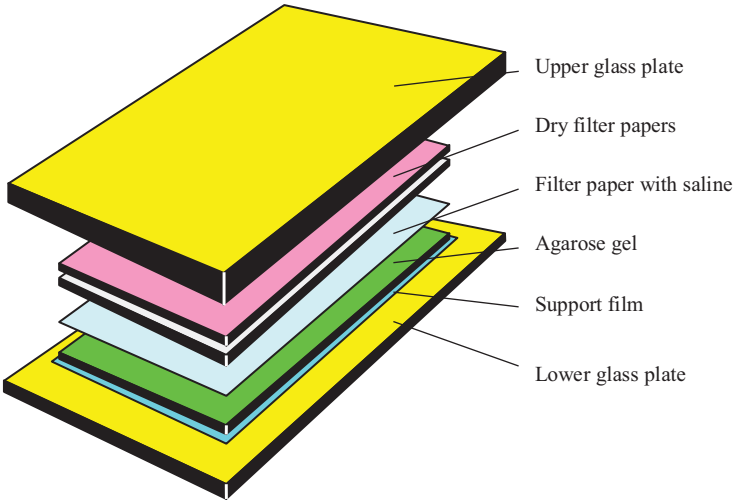

Figure 2.3-10: Dry pressing of an agarose gel.

Staining the protein bands

- Stain the gel in 0.2 g/dl Coomassie violet R-200 or 0.2 g/dl Amido black in methanol-acetic acid-water (3:1:6, *V:V:V*) for 10 min.
- Destain the gel 2–3 times for 2–3 min each in 0.5 g/dl citric acid until the background is clear.
- Dry the gel with a dryer or in a drying oven.

Evaluation

The analysis of results is performed by comparing the precipitation bands in the IFE positions with the ELP reference bands. The lower detection limit for monoclonal IgG, IgA, IgM and κ chains is at 0.5 to 1.5 mg/ml, for λ chains is at 1 to 2 mg/ml.

2.3.8.5 Immunofixation of Bence Jones proteins

Materials and equipment

TRIS-glycinate or TRIS-taurinate buffer

Agarose gels for immunofixation of Bence Jones proteins (light chains of paraproteins). They are located between two films. The lower film is firmly bound to the gel, while the upper film can be easily detached from it.

Coomassie violet R-200 or Amido black 10B

Procedure

Sample preparing and applying

- Preferably use fresh urine samples. Due to the low concentration of Bence Jones proteins, the urine should be concentrated. A quick method for this purpose is its centrifugation in microcentrifuge concentrators. The final concentration of proteins in the urine samples should be 1.5–3.5 g/l.
- Blot with a thin filter paper the gel surface where the samples will be applied.
- Place a polyester template onto the blotted gel surface, according to the both markers on the support film (Figure 2.3-11) and press gently the template to remove the air bubbles underneath.
- Apply 2–3 µl of the sample onto the ELP slot as well as onto the slots for GAM, κ, λ, κ free, and λ free immunoglobulins.
- Wait 5 min until the samples diffuse into the gel.
- Blot the exceeded samples with a filter paper and remove it, together with the template, from the gel.

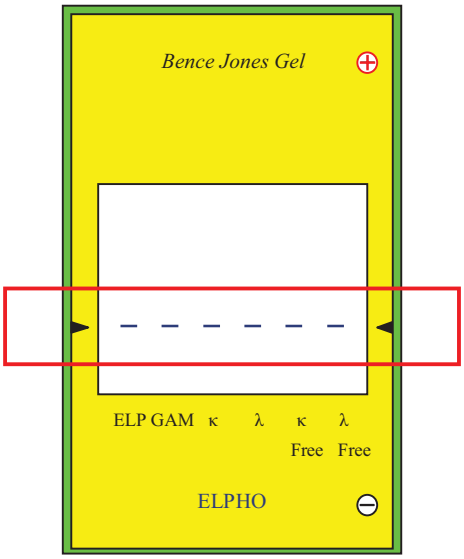

Figure 2.3-11: Placing a sample template onto a gel.

Electrophoresis

- Hang the support film, with the gel side down, onto the bridge of an electrophoretic cell.
- Fill the tanks of the electrophoretic cell with electrode buffers and insert the bridge into them. Thus, the gel is directly connected to the electrode buffers (s. above).
- Close the electrophoretic cell with the lid and turn on the power supply.

– Run electrophoresis at constant voltage (80 V) or constant electric current (20–25 mA) for 20–25 min at room temperature.

Immunoprecipitation

– After electrophoresis, blot the gel surface with a thin filter paper.
– Place an antiserum template onto the gel and press it gently (Figure 2.3-12).
– Apply 50 µl polyvalent antiserum into the ELP reference bed and 50 µl of the appropriate antisera (GAM, *κ*, *κ*-free, *λ* and *λ*-free) into the test beds.
– Incubate the gel with the antisera for 30 min on wet filter paper at room temperature.
– After incubation, remove the antiserum template and press the gel dry for 10 min by placing a thin filter paper, 4–5 thick filter papers, a glass plate, and a weight of 1–2 kg on it (s. above).
– Soak the gel in saline for 10 min.
– Press the gel dry and soak it in saline another 2 times.
– Dry the gel with a hairdryer or at room temperature.

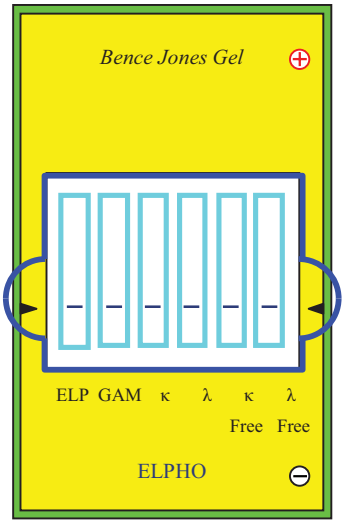

Figure 2.3-12: Placing an antiserum template onto the gel.

Staining the immune complexes

– Stain the immune complexes in the gel with Coomassie violet R-200 or Amido black blue 10B (s. above) for 10 min.
– Destain the gel background in a destaining solution (s. above).
– Dry the gel with a hairdryer or at room temperature.

Evaluation

The evaluating of the results can be done by comparing the precipitation strips in the test beds with the precipitation strips in the reference ELP bed. The lower concentration limit for seeing monoclonal κ-chains is 0.5–1.5 mg/ml, and for α-chains is 1–2 mg/ml.

2.3.8.6 Immunoprobing with avidin-biotin coupling to secondary antibody

Materials and equipment

TBS (TRIS-**b**uffered **s**aline)
TBST – mixture of **TBS** and **T**ween 20
Avidin
Biotinylated HRP (**h**orse**r**adish **p**eroxidase) or ALP (**al**kaline **p**hosphatase)
Primary antibody
Biotinylated secondary antibody
Plastic bag
Plastic box

Procedure

- Prepare primary antibody in 5 ml TBST (for nitrocellulose or PVDF membranes) or TBS (for nylon membrane).
- Place the membrane with the transferred and blotted proteins into a bag containing the primary antibody solution.
- Incubate at room temperature for 30 min.
- Transfer the membrane from the plastic bag onto a plastic box.
- Wash the membrane three times for 15 min each in TBST (for nitrocellulose or PVDF membranes) or TBS (for nylon membranes). Add buffer to cover the membrane.
- Transfer the membrane into a plastic bag containing diluted secondary antibody solution: 50 to 100 ml of TBST (for nitrocellulose or PVDF membranes) or TBS (for nylon membranes).
- Incubate at room temperature for 30 min, then wash.
- Prepare avidin-biotin-HRP (or ALP) complex.
- Mix two drops of avidin solution and two drops of biotinylated HRP (or ALP) solution into 10 ml TBST (for nitrocellulose or PVDF membranes) or TBS (for nylon membranes).
- Incubate at room temperature for 30 min, then dilute to 50 ml with TBST or TBS.
- Transfer the washed membrane to the avidin-biotin-enzyme solution.
- Incubate at room temperature for 30 min.
- Wash for 30 min.
- Develop according to an appropriate visualization protocol.

2.3.9 Troubleshooting

Problem	Cause	Solution
Prior to electrophoresis		
The consistency of the agarose gel is too soft.	The agarose concentration was too low. The gelling time was too short.	Check the recipe. Increase the agarose concentration. Let the solution gel at least 60 min at room temperature. It would be better, if the agarose gel is stored in the refrigerator overnight at 4 °C.
	Urea changed the agarose structure.	Let the agarose solution gel longer or use rehydratable agarose gels.
The gel separates from the support film.	An incorrect support film was used. The gel was cast on the wrong side of the support film.	Do not exchange the support films for agarose and polyacrylamide gels. Cast the gel on the hydrophilic side of the support film. Check the sides with water drops.
There are air bubbles in the agarose gel.	The glass plate or the support film of the casting cassette was unclean.	Wash the glass plate thoroughly before use. Do not touch the hydrophilic side of the support film with fingers.
	The glass plates or the agarose solution were too cold.	Preheat the glass plates to 60–70 °C before casting the agarose solution.
Gel separates from the support film and adheres to the glass plate.	An incorrect support film was used. The gel was cast on the wrong side of the support film. The glass plate was too hydrophilic.	Do not exchange the support films for agarose and polyacrylamide gels. Cast the agarose solution on the hydrophilic side of the support film; check the sides with water drops. Treat the glass plate with Repel-silane before casting.
The gel does not stick to the support film, but to the glass plate.	The glass plate was too hydrophilic.	Clean the glass plate and coat it with Repel-silane.
During electrophoresis		
Electric current does not flow or flows too little during the electrophoresis.	Some of the power connections has no or poor contact.	Check all power connections. Weight the electrode strips with a glass plate.

(continued)

Problem	Cause	Solution
The gel “evaporates” (the lid of the separation chamber is covered with condensed water vapor).	The cooling is insufficient.	Check the coolant temperature (10–15 °C are recommended). Give contact liquid (kerosene) between the cooling block and support film. Cooling blocks should be made of glass, metal or preferably of ceramic.
	The electric voltage or power is too high.	Reduce the electric voltage or power.
The gel “sweats” (is covered with water drops during the electrophoresis).	No or too little glycerol in the rehydration solution.	Check the recipe for the rehydrating solution. Add 20 g/dl glycerol, 10 g/dl sucrose, or 10 g/dl of sorbitol.
	The agarose gel gives off water for the sake of electroosmosis.	Prior to casting add to the agarose solution 20 g/dl glycerol or 10 g/dl sorbitol
Gel sparks and burns.	Thin areas in the gel because the support film was not fixed on the glass plate prior to gel casting.	Roll strongly the support film on the glass plate of the casting cassette.
	Poor contact between the gel and the electrode bridges.	Weight the electrodes bridges on the gel with a glass plate.
After electrophoresis		
There are no protein bands in the gel.	The proteins have left the gel.	Monitor the movement of the dye front.
Some bands are missing.	The proteins built complexes.	Give more than 7 mol/l urea or EDTA, or a nonionic detergent (Triton X-100, Nonidet NP-40) in the sample.
The protein bands are pale.	The protein concentration or sample volume was small.	Concentrate the sample, or apply more sample volume.
	The sample was not completely dissolved.	Treat the sample with ultrasound; centrifuge, if there is opacity.
The protein bands have tails.	There were solids in the sample.	Centrifuge the sample before applying.
	The sample contained too much protein.	Reduce the sample volume or dilute the sample.
	The sample is denatured.	Make the sample preparation before electrophoresis. Store the samples at –20 °C.
	The samples were old.	Use fresh samples.
	Diffusion after the electrophoresis.	Stain the protein bands after fixation and drying of the agarose gel.

(continued)

Problem	Cause	Solution
The samples from adjacent tracks run into each other.	The sample volumes were too large. The application template did not lay tight on the gel.	Concentrate the samples and apply smaller volumes. Press lightly the application template toward the gel to remove any air bubbles between the template and gel.
The bands on the side margins of the gel are distorted.	The sample was applied too close to the side margins of the gel.	Apply samples 1 cm away from the side margins of the gel.
The gel dissolves from the support film in the staining procedure.	The bonds between the support film and the gel are partially hydrolyzed by strong acids (TCA) in the staining solutions.	Use weaker acids in the staining solution.
The support film rolls on during drying.	Gels contract in all directions.	Add 5 g/dl glycerol to the final wash solution to make the gel elastic.

References

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2.4 Affinity electrophoresis

The affinity electrophoresis [1,2] was developed by Nakamura *et al.* [3,4]. It allows simultaneous testing of several samples. The affinity electrophoresis is faster than the affinity chromatography [5]. It can be used for estimating the binding constants, or for characterization of polyions. The affinity electrophoresis is suitable also for studying the isoenzymes (isozymes), for example, lactate dehydrogenase [6], alcohol dehydrogenase [7], and plasminogen [8], and above all alkaline phosphatase [9]. It can be run in agarose or polyacrylamide gels.

2.4.1 Theory of affinity electrophoresis

Affinity electrophoresis is a zone gel electrophoresis in which proteins bind to ligands (specific macromolecules). Therefore, affinity electrophoresis and immonoelectrophoresis (s. above) are known as *ligand electrophoresis*. The ligands are lectins, enzyme substrates, or other substances. They are not bound to agarose or polyacrylamide gel, opposite to the ligands in affinity chromatography, which are immobilized on a matrix. The ligands bind to corresponding polyions and form complexes with them. The complexes have other electric charges and as a result other mobilities.

A few methods of affinity electrophoresis are known: lectin electrophoresis, saccharide affinity electrophoresis, affinity supported molecular matrix electrophoresis, phosphate affinity electrophoresis, capillary affinity electrophoresis, affinity-trap electrophoresis, charge shift electrophoresis, and mobility shift electrophoresis.

2.4.2 Lectin affinity electrophoresis

Lectins (phytohemagglutinins) are plant carbohydrate-binding proteins. They show high specificity for foreign glucoconjugates [10,11,12] and are used in blood cell testing. Besides, lectins mediate the attachment and binding of bacteria and viruses to their targets. For example, hepatitis C viral glycoproteins attach to C-type lectins on the surface of liver cells to start an infection [13]. In affinity electrophoresis, wheat germ lectin (**w**heat **g**erm **a**gglutinin, WGA) is used.

The lectin electrophoresis is used for separating the alkaline phosphatase isoenzymes.

The **al**kaline **p**hosphatase (ALP, orthophosphate monoester hydrolase, EC 3.1.3.1) is a glycoprotein, which is composed of two monomers connected by a Zn^{2+}. It catalyzes the hydrolytic splitting of terminal phosphate groups from organic phosphoric esters in alkaline solutions (Figure 2.4-1). The pH value of the solutions must be above pH = 10.0, but this varies with the particular substrate.

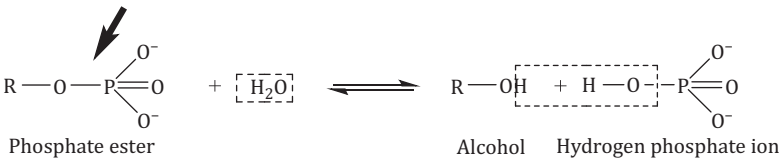

Figure 2.4-1: Hydrolysis of a phosphate ester by alkaline phosphatase.

The alkaline phosphatase consists of two polypeptide chains, which are chelated by Zn^{2+}. The polypeptide chains show differences in their glycostructures. They are encoded by three genes: a tissue-unspecific gene, a colon gene, and a placental gene. The polypeptide chains are bound two by two forming a few isoenzymes of alkaline phosphatase (ALP isoenzymes). For its action, the alkaline phosphatase needs Mg^{2+}.

2.4.2.1 Electrophoresis of alkaline phosphatase isoenzymes in lectin agarose gels

The agarose gels for ALP isoenzyme electrophoresis (ELPHO, Nuremberg) are located between two polyester films. The support film is chemically bound to the agarose gel, whereas the cover film can be easily removed from it. Beside of wheat germ, the gels contain the nonionic detergent Triton X-100. Triton X-100 frees the ALP isoenzymes from lipoproteins.

With the help of lectin electrophoresis, the following serum ALP isoenzymes are established: liver, bone, intestinal, placental, and macro-ALP isoenzyme (Figure 2.4-2).

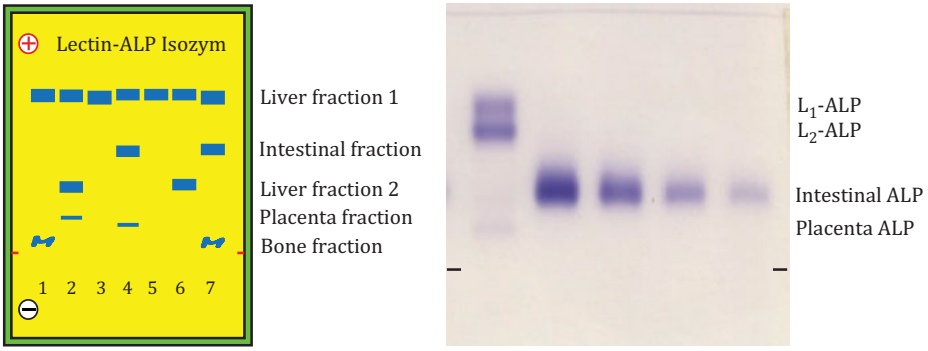

Figure 2.4-2: Alkaline phosphatase isoenzymes separated by affinity electrophoresis.

Liver ALP Isoenzyme. The liver ALP isoenzyme contains N-acetylneuraminic (sialic) acid in low concentration. It is heat stable and is present in two forms: L_1 or fast liver ALP isoenzyme, and L_2 or slow liver ALP isoenzyme. The L_1-isoenzyme, together with the intestinal ALP isoenzyme, is found in healthy adult people. The L_2-isoenzyme is rarely detected. It is located at the cathode side of L_1. The L_2 correlates with malignant diseases with or without metastases.

Bone ALP isoenzyme. The bone ALP isoenzyme contains residues of N-acetylneuraminic (sialic) acid in high concentration. It is heat labile and its mass is almost twice greater than the mass of the liver ALP isoenzyme in both genders.

Intestinal ALP isoenzyme. The intestinal ALP isoenzyme is found in 10% of human population. Up to 3 bands of it can be detected in blood serum.

Placental ALP isoenzyme. The placental ALP isoenzyme exists in two forms: P_1 (90%) and P_2 (10%). They appear in the blood serum during pregnancy or at cancer of the ovary, pancreas, stomach, and colon, as well as at sarcomas [14,15].

Macro-ALP isoenzyme. The macro ALP isoenzyme is rarely found. It is a complex between alkaline phosphatase and immunoglobulins. Since its molecular mass is too large, the macro ALP isoenzyme remains on the application site.

As pointed out, the liver and bone ALP isoenzymes differ only by the concentration of their N-acetylneuraminic acid. They can be separated from each other by agarose gel electrophoresis in the presence of Triton X-100 and wheat germ lectin. Triton X-100 sets free the ALP isoenzymes from membrane lipoproteins, whereas wheat germ lectin binds specifically to the residues of N-acetylneuraminic acid in the bone ALP isoenzyme. As a result, the bone ALP isoenzyme comes to a standstill near the sample application place and forms a tooth-shaped precipitate [16,17].

After electrophoresis, colorimetric detection of the resolved isoenzyme bands can be carried out. For this purpose, the colorless disodium salt of 5-**b**romo-4-**c**hloro-3-**i**ndolyl **p**hosphate (Na_2BCIP) is hydrolyzed by the alkaline phosphatase giving after oxidation with **n**itro **b**lue **t**etrazolium chloride (NBT) the blue dimer

$(BCI)_2$ (5,5′-dibromo-4,4′-dichloro-indigo) and disodium hydrogen phosphate [18] (Figure 2.4-3). The inorganic phosphate esterifies a serine residue in the neighborhood of the active site of the alkaline phosphatase, which later transfers the inorganic phosphate onto the aminoalcohol 2-**a**mino-2-**m**ethyl-1-**pro**panol (AMPro). Thus the enzyme inhibition is avoided. The colored dimer shows which protein bands contain ALP activity.

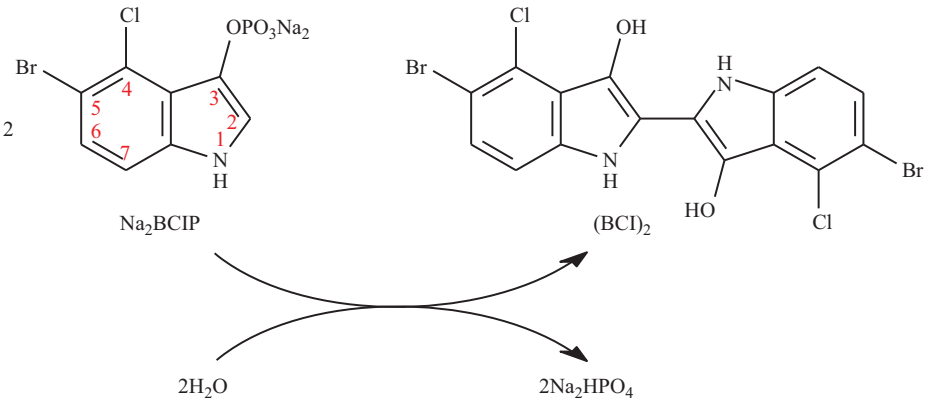

Figure 2.4-3: Hydrolysis of Na_2BCIP giving a dimer $(BCI)_2$ and disodium hydrogen phosphate.

After the incubation, the enzyme reaction is stopped in a mixture of methanol, acetic acid, and water, and the gel is washed first with tap water, then with deionized water. At the end, it is dried and the ALP isoenzyme bands are evaluated visually, or with scanner or densitometer.

In addition, a facile fluorescence turn-on approach for the detection of ALP activity was developed [19]: L-**a**scorbic **a**cid-2-**p**hosphate (AAP) could be hydrolyzed by ALP to give L-ascorbic acid. L-ascorbic acid then reduced resazurin to resorufin which results in a turn-on fluorescence signal (Figure 2.4-4). The assay is very sensitive, as 0.12 mU/ml ALP could be detected.

The alkaline phosphatase is widely distributed in the human body. Its concentration is age-dependent and is elevated during the active bone growth. The reference values of serum ALP in childhood are about 2–3 times higher than the reference values in adults: 60–470 U/l vs. 50–170 U/l.

The activity of serum ALP is high in all bone disorders accompanied by increased osteoblastic synthesis, for example, Paget's disease (osteitis deformans), hyperparathyroidism, rickets, osteomalacia, and osteoblastic tumors with metastases [20]. Moderate elevations of the ALP activity may be attributed to pregnancy, Hodgkin's disease, and more. Low serum ALP activity is found in hypothyroidism, hypophosphatasemia, pernicious anemia, and dwarfism.

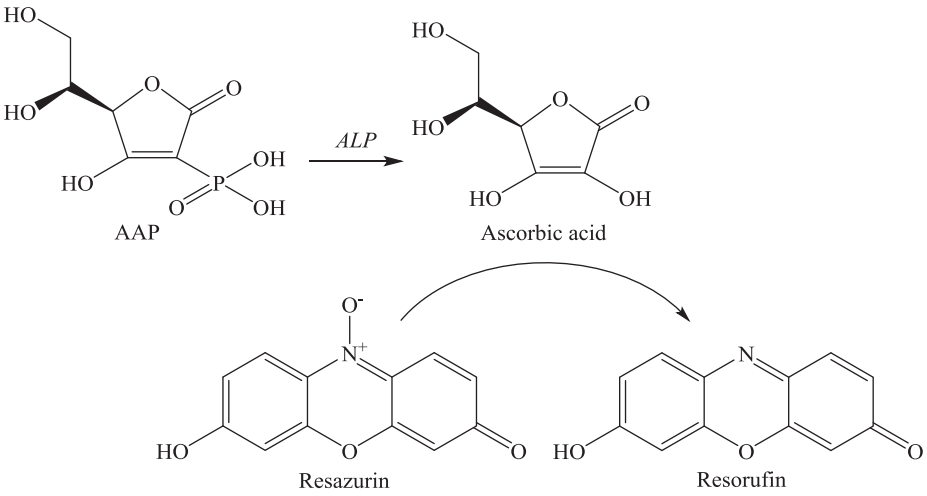

Figure 2.4-4: L-ascorbic acid-2-phosphate (AAP) is hydrolyzed by alkaline phosphatase (ALP) to give L-ascorbic acid. It reduced resazurin to resorufin, which results in a turn-on fluorescence signal.

2.4.3 Saccharide affinity electrophoresis

Glycosylation plays an important role in protein modification. To separate glycoproteins, Jackson *et al*. [21] used the boron compound [3-(**m**ethacryloylamino)-**p**henyl] **b**oronic **a**cid (MPBA) as ligand (probe) (Figure 2.4-5).

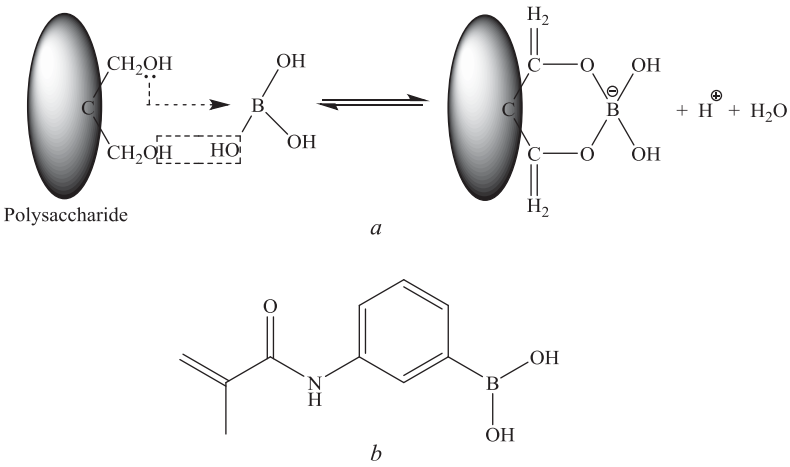

Figure 2.4-5: Saccharide affinity electrophoresis.
a) Reversible bonding between a boron compound and a polysaccharide;
b) MPBA [(3-**m**ethacryloylamino)**p**henyl]**b**oronic **a**cid

In **b**oronate **a**ffinity **s**accharide **e**lectrophoresis (BASE), MPBA (in concentration of 0.5–1 g/dl) is immobilized by copolymerization in a polyacrylamide gel. It operates as an affinity probe for fructose and linear polyalcohols of saccharide derivatives containing sialic acids. Phenyl boronic acids function as saccharide receptors in aqueous solution [22,23,24] and in polyacrylamide gels when incorporated there [25,26,27]. They form cyclic boronic esters with various carbohydrates by reversible covalent interactions. As a result, the saccharides migrate more slowly during electrophoresis and are resolved in the gel. Morais *et al*. applied the same technique for serum glycoproteins of diabetic patients [28]. DNA and RNA that contain carbohydrates can also be analyzed by this method.

2.4.4 Affinity supported molecular matrix electrophoresis

Mucins are viscous giant glycoproteins produced by epithelial cells. They have relative molecular mass of several millions and sugar chains, which represents up to 50–80% of the molecules.

To resolve mucins, Matsuno *et al*. established **s**upported **m**olecular **m**atrix **e**lectrophoresis (SMME) [29,30,31]. In this method, fibers of a porous hydrophobic **p**oly**v**inylidene **dif**luoride (PVDF) membrane, used as a support matrix, are coated with a hydrophilic polymer. As a result, proteins or lipids are resolved during the electrophoresis. Later Matsuno and Kameyama [32] combined SMME with affinity electrophoresis. For this purpose they bound to the hydrophobic fibers probes as lectins, glycolipids or antibodies, and hydrophilized the fibers. This technique was called **a**ffinity **SMME** (ASMME). Migrating through this system, polyions interact with the probes and resolve (Figure 2.4-6).

The affinity-SMME is suitable for analyzing glycoprotein markers of some diseases. Using this technique and subsequent mass spectrometric analysis, Matsuno *et al*. showed that the sugar chain profiles of mucins are different in different tumor types.

2.4.5 Phosphate affinity electrophoresis

In **phos**phate affinity electrophoresis (Phos-tag PAGE), Phos-tag [33] is used as an affinity probe that binds specifically to divalent phosphate ions in neutral solutions (Figure 2.4-7). Phos-tag (M_r = 595) is developed by the Department of Functional Molecular Science at Hiroshima University for simultaneous analysis of a phosphoprotein isoform and its non-phosphorylated counterpart.

The phosphate affinity electrophoresis is run in a gel that contains the copolymerized acrylamide-pendent Phos-tag monomer. During the electrophoresis, the phosphorylated proteins interact with the Phos-tag moieties in the gel and, as a

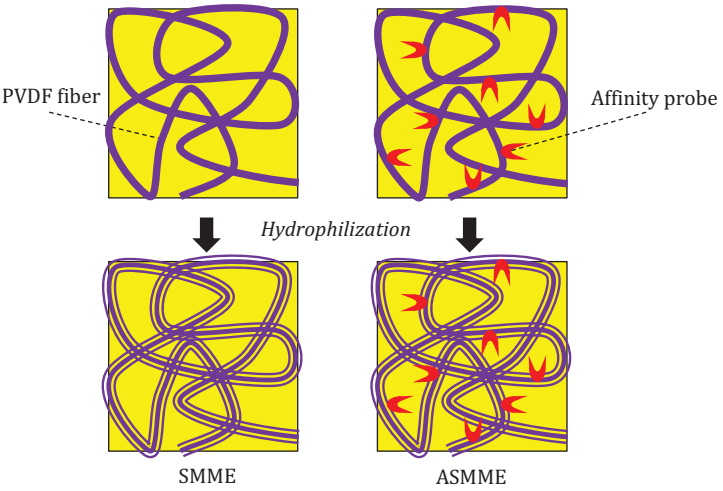

Figure 2.4-6: Supported molecular matrix electrophoresis (SMME) and affinity SMME (ASMME). *Left*. A hydrophobic membrane (PVDF) becomes a separation medium after hydrophilization. *Right*. Biological components, such as proteins, are adsorbed on PVDF fibers and act as affinity probes after the PVDF membrane is hydrophilized.

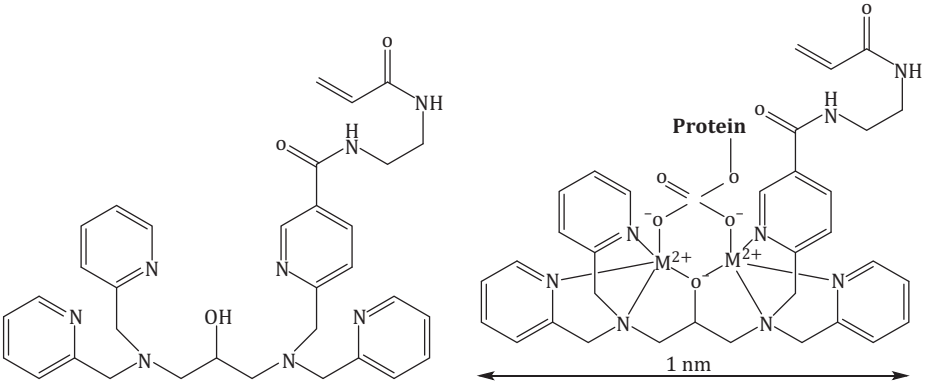

Figure 2.4-7: Phos-tag (*left*) and Phos-tag bound to phosphoprotein (*right*).

result, they migrate more slowly than non-phosphorylated proteins. Even protein polyions with identical numbers of phosphorylated amino acid residues, but phosphorylated at different sites, exhibit different mobilities and make separate bands [34,35].

By applying the Laemmli SDS system [36] in Phos-tag gels, the Phos-tag SDS-PAGE is developed [37,38]. During it, a M^{2+}-Phos-tag complex (Figure 2.4-8) is formed, which binds to phosphate groups in gels with pH > 9.0.

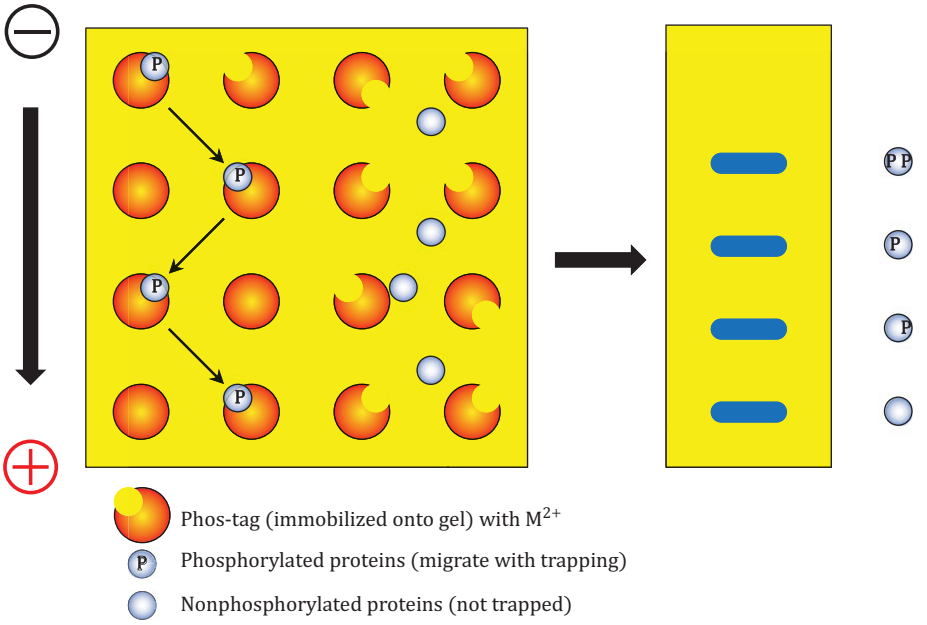

Figure 2.4-8: Phosphate affinity SDS electrophoresis (Phos-tag SDS-PAGE).
The copolymerization of Phos-tag with acrylamide provides a gel that binds phosphorylated proteins and resolves them. $M^{2+} = Zn^{2+}$ or Mn^{2+}.

Kimura *et al.* characterized the phosphorylation states of posttranslational proteins by 2D-electrophoresis using isoelectric focusing in the first dimension, Mn^{2+}-Phos-tag SDS-PAGE (in Figure 2.4-8, $M^{2+} = Mn^{2+}$) in the second dimension, and mass spectrometry after the electrophoresis [39]. They examined the **h**eterogeneous **n**uclear **r**ibo**n**ucleo**p**rotein **K** (hnRNP K) that is involved in the chromatin remodeling, transcription, and translation, and revealed that it exists in different phosphorylation states. Hosokawa *et al.* [40] studied *in vivo* the phosphorylation states of **c**yclin-**d**ependent **k**inase **5** activator **p35** (Cdk5-p35) which regulates the cell cycle.

For better resolution, Zn^{2+} are used in the SDS-PAGE system because they form Zn^{2+}-Phos-tag complexes. These complexes bind optimally to phosphate groups in neutral solutions (in Figure 2.4-8, $M^{2+} = Zn^{2+}$).

2.4.5.1 Analysis of chemically unstable histidine- or aspartic acid-phosphorylated proteins

The bacteria and plants possess two-component signaling systems that are mediated by phosphorylation of histidine (His) and aspartic acid (Asp) residues. The phosphorylated His and Asp residues are chemically unstable. They were quantitative analyzed by Mn^{2+}- and Zn^{2+}-Phos-tag SDS-PAGE [41,42,43].

Additionally, using ATPyS, a sulfur analogue of ATP, the transphosphorylation of histidine kinase was examined and analyzed by Phos-tag SDS-PAGE. As phosphorylated residues, corresponding thiophosphorylated residues bind to Phos-tag too [44]. So, phosphate groups are detected on His-residues in the case of thiophosphate groups. A combination of the Phos-tag SDS-PAGE with thiophosphorylation can be applied for detecting the transphosphorylation between enzymes and their substrates.

2.4.6 Capillary affinity electrophoresis

Capillary **a**ffinity **e**lectrophoresis (CAE) combines capillary electrophoresis with intermolecular interactions. It uses fluorophore-labeled molecules as affinity probes. The samples and the probes are mixed and the resulting complexes are separated by capillary electrophoresis (Figure 2.4-9). Polyions that are bound to their probes migrate; the rest of the polyions does not migrate. A detection system based on scanning laser-induced fluorescence detects the sample bands.

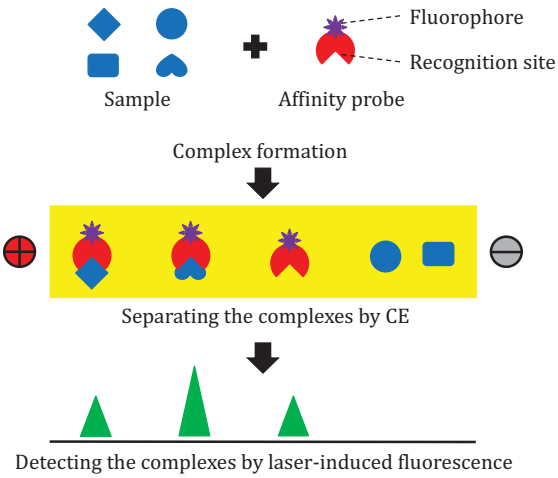

Figure 2.4-9: Principle of capillary affinity electrophoresis.

The affinity probes for CAE can be prepared for macromolecules as well as for low-molecular-mass compounds. Besides, CAE separations can be made in short periods of time, to detect short-lived complexes that cannot be detected by other methods.

Shimura and Karger used the fluorophore-labeled **f**ragment **a**ntigen-**b**inding (Fab) region of an immunoglobulin as an affinity probe for capillary affinity electrophoresis of human growth hormone [45]. For this purpose, they labeled fluorescently

a cysteine residue on the Fab region. Shimura and Kasai prepared an affinity probe from a recombinant immunoglobulin against human insulin [46]. Then they used CAE to separate insulin from its complex with the affinity probe.

2.4.7 Affinity-trap electrophoresis

The affinity-trap electrophoresis (**a**ffinity-**t**rap PAGE, AT-PAGE) was developed by Awada *et al.* [47]. In this method, protein samples are separated by normal polyacrylamide electrophoresis and then are transferred onto an affinity-trap polyacrylamide gel with immobilized affinity probes. Proteins that do not have an affinity for the probes pass through the affinity-trap gel; proteins that interact with the probes are trapped and can be stained, or identified by Western blotting or mass spectrometry after in-gel digestion (Figure 2.4-10).

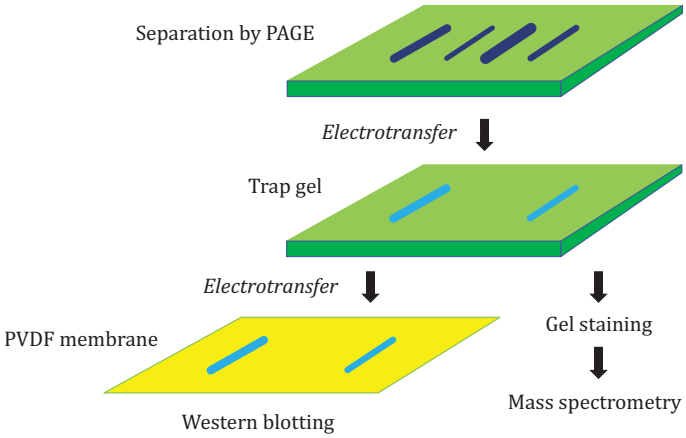

Figure 2.4-10: Principle of affinity-trap polyacrylamide gel electrophoresis.

The affinity-trap electrophoresis is used to analyze expressional proteins and post-translational protein modifications.

2.4.8 Charge shift electrophoresis

Charge **s**hift **e**lectrophoresis (CSE) is a simple, rapid, and sensitive method for distinguishing hydrophilic and amphiphilic proteins. It was introduced by Helenius and Simons [48]. They separated 17 hydrophilic proteins and 5 amphiphilic membrane proteins using agarose gel electrophoresis in three detergent solutions: in the presence of a nonionic detergent (Triton X-100); in a mixture of a nonionic and an

anionic detergent (Triton X-100 and sodium deoxycholate); and in a mixture of a nonionic and a cationic detergent (Triton X-100 and cetyltrimethylammonium bromide). The electrophoretic mobility of the hydrophilic proteins was unaffected in the three detergent solutions; however, the mobility of the amphiphilic proteins was shifted anodally in the Triton X-100-deoxycholate system and cathodally in the Triton X-100-cetyltrimethylammonium bromide system.

2.4.9 Mobility shift electrophoresis

Mobility **s**hift **e**lectrophoresis (MSE, electrophoretic mobility shift assay, band shift assay) is an affinity electrophoresis technique used to study if a protein or mixture of proteins is capable to bind a given DNA or RNA sequence. With the help of mobility shift electrophoresis, the affinity, abundance, association, and dissociation rate constants, binding specificity of DNA-binding proteins, transcription initiation, DNA replication, DNA repair, or RNA processing and maturation can be quantitatively determined. It is described by Garner and Revzin [49], and Fried and Crothers [50].

Mobility shift electrophoresis is carried out in a 15–20 cm agarose or polyacrylamide gel for 1.5–2 h [51]. The control lane contains unbound DNA or RNA fragment. The protein-nucleate complex is larger than the single protein polyion, therefore it moves more slowly and form another band that is shifted up in the gel (Figure 2.4-11).

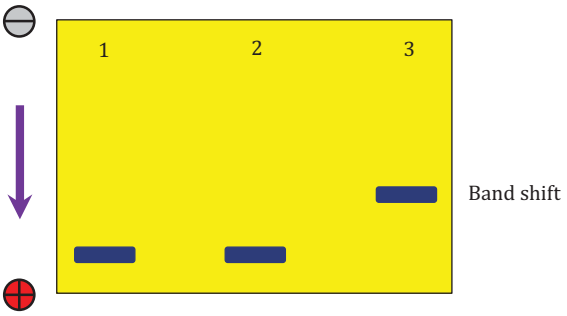

Figure 2.4-11: Mobility shift electrophoresis.
Lane 1 contains genetic material. Lane 2 contains a protein as well as a DNA fragment that, based on its sequence, does not interact with the protein. Lane 3 contains a protein and a DNA fragment that does react. The resulting complex is larger, heavier, and slower-moving. If not all DNA is bound to the protein, a second band might be seen reflecting the presence of free DNA.

The most common buffers used in this method are TRIS-acetate, TRIS-glycinate, and TRIS-borate buffers. Ion concentration and pH value affect the binding. Inclusion of **b**ovine **s**erum **a**lbumin (BSA) or other carrier protein in the gel improves the stability of some complexes during electrophoresis. Other reagents, which affect the formation

and stability of some protein-DNA complexes, are: nonionic detergents; polycations, such as spermine or spermidine; and ATP.

The mobility shift electrophoresis is carried out in four steps:

- Preparation of a radioactively labeled DNA probe containing a protein binding site.
- Preparation of a native gel. Typical gels for this assay are agarose or polyacrylamide gels in the range of $T = 4–5$ g/dl, and $C = 0.025$ (40:1 acrylamide/bisacrylamide ratio which gives a more cohesive gel).
- Binding of a protein mixture to a DNA probe.
- Electrophoresis of protein-DNA complexes in a gel, which is later dried and autoradiographed.

If the starting concentrations of protein and probe, and the stoichiometry of the complex is known, the apparent affinity of a protein for a nucleic acid sequence may be determined [52]. If the protein concentration is not known but the complex stoichiometry is known, the protein concentration can be determined by increasing the concentration of DNA probe until further increments do not increase the fraction of bound protein. By comparison with a set of standard dilutions of free probe run on the same gel, the number of moles of protein can be calculated.

To create a larger complex with a greater shift, an antibody against the protein can be added to the protein-nucleate mixture. This method is referred to as a *mobility supershift electrophoresis*, and is used to identify a protein in a protein-nucleic acid complex.

For visualization of the bands obtained by mobility shift electrophoresis, the standard ethidium bromide staining is less sensitive. The nucleic acid fragment is usually labeled with a radioactive agent, fluorescent, or biotin. When using a biotin label, streptavidin conjugated to an enzyme such as horseradish peroxidase detects the DNA fragment. The isotopic DNA labeling has little or no effect on protein binding affinity, but non-isotopic labels including flurophores or biotin can alter the affinity and stoichiometry of the protein interaction of interest.

2.4.10 Protocols

2.4.10.1 Electrophoresis of ALP isoenzymes in lectin agarose gels

Materials and equipment

Ready-to-use lectin agarose gels
Sample buffer
Electrode buffer
Application template
Electrophoresis unit
Power supply

Procedure

- Remove the cover film from a lectin agarose gel.
- Blot the gel briefly with a filter paper strip where samples will be applied.
- Place an application template on the blotted place adjusting it according to both marks on the support film.
- Press gently the application template with fingers to push out air bubbles between it and the gel.
- Dilute the sera up to an ALP activity of 200–1,000 U/l and mix them with a sample buffer in a volume ratio of 1:1. If cerebrospinal fluid or urine is analyzed, concentrate it up to the same activity prior to mixing it with the sample buffer.
- Apply 5 µl of serum, cerebrospinal fluid, or urine in the template slots and let them diffuse in the gel for 5 min.
- Blot the rest of the samples with a filter paper strip and remove the template and the strip.
- Fill the tanks of an electrophoresis cell with electrode buffer.
- Hang the film supported gel onto the bridge of an appropriate electrophoresis cell, the gel side down.
- Place the bridge with the gel into the electrophoresis cell. So the gel ends will contact directly with the electrode buffer in both electrode tanks.
- Run the ALP electrophoresis at constant direct voltage of 80–100 V for 25–30 min.

2.4.10.2 Staining ALP isoenzymes

Materials and equipment

*To stain ALP isoenzymes, two substrate solutions are needed: Solution 1 and Solution 2. Solution 1 is an AMPro-Cl buffer (AMPro is 2-**a**mino-2-**m**ethyl-1-**pro**panol) with pH = 10.2. Solution 2 contains NBT (**n**itro **b**lue **t**etrazolium chloride, 4-nitrotetrazolium blue chloride). The solutions are made according to Table 2.4-1*:

Table 2.4-1: Solutions for ALP isoenzymes.

Solution 1, 6x		***Solution 2, 24x***	
AMPro	14.23 ml	98% NBT**	0.83 g
37 g/dl HCl	4.14 ml	Methanol to	100.00 ml
0.1 g/dl $ZnCl_2$	6.82 ml		
10 g/dl $MgCl_2$	1.02 ml		
10 g/dl Na_2BCIP*	2.57 ml		
NaN_3	0.10 g		
Deionized water to	100.00 ml		

*Na_2BCIP = 5-**b**romo-4-**c**hloro-3-**i**ndolyl **p**hosphate disodium salt
NBT = **nitro **b**lue **t**etrazolium chloride

Inactivating solution

Methanol	40.0 ml
Acetic acid	10.0 ml
Deionized water to	100.0 ml

Procedure

- Make substrate solution (solution 1: solution 2: deionized water, 4:1:5, *V:V:V*).
- Immerse the gel (gel surface upward) in the substrate solution.
- Incubate at 37 °C for 30 min.
- Inactivate the ALP isoenzymes in the inactivating solution.
- Wash the gel in tap water for 5 min and rinse 3 times in deionized water.
- Dry the gel using a hair dryer at room temperature.
- Document the ALP isoenzyme bands using a scanner, photo camera, or densitometer at 580 nm.

2.4.10.3 Visualization with luminescent substrates

Materials and equipment

TBS (**T**RIS-**b**uffered **s**aline): 0.02 mol/l TRIS, 0.15 mol/l NaCl; adjust pH with HCl to pH = 7.4.
Nitro-Block (for ALP)
Nitrocellulose or PVDF membrane
Luminescent visualization solution
Plastic wrap

Substrate buffer

50 mmol/l TRIS-HCl, pH = 7.5 (for HRP) or
Dioxetane phosphate substrate buffer (for ALP): dioxetane in 0.05 mol/l phosphate buffer with pH = 7.4 (0.01 mol/l Na_2PO_4, 0.0018 mol/l KH_2PO_4)

Procedure

- Equilibrate the membrane twice in 50 ml substrate buffer for 15 min.
- Incubate in 50 ml freshly prepared 5 ml/dl Nitro-Block (for ALP) in dioxetane phosphate substrate buffer for 5 min.
- Incubate in 50 ml substrate buffer alone for 5 min.
- Transfer the membrane into 50 ml luminescent visualization solution.
- Soak 30 s (HRP) to 5 min (ALP).
- Remove the membrane, drain, and place face down onto a sheet of the plastic wrap. Fold the wrap on the membrane to form a liquid-tight enclosure.
- Place the membrane face down onto a film in a darkroom.

- Expose the film for a few seconds to several hours.
- Wash the membrane twice in 50 ml TBS for 15 min and process for chromogenic development.

2.4.10.4 Mobility shift electrophoresis

Materials and equipment

10x electrophoresis buffer (TAE, TBE, or TRIS-glycinate)
Native monomer solution
TMEDA
APS
Bulk carrier DNA
Bovine serum albumin
DNA-binding protein
10x sample buffer with dye
Water bath
Two-head peristaltic pump
10 µl glass capillary pipette
Filter paper
DNA fragments (20 to 300 bp long) as probes

Preparing of DNA probes

- *For restriction endonuclease fragments*
  Isolate a small DNA fragment containing the binding site of interest from a plasmid using a restriction endonuclease. Label the fragment by filing in a 5′-overhang with the Klenow fragment of *Escherichia coli* DNA polymerase and ^{32}P-labeled nucleotide or by end labeling using polynucleotide kinase. Separate the fragment from the plasmid by gel electrophoresis.
- *For PCR fragments*
  Generate a DNA fragment containing the binding site of interest by PCR. End-label one of the primers with polynucleotide kinase before the PCR reaction, or label the double-stranded PCR product.
- *For synthetic olygonucleotides*
  Synthesize and anneal complementary olygonucleotides to generate a double-stranded DNA fragment containing the binding site of interest. Label the probe using polynucleotide kinase.

Procedure

- Determine the concentration of the probe by ethidium bromide dot quantitation.
- Count 1 µl for Cerenkov counts in a scintillation counter to determine specific activity (cpm/µl).

A binding reaction will contain about 5,000 to 20,000 cpm and about 10 to 1,000 fmol probe. (l0 fmol DNA in a final reaction volume of 10 µl gives a total DNA concentration of 1 nmol/l).

- Dilute the 10x electrophoresis buffer to prepare electrophoresis buffer for the tanks.
- Assemble glass plates and 1.5 mm spacers for preparing a casting cassette.
- Add 0.70 ml of 10 g/dl TMEDA and 0.15 ml of 30 g/dl APS to 60 ml monomer solution. Swirl to mix.
- Cast the mixture in the casting cassette and insert a comb with teeth. Polymerize for 30 min.
- Remove the comb and spacers.
- Fill the lower tank with electrophoresis buffer.
- Attach the plates to the electrophoresis unit.
- Fill the upper tank with electrophoresis buffer.
- Prerun the gel at 100 V for 30 to 60 min. For low-ionic-strength buffers, use the two-head peristaltic pump and a flow rate of 5 to 30 ml per min to exchange the buffer between the upper and lower tanks.
- While the gel is prerunning, assemble the binding reaction by combining the following in a 0.5 ml or 1.5 ml microcentrifuge tube, adding protein last:
  5,000 to 20,000 cpm radiolabeled probe DNA (0.1 to 0.5 ng, > 10 fmol)
  0.1 to 2 µg nonspecific carrier DNA
  300/ml BSA
  10% Glycerol
  Appropriate buffer and salt
  DNA-binding protein (5 to 25 ng purified protein or 15 µg crude extract).
  Adjust the final reaction volume to 10 to 15 µl with buffer.
- Mix by tapping the bottom of the tube.
- Incubate the mixture at 37 °C for 15 to 30 min in a water bath.
- Apply each binding reaction into an appropriate well of the gel using a pipette. Add a dye into a separate well.
- Run electrophoresis at 30 to 35 mA. Stop before Bromophenol blue reaches the bottom of the gel (1.5–2 h for a 15–20 cm gel).
- Remove the glass plates from the gel box and the side spacers.
- Prize the glass plates apart with a spatula.
- Lay the glass plate with the gel attached and facing up.
- Place three sheets of chromatographically clear filter paper with the same dimensions on top of the gel.
- Lift up the glass plate. Peel the filter paper together with the gel from the plate.
- Cover the gel-filter paper sandwich with plastic wrap and dry it under vacuum.
- Autoradiograph the dried gel-filter paper sandwich overnight without an intensifying screen or 2–3 h with an intensifying screen.

2.4.11 Troubleshooting

Problem	Cause	Solution
Prior to electrophoresis		
The consistency of the agarose gel is too soft.	Agarose concentration is very low. Gelation time was too short.	Increase agarose concentration. Let agarose solution gel at least 60 min at room temperature, or better overnight at 4 °C.
Air bubbles in the agarose gel.	The glass plate or support film of the casting cassette was unclean. The glass plates were too cold.	Wash the glass plate before use. Do not touch the hydrophilic side of the support film with fingers. Heat the glass plates to 60–70 °C before casting the agarose gel.
The gel separates from the support film and adheres to the glass plate.	An incorrect support film was used. The gel was cast onto the wrong side of the support film. The glass plate was hydrophilic.	Do not exchange support films for agarose and polyacrylamide gels. Cast the agarose solution on the hydrophilic side of the support film. Treat the glass plate with Repel-silane before casting.
During electrophoresis		
Electric current does not flow during electrophoresis.	Some of power connections has no or poor contact.	Check all power connections.
The gel evaporates (the lid of the electrophoresis cell is covered with water condensation).	The electric power was too high. The cooling was insufficient.	Reduce the electric power. Check the cooling temperature. The cooling plate should be made of glass, metal or best of ceramic.
Gel “sweats” (is covered with water drops).	Strong electroosmosis.	Prior to gel casting add 20 g/dl glycerol or 10 g/dl sorbitol to the agarose solution.
The gel sparks and burns.	Thin areas in the gel, because the support film was not fixed on the glass plate during the gel casting. Poor contact between the gel and electrode strips.	Roll strongly the support film on the glass plate of the casting cassete. Put a glass plate on the electrode strips.

(continued)

Problem	Cause	Solution
After electrophoresis		
No protein bands in the gel.	The proteins have left the gel.	Monitor the moving of dye front.
The protein bands are pale.	The protein concentration or the sample volume was insufficient. The sample was not completely dissolved.	Concentrate the sample, or apply more sample volume. Treat the sample with ultrasound and centrifuge, if turbid.
The protein bands are not sharp or have tails.	The sample contained too much protein. The bands have diffused. The sample had solids.	Reduce the sample volume or dilute the sample. Fix the protein bands immediately after the electrophoresis. Centrifuge the sample before applying.
The adjacent samples run into each other.	The sample volume was too much. The application template did not lie closely on the gel.	Concentrate the samples and apply less volume. Press gently the application template toward the gel to remove air bubbles between the template and gel.

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2.5 Polyacrylamide gel zone electrophoresis of proteins

Poly**a**crylamide **g**el **e**lectrophoresis (PAGE) is used for separating proteins ranging in relative molecular masses M_r from $5 \bullet 10^3$ to $2 \bullet 10^6$. The pore size of the gel is controlled by modulating the concentrations of acrylamide and BIS during the gelling.

The *native PAGE* is used to separate proteins in their native states, for example, enzymes. It is carried out at near neutral pH value to avoid acidic or alkaline denaturation of proteins. The separation depends on the protein mobilities and the polyacrylamide gel properties. It gives also information about protein isomers.

The *denaturing PAGE* is used to separate proteins, denatured by SDS or other detergents.

2.5.1 Theory of polyacrylamide gel zone electrophoresis of proteins

The resolution of the polyacrylamide gel zone electrophoresis depends on the volume and electric charge of proteins. The electric charge of a protein is a function of the dissociation degree of its chemical groups and depends on the buffer pH value and its ionic strength.

2.5.1.1 Continuous buffers

The polyacrylamide gel zone electrophoresis is running in one buffer. It is contained in the gel, sample, and electrode tanks.

The ionic strength of a buffer should be relative low to keep heat generation at a minimum. On the other hand, the too low ionic strength buffers have lower electrophoretic resolution. In general, the ionic strength of the electrophoresis buffers should be in the range of 0.01–0.1 mol/l.

The polyacrylamide gel zone electrophoresis can be carried out in alkaline or acidic buffers. In alkaline buffers, the proteins migrate to the anode, since they are negatively charged; in acidic buffers they migrate to the cathode, since

they are positively charges. The sample is applied on the cathode or anode side of the gel, respectively.

The electrode buffer concentration can be equal to the resolving buffer concentration or greater. Usually, the buffers used contain TRIS, Bis-TRIS, imidazole, or histidine as a buffer base.

In polyacrylamide gel zone electrophoresis in alkaline buffers, Bromophenol blue Na salt is added to the sample; and in acidic buffers, Fuchsine red is added to the sample. These dyes overran the proteins and show their movement in the gel. The electrophoresis should be stopped after the dye front has reached the opposite gel end.

The preparation of polyacrylamide gels of different concentrations for polyacrylamide gel zone electrophoresis is shown in Table 2.5-1.

Table 2.5-1: Contents of homogeneous polyacrylamide gels of different *T*-concentrations, but same dimensions (120 × 250 × 0.5 mm). c_{APS} was calculated according to the formula $c_{APS} = 0.32/T$, which we propose.

Solutions	Concentrations	T = 5 g/dl	T = 12.5 g/dl	T = 20 g/dl
Buffer-gel system for acidic proteins				
TRIS-glycinate buffer, 4x (0.8 mol/l, pH = 8.3) or	0.2 mol/l	4.0 ml	4.0 ml	4.0 ml
TRIS-chloride buffer, 4x (1.5 mol/l, pH = 8.8)	0.375 mol/l	4.0 ml	4.0 ml	4.0 ml
Monomeric solution (T = 50 g/dl, C = 0.03)	T = 5–20 g/dl	1.6 ml	4.0 ml	6.4 ml
87% glycerol	0.2 mol/l	2.8 ml	2.8 ml	2.8 ml
10 g/dl TMEDA	0.4 mmol/l	74.4 µl	74.4 µl	74.4 µl
10 g/dl APS	0.2 to 0.15 mmol/l	64.0 µl	25.6 µl	16.0 µl
Deionized water to 16.0 ml	–	7.5 ml	5.1 ml	2.7 ml
Buffer-gel system for alkaline proteins				
TRIS-acetate buffer, 4x (0.8 mol/l, pH = 4.7)	0.2 mol/l	4.0 ml	4.0 ml	4.0 ml
Monomeric solution (T = 50 g/dl, C = 0.03)	T = 5–20 g/dl	1.6 ml	4.0 ml	6.4 ml
87% glycerol	0.2 mol/l	2.8 ml	2.8 ml	2.8 ml
10 g/dl TMEDA	0.4 mmol/l	74.4 µl	74.4 µl	74.4 µl
10 g/dl APS	0.2 to 0.15 mmol/l	64.0 µl	25.6 µl	16.0 µl
Deionized water to 16.0 ml	–	7.5 ml	5.1 ml	2.7 ml

McLellan buffers

McLellan proposed a set of buffers, which are applicable for running electrophoresis of native proteins [1]. These buffers cover the pH range from 3.8 to 10.2, and have relatively low conductivity (Table 2.5-2).

Table 2.5-2: Buffers for continuous electrophoresis of native proteins.

Buffer, pH	Basic component, concentration	Concentration for 5x solution	Acidic component, concentration	Concentration for 5x solution
3.8	β-Alanine, 30 mmol/l	13.36 g/l	Lactic acid, 20 mmol/l	7.45 ml/l
4.4	β-Alanine, 80 mmol/l	35.64 g/l	Acetic acid, 40 mmol/l	11.50 ml/l
4.8	GABA, 80 mmol/l	41.24 g/l	Acetic acid, 20 mmol/l	5.75 ml/l
6.1	Histidine, 30 mmol/l	23.28 g/l	MES, 30 mmol/l	29.28 g/l
6.6	Histidine, 25 mmol/l	19.40 g/l	MOPS, 30 mmol/l	31.40 g/l
7.4	Imidazole, 43 mmol/l	14.64 g/l	HEPES, 35 mmol/l	41.71 g/l
8.1	TRIS, 32 mmol/l	19.38 g/l	HEPPS, 30 mmol/l	37.85 g/l
8.7	TRIS, 50 mmol/l	30.29 g/l	Boric acid, 25 mmol/l	7.73 g/l
9.4	TRIS, 60 mmol/l	36.34 g/l	CAPS, 40 mmol/l	44.26 g/l
10.2	Ammonia, 37 mmol/l	12.50 ml/l	CAPS, 20 mmol/l	22.13 g/l

Using McLellan buffers, the effect of pH values on the mobility of hemoglobin (Hb) variants A and C on gel was examined (Figure 2.5-1). HbA and HbC have isoelectric points pI equal to 7.1, and 7.4, respectively. Therefore, they have not enough electric charge to enter the gel at pH = 7.4. In acidic buffers, both hemoglobin variants are positively charged, however HbC has greater electric charge than HbA; as a result it moves further than HbA. In basic buffer, both hemoglobin variants are negatively charged, however HbC has less electric charge than HbA; as a result it moves slower than HbA.

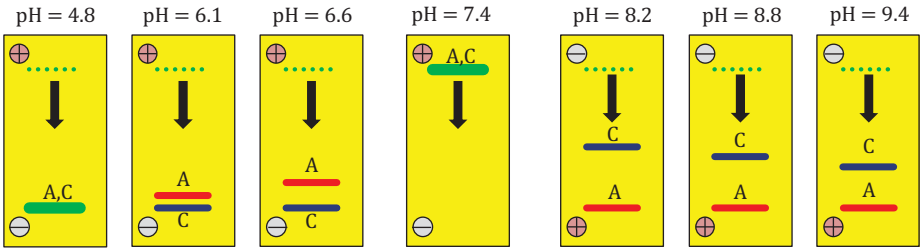

Figure 2.5-1: Effect of pH values on protein mobility of hemoglobins A and C.
A – hemoglobin A; C – hemoglobin C.
At pH = 7.4 the hemoglobins do not enter the gel.

Other buffers that have been used for native continuous electrophoresis are TRIS-glycinate (pH range 8.3–9.5) [2], TRIS-acetate (pH range 7.2–8.5) [3], and TRIS-borate (pH range 8.3–9.3) [4]. Borate ions [$B(OH)_4^-$] form complexes with some sugars and therefore influence the resolution of glycoproteins.

2.5.1.2 Running electrophoresis

Because the bandwidths depend on the volume of the sample, it must be kept as small as possible. It is useful also to dilute the sample buffer to at least half ionic strength of gel buffer. The dilution decreases the sample buffer conductivity as a result of which the voltage across the sample will be higher, what will concentrate the sample before entering the gel.

The contact between the electrodes and the polyacrylamide gel can be realized *via* filter paper bridges impregnated with electrode buffer (Figure 2.5-2). They must be parallel to the gel ends, and overlap 1 cm of its ends.

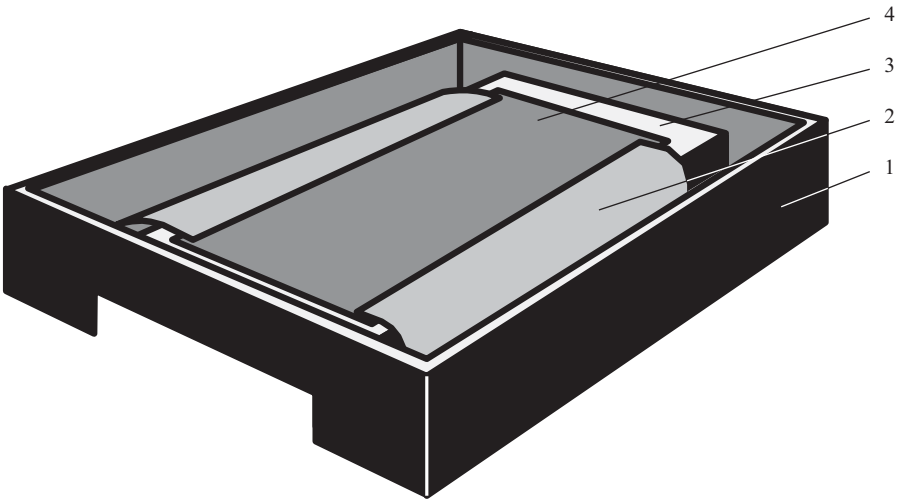

Figure 2.5-2: Filter paper bridges as contacts between the gel and electrode buffers.
1. Separation chamber; 2. Filter paper bridge; 3. Cooling plate; 4. Gel.

An alternative to the paper bridges are the electrode strips. They are composed of gel (usually polyacrylamide gel) or thick filter paper. The strips contain electrode buffer. The electrodes are placed onto the electrode strips (Figure 2.5-3).

The electrophoretic conditions depend on buffer composition and gel thickness. The voltage can be in the range 200 to 500 V and should not be changed during the

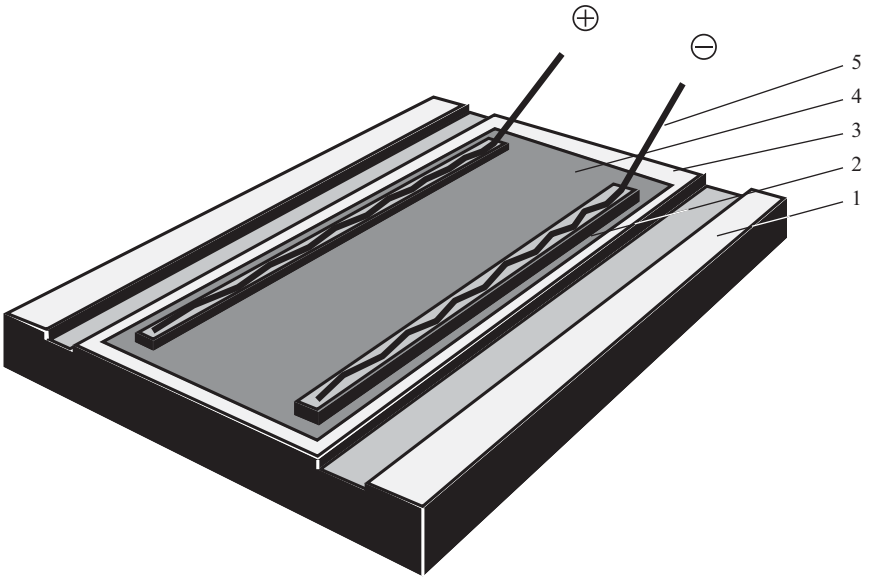

Figure 2.5-3: Electrode strips as contacts between the gel and the electrodes.
1. Separation chamber; 2. Electrode strips; 3. Cooling plate; 4. Gel; 5. Electrode.

electrophoresis. The cooling temperature is recommended to be 10 °C. If required, the gels can be cooled to 2–4 °C.

The polyacrylamide gel zone electrophoresis of proteins can be carried out in homogeneous or gradient gels. The gels can be situated vertically or horizontally. The vertical zone electrophoresis is carried out in slab or round gels. The horizontal zone electrophoresis takes place in slab gels.

2.5.2 Homogeneous zone electrophoresis

The gels for *vertical electrophoresis* can be used together with their casting cassettes or casting tubes. So they become direct contact with the tank buffers. The samples are pipetted in gel wells under the upper electrode buffer. The wells are produced by a comb, which was inserted during the polymerization process. The samples must contain 10–20 g/dl glycerol or sucrose to delay their diffusion in the upper electrode buffer.

The vertical PAGE on polyacrylamide gels under native conditions was used for studying the interaction between the domains of the oncosuppressor p53 and its protein ligands MDM2/X (**m**urine **d**ouble **m**inute) [5]. While the two proteins MDM2/X alone were able to enter the gel, the binary complex between p53 and MDM2/X could not entry the gel. This technique is reliable for determining the different affinity

elicited by MDM2 or MDMX toward p53, and can be useful for analyzing the dissociation power by diverse molecules on the p53–MDM2/X complex.

The gels for *horizontal electrophoresis* are usually cast on a support film. The samples are applied generally onto the gel surface with the help of a silicone template.

The horizontal electrophoresis in thin homogeneous or gradient gels cast on support films has many advantages over the vertical electrophoresis [6]: the gels are easy to handle during electrophoresis; permit an usage of electrode strips with buffers; need small sample volumes and adequate cooling; the resolved protein bands are quickly stained; and the electrophoresis can be automated.

2.5.3 Protocols

2.5.3.1 Casting homogeneous polyacrylamide gels on a support film

Support films are hydrophilic, however, the upper glass plate, which comes in contact with the gel, must be hydrophobic. Therefore, it should be coated with Repel-silane (Pharmacia Biotech, Uppsala) or dichlorodimethyl silane (Merck, Darmstadt) to prevent the gel to adhere (Figure 2.5-4).

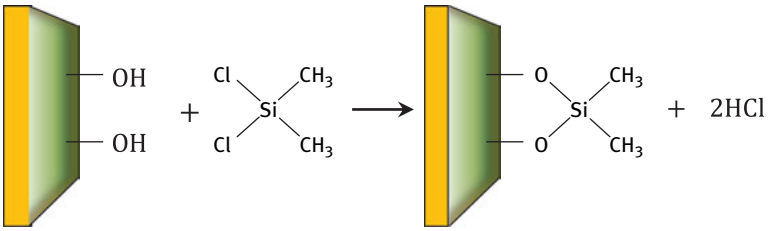

Figure 2.5-4: Binding of dichlorodimethyl silane (Repel-silane) to a glass surface.

The silanization is carried out in the following way:

- Smear a glass plate with 2 ml/dl Repel-silane in chloroform or with 2 ml/dl dichlorodimethyl silane in 1,1,1-trichloroethane.
- Dry the plate until the solution evaporates.
- Wash the plate with running and deionized water and dry. So hydrochloric acid, gained at the silanization, is removed.

Casting thin PAA gels by cassette technique

Materials and equipment

Glass plates
Repel-silane

Support film for PAG
Photo roller
U-form silicone gasket
Clamps
Monomeric solution

Procedure

The cassette technique [7] is used for casting of 0.5–1.0 mm gels. It is carried out in the following way (Figure 2.5-5):

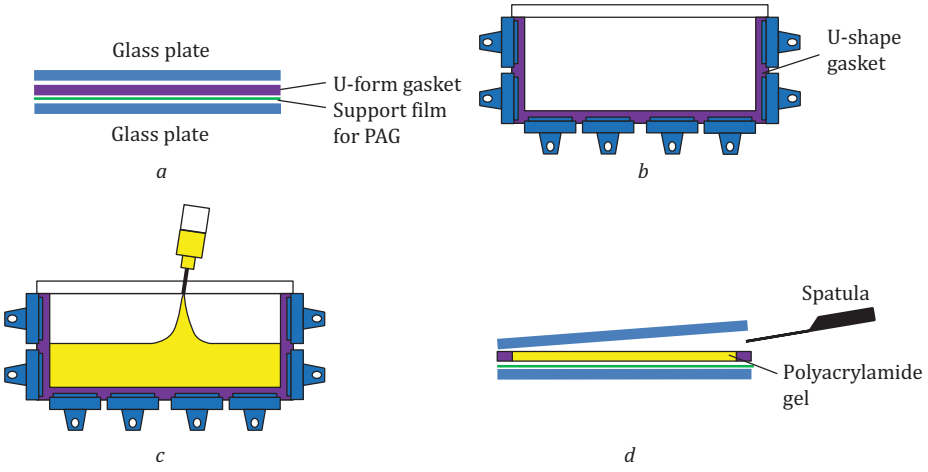

Figure 2.5-5: Steps (*a, b, c*, and *d*) of casting polyacrylamide gel by cassette technique.

- Apply a few drops of water onto a glass plate, place a support film over it, with its hydrophobic side down, and roll the film with a photo roller to remove the air bubbles. Thus, a thin film of water is created between the glass plate and the support film, which holds them together (Figure 2.5-6). Remove the excess of water with a filter paper.
- Place a 0.5–1.0 mm thick U-shaped silicone gasket on the margins of the support film and press it slightly down.
- Smear a second glass plate of same dimensions (cover glass) with a repelling solution.
- Gather the two glasses in a glass cassette, fix it with clamps, and place it vertically.
- Cast the monomeric solution into the cassette using a syringe or a pipette. After 1 hour, remove the clamps from the cassette, disassemble it, and take the gel from the cassette. The gel can be used immediately for electrophoresis or can be stored in a plastic envelope for weeks in the refrigerator at 4 °C.

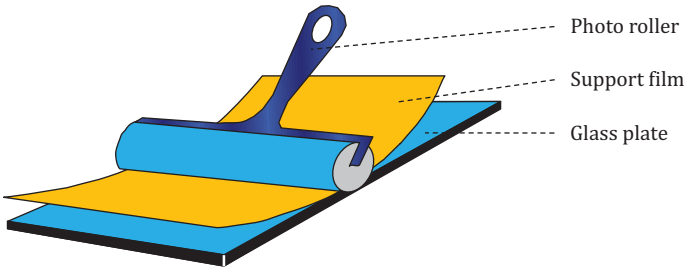

Figure 2.5-6: Rolling a support film.

Casting ultrathin PAA gels by flap technique

Materials and equipment

Glass plates
Repel-silane
Support film for PAG
Silicone spacers
Monomeric solution
Spatula

Procedure

The flap technique [8] is suitable for production of ultrathin (0.05–0.30 mm) polyacrylamide gels, even with dimensions of 40 × 20 cm. It is carried out in the following way (Figure 2.5-7):

– Put a few drops of water onto a horizontal 4 mm thick glass plate (lower glass plate) and place on it a support film with its hydrophobic side down. Roll the support film with a photo roller to remove the air bubbles.

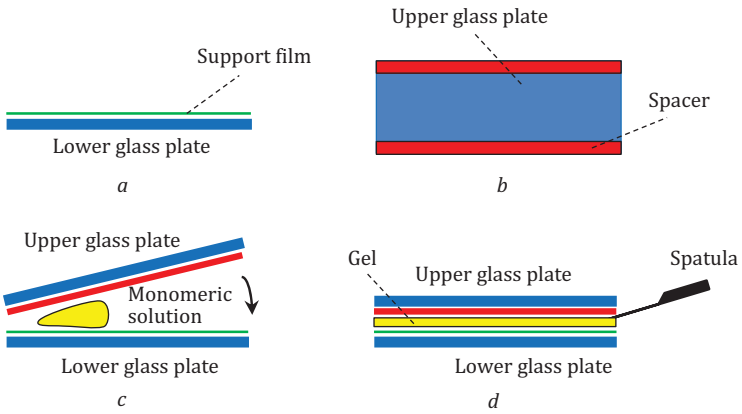

Figure 2.5-7: Steps (*a*, *b*, *c*, and *d*) of polyacrylamide gel casting by flap technique.

- Smear over a second glass plate (upper glass plate) using a fluff-free cloth soaked with repelling solution to make the glass surface hydrophobic.
- Mount an adhesive tape onto the longer sides of the upper glass plate as spacers, using one or more layers depending on the thickness of the gel required (one layer of the tape provides 0.15 mm thickness).
- Apply a degassed monomeric solution onto the middle of the support film (approx. 6 ml to cast a gel of 240 × 110 × 0.15 mm).
- Place slowly the upper glass plate onto the support film, starting at a short end of the lower plate, and avoiding air bubbles.
- Let the monomeric solution polymerize in the resulting sandwich for 60 min at room temperature. Remove the gel with the support film from the glass plates to use, or envelope it into a plastic bag and store in the refrigerator at 4 °C for up to several weeks.

2.5.3.2 Casting homogeneous polyacrylamide gels on a support fabric

Materials and equipment

Glass plates
Repel-silane
Hydrophobic support film
Photo roller
Support net for PAG
U-shape silicone gasket
Clamps
Monomeric solution

Procedure

The casting of polyacrylamide gels on support fabric is similar to the casting of film-supported gels:

- Apply a few water drops on a glass plate, place a hydrophobic polyester film on it, and roll it with a photo roller.
- Place a hydrophilic polyester fabric (net) on the hydrophobic film and soak it with buffer containing 30 g/dl glycerol. Blot the buffer excess with a fluff-free filter paper.
- Place a U-shape silicone gasket on the polyester film 1–2 mm away from the fabric margins.
- Cover the gasket with a silanized glass plate.
- Hold together the so built casting cassette with clamps and fill it with monomeric solution.
- After copolymerization of the monomeric solution, disassemble the cassette, and take out the net gel.

2.5.3.3 Casting two mini gels for vertical homogeneous zone electrophoresis

Materials and equipment

TRIS
HCl
Acrylamide-BIS mixture (T = 40 g/dl, C = 0.03)
TMEDA
APS
Combs

Gel buffer, 3x (TRIS-HCl, pH = 8.5)

TRIS	36.3 g in 80 ml of deionized water
Adjust the solution with HCl to pH = 8.5.	
Deionized water to	100.0 ml

Store the solution in the refrigerator.

Procedure

Resolving mini-gel, 2x (T = 16.4 g/dl)

3x gel buffer	6.66 ml
Acrylamide-BIS mixture	8.20 ml
87% Glycerol	2.30 ml
10 g/dl TMEDA	0.10 ml
10 g/dl APS	0.10 ml
Deionized water	2.64 ml

Mix the components and transfer the mixture into two casting cassettes using a pipette with a bulb. Leave space in the cassettes for the stacking gels. Allow the gels to polymerize at room temperature for 1 h.

Stacking gel (T = 4.0 g/dl)

3x gel buffer	3.33 ml
Acrylamide-BIS mixture	1.00 ml
10 g/dl TMEDA	0.10 ml
10 g/dl APS	0.05 ml
Deionized water	5.52 ml

Swirl the solution to mix and transfer into the casting cassettes over the resolving gel using a pipette with a bulb. Insert combs in the solution to produce application wells. Use the total gel an hour after the polymerization of stacking gel.

2.5.4 Troubleshooting

Problem	Cause	Solution
Prior to electrophoresis		
The monomeric solution does not polymerize, or polymerizes to slow.	The concentration of TMEDA or APS in the monomeric solution was too low.	Use at least 5 µl 10 ml/dl TMEDA and 5 µl 10 g/dl APS per 1 ml monomeric solution.
	The APS solution was too old.	Use new APS solution. Keep the APS solution in the refrigerator.
	The polymerization temperature was low.	Cast the gel at 20–25 °C.
	Too much oxygen in the monomeric solution.	Vent the monomeric solution using a water-jet pump.
	The pH value was not optimal for gel polymerization.	When pH value is low, increase the concentration of TMEDA.
A part of the gel is not polymerized.	The APS solution was partially dissolved in the monomeric solution.	Use a magnetic stirrer after APS was added, and then cast the solution.
The polyacrylamide gel is too soft.	The concentration of the monomeric solution was too low.	Increase the concentration of the monomeric solution.
	The APS solution was too old or stored improperly.	Use a new APS solution. Store the APS solution in the refrigerator.
The gel margins are polymerized insufficient.	The atmospheric oxygen has inhibited the polymerization of the gel margins.	Deaerate the monomeric solution and overlay it with deionized water after casting.
The gel polymerizes too rapidly.	The concentration of TMEDA or APS in the monomeric solution was too high.	Reduce the concentration of TMEDA or APS.
The gel separates from the support film.	An incorrect support film was used.	Do not exchange the support films for polyacrylamide and agarose gels.
	The gel was cast onto the wrong side of the support film.	Cast the gel on the hydrophilic side of a support film; prior to casting check its hydrophilic side with water drops.
	The support film was too old.	Use newly prepared support film.

(continued)

Problem	Cause	Solution
The gel does not stick to the support film, but to the glass plate.	The glass plate was too hydrophilic. The gel concentration was too low.	Clean the glass plate and coat it with Repel-silane. The lowest polyacrylamide concentration *T* should not be less than 4 g/dl.
There are bubbles in the gel.	The glass plate was dirty. The support film was unclean. There were bubbles in the outlet tube of the gradient mixer.	Wash the glass plate before use with detergent and ethanol. Do not touch the hydrophilic side of the film with fingers. Keep the outlet tube clean and dry. Remove air bubbles in the casting cassette by knocking on it.
There is a liquid on the gel surface.	The polyacrylamide gel has hydrolyzed.	Store the polyacrylamide gels with alkaline buffers in a refrigerator.
During electrophoresis		
It does not flow or flows too little electric current.	One of the connectors had no or poor contact. Poor contact between electrodes and resolving gel.	Check all connections. Check the uniform laying of the electrodes; eventually complain them with a glass plate.
The gel “evaporates” (the lid of the electrophoresis cell is covered with condensed water).	The cooling is insufficient. The electric voltage is too high.	Check the gel temperature. The cooling blocks should be made of glass, metal, or best of ceramic. Reduce the electric voltage.
The gel sparks and burns.	There are thin areas in the gel because the support film was not fixed on the glass plate during the casting.	Roll strongly the support film on the glass plate of the casting cassette.
There are wavy iso-pH lines.	The APS concentration was too high during the polymerization. The urea in the urea-containing gels was destroyed to isocyanate.	Swell rehydratable gels in carrier ampholyte solution. Use urea-containing gels immediately after preparation.

(continued)

Problem	Cause	Solution
After electrophoresis		
There are no protein bands in the gel.	The cathode and anode were exchanged.	Check the connections between the electrophoresis cell and power supply.
	The proteins have left the gel.	Monitor the movement of the dye front.
Some bands are missing.	The proteins built complexes.	Give 7 mol/l urea or EDTA, or a nonionic detergent (Triton X-100, or Nonidet NP-40) into the sample.
The protein bands are very weak.	The protein concentration or the volume of sample was too small.	Concentrate the sample or proof more sensitive staining method.
	The sample was not completely dissolved.	Treat the sample with ultrasound or use additives to dissolve it.
Protein bands are blurred.	Too much protein in the sample.	Apply smaller volume or dilute the sample.
Protein bands form tails.	Components in the sample.	Centrifuge the sample before applying.
The samples of adjacent tracks run into each other.	The application template was not tight on the gel.	Press gently the application template before applying the sample to put out air bubbles.
The bands on applying the side gel margins are distorted.	The sample was applied too close to the side margins.	Apply the samples 1 cm away from the side margins.
The gel dissolves from the support film during the staining.	The bonds between the support film and gel were partially hydrolyzed by strongly acids (TCA) in some staining solutions.	The concentrated gels ($T > 10$ g/dl) must have cross-linking degree of $C = 0.02$.
The gel rolls on during drying.	The gel contracts.	Add 5 g/dl glycerol in the final wash solution.

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2.6 Gradient polyacrylamide gel electrophoresis of proteins

The gradient polyacrylamide gels were introduced by Kolin [1] and improved by Margolis and Kenrick [2]. Their *T*-concentration varies continuously from one to other gel end, which results in a continuous decrease of the pore diameters. These gels can be used to identify the molecular masses and radii of native proteins [3]. For this purpose, the velocities of tested proteins are compared with the velocities of proteins with known masses and radii.

The gradient zone electrophoresis is carried out in same buffers. The samples are applied on the large-porous gel end. In the beginning they move fast through the gel; later, however, the velocity of their movement decreases more and more because they begin to migrate through smaller and smaller gel pores.

2.6.1 Theory of the gradient gel zone electrophoresis

To explain the events in the gradient gel electrophoresis, we should consider the movement of a charged particle in a linear concentration gradient. The velocity v of a particle equals the ratio between the distance $l = l_2 - l_1$ and the time $t = t_2 - t_1$. If we express the ratio v/l as a, then

$$v = \frac{dl}{dt} = al \tag{2.6-1}$$

hence

$$\frac{dl}{l} = adt \tag{2.6-2}$$

The integration of eq. (2.6-1) in the intervals l_1, l_2 and t_1, t_2 gives the expressions

$$\int_{l_1}^{l_2} \frac{dl}{l} = \int_{t_2}^{t_1} a dt \tag{2.6-3}$$

and

$$\ln \frac{l_2}{l_1} = \mathrm{at} \tag{2.6-4}$$

which can be transformed in

$$e^{at} = \frac{l_2}{l_1} \tag{2.6-5}$$

It follows from eq. (2.6-1) and (2.6-5) that

$$l_1 = \frac{v_1}{a} \tag{2.6-6}$$

and

$$l_2 = \frac{v_1}{a} e^{at} \tag{2.6-7}$$

So, the covered distance

$$l_2 - l_1 = \frac{v_1}{a} \left(e^{at} - 1\right) \tag{2.6-8}$$

and the modified linear velocity

$$v_2 - v_1 = v_1 \left(e^{at} - 1\right) \tag{2.6-9}$$

Let us now analyze the movement of the zone $l_1 l_1'$ through the linear concentration gradient, the initial zone width being $l_1' - l_1 = d_1$, and final zone width being $l_2' - l_2 = d_2$. It follows from eq. (2.6-5) and eq. (2.6-9) that

$$d_2 = e^{at} \left(l_1' - l_1\right) = d_1 e^{at} = d_1 \frac{v_2}{v_1} \tag{2.6-10}$$

The polyionic velocity decreases during electrophoresis, that is, $v_2 < v_1$. Therefore, according to eq. (2.6-10), the final width of the band is narrower than its initial width, that is, $d_2 < d_1$.

The gradient gel has typically T = 4–30 g/dl (0.5 to 4.22 mol/l) (Figure 2.6-1). Margolis and Kenrick used TRIS-borate-EDTA buffer with pH = 8.3 for the resolving gel as well as in the electrode tank. Other buffers for gradient zone electrophoresis are TRIS-glycinate, TRIS-barbitalate, and TRIS-taurinate.

The separated protein bands can be detected by staining, enzyme-substrate reactions, immunoprecipitation, autoradiography, fluorography, additional blotting, or immune printing.

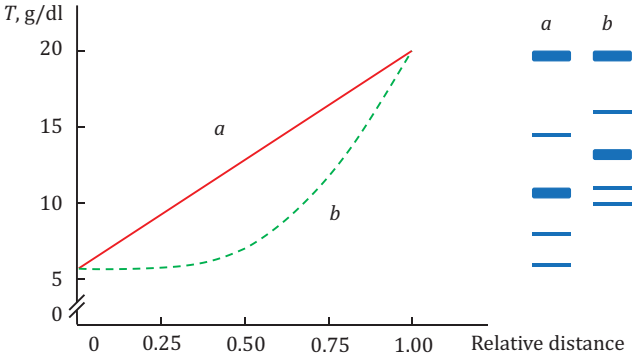

Figure 2.6-1: Proteins separated in a linear (*a*) and an exponential (*b*) gradient gels. The polyacrylamide concentration *T* is 5–20 g/dl.

The resolution of the gradient polyacrylamide electrophoresis is high, but the separation takes a long time. Besides, there are difficulties while treating the gel after the electrophoresis – it deforms during drying.

2.6.2 Ferguson plots

As native proteins migrate through a polyacrylamide gel with increasing concentration, they migrate through regions of ever smaller pore sizes and their mobilities decrease. At the end each protein reaches its pore-limit where it stops. The graphic dependence of the polyionic mobilities on the gel concentration was studied by Kenneth Ferguson while he analyzed the movement of the pituitary hormone in starch gels [4]. On the basis of the extended Ogston theory that explains the migration of a particle in an inert fiber network, he described the linear dependence of the mobility of polyions on their masses, now referred to as Ferguson plots.

If different polyions are separated under same conditions (in a same buffer at same temperature and in same electric field strength), but at different gel concentrations different distances *d* are run, that is, they show different relative mobilities.

The relative mobility μ_r of a polyion is defined as the ratio between the migration distance *d* of the polyion (or its mobility μ) in the gel, and the migration distance of the polyion at free electrophoresis d_0 (or its absolute mobility μ_0). The relative mobility ought to be calculated at least in three different gel concentrations according to the equation

$$\mu_r = \frac{d}{d_0} = \frac{\mu}{\mu_0} \tag{2.6-11}$$

The absolute mobility μ_0 can be determined by extrapolation of the gel concentration to 0. The line slope is referred to as the retardation coefficient K_R, that is, the

extent in which the gel matrix affects the polyionic mobility at a certain pH value, ionic strength, and temperature. The absolute mobility μ_0 is related to the electric charge of a protein, while K_R is related to its hydrodynamic properties, which are dependent on the peptide chain length (its mass).

The relationship between the relative protein mobility and the gel concentration may be expressed by the equation

$$\log \mu_r = \log \mu_{0(r)} - K_R T_r \tag{2.6-12}$$

where $\mu_{0(r)}$ is the relative absolute mobility of the protein in the absence of any sieving matrix, and T_r is the relative total concentration of the gel, equal to the ratio between T, in g/dl, and 1 g/dl. μ_r, $\mu_{0(r)}$, K_R, and T_r are dimensionless magnetutes. If one of these parameters is changed, new Ferguson plots appear.

In the presence of SDS, all proteins have same μ_0. Under these conditions, K_R is related to protein molecular masses at any given T, and allows their calculation. In other words, SDS treated proteins have identical charge-to-mass ratio and therefore migrate with same speed.

According to the location of Ferguson plots, following statements can be made:

- If the lines are parallel, the polyions have identical compositions, but different charges, as the isoenzymes.
- If two non-parallel lines do not intersect, the polyion of the upper straight line is smaller and has a higher net charge than the polyion of the lower line.
- If the plots cross, the polyion whose plot crosses the *y*-axis high up has bigger mass but smaller charge.
- If a lot of plots cross in one point, the protein builds various polymers (Figure 2.6-2).

For globular proteins, a linear relationship between K_R and the molecular radius r_s (Stokes radius) exists. So the molecular size can be calculated from the slope of the plot. Once the free mobility and the molecular radius are known, the net charge can also be calculated.

The Ferguson plots are characteristic also for agarose gels. At agarose gel concentration of 0.75 to 1.5 g/dl, they are linear for proteins with M_r = 500–4,000•10^3 [5].

2.6.3 Determination of Stokes radii and masses of native proteins

The Ferguson plots can be used for calculating K_R and the Stokes radii r_s of proteins. In case of globular proteins, a linear relationship exists between K_R and r_s of proteins with relative molecular masses M_r in the range from 10,000 to 100,000. After the mobility and radius of a protein are known, its electric charge can also be determined [6].

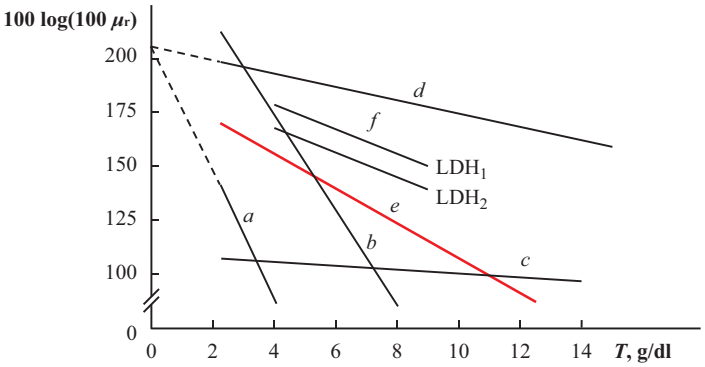

Figure 2.6-2: Ferguson plots: relative polyionic mobilities as a function of the polyacrylamide gel concentration *T*. The slopes of the lines are equal to K_R.
a) A polyion with large mass and low charge; *b*) A polyion with large mass and large charge; *c*) A polyion with small mass and small charge; *d*) A polyion with small mass and large charge; *e*) A polyion with intermediate mass and intermediate charge (typical case); *f*) LDH_1 and LDH_2 – isoenzymes of lactate dehydrogenase, which have same masses but different charges.

The Stokes radius r_s of a native protein is linked to its relative maximum migration distance $d_{r(max)}$ in a gel according to the equation

$$\{[\ln(\ln d_r)]_{max}\}^{-1} = \alpha r_s + \beta \tag{2.6-13}$$

where α and β are constants. In order to determine the relative maximum migration distance, it is necessary to use linear gradient gels with T = 3–30 g/dl and constant concentration of the cross-linker (BIS). Simultaneously the Stokes radius of a native protein is linked to its relative molecular mass M_r over the relationship

$$r_s = \varepsilon M_r^{1/3} \tag{2.6-14}$$

where ε is a constant. Substituting eq. (2.06-13) in eq. (2.06-14), the following equation is obtained:

$$\ln\{[\ln(\ln d_r)]_{max}\}^{-1} = \gamma(M_r)^{1/3} + \delta \tag{2.6-15}$$

where γ and δ are constants. The last equation shows that the natural logarithm of the reciprocal of $d_{r(max)}$ is linearly correlated with the third root of M_r. If the logarithm of $d_{r(max)}$ of calibration proteins is plotted against the logarithm of their relative molecular masses, a straight line is obtained. It allows calculating the relative molecular mass of M_r of a protein using the relative maximum migration distance $d_{r(max)}$ (Figure 2.6-3).

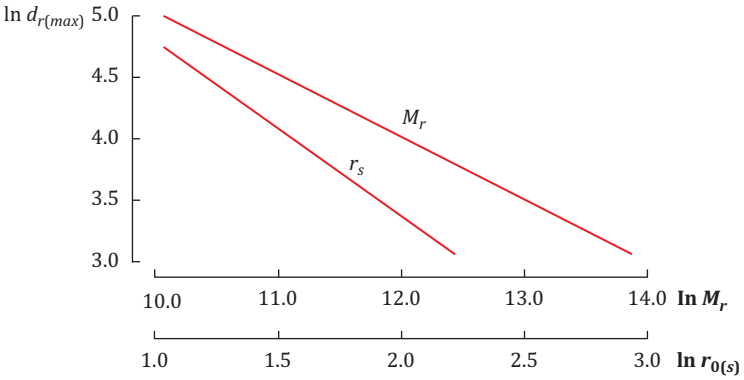

Figure 2.6-3: Calibration straight lines used for determination of Stokes radii r_s or relative molecular masses M_r of carbonic anhydrase from mammalian erythrocytes.
$r_{0(s)}$ – Relative Stokes radii (dimensionless)

The determination of protein relative molecular mass proceeds in two steps [7]: In the first step, the protein maximum migration distance is calculated by the relationship between the migration distance and separation time. In the second step, the maximum migration distances of calibration proteins are obtained in relation to their volumes. Then the relative molecular mass and the radius of the protein are calculated from the calibration straight line (Table 2.6-1).

Table 2.6-1: Relative molecular masses M_r and Stokes radii r_s obtained using gel electrophoresis [8].

Protein	M_r	r_s, nm
Cytochrome *c*	12,400	1.70
Ribonuclease	12,640	1.64
Lysozyme, chicken	14,300	1.91
Myoglobin	17,000	1.86
α-Chymotrypsinogen, bovine	24,000	2.09
β-Lactoglobulin, bovine	35,000	2.73
Ovalbumin	45,000	2.73
α-Amylase	48,580	3.17
Prealbumin (transthyretin)	61,000	3.36
Hemoglobin	67,360	3.13
Serum albumin, human	66,500	3.55
Serum albumin, bovine	68,000	3.53
Alcohol dehydrogenase from liver	80,050	3.50
Transferrin, human	81,000	3.67

Table 2.6-1 (continued)

Protein	M_r	r_s, nm
Lipoxidase	97,440	3.85
Lactate dehydrogenase from pig	109,000	3.60
Phosphoglycerate mutase from yeast	112,000	4.05
Alcohol dehydrogenase from yeast	150,000	4.58
β-Amylase	152,000	4.97
Ceruloplasmin, human	152,000	4.42
Aldolase	164,500	4.81
Fumarate hydratase from pig	204,000	5.47
Isocitrate lyase	222,000	5.53
Catalase	230,000	5.25
Glycerol kinase	251,000	5.10
Leucine aminopeptidase	326,000	5.26
Ferritin	440,000	6.10
Apoferritin	473,450	7.40
Urease	478,600	6.15
β-Galactosidase	518,150	6.90
Thyroglobulin	660,000	8.51
α-Crystalline	770,000	9.30
α_2-Macroglobulin, human	797,750	9.60
Glutamate dehydrogenase	1,007,000	8.40
β-Lipoprotein, human	2,663,000	12.40
Hemocyanine from Helix pomatia	4,310,000	15.20
Hemocyanine from Paludia vivipara	8,699,000	30.20

Gradient gels give a better separation and sharpening of individual protein bands [9] under non-denaturing conditions. Best results are obtained in 3–30 g/dl gels [10] where the relative molecular masses of proteins, in the range of 13,000 to 950,000, can be determined.

2.6.4 Gradient polyacrylamide gel zone electrophoresis of mass and charge isomers of proteins

The protein isomers can be subdivided in mass and charge isomers. The mass isomers have same mass, but different net charges. The charge isomers have same net charge, but different masses.

The resolution of mass or charge isomers is carried out in gradient gels. Later, the natural logarithm of protein mobility is set as a function of the mobility of

Bromophenol blue moving in front of the proteins. If at $T = 0$ g/dl parallel lines are resulting, mass isomers are moving in the gel; if non-parallel lines are resulting, which intersect in one point, charge isomers are moving in the gel.

The mass isomers migrate in the beginning of electrophoresis with different velocities in the low gel concentration, since the migration velocity is determined only by the electric charge of proteins. Later, their velocities decrease with the increase of the gel concentration until they become equal to zero and form bands.

Typical mass isomers are the lactate dehydrogenase isoenzymes [11] and the isoenzymes of carbonic anhydrase (carbonate dehydratase, EC 4.2.1.1) from mammalian erythrocytes [12,13,14]. The carbonic anhydrases from different mammalian erythrocytes (Figure 2.6-4) show various lines, which intersect in one point on the ordinate axis, because they have only one monomer with M_r = 30,000 to 38,000, but different net charges.

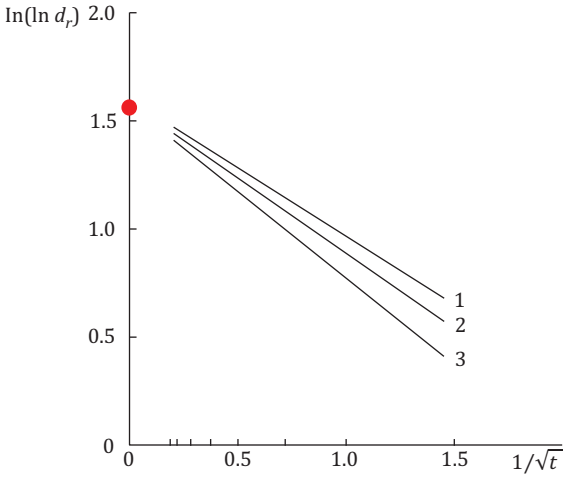

Figure 2.6-4: Carbonic anhydrases from different mammalian erythrocytes (1 – bovine; 2 – rabbit; 3 – human). All lines of various carbonic anhydrases intersect in one point on the ordinate axis.

In the beginning of electrophoresis, the charge isomers migrate in a gradient polyacrylamide gel with same speed, but later they form two or more bands. Their separation in several bands may also depend on the fact that they have same mass, but different conformation: elongated polyions migrate faster than spherical polyions [15].

2.6.5 Blue native polyacrylamide gel electrophoresis

The **m**ulti**p**rotein **c**omplexes (MPC) play a regulatory role in cell signaling [16]. To be analyzed, they must be separated under native conditions. For this purpose,

blue **n**ative **p**oly**a**crylamide **g**el **e**lectrophoresis (BN-PAGE) is used. It is a resolving technique in native gels that uses the anionic blue dye Coomassie brilliant blue G-250 instead of a detergent. Because of its hydrophobic properties, Coomassie brilliant blue G-250 binds unspecifically to all proteins, also to membrane proteins, and covers them with negative charges. The membrane proteins lose their hydrophobic character and convert into water-soluble proteins. At neutral pH values, they move through gradient gels toward the anode as blue bands gradually decelerating with the decreasing pore size of the gel [17,18]. So BN-PAGE allows separation and determination of size and composition of multiprotein complexes at native conditions [19,20].

Beside analyzing the composition of MPC, BN-PAGE helps determine the stoichiometry of MPC. For this purpose, the **n**ative **a**ntibody-based **mo**bility-**s**hift (NAMOS) assay is performed [21,22,23]. As in the antibody-based gel shift assay, the cellular lysates are incubated with monoclonal subunit-specific antibodies. This leads to electrophoretic immunoshifts in the BN-gels, which allows determining of MPC stoichiometry.

The adjustment of salt concentration and the removal of low molecular mass impurities are crucial for the high resolution of BN-PAGE. Therefore, the dialysis of lysates is required prior to electrophoresis [24,25].

After BN-PAGE, the visualization of MPC can be achieved by additional Coomassie brilliant blue staining, silver staining, or immunoblotting. Protein bands visualized by Coomassie brilliant blue or silver are suitable for further analysis by mass spectrometry [26].

BN-PAGE allows also detection, quantitation, and purification of the NADPH-producing enzymes: glucose 6-phosphate dehydrogenase (EC 1.1.1.49), malic enzyme (EC 1.1.1.40), and NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42) [27]. These enzymes, together with the phosphogluconate dehydrogenase (EC 1.1.1.44), are involved in the NADPH production. NADPH is a pivot in the lipid synthesis, cellular replication, and functioning of the antioxidative enzymes catalase and glutathione reductase [28,29,30]. So the NADPH-producing enzymes play a key role in biosynthetic processes and in regulating the cellular redox status [31].

The staining of proteins prior to electrophoresis is not always advantageous, because the preparative applications after electrophoresis demand a removal of the dye [32,33]. Therefore, BN gels with anionic detergents were established. They are a part of the so-called **c**olorless **n**ative (CN) electrophoresis [34]. CN-gels can contain, for example, the detergent taurodeoxycholic acid and a buffer with pH = 7.5 [35]. This alternative however is not so functional as the blue variant.

2.6.6 Protocols

2.6.6.1 Casting gradient polyacrylamide gels for vertical electrophoresis

Gradient gels are cast by a gradient maker connected to a casting cassette.

Materials and equipment

Appropriate buffer
Monomeric solution (T = 50 g/dl, C = 0.03)
Glycerol
TMEDA
APS
Gradient maker

Table 2.6-2: Solutions for a gradient gel (T = 5–20 g/dl), according to Michov (s. above).

Composition	Light solution T = 5 g/dl, C = 0.03	Heavy solution T = 20 g/dl, C = 0.03
Buffer, 4x	1.00 ml	2.00 ml
Monomeric solution (T = 50 g/dl, C = 0.03)	0.40 ml	3.20 ml
87% glycerol*	–	2.19 ml
10 g/dl TMEDA	0.02 ml	0.04 ml
10 g/dl APS	0.03 ml	0.01 ml
Deionized water to	4.00 ml	8.00 ml

*The concentration of glycerol in the heavy solution is 30 g/dl, in the light solution is 0 g/dl.

Procedure

The most common gradient gels are linear. Exponential gradients are rarely used.

Linear gradient gels

A linear gradient can be created when the mixing chamber and the reservoir of the gradient maker are in open connection. Thus, the two solutions flow out keeping the same level during the casting process.

- Assemble the casting cassette and place it minimum 5 cm under the gradient maker.
- Introduce the outlet tubing of the gradient maker into the top end of the casting cassette.
- Close the valve between both tubes and the pinchcock between the gradient maker and the casting cassette.

- Pipette 8.0 ml of the *heavy solution* (the monomeric solution containing glycerol in a high concentration and the catalysts) into the mixing chamber of the gradient maker (Figure 2.6-5).
- Pipette 8.0 ml of the *light solution* (the monomeric solution containing glycerol in a low concentration and the catalysts) into the reservoir of the gradient maker.
- Turn on the magnetic stirrer to stir the magnetic bar in the mixing chamber.
- Open the valve between both tubes, then the pinchcock. The heavy solution begins to mix with the incoming light solution in the mixing chamber, and then flows down into the casting cassette so that the monomer concentration decreases linearly from the bottom to the top of the casting cassette.
- Stop the casting after the gradient solution has reached the top end of the casting cassette.
- Put a comb between the two glass plates. So wells for sample application on the top of the gel are generated.
- Let the solution polymerize at a room temperature for 60 min.

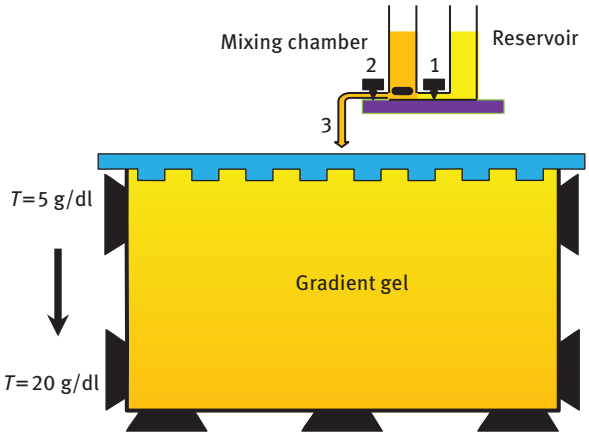

Figure 2.6-5: Casting a gradient gel for vertical electrophoresis.
1. Valve; 2. Pinchcock; 3. Outlet tubing

Exponential gradient gels

An exponential gradient can be created when the solution volume in the mixing chamber remains constant during the casting process. To do this, the mixing chamber must be closed with a plug. If so, the solution flows from the reservoir through the mixing chamber to the outlet tubing. Two types of exponential gradient gels can be created: concave or convex.

2.6.6.2 Horizontal electrophoresis of proteins on gradient gels

Materials and equipment

TRIS
HCl
Light and heavy monomeric (acrylamide/BIS) solutions
Sucrose
TMEDA
APS
Tubing with micropipette tip
Casting cassette with plates, spacers, clamps, and possibly combs
Gradient maker

4x TRIS-HCl buffer, pH = 8.8

TRIS	181.7 g (1.5 mol/l)
Deionized water	600.0 ml
Adjust with HCl to pH = 8.8.	
Deionized water to	1,000.0 ml

Procedure

- Assemble the casting cassette.
- Set up the gradient maker, close all its valves, and place a magnetic bar in the mixing chamber.
- Place the gradient maker on a magnetic stirrer.
- Connect the outlet port of the gradient maker to the tubing with the micropipette tip and insert the tip into the casting cassette.
- Pipette the high-concentration (heavy) solution into the mixing chamber and the low-concentration (light) solution into the reservoir and add APS at last.
- Open the interconnecting valve and turn on the magnetic stirrer.
- Open the outlet valve and fill the casting cassette from the top. The heavy solution will flow first into the cassette, followed by the light solution.
- Overlay the gradient gel solution with deionized water and allow it to polymerize for 1 h.
- Remove the overplayed water.
- Dissemble the casting cassette and take out the gel on its support film.
- Place the gel on a horizontal separation chamber.
- Put onto the gel poles polyacrylamide or paper strips soaked with buffers.
- Place on the gel before the cathode strip a silicone template.
- Add to samples 10–20 g/dl glycerol and apply them in the template slots.
- Run the electrophoresis.
- Stain the protein bands with Coomassie brilliant blue or silver.

Table 2.6-3: Light monomeric solutions, according to Michov (s. above).

Stock solution	Light monomeric solution (g/dl), 15 ml									
	5	6	7	8	9	10	11	12	13	14
4x TRIS-HCl, pH = 8.8, ml	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
30 g/dl Acrylamide/ 0.8 g/dl BIS, ml	2.50	3.00	3.50	4.00	4.50	5.00	5.50	6.00	6.50	7.00
10 g/dl TMEDA, ml	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
10 g/dl APS, ml	0.10	0.09	0.07	0.06	0.05	0.05	0.04	0.04	0.04	0.03
Deionized water, ml, to	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00

Table 2.6-4: Heavy monomeric solutions, according to Michov (s. above).

Stock solution	Heavy monomeric solution (g/dl), 15 ml										
	10	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
4x TRIS-HCl, pH = 8.8, ml	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
30 g/dl Acrylamide 0.8 g/dl BIS, ml	5.00	5.50	6.00	6.50	7.00	7.50	8.00	8.50	9.00	9.50	10.00
Sucrose, g	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25
10 g/dl TMEDA, ml	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
10 g/dl APS, ml	0.05	0.04	0.04	0.04	0.03	0.03	0.03	0.03	0.03	0.03	0.02
Deionized water, ml, to	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00

2.6.6.3 Blue native polyacrylamide gel electrophoresis

Preparation of dialyzed cell lysate

Materials and equipment
BISTRIS
6-Aminohexanoic acid (ε-aminocaproic acid)
Sodium chloride
Brij 96
Digitonin
Triton X-100
Dialysis membrane for M_r = 10,000–50,000
Centrifuge
Microcentrifuge tubes
Parafilm

Phosphate-buffered saline (PBS) buffer, pH = 7.4

Na_2HPO_4	1.42 g (10 mmol/l))
KH_2PO_4	0.24 g (1.8 mmol/l)
NaCl	8.00 g (137 mmol/l)
KCl	0.20 g (2.7 mmol/l)
Deionized water to	1,000.00 ml

Buffer should have pH = 7.4, if prepared properly.

BN-lysis (BN-dialysis) buffer, pH = 7.0

BISTRIS	4.18 g/l (20 mmol/l)
ε-Aminocaproic acid	65.59 g/l (500 mmol/l)
EDTA · Na_2	0.67 g/l (2 mmol/l)
NaCl	1.17 g/l (20 mmol/l)
Glycerol	100.00 ml/l

Adjust to pH = 7.0 with HCl. Store at 4 °C.

Detergent

0.5 to 1.0 g/dl Digitonin, 0.1 to 0.5 g/dl Brij 96, 0.1 to 0.5 ml/dl Triton X-100, 0.1 to 0.5 g/dl dodecylmaltoside
The detergent must be determined empirically. Digitonin must be added before use from a 2 g/dl stock solution. Store in 5 ml aliquots at –20 °C.

Protease and phosphatase inhibitors

PMSF (**p**henyl**m**ethyl**s**ulfonyl **f**luoride)	0.02 g/dl (1.0 mmol/l)
Sodium orthovanadate	0.01 g/dl (0.5 mmol/l)

Protocol

- Pellet by centrifugation 10 • 10^6 cells at 350 g and 4 °C for 5 min.
- Wash the cell pellet three times with ice-cold PBS buffer and centrifuge.
- Resuspend the pellet in 250 µl of ice-cold BN-lysis buffer.
- Centrifuge at 13,000 g at 4 °C for 15 min to remove insoluble material.
- Melt a hole in the cap of a 1.5 ml microcentrifuge tube using the heated large diameter side of a Pasteur pipette, then place the tube on ice to cool down to 4 °C.
- Transfer supernatant into the chilled tube with the hole in the cap.
- Place a dialysis membrane for M_r = 10,000 with forceps on top of the opened tube, close the cap, and cut off excess dialysis membrane that sticks out.
- Seal the cap on the side with Parafilm.
- Invert the tubes and centrifuge upside-down at the lowest speed possible in a cell culture centrifuge at 4 °C for 10 s.
- Prepare a 100 ml beaker with 10 ml of cold BN-dialysis buffer per 100 µl sample.
- Affix the tube with tape and remove air bubbles from the hole beneath the cap using a Pasteur pipette.
- Place beaker onto a magnet stirrer, switch on the stirrer, and leave it in the cold room for 6 hours or overnight.
- Collect the dialyzed cell lysate in a new chilled microcentrifuge tube.

Casting a gradient BN gel

Materials and equipment

BISTRIS
HCl
Acrylamide/BIS solution (T = 40 g/dl, C = 0.03)
TMEDA
APS
Gradient maker

BN gel buffer, pH = 7.0

BISTRIS	0.05 mol/l

Prepare 80 ml as a 3x stock, adjust pH to 7.0 with HCl, and add deionized water to 100 ml.

Stacking gel (T = 4.0 g/dl)

3x BN-gel buffer	3.00 ml
Acrylamide/BIS	0.90 ml
10 g/dl TMEDA	0.04 ml
10 g/dl APS	0.07 ml
Deionized water to	9.00 ml

Light separating gel solution (T = 5 g/dl)

3x BN-gel buffer	5.00 ml
Acrylamide/BIS	1.88 ml
10 g/dl TMEDA	0.04 ml
10 g/dl APS	0.10 ml
Deionized water to	15.00 ml

Heavy separating gel solution (T = 15 g/dl)

3x BN-gel buffer	5.00 ml
Acrylamide/BIS	5.63 ml
87% Glycerol*	4.10 ml
10 g/dl TMEDA	0.04 ml
10 g/dl APS	0.03 ml
Deionized water to	15.00 ml

**The concentration of glycerol is 30%.*

Protocol

- Place the gradient maker on a stir plate and attach it to flexible tubing. Close the channel between the cylinders and the tubing clamp. Place a magnetic stirrer into the cylinder connected to the tubing.
- Place a syringe needle between the two glass plates of the casting cassette.
- Add to the light 5 g/dl gel solution and to the heavy 15 g/dl gel APS solution.
- Pour the 15 g/dl solution into the mixing cylinder, and the 5 g/dl solution into the reservoir of the gradient maker.
- Open the clamps and force out the air bubble inside the channel between the cylinders.
- Switch on the magnetic stirrer.
- Remove the clamp and let the mixed solution flow slowly between the glass plates.
- After all solution is in the glass cassette, overlay it with deionized water.
- Let the gel solution polymerize at room temperature for 60 min.
- Add to the 4 g/dl stacking gel solution APS solution.
- Pour with a pipette the stacking gel solution onto the resolving gel.
- Overlay the stacking gel solution with deionized water.

Running BN electrophoresis

Materials and equipment

BISTRIS
TRICINE
Ready-made polyacrylamide gel (T = 40 g/dl, C = 0.03) for BN electrophoresis
Coomassie **b**rilliant **b**lue G-250 (CBB G250)
Silicone template
Gel electrophoresis unit

Electrode buffer, pH = 7.0

BISTRIS	3.14 g (15 mmol/l)
TRICINE	8.96 g (50 mmol/l)
CBB G250	0.20 g
Deionized water to	1,000.00 ml

Store at 4 °C.

Protocol

- Place on the anode and cathode ends of the BN gel paper strips soaked with electrode buffer.
- Place the silicone template onto the stacking gel befor the cathode strip.
- Load 1 to 40 µl of the dialyzed lysate and 10 to 20 µl of marker mix in the wells of the silicone template.
- Apply 100 V to a minigel or 150 V to a large gel, until the samples have entered the separating gel.
- Increase the voltage to 180 V (minigel) or 400 V (large gel) and run the electrophoresis until the dye front reaches the end of the gel (3 to 4 h for a minigel, and 18 to 24 h for a large gel).
- Stain, if necessary.

2.6.7 Troubleshooting

Problem	Cause	Solution
Prior to electrophoresis		
The monomeric solution does not polymerize, or polymerizes to slow.	The concentration of TMEDA or APS in the monomeric solution is too low.	Use at least 5 µl 10 g/dl TMEDA and 5 µl 10 g/dl APS per 1 ml monomeric solution.
	The APS solution is too old.	Use new APS solution. Keep the APS solution in the refrigerator.
	The casting temperature is too low	Cast the gel at 20–25 °C.
	Too much oxygen in the monomeric solution.	Vent the monomeric solution with a water-jet pump.
	The pH value for gel polymerization is not optimal.	Increase the concentration of TMEDA, if the pH value of the monomeric solution is too low.
The gel is not total polymerized.	The APS solution has not spread in the total monomeric solutions, especially in the heavy solution.	After the APS is added, turn on the magnetic stirrer, then cast.

(continued)

Problem	Cause	Solution
The gel polymerizes too rapidly.	The concentration of TMEDA or APS in the monomeric solutions is too high.	Reduce the concentrations of TMEDA or APS.
Bubbles in the polyacrylamide gel.	The glass plate was dirty.	Wash the glass plate with detergent and ethanol before use.
	The support film was unclean.	Do not touch the hydrophilic side of the support film with fingers.
	There were bubbles in the outlet tube of the gradient maker or in the casting cassette.	Keep the outlet tube clean and dry. Remove air bubble in the casting cassette by vigorous knocking on it.
The gel separates from the support film.	An incorrect support film was used. The gel was cast on the wrong side of the support film.	Do not change the support films for polyacrylamide and agarose gels. Cast the gel on the hydrophilic side of the support film; prior to use check the hydrophilic side with water drops.
The gel margins are polymerized insufficient.	The atmospheric oxygen has inhibited the polymerization of the gel.	Deaerate the monomeric solutions and overlay the mixture with deionized water after casting.
The gel does not stick to the support film, but to the glass plate.	The glass plate was too hydrophilic.	Prior to use coat the glass plate with Repel-silane.
There is liquid on the gel surface.	The polyacrylamide gel hydrolyzes in alkaline buffers.	Store polyacrylamide gels that contain alkaline buffers in the refrigerator.
During electrophoresis		
It does not flow or flows too little electric current.	One of the connectors has no or poor contact.	Check all connections.
	There is poor contact at the electrode gels or strips.	Check the uniform laying of the electrodes; complain them with glass plates.
The gel “evaporates” (the cover of the separation chamber is covered with condensed water).	The cooling is insufficient.	Check the temperature. The cooling blocks should be made of glass, metal, or best of ceramic.
	The voltage is too high.	Reduce the voltage.
The gel sparks and burns.	There are thin areas in the gel – the support film was not fixed on the glass plate during gel casting.	Roll strongly the support film on the glass plate during gel casting.

(continued)

Problem	Cause	Solution
After electrophoresis		
No protein bands in the gel.	The cathode and anode were exchanged.	Check the connections between the electrophoresis unit and power supply.
	The proteins have left the gel.	Monitor the movement of the dye front.
The protein bands are very weak.	The protein concentration or the volume of sample was too small.	Concentrate the sample, apply more volume of the sample, or proof more sensitive staining method.
	The sample was not completely dissolved.	Treat the sample with ultrasound; centrifuge at opacities; use additives to dissolve the sample.
The protein bands are blurred.	The sample contained too much protein.	Apply less volume or dilute the sample.
The protein bands form tails.	Solid components in the sample.	Centrifuge the sample before applying.
The samples of adjacent tracks run into each other.	The application template was not tight on the gel.	Press gently the application template onto the gel to put way air bubbles between it and the gel.
The gel rolls on.	The gel contracts in all directions.	Give 5–10 g/dl of glycerol in the final wash solution.

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2.7 Isotachophoresis of proteins

Iso**t**acho**p**horesis (ITP, from the Greek verbs *iso* = same, *tachos* = speed, and *phoresis* = migration) is electrophoresis, where all co-ions (ions of same sign charges) migrate with same velocities.

2.7.1 Theory of isotachophoresis of proteins

The isotachophoresis is based on the theory of Kohlrausch [1]. In 1897, he studied a system of two strong electrolyte solutions – the solution containing the faster ion (the leading ion) located in front of the solution containing the slower ion (the trailing ion). Kohlrausch noted that, if electric current was passed through the solution system, a moving ionic boundary was formed between the two solutions over the time. He designated this phenomenon as *persistent function* (now bearing his name – *Kohlrausch regulating function*) and derived electrochemical equations for the ion concentrations at the boundary.

Later Longsworth [2], Svensson [3], Alberty [4], and Dismukes and Alberty [5] obtained equations for moving boundary composed of weak electrolytes. In 1964 Ornstein [6] stretched the Kohlrausch regulating function onto buffers, and in 1973 Jovin [7] derived equations for moving boundaries between univalent acids and univalent bases.

Isotachophoresis is running in a system of two buffers: the front buffer contains the leading ion, and the back buffer contains the trailing ion. The leading ions are usually small ones, such as chloride, and the trailing ions are larger ones, such as histidine ions. Between the buffers, a migrating ionic boundary is formed. The sample with proteins or nucleates is to be placed between the two buffers, or into the leading buffer.

If voltage is applied, the analytes separate into zones (bands) according to their mobilities. When a steady state is reached, all bands have ionic concentration with same resistance R. The concentrations of proteins or nucleates increase according to the concentration of the leading ion.

2.7.1.1 Kohlrausch regulating function

Let us analyze a buffer system consisting of a leading buffer (a) and a trailing buffer (b). The leading buffer contains the strong electrolyte HA; the trailing

buffer contains the weak electrolyte HB. The electrolytes dissociate the ions A^- and B^-, respectively, whereas the mobility of ion A^- is higher than the mobility of ion B^-, that is, $|\mu_{A^-}| > |\mu_{B^-}|$. Both buffers contain the same base C, which associates a proton to build the counterion HC^+ (Figure 2.7-1).

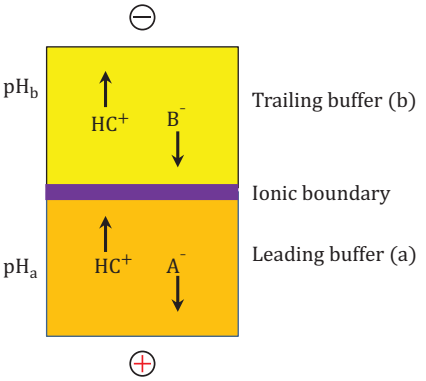

Figure 2.7-1: Scheme of an isotachophoresis buffer system.
A^- – leading ion; B^- – trailing ion; HC^+ – counterion; pH_a and pH_b – pH values of the leading and trailing buffers, respectively.

Let us determine under which conditions ions A^- and B^- will move with the same effective velocity v', that is, under which conditions

$$v'_{A^-} = v'_{B^-} \tag{2.7-1}$$

It is known that the effective velocity of an ion is equal to the product of its effective mobility μ' and the electric field strength E, that is

$$v'_{A^-(B^-)} = \mu'_{A^-(B^-)} E_{a(b)} = \alpha_{HA(HB)} \mu_{A^-(B^-)} E_{a(b)} \tag{2.7-2}$$

where $\alpha_{HA(HB)}$ is the dissociation degree of acid HA (HB) ($\alpha_{HA} = 1$, since HA is a strong acid); $\mu_{A^-(B^-)}$ is the mobility of ion $A^-(B^-)$, in $m^2/(sV)$; and $E_{a(b)}$ is the electric field strength in the leading (trailing) buffer. The strength of the electric field is given by the equation

$$E_{a(b)} = \frac{J}{\gamma_{a(b)}} \tag{2.7-3}$$

where J is the density of the total ionic current, in A/m^2; and $\gamma_{a(b)}$ is the specific electric conductivity of the leading (trailing) buffer, in S/m.

It follows from eqs. (2.7-2) and (2.7-3) that

$$\frac{\alpha_{HB}\mu_{B^-}}{\gamma_b} = \frac{\alpha_{HA}\mu_{A^-}}{\gamma_a} \tag{2.7-4}$$

The specific conductivity is equal to

$$\gamma_{a(b)} = F\Sigma c_{A^-(B^-)} z_{A^-(B^-)} \mu_{A^-(B^-)} \tag{2.7-5}$$

where $c_{A^-(B^-)}$ is the concentration of ion $A^-(B^-)$, in mol/l; and $z_{A^-(B^-)}$ is the number of electric charges (electrovalence) of ion A^- (B^-).

Equations (2.7-4) and (2.7-5) result in

$$\frac{\alpha_{HB}\mu_{B^-}}{\alpha_{HB}c_{HB}z_{B^-}\mu_{B^-} + \alpha_{Cb}c_{Cb}z_{HC^+}\mu_{HC^+}} = \frac{\alpha_{HA}\mu_{A^-}}{\alpha_{HA}c_{HA}z_{A^-}\mu_{A^-} + \alpha_{Ca}c_{Ca}z_{HC^+}\mu_{HC^+}} \tag{2.7-6}$$

where $c_{Ca(b)}$ is the concentration of base C, in mol/l, in the leading (trailing) buffer.

According to the law of electric neutrality of a chemical solution,

$$\alpha_{HA(HB)}c_{HA(HB)}z_{A^-(B^-)} + \alpha_{Ca(b)}c_{Ca(b)}z_{HC^+} = 0 \tag{2.7-7}$$

Hence, eq. (2.7-6) can be transformed into the equation of Ornstein

$$\frac{c_{HB}}{c_{HA}} = \frac{z_{A^-}\mu_{B^-}\left(\mu_{A^-} - \mu_{HC^+}\right)}{z_{B^-}\mu_{A^-}\left(\mu_{B^-} - \mu_{HC^+}\right)} = F_k \tag{2.7-8}$$

where F_k is the Kohlrausch regulating function.

The equation of Ornstein can be applied not only for anionic but also for cationic buffer systems. If a cationic buffer system is assembled by the leading base A, trailing base B, and the common acid HC, the Ornstein equation obtains the following form:

$$\frac{c_B}{c_A} = \frac{z_{HA^+}\mu_{HA^+}\left(\mu_{HA^+} - \mu_{C^-}\right)}{z_{HB^+}\mu_{HA^+}\left(\mu_{HB^+} - \mu_{C^-}\right)} = F_k \tag{2.7-9}$$

At first glance, the Kohlrausch regulating function, that is, the dependence of the ionic concentration on the ionic mobility, looks unexplained. However, it can be explained with the discontinuous distributed electric field strength: the electric field strength in the zone of the trailing electrolyte is higher than in the zone of the leading electrolyte; so a potential gradient is formed in the ionic boundary between the leading and trailing ions.

If a leading ion enters the zone of the trailing electrolyte, it begins to move quicker at the higher voltage here and overtakes the ionic boundary. If a trailing ion penetrates into the zone of the leading electrolyte, its velocity decreases due to the lower voltage in the zone of leading electrolyte and the ionic boundary overtakes it.

When the concentrations of the leading and trailing electrolytes are in accordance with the Kohlrausch regulating function or differ by no more than 10%, polyions with intermediate effective mobilities between the effective mobilities of the leading and trailing ions staple in a decreasing turn and build a migration sandwich-like structure together in the ionic boundary. At the same time, the product of the effective mobility of each zone and its electric field strength remains constant (Figure 2.7-2).

To concentrate a polyion, the effective mobility of the leading ion A^- has to be greater, and the effective mobility of the trailing ion B^- has to be smaller than the effective mobility of the polyion, hence, the following inequation

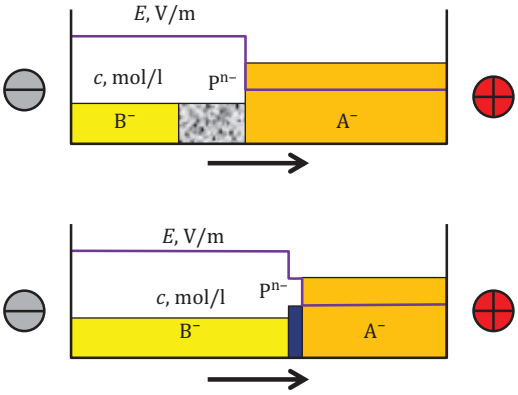

Figure 2.7-2: Start of electrophoresis (top) and concentrating of polyion P^{n-} in the moving ionic boundary (bottom).
A^- – leading ion; B^- – trailing ion

$$|\alpha_{HA}\mu_{A^-}| > |\alpha_{H_nP}\mu_{P^{n-}}| > |\alpha_{HA}\mu_{B^-}| \tag{2.7-10}$$

should be respected, where P^{n-} is the polyion to be examined. For example, if the protein polyion P^{20-} ($\mu_{P20-} = -5 \bullet 10^{-9}$ m^2/(sV)) has to be concentrated at a front of chloride ion ($\mu_{Cl^-} = -37 \bullet 10^{-9}$ m^2/(sV)) at 0 °C against TRIS-ion ($\mu_{HT^+} = 9 \bullet 10^{-9}$ m^2/(sV)) at 0 °C, the Kohlrausch regulating function will be

$$F_k = \frac{c_{H_{20}P}}{c_{HCl}} = \frac{(-1)(-5 \bullet 10^{-9})(-37 \bullet 10^{-9} + 9 \bullet 10^{-9})}{(-20)(-37 \bullet 10^{-9})(-5 \bullet 10^{-9} + 9 \bullet 10^{-9})} = 0.022$$

This means that, if the concentration of HCl is 0.06 mol/l, the protein concentration will reach the concentration

$$0.022 \bullet 0.06 = 0.0013 \text{ mol/l}$$

According to the Ornstein equation, all concentrations in the ionic boundary, including the polyion to be separated, depend on the concentration of the leading electrolyte. The higher its concentration, the more focused the polyion. Moreover, the concentration of each zone is proportional to its length. This makes it possible to compute the polyion concentration according to its band length.

The obtained equations show that the velocity of the migrating ions remains constant when the electric current density J is constant within the buffer system. Therefore, the isotachophoresis is carried out at a constant electric current I.

During isotachophoresis, the polyions concentrate in extremely thin zones. Such a concentration is not known in any other methods, except in isoelectric focusing. Additionally, the polyion bands can be separated from each other using carrier ampholytes.

2.7.2 Troubleshooting

Problem	Cause	Solution
Prior to electrophoresis		
The monomeric solution polymerizes too slow or does not polymerize.	The concentration of TMEDA or APS in the monomeric solution was too low.	Check the content of the monomeric solution.
	The APS solution was too old or stored improperly.	Use a new APS solution. Store the APS solution in the refrigerator.
	The room temperature was too low.	Cast gels at 20–25 °C.
The polyacrylamide gel is sticky.	The concentration of acrylamide or BIS in the monomeric solution was too low.	Check the content of the monomeric solution.
	The concentration of TMEDA or APS in the monomeric solution was too low.	Use at least 5 µl 10 g/dl TMEDA and 4 µl 10 g/dl APS per 1 ml monomeric solution.
	The APS solution was too old or stored improperly.	The usable life of 10 g/dl APS is a week. Store the APS solution in the refrigerator.
	The atmospheric oxygen inhibits the polymerization of the gel surface.	Deaerate the monomeric solution with a water-jet pump. Overlay the monomeric solution with deionized water before gelling.
The monomeric solution polymerizes too quickly.	The concentration of TMEDA or APS in the monomeric solution is too high.	Diminish the concentration of TMEDA or APS.
	The room temperature is very high.	Cast gel at 20–25 °C.
There are bubbles in the gel.	The glass plate that comes into contact with the monomeric solution was unclean.	Wash the glass plate with dishwashing detergent and ethanol prior to use.
	The support film was unclean.	Do not touch the hydrophilic side of the support film with fingers.
The gel separates from the support film.	A wrong support film was used.	Do not exchange the support films for polyacrylamide and agarose gels.
	The gel was cast onto the wrong side of the support film.	Cast gel on the hydrophilic side of the support film; test the film site with drops of water.
The gel does not stick to the support film, but to the glass plate.	The glass plate is too hydrophilic.	Coat the glass plate with Repel-silane.
	The gel was left too long in the casting cassette.	Remove the gel from the casting cassette after the polymerization.

(continued)

Problem	Cause	Solution
During electrophoresis		
Electric current does not flow or too little electric current flows through the gel.	One of the connectors has no or poor contact. Poor contact between the electrodes and the gel.	Check all connections. Check the uniform lying of the electrodes on the gel. Put above them a glass plate.
The gel "evaporates" (The lid of the electrophoresis cell is covered with condensed water).	The cooling is insufficient. The electric voltage is too high.	Check the temperature. The cooling block should be made of glass, metal or best of ceramic. Reduce the electric voltage.
The gel sparks and burns.	There are thin spots in the horizontal gel. Poor contact between the gel and electrodes.	Roll strongly the support film on the glass plate of the casting cassette before gel casting. Place the electrodes parallel to the gel margins and put above them a glass plate.
After electrophoresis		
No protein bands in the gel.	The protein concentration was too low. The detection sensitivity of the staining method was too low. The cathode and anode were exchanged.	Apply more sample volume or concentrate the sample. Use another staining method, e.g., silver staining. Check the connection of the electrophoresis cell to the power supply.
The protein bands seem too weak. The protein bands are blurred.	The protein concentration or the sample volume was too low. Proteolytic degradation of proteins in the sample. Diffusion after the separation.	Concentrate the sample or apply larger volume of the sample. Mix the sample with protease inhibitors (EDTA, *etc*.). Fix the protein bands immediately after the end of electrophoresis.
The protein bands form tails.	Precipitates or solid components in the sample. The sample contained too much protein.	Centrifuge the sample prior to application. Apply less sample volume or dilute the sample.
The adjacent samples are running into each other.	The sample volumes were too big. The application template was not tight on the gel.	Concentrate the samples and apply less volumes. Press gently the application template onto the gel to push away air bubbles between it and the gel.

(continued)

Problem	Cause	Solution
The gel rolls on.	The gel contracted in all directions.	Give 5–10 g/dl glycerol in the final wash solution.

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2.8 Disc-electrophoresis of proteins

Disc-electrophoresis is carried out in two or more buffers. The buffers differ in their composition, ionic strength, and pH value, so that the strength of the applied electric field distributes **disc**ontinuously (disc).

The term *discontinuous* buffer system was introduced in 1957 by Poulik [1] after he found that the electrophoretic resolution increases, if the gel used in starch gel electrophoresis contains a citrate buffer and the electrode tanks contain a borate buffer. In our view, one buffer can also form a discontinuous buffer system, if a same buffer is divided in two or more parts of different composition (different ionic strengths) and different pH values.

During disc-electrophoresis the sample polyions are stacked at first in isotachophoresis, and then are resolved in zone electrophoresis. Isotachophoresis takes place in a large-pore polyacrylamide gel (stacking gel) with a *T*-concentration of 0.4–0.7 mol/l (3–5 g/dl); zone electrophoresis takes place in a small-pore polyacrylamide gel (resolving gel) with a *T*-concentration of 0.7 to 4.2 mol/l (5–30 g/dl).

The zone electrophoresis is carried out in a homogeneous gel. A gradient gel can also be used, where the protein bands become sharper since their diffusion is limited by the increasing polyacrylamide concentration [2]. However, the gradient gel has also disadvantages, such as delayed electrophoresis and difficulties with the band detection after electrophoresis.

A disc-buffer system forms moving ionic boundary between leading and trailing ions. The *leading ions* move before the sample polyions; the *trailing ions* move after them. Protein polyions concentrate in the moving ionic boundary in the stacking gel. They arrange themselves according to their decreasing mobilities (stacking effect). The

stack width is not more than a few hundred micrometers, where the protein concentration can approach 100 mg/ml [3].

Later the proteins migrate through the resolving gel, where their velocities diminish, since the viscosity of the resolving gel exceeds the viscosity of the stacking gel. As a result, the ionic boundary overtakes the concentrated proteins, and they separate from each other depending on their electric charges and masses.

The sample must be diluted or desalted, if it contains too much salt (the salt concentration should be less than 50 mmol/l). When an appropriate dye is added to the sample, a dye front can be monitored during the electrophoresis before the fastest anionic proteins (Bromophenol blue), or before the fastest cationic proteins (pyronine). After disc-electrophoresis, the protein bands can be stained or blotted.

The bands obtained can be quantitatively analyzed by a gel imaging device or by visualizing with UV light. The image is recorded with a computer-operated camera, and then the intensity of the bands is measured with specialized software and compared against marker images separated on the same gel.

Disc-electrophoresis is used in medicine, molecular biology, genetics, microbiology, biochemistry, and forensics for separating different proteins, for example, in blood sera, cerebrospinal fluids, urines, or tissue extracts.

Two disc-electrophoresis methods are known: according to Ornstein–Davis, and according to Allen *et al*. We have created a third disc-electrophoresis method, which we named disc-electrophoresis in one buffer at two pH values.

2.8.1 Theory of disc-electrophoresis

When the concentration of the leading electrolyte, for example, the leading acid, is given, the concentration of the following electrolyte, for example, the trailing acid can be calculated using the Ornstein equation. To do this, it is necessary to know the mobilities of the leading, trailing, and counterions. Thereafter, the pH values and the concentrations of the base (acid) in both buffers can be determined.

Let us analyze an acidic buffer system. To determine the pH value of the trailing buffer, the dissociation degree α_{HB} of the trailing acid HB should be calculated. For this purpose, the equation

$$\alpha_{HB} = \frac{\mu'_{B^-}}{\mu_{B^-}} \tag{2.8-1}$$

is used, where μ'_{B^-} is the effective mobility of the trailing ion B^-.

The pH_b value of the trailing buffer can be calculated from the Henderson–Hasselbalch equation, according to which

$$pH_b = pK_{c_{HB}} + \log\frac{\alpha_{HB}}{1-\alpha_{HB}} \tag{2.8-2}$$

The dissociation degree of the base C in the trailing buffer, α_{Cb}, can also be determined from the Henderson–Hasselbalch equation that gives

$$\alpha_{C_b} = \left(1 + 10^{pH_b - pK_{c_{HB^+}}}\right)^{-1} \tag{2.8-3}$$

The concentration of the base in the trailing buffer can be calculated from the equation of electric neutrality of a solution, according to which

$$c_{C_b} = -c_{HB}\frac{\alpha_{HB} z_{B^-}}{\alpha_{C_b} z_{HC^+}} \tag{2.8-4}$$

It follows from the theory of moving ionic boundary and eq. (2.8-4) that

$$\frac{\alpha_{HB} c_{HB}}{c_{HA}} = \alpha_{HB} F_k = \frac{[H_3O^+{}_b]}{[H_3O^+{}_a]} \tag{2.8-5}$$

hence

$$\mathrm{pH_a} = \mathrm{pH_b} + \log(\alpha_{HB} F_k) \tag{2.8-6}$$

The dissociation degree of the base in the leading buffer, α_{Ca}, can be also calculated from the Henderson–Hasselbalch equation, which gives

$$\alpha_{C_a} = \left(1 + 10^{pH_a - pK_{c_{HC^+a}}}\right)^{-1} \tag{2.8-7}$$

The concentration of the base in the leading buffer can be obtained from the equation of electric neutrality of a solution, too:

$$c_{C_a} = -c_{HA}\frac{\alpha_{HA} z_{A^-}}{\alpha_{C_a} z_{HC^+}} \tag{2.8-8}$$

All equations derived so far are for anionic buffer systems. The cationic buffer systems, which consist of the bases A and B, and a common acid HC, obey similar equations.

2.8.2 Disc-electrophoresis according to Ornstein and Davis

The disc-electrophoresis according to Ornstein–Davis is a milestone in the electrophoretic science. Its theoretical basis was developed by Leonard Ornstein [4], and its practical implementation by Baruch Davis [5]. The performance of the disc-electrophoresis requires three different buffers: stacking, resolving, and electrode buffers.

To explain the effects in the disc-electrophoresis according to Ornstein–Davis, we assume that the stacking and resolving buffer are assembled by the strong acid HA and weak base C; and the electrode buffer is composed of the weak acid HB and same base. Hence, the stacking and resolving buffers contain the leading ion A^- and counterion HC^+, and the electrode buffer contains the trailing ion B^- and same counterion HC^+ (Figure 2.8-1).

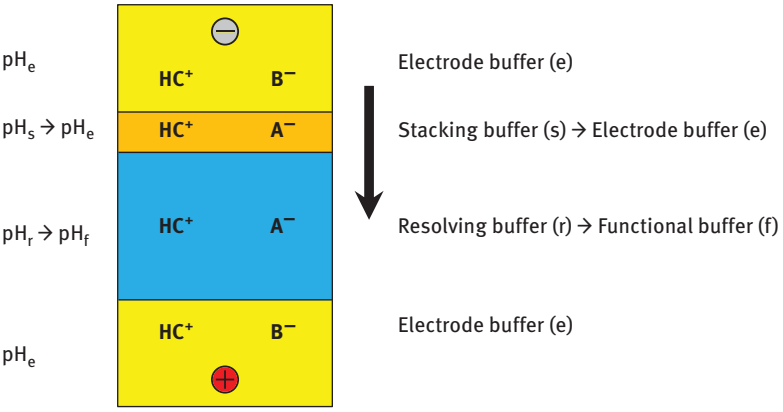

Figure 2.8-1: Scheme of a buffer system for disc-electrophoresis according to Ornstein–Davis. A^- – leading ion; B^- – trailing ion; HC^+ – counterion; pH_e, pH_s, pH_r, and pH_f – pH values of the electrode, stacking, resolving, and functional buffer, respectively. The two electrode buffers can be different or same (as on the figure).

The preparation of a gel-buffer system for disc-electrophoresis according to Ornstein–Davis needs selection of buffer components and calculation of the concentrations and pH values of the electrode, stacking, and resolving buffer.

Let us calculate the concentrations and pH values of a buffer system that is suitable for disc-electrophoresis of serum proteins at 0 °C. Most serum proteins have negative total charges at the physiological pH value of the blood, that is, at pH = 7.40. To charge all of them negatively, it is necessary to dissolve them in a buffer with pH = 9.0–10.0. This buffer should be referred to as functional buffer (f).

The required pH value can be assured by glycine (HG), since the pK value of its protonated amino group is approximately equal to 9.8. At pH = 8.0 and 0 °C, the effective mobilities of serum proteins are between $-0.6 \bullet 10^{-9}$ and $-7.5 \bullet 10^{-9}$ $m^2/(sV)$. This means that the effective mobility of glycinate ion, which serves as a trailing ion in the electrode buffer, must be $-0.5 \bullet 10^{-9}$ $m^2/(sV)$, and in the functional buffer must be $-10 \bullet 10^{-9}$ m^2 /(sV). Under these conditions, the glycinate ion will follow the proteinates in the electrode buffer and will overtake them in the resolving buffer. However, the effective mobility of the proteins in the resolving gel falls by half, so it is sufficient, if the effective mobility of glycinate ion in the resolving gel is $-5 \bullet 10^{-9}$ $m^2/(sV)$.

The mobility of the glycinate ion μ_{G^-} at 0 °C is equal to $-15 \bullet 10^{-9}$ $m^2/(sV)$. So, the dissociation degree of glycine in the electrode buffer must be

$$\alpha_{HGe} = \frac{-0.5 \bullet 10^{-9}}{-15 \bullet 10^{-9}} = \frac{1}{30}$$

and the dissociation degree of glycine in the functional buffer must be

$$\alpha_{HGf} = \frac{-5 \bullet 10^{-9}}{-15 \bullet 10^{-9}} = \frac{1}{3}$$

From the equations derived above can be calculated that pH_e should be 8.3, and pH_f should be 9.5 (Figure 2.8-2).

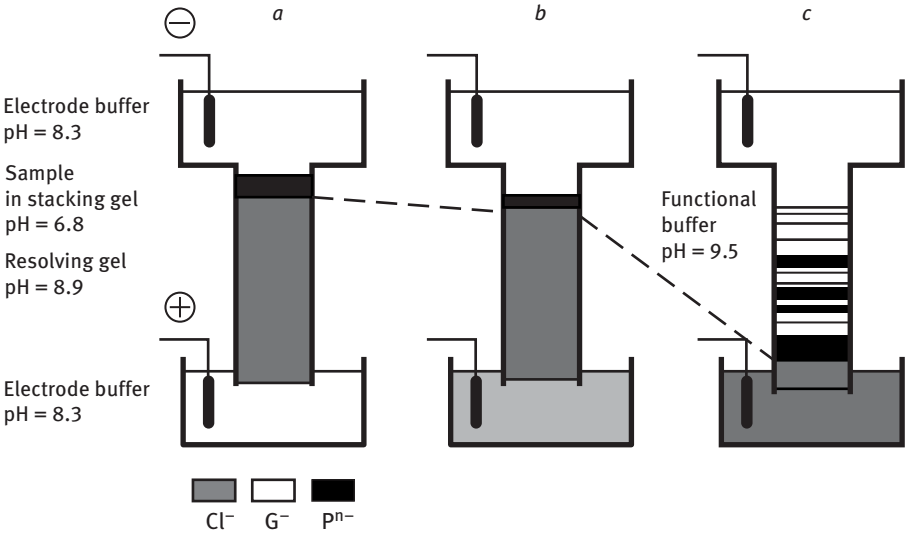

Figure 2.8-2: Scheme illustrating disc-electrophoresis of serum proteins.
a) Starting the disc-electrophoresis; *b*) Concentrating the proteins in the stacking gel;
c) Separating the proteins in the resolving gel.
Cl^- – chloride ion; G^- – glycinate ion; P^{n-} – protein polyions (polyanions)

TRIS (T) is a suitable buffering base because the p*K* value of its protonated form (8.1), namely pK_{HT^+}, is approximately equal to the calculated pH_e value. Considering that at 0 °C $\mu_{HT^+} = 9 \bullet 10^{-9}$ m^2/(sV), and if leading acid is HCl [$\mu_{Cl^-} = -37 \bullet 10^{-9}$ m^2/(sV) at 0 °C], can be calculated from the above equations that Kohlrausch function $F_k = 0.78$.

It is found *a priori* that good electrophoretic results are obtained when the concentration of the strong acid is 0.05–0.06 mol/l. If $c_{HCl} = 0.06$ mol/l, it follows from the above equations that $c_{HG} = 0.047$ mol/l. It can also be calculated that $pH_s = 6.8$, and $pH_r = 8.9$, $\alpha_{Ce} = 0.37$, $c_{Te} = 0.004$ mol/l, $\alpha_{Cs} = 0.96$, $c_{Ts} = 0.062$ mol/l, $\alpha_{Cr} = 0.14$, and $c_{Tr} = 0.439$ mol/l.

2.8.2.1 Buffer-gel systems for disc-electrophoresis according to Ornstein–Davis

A number of disc-buffer-gel systems have been developed [6] for separation of acidic and basic proteins. The oldest and most widely used of them is the alkaline buffer-gel system of Davis. An acidic disc-buffer-gel system was described by Reisfeld *et al.* [7].

Later a buffer system for disc-electrophoresis was developed that also used chloride ion, as a leading ion, but histidinate ion, instead of glycinate ion, as a trailing ion. It is also suitable for separation of basic proteins [8].

The buffer-gel system of Davis is used for separating acidic proteins, stable at pH = 8.9 and with relative molecular masses M_r of 10^{4-6}, in 1 mol/l (7 g/dl) polyacrylamide gel. Such proteins are the serum proteins. They are resolved at pH = 9.5.

The buffer-gel system of Reisfeld *et al.* is used for separating alkaline proteins, stable at pH = 4.3 and with relative molecular masses M_r of approximately $2 \bullet 10^4$ in 2.1 mol/l (15 g/dl) polyacrylamide gel. Such proteins are histones. They are separated at pH = 3.8.

The sample buffer for the disc-electrophoresis of Ornstein–Davis contains: stacking buffer, 10 g/dl glycerol, and 0.01 g/dl Bromophenol blue Na salt.

The disc-electrophoresis was originally carried out in vertical round gels. Nowadays, horizontal gels are preferred, because they are cast on support film or fabric. To achieve a sharp boundary between the stacking and resolving gels, the resolving gel solution, containing 10 g/dl glycerol, is overlaid with deionized water or resolving buffer. After the resolving gel has polymerized, the water (buffer) is sucked out and the stacking gel solution, containing 30 g/dl glycerol, is cast.

On Figure 2.8-3 is shown a disc-pherogram obtained with the buffer-gel system of Davis.

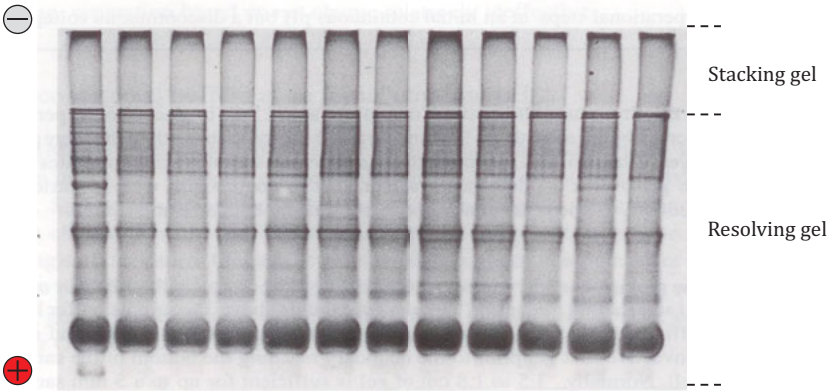

Figure 2.8-3: Disc-pherogram of serum proteins according to Davis.

2.8.3 Disc-electrophoresis according to Allen *et al.*

During the disc-electrophoresis according to Allen *et al.* [9,10], the samples are concentrated by Hjerten effect and then separated in a step-gradient gel.

2.8.3.1 Effect of Hjerten

Hjerten *et al.* [11] proved that biological solutions can be concentrated, if they cross a boundary between a buffer of low ionic strength and a buffer of high ionic strength. Under these conditions, the polyions move quickly through the buffer of low ionic strength (the stacking buffer) and diminish their velocity (focus) on the boundary with the buffer of high ionic strength (the resolving buffer).

2.8.3.2 Buffer-gel systems for disc-electrophoresis according to Allen *et al.*

The buffer system of disc-electrophoresis according to Allen *et al.* also contains a leading and a trailing ions, but the pH value of the buffer system is everywhere constant. The stacking possibility of this electrophoresis is lower than that of disc-electrophoresis according to Ornstein–Davis. However, the gradient resolving gel of a concentration of 0.4 to 1.7 mol/l (3 to 12 g/dl) sharpens the resolved bands. In addition, a cover gel is recommended, which prevents the sample to diffuse and reduce the velocity of the trailing ion (Figure 2.8-4).

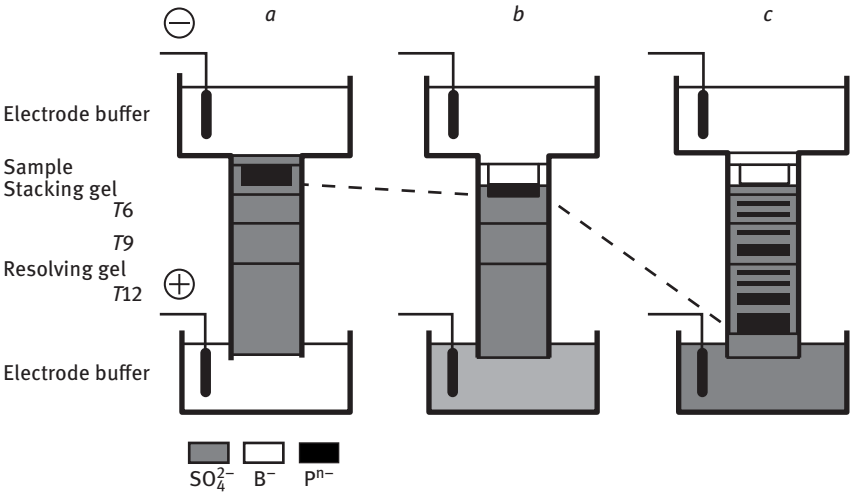

Figure 2.8-4: Effects during disc-electrophoresis according to Allen *et al.*
a) Starting the electrophoresis; *b*) Protein polyions are stacked by a moving ionic boundary;
c) Protein polyions are resolved in a step-gradient gel.
SO_4^{2-} – sulfate ion; B^- – borate ion; P^{n-} – proteinate polyions

A few buffer-gel systems for this electrophoresis are created [12]: A sulfate-borate buffer system (pH = 9.0) for resolving plasma and tissue polyions, enzymes and nucleates; a citrate-borate buffer system (pH = 9.0) for resolving plasma lipoproteins, which show tendency to aggregate (citrate and borate ions build chelates that reduce

the lipoprotein aggregation; the non-ionic detergent Brij 35 helps also here); a chloride-glycinate buffer system (pH = 8.5) for resolving enzymes, for example, lactate dehydrogenase isoenzymes; and a potassium-β-alanine buffer system (pH = 4.0) for resolving basic proteins, for example, histones and ribosomal proteins.

The disc-electrophoresis according to Allen is characterized by high resolution. However, the usage of cover, sample, and step-gradient gel complicates the method and makes the uniform drying of the polyacrylamide gel difficult.

2.8.4 Disc-electrophoresis in one buffer at two pH values

In the disc-electrophoresis in one buffer at two pH values according to Michov [13], the polyions stack between same leading and trailing ions and are resolving in a zone electrophoresis.

2.8.4.1 Theory of disc-electrophoresis in one buffer at two pH values

The electrophoresis in one buffer at two pH values is similar to the disc-electrophoresis according to Ornstein–Davis. However, its buffer system does not form a moving ionic boundary, but a stationary ionic boundary between a stacking and a resolving buffer, made by a same ion of the polyion's polarity. The polyions are concentrated at this boundary and then are separated from each other in the resolving gel (Figure 2.8-5).

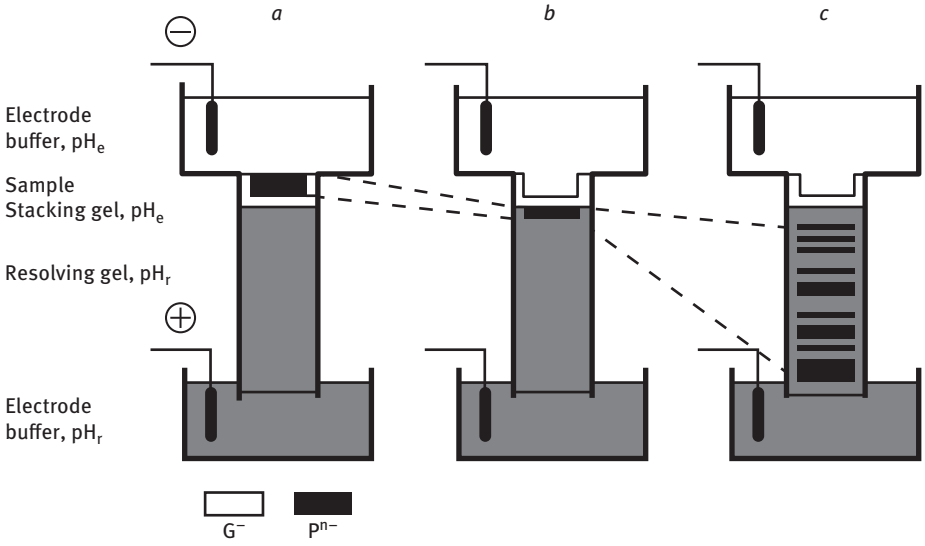

Figure 2.8-5: Disc-electrophoresis in one buffer at two pH values.
a) Starting the disc-electrophoresis; *b*) Stacking the polyions in a stationary ionic boundary between the stacking and resolving gels; *c*) Resolving the polyions in a resolving gel.
G^- – glycinate ion; P^{n-} – proteinate polyions

In the methods of Ornstein–Davis, and Allen *et al*., as well as in the theoretical works of Jovin [14] and Chrambach *et al*. [15], different leading and trailing ions are used for disc-electrophoresis, for example, chloride and glycinate ions, or sulfate and borate ions. On the contrary, we have proved that the leading and trailing ions can be the same, for example, only glycinate or borate ion. So, the polyions are stacking and resolving in only one buffer system consisting of the weak acid HA and the weak base B, but having two pH values.

Let us determine the conditions under which the same leading and trailing ion A^- can form an ionic boundary. To answer this question, we should find when its velocity v_{A^-} in the resolving buffer (*a*) and in the electrode buffer (*b*) is the same, hence when

$$v_{A^-a} = v_{A^-b} \tag{2.8-9}$$

As mentioned, the velocity of ion *i* can be expressed by the equation

$$v_i = \mu'_i E = \alpha \mu_i E \tag{2.8-10}$$

where μ_i and μ'_i are its mobility and effective mobility, respectively; E is the electric field strength; and α is the ionization degree of the electrolyte that creates ion *i*. If the field strength is represented as a ratio between the density of electric current J and the specific conductivity of the solution γ, it follows that

$$\frac{\alpha_{HAa}}{\gamma_a} = \frac{\alpha_{HAb}}{\gamma_b} \tag{2.8-11}$$

The specific conductivity corresponds to the sum $F\Sigma c_i z_i \mu_i$, where F is the Faraday constant (96,485.332 12 C/mol), c_i is the concentration, and z_i is the number of the electric charges (electrovalency) of ion *i*. Using this sum and the equation of electric neutrality of a solution, eq. (2.8-11) can be simplified into

$$c_{HAa} = c_{HAb} = c_{HA} \tag{2.8-12}$$

The last equation shows that ion A^- moves with same velocity in all parts of a buffer system, if the concentration of the electrolyte, which builds it, is the same everywhere.

From eqs. (2.8-11) and (2.8-12) follows the equation

$$\frac{c_{Ba}}{c_{Bb}} = \frac{\alpha_{HAa}\alpha_{Bb}}{\alpha_{HAb}\alpha_{Ba}} \tag{2.8-13}$$

which describes the stationary ionic boundary.

The stationary ionic boundary can be extended over the whole buffer system [16]. The concentration of the weak acid must be constant, however, the concentration of the weak base should change continuously, which changes the pH value over the buffer system.

To concentrate polyions, μ'_{A^-a} and μ'_{A^-b} should be higher and lower, respectively, than the effective mobilities of the polyions. For example, if the protein polyion P^{n-} should be concentrated, then

$$\left|\mu'_{A^-a}\right| > \left|\mu'_{P^{n-}}\right| > \left|\mu'_{A^-b}\right| \tag{2.8-14}$$

When these conditions are available, the polyions arrange themselves in the stationary boundary until the equation

$$c_{H_nP} = c_{HA}\frac{\alpha_{HA}z_{A^-}}{\alpha_{H_nP}z_{P^{n-}}} \tag{2.8-15}$$

is fulfilled. In this equation, c_{H_nP} is the concentration of the stacked polyion, α_{H_nP} is its ionization degree, and $z_{P^{n-}}$ is its electrovalency.

The values of α_{HAa} and α_{HAb} can be calculated from the values of the effective mobilities of ion A^- in the resolving and electrode buffer, that is, from μ'_{A^-a} and μ'_{A^-b}, respectively. Then the concentration of the base in the resolving gel and electrode buffer can be determined. The value of $\alpha_{Ba(b)}$ can be computed from the Henderson–Hasselbalch equation.

2.8.4.2 Buffer-gel systems for disc-electrophoresis in one buffer at two pH values

Let us derive a buffer system, which is suitable for resolving serum proteins at 25 °C.

It is known that at 25 °C and pH ≥ 8.0 the effective mobilities of serum protein polyions $\mu'_{P^{n-}}$ are between $|15\bullet10^{-9}|$ and $|1.2\bullet10^{-9}|$ $m^2/(sV)$. Since the value of $\mu'_{P^{n-}(max)}$ decreases in the resolving gel, we can assume that the mobility of the same-polar ion in the resolving gel μ'_{A^-a} should be $-10\bullet10^{-9}$ $m^2/(sV)$; and in the stacking gel μ'_{A^-b} should be -10^{-9} $m^2/(sV)$.

These conditions correspond to the TRIS-glycinate buffer. It consists of the weak base TRIS (T) and the weak acid glycine (HG). At 25 °C and an ionic strength of 0.1 mol/l, the absolute mobility of the glycinate ion $\mu_{G^-} = -27.87\bullet10^{-9}$ $m^2/(sV)$ [17]. Therefore, it follows from the above equations that $\alpha_{HGa} = 0.3588$ and $\alpha_{HGb} = 0.0359$. If $I_a = 0.10$ mol/l, c_{HG} should be 0.2787 mol/l, and I_b should be 0.01 mol/l.

At 25 °C, $pK_{HG} = 9.78$, and $pK_{HT^+} = 8.07$. Therefore, it follows from the derived equations that $pK_{HGa} = 9.54$, $pK_{HGb} = 9.69$, $pK_{HT^+a} = 8.31$, and $pK_{HT^+b} = 8.16$, where $pK_{HGa(b)}$ and $pK_{HT^+a(b)}$ are the negative decimal logarithms of the concentration ionization constants of glycine and TRIS-ion in the resolving and electrode buffers, respectively. After this we can calculate that $pH_a = 9.29$, $pH_b = 8.26$, $\alpha_{Ta} = 0.0952$, and $\alpha_{Tb} = 0.4420$, and that the concentrations of TRIS in the resolving buffer $c_{Ta} = 1.050$ mol/l, and in the electrode buffer $c_{Tb} = 0.023$ mol/l.

The APS solution must be freshly prepared and last added. We propose that its concentration (in g/dl) should be calculated according to the formula

$$c_{APS} = \frac{0.32(\mathrm{g/dl})^2}{T} \quad [\mathrm{g/dl}] \tag{2.8–16}$$

where T is the total monomer concentration in g/dl.

The disc-electrophoresis in one buffer at two pH values is carried out for 1 to 2 h. Then the protein bands are fixed in a mixture of 1.0 mol/l (16.34 g/dl) trichloroacetic acid and 0.2 mol/l (5.08 g/dl) 5-sulfosalicylic acid dehydrate for 30 min. Afterwards they are stained in filtered 0.001 mol/l (0.17 g/dl) Coomassie brilliant blue R-250 dissolved in the destaining solution for 30 min. The destaining solution contains methanol, acetic acid and deionized water in a volume ratio of 3:1:6. The destaining solution is used for destaing of the gel background (Figure 2.8-6).

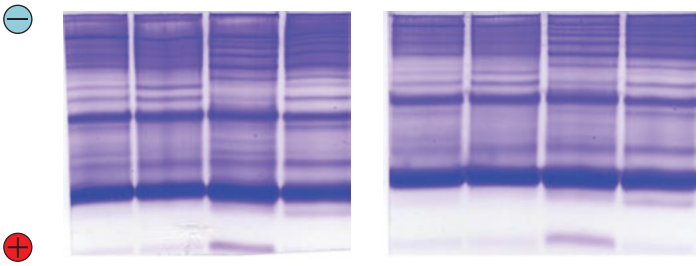

Figure 2.8-6: Serum proteins were separated in a TRIS-glycinate buffer expanded stationary boundary of pH = 7.89–9.13, obtained in a 7 g/dl polyacrylamide slab gel. Staining with Coomassie brilliant blue R-250.

2.8.5 Protocols

2.8.5.1 Casting homogeneous polyacrylamide gels for horizontal disc-electrophoresis

Materials and equipment

Appropriate buffer
Monomer solution (T = 50 g/dl, C = 0.03)
Glycerol
TMEDA
APS
Support film for polyacrylamide gel
250 ml side-arm flask for degassing gel solutions
Glass plates
0.5 mm U-form silicone gasket
Clamps
Water-jet pump

Table 2.8-1: Solutions for a homogeneous gel for disc-electrophoresis.

Composition	Stacking solution $T = 4$ g/dl, $C = 0.03$	Resolving solution $T = 8$ g/dl, $C = 0.03$
Buffer, 4x	1.00 ml	3.00 ml
Monomer solution ($T = 50$ g/dl, $C = 0.03$)	0.32 ml	1.92 ml
87% glycerol*	1.09 ml	1.09 ml
10 g/dl TMEDA	0.02 ml	0.06 ml
10 g/dl APS	0.03 ml	0.05 ml
Deionized water to	4.00 ml	12.00 ml

*The concentration of glycerol in the stacking and resolving solutions is 10 g/dl.

Procedure

- Assemble a casting cassette: place between two glass plates a 0.5 mm U-form silicone gasket and fix them with clamps.
- Make the resolving solution in the side-arm flask and degas it with the water-jet pump.
- Pipette the resolving solution with the catalysts into the casting cassette until the solution fills ¾ of the cassette (Figure 2.8-7).
- Pipette 2.0 ml deionized water or resolving buffer over the resolving solution.
- Let the resolving solution polymerize at a room temperature for 45 min.
- Soak the liquid over the resolving gel using the water-jet pump.
- Pipette stacking solution over the resolving gel.
- Let the stacking solution polymerize at a room temperature for another 45 min.
- Dissemble the casting cassette und put out the polyacrylamide gel.

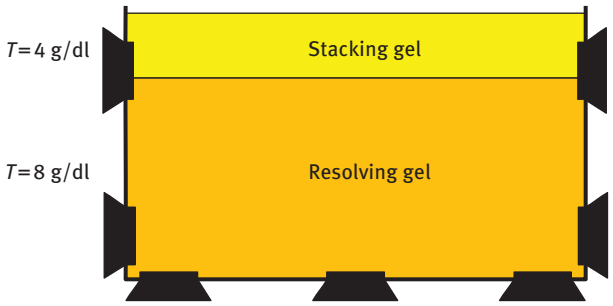

Figure 2.8-7: Casting a homogeneous gel for disc-electrophoresis.

2.8.5.2 Disc-electrophoresis in alkaline buffer-gel system according to Ornstein–Davis

Materials and equipment

Buffers
Monomer solutions for stacking and resolving gels
TMEDA
APS
Tri**c**hloro**a**cetic acid (TCA)
5-Sulfosalicylic acid
Coomassie brilliant blue R-250
Methanol
Acetic acid
0.5 mm U-form silicone gasket
Support films for polyacrylamide gel
250 ml side-arm flask for degassing gel solutions
Glass plates
Clamps
Water-jet pump

Procedure

Table 2.8-2: Stacking gel, $T = 4$ g/dl, $C = 0.03$.

Solutions	Preparation	Ingredients
TRIS-chloride buffer, 4x 0.25 mol/l TRIS HCl to pH = 6.8	Dissolve 3.03 g TRIS in 80 ml deionized water, titrate with 1 mol/l HCl to pH = 6.8, and fill up with deionized water to 100.0 ml.	25.00 ml
Monomer solution $T = 50$ g/dl, $C = 0.03$	Dissolve 48.5 g acrylamide and 1.50 g BIS in deionized water and fill up to 100.0 ml. Add 1.0 g Amberlite MB-1.	8.00 ml
87% Glycerol*		9.11 ml
10 g/dl TMEDA		0.46 ml
10 g/dl APS		0.80 ml
Deionized water to		100.00 ml

*The concentration of glycerol is 10 g/dl.

Procedure

- Assemble a casting cassette: place between two glass plates the 0.5 mm U-form silicone gasket and fix them with clamps.
- Make the resolving solution in the side-arm flask and degas it with the water-jet pump.

Table 2.8-3: Resolving gel, T = 12.5 g/dl, C = 0.03.

Solutions	Preparation	Ingredients
TRIS-chloride buffer, 4x 1.5 mol/l TRIS HCl to pH = 8.8	Dissolve 18.17 g TRIS in 80 ml deionized water, titrate with 1 mol/l HCl to pH = 8.8, and fill up with deionized water to 100.0 ml.	25.00 ml
Monomer solution T = 50 g/dl, C = 0.03	Dissolve 48.5 g acrylamide and 1.5 g BIS in deionized water, and fill up to 100.0 ml. Add 1.0 g Amberlite MB-1.	25.00 ml
87% Glycerol*		9.11 ml
10 g/dl TMEDA		0.46 ml
10 g/dl APS		0.26 ml
Deionized water to		100.00 ml

*The concentration of glycerol is 10 g/dl.

Table 2.8-4: Electrode buffer.

Solutions	Preparation	Ingredients
TRIS-glycinate buffer, 10x 0.025 mol/l TRIS 0.192 mol/l glycine pH = 8.3	Dissolve 3.03 g TRIS and 14.41 g glycine in deionized water and fill up to 1,000.0 ml.	100.00 ml
Deionized water to		1,000.00 ml

- Pipette the resolving solution with the catalysts into the casting cassette until the solution fills ¾ of the cassette (Figure 2.8-7).
- Pipette 2.0 ml deionized water or resolving buffer over the resolving solution.
- Let the resolving solution polymerize at room temperature for 45 min.
- Soak the liquid over the resolving gel using the water-jet pump.
- Pipette the stacking solution over the resolving gel.
- Let the stacking solution polymerize at room temperature for another 45 min.
- Dissemble the casting cassette und put out the polyacrylamide gel.
- Run disc-electrophoresis for 1 to 2 h.
- Fix the protein bands in a mixture of 1.0 mol/l (16.34 g/dl) trichloroacetic acid and 0.2 mol/l 5-sulfosalicylic acid (5.08 g/dl 5-sulfosalicylic acid dehydrate) for 30 min.

- Stain the bands in a filtered 0.001 mol/l (0.17 g/dl) Coomassie brilliant blue R-250 dissolved in the destaining solution for 30 min.
- Destain the gel background in the destaining solution (methanol-acetic acid-deionized water, 3:1:6, *V:V:V*).
- Dry the gel in air or in an oven.

2.8.5.3 Disc-electrophoresis in acidic buffer-gel system according to Reisfeld *et al.*

Materials and equipment

Buffers
Monomer solutions for stacking and resolving gels
TMEDA
APS
Trichloroacetic acid
5-Sulfosalicylic acid
Coomassie brilliant blue R-250
Methanol
Acetic acid
250 ml side-arm flask for degassing gel solutions
Support films for polyacrylamide gel
Glass plates
0.5 mm U-form silicone gasket
Clamps
Water-jet pump

Procedure

Table 2.8-5: Stacking gel, $T = 4$ g/dl, $C = 0.03$.

Solutions	Preparation	Ingredients
Acetate buffer, 4x 0.24 mol/l KOH CH_3COOH to pH = 6.8	Dissolve 1.35 g KOH in 80 ml deionized water, titrate with CH_3COOH to pH = 6.8, and fill up with deionized water to 100.0 ml.	25.00 ml
Monomer solution $T = 50$ g/dl, $C = 0.03$	Dissolve 48.5 g acrylamide and 1.5 g BIS in deionized water, and fill up to 100.0 ml. Add 1.0 g Amberlite MB-1.	8.00 ml
87% Glycerol *		9.11 ml
10 g/dl TMEDA		0.92 ml
10 g/dl APS		0.80 ml
Deionized water to		100.00 ml

*The concentration of glycerol is 10 g/dl.

Table 2.8-6: Resolving gel, T = 12.5 g/dl, C = 0.03.

Solutions	Preparation	Ingredients
Acetate buffer, 4x 0.24 mol/l KOH CH_3COOH to pH = 4.3	Dissolve 1.35 g KOH in 80 ml deionized water, titrate to pH = 4.3 with CH_3COOH, and fill up with deionized water to 100.0 ml.	25.00 ml
Monomer solution T = 50 g/dl, C = 0.03	Dissolve 48.5 g acrylamide and 1.5 g BIS in deionized water and fill up to 100.0 ml. Add 1.0 g Amberlite MB-1.	25.00 ml
87% Glycerol*		9.11 ml
10 g/dl TMEDA		0.92 ml
10 g/dl APS		0.26 ml
Deionized water to		100.00 ml

*The concentration of glycerol is 10 g/dl.

Table 2.8-7: Electrode buffer.

Solutions	Preparation	Ingredients
β-Alanine-acetate buffer, 10x 0.35 mol/l β-alanine 0.14 mol/l CH_3COOH pH = 4.5	Dissolve 31.19 g β-alanine in 800 ml deionized water, titrate with CH_3COOH to pH = 4.5, and fill up with deionized water to 1,000.0 ml.	100.00 ml
Deionized water to		1,000.00 ml

Electrophoresis, staining, destaining, and drying as in the method according to Ornstein–Davis.

2.8.5.4 Disc-electrophoresis in one buffer at two pH values

Materials and equipment

Buffers
Monomer solutions for stacking and resolving gels
TMEDA
APS
Trichloroacetic acid
5-Sulfosalicylic acid
Coomassie brilliant blue R-250
Methanol
Acetic acid

250 ml side-arm flask for degassing gel solutions
Support films for polyacrylamide gel
Glass plates
0.5 mm U-form silicone gasket
Clamps
Water-jet pump

Table 2.8-8: Solutions 1 and 2 are double concentrated resolving and electrode buffer, respectively. For casting the resolving and stacking gels, solution 3 is also needed, where the total monomer concentration $T = 50$ g/dl, and the crosslinking degree $C = 0.03$.

	Solution 1, 2x, pH = 9.3	Solution 2, 2x, pH = 8.3	Solution 3
TRIS	12.72 g	2.79 g	–
Glycine	2.09 g	20.92 g	–
Acrylamide	–	–	48.50 g
BIS	–	–	1.50 g
Deionized water to	100.00 ml	1,000.00 ml	100.00 ml

Procedure

- Assemble a casting cassette: place between two glass plates the 0.5 mm U-form silicone gasket and fix them with clamps.
- Make the resolving solution in the side-arm flask and degas it with the water-jet pump.
- Pipette the resolving solution with the catalysts into the casting cassette until the solution fills ¾ of the cassette (Figure 2.8–7).
- Pipette 2.0 ml deionized water or resolving buffer over the resolving solution.
- Let the resolving solution polymerize at room temperature for 45 min.
- Soak the liquid on the resolving gel using the water-jet pump.
- Pipette stacking solution with the catalysts onto the resolving gel in the cassette.
- Let the stacking solution polymerize at room temperature for another 45 min.
- Dissemble the casting cassette und put out the polyacrylamide gel.
- Run disc-electrophoresis for 1 to 2 h.
- Fix the protein bands in a mixture of 1.0 mol/l (16.34 g/dl) trichloroacetic acid and 0.2 mol/l 5-sulfosalicylic acid (5.08 g/dl 5-sulfosalicylic acid dehydrate) for 30 min.
- Stain the bands in a filtered 0.001 mol/l (0.17 g/dl) Coomassie brilliant blue R-250 dissolved in the destaining solution for 30 min.
- Destain the gel background in the destaining solution (methanol-acetic acid-deionized water, 3:1:6, *V:V:V*).
- Dry the gel in air or in an oven.

Table 2.8-9: Preparing gels and buffers used for disc-electrophoresis in one buffer at two pH values.

	Electrode buffer pH = 8.3	Stacking gel *T* = 4 g/dl, pH = 8.3	Resolving gel *T* = 7 g/dl, pH = 9.3	Sample buffer pH = 8.3
Solution 1	–	–	50.00 ml	–
Solution 2	500.00 ml	50.00 ml	–	50.00 ml
Solution 3	–	8.00 ml	14.00 ml	–
87% Glycerol*	–	9.11 ml	9.11 ml	–
Bromophenol blue Na salt	–	–	–	0.001 g
10 g/dl TMEDA	–	0.40 ml	0.40 ml	–
10 g/dl APS	–	0.80 ml	0.46 ml	–
Deionized water to	1,000.00 ml	100.00 ml	100.00 ml	100.00 ml

*The concentration of glycerol in the stacking and resolving gel is 10 g/dl

2.8.6 Troubleshooting

Problem	Cause	Solution
Prior to electrophoresis		
The monomer solution polymerizes too slowly or does not polymerize.	The concentration of TMEDA or APS in the monomer solution is too low.	Increase the concentration of TMEDA or APS in the monomer solution.
	The APS solution is too old or stored improperly.	Use a new APS solution. Store the APS solution in the refrigerator.
	The gelling temperature is too low.	Cast gels at 20–25 °C.
The polyacrylamide gel is sticky.	The concentration of acrylamide or BIS in the monomer solution was too low.	Check the composition of the monomer solution.
	The concentration of TMEDA or APS in the monomer solution was too low.	Use at least 5 µl 10 g/dl TMEDA and 4 µl 10 g/dl APS per 1 ml monomer solution.
	The APS solution was too old or stored improperly.	The usable life of an APS solution is a week. Store APS solution in the refrigerator.
	The atmospheric oxygen inhibited the polymerization of the gel surface.	Degas the monomer solution with a water-jet pump and overlay the monomer solution with deionized water after casting.

(continued)

Problem	Cause	Solution
The monomer solution polymerizes too quickly.	The concentration of TMEDA or APS in the monomer solution is too high.	Decrease the concentration of TMEDA or APS.
	The gelling temperature is very high.	Cast gels at 20–25 °C.
Bubbles in the gel.	The glass plate that comes into contact with the monomer solution was unclean.	Wash the glass plate with detergent and ethanol prior to use.
	The support film was unclean.	Do not touch the hydrophilic side of the support film with fingers.
The gel separates from the support film.	A wrong support film was used.	Do not exchange the support films for polyacrylamide and agarose gels.
	The gel was cast on the wrong side of the support film.	Cast gel on the hydrophilic side of the support film; test the sides with water drops.
The gel does not stick to the support film, but to the glass plate.	The glass plate was too hydrophilic.	Coat the glass plate with Repel-silane.
	The gel was left too long in the casting cassette.	Remove the gel from the casting cassette after polymerization.
During electrophoresis		
Electric current does not flow or flows too little through the gel.	One of the connectors has no or poor contact.	Check all connections.
	The contact between the electrode strips and the gel is poor.	Check the uniform lying of the electrodes strips on the gel; put a glass plate on them.
The gel “evaporates” (the lid of the electrophoresis unit is covered with condensed water).	the cooling is insufficient.	Check the temperature. The cooling block should be made of glass, metal or best of ceramic.
	The voltage is too high.	Reduce the voltage.
The gel sparks and burns.	There are thin spots in the horizontal gel.	Roll strongly the support film on the glass plate prior to casting.
	There is a poor contact between the gel and electrode strips.	Put a glass plate on the electrode strips.

(continued)

Problem	Cause	Solution
After electrophoresis		
No protein bands in the gel.	The protein concentration in the sample was too low. The cathode and anode were exchanged. The detection sensitivity of the staining method is too low.	Apply more sample volume or concentrate the sample. Check the connections to the power supply. Use another staining method, e.g., silver staining.
The protein bands are too weak.	The protein concentration was too low. The sample volume was too little.	Concentrate the sample before applying. Apply larger volume of the sample.
The protein bands are blurred.	The sample proteins were degraded. The separated proteins diffused in the gel.	Mix the sample with protease inhibitors (EDTA, *etc.*). Fix the proteins immediately after electrophoresis.
The protein bands form tails.	There are precipitates or solid components in the sample. The sample containes too much protein.	Centrifuge the sample prior to application. Apply less sample volume or dilute the sample.
The adjacent samples are running into each other.	The sample volumes were too big. The application template was not tight on the gel.	Concentrate the samples and apply less volume. Press gently the application template against the gel to push away air bubbles between the template and gel.
The gel rolls on.	The gel contracts in all directions.	Give 5–10 g/dl glycerol in the last wash solution.

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2.9 SDS electrophoresis of proteins

SDS electrophoresis is usually carried out on polyacrylamide gel. The agarose gel is the second matrix suitable for high resolution of many biomolecules up to huge cellular structures [1,2,3].

SDS poly**a**crylamide **g**el **e**lectrophoresis (SDS-PAGE) is a technique widely used in biochemistry, forensics, genetics, molecular biology, and biotechnology, for separating of denatured biological macromolecules, usually proteins or nucleic acids. Whereas the mobility of native polyions depends on their conformation and charge-to-mass ratio, the mobility of denatured polyions depends only on their charge-to-mass ratio. SDS-PAGE is run common in homogeneous gels, but the introduction of gradient gels improved the resolution of electrophoresis [4].

SDS polyacrylamide gel electrophoresis was introduced by Shapiro *et al.* [5]. It is the most widespread electrophoresis of proteins. The method can be used both for insoluble and soluble polyions – practically for all proteins from prokaryotic or eukaryotic cells, tissues, or viruses.

2.9.1 Theory of detergent electrophoresis of proteins

Biological membranes, cells, and tissues contain many lipoproteins. Most lipoproteins are insoluble in water, so they cannot be separated electrophoretically. Therefore, they

should be first solubilized. This happens in the presence of detergents, which denature the lipoproteins and give them rod-like but soluble conformation.

2.9.1.1 Detergents

The detergents [6,7], as all surfactants, lower the surface tension. They are employed in electrophoresis to disrupt protein-lipid and protein-protein bonding. The detergents build micelles in water, whose structure depends on their critical micelle concentration and aggregation number (Figure 2.9-1).

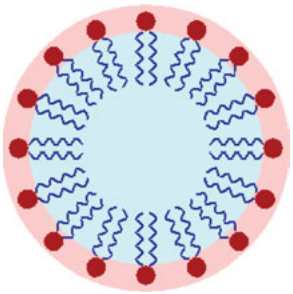


Figure 2.9-1: Schematic structure of an oil micelle in water. The detergent hydrophobic tails project into the oil, while the hydrophilic heads remain in contact with water.

Critical **m**icelle **c**oncentration (CMC) is the concentration of a detergent, in which it forms micelles. The lower the CMC value, the greater is the detergent strength. The **a**ggregation **n**umber (N_a) is the number of detergent molecules, which form a micelle. It is a criterion of the micelle volume. The smaller N_a, the less the lipoprotein mass is increased. The critical micelle concentration and the aggregation number are influenced by the pH value, temperature, and ionic strength.

Depending on their electric charges, the detergents can be subdivided in three groups: anionic, cationic, and zwitterionic detergents. Additionally, there are detergents that do not have electric charges – nonionic detergents (Table 2.9-1).

SDS precipitates under cold room conditions. Unlike it, **l**ithium **d**odecyl **s**ulfate (LDS) does not form precipitates at 4 °C and therefore is an alternative to SDS for isolating photosynthetic complexes by electrophoresis [8].

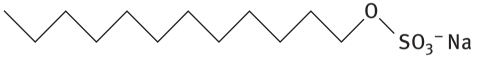
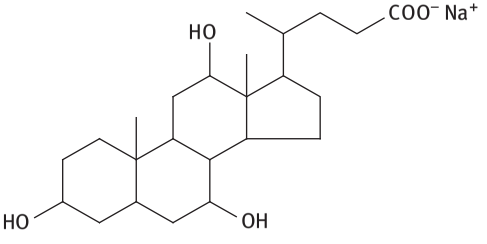
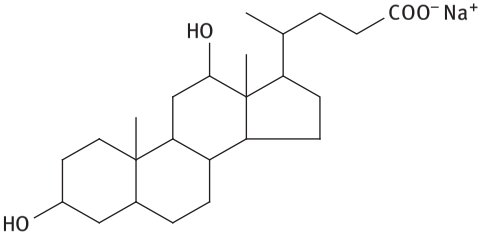
Detergents, which are sufficiently mild to preserve protein complexes during electrophoresis, are sodium **d**e**o**xy**c**holate (DOC) [9,10], sodium oligooxyethylene **a**lkyl **e**ther **s**ulfate (AES) [11], or a combination of the latter with **d**imethyl **d**odecyl**a**mine **o**xide (DDAO) [12].

Sodium dodecyl sulfate

The most commonly used detergent is **s**odium **d**odecyl **s**ulfate (SDS, **s**odium **l**auryl **s**ulfate, SLS, sodium dodecane sulfate, M_r = 288.37) (Figure 2.9-2).

SDS is a strong anionic surfactant that denatures the secondary and non-disulfide-linked tertiary structures of native proteins. It consists of a 12-carbon tail attached to a

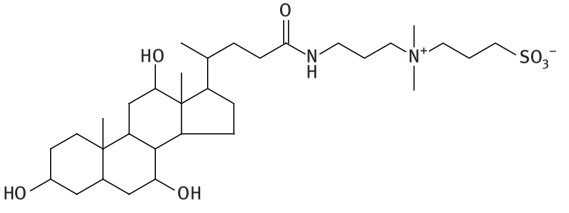
Table 2.9-1: Detergents of importance for electrophoresis.

Detergents	Chemical formulas	Properties at 25 °C
Anionic detergents		
SDS (**s**odium **d**odecyl **s**ulfate)		M_r = 288.37 CMC = $8.2 \bullet 10^{-3}$ mol/l N_a = 62
LDS (**l**ithium **d**odecyl **s**ulfate)		M_r = 272.33 CMC = $8.77 \bullet 10^{-3}$ mol/l
Sodium cholate		M_r = 430.57 CMC = $1.4 \bullet 10^{-2}$ mol/l N_a = 3
DOC (sodium **deoxyc**holate)		M_r = 414.57 CMC = $5 \bullet 10^{-3}$ mol/l N_a = 4–10

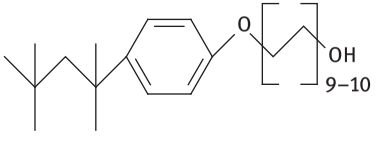
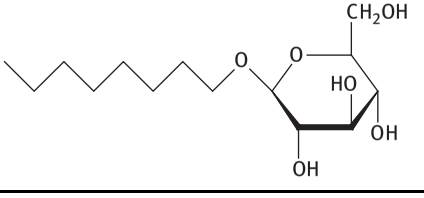
Cationic detergents

CTAB (N-**C**etyl-N, N, N-**t**rimethyl**a**mmonium **b**romide)		M_r = 364.46 CMC = 0.2•10^{-4} mol/l N_a = 169

Zwitterionic detergents

CHAPS (3-[(3-**ch**olamidopropyl)-dimethyl**a**mmonio]-1-**p**ropane**s**ulfonate)		M_r = 614.89 CMC = 4.2•10^{-3} mol/l N_a = 9–10

Nonionic detergents

Triton X-100		M_r = 646.87 CMC = 3.1•10^{-4} mol/l N_a = 143
Octyl-β-glucoside (Octyl-β-D-glucopyranoside)		M_r = 292.37 CMC = 2.5•10^{-2} mol/l N_a = 84

M_r = relative molecular mass, CMC = critical micelle concentration, N_a = aggregation number

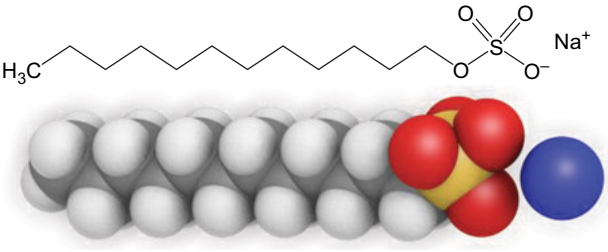


Figure 2.9-2: Chemical formula ($NaC_{12}H_{25}SO_4$) and structure of SDS.

sulfate group that is electrostatically connected to a sodium ion. This means that it is the sodium salt of *dodecyl hydrogen sulfate*, the ester of dodecyl alcohol and sulfuric acid. So the hydrophobic hydrocarbon tail of SDS is combined with its hydrophilic sulfate group. This structure gives the compound amphiphilic properties that allow it to form micelles. When a protein mixture is heated to 100 °C in the presence of SDS, SDS wraps around the proteins and unfolds them giving them negative electric charges.

CMC of SDS in pure water is 8.2 mmol/l at 25 °C [13], its aggregation number is about 62 [14], and its degree of micelle ionization α is around 0.3 (30 g/dl) [15,16].

SDS is synthesized by treating lauryl alcohol with SO_3 or chlorosulfuric acid, and neutralizing the product adding NaOH or Na_2CO_3. Lauryl alcohol can be derived from either coconut or palm kernel oil by hydrolysis, followed by hydrogenation.

SDS ions cover the protein molecules and form with them SDS-protein complexes. The SDS-protein complexes are characterized by a constant ratio between the masses of SDS and proteins. Approximately 1.4 g of SDS are connected with one gram of protein (one SDS ion is connected with two amino acid residues). So the proteins constitute about 42% of the total mass of the SDS-protein complex [17]. The hydrophobic membrane proteins bind up to 4.5 g of SDS per gram of protein [18], hence they represent there only 19% of the total mass of the SDS-protein complex. The glycoproteins depart from this rule since their hydrophilic glycan moiety reduces the hydrophobic interactions between the protein and SDS.

The hydrophobic tails of SDS ions remain inside the SDS-protein complex, the heads facing outward to water dipoles. The hydrophobic interactions and the hydrogen bonds of the proteins are broken down and the individual charge differences between the proteins disappear. The protein polypeptide chains unfold and linearize to become rod-like structures [19,20] (Figure 2.9-3). The charge heterogeneity of the isoenzymes cancels and they form one electrophoretic band. With another words, SDS denatures the secondary and tertiary protein structure.

The protein polyions obtain certain numbers of negative electric charges per mass (in C/g) [21,22], and all SDS-protein complexes run to the anode. Since the conformation of all denatured proteins, except of histones [23], is similar, the protein velocity through a polyacrylamide gel will depend only on their masses in a linear function.

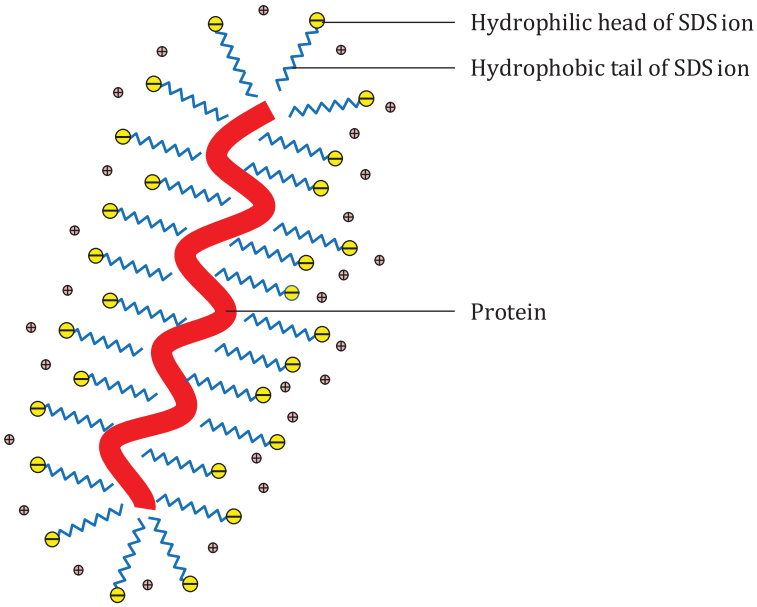


Figure 2.9-3: Structure of a SDS-protein complex.

The cationic detergent **c**etyl**t**rimethyl**a**mmonium **b**romide (CTAB) was used as an alternative to SDS for gel electrophoresis of proteins. It coats the proteins thoroughly enough and therefore can also be used for molecular mass determinations.

2.9.2 Sample preparation for SDS polyacrylamide gel electrophoresis

If proteins are a part of a solid tissue, the solid tissue must be homogenized, or sonicated, and then filtered and centrifuged. Afterward the proteins are mixed with sufficient (1–2 g/dl) SDS, and heated at 90–100 °C for 2–5 min [24,25].

Except SDS, the samples must contain: gel buffer, glycerol, and Bromophenol blue as a trailing dye. Glycerol provides density for applying the sample in the wells under electrode buffer. The trailing dye is necessary to monitor the progress of the protein run during electrophoresis.

For the quality of SDS electrophoresis, the concentrations of proteins and salts in the samples play an important role.

Protein concentration in the sample. The protein concentration in the samples should be 1 g/dl for Coomassie brilliant blue staining, or less than 0.05 g/dl for silver staining. Samples, whose protein concentration is too low, can be concentrated prior to electrophoresis by lyophilization, precipitation, gel filtration, or ultrafiltration.

Lyophilization is referred to as protein concentrating by freeze-drying in the lyophilizer.

The precipitation can be made with solutions of ethanol, acetone, trichloroacetic acid, or ammonium sulfate.

In gel filtration, the protein solution passes through a column filled with a swollen cross-linked dextran gel, named Sephadex. During this, the accompanying ions penetrate, due to their small masses, in the cavities of the Sephadex gel and migrate slower than the proteins. So 5–20 fold concentration is possible.

In ultrafiltration, special filter devices are used which retain the proteins on a filter whereas the solvent molecules and the dissolved ions pass. Using this method, a sample can be concentrated 100–1,000 fold.

Salt concentration in the sample. The salt concentration in the samples, that is, the ionic strength of the sample, should be the same or less than the salt concentration in the gel buffer. If the salt concentration is greater, the sample should be desalted. The desalting is carried out by dialysis. It is performed overnight in small dialysis bags, which are dipped in an at least 100-fold larger volume of a dialysis buffer. The dialysis buffer should contain 1 g/dl SDS. Besides, the sample should contain neither potassium nor divalent metal ions [26], as they form insoluble precipitates with the SDS-ions.

Depending on the additional chemicals (reduction or alkylation agents) in the sample solution, non-reducing and reducing sample preparations are distinguished. In a third way of sample preparation, the reduced polypeptide chains can be alkylated.

2.9.2.1 Non-reducing sample preparation

During the non-reducing sample preparation, the disulfide bonds in the proteins (for example, in immunoglobulins) remain intact (Figure 2.9-4). This preparation is carried out in a non-reducing buffer, where the proteins, in a concentration of

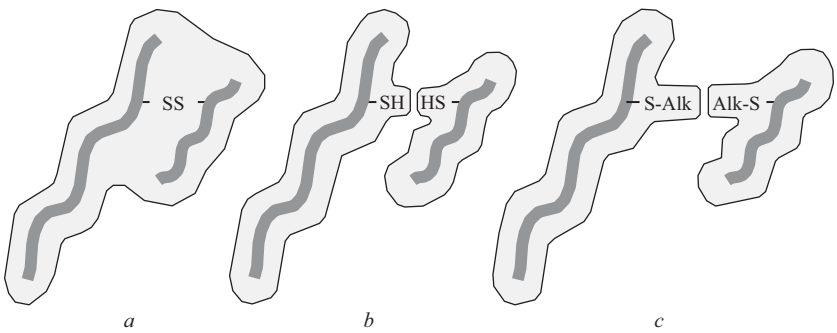


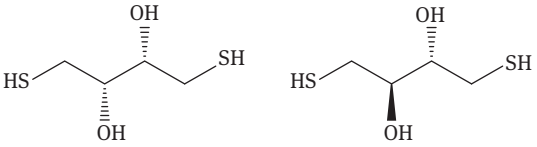
Figure 2.9-4: SDS treated proteins. The polypeptide chains are presented as thick lines surrounded by SDS ions.
a) Without reduction; *b*) With reduction; *c*) With reduction and alkylation
–SS–, disulfide bridge; –SH, thiol group; Alk, alkyl group

0.5–1.0 mg/ml, are incubated at room temperature for 1 h. They should not be cooked, because cooking can fragment the proteins. Thereafter the sample must be centrifuged to remove debris.

The non-reducing sample preparation can also take place in the presence of hydrophobic reagents (e.g., Nonidet P-40).

2.9.2.2 Reducing sample preparation

The reducing sample preparation takes place, if a reducing agent, most often 1,4-**d**i **t**hio**t**hreitol (DTT), or 2-mercaptoethanol (**b**eta-**m**ercapto**e**thanol, BME), or 1,4-**d**i**t**hio**e**rythritol (DTE) in concentrations of 0.1 to 1.0 g/dl is added to the sample buffer.



The mixture is briefly boiled, and the disulfide bridges between the cysteine residues of the proteins are cleaved (Figure 2.9-5). As a result, the proteins unfold, stretch their polypeptide chains, and form rod-like structures with the SDS ions. The quaternary protein structure (oligomeric structure) breaks too.

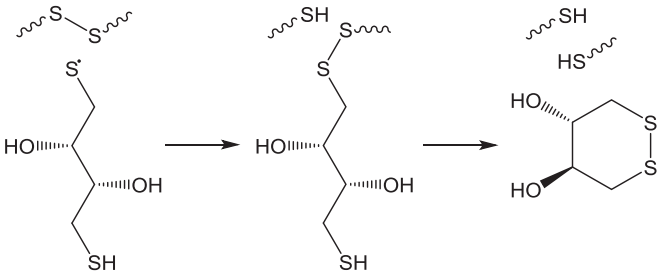


Figure 2.9-5: Reducing a disulfide bond by DTT *via* two sequential thiol-disulfide exchange reactions.

When reduced, the samples must be boiled in a closed vessel with 1 g/dl SDS and 5 mmol/l 1,4-dithiothreitol for 3 to 5 min. If the proteins should not be heated at 100 °C, the samples are left at room temperature for a few hours or overnight.

The SDS sample may be stored at –20 °C and applied on a gel after weeks, even months later. However, it is better to incubate the sample once more in a boiling water bath for 3 min prior to electrophoresis. If the samples are turbid, they should be centrifuged.

2.9.2.3 Reducing sample preparation with alkylation

The SH-groups that occur after the reducing treatment can be oxidized again by atmospheric oxygen or other oxidizing agents to form again disulfide bridges. Therefore, they should be alkylated (to be connected to difficult reacting alkyl groups), which prevents the rebuilding of disulfide bonds.

The alkylation can be carried out with iodoacetamide, iodoacetic acid, or vinylpyridine [27] (Figure 2.9-6). After alkylation, proteins with a high content of sulfur-containing amino acids increase slightly their molecular masses, as the relative molecular mass M_r of iodoacetamide is 184.96.

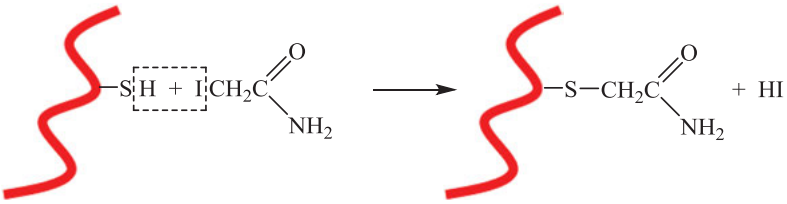


Figure 2.9-6: Alkylation of a thiol group with iodoacetamide.
The polypeptide chain is shown as a thick line.

The alkylation is carried out in the following way: 20 g/dl iodoacetamide is added to cool reduced proteins, and after incubation at room temperature for 30 min the sample is centrifuged. The alkylation is carried out at pH = 8.0.

2.9.3 SDS polyacrylamide gel electrophoresis

The classical SDS electrophoresis was originally carried out in vertical round gels [28], and later in vertical slab gels [29,30]. The wells in the vertical slab gels are formed by a comb and are filled with glycerol containing samples under the upper (cathode) buffer.

The introduction of thin (0.5–1.0 mm) and ultra-thin (0.1–0.5 mm) horizontal polyacrylamide gels [31] on support films [32,33], for example, on pretreated polyester films [34], was an important step forward. These SDS gels can be effectively cooled, because they do not form a significant temperature gradient, and permit multiple samples to be separated under the same conditions.

A tracking dye may be added to the sample. Its higher electrophoretic mobility than the proteins allows monitoring of the progress of electrophoresis.

If necessary, a mixture of protein markers can be used. It can contain: lysozyme from chicken egg white (M_r = 14,400), β-lactoglobulin from bovine milk (M_r = 18,400), restriction endonuclease Bsp981 from E. coli (M_r = 25,000), lactate dehydrogenase from porcine muscle (M_r = 35,000), ovalbumin from chicken egg white (M_r = 45,000), bovine

serum albumin from bovine plasma (M_r = 66,200), and β-galactosidase from E. coli (M_r = 116,000).

The concentration of SDS in the gel should be 0.1 g/dl.

The electrode buffer for horizontal electrophoresis can be in the tanks of electrophoresis cell or in porous electrode strips. The porous electrode strips can be constructed from gel (polyacrylamide or agarose gel) or paper. In the gel strips, the electrode buffer must be added prior to the gelation; the paper strips are impregnated with it. The gel and paper strips are 1–2 cm wide and come in direct contact with the electrodes. Since the volume of the strips is smaller than the volume of the electrode tanks, the electrode buffer in the strips must be concentrated.

The most SDS-PAGE separations are carried out under constant electric current. The resistance of the gel increases during SDS-PAGE, as result of which the voltage increases too.

As the native electrophoresis, SDS electrophoresis can be carried out in one buffer or in a system of two or more buffers. The first method is referred to as SDS zone electrophoresis (or continuous SDS electrophoresis); the second method is referred to as **disc**ontinuous SDS electrophoresis (SDS disc-electrophoresis) [35,36].

2.9.3.1 SDS zone electrophoresis

The SDS zone electrophoresis is performed most common in hydrogen phosphate buffer (Table 2.9-2).

Table 2.9-2: Buffers for SDS zone electrophoresis of proteins.

pH	Gel buffer	*T*, g/dl	*C*	Electrode buffer	M_r	Inventors
6.8	0.1 mol/l TRIS-hydrogen phosphate buffer 1 g/l SDS 8 mol/l urea	12.5	0.10	0.1 mol/l TRIS-hydrogen phosphate buffer 1 g/l SDS	$1–10•10^3$	Swank and Munkres [37]
7.0	0.1 mol/l TRIS-hydrogen phosphate buffer 2 g/l SDS	10.0	0.027	0.05 mol/l TRIS-hydrogen phosphate buffer 1 g/l SDS	$15–200•10^3$	Weber and Osborn [38]
7.1	0.1 mol/l TRIS-hydrogen phosphate buffer 1 g/l SDS	5.0	0.026	0.1 mol/l TRIS-hydrogen phosphate buffer 1 g/l SDS	$15–200•10^3$	Shapiro *et al.* [39]
7.2	0.1 mol/l TRIS-hydrogen phosphate buffer 1 g/l SDS	5.0 10.0 15.0	0.034 0,034 0.034	0.1 mol/l TRIS-hydrogen phosphate buffer 1 g/l SDS	$20–350•10^3$ $10–100•10^3$ $10–60•10^3$	Dunker and Rueckert [40]

2.9.3.2 SDS disc-electrophoresis

The SDS disc-electrophoresis does not distinguish from the known disc-electrophoresis developed by Ornstein [41], and Davis [42] (s. there). The only difference is that it contains SDS in addition.

In the SDS disc-electrophoresis, the SDS-protein complexes concentrate by a moving ionic boundary in the stacking gel and also arrange themselves according to their decreasing mobilities. Then the SDS-protein complexes migrate through the resolving gel, where their rates diminish, since the viscosity of the resolving gel exceeds the viscosity of the stacking gel. As a result, the moving ionic boundary overtakes the concentrated proteins leaving them to separate from each other depending on their electric charges and masses.

SDS disc-electrophoresis in homogeneous gels

Currently, tree buffer systems are used for SDS disc-electrophoresis in homogeneous gels: TRIS-chloride-glycinate system according to Laemmli, TRIS-acetate-TRICINEate system accordinbg to Schägger–Jagow, and TRIS-formate-taurinate buffer system according to Michov. In addition, SDS disc-electrophoresis can be carried out in one buffer at two pH values.

SDS disc-electrophoresis in a TRIS-chloride-glycinate buffer system according to Laemmli

The SDS disc electrophoresis was described in 1970 by Laemmli [43], who added SDS to the discontinuous buffer system of Ornstein and Davis. It is characterized by a moving ionic boundary between the chloride ion, as leading ion, and the glycinate ion, as trailing ion, against the TRIS ion as counter ion, and is running in two gels: a stacking and a resolving gel (Figure 2.9-7).

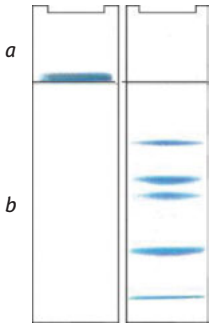


Figure 2.9-7: Laemmli electrophoresis.
a) Stacking gel; *b*) Resolving gel

The stacking polyacrylamide gel is cast in front of (over) the resolving gel. It is a large pore gel with T = 4 g/dl, which contains TRIS-HCl buffer with pH = 6.8. The resolving polyacrylamide gel is a small pore gel with T = 8–30 g/dl, which contains TRIS-HCl

buffer with pH = 8.8. In both gels, the ratio between acrylamide and BIS concentrations is 30:1. The electrode buffer is a TRIS-glycinate buffer with pH = 8.3.

The Laemmli system is used for SDS-denatured proteins with molecular masses of 15–150•10^3. It was originally used in cylindrical gels: The monomer solutions for the resolving and stacking gel polymerized in glass tubes with a diameter of 3–5 mm and a length of 7–12 cm. Usually the resolving gel is about 8 cm long and the stacking gel is about 2 cm long. The gel-filled tubes stay vertical and have contact with the upper and lower electrode buffers.

Protein samples are dissolved in 50 mmol/l TRIS-HCI, pH = 6.8, containing 2 mmol/l EDTA, 10 mmol/l DTT, 2 g/dl SDS, 10 ml/dl glycerol, and 0.01 g/dl Bromophenol blue Na salt. The proteins, in concentrations of 0.2–0.6 mg/ml, are dissolved by heating.

The polyacrylamide gels contain 0.0625 mol/l TRIS-chloride buffer with pH = 6.8, 0.1 g/dl SDS, 5 g/dl 2-mercaptoethanol, 10 ml/dl glycerol, and 0.001 g/dl Bromophenol blue Na salt. After applying 10–50 µl of the sample on the stacking gel, the electrophoresis is performed at electric current of 3 mA per tube until Bromophenol blue front reaches the lower end of the gel.

The separation of SDS-protein complexes depends only on the polypeptide chain length. Therefore, it is easier to choose appropriate gel concentrations for SDS-PAGE than for native electrophoresis. Laemmli gels with T = 7.5 g/dl resolve proteins in the 40,000 to 200,000 range, those with T = 10 g/dl resolve proteins in the 20,000 to 200,000 range, those with T = 12 g/dl resolve proteins in the 15,000 to 100,000 range, and those with T = 15 g/dl resolve proteins in the 6,000 to 90,000 range. The electrophoresis is run at 100–500 V and 20–100 mA. The cooling temperature should be 10–15 °C. The velocity of Bromophenol blue, added to the sample, should be 4–5 cm/h.

Some types of proteins do not behave themselves "normally" during the SDS-PAGE in Laemmli system [44,45]. The glycoproteins, for example, move too slowly, because their sugar residues bind to SDS. This can be avoided, if a TRIS-borate-EDTA buffer is used: the borate ions build negatively charged complex compounds with the sugar residues, which increase the electrophoretic mobility of proteins [46]. Strongly acidic proteins also bind to SDS. An alternative for this case is the usage of the cationic detergent CTAB (Table 2.9-1) instead of SDS in an acidic buffer system of pH = 3–5 [47]. CTAB denatures proteins far less than SDS so that CTAB electrophoresis is considered to be a native electrophoresis [48].

Proteins whose M_r is smaller than 12,000 do not resolve in the Laemmli system because they do not leave the band of SDS micelles that is formed behind the leading ion front. They separate from each other better in the TRICINEate-buffer system (s. below).

In addition to Laemmli system, there are other SDS systems. Neville [49] adapted a TRIS-sulfate-borate buffer system for fractionation of SDS-saturated proteins in the M_r range of 2–300•10^3. The system produces sharp protein bands.

Wykoff *et al.* [50] replaced TRIS in the Laemmli SDS-PAGE system with its analog ammediol (2-**am**ino-2-**me**thyl-1,3-propane**diol**). The ammediol system resolves the proteins better in the M_r range of 1–10•10^3 than the Laemmli or Neville systems, but the bands are not so sharp.

SDS disc-electrophoresis in a TRIS-acetate-TRICINEate buffer system according to Schägger–Jagow

A SDS disc-electrophoresis in gradient gels, which is run in a TRIS-acetate-TRICINEate buffer system, was empirically developed by Schägger and Jagow [51,52]. It gives better results than the classic TRIS-chloride-glycinate buffer system of Laemmli when low molecular mass proteins (M_r < 14,000) are separated (Figure 2.9-8). In addition, the gels show a higher storage stability because they contain a TRIS-acetate buffer with pH = 6.4 – a pH value at which the polyacrylamide gels hydrolyze very slowly. The TRIS-acetate-TRICINEate buffer system uses the TRICINEate ion as a trailing ion in place of the glycinate ion in the Laemmli system.

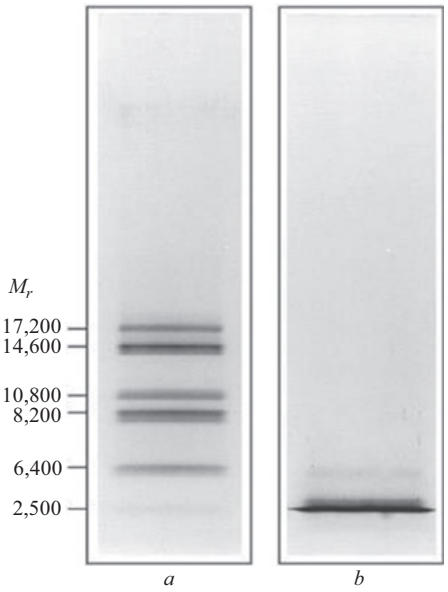


Figure 2.9-8: Comparison of TRICINE-SDS-PAGE with Laemmli-SDS-PAGE.
Resolution of cyanogen bromide fragments of myoglobin by TRICINE-SDS-PAGE (*a*) and Laemmli-SDS-PAGE (*b*) using gels of T = 10 g/dl and C = 0.03. Modified from Schägger and Jagow (Schägger H, Jagow G. *Anal Biochem*, 1987, **166**, 368–379).
a) Stacking gel; *b*) Resolving gel

The TRIS-acetate-TRICINEate buffer system was used in the ExcelGel system (Pharmacia Biotech, Uppsala). The ExcelGel system consists of a horizontal slab gel with a stacking and a resolving part, and two polyacrylamide gel electrodes.

The stacking gel is homogeneous (T = 5 g/dl, C = 0.03), whereas the resolving gel is linearly gradual (T = 8–18 g/dl) at the same C value. Both gels contain a TRIS-acetate buffer with pH = 6.4 (0.12 mol/l TRIS, 0.12 mol/l acetic acid, and 1 g/dl SDS).

The anode electrode gel (T = 12 g/dl, C = 0.03) contains a TRIS-acetate buffer with pH = 6.6 (0.45 mol/l TRIS, 0.45 mol/l acetic acid, 0.4 g/dl SDS, and 0.05 g/dl orange G), and the cathode electrode gel (T = 12 g/dl, C = 0.03) contains TRIS-TRICINEate buffer with pH = 7.1 (0.08 mol/l TRIS, 0.8 mol/l TRICINE, and 0.6 g/dl SDS).

An ExcelGel is placed on the cooling plate of a horizontal electrophoretic unit, and onto its ends are placed electrode gels, whereas the cathode gel must be placed on the stacking gel. At a distance of 0.5–1.0 cm from the cathode gel, an application template is placed on the stacking gel and 5–10 µl of the pretreated samples each are applied in its slots.

A disadvantage of the gradient gels according to Schägger and Jagow is that their preparation is more difficult than that of homogeneous gels. In addition, the staining and destaining of the gradient gels are irregular and the gels roll up during drying.

SDS disc-electrophoresis in a TRIS-formate-taurinate buffer system according to Michov

To eliminate the disadvantages of the gradient gels, a new SDS disc-electrophoresis was developed in homogeneous slab gels [53]. This electrophoresis is carried out in a TRIS-formate-taurinate buffer system that has a higher buffer capacity than the former buffer systems, and is suitable for resolving of proteins with relative molecular masses of 6,000–450,000.

The horizontal SDS gel disc-electrophoresis in a TRIS-formate-taurinate buffer system does not require gel electrode strips, but paper electrode strips. To handle paper electrode strips is easier than gel electrode strips. Besides, they are non-toxic, while the gel electrode strips contain traces of acrylamide. This method can also be used for vertical separation. Then electrode strips are not needed because the gel comes into direct contact with the electrode buffers.

The stacking gel, the resolving gel, and the anode strip contain TRIS-formate buffer; the cathode strip contains TRIS-taurinate buffer. Formic acid (HA) forms the leading formate ion (A^-), taurine (HB) forms the trailing taurinate ion (B^-), and TRIS forms the counter TRIS-ion (HC^+). At 25 °C and an ionic strength of 0.1 mol/l, the mobilities of the formate, taurinate, and TRIS-ion are as follows: $\mu_{A^-} = -44.22 \bullet 10^{-9}$, $\mu_{B^-} = -23.53 \bullet 10^{-9}$, and $\mu_{HC^+} = 17.27 \bullet 10^{-9}$ m^2/(V s) [54].

In order to achieve high resolution, the effective mobilities of the SDS-protein complexes must be higher than the effective mobility of the trailing ion in the stacking gel, but lower in the resolving gel. As a result, the moving ionic boundary between the leading and trailing ion will follow the SDS-protein complexes in the stacking gel, but will gradually overtake them in the resolving gel. Thus the proteins will be concentrated and resolved during the electrophoresis.

To assemble the TRIS-formate-taurinate buffer system, we must calculate the function of Kohlrausch [55]; the degree of taurine dissociation, and the pH value of

the trailing buffer (pH_b) in the cathode strip; and the pH value of the leading buffer (pH_a) in the stacking and resolving gel and in the anode strip.

It follows from the above equations that the function of Kohlrausch $F_k = c_{HTa}/c_{HFo}$ = 0.802, where c_{HB} and c_{HA} are the molar concentrations of formic acid and taurine. If we assume that c_{HA} = 0.10 mol/l, then c_{HB} = 0.0802 mol/l.

The SDS-protein complexes move in a *T*13-resolving gel with effective mobilities lower than $-4 \bullet 10^{-9}$ $m^2/(sV)$. Therefore, the effective mobility of taurinate ion $\mu_{B^-}{}'$ in the stacking gel as well as in the resolving gel should be $-4 \bullet 10^{-9}$ $m^2/(sV)$; the dissociation degree of taurine in the trailing buffer (cathode buffer) should be 0.17; and its ionic strength I_b should be 0.17•0.0802 = 0.0136 mol/l.

According to the theory of Debye–Hückel [56], at I_b = 0.0136 mol/l pK_{cHC^+b} is equal to 8.17 and pK_{cHB} is equal to 8.96. Then it follows from the Henderson–Hasselbalch equation that pH_b = 8.3 and pH_a = 7.4.

The gels for SDS gel disc-electrophoresis in a TRIS-formate-taurinate buffer system consist of a stacking and a resolving gel. The stacking gel forms approximately 1/3, and the resolving gel forms approximately 2/3 of the gel volume (Figure 2.9-9).

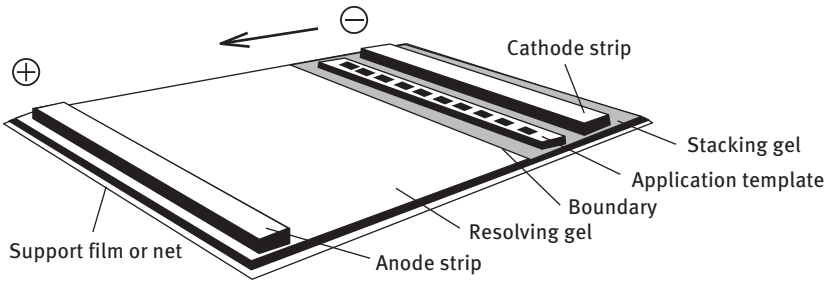


Figure 2.9-9: Scheme of a gel for SDS gel disc-electrophoresis in a TRIS-formate-taurinate buffer system.

Prior to electrophoresis, 0.5–1.0 ml kerosene or silicone oil DC 200 is applied on the cooling plate of an electrophoretic unit and a precast gel with its support film down and its cover film upward is placed on it. The cover film is carefully rolled over with a roller to push out the air bubbles between the support film and the cooling plate and to form a thin liquid layer, which will mediate the cooling process.

The two gel ends are covered with the corresponding paper electrode strips that contain the anode and cathode buffer. Onto the stacking gel, before the cathode strips, an application template, best of silicone, is placed and lightly pressed to create a good contact with the gel. The distance between the application template and the cathode strip should be at least 10 mm.

In each slot, 5–10 µl SDS-sample is applied. The samples should contain 20 ml/dl glycerol. Thereafter, the electrodes are placed on the electrode paper strips and the electrophoresis is started.

The application of samples in the slots is of great importance for the quality of the electrophoresis. We propose that the samples in the slots be overlaid with 2–3 µl deionized water each. Thus the sample surface becomes horizontal (Figure 2.9-10) while, without water, it is concave. There is no danger to mix samples with water because the samples contain glycerol in high concentration.

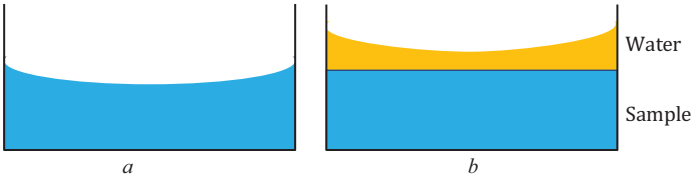


Figure 2.9-10: The sample surface without water (*a*) and with overlaid water (*b*).

The fixation and staining of the protein bands, as well as the destaining of the gel background, take place faster and evenly in homogeneous gels whereas these processes are slower and uneven in gradient gels. In addition, the homogeneous SDS-gels reserve their original shape after drying, whereas the gradient SDS-gels roll themselves. Results obtained by this method are shown on Figure 2.9-11.

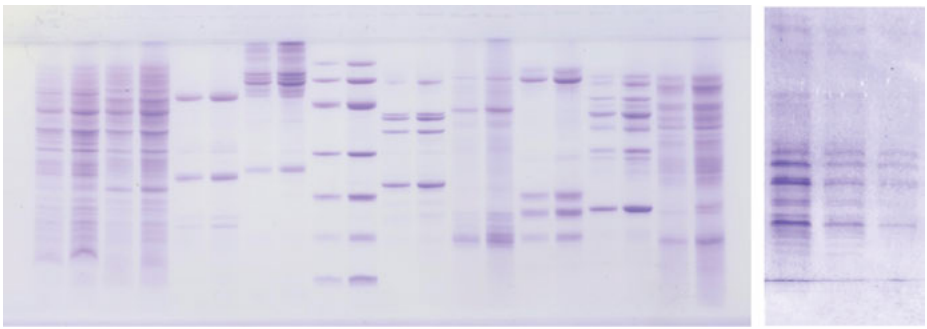


Figure 2.9-11: Electrophoretic results in a 13 g/dl homogeneous SDS slab gel according to Michov. Left – on polyacrylamide gel on support film; Right – on polyacrylamide gel on net
Applied volumes of 5 µl. Electrophoresis at constant electric current of 80 mA (100–500 V) and temperature of 10 °C. Running time 90 min. Staining with Coomassie brilliant blue R-250.

Another buffer system, which includes taurine, is the TRIS-sulfate-taurinate buffer system [57]. Here, the sulfate ion is the leading ion. The effective pH of this system is 8.9, which is close to the pK_c value of taurine but is far away from the pK_c value of TRIS-ion. The capacity of this buffer system is relatively low.

Later a horizontal SDS disc-electrophoresis in a 150 µm polyacrylamide slab gels was described [58]. It uses the resolving system ammediol (2-**a**mino-2-**m**ethyl-1,3-**p**ropane**d**iol, AMPD)-glycinate buffer. A film-supported gel is placed on the cooling plate and 7 cm wide filter paper strips soaked in electrode buffers (cathode buffer: 50 mmol/l TRIS-glycine, pH = 8.2, 0.1 g/dl SDS; anode buffer: 250 mmol/l ammediol-glycine, pH = 8.8, 0.1 g/dl SDS) are arranged in parallel on the gel. The electrophoresis is performed at 15 °C, 350 V, and 7 mA for 3 h.

SDS disc-electrophoresis in one buffer at two pH values

In the SDS disc-electrophoresis in one buffer at two pH values after Michov [59], the denatured polyions are stacking between same leading and trailing ions and are resolving in a zone electrophoresis. This electrophoresis is similar to the disc-electrophoresis after Laemmli. However, its buffer system does not form a moving ionic boundary, but a stationary ionic boundary between a stacking and a resolving buffer, made by a same ion with the polarity of the polyions. The polyions are concentrated at this boundary and then are separated from each other in the resolving gel (s. *Native disc-electrophoresis in one buffer at two pH values*).

In the methods of Laemmli, and Schägger–Jagow, different leading and trailing ions are used for SDS disc-electrophoresis: chloride and glycinate ions, and chloride and acetate ions, respectively. On the contrary, in the SDS disc-electrophoresis in one buffer at two pH values the leading and the trailing ions are the same, for example, glycinate or borate ion. So the polyions are stacking and resolving in only one buffer system consisting of a weak acid and a weak base, but having two pH values.

After SDS disc-electrophoresis in one buffer at two pH values the protein bands are fixed in a mixture of 1.0 mol/l (16.34 g/dl) trichloroacetic acid and 0.2 mol/l 5-sulfosalicylic acid (5.08 g/dl 5-sulfosalicylic acid dehydrate) for 30 min. Then they are stained for 30 min in 0.001 mol/l (0.17 g/dl) Coomassie brilliant blue R-250 dissolved in a destaining solution. The destaining solution contains methanol, acetic acid, and water (3:1:6, *V:V:V*). It is used for destaining the gel background after the staining procedure.

SDS disc-electrophoresis in gradient gels

Pore-gradient gels are also used in SDS-PAGE applications where they have some advantages over homogeneous gels. Usually the polyacrylamide concentration of the gradient gels increases linearly so that the gel pores get smaller. As proteins move through a gradient gel from a region of large pores to a region of small pores, the small proteins move much longer than big ones.

Discontinuous buffer systems in gradient gels, based on the Laemmli buffer, give high resolution: Gradient gels with T = 4–15 g/dl are suitable for SDS-proteins with M_r = 40,000 to 200,000; and with T = 4–20 g/dl are suitable for SDS-proteins with M_r = 10,000 to 100,000.

An interesting method for separation of proteins with very high molecular masses is proposed by Issa *et al.* [60]. They resolved mucosal mucins (M_r = 0.2–2•10^6) using gradient SDS **ag**arose/**p**oly**a**crylamide **g**el **e**lectrophoresis (SDS-AgPAGE). An inclusion of urea in the gels allowed gel casting at room temperature. The urea addition had no effect on western and lectin blotting.

Mucins are secreted proteins present on all mucosal surfaces. Two kinds of mucins are known: large gel-forming mucins and small mucins. The gel-forming mucins are composed of mucin subunits joined by disulfide bonds [61,62]. Treatment with reducing agents releases the subunits and causes unfolding of regions stabilized by intramolecular disulfide bonds. The small mucins are generally associated with the cell membranes.

Mucin glycosylation is a dynamic event on mucosal surfaces [63]. Glycosylation is the major post-translational modification in biology, from aiding protein folding and protein stabilization [64,65] to structural support [66] and antimicrobial activity [67]. It is linked to various diseases such as cancer, inflammation bowel diseases, and cystic fibrosis [68,69,70]. Specific carbohydrate structures are also involved in tumor metastasis [71], and inflammation and immune processes [72].

2.9.3.3 Staining of SDS-protein bands

After Bromophenol blue has reached the anodic end of the gel, the electrophoresis is stopped and the protein bands in the gel are stained by Coomassie brilliant blue or silver. A protein band have to contain approximately 1 µg of protein to be stained with Coomassie brilliant blue R-250 or 0.1 µg of protein to be stained with silver. Both Coomassie brilliant blue staining and silver staining can take place, if Coomassie brilliant blue staining is done first. Alternatively, the proteins can be transferred onto a PVDF membrane by electroblotting.

The separated proteins are stained commonly with Coomassie brilliant blue R-250. The Coomassie brilliant blue and SDS-ions compete for the same binding sites on proteins. Therefore, SDS-ions have to be removed from the gel prior to staining. To accelerate this process, the bands can be fixed in methanol–acetic acid. Methanol increases the CMC value (s. there) of SDS, so it can be easily washed. However, the low pH values reduce the solubility of SDS, so that the SDS removal proceeds slowly.

Other staining method that can be used is the colloidal staining. The colloidal staining has a higher detection sensitivity (approximately 30 ng of protein per band) and the gel background remains clear, but proceeds overnight [73].

The silver staining offers the highest sensitivity, but shows a lack of reproducibility. Silver-stained SDS gels show sometimes artifact bands in the M_r range of 50,000 to 70,000. These artifacts are attributed to contamination with skin keratin [74]. To avoid keratin introduction into the sample, gloves must be wearied during the sample preparing. The gel apparatus must be clean, too.

2.9.4 Determination of molecular masses of SDS-denatured proteins

SDS-PAGE has become the most popular electrophoresis method because it can be used to estimate the relative molecular masses M_r of denatured proteins [75,76,77]. This is based on the dependence of protein mobility μ on the total polyacrylamide gel concentration T (in g/dl), as expressed by the equation

$$\log M_r = a \log T + b \tag{2.9-1}$$

where a and b are constants. The larger the protein, the slower it migrates in a gel. In the practice, the mobilities of investigated proteins are compared to the mobilities of marker proteins (Table 2.9-3) [78,79].

Table 2.9-3: Relative molecular masses (M_r) of marker proteins used for electrophoretic determination of the relative molecular masses of SDS-denatured proteins.

Proteins	M_r	Proteins	M_r
Immunoglobulin M	950,000	H chain of IgG	50,000
2-Macroglobulin	380,000	Ovalbumin	45,000
Thyroglobulin	355,000	Aldolase	40,000
Ferritin	220,000	Alcohol dehydrogenase (liver)	39,800
Myosin (rabbit muscle)	205,000	Lactate dehydrogenase (porcine heart)	36,000
α-Macroglobulin (reduced)	190,000	Pepsin	34,700
Immunoglobulin A	160,000	Carbonic anhydrase	31,000
RNA polymerase (E. coli)	160,000	Carbonic anhydrase (bovine Ery)	29,000
Immunoglobulin G	150,000	Trypsinogen, PMSF treated	24,000
Ceruloplasmin	124,000	Trypsin inhibitor (soybean)	20,100
β-Galactosidase	116,300	β-Lactoglobulin	18,400
Phosphorylase *a* (muscle)	100,000	Myoglobin (sperm whale)	16,800
Phosphorylase *b* (rabbit muscle)	97,400	Hemoglobin	15,500
Lactoperoxidase	93,000	Ribonuclease *b*	14,700
Plasminogen	81,000	Lysozyme (hen egg white)	14,300
Transferrin	76,000	α-Lactalbumin	14,200
Bovine serum albumin	66,200	Cytochrome *c* (muscle)	11,700
Catalase (liver)	57,500	Insulin (reduced)	6,600
Glutamate dehydrogenase (liver)	53,000	Glucagon	3,500

The relative mobilities R_f of the marker proteins are transmitted onto the abscissa axis of a coordinate system, and the logarithms of protein relative molecular masses are plotted on the ordinate axis to build a semi-logarithm calibration straight line. The relative mobility is defined as the mobility of a protein divided by the mobility of the ionic front and is calculated as the distance traveled by a

protein in the resolving gel divided by the distance traveled by the front (the distance of the trailing dye). When the R_f values of unknown proteins are plotted on the calibration straight line, the logarithm of their relative molecular masses can be obtained (Figure 2.9-12).

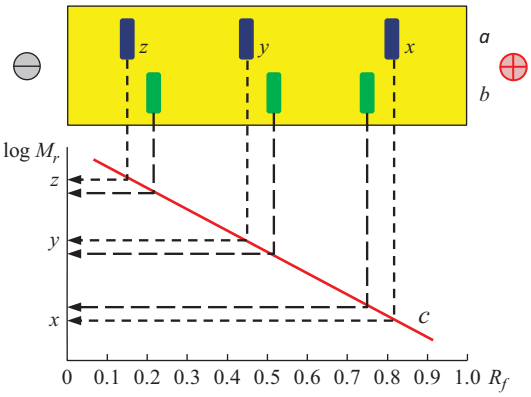


Figure 2.9-12: Determination of relative molecular masses of proteins by SDS electrophoresis using marker proteins.
a) Protein bands *x*, *y*, and *z* in the pherogram; *b*) Bands of marker proteins; *c*) Calibration straight line
M_r – Relative molecular mass; R_f – Relative migration distance

The useful SDS homogeneous gels for estimating the relative molecular masses of proteins in SDS-PAGE are as follows: T = 7.5 g/dl for M_r = 40–200•10^3; T = 10 g/dl for M_r = 30–100•10^3; T = 12 g/dl for M_r = 15–90•10^3; and T = 15 g/dl for M_r = 10–70•10^3. In gradient SDS gels, the relative molecular masses of papain, pepsin, ribonuclease, lysozyme, and more were determined [80].

2.9.5 Fast parallel proteolysis

SDS electrophoresis is related to another electrophoretic method: **fast p**arallel **p**roteolysis (FASTpp). It is a technique for determining the thermostability of proteins [81]. FASTpp is carried out in the following way: At first, a protein mixture is parted after being exposed to different temperatures and a thermostable protease which cleaves specifically at hydrophobic amino acid residues. Then the products obtained are separated by SDS-PAGE (Figure 2.9-13).

Proteolysis is widely used in biochemistry and cell biology to probe protein structure [82]. In *limited trypsin proteolysis*, the enzyme trypsin digests both folded and unfolded proteins but at largely different rates: unstructured proteins are cut rapidly, while structured proteins are cut slowly. Fast parallel proteolysis probes the lysate effect on protein stability, protein ligand binding, mutation effects on proteins, for

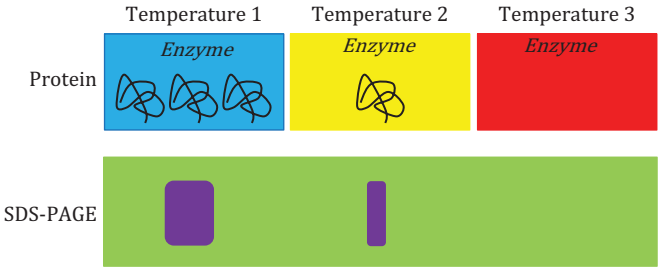


Figure 2.9-13: Determination of relative molecular masses of proteins by SDS electrophoresis using marker proteins.
a) Protein bands x, y, and z in the pherogram; *b*) Bands of marker proteins; *c*) Calibration straight line
M_r – Relative molecular mass; R_f – Relative migration distance

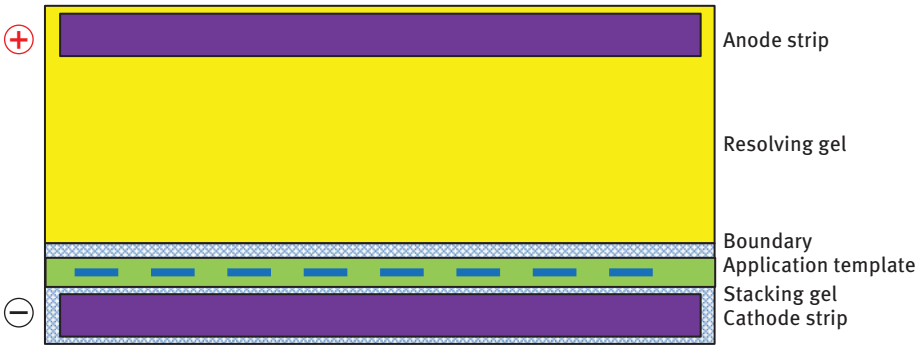


Figure 2.9-14: Setup of horizontal polyacrylamide gel electrophoresis.

example, point mutations [83,84,85], and kinetic stability of proteins [86]. It removes the unfolded fraction. The proteasomes have a similar effect on unfolded proteins in the eukaryotic cells: Its 20S core degrades unfolded proteins without energy supply [87].

2.9.6 Protocols

2.9.6.1 Continuous SDS-PAGE

Materials and equipment
Hydrogen phosphate/SDS buffer, pH = 7.3
Acrylamide
BIS
SDS

Glycerol
TMEDA
APS
Casting cassette
Application template
Unit for horizontal electrophoresis
Power supply

4x Hydrogen phosphate-buffer, 0.05 mol/l, pH = 7.3

$Na_2HPO_4 \cdot 7H_2O$	9.51 g (0.036 mol/l)
$NaH_2PO_4 \cdot H_2O$	2.00 g (0.014 mol/l)
SDS	4.00 g (0.014 mol/l)
Deionized water to	1,000.00 ml

Table 2.9-4: Acrylamide-BIS solutions.

Stock solution	Monomer concentration in the resolving gel (*T*, g/dl)										
	5	6	7	8	9	10	11	12	13	14	15
4x Phosphate/SDS buffer, pH = 7.3, ml	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
30 g/dl acrylamide/ 0.8 g/dl BIS, ml	2.50	3.00	3.50	4.00	4.50	5.00	5.50	6.00	6.50	7.00	7.50
10 g/dl TMEDA, ml	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
10 g/dl APS, ml	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Deionized water, ml	8.60	8.10	7.60	7.10	6.60	6.10	5.60	5.10	4.60	4.10	3.60

The recipes (for 15 ml gel) are based on the continuous phosphate buffer system of Weber et al. [88].

Procedure

- Prepare acrylamide/BIS mixture.
- Degas under vacuum for 5 min.
- Add 10 g/dl APS and mix.
- Pour the mixture in the casting cassette and allow the mixture to polymerize at room temperature for 60 min.
- Place the polyacrylamide gel onto the cooling plate of the horizontal electrophoresis unit.
- Fill the electrode tanks of the electrophoresis unit with hydrogen phosphate/ SDS buffer.
- Mix the samples with 2x hydrogen phosphate/SDS sample buffer and trailing dye, and heat at 100 °C for 2 min.
- Place the application template onto the cathode end of the gel and apply the sample in its slots.

- Connect the power supply and start the electrophoresis at constant electric current until the trailing dye has reached the opposite gel end.
- Control temperature to maintain 15 to 20 °C.

2.9.6.2 Gel casting and horizontal SDS disc-electrophoresis according to Laemmli

Materials and equipment

TRIS
HCl
Acrylamide
BIS
SDS
Glycerol
TMEDA
APS
Glass plates
Support films
0.5 mm U-form spacer
Clamps
Kerosene
Paper strip electrodes
Electrophoresis unit
Constant current power supply
Coomassie brilliant blue or silver staining

4x Stacking gel buffer, pH = 6.8

TRIS	6.06 g (0.5 mol/l)
Adjust with 4 mol/l HCl to pH = 6.8.	
SDS	0.40 g (0.014 mol/l)
Sodium azide	0.01 g (0.002 mol/l)
Deionized water to	100.00 ml

Filter and store at 4 °C up to 2 weeks.

4x Resolving gel buffer, pH = 8.8

TRIS	18.20 g (1.5 mol/l)
Adjust with 4 mol/l HCl to pH = 8.8	
SDS	0.40 g (0.014 mol/l)
Sodium azide	0.01 g (0.002 mol/l)
Deionized water to	100.00 ml

Filter and store at 4 °C up to 2 weeks.

Acrylamide/BIS solution (40 g/dl T, 0.03C)

Acrylamide	38.8 g
BIS	1.2 g
Deionized water to	100.0 ml

Add 1 g Amberlite MB-1, stir for 10 min and filter. The solution can be stored up to 2 weeks in the refrigerator.

Table 2.9-5: Stacking and resolving gels for Laemmli electrophoresis for one casting cassette.

Stock solution	Stack. gel	Resolving gels						
	4	5	7	8	10	12	13	15
4x Stacking gel buffer, pH = 6.8, ml	3.75	–	–	–	–	–	–	–
4x Resolving gel buffer, pH = 8.8, ml	–	3.75	3.75	3.75	3.75	3.75	3.75	3.75
Acrylamide/BIS, ml	1.88	1.88	2.63	3.00	3.75	4.50	4.88	5.63
87% Glycerol	5.17	5.17	5.17	5.17	5.17	5.17	5.17	5.17
10 g/dl TMEDA, ml	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
10 g/dl APS, ml	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Deionized water to, ml	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00

Use 5 g/dl gel for SDS-denatured proteins with M_r = 60,000 to 200,000, 10 g/dl gel for proteins with M_r = 16,000 to 70,000, and 15 g/dl gel for proteins with M_r = 12,000 to 45,000.

10x Electrode buffer, pH = 8.3

TRIS	30.28 g (0.25 mol/l)
Glycine	144.00 g (1.92 mol/l)
SDS	10.00 g (0.03 mol/l)
Sodium azide	1.00 g (0.02 mol/l)
Deionized water to	1,000.00 ml

Filter and keep at room temperature for up to 2 weeks. Before use, mix 100 ml of the electrode buffer with 900 ml of deionized water.

Procedure

- Assemble a sandwich of two glass plates and the 0.5 mm spacer.
- Cast 10 ml of the resolving gel solution using a syringe or pipette in the casting cassette and overlay with deionized water.
- Allow the solution to polymerize for 30 min.
- Drain off the overlaid water.
- Cast 5 ml of the stacking gel solution using a syringe or pipette in the casting cassette and overlay with deionized water.
- Allow the solution to polymerize for 60 min at room temperature or at 40 °C in a drying oven.

- Remove the gel from the casting cassette.
- Mix the protein samples with 2x SDS stacking gel buffer (1:1, *V:V*) and heat in a sealed screw-cap microcentrifuge tube at 100 °C for 3 to 5 min. Dissolve molecular mass standards in 1x SDS stacking gel buffer.
- Pipette a few ml of kerosene on the cooling block of the electrophoresis unit.
- Place the film supported gel onto the horizontal electrophoresis unit.
- Place on the gel ends gel or paper strip electrodes soaked with 4x electrode buffer.
- Place a silicon applicator template onto the gel, in front of the cathode.
- Apply the samples in the template slots using a micropipette with thin tips.
- Connect the electrophoresis unit to the power supply and run electrophoresis at 10 mA constant electric current until Bromophenol blue enters the resolving gel. Increase then the electric current to 15 mA and run the electrophoresis until Bromophenol blue has reached the anode end of the gel.
- Turn off the power supply.
- Fix the gel in a mixture of ethanol–acetic acid–water (4:1:5, *V:V:V*) for one hour.
- Stain with either Coomassie brilliant blue or silver, or electroblot the proteins onto a membrane for immunoblotting. If the proteins are radiolabeled, they can be detected by autoradiography.

2.9.6.3 Gel casting and gradient SDS-PAGE in TRIS-acetate-TRICINEate buffer system according to Schägger–Jagow

Materials and equipment

TRIS
Acetic acid
TRICINE
SDS
87% Glycerol
Bromophenol blue Na salt
2-Mercaptoethanol
TMEDA
APS
Coomassie brilliant blue G-250
Support film
Glass plates
Silicon U-frame
Clamps
Horizontal electrophoresis unit
Power supply (600 V, 500 mA) for SDS-PAGE and electroblotting

Nonreducing sample buffer, pH = 7.0

TRIS	0.18 g (0.15 mol/l)
Adjust with 4 mol/l HCl to pH = 7.0.	
SDS	1.20 g
87% Glycerol	3.00 ml
Coomassie brilliant blue G-250	0.005 g
Deionized water to	10.00 ml

Reducing sample buffer, pH = 7.0

TRIS	0.18 g (0.15 mol/l)
Adjust with 4 mol/l HCl to pH = 7.0.	
SDS	1.20 g
2-Mercaptoethanol	0.60 ml
87% Glycerol	3.00 ml
Coomassie brilliant blue G-250	0.005 g
Deionized water to	10.00 ml
Store at room temperature.	

4x SDS gel buffer, pH = 8.45

TRIS	1.82 g (0.15 mol/l)
Adjust with 4 mol/l HCl to pH = 8.45.	
SDS	0.40 g
Deionized water to	100.00 ml
Store at room temperature.	

SDS TRIS-TRICINEate electrode buffer, pH = 8.3

TRIS	12.1 g (0.10 mol/l)
TRICINE	17.9 g (0.10 mol/l)
SDS	10.0 g (0.03 mol/l)
Deionized water to	1,000.0 ml

Table 2.9-6: Solutions for stacking and gradient gel.

	Stacking gel (T = 4 g/dl)	Light resolving gel (T = 8 g/dl)	Heavy resolving gel (T = 16 g/dl)
4x SDS gel buffer, ml	25.0	25.0	25.0
Monomer solution, ml	8.0	16.0	32.0
87% Glycerol, ml	30.0	–	20.0
10 g/dl TMEDA, ml	0.8	0.5	0.4
10 g/dl APS, ml	0.8	0.5	0.4
Deionized water to, ml	100.0	100.0	100.0

Monomer solution (T = 50 g/dl, C = 0.03)

Acrylamide	48.5 g
BIS	1.5 g
Deionized water to	100.0 ml

Procedure

- Pipette a few drops of water onto a glass plate.
- Place a support film for polyacrylamide gel, and roll over with a roller.
- Put a silicon U-frame (0.5 mm thick) on the support film, place over a second glass plate, and hold all parts with clamps to assemble a casting cassette.
- Place the cassette vertically.
- Cast a polyacrylamide gradient gel mixing the heavy resolving gel solution with same volume of the light resolving gel solution in a gradient maker.
- Overlay the gel solution with deionized water to obtain a smooth top surface.
- Remove the deionized water using a water-jet pump.
- Allow the resolving solution to polymerize at room temperature for 45 min.
- Pour the stacking gel solution onto the resolving gel and allow it to polymerize at room temperature for another 45 min.
- Mix the sample solutions with the 2x sample buffer (1:1, *V:V*) and heat at 40 °C for 30 to 60 min. If proteolytic activity is a problem, heat samples to 100 °C for 3 to 5 min.
- Pipette a few drops of kerosene on the cooling block of the horizontal electrophoresis unit.
- Place the film supported gel onto the electrophoresis unit.
- Place gel or paper strip electrodes soaked with the electrode buffer onto the gel ends.
- Place a silicon applicator template onto the gel, in front of the cathode.
- Apply the samples in the slots of the template using a micropipette with thin tips.
- Connect the electrophoresis unit to the power supply and run electrophoresis at 10 mA constant electric current (for a 0.5 mm slab gel) until Coomassie brilliant blue G-250 enters the resolving gel. Increase then the electric current to 15 mA and run the electrophoresis until Coomassie brilliant blue G-250 has reached the anode of the gel.
- Stain the proteins in the gel with a Coomassie brilliant blue G-250 solution for 1 h.
- Destain the gel background with 10 ml/dl acetic acid. If higher sensitivity is needed, use silver staining.

2.9.6.4 Gel casting and SDS-PAGE in TRIS-formate-taurinate buffer system according to Michov

Materials and equipment

TRIS
Formic acid
Taurine
SDS
87% Glycerol
TMEDA
APS
SDS containing sample buffer
Glass plates
0.5 mm U-shaped gasket
Clamps
Horizontal electrophoresis unit
Power supply

Table 2.9-7: Electrode solutions for SDS disc-electrophoresis in a TRIS-formate-taurinate buffer system.

	4x Anode buffer pH = 7.8, *I* = 4•0.30 mol/l	**4x Cathode buffer pH = 8.5, *I* = 4•0.06 mol/l**
TRIS	19.02 g	8.71 g
99 g/dl Formic acid	4.57 ml	–
99% Taurine	–	12.17 g
SDS	0.24 g	0.24 g
0.1 g/dl BPB*	–	0.69 g
Deionized water to	100.00 ml	100.00 ml

BPB = **B**romo**p**henol**b**lue Na salt

Nonreducing sample buffer, pH = 7.8

4x Anode buffer, pH = 7.8	2.50 ml
SDS	0.10 g
87% Glycerol	3.00 ml
Deionized water to	10.00 ml

Monomer solution (T = 50 g/dl, C = 0.03)

Acrylamide	48.5 g
BIS	1.5 g
Deionized water to	100.0 ml

Procedure

- Place a support film with its hydrophobic side down onto a glass plate, using water as an adhesive agent.
- Roll the support film with a photo roller to remove the air bubbles between the film and glass.
- Place a 0.5 mm U-shaped gasket onto the margins of the support film.
- Smear a second glass plate of same dimensions with a repelling solution to make it hydrophobic.
- Place the second glass plate over and fix all parts with clamps to build a cassette with a volume of 15 ml.
- Cast 10 ml of the resolving gel solution using a syringe or pipette into the casting cassette and overlay with deionized water.
- Allow the solution to polymerize for 30 min.
- Drain off the overlaid water.
- Cast 5 ml of the stacking gel solution using a syringe or pipette into the cassette.
- Allow the solution to polymerize at room temperature for 60 min.
- Remove the gel from the casting cassette.
- Dissolve protein samples and mass standards in the sample buffer.
- Heat the mixture in a sealed screw-cap microcentrifuge tube at 100 °C for 3 to 5 min.
- Apply prior to electrophoresis 0.5–1.0 ml kerosene or silicone oil DC 200 onto the cooling plate of the electrophoretic unit.
- Place the gel with its support film down and its cover film upward onto the cooling plate.
- Roll gently the cover film with a roller to push away the air bubbles between the support film and the cooling plate. So a thin liquid layer is formed, which mediates the cooling process.
- Remove the cover film.
- Place paper strips with the 4x anode and 4x cathode buffer onto the two opposite gel ends.
- Place onto the stacking gel, 10 mm before the cathode strip, an application template, best of silicone, and press it gently to create a good contact with the gel.
- Apply 5 µl SDS-sample in a slot each using a micropipette with thin tips or a microliter syringe.
- Place the electrodes onto the electrode paper strips.
- Connect the electrophoresis cell to the power supply and run electrophoresis at constant electric current density of 0.65 mA/mm^2, an electric voltage of 100–500 V, and temperature of 10 °C until Bromophenol blue has reached the anode electrode strip.

– Stain the gel with Coomassie brilliant blue or silver, or electroblot the proteins onto a membrane for immunoblotting. If the proteins are radiolabeled, detect them by autoradiography.

Table 2.9-8: Stacking and resolving gels.

	Stacking gel $T = 5$ g/dl	Resolving gel $T = 9$ g/dl	Resolving gel $T = 11$ g/dl	Resolving gel $T = 13$ g/dl	Resolving gel $T = 15$ g/dl	Resolving gel $T = 17$ g/dl
4x Anode buffer pH = 7.8, ml	25.00	25.00	25.00	25.00	25.00	25.00
Monomer solution, ml	10.00	18.00	22.00	26.00	30.00	34.00
87% Glycerol, ml	30.00	10.00	10.00	10.00	10.00	10.00
10 g/dl TMEDA, ml	0.45	0.45	0.45	0.45	0.45	0.45
10 g/dl APS, ml	0.36	0.20	0.16	0.14	0.12	0.11
Deionized water to, ml	100.00	100.00	100.00	100.00	100.00	100.00

Table 2.9-9: Gradient gels.

	Light solution $T = 6$ g/dl, $C = 0.03$	Heavy solution $T = 20$ g/dl, $C = 0.03$
4x Gel buffer, pH = 7.8 $I = 0.30$ mol/l, ml	25.50	25.50
Monomer solution, ml	12.00	40.00
87% Glycerol, ml	-	30.00
10 g/dl TMEDA, ml	0.45	0.45
10 g/dl APS, ml	0.36	0.09
Deionized water to, ml	100.00	100.00

Gradient gels ($T = 8$–18 g/dl) are cast using a gradient mixer. The mixing chamber of the gradient mixer is filled with the heavy solution; its reservoir is filled with the light solution.

2.9.6.5 Coomassie brilliant blue R-250 staining of proteins resolved on SDS gels

Materials and equipment

Fixing solution (5 g/l glutaraldehyde in 30 ml/dl ethanol)
Coomassie brilliant blue R-250
Ethanol
Acetic acid

Procedure

- Incubate a SDS polyacrylamide gel with separated proteins in the fixing solution: 10 min for 0.5 mm polyacrylamide gels with T = 10 g/dl; 20 min for 0.5 mm polyacrylamide gels with T = 16 g/dl. During fixation SDS is removed.
- Mix equal volumes of 0.4 g/dl Coomassie brilliant blue R-250 in ethanol-water (6:4, *V:V*) and 20 ml/dl acetic acid to make the staining solution.
- Stain for twice the time used for fixing.
- Destain the gel several times 30 min each in ethanol-acetic acid-glycerol-water (3: 1: 1: 5, *V:V:V:V*).
- Dry the polyacrylamide gel under a cellophane membrane (the agarose gels without cellophane membrane) at room temperature, under a hair dryer or in a dry oven.

  If polyacrylamide gels are cast on a net, the times must be halved.

2.9.6.6 Silver staining of proteins resolved on SDS gels

Materials and equipment

Fixing solution
Sodium thiosulfate ($Na_2S_2O_3$)
Silver nitrate ($AgNO_3$)
Developing solution (0.036 g/dl formaldehyde, 2 g/dl sodium carbonate)
Stopping solution (50 mmol/l Na_2EDTA)
Gloves for avoiding fingerprint contamination

Procedure

- Incubate a polyacrylamide gel with resolved bands in the fixing solution: 10 min for 0.5 mm polyacrylamide gels with T = 10 g/dl; and 20 min for 0.5 mm polyacrylamide gels with T = 16 g/dl. During fixation SDS is removed.
- Wash the gel twice with deionized water.
- Sensitize the gel by incubating with 0.005 g/dl sodium thiosulfate for the same time as the time for incubation in the fixing solution (10–20 min).
- Incubate the gel in 0.1 g/dl silver nitrate for the same time as the time for incubation in the fixing solution (10–20 min).
- Wash the gel with deionized water.
- Add the developing solution for 1–2 min.
- Stop development by incubating the gel in the stopping solution for 10–20 min.
- Wash the gel twice with deionized water.

2.9.7 Troubleshooting

Problem	Cause	Solution
Prior to electrophoresis		
The polyacrylamide gel does not polymerize or is sticky after polymerization.	The concentration of TMEDA or APS in the monomer solution is too low.	Check the recipe of the monomer solution. Use 50 µl of 10 g/dl TMEDA and 50 µl of 10 g/dl APS per 1 ml monomer solution.
	The APS solution was too old or was stored improperly.	Use fresh APS solution. Store the APS solution at 4–8 °C for a week.
	The polymerization temperature was too low.	Polymerize the monomer solution at room temperature (25 °C).
The gel margins are sticky and detach from the support film.	The atmospheric oxygen inhibited polymerization of gel margins.	Overlay the monomer solution with deionized water after casting.
A part of gel is not polymerized.	APS solution was not mixed well with the monomer solution (usually when casting gradient gel).	Mix well APS solution with the monomer solution.
The monomer solution polymerizes too quickly.	The concentration of TMEDA or APS in the monomer solution is too high.	Reduce the concentration of TMEDA or APS in the monomer solution.
Air bubbles in the gel.	Air bubbles came from the outlet tubing of the gradient mixer.	Push air bubbles out of the monomer solution knocking on the casting cassette.
	The support film or glass plate, which contacted the monomer solution, was unclean.	Do not touch the hydrophilic side of the support film and glass plate with fingers. Prior to use wash the glass plate with a detergent.
The gel separates from the support film.	An incorrect support film was used.	Do not exchange the support films for polyacrylamide and agarose gel.
	The gel was cast on the wrong side of the support film.	Cast gel on the hydrophilic side of the support film. Check the sides of the support film with water drops before casting.
The gel does not stick to the support film, but to the glass plate.	The monomer concentration was too low.	Increase the acrylamide or BIS concentration.
	The glass plate was too hydrophilic.	Coat the glass plate with Repel-silane.
	The gel was too long in the casting cassette.	Take the gel out of the casting cassette after polymerization.
There is a liquid on the polyacrylamide gel surface.	The polyacrylamide gel hydrolyzes in alkaline buffers.	Use polyacrylamide gels with alkaline buffers in 2 weeks.

(continued)

Problem	Cause	Solution
During electrophoresis		
It does not flow or flows too little electric current.	One of the connectors has no or poor contact.	Check all connections.
The electrophoresis is running too long time (the front moves too slowly).	The electric current flows partially under the support film.	Use kerosene or silicone oil DC-200 as a contact liquid.
The front moves curved in gradient gel.	The gradient gel polymerized very quickly, before the horizontal offset of the density gradient.	Reduce the APS concentration in the monomer solutions.
The lid of the electrophoresis cell is covered with condensed water.	The cooling is insufficient.	Reduce the cooling temperature. The cooling blocks should be made of glass, metal, or best of ceramic.
	The voltage is too high.	Reduce the voltage.
The gel sparks and burns.	There are thin spots in the gel – the support film was not rolled strongly on the glass plate of casting cassette.	Roll the support film strongly on the glass plate of casting cassette.
After electrophoresis		
No bands in the gel.	The cathode and anode were exchanged.	Check the connection of the electrodes to the power supply.
	The proteins have left the gel.	Give Bromophenol blue Na salt to the sample (in alkaline buffers) and monitor its movement. Use a higher concentration polyacrylamide gel.
	Load quantity was below the detection level of the stain.	Increase sample concentration. Use more sensitive stain (e.g., silver stain).
	Proteins were not fixed in the gel.	Fix longer the proteins. Fix the gel in 5 ml/dl glutaraldehyde.
	Proteins are degraded.	Avoid protease contamination.
Protein precipitates on the slot edge.	The proteins concentration was too high.	Dilute the sample.
	SH groups of polypeptide chains have bound to each other through sulfur bridges forming protein aggregates, which cannot penetrate into the gel.	Add to the sample iodoacetamide.
	Hydrophobic proteins.	Add 4–8 mol/l urea to the sample.
Poor band resolution.	Concentration of protein is high.	Load 10 µg of protein or 100 µg of protein extract.

(continued)

Problem	Cause	Solution
	Old gel.	Use fresh precast gel or cast a fresh gel.
Protein bands are blurred.	SDS-protein complexes were not formed.	SDS concentration should not be lower than 1 g/dl in the sample, and 0.1 g/dl in the gel.
	The samples are too old.	Use fresh samples or boil again ancient samples with SDS.
	The separated bands diffused in the gel.	Fix the proteins immediately after electrophoresis.
The protein bands in the gel are poor stained.	The protein concentration was too low.	Concentrate the sample.
	The sample was not completely dissolved.	Treat the sample with ultrasound, increase SDS concentration.
	The detection sensitivity of the staining method is too low.	Use silver staining.
Band smearing.	High salt concentration.	Dialyze sample. Precipitate the protein with TCA or use desalting column.
	Concentration of protein is high.	Load 10 µg of protein or 100 µg of protein extract.
	Protein aggregation.	Add 4–8 mol/l urea to the sample.
The protein bands have tails.	Solid components in the sample.	Centrifuge the samples before applying.
The protein bands have run into each other.	The sample volume was too large.	Concentrate the samples and apply less volume.
	The application template was not tight on the gel.	Press lightly the application template with fingers onto the gel to push out air bubbles between the template and gel.
The protein bands in a gradient gel are arcuate.	The gel polymerized at heat convection.	Give less APS to the monomer solutions.
The Coomassie brilliant blue R-250 staining has insufficient intensity.	SDS was insufficiently removed from proteins.	Wash off SDS prior to staining. Stain longer.
	The alcohol concentration in the destaining solution is too high.	Reduce the concentration of ethanol or methanol in the destaining solution.
The gel separates from the support film at staining.	Strong acids (TCA) in some staining solutions hydrolyze the bonding between the support film and gel.	Use crosslinking degree $C = 0.02$ instead of usually $C = 0.03$ in highly concentrated gels ($T > 10$ g/dl).
The support film rolls on.	The gel contracts.	Put into the last wash solution 5–10 g/dl glycerol to make the gel elastic.

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2.10 Free-flow electrophoresis of proteins

Free-**f**low **e**lectrophoresis (FFE) is a matrix-free electrophoretic technique, which resembles the capillary electrophoresis and has high resolution. It is characterized by protein flow through a planar channel, which is located perpendicularly to an applied electric field.

Free-flow electrophoresis was developed by Grassmann and Hannig [1,2,3]. Until the 1980s, it was a technology for separation of cells and organelles, but is now used for separation of proteins and other charged particles. Besides the (macro) FFE, miniaturized versions of FFE, named micro FFE, are also known [4].

The advantage of FFE is the fast separation of proteins, which do not adhere to any matrix structure like agarose or polyacrylamide gel. The separations are highly reproducible and can be performed under native or denaturing conditions [5]. They can be carried out with volumes from around 50 µl up to several milliliters. The electrophoresis continues several minutes; therefore, most proteins, for example, enzymes, preserve their activity. To succeed this, the pH value during electrophoresis must be kept within physiological limits, and sucrose, glucose, or other compounds should be added to maintain appropriate isoosmotic pressure.

2.10.1 General theory of free-flow electrophoresis

In free-flow electrophoresis, the sample is continuously injected in a buffer layer (less than 1 mm) that flows through a separation channel formed between two parallel plates [6,7]. An electric field is applied perpendicularly to the flow in the separation channel in order to deflect the charged components at different angles according to their electrophoretic mobilities. The separated components can be collected continuously as fractions.

The theory of free-flow electrophoresis is based on the fact that two perpendicular forces act on the sample to be analyzed: the electric field on the abscissa axis, and the buffer flow on the ordinate axis. As a result, the sample runs the distances x and y after the time t. The ratio between these distances is referred to as $tg\,\theta$, where θ is the angle between the resultant and the y-axis (deflection angle). The deflection angle increases with the increase in the electric field strength and decreases with the increase of the buffer flow velocity (Figure 2.10-1). The magnitude of $tg\,\theta$ is equal to the ratio between the distance x and the distance y, hence, between the sample velocity v_s and the buffer velocity v_b:

$$tg\,\theta = \frac{x}{y} = \frac{v_s}{v_b} \tag{2.10-1}$$

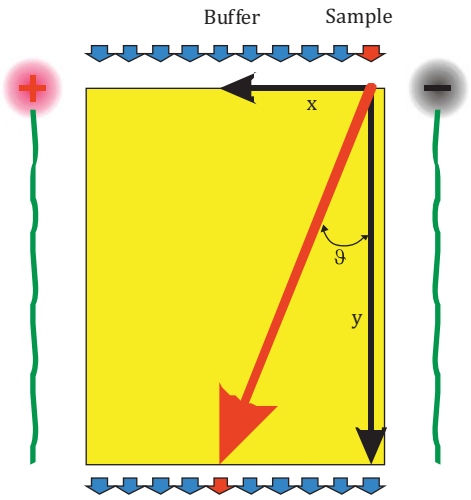

Figure 2.10-1: Scheme of free-flow electrophoresis.

The electrophoretic velocity of the sample in an electric field is given by the equation

$$v_s = \mu_s E = \mu_s \frac{J}{\gamma} = \mu_s \frac{I}{q\gamma} \tag{2.10-2}$$

where μ_s is the sample mobility, E is the electric field strength, J is the density of the electric current, γ is the specific conductivity of the buffer, I is the strength of the electric current, and q is the cross-section of the electrophoretic channel. From eqs. (2.10-1) and (2.10-2) follows that

$$tg\,\theta = \frac{\mu_s I}{q\gamma v_b} \tag{2.10-3}$$

As the strength of the electric field, the cross-section of the electrophoretic channel, the specific conductivity of the buffer, and the velocity of the buffer flow remain constant during the electrophoresis, the resolution of free-flow electrophoresis depends only on the sample mobility.

The residence time of the sample in the separation chamber is determined by different factors. Too short residence time yields too short migration distance. Too long residence time, however, leads to results that are not optimal because of the thermal convection. The optimal residence time is 5–10 min or more at 10–20 °C, at electric field strength of 200–250 V/cm.

2.10.2 Types of free-flow electrophoresis

According to the electrophoretic principles, three types of free-flow electrophoresis are distinguished: free-flow zone electrophoresis, free-flow isotachophoresis, and free-flow isoelectric focusing.

2.10.2.1 Free-flow zone electrophoresis

Free-**f**low **z**one **e**lectrophoresis (FFZE) is carried out in one (continuous) buffer. The buffer must have sufficient capacity and an appropriate pH value, preferably in neutral range (pH = 7.0–7.4) or in slightly alkaline range (pH = 8.0–9.0). Its components should not react with added substances, for example, viscous additives, such as sucrose or glycerol. In this connection, the borate buffer should be mentioned because boric acid forms complexes with polyols [8].

During the electrophoresis, the sample particles are deflected at different angles from the flow direction because of the different velocities they have in the electric field (Figure 2.10-2).

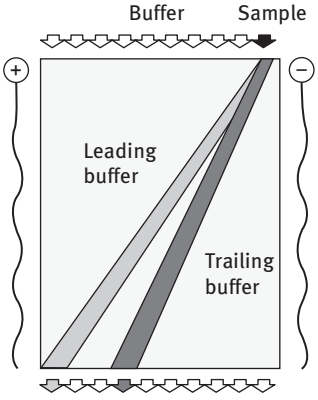

Figure 2.10-2: Free-flow zone electrophoresis of two sample components.

Theory of free-flow zone electrophoresis

Free-flow zone electrophoresis uses a buffer of constant pH value, and electric field with continuous strength. Under these circumstances, the components are separated according to their mobilities determined by the charge-to-mass ratio. The analyte to be separated is deflected linearly under a constant angle determined by the electric field strength, the analyte mobility, and the flow velocity. The migration distance d of the analyte moving through the electric field is given by the equation

$$d = \mu_p E t \tag{2.10-4}$$

where μ_p is the electrophoretic mobility of the analyte, E is the electric field strength, and t is the residence time of the analyte in the separation chamber [9].

Several phenomena influence negatively the band broadening at FFZE: the width of the injected sample σ_{INJ}, diffusional broadening (σ_D), hydrodynamic broadening (σ_{HD}), electrodynamic broadening (σ_{ED}), Joule heating (σ_{JH}), and electromigration dispersion (σ_{EMD}) [10,11]:

$$\sigma_T^2 = \sigma_{INJ}^2 + \sigma_D^2 + \sigma_{HD}^2 + \sigma_{ED}^2 + \sigma_{JH}^2 + \sigma_{EMD}^2 \tag{2.10-5}$$

The variance due to the sample injection width w_i^2 [12] is expressed by

$$\sigma_{INJ}^2 = \frac{w_i^2}{12} \tag{2.10-6}$$

Reduction of the sample injection width in microfluidic systems leads to better results [13]. The diffusional broadening is directly related to the residence time t of the analyte in the separation chamber, and can be expressed by

$$\sigma_D^2 = 2Dt \tag{2.10-7}$$

where D is the analyte diffusion coefficient [14]. Short residence times, as usually involved in μ-FFE systems, reduce this effect too.

Free-flow field step electrophoresis

A variant of free-flow zone electrophoresis is **f**ree-**f**low **f**ield **s**tep **e**lectrophoresis (FFFSE). It needs two buffers: diluted and concentrated buffer, which build a boundary between them. The electric field strength in the diluted buffer is higher than the electric field strength in the concentrated buffer. As a result, the analytes to be separated move fast through the diluted buffer until they reach the boundary between the

buffers where they concentrate [15]. This mechanism of concentration is known as effect of Hjerten (s. there).

2.10.2.2 Free-flow isotachophoresis

In **f**ree-**f**low **i**so**t**acho**p**horesis (FFITP), discontinuous buffer system is used. The sample components arrange themselves in it according to their mobilities (Figure 2.10-3).

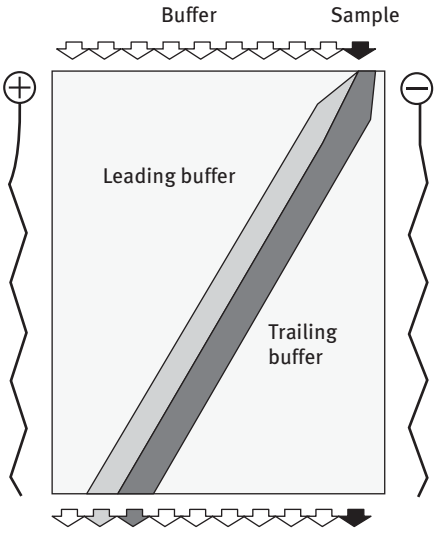

Figure 2.10-3: Scheme of free-flow isotachophoresis of two negatively charged particles.

Theory of free-flow isotachophoresis

The discontinuous buffer system of free-flow isotachophoresis consist of a leading and trailing buffer. The mobility of the leading ion must be greater, and the mobility of the trailing ion must be smaller than the mobility of the particles to be resolved. The counterion is usually an ion of a weak base or acid, which has high buffer capacity in the necessary pH range. After electric field is applied, low field strength in the area of the leading buffer, high field strength in the area of the trailing buffer, and mean field strength in the sample region are created. During the electrophoresis run, the sample components arrange themselves according to their descending mobility and form sharp bands whose concentrations depend on the concentration of the leading ion.

To keep the particles to be resolved away from each other, spacer substances are added into the sample. For example, when proteins are to be separated, amino acids are used whose mobility values are between those of the proteins.

2.10.2.3 Free-flow isoelectric focusing

In **fr**ee-**f**low **i**so**e**lectric **f**ocusing (FFIEF), the sample is fractionated in a linear pH gradient [16] created by carrier ampholytes (Figure 2.10-4). The proteins to be re-

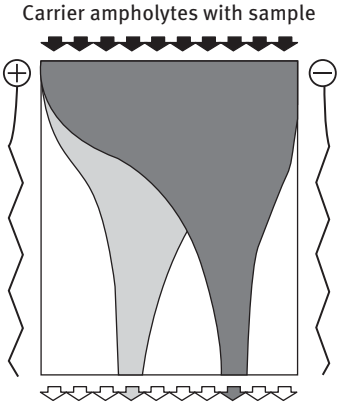

Figure 2.10-4: Free-flow isoelectric focusing in a carrier ampholytes pH gradient.

solved can be added either as a narrow zone, or can be dissolved in the carrier ampholyte solution. When an electric field is applied, a pH gradient is formed, where the proteins are focused at their isoelectric points (pI).

Theory of free-flow isoelectric focusing

In FFIEF, the ampholytes used form a linear pH gradient that is perpendicular to the flow direction. Sample components migrate through the pH gradient due to the electric field until they reach that point of the pH gradient that is equal to their pI. There they loss their total electric charge and focus [17]. Therefore, the width of the sample injected play a minor role in FFIEF.

The equilibrium between the electrophoretic and diffusional transport during IEF is described by the following differential equation, valid under steady state conditions [18]:

$$\frac{d(c\mu E)}{dx} = \frac{d}{dx} D \frac{dc}{dx} \tag{2.10-8}$$

where c (in mol/l) is the analyte concentration at position x in the separation chamber, μ [in $m^2/(sV)$] is its mobility at that point, E (in V/m) is the electric field strength, and D (in m^2/s) is the diffusion coefficient.

Beside carrier ampholytes, other electrolytes can be used, for example, Good's buffers [19]. It is important to know that the difference between the pI and pK values of an electrolyte must be as small as possible, and the number of buffering electrolytes must be as large as possible. The sample solutions should be desalted. If this is not the case, precipitates may appear.

2.10.3 Device technology of free-flow electrophoresis

A free-flow electrophoresis device is built by a front and a back plate. The front plate is made of **p**oly(**m**ethyl **m**eth**a**crylate) (PMMA, Plexiglas). The back plate consists of an aluminum block, covered with a glass mirror, which in turn is covered with a plastic. The front plate contains inlets for samples and buffers, and outlets for fractionation tubes. The distance between the front and back plates can be adjusted by membrane spacers, for example, of cellulose nitrate, and is usually 0.1–0.5 mm. They define the separation chamber height.

The separation chamber is divided in three regions: separation region, anode bed, and cathode bed. Electrodes are placed outside the separation region avoiding the disturbance of generated oxygen and hydrogen bubbles. Usually, the device is placed on a cooled plate in order to remove heat generated by the electric current. This principle can be applied for smaller FFE systems that use injection molding and milling [20].

The buffer is fed with the help of a tubing pump and flows vertical in the narrow gap between the two plates [21,22,23]. The sample is applied in the upper part of the device with the aid of a dossier pump. A high voltage electric field crosses the flow direction perpendicularly. The electric current is maintained by filter paper strips, which are immersed in lateral electrodes tanks. The separated proteins are collected at the lower end of the device. The separation device is cooled with water.

For the FFE microdevices, three aspects are of importance: 1. stable electric field over the separation chamber; 2. efficient removal of gas and other side products formed during electrolysis; and 3. good voltage efficiency. The voltage efficiency η_V is defined by

$$\eta_V = \frac{V_{eff}}{V_{total}} \tag{2.10-9}$$

where V_{total} is the total applied voltage and V_{eff} is the voltage effectively utilized for separation.

Electrode side channels can be open or closed.

The *open electrode side channels* allow for ventilation of gas formed during electrolysis. This design, however, results in a pressure gradient from the separation chamber toward the electrode side beds, which can cause fluid leakage. The fluid leakage has to be compensated by proper shielding of the separation chamber with membrane equivalent structures with high hydrodynamic resistance.

The *closed electrode side channels* require additional flow streams ensuring a removal of produced gas bubbles and chemical side products. Besides, the flow balancing of the fluid flow streams in the electrode and separation chamber is important to avoid contamination of fluids between the electrode channels and the separation chamber.

2.10.4 Detection system of free-flow electrophoresis

The separated proteins in free-flow electrophoresis are analyzed usually by a detection system. The detection system consists of a scanner and computer with printer. The scanner measures the absorption of the buffer flowing with the proteins in UV light. Prior to each electrophoretic separation, the absorbance of the pure buffer is measured to determine a baseline, which must be subtracted from the values obtained. The measurement signals are sent to a computer with appropriate software.

2.10.5 Applications of free-flow electrophoresis

Free-flow electrophoresis is an efficient analytical and preparative method in chemistry and biochemistry [24]. It can be used for analytical separation of proteins, inclusive enzymes, of membranes, vesicles, lysosomes, mitochondria, ribosomes, chloroplasts, viruses, bacteria, and cells as platelets, red cells, thymus cells, tumor cells, and so on. [25,26]. Free-flow electrophoresis is an excellent method for analyzing human plasma proteins [27] and an useful technique for preparative separations of peptides [28,29,30], proteins [31,32,33], enzymes [34,35], cellular components [36], and cells [37,38,39]. In addition, it is applied for characterization of liposomes [40], human smooth muscle, or thyroid cells [41]. However, FFE is rarely used for separation of nucleosides, nucleotides, or nucleates.

Objects of free-flow zone electrophoresis are cells (lymphocytes, kidney cells, tumor cells and more), and cell organelles (lysosomes, mitochondria and more) [42]. An example of free-flow zone electrophoresis is the separation of human T and B lymphocytes (Figure 2.10-5). Although they hardly distinguish in size, it is possible to separate them from each other due to their different surface charges [43].

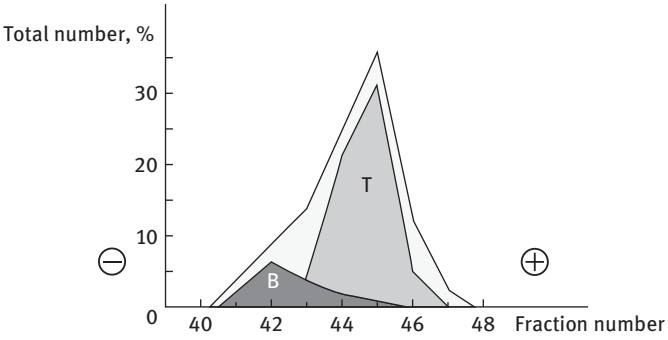

Figure 2.10-5: Free-flow zone electrophoresis of T and B lymphocytes.

Free-flow isotachophoresis is a method for analyzing organic acids, antibiotics, amino acids, oligopeptides, and proteins, for example, immunoglobulins G, A, and M. Using free-flow isotachophoresis, human transferrin was isolated [44], and the purification of ovalbumin, lysozyme [45], and monoclonal antibodies [46] was reported. Janasek *et al.* [47] focused fluorescein applying 150 V (electric field strength of 18,000 V/m inside the separation chamber), and a flow rate of 20 µl/min.

Free-flow isoelectric focusing is used for separation of amphoteric substances, for example, proteins [48], amino acids, peptides, peptide hormones, enzymes, nucleoproteins, and viruses. In a pH gradient, generated with carrier ampholytes, proteins from human cell lysates were separated into 80 fractions [49].

By making use of narrow pH gradients, free-flow isoelectric focusing can also separate protein isoforms (e.g., antibody isoforms), that vary by less than 0.02 pH units, with a throughput of around 3 mg/h [50].

2.10.6 Protocols

2.10.6.1 Free-flow zone electrophoresis of human T and B lymphocytes

Materials and equipment

TRIS
Phosphoric acid Na salt
NaCl
KCl
T and B lymphocytes
Apparatus for free-flow electrophoresis

Resolving buffer, pH = 7.4

$NaH_2PO_4 \cdot H_2O$	1.38 g (0.01 mol/l)
Adjust with TRIS to pH = 7.4.	
NaCl	8.77 g (0.15 mol/l)
KCl	0.22 g (0.003 mol/l)
Deionized water to	1,000.00 ml

Cathode buffer, pH = 7.4

$NaH_2PO_4 \cdot H_2O$	4.14 g (0.03 mol/l)
Adjust with TRIS to pH = 7.4.	
NaCl	26.30 g (0.45 mol/l)
KCl	0.60 g (0.008 mol/l)
Deionized water to	1,000.00 ml

Anode buffer, pH = 7.0

$NaH_2PO_4 \cdot H_2O$	2.76 g (0.02 mol/l)
Adjust with TRIS to pH = 7.0.	
Deionized water to	1,000.00 ml

Procedure

- Suspend $2x10^7$ cells of a sample in 1 ml resolving buffer.
- Fill the cathode and anode tanks with cathode and anode buffer, respectively.
- Give free-flow zone electrophoresis 240 V/cm field strength at 20 °C.
- Run the free-flow electrophoresis for 6 min.

2.10.7 Troubleshooting

Problem	Cause	Solution
During electrophoresis		
It does not flow or flows too little electric current.	No or poor contact to the power supply.	Check all connections.
The electric current strength increases during FFE.	The cooling is insufficient.	Check the cooling water flow. If necessary, reduce the power.
After electrophoresis		
The concentration of proteins is low.	The proteins were digested by proteolytic enzymes.	Check the sample preparation. Add inhibitors, e.g., EDTA.

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2.11 Isoelectric focusing of proteins

Iso**e**lectric **f**ocusing (IEF), also known as *electrofocusing*, is an electrophoresis technique for separating proteins in pH gradients. Moving through the pH gradients, the proteins reach pH values (their **i**soelectric **p**oints, pI, pH(I)) where they lose their net electric charge and stop. Using isoelectric focusing, proteins that differ in pI values by as little as 0.01 pH units can be resolved. Usually pH gradients are obtained in polyacrylamide gels with low concentration, which eliminate any sieving effect.

The isoelectric focusing on polyacrylamide gels can be carried out in round gels [1,2] and slab gels [3,4]. The importance of the vertical round or slab gels diminishes at the expence of horizontal slab gels. The reason is that the horizontal slab gels have, compared to the vertical gels, important advantages:

- They enable a direct comparison of many samples in a gel (in the vertical slab gels, this is also possible);
- The place of sample application can be varied between the cathode and anode; and
- The pH gradient can be measured directly with a surface electrode.

The history of IEF began with Kolin [5]. He obtained unstable pH gradients via diffusion of two buffers with different pH values and received sharp protein bands during electrophoresis. Later in the early 1960s, Svensson (Rilbe) published his classical theoretical works on the carrier (free) ampholytes [6,7]: when hundreds of ampholytes with different isoelectric points are in an electric field, they form a pH gradient. To obtain stable pH gradients with carrier ampholytes, he checked amino acids, synthetic peptides, and hydrolyzed proteins [8], but the problem still persisted.

The first successful synthesis of carrier ampholytes was performed by Vesterberg [9,10,11], a student of Svensson. He synthesized a mixture of carrier ampholytes from residues of carboxylic acids and polyethylene amines.

Next Grubhofer filed two patents for producing carrier ampholytes from oligoamino and oligosulfonic acid [12], and marketed them under the trademark of *Servalyt*. The next attempt came from the Pharmacia scientists Williams and Söderberg [13,14] who established carrier ampholytes under the trademark of *Pharmalyte*. Later, carrier ampholytes were synthesized that contained in addition residues of phosphoric and sulfuric acid [15]. LKB offered them under the trade name of *Ampholine*.

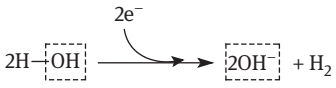
The next step was the binding of ampholytes to polyacrylamide gel as a result of which **i**mmobilized **p**H **g**radients (IPG) were created. Nowadays, the IPG gels on plastic strips are preferred, because they can also be used for the second SDS dimension of the two dimensional electrophoresis or in the mass spectrometry.

2.11.1 Theory of isoelectric focusing

During isoelectric focusing the amphoteric polyions lose their net charges gradually until they stop at their isoelectric points (Figure 2.11-1).

At pH values above pI, the carboxyl groups are negatively charged (COO^-), but the amino groups ($-NH_2$) carry no charges. As a result, the protein polyions have negative net charges. At pH values below pI the carboxyl groups (–COOH) do not bear any charges, however, the amino groups are positively charged ($-NH_3^+$). As a result, the protein polyions have positive net charges. At the isoelectric points the net charges of proteins are equal to zero.

During the isoelectric focusing electrolytic processes take place at the electrodes. At the cathode, where electron excess exists, hydroxide ions are formed:



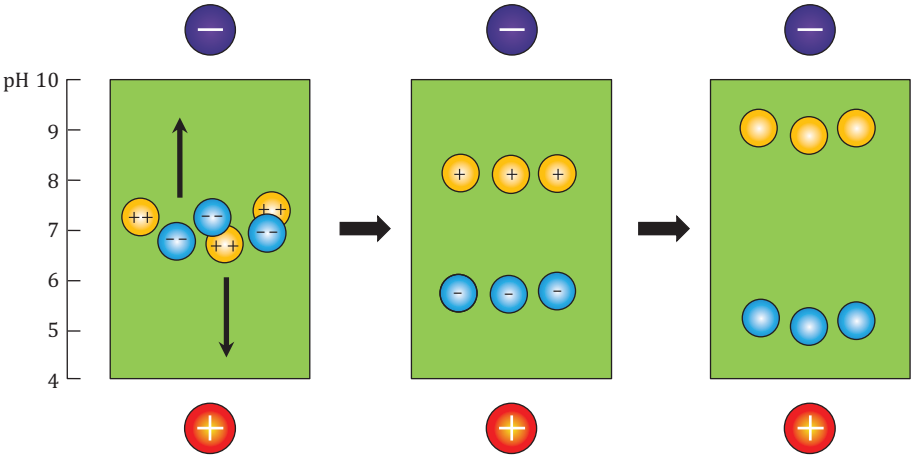


Figure 2.11-1: Isoelectric focusing of two proteins with pI values of 9.0 and 5.0.

and at the anode, where electron deficiency exists, protons are formed:

$$H_2O \xrightarrow{2e^-} 2H^+ + 1/2O_2$$

As a result, the pH value at the anodic end of the gel decreases, and the pH value at the cathodic end of the gel increases. To keep the pH gradient stable in the gel, corresponding electrode solutions are used – an acid at the anode end, and a base at the cathode end. When an acidic carrier ampholyte meets the anode solution, its basic groups charge positively and will be attracted by the cathode, and vice versa.

The direction and the velocity of moving of an amphoteric polyion in a pH gradient depends on its net charge at a given pH value. For example, a protein with a positive net charge migrates toward the cathode through the increasing pH value and loses gradually its net charge by deprotonation. This continues until the numbers of its negative and positive charges become equal and, as a result, its net charge will be equal to zero. If the polyion moves away from its pI point, for example, by diffusion, it obtains again positive or negative charges, and moves again to its pI point.

The isoelectric points of most proteins and peptides find themselves in the pH range of 3 to 12, but most of them are between pH = 4.0 and 7.0 [16,17]. Narrow pH gradients can also be obtained. For example, if proteins have pI values from 5.2 to 5.8, they can be focused in a pH gradient in the range 5.0 to 6.0.

Using isoelectric focusing, protein polyions with isoelectric points differing by 0.0025 pH units can be separated [18].

2.11.1.1 Resolution of isoelectric focusing

According to the theory of Svensson, the standard deviation (approximately 1/4 of the bandwidth, in $(Vm)^{1/2}$) of a protein band

$$\sigma = \sqrt{\frac{D}{(d\mu/d\mathrm{pH})(d\mathrm{pH}/dx)}} \tag{2.11-1}$$

where D is the diffusion coefficient of the protein (in m^2/s), $d\mu/d\mathrm{pH}$ is the increase of the mobility (in $m^2/(sV)$), and $d\mathrm{pH}/dx$ is the increase of the pH gradient (in m^{-1}).

Later Svensson created another equation that describes the resolution of two proteins with same concentration forming two adjacent bands:

$$\Delta\mathrm{pI} = 3.07\sqrt{\frac{D(d\mathrm{pH}/dx)}{E(-d\mu/d\mathrm{pH})}} \tag{2.11-2}$$

where ΔpI is the required difference between the pI values of both proteins, and E is the strength (intensity) of the electric field at the pI values, in V/m. This equation defines the resolution of isoelectric focusing as the smallest difference between two ampholytes, which can be separated from each other. The formula also shows that the resolution increases when the diffusion coefficient is reduced and the field strength is increased. The resolution is higher, that is, ΔpI is smaller, if the field strength is higher and the pH difference is smaller.

The peak capacity n of isoelectric focusing, that is, the maximum number of separable polyions, can be determined [19] by the equation

$$n = \frac{l}{4}\sqrt{\frac{FE(dQ/d\mathrm{pH})(d\mathrm{pH}/dx)}{RT}} \tag{2.11-3}$$

where l is the length of the distance (in m), F is the Faraday constant (in C/mol), Q is the electric charge (in C), R is the molar gas constant [in J/(mol K)], and T is the absolute temperature (in K). The peak capacity is determined by the length of the separation distance, the field strength, and the slope of the electric charge or pH gradient.

The velocity v of the proteins is proportional to the field strength E according to the equation

$$v = \mu E = \frac{l}{t} \tag{2.11-4}$$

where l is the distance passed for the time t.

The processes accompanying the isoelectric focusing were analyzed by high-resolution computer simulation [365].

There are two ways of forming a pH gradient: using free ampholytes (carrier ampholytes), which build a pH gradient in an electric field; or using immobilized ampholytes (immobilines) attached covalently to a polyacrylamide gel.

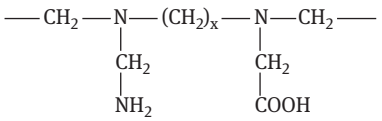
2.11.2 Isoelectric focusing with carrier ampholytes

Isoelectric focusing with carrier ampholytes can be carried out in different separation media such as polyacrylamide gel [20], agarose gel [21,22], acetyl cellulose films [23], and granulated gels [24,25]. They should not have a molecular sieve effect.

Isoelectric focusing with carrier ampholytes on polyacrylamide gels containing urea is referred to as denaturing isoelectric focusing with carrier ampholytes.

2.11.2.1 Properties of carrier ampholytes

The carrier ampholytes represent a heterogeneous mixture of different low-molecular aliphatic oligoamino-oligocarboxylic acids (ampholytes, zwitterions) with closely pI points from 2.5 to 11:



They arrange themselves in an electric field according to their pI values and build a pH gradient. In order to ensure a stable pH gradient, the carrier ampholytes should:

- Be made of many components;
- Have low molecular masses (M_r < 1,000) to reach quickly their pI points;
- Have sufficient buffering capacity at their pI points. The buffering capacity is important for the pH gradients in the presence of other charged ions, for example, protein polyions. Svensson noted that the difference (pI – pK_c) should be less than 0.5 pH units. Therefore, the amino acids are not suitable as carrier ampholytes;
- Have low and evenly distributed electric conductivity along the pH gradient. If a lower conductivity point in a pH gradient is present, it couses a higher voltage here; as a result, the temperature rises at this point and a hot spot is formed in the gel, where proteins can denature.

Since the pK values of carrier ampholytes and proteins to be analyzed are temperature dependent, the isoelectric focusing should be carried out at a constant temperature, preferably at 10 °C.

2.11.2.2 Formation of a pH gradient by carrier ampholytes

Prior to an electric field is applied, carrier ampholytes are in homogeneous distribution in the gel. Then a pH gradient occurs in three steps (Figure 2.11-2):

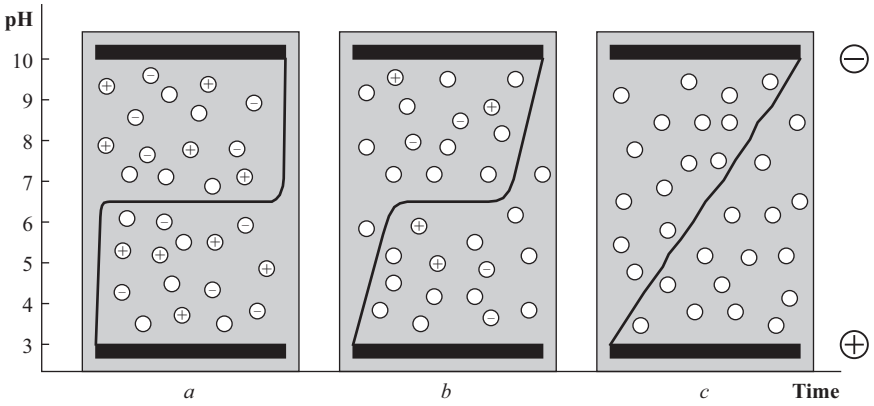


Figure 2.11-2: Steps (*a, b*, and *c*) of pH gradient building by carrier ampholytes.

- The carrier ampholytes begin to migrate according to their net charges to opposite electrodes. Carrier ampholytes with pI lower than the pH value of the separation medium carry negative charges and therefore migrate to the anode. Carrier ampholytes with pI higher than the pH value of the separation medium have positive charges and migrate to the cathode. As a result, two steep pH gradients are created with a pH plateau between them.
- The pH plateau disappears and
- Both pH gradients form a continuous linear pH gradient.

Optimal pH gradients are formed by carrier ampholytes with relative molecular masses M_r between 300 and 1,000. If M_r are less than 300, the pH gradient is unstable; if they are larger, they cannot be distinguished from proteins to be resolved.

The pH gradient also depends on the number of carrier ampholytes molecules. The larger it is, the "smoother" the pH gradient. Ampholyte concentrations of about 2 g/dl give stable pH gradients. If the ampholyte concentrations are above 3 g/dl, the ampholytes cannot be totally removed from the gels and can interfere with protein dyes.

The carrier ampholytes are nowhere fixed and can move freely in the pH gradient. So they are exposed to two harmful effects: electroosmosis and external buffers. The electroosmosis causes an water drift to the cathode, which results in deformation of the pH gradient. The external buffers in the protein samples can cause, especially at high concentrations, "crooked" protein bands (Figure 2.11-3).

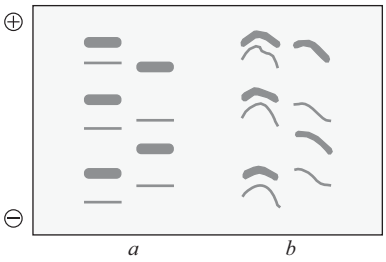


Figure 2.11-3: Influence of sample ionic strength on the bands formed in electrofocusing.
a) Samples with low ionic strength; *b*) Samples with high ionic strength

Since the buffering capacity is low, the pH gradient is affected by air carbon dioxide. It binds to water in the gel and forms carbonic acid, which, at pH > 8.2, lowers the pH values of the pH gradient with 0.2–0.3 pH units [26]. Besides, the hydrogen carbonate ions HCO_3^- cause an anode drift of the pH gradient.

The influence of carbon dioxide on the pH gradient can be minimized by the following ways:

- The gel surface can be covered with a thin polyester film;
- The electrofocusing can be run in closed units under a nitrogen atmosphere; or
- In the separation unit, filter cartons impregnated with concentrated KOH can be placed, because KOH binds carbon dioxide.

In addition, glycerol, sorbitol, or sucrose can be added to the mixture, but they change the pH gradients, if their concentrations are higher than 20 g/dl.

Diverse pH gradients are used: acidic gradients (pH = 4.0–6.0), very acidic gradients (pH = 2.0–4.0), neutral gradients (pH = 6.0–8.0), basic gradients (pH = 8.0–10.0), and very basic gradients (pH = 9.0–11.0).

pH Measurement

The pH value in a gel can be measured with surface electrodes. Another slower method is the pH measurement of eluates from gel pieces: The gel is cut into equal pieces, every piece is eluted in a small volume of 10 mol/l KCl, and the pH values of the solutions are measured. A third method for measurement of a pH gradient may be carried out after creating a calibration curve obtained by focused marker proteins with known pl values. On this curve, the pI values of proteins under investigation can be extrapolated. The most usable marker proteins are listed in Table 2.11-1.

Separator electrofocusing

The resolution of a pH gradient can be increased by addition of appropriate separators, also known as pH gradient modifiers [27,28]. The separators are amino acids or

Table 2.11-1: Marker proteins.

Marker proteins	pI values
Amyloglucosidase from Aspergillus niger	3.6
Ferritin from horse	4.2, 4.3, 4.5
Bovine serum albumin	4.8
β-Lactoglobulin from bovine milk	5.1
Conalbumin	5.9
Myoglobin from horse	6.8, 7.2
Myoglobin from whale	7.7, 8.2
LDH from rabbit muscle	8.6
Ribonuclease	9.5
Cytochrome *c*	10.7

amphoteric electrolytes, which flatten the pH gradient near their isoelectric points, and so increase the resolution in the pH range. Separators are, for example, tetraglycine (pI = 5.2), proline (pI = 6.3), threonine (pI = 6.5), β-alanine (pI = 6.9), 5-aminovaleric acid (pI = 7.5), 7-aminocapryl acid, histidine (pI = 7.6), and 6-aminocaproic acid (pI = 8.0). With the help of 0.33 mol/l β-alanine in a pH gradient of 6–8, the band of the glycated HbA_{1c} was separated from the band of the closely adjacent HbA, the major hemoglobin band [29].

Carrier ampholytes IEF can be run on polyacrylamide or agarose gels.

2.11.2.3 IEF with carrier ampholytes on polyacrylamide gels

Carrier **a**mpholytes **IEF** (CA-IEF) is carried out usually on polyacrylamide gels: at T = 4–5 g/dl and C = 0.03 for electrofocusing of proteins; and at T = 10 g/dl and C = 0.025 for electrofocusing of oligopeptides. In polyacrylamide gels, very high field strengths can be used because they have no polar groups and as a result no electroosmotic effects. However, the polyacrylamide gels are not suitable for separation of macromolecules with molecular masses more than 500,000. These polyions can be focused only on agarose gels.

Thin and ultrathin polyacrylamide gels for IEF with carrier ampholytes

A problem of IEF is the Joule heating, which is created during the electrophoresis. This problem was solved after the gel thickness was reduced and thin (thickness of 0.5 mm) [30,31] and ultrathin (thickness less than 0.5 mm) [32,33] slab gels were produced on pretreated plastic sheets (support films) (Table 2.11-2). Because of their higher surface-to-volume ratio, the thin polyacrylamide or agarose gels dissipate

Table 2.11-2: Recipes for casting of polyacrylamide gels for isoelectric focusing with carrier ampholytes. The internal dimensions of the casting cassette are 120×250×0.5mm.

Components	Concentration	Cartridge technique 16 ml mixture Gel thickness 0.5 mm	Clams technique 6 ml mixture Gel thickness 0.15 mm
For water-soluble proteins			
Monomer solution (T = 50 g/dl, C = 0.03)	10.0 ml	1.60 ml	0.60 ml
40 g/dl carrier ampholytes	5.0 ml	0.80 ml	0.30 ml
87% Glycerol	20.0 ml	3.20 ml	1.20 ml
10 g/dl TMEDA	0.5 ml	0.08 ml	0.03 ml
10 g/dl APS	0.5 ml	0.08 ml	0.03 ml
Deionized water to	100.0 ml	16.00 ml	6.00 ml
For water-insoluble proteins			
Monomer solution (T = 50 g/dl, C = 0.03)	10.0 ml	1.60 ml	0.60 ml
40 g/dl carrier ampholytes	5.0 ml	0.80 ml	0.30 ml
Urea	48.1 g	7.69 g	2.88 g
87% Glycerol	20.0 ml	3.20 ml	1.20 ml
10 g/dl TMEDA	0.5 ml	0.08 ml	0.03 ml
10 g/dl APS	0.5 ml	0.08 ml	0.03 ml
Deionized water to	100.0 ml	16.00 ml	6.00 ml

the Joule heating better than the thick ones and as a result can be subjected to higher voltage. This leads to faster and sharper resolving. Moreover, the sample volume is reduced, and the times for staining, destaining, and drying of the gel are shorter.

Rehydratable polyacrylamide gels for IEF with carrier ampholytes

The polymerization of a monomer solution in the presence of carrier ampholytes requires higher concentrations of APS. In addition, the carrier ampholytes interfere with the adhesion of the gel to the support film: When a gel is polymerized together with carrier ampholytes, it dissolves partially or completely from the support film in an acidic solution, for example, of TCA. Nonionic detergents, which are often added to the monomer solution to increase the solubility of hydrophobic proteins, also inhibit the adhesion between the gel and film. To eliminate the APS and the

monomer residues, and to improve the adhesion of the gel to the support film, rehydratable gels are used [34,35].

The rehydratable gels polymerize without addition of carrier ampholytes, and then are washed with deionized water and dried. Before IEF they are swelled (rehydrated) in 2 g/dl carrier ampholytes with 20–30 ml/dl glycerol to their original thickness.

The rehydratable gels have the following advantages against the wet gels: They do not contain more catalysts (TMEDA and APS) and monomer (acrylamide and BIS) residues. The APS ions and acrylic acid affect negatively the pH gradient, because they cause electroosmosis and as a result iso-pH lines during electrofocusing. When carrier ampholytes, eventually Triton X-100, Nonidet NP-40, or zwitterionic detergents, such as CHAPS, are added later to the dried polyacrylamide gel, the adhesion between the gel and the support film remains strong. The rehydratable gels can be stored indefinitely at −20 °C, too.

However, the rehydratable gels also have some disadvantages: Their production (washing and drying) and their rehydrating take time. Besides, they free water on their surface during electrofocusing.

The rehydration (swelling) time for 0.5 mm rehydratable gels comes generally to two hours. In the presence of urea and nonionic or zwitterionic detergents it should be extended to overnight (Table 2.11-3).

Table 2.11-3: Rehydration solutions and their application.

Rehydration solutions	Time	Usage
4 g/dl Ampholine, 2 ml/dl Nonidet NP-40	Overnight	Membrane proteins [36], alkaline phosphatase [37]
8 mol/l urea, 0.5 g/dl Ampholine, 30 ml/dl glycerol	Overnight	Serum VLDL, apolipoprotein E [38]
8 mol/l urea, 0.5 g/dl Ampholine, 50 mmol/l DTT	Overnight	Globins, plasma proteins, proteins in erythrocyte lysates [39]
9 mol/l urea, 0.5 ml/dl Nonidet NP-40	Overnight	Hydrophobic proteins, complex proteins, 2D-electrophoresis [40]

The rehydratable gels should absorb all rehydration solution. If the rehydration solution contains a nonionic detergent, for example, Nonidet NP-40 or Triton X-100 at a concentration of more than 1 ml/dl, the gel surface could become greasy. In this case, the gel surface must be dried with a lint-free filter paper.

2.11.2.4 IEF with carrier ampholytes on agarose gels

The agarose gels are an alternative to the polyacrylamide gels for isoelectric focusing of high molecular mass proteins (with M_r of 100,000 to several millions) [41,42].

The IEF is carried out usually in 0.8–1.0 g/dl agarose gels, which contain additionally 2.0 g/dl carrier ampholytes and 10 g/dl sorbitol or 30 g/dl glycerol. They have important advantages over the polyacrylamide gels: The agarose gels are non-toxic and their manufacturing is a simple process that does not require catalysts. They have large pores, and show high stability at low concentrations. By addition of linear polyacrylamide, rehydratable agarose gels can also be produced [43].

Despite these advantages, the agarose gels have disadvantages that limit their usage as a separation medium for isoelectric focusing: First of all, the charged groups in the agarose gels cause electroosmotic flow. To avoid this, the agaropectin residues, which carry electric charges, are removed [44,45]. Nevertheless, the agarose gels contain still charged carboxyl and sulfate groups. In addition, urea-agarose gels are produced difficult because urea influences the normal formation of the gel-helix structure. Thin [46,47] and ultrathin [48] agarose gels win, similarly to polyacrylamide gels, more and more popularity.

2.11.2.5 Sample preparation and application

The proteins in the samples should be completely dissolved and have a concentration at which the pherogram bands will be visible. For Coomassie Brilliant Blue R-250 staining, it should be sufficient, if the total protein concentration in the sample is about 0.1 g/dl (1 mg/ml); for silver staining about 0.01 g/dl (0.1 mg/ml). The application volume of a sample should be between 5 and 10 µl. Highly diluted protein solutions can be concentrated by ultrafiltration.

The hydrophobic proteins, such as membrane proteins, should not be resolved with the help of ionic detergents, such as SDS, but with the help of 8–9 mol/l of urea. If the proteins are still unresolved, nonionic detergents (Nonidet NP-40, Triton X-100) at concentrations between 0.5 and 2 ml/dl or 1–2 g/dl of zwitterionic detergents (CHAPS) come into account [49,50]. Some proteins may require as high as 4 g/dl of detergent for their solubility. Urea and detergents destroy the quaternary structure of the proteins and unfold the polypeptide chains. The disulfide bonds between the polypeptide chains are broken when 1,4-dithiothreitol is added.

The ionic strength (a function of the salt concentration) of the sample should be reduced to a minimum, since when the ionic strength is higher than that in the gel, local gradient drifts appear in the gel. As a critical upper limit of the salt concentration in a protein sample, 20 mmol/l was specified [51], but it is better to be decreased to 5 mmol/l. If necessary, the samples can be desalted by dialysis, gel filtration, or ultrafiltration.

The sample can be applied onto the gel surface in different ways: as drops into template slots; into slots in the gel; onto application platelets, and so on (Figure 2.11-4).

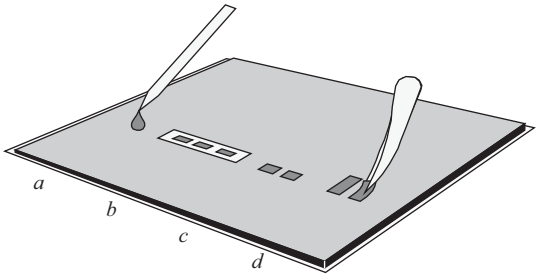


Figure 2.11-4: Sample application in IEF.
a) As drops onto the gel surface; *b*) Into template slots; *c*) Into slots in the gel; *d*) Onto application platelets

Among all these methods, the application of samples into template slots is the most convenient technique. The template must be removed 10 mm after the application. If residual sample is still on the gel, it should be blotted with blotting paper.

2.11.2.6 Electrode solutions

The contact between gel and electrodes is realized *via* electrode solutions whose pH values are higher and lower than the minimum and maximum pI points of the carrier ampholytes. The electrode solutions are dropped onto 5–7 mm wide strips of thick filter paper, placed on a glass plate. The excess of the electrode solutions is blotted and they are put onto the gel.

The anode strip contains acidic solution; the cathode strip contains basic solution. As catholyte, a mixture of 20 mmol/l arginine and 20 mmol/l lysine (0.34 g/dl arginine and 0.36 g/dl lysine) is recommended; as anolyte 70 mmol/l H_3PO_4 is recommended, but it can be substituted by a mixture of 20 mmol/l aspartic acid and 20 mmol/l glutamic acid (0.26 g/dl aspartic acid and 0.29 g/dl glutamic acid). Additionally, the electrode solutions may contain 30–40 ml/dl glycerol, which prevents the drying of electrode strips (Table 2.11-4).

2.11.2.7 Running isoelectric focusing with carrier ampholytes

There is almost no difference between the isoelectric focusing on polyacrylamide and agarose gels (Table 2.11-5). It is important that prior to isoelectric focusing, the surface of agarose gel is dried with a filter paper to remove the superficial liquid film. A prefocusing on agarose gels should be avoided because here the cathode drift is much stronger than in polyacrylamide gels.

The constant power is the most comfortable setting for isoelectric focusing. At the beginning of electrofocussing, the carrier ampholytes wear high effective charges, so the gel has a high conductivity. With the approach to their isoelectric points and formation of a pH gradient, they lose their charges and as a result their

Table 2.11-4: Electrode solutions for isoelectric focusing with carrier ampholytes on polyacrylamide and agarose gels.

	pH gradient	Anode solution	Cathode solution
Polyacrylamide gel	3–10	0.5 mol/l H_3PO_4 0.25 mol/l acetic acid 0.025 mol/l aspartic acid, 0.025 mol/l glutamic acid	0.5 mol/l NaOH 0.25 mol/l NaOH 0.025 mol/l lysine, 0.025 mol/l arginine
	5–8	0.04 mol/l glutamic acid	0.25 mol/l NaOH
	3–7	0.04 mol/l glutamic acid	0.2 mol/l histidine
	6–8	0.25 mol/l HEPES	0.2 mol/l histidine
Agarose gel	3–10	0.5 mol/l acetic acid	0.5 mol/l NaOH

Table 2.11-5: Electrophoretic conditions for isoelectric focusing on polyacrylamide and agarose gels containing 2 g/dl carrier ampholytes at 10 °C and at a separation distance of 10 cm.

pH interval	Time	Power	Voltage	Electric current
Polyacrylamide gels				
Prefocusing				
pH = 3.0–10.0	60 min	So high that the voltage reaches 300 V.	Maximum	Maximum
Narrow pH gradients (2 pH units)	90 min	So high that the voltage reaches 300 V.	Maximum	Maximum
Focusing				
pH = 3.0–10.0	10 min	As the prefocusing.	200 V	Maximum
	120 min	Increase the power by a factor of 2.5.	Maximum	Maximum
Narrow pH gradients (2 pH units)	15 min	As the prefocusing.	200 V	Maximum
	180 min	Increase the power by a factor of 2.5.	Maximum	Maximum
Agarose gels				
Focusing				
pH = 3.0–10.0	30 min	So high that the voltage reaches 500 V.	Maximum	Maximum
Narrow pH gradients (2 pH units)	60 min	So high that the voltage reaches 500 V.	Maximum	Maximum

mobilities. Therefore, the conductivity of the carrier ampholytes lowers, which causes the voltage to increase. Thus, a typical current-voltage relationship takes place: the electric current falls, the voltage increases, and the electric power remains constant (Figure 2.11-5).

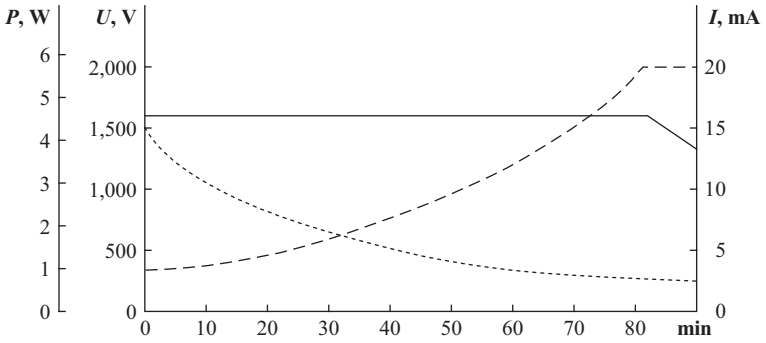


Figure 2.11-5: Electric power (*P*), voltage (*U*), and electric current (*I*) during isoelectric focusing in a pH gradient of 3–10 obtained with carrier ampholytes in a polyacrylamide gel with dimensions of 120 × 250 × 0.25 mm, and a run at 10 °C for 90 min. The limit of the electric power is 5 W, of the voltage is 2,000 V, and of the electric current is 20 mA.
— *P*, --- *U*, · · · *I*

Electric fields used in IEF are of the order of 100 V/cm. During focusing, the electric current drops to nearly zero since the carrier ampholytes stop moving. The isoelectric focusing in a standard gel (120 × 250 × 0.5 mm) is carried out at 10 °C and at a constant power of 8–12 W for about 3 h and a limiting voltage of 2,000–3,000 V. In the presence of 8–9 mol/l urea, the temperature should not be below 15 °C to avoid the crystallization of urea.

2.11.2.8 Staining of separated protein bands

After isoelectric focusing, the gels are stained usually with Coomassie brilliant blue R-250, Serva violet 17, or silver (s. there). In agarose gels, the silver staining is not very successful because of the electroosmosis.

2.11.3 Isoelectric focusing in immobilized pH gradients

Gasparic, Bjellqvist, and Rosengren introduced the **i**mmobilized **p**H **g**radients (IPG) [52]. First they were produced by LKB Produkter AB and offered under the trade name *Immobiline*. The immobilines are acrylamido buffers that are copolymerized by their vinyl groups into the polyacrylamide matrix to form IPG gels [53,54] (Figure 2.11-6).

The distribution of immobilines in polyacrylamide gels takes place during gel casting. Linear pH gradients are prepared using a gradient mixer and two mixtures of acrylamido buffers: one basic and one acidic. Ultra-narrow pH gradients (up to 0.01 pH/cm) can also be produced. They have resolution up to 0.002 pH units [55,56].

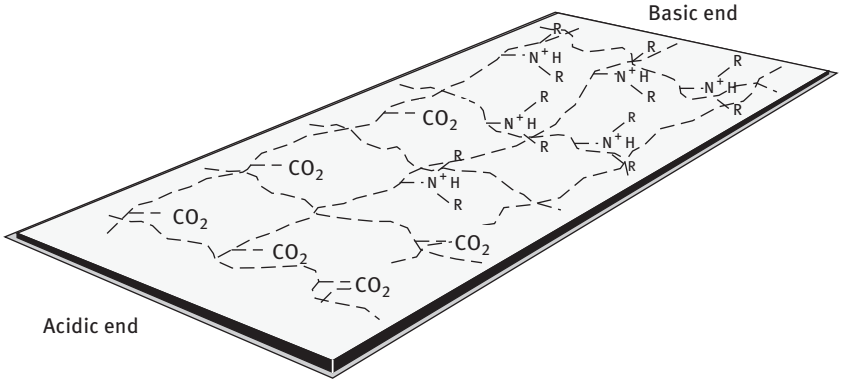


Figure 2.11-6: Polyacrylamide gel copolymerized with immobilines.

The immobilized pH gradients have several advantages over the pH gradients obtained with carrier ampholytes:

- The IPG are stable pH gradients, which do not deform by any external influences (electroosmosis, high ionic strength of the samples, or protein overloads). Therefore, they do no form warped iso-pH lines.
- The immobilized pH gradients have a uniform buffering capacity and conductivity along the gel. This cannot be guaranteed by the carrier ampholytes pH gradients, because not all of their components are present in same concentration.
- The IPG gels have a low conductivity, as the immobilines cannot move freely. As a result, little Joule heating is developed during IEF, even at very high voltages.
- In the IPG gels, the cathode drift is excluded, which is available in IEF with carrier ampholytes. This is especially important for IEF in narrow pH gradients where the cathode drift is noticeable.
- The resolution of IEF in IPG is extremely high. It exceeds the resolution of IEF with carrier ampholytes to 10–20 times and allows to resolve proteins, which differ from each other by pI values of only 0.001–0.002 pH units [57]. While the resolution of IEF with carrier ampholytes is more than 0.02 pH units/cm, the resolution of IEF in IPG gels may reach 0.001 pH units/cm.

The isoelectric focusing in IPG gels, however, has some disadvantages:

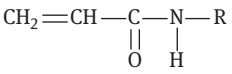
- The preparation of IPG is a complex process.
- The IPG gels can cause protein adsorption.
- During IEF, very high voltage is required.
- The electrophoretic separation takes too long time.

Isoelectric focusing in immobilized pH gradients containing carrier ampholytes is also known. It is referred to as hybrid IEF [58,59] or **IPG**-**c**arrier **a**mpholytes (IPG-CA). In this method, the carrier ampholytes reduce the tendency of protein polyions to be adsorbed by the immobilized pH gradients [60,61].

Denaturing isoelectric focusing in immobilized pH gradient is an isoelectric focusing in immobilized pH gradients that is run in urea-containing polyacrylamide gels.

2.11.3.1 Properties of immobilines

The immobilines are low molecular mass acrylamide derivatives, which carry buffering groups. Their general formula is



where the residue R is either an acidic group (carboxyl group) or a basic group (tertiary amino group). The immobilines take part in the copolymerization of acrylamide and BIS by their methylene group.

The immobilines are supplied in concentrations of 0.2 mol/l. They polymerize and hydrolyze spontaneously [62], so it is recommended [63] that 0.005 mg/ml hydroquinone monomethyl is added to the acidic immobilines (with pK = 1.0, 3.6, 4.4, and 4.6), and the predominantly basic immobilines (with pK = 6.2, 7.0, 8.5, and 9.3) to be dissolved in n-propanol. Additional acrylamido buffers, in particular basic acrylamido buffer with pK of 10.3, and > 12.0, as well as a strongly acidic sulfonic acid derivative and a strongly basic quaternary amine derivative, also came into use. The immobiline solutions can be stored for months and years at 4 °C. They should not be frozen.

The structures of the acrylamido buffers used for preparing immobilized pH gradients are shown in Table 2.11-6.

The preparation of immobilized pH gradients represents an acid-base titration between two immobiline solutions, which have same concentration of acrylamide and BIS. The resulting pH value at each point of the pH gradients can be calculated by the Henderson–Hasselbalch equation. If the immobiline is an acid, the equation

$$\mathrm{pH} = \mathrm{p}K_c + \log \frac{c_B}{c_{HA} - c_B} \tag{2.11-5}$$

can be used; if it is a base, the equation

$$\mathrm{pH} = \mathrm{p}K_c + \log \frac{c_B - c_{HA}}{c_{HA}} \tag{2.11-6}$$

can be used, where $\mathrm{p}K_c$ is the dissociation constant of the buffering immobiline group, and c_{HA} and c_B are the molar concentrations of the acidic or basic immobiline.

Table 2.11-6: Structures of the acrylamido buffers used for preparing immobilized pH gradients.

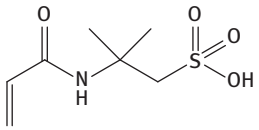
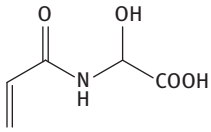
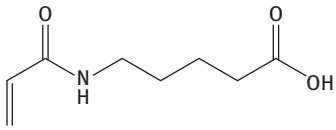
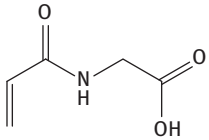
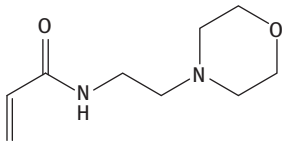
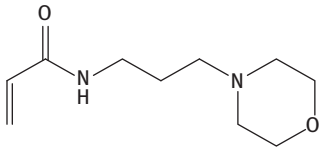
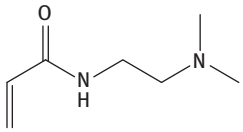
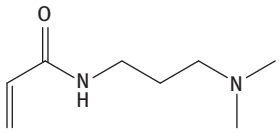
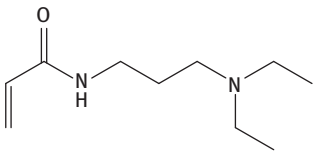
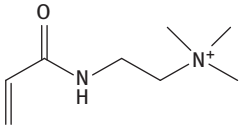
pH	Chemical formula	Name	M_r
Acidic acrylamido buffers			
1.2		2-Acrylamido-2-methylpropane sulfonic acid	207
3.1		2-Acrylamido-glycolic acid	145
3.6		N-Acryloil-glycine	129
4.6		4-Acrylamido-butyric acid	157
Basic acrylamido buffers			
6.2		2-Morpholino propylacrylamide	184
7.0		3-Morpholino propylacrylamide	199
8.5		N,N-Dimethyl aminoethyl acrylamide	142
9.3		N,N-Dimethyl aminopropyl acrylamide	156

Table 2.11-6 (continued)

pH	Chemical formula	Name	M_r
10.3		N,N-Diethyl aminopropyl acrylamide	184
> 12		N,N,N-Triethyl aminoethyl acrylamide	198

2.11.3.2 Casting IPG gels

The IPG gels are cast as other gradient gels using a gradient mixer [64], wherein the heavy (with 25 ml/dl glycerol) and light (with 5 ml/dl glycerol) solutions contain different immobilines in different concentrations. The heavy solution is acidic and builds the lower part of the immobilized pH gradient; the light solution is basic and builds the upper part of the pH gradient. The mixing chamber is filled with the acidic solution; the reservoir is filled with the basic solution. For IEF of proteins, gels with T = 10 g/dl and C = 0.025 are recommended; for IEF of oligopeptides, gels with T = 4 g/dl and C = 0.03 are recommended.

The casting cassette is identical to the common casting cassette described above. The volume of a standard casting cassette (with interior dimensions of 120 × 250 × 0.5 mm) is about 15 ml. Therefore, the mixing chamber of a gradient mixer is filled with 8 ml of the heavy solution, and its reservoir is filled with 8 ml of the light solution.

After the two immobiline solutions are prepared, the acidic solution is titrated with TMEDA, and the basic solution is titrated with HCl to pH = 7.0. The pH-value of 7.0 ensures a uniform copolymerization of the monomers and immobilines. The required pH = 7.0 can also be produced by a 0.125 mol/l TRIS-hydrogen phosphate buffer with pH = 7.0. After the polymerization, HCl, TMEDA, or TRIS-hydrogen phosphate buffer are washed out and the gel is dried (s. below).

An example of preparing a monomer-immobiline solution is shown in Table 2.11-7. In the practice, thin (0.5 mm) as well ultrathin (<0.5 mm) IPG gels are used [65].

Rehydratable IPG gels

After casting, an IPG gel can be used immediately or dried. Before drying, the gel must be washed 3 times for 20 min each in deionized water and once in 2–3 ml/dl

Table 2.11-7: Preparation of solutions for a 120 × 250 × 0.5 mm IPG gel (T = 4 g/dl, C = 0.03) with a pH gradient 4–10, 4–7, 4–9, and 6–10.

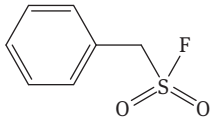
Linear pH gradient	**pH = 4–10**		**pH = 4–7**		**pH = 4–9**		**pH = 6–10**	
	Acidic solution pH = 4.0	**Basic solution pH = 10.0**	**Acidic solution pH = 4**	**Basic solution pH = 7**	**Acidic solution pH = 4**	**Basic solution pH = 9**	**Acidic solution pH = 6**	**Basic solution pH = 10**
Immobiline pK = 3.6	588 µl	–	308 µl	161 µl	442 µl	78 µl	502 µl	53 µl
Immobiline pK = 4.6	–	61 µl	59 µl	394 µl	125 µl	226 µl	–	–
Immobiline pK = 6.2	243 µl	27 µl	240 µl	81 µl	124 µl	192 µl	146 µl	178 µl
Immobiline pK = 7.0	47 µl	260 µl	–	143 µl	12 µl	158 µl	130 µl	193 µl
Immobiline pK = 8.5	178 µl	84 µl	–	–	133 µl	38 µl	139 µl	127 µl
Immobiline pK = 9.3	–	190 µl	–	467 µl	118 µl	354 µl	150 µl	174 µl
Acrylamide/BIS (28.8/1.2)	1.07 ml	1.07 ml	1.07 ml	1.07 ml	1.07 ml	1.07 ml	1.07 ml	1.07 ml
87% Glycerol	2.0 ml	0.4 ml	2.0 ml	0.4 ml	2.0 ml	0.4 ml	2.0 ml	0.4 ml
10 g/dl TMEDA	80 µl	80 µl	80 µl	80 µl	80 µl	80 µl	80 µl	80 µl
10 g/dl APS	80 µl	80 µl	80 µl	80 µl	80 µl	80 µl	80 µl	80 µl
Deionized water to	8.0 ml	8.0 ml	8.0 ml	8.0 ml	8.0 ml	8.0 ml	8.0 ml	8.0 ml

glycerol for 20 min. Then it is dried under a hair dryer. The dried gel can be sealed in a plastic wrap and stored in the refrigerator at 4–8 °C for several days or in the freezer at –20 °C indefinitely. The dried gel, which is referred to as a *rehydratable gel*, can be rehydrated (reconstituted) later with suitable solutions.

2.11.3.3 Running isoelectric focusing in IPG gels

The sample preparation for isoelectric focusing in IPG gels does not differ from that for IEF with carrier ampholytes. Bjellquist *et al.* [66] suggest that the salt concentration at IEF in IPG gels does not exceed 100 mmol/l, which is 5 times higher than the critical salt concentration at IEF with carrier ampholytes. To remove salts out, IPG strips were subjected to a low voltage for several hours or the electrode paper wicks were changed periodically during IEF [67]. It was proved [68] that, if a rehydrated IPG strip is washed three times 10 min each, the contaminating salts are removed from the gel.

When cell lysates are prepared, 8 mmol/l of PMSF (**p**henyl**m**ethyl**s**ulfonyl**f**luoride) as a protease inhibitor [69] is recommended to be added.



PMSF

The sample application on IPG gels does not differ from the sample application on gels with carrier ampholytes. For this purpose silicone application templates are comfortable. However, sample precipitation occurs more often on immobilized pH gradient gels than on gels with carrier ampholytes. This can be avoided, if hybrid isoelectric focusing (s. above) is performed. In this case, samples should contain carrier ampholytes, too [70].

As electrode solutions for IEF in IPG gels, also for hybrid IEF, 0.1 g/dl NaOH or 0.01 mol/l lysine (as a cathode solution), and 0.1 g/dl H_3PO_4 or 0.01 mol/l glutamic acid (as an anode solution) are used.

The immobilized pH gradient IEF is carried out in the following way: The IEF in IPG gels should be performed at 10 °C. The gel end with the low pH value should be connected to the anode, whereas the gel end with the high pH value should be connected to the cathode. If the gel contains urea, the separation temperature should be 15 °C, since urea crystallizes at lower temperatures. The IPG gels do not require prefocusing, because the pH gradient is preformed.

The isoelectric focusing itself proceeds in two phases: During the *first phase*, the proteins migrate at a constant voltage into the gel. The electric power and the electric current are set at the maximum values, but the voltage is set on 120–300 V,

so that the field strength is as low as possible (up to 40 V/cm). If the initial field strength is too high, the proteins may precipitate partially. Since the buffering groups of immobilized pH gradients are anchored in the gel and do not move freely, the IPG gels have low conductivity and the electric current strength is only 1–2 mA (Figure 2.11-7). This phase continues 60 min.

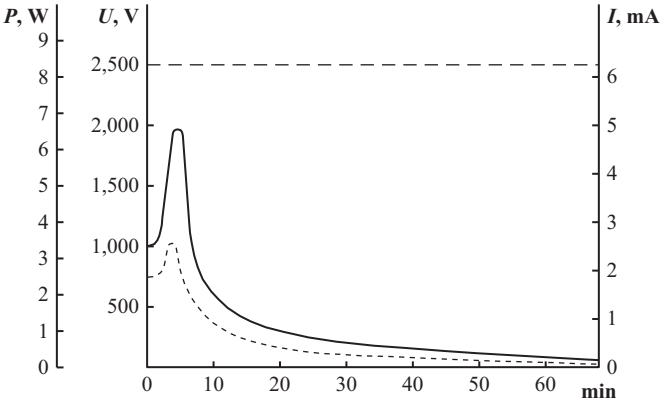


Figure 2.11-7: Electric power (*P*), voltage (*U*), and electric current (*I*) during the first 60 min of IEF in a narrow immobilized pH gradient (pH = 4.5–4.8).
— *P*, –– *U*, · · · *I*

In the *second phase* of IEF, the voltage is set on the maximum value of 5,000 V (2,000 V at hybrid IEF), the power is set on the maximum value of 10 W, and the electric current reaches usually 15 mA. The electric resistance in the IPG gels is 50–100 times higher than that in gels for IEF with carrier ampholytes. Therefore, the voltage during the isoelectric focusing in IPG gels should be higher and IEF should continue longer.

The electrofocusing time is reciprocally proportional to the pH units, which are contained in a pH gradient: 3–4 h for broad pH gradients (4–7 pH units), 8 h for narrow pH gradients (2 pH units), and 16 h (overnight) for ultra-narrow pH gradients (one pH unit or less). After one third of the electrofocussing is run, the application template should be removed and the fluid on the application site should be blotted with a filter paper.

When rehydratable gels are used, the immobilized pH gradient IEF is carried out in the following steps:

– The IPG gel is removed from the freezer and left for a while at room temperature. Then it is rehydrated in a rehydration cassette until it reaches its original thickness.

- A few drops of kerosene (boiling point 70 °C) or another low volatile alkane is pipetted onto the cooling plate of an electrophoresis unit and the gel is placed on with the support film down to form an air-free layer with the cooling plate. This guarantees a uniform heat exchange.
- The solution-soaked electrode strips are placed on the cathodic and anodic end of the gel.
- The samples are applied.
- The electrophoresis unit is covered with its lid and the power supply is switched on.

On a IPG gel, the pH gradient cannot be measured with a contact electrode because the gel conductivity is extremely low. The pH gradient is determined usually with pI markers.

After electrofocusing, the proteins in an IPG gel can be stained with conventional or colloidal Coomassie brilliant blue in phosphoric or sulfuric acid. The solutions used for the colloidal staining are almost odorless and the background of the gel remains clear. If higher sensitivity is desired, the IPG gels can be stained with silver.

After the isoelectric focusing, the gels can be blotted. Qualified blotting can be performed, if the IPG gels are net-supported.

In the last years, an IEF apparatus operating at an unprecedented 12,000 V maximum voltage was described, enabling to run high speed isoelectric focusing of proteins within three hours [71]. The IEF-100 is a microprocessor controlled IEF apparatus which can be outputted by computer interface. In its standard configuration, up to six IPG strips can be run.

2.11.4 Isoelectric focusing by living cells

According to some opinions [72,73], living eukaryotic cells also perform isoelectric focusing of proteins in their interior. They can modify, for example, the isoelectric point of a particular enzyme by phosphorylation or dephosphorylation, and can transfer their substrates between different parts of the cell. So the rate of particular biochemical pathways is increased by several orders of magnitude, which can switch on or switch off particular biochemical processes.

2.11.5 Electric field gradient focusing

Simmilar to IEF is the **e**lectric **f**ield **g**radient **f**ocusing (EFGF) (known also as *electromobility focusing*). It uses an electric field gradient and a hydrodynamic counter flow to separate and focus charged analytes in a channel.

Previously, most EFGF devices were designed to form a linear field gradient in the channel. However, the peak capacity obtained using a linear gradient is not better than conventional capillary electrophoresis. Improvement of peak capacity in EFGF can be achieved by using a nonlinear gradient.

Koegler and Ivory [74] first demonstrated a design in which a curved electric field was produced by a continuously narrowing flow channel. The design included an array of electrodes, separated from the separation channel by a dialysis membrane, and producing an electric field, which could be shaped at will [75]. Because of the capacity to change the electric field during a run, this version of the technique was dubbed **d**ynamic **f**ield **g**radient **f**ocusing (DFGF). The analyte focus can be moved up and down, and eluted from the chamber as tightly focused bands.

Next, Greenlee and Ivory [76] demonstrated an apparatus where the gradient was formed by a flat dialysis membrane sandwiched between two channels. Later an EFGF device with convex bilinear gradient was fabricated from **p**oly(**e**thylene **g**lycol) (PEG)-functionalized acrylic copolymers [77]. By increasing the flow rate, stacked proteins that were not resolved after focusing in the steep gradient segment moved into the shallow gradient segment, where the analyte peak resolution increased significantly.

Electric field gradient focusing was used to separate the two oxidation forms of myoglobin [78].

2.11.6 Applications of isoelectric focusing

Isoelectric focusing has many aplications. Using it, a titration curve analysis can be made. Isoelectric focusing is used also in the clinical laboratory.

2.11.6.1 Titration curve analysis

Focusing gels with carrier ampholytes can be used for determination of protein titration curves. So information about the isoelectric point of a protein as well as its mobility at different pH values can be obtained.

The titration curve analysis [79] is performed as follows: In a square slab gel with carrier ampholytes and a narrow groove in the center, isoelectric focusing is run along the groove until a pH gradient is formed. Then, the electrode strips are removed and the gel is rotated at 90°. A protein solution is pipetted into the narrow groove and electrophoresis is run perpendicularly to the pH gradient at voltage of 600 V for 10 to 45 min. During the electrophoresis, the carrier ampholytes remain in place, as they have no net charges at their pI values. However, the protein polyions migrate with different mobilities, depending on the local pH value of the pH gradient, and form titration curves [80,81] (Figure 2.11-8).

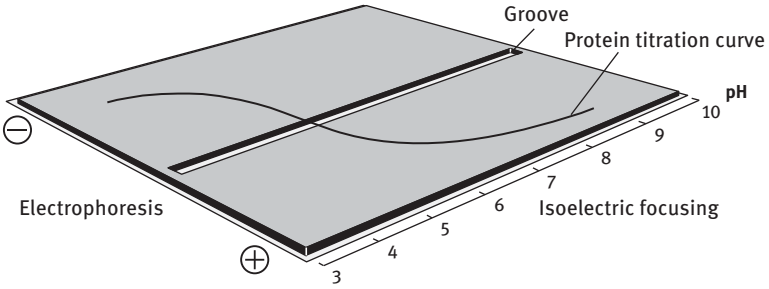


Figure 2.11-8: Titration curve analysis.

The obtained titration curve is similar to the classic titration curve. The pI value of the protein is located on the groove where it cuts off the titration curve.

2.11.6.2 Isoelectric focusing in the clinical laboratory

With the help of isoelectric focusing on polyacrylamide gels more than 300 hemoglobin variants [82,83,84], lipoproteins [85,86,87], phenotypes of α_1-antithrypsin [88,89], urinary proteins [90,91], globulins [92,93], salivary proteins [94,95], catalase [96], and many other clinically important proteins were analyzed. Besides, genetic investigations in forensic medicine [97] were performed. In addition, meat proteins [98,99], potato varieties [100,101], and wheat proteins [102] were studied.

2.11.7 Protocols

2.11.7.1 Casting gels for isoelectric focusing with carrier ampholytes

Materials and equipment

Acrylamide
BIS
TMEDA
APS
40 g/dl Carrier ampholytes
87% Glycerol
0.1 g/dl Riboflavin-5′-phosphate (FMN)
Gel cassette
Fluorescent lamp

Acrylamide/BIS stock solution (T = 30 g/dl, C = 0.03)

Acrylamide	29.1 g
Bisacrylamide	0.9 g
Deionized water to	100.0 ml

Monomer solution

Acrylamide/BIS stock solution	16.7 ml
40 g/dl Carrier ampholytes	5.0 ml
87% Glycerol	10.0 ml
0.1 g/dl FMN	0.5 ml
10 g/dl TMEDA	0.4 ml
10 g/dl APS	0.3 ml
Deionized water to	100.0 ml

Procedure

- Transfer the monomer solution into the casting cassette.
- Illuminate the monomer solution in the cassette with a fluorescent lamp for one hour.
- Open the gel cassette and illuminate once more the gel face with the fluorescent lamp for 30 min.
- Use the gel immediately or cover it with a polyethylene sheet and store at 4 °C for several days.

2.11.7.2 Running isoelectric focusing with carrier ampholytes

The isoelectric focusing with carrier ampholytes in film-supported gels is carried out in the following steps:

- Pipette 0.5–1.0 ml of kerosene, silicone oil DC 200 or other less volatile alkanes onto the cooling plate of an electrophoretic unit.
- Place a gel with its support film down, so that no air bubbles are formed between the film and cooling plate.
- Place the electrode strips with the anode and cathode solutions on both ends of the gel.
- Placed an application template onto the gel centrum, and press it gently to provide a good contact.
- Prefocuse the gel, so that a pH gradient is formed, and the unwanted ions, for example, APS and TMEDA ions, migrate toward the electrode strips. The prefocusing is carried out at 400–500–1,000 V per 10 cm gel for 30–60 min.

  Gels with higher glycerol concentration, and the rehydrated gels do not require prefocusing.
- After the prefocusing, place an application template onto the gel.
- Apply in the template slots 5–10 µl of desalted samples containing 0.1–0.5 mg/ml proteins (Figure 2.11-9).
- Increase gradually the field strength from 50–100 V/cm to 300–500 V/cm, depending on the pH range, concentration of the carrier ampholytes, gel thickness, glycerol concentration, and cooling temperature. For most proteins, 5,000 to 20,000 Vh per 10 cm gel are used.

- Interrupt the isoelectric focusing after the half of electrofocusing time is over.
- Remove the application template, and blot the excess solution on the electrode strips with filter paper.
- Continue with the electrofocusing.

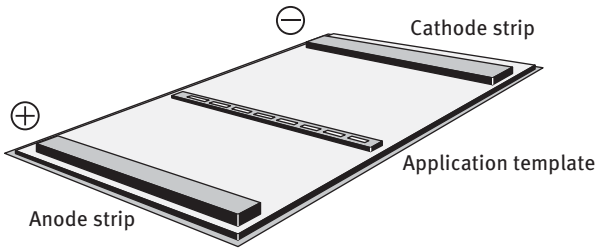


Figure 2.11-9: Gel with carrier ampholytes, paper electrode strips, and an application template.

The isoelectric focusing with carrier ampholytes in net-supported gels is carried out in the following steps:

- Place the net- supported gel onto the cooled to 15 °C cooling plate of an electrophoretic unit.
- Place a gel with its support net down, so that no air bubbles are formed between the net and cooling plate.
- Place the electrode strips with the anode and cathode solutions on both ends of the gel.
- Place an application template onto the gel centrum, and press it gently to provide a good contact.
- Fill the slots of the application template with 5–10 µl of the samples each.
- Start the isoelectric focusing at 200 V (for gels with a separation distance of 10 cm).
  Because of the better heat dissipation, net-supported gels tolerate higher field strengths than film-supported gels.
- Increase every 30 min the electric voltage by 400 V to 1,000 V until a maximum voltage of 3,000 V is reached.
  In net-gels with high glycerol concentration, the voltage can reach 6,000 V.
- Continue the electric focusing until the desired Vh product.

2.11.7.3 Isoelectric focusing of strong acidic proteins

Materials and equipment

Acrylamide/BIS solution
Ampholytes, pH = 2.5 to 4

Ampholytes, pH = 2 to 11
Sodium hydroxide
Sulfuric acid
Urea
NP-40
Paper electrode strips
Application template
Electrofocusing unit
Power supply

Monomer solution

Acrylamide	6.00 g
BIS	0.36 g
Ampholytes, pH = 2.5 to 4	10.00 ml
Ampholytes, pH = 2 to 11	3.00 ml
Urea	82.50 g
Deionized water to	100.00 ml

Procedure

- Place a support film with its hydrophobic side down onto a glass plate, using water as an adhesive agent, and roll the support film with a photo roller to remove the air bubbles between the film and the glass.
- Place a 0.5 mm U-shaped gasket onto the margins of the support film.
- Smear a second glass plate of same dimensions with a repelling solution to make it hydrophobic.
- Place the second glass plate over and fix all parts with clamps to build a cassette with a volume of 15 ml.
- Deaerate 15 ml of the monomer solution by vacuum pump for 2 to 3 min.
- Add 0.3 ml NP-40, 0.1 ml 10 g/dl TMEDA, and 0.07 ml of 10 g/dl APS, and swirl.
- Cast the monomer solution using a syringe or pipette into the casting cassette and overlay with deionized water.
- Allow the solution to polymerize for 60 min.
- Drain off the overlaid water.
- Remove the gel from the casting cassette.
- Add 0.15 ml concentrated sulfuric acid to 100 ml of deionized water and soak an electrode paper strip.
- Add 3 ml ampholyte, pH = 2 to 11, to 100 ml deionized water and soak the another electrode paper strip.
- Place the application template on the gel and pipette 10 to 30 µl protein samples into its slots.
- Prefocus the gel at 200 V for 1 h.
- Run electrofocusing at 800 V for 4 to 5 h or at 250 V for 16 h.
- Continue with the staining of the bands.

2.11.7.4 Isoelectric focusing of strong basic proteins

Materials and equipment

Acrylamide
BIS
Ampholytes, pH = 9.0 to 10.5
Ampholytes, pH = 2 to 11
Sodium hydroxide
Phosphoric acid
Urea
NP-40
Paper electrode strips
Application template
Electrofocusing unit
Power supply

Gel solution

Acrylamide	6.00 g
BIS	0.36 g
Ampholytes, pH = 9 to 10.5	10.00 ml
Ampholytes, pH = 2 to 11	3.00 ml
Urea	82.50 g
Deionized water to	100.00 ml

Procedure

- Place a support film with its hydrophobic side down onto a glass plate, using water as an adhesive agent, and roll the support film with a photo roller to remove the air bubbles between the film and the glass.
- Place a 0.5 mm U-shaped gasket onto the margins of the support film.
- Smear a second glass plate of same dimensions with a repelling solution to make it hydrophobic.
- Place the second glass plate over and fix all parts with clamps to build a cassette with a volume of 15 ml.
- Deaerate 15 ml of the monomer solution by vacuum pump for 2 to 3 min.
- Add 0.3 ml NP-40, 0.1 ml 10 g/dl TMEDA, and 0.07 ml of 10 g/dl APS, and swirl.
- Cast the monomer solution using a syringe or pipette into the cast cassette and overlay with deionized water.
- Allow the solution to polymerize for 60 min.
- Drain off the overlaid water.
- Remove the gel from the casting cassette.
- Soak both electrode strips with 0.02 mol/l NaOH and 0.01 mol/l phosphoric acid, respectively, and place them onto the gel ends.
- Place the application template on the gel and pipette the samples into its slots.

– Prefocus at 400 V for 1 h, and focus at 800 V for 1 h, later at 4,000 V for 4 to 5 h.
– Continue with the staining of the bands.

2.11.7.5 Casting immobiline gels

IPG slab gels with linear gradients of pH = 4–7, 4–9, or 6–10 are cast according to the recipes of Righetti [103].

In Tables 2.11-8 and 2.11-9 recipes are given for producing diverse immobilized pH gradients.

Table 2.11-8: Volumes of the acidic (heavy) and basic (light) immobiline solution, which are used for casting of 15 ml gels (120 × 250 × 0.5 mm) with wide pH gradients (2–6 pH units) [104,105]. Both solutions contain monomers in same concentration but the heavy solution contains more glycerol.

Wide pH ranges												
Acidic (heavy) solution (0.2 mol/l immobilines with diverse p*K*), in µl							Basic (light) solution (0.2 mol/l immobilines with diverse p*K*), in µl					
3.6	**4.6**	**6.2**	**7.0**	**8.5**	**9.3**	**pH range**	**3.6**	**4.6**	**6.2**	**7.0**	**8.5**	**9.3**
159	119	84	–	–	–	3.5–5.0	113	165	248	–	–	–
303	53	234	–	–	–	4.0–6.0	208	278	147	–	–	385
221	128	266	–	–	–	4.5–6.5	–	304	130	125	–	158
37	228	221	–	–	–	5.0–7.0	–	253	144	117	–	171
–	240	189	60	–	–	5.5–7.5	185	–	126	153	151	–
232	–	172	111	23	–	6.0–8.0	153	–	93	173	175	–
411	–	147	99	287	–	6.5–8.5	102	–	82	148	193	–
719	–	–	145	198	451	7.0–9.0	258	–	–	124	101	291
356	–	–	237	121	186	7.5–9.5	110	–	–	493	74	185
213	–	–	194	189	50	8.0–10.0	49	–	–	175	195	154
308	59	240	–	–	–	4.0–7.0	161	394	81	143	–	467
374	135	222	71	185	–	5.0–8.0	93	66	70	184	185	–
415	–	214	50	194	43	6.0–9.0	129	–	86	239	126	120
289	–	–	202	187	–	7.0–10.0	48	–	–	173	187	149
314	135	125	62	91	–	4.0–8.0	–	295	192	76	178	154
442	310	116	74	424	65	5.0–9.0	–	133	140	113	156	122
502	–	146	130	139	150	6.0–10.0	53	–	178	193	127	174
442	125	124	12	133	118	4.0–9.0	78	226	192	158	38	354
300	247	159	146	121	68	5.0–10.0	11	31	18	224	165	146
588	–	243	47	178	–	4.0–10.0	–	61	27	260	84	190

Table 2.11-9: Volumes of the acidic (heavy), and basic (light) immobiline solution, which are needed for requiring a 15 ml gel (120 × 250 × 0.5 mm) having a narrow immobilized pH gradients of 1 pH unit in the pH interval from 3.8 to 10.5 [106]. Both solutions contain monomers in same concentration; the heavy solution contains more glycerol.

Narrow pH ranges												
Acidic (heavy) solution (0.2 mol/l immobilines with diverse p*K*), in µl							Basic (light) solution (0.2 mol/l immobilines with diverse p*K*), in µl					
3.6	4.6	6.2	7.0	8.5	9.3	pH range	3.6	4.6	6.2	7.0	8.5	9.3
–	482	–	–	–	69	3.8–4.8	–	195	–	–	–	135
–	436	–	–	–	75	3.9–4.9	–	201	–	–	–	149
–	403	–	–	–	84	4.0–5.0	–	397	–	–	–	311
–	380	–	–	–	94	4.1–5.1	–	428	–	–	–	351
–	367	–	–	–	108	4.2–5.2	–	471	–	–	–	402
–	364	–	–	–	125	4.3–5.3	–	529	–	–	–	465
–	369	–	–	–	147	4.4–5.4	–	604	–	–	–	545
–	382	–	–	–	173	4.5–5.5	–	701	–	–	–	644
300	320	460	–	–	–	4.6–5.6	–	460	460	–	–	56
244	360	460	–	–	–	4.7–5.7	–	460	460	–	–	80
188	400	460	–	–	–	4.8–5.8	–	460	460	–	–	108
116	460	460	–	–	–	4.9–5.9	–	460	460	–	–	132
84	460	460	–	–	–	5.0–6.0	–	460	428	–	–	180
60	460	460	–	–	–	5.1–6.1	–	460	380	–	–	236
667	–	723	–	–	–	5.2–6.2	180	–	386	–	–	–
563	–	621	–	–	–	5.3–6.3	151	–	370	–	–	–
479	–	542	–	–	–	5.4–6.4	129	–	364	–	–	–
413	–	482	–	–	–	5.5–6.5	111	–	366	–	–	–
361	–	436	–	–	–	5.6–6.6	97	–	377	–	–	–
319	–	404	–	–	–	5.7–6.7	86	–	397	–	–	–
286	–	380	–	–	–	5.8–6.8	77	–	428	–	–	–
259	–	367	–	–	–	5.9–6.9	70	–	471	–	–	–
238	–	364	–	–	–	6.0–7.0	64	–	529	–	–	–
222	–	369	–	–	–	6.1–7.1	60	–	604	–	–	–
518	–	–	579	–	–	6.2–7.2	140	–	–	366	–	–
444	–	–	510	–	–	6.3–7.3	119	–	–	364	–	–
385	–	–	457	–	–	6.4–7.4	104	–	–	370	–	–
339	–	–	418	–	–	6.5–7.5	91	–	–	386	–	–
301	–	–	390	–	–	6.6–7.6	81	–	–	411	–	–
271	–	–	373	–	–	6.7–7.7	73	–	–	448	–	–
248	–	–	364	–	–	6.8–7.8	67	–	–	498	–	–
229	–	–	365	–	–	6.9–7.9	62	–	–	564	–	–
215	–	–	374	–	–	7.0–8.0	58	–	–	649	–	–
203	–	–	393	–	–	7.1–8.1	55	–	–	758	–	–
548	–	–	400	400	–	7.2–8.2	292	–	–	400	400	–
524	–	–	400	400	–	7.3–8.3	268	–	–	400	400	–

Table 2.11-9 (continued)

Narrow pH ranges												
Acidic (heavy) solution (0.2 mol/l immobilines with diverse p*K*), in µl							Basic (light) solution (0.2 mol/l immobilines with diverse p*K*), in µl					
3.6	**4.6**	**6.2**	**7.0**	**8.5**	**9.3**	**pH range**	**3.6**	**4.6**	**6.2**	**7.0**	**8.5**	**9.3**
500	–	–	400	400	–	7.4–8.4	244	–	–	400	400	–
656	–	–	–	711	–	7.5–8.5	177	–	–	–	384	–
553	–	–	–	613	–	7.6–8.6	149	–	–	–	369	–
472	–	–	–	535	–	7.7–8.7	127	–	–	–	364	–
407	–	–	–	476	–	7.8–8.8	110	–	–	–	366	–
356	–	–	–	432	–	7.9–8.9	96	–	–	–	379	–
315	–	–	–	400	–	8.0–9.0	85	–	–	–	400	–
283	–	–	–	379	–	8.1–9.1	76	–	–	–	432	–
257	–	–	–	366	–	8.2–9.2	69	–	–	–	476	–
236	–	–	–	364	–	8.3–9.3	63	–	–	–	535	–
220	–	–	–	369	–	8.4–9.4	59	–	–	–	613	–
207	–	–	–	384	–	8.5–9.5	56	–	–	–	711	–
644	–	–	–	–	701	8.6–9.6	173	–	–	–	–	382
545	–	–	–	–	604	8.7–9.7	147	–	–	–	–	369
465	–	–	–	–	529	8.8–9.8	125	–	–	–	–	364
402	–	–	–	–	471	8.9–9.9	108	–	–	–	–	367
351	–	–	–	–	428	9.0–10.0	94	–	–	–	–	380
311	–	–	–	–	397	9.1–10.1	84	–	–	–	–	403
280	–	–	–	–	377	9.2–10.2	75	–	–	–	–	436
255	–	–	–	–	366	9.3–10.3	69	–	–	–	–	482
235	–	–	–	–	364	9.4–10.4	63	–	–	–	–	542
219	–	–	–	–	370	9.5–10.5	59	–	–	–	–	621

The recipes in Tables 2.11-8 and 2.11–9 can be used for producing any immobilized pH gradient in the pH range from 3.5 to 10.5. For this purpose, graphically interpolation is needed. If, for example, a 4.4–4.9 pH gradient should be cast, the immobiline volumes can be found in the following way:

- Take from 2.11–9 the recipe, which includes the desired pH gradient of 4.2–5.2.
- Carry on a graph paper the volumes of the acidic and basic immobiline solutions for the 4.2–5.2 pH gradient and connect them by straight lines (Figure 2.11-10).

The graph shows that for obtaining 8.0 ml acidic (heavy) solution with pH = 4.4, 388 µl immobiline stock solution with p*K* = 4.6 and 154 µl immobiline stock solution with p*K* = 9.3 are needed. It follows from the same graph that for obtaining of 8.0 ml basic (light) solution with pH = 4.9, 440 µl immobiline stock solution with p*K* = 4.6 and 308 µl immobiline stock solution with p*K* = 9.3 are needed.

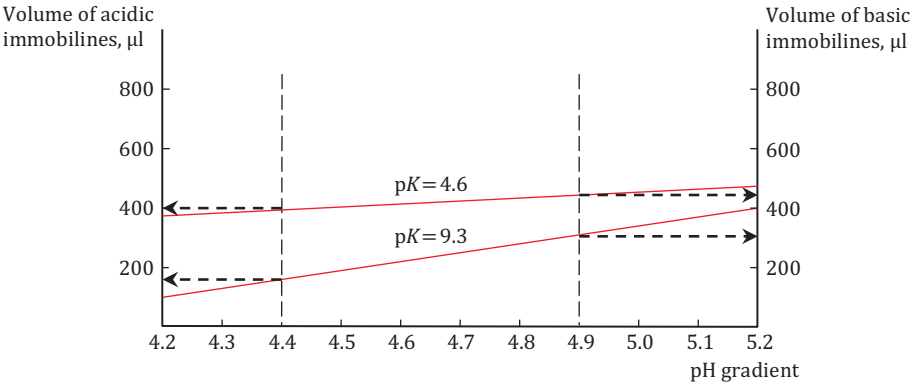


Figure 2.11-10: Graphical interpolation for obtaining the solution for immobilized pH gradient from 4.4 to 4.9. The volumes of the immobiline solutions are plotted on the ordinate axis.

- Mark the acidic and basic endpoints of the desired gradient on the pH gradient-axis, and draw vertical lines through them.
- Take the volumes of the solutions necessary for obtaining the desired 4.4–4.9 immobiline pH gradient from the the point of intersection between the vertical and connecting lines.
- After polymerization, keep the casting cassette at room temperature for 30 min.
- Remove the IPG gel from the cassette, wash with 2 ml/dl glycerol, and dry at room temperature.
- Cover the gel with a plastic film and store at –20 °C.

2.11.7.6 IEF with IPG gel strips

Materials and equipment

Dry IPG strips
Rehydration solution
Rehydration cassette
IEF unit
Power supply

Sample solubilization buffer

Urea	54.05 g (9.0 mol/l)
CHAPS	2.00 g
40 g/dl Pharmalyte 3–10	5.00 ml
DTT	0.23 g (15.0 mmol/l)
Deionized water to	100.00 ml

Rehydration (reswelling) solution

Urea	48.05 g (8 mol/l)
CHAPS	0.50 g
DTT	0.23 g (15 mmol/l)
40 g/dl Pharmalyte, pH = 3–10	5.00 ml
Deionized water to	100.00 ml

Procedure

- Assemble the rehydration cassette with a 0.7 mm thick U-frame (0.5 mm of the plate's U-frame and two layers of Parafilm, 0.1 mm each).
- Clamp the cassette and pipette in the rehydration solution.
- Cut a dry IPG gel into 3 mm wide strips with the help of a paper cutter. Alternatively, use ready-made IPG strips.
- Introduce the IPG gel strips in the rehydration cassette and rehydrate them in the rehydration solution overnight at room temperature to their original thickness (0.5 mm) (Figure. 2.11-11).
- Take the rehydrated gel strips out of the cassette and place them gel side up on a water-saturated filter paper.
- Blot the gel strips gently to remove excess rehydration solution in order to prevent urea crystallization on the gel surface during electrofocusing.
- Pipette 2–3 ml of kerosene onto the flat-bed cooling block and place the IPG gel strips on it, 2 mm apart from each other, the acidic end of the IPG gel strips facing toward the anode.
- Cut two 3 mm thick filter paper to the length of all IPG gel strips in the tray and use them as electrodes. Soak the electrode strips with electrode solutions and remove excessive fluid by blotting with filter paper.
- Place the IEF electrode strips on the cathode and anode ends of the IPG gel strips.
- Connect the electrode leads on the tray to the IEF unit.
- Orientate the acidic end of the IPG gel strips toward anode on the cooling block of the IEF apparatus.
- Set the temperature of the cooling block at 20 °C.
- Dilute samples with the sample buffer and apply 20 µl each. The protein concentration should not exceed 5–10 mg/ml.
- Apply the samples with a pipette.
- Place the lid on the electrofocusing unit and connect the cables to the power supply.
- Start IEF at low voltage: 150 V for 30 min, then 300 V for 60 min. Raise the voltage at the end to 3500 V overnight (Table 2.11-10).

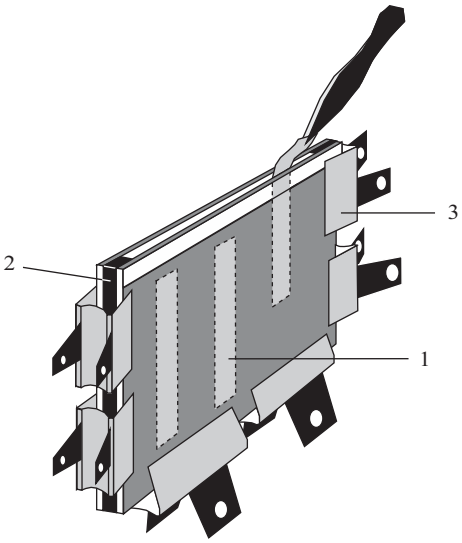


Figure 2.11-11: Rehydration of IPG gel strips. 1. Gel strip; 2. U-formed gasket; 3. Clamp

Table 2.11-10: Running conditions of IEF with IPG gel strips at different pH gradient. The maximum electric current is 0.05 mA, and the maximum power is 5 W per strip.

Separation distance	11 cm	18 cm
IPG 4–7	22,000 Vh	42,000 Vh
IPG 4–9	17,000 Vh	30,000 Vh
IPG 6–10	21,000 Vh	35,000 Vh
IPG 3–10.5	11,000 Vh	18,000 Vh

Coomassie staining

Staining solution

Coomassie brilliant blue R-250	0.2 g
Methanol	30.0 ml
Acetic acid	10.0 ml
Deionized water to	100.0 ml

Destaining solution

Ethanol	40.0 ml
Acetic acid	10.0 ml
Deionized water to	100.0 ml

Procedure

- Fix 2 times for 10 min each in the destaining solution.
- Stain in the staining solution for 20 min.
- Destain several times in the destaining solution.
- Rinse in 10 ml/dl 87% glycerol for 10 min.
- Dry the gel under a cellophane membrane at room temperature or under a hair dryer.

Silver staining

Sensitizer

$AgNO_3$	0.20 g (0.012 mol/l)
NH_4NO_3	0.20 g (0.024 mol/l)
Tungstic silicic acid · aq	1.00 g (0.003 mol/l)
37% HCHO	0.89 ml (0.12 mol/l)
Deionized water to	100.00 ml

Developer

Na_2CO_3	8.0 g (0.755 mol/l)
Deionized water to	100.0 ml

Procedure

- Place the gel in a mixture of ethanol-acetic acid-water (4:1:5, *V:V:V*) for 10 min.
- Rinse twice in deionized water for 5 min each.
- Dry with a hair dryer or in a dryer.
- Place the gel in 0.012 mol/l $Na_2S_2O_3$ (0.30 g/dl $Na_2S_2O_3$) for 10 min.
- Rinse twice with deionized water.
- Put the gel in the sensitizer for 5 min.
  Do not throw away the sensitizer.
- Add an equal volume of the developer.
- Shake the gel in the mixture until the color of the fractions reaches the desired intensity.
- Place in 2 ml/dl acetic acid for 5 min.
- Rinse with tap water a few times.
- Dry with a hair dryer or at room temperature.
- Clean the metallic silver on the film back with a cotton swab.
  When using net gels, the staining time should be halved.

2.11.8 Troubleshooting

Problem	Cause	Solution
Prior to isoelectric focusing		
The polyacrylamide gel is not polymerized or is sticky.	The concentration of monomers was too low.	Increase monomer concentration.
	The concentration of TMEDA or APS in the monomer solution was too low.	Use 5 µl of 10 g/dl TMEDA and 5 µl of 10 g/dl APS per 1 ml of monomer solution.
	The APS solution was too old or improperly stored.	Use a new APS solution. Store the APS solution in the refrigerator at 4–8 °C for a week.
	The concentration of air oxygen in the monomer solution was too high.	Degas the monomer solution with a water-jet pump.
	The high carrier ampholytes concentration (more than 3 g/dl) has retarded the polymerization.	Decrease the carrier ampholyte concentration or polymerize the gel solution in the absence of carrier ampholytes and later rehydrate it with carrier ampholytes.
The gel surface is sticky and separates itself from the support film.	The atmospheric oxygen has inhibited the polymerization of the gel solution.	After casting, overlay the monomer solution with deionized water.
The monomer solution polymerized too fast.	The concentration of TMEDA or APS in the monomer solution was too high.	Check the recipe for gel casting.
	The environment temperature was too high.	Cast gel at 20–25 °C.
Air bubbles in the gel.	The glass plate, which had come in contact with the monomer solution was unclean.	Wash the glass plate before use.
	The support film was unclean.	Do not touch the hydrophilic side of the support film with fingers.
The gel separates from the support film.	A wrong support film was used.	Do not exchange the support films for polyacrylamide and agarose gels.
	The gel was cast on the wrong side of the support film.	Cast gel solution only on the hydrophilic side of the support film. Check the film sites with drops of water.

(continued)

Problem	Cause	Solution
	The monomer solution contained high concentrations of the non-ionic detergent Triton X-100 or Nonidet NP-40.	Polymerize the gel solution in absence of detergents. The detergents can be brought into the gel during rehydration.
The gel does not adhere to the support film, but to the glass plate.	The glass plate is hydrophilic.	Clean the glass plate and treate it with Repel-silane.
	The gel was too long time in the casting cassette.	Take the gel from the casting cassette not later than 1 hour after polymerization.
During isoelectric focusing		
It does not flow or flow too little electric current.	One of the connectors has no or poor contact.	Check all connections. The default setting for IEF in an IPG gel is 5,000 V, 1.0 mA, and 5.0 W.
	There is a bad contact with the electrode strips.	Check the uniform laying of the electrodes on the gel, put on a glass plate.
The electric current intensity increases during IEF.	The electrode solutions are changed.	Use basic solution for the cathode; and acidic solution for the anode.
Water condensation on the lid of the separation chamber.	The electric power is too high.	The electric power should not exceed 2.5 W/ml gel.
	The cooling is insufficient.	Check the cooling temperature. The cooling block should be made of glass, metal, or best of ceramic. Give kerosene or silicone oil DC 200 between the cooling block and support film.
The gel “sweats” (is covered with drops of water).	No water-binding additives in the gel.	Add 20 g/dl glycerol, 10 g/dl sucrose, or 10 g/dl of sorbitol to the monomer solution.
	Too little or no glycerol in the rehydration solution.	Add to the rehydration solution 20 g/dl glycerol.
Water condensation on specific points of the gel.	The contact liquid between the support film and cooling block contains air bubbles, which lead to local overheating.	Remove the air bubbles.

(continued)

Problem	Cause	Solution
The gel sparks and burns.	The support film was not fixed on the glass plate during the gel casting. Poor cooling of the gel.	Prior to gel casting, roll strongly the support film on the glass plate of the casting cassette. Give kerosene or silicone oil DC 200 between the support film and cooling plate.
The urea in the IPG gels crystallizes.	The IEF temperature is too low.	Focus at 10–15 °C.
	The surface dries out.	Add 0.5 ml/dl Nonidet NP-40 to the rehydration solution.
Iso-pH lines in the gel.	The salt concentration in the sample is too high.	Desalinate the samples.
	The concentration of APS was too high.	Reduce the APS concentration or use rehydratable gels.
After isoelectric focusing		
The protein bands are unclear.	The protein concentration was too low. The sample was not completely dissolved.	Concentrate the sample or apply more sample volume. Treat the sample with ultrasound or use additives to dissolve it.
The protein bands are not sharp.	The concentration of the carrier ampholyte was too low.	The concentration of a carrier ampholyte in the gel must be at least 2 g/dl.
	The electrofocusing time was too short.	Prolong the electrofocusing time.
The bands pull tails.	Precipitates or particles in the sample.	Centrifuge the sample.
The bands are crooked.	The salt concentration in the samples is too high.	Desalinate the samples.
The samples from adjacent tracks run into each other.	The sample volume was too large. The application template was not placed tight on the gel.	Concentrate the samples and apply less volume. Press gently the application template onto the gel to remove any air bubbles between the template and the gel.
Wavy iso-pH lines.	The APS concentration was too high.	Use rehydratable gels.

(continued)

Problem	Cause	Solution
	The urea in the gel has destroyed to isocyanate.	Use urea gels immediately after their preparation.
The bands at gel margins are distorted.	The samples have been applied too close to the gel margins.	Apply the samples 1 cm from the gel margines.
After Coomassie staining the background remains blue.	Basic immobiline groups bind to Coomassie dyes.	Use a colloidal or a silver staining method.

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2.12 Capillary electrophoresis of proteins

Capillary **e**lectrophoresis (CE) includes different resolving techniques [1,2]. They can be carried out only in buffers or on gels containing native or denaturing buffers [3]. The interest in capillary electrophoresis is based on its high resolution and speed. CE separations are simpler in both operation and equipment, and faster. With the help of capillary electrophoresis is possible to separate reproducibly attomole quantities (10^{-18} to 10^{-21} mol) of proteins, nucleic acids, carbohydrates, and other compounds in minutes up to an hour [4,5]. There are more than 5,000 publications describing separations of more than 10,000 compounds, even enantiomers.

Compared to **h**igh-**p**erformance **l**iquid **c**hromatography (HPLC), CE achieves better resolution – 30% more peaks have been obtained by CE than with the gradient HPLC under similar conditions. However, the sensitivity of CE is lower than the sensitivity of HPLC. In addition, CE instrumentation is more expensive than HPLC, however, the running costs are considerably lower: CE uses less sample and reagents than HPLC.

2.12.1 Theory of capillary electrophoresis of proteins

The theory of capillary electrophoresis does not differ from the general electrophoretic theory [6,7]. The analytes migrate in an electric field E with the velocity

$$v_{ep} = \mu_{ep} E = = \frac{QE}{6\pi r\eta} \tag{2.12-1}$$

where μ_{ep} is the electrophoretic mobility, E is the field strength, Q is the net charge of the analyte, r is its Stokes radius, and η is the dynamic viscosity of the solvent. Hence, when a voltage is applied to the system, analytes migrate with velocities that are generally determined by their charges and masses. Other variables such as shape and hydrophobicity have also been shown to affect the mobility [8].

In a fused silica capillary, silanol (–Si–OH) groups (p$K \approx 3$) attached to the internal wall of the capillary are ionized to negatively charged silanolate groups (–Si–O^-). The positive ions of the buffer are attracted to the negative silanolate groups. Therefore, an electric double layer with fixed and diffuse positive counterions is built (Figure 2.12-1). When an electric field is applied in a capillary, the diffuse positive counterions (cations) migrate in the direction of the negatively charged cathode through an electric field. Since these cations are hydrated, the water of the buffer migrates too, which causes the **e**lectro**o**smotic **f**low (EOF). Therefore, in the capillary electrophoresis, two opposite processes are acting: the electrophoretic mobility of polyions, and the electroosmotic flow of the buffer [9].

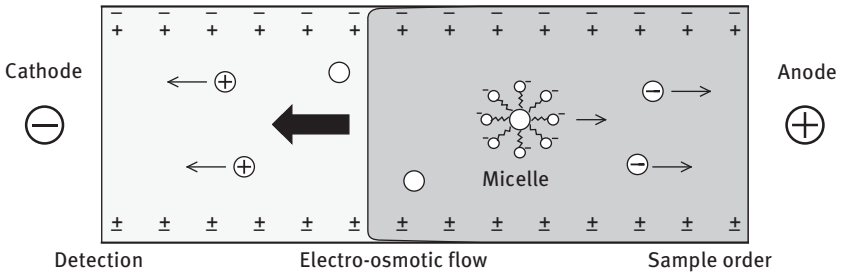


Figure 2.12-1: Depiction of the interior of a fused silica capillary filled with a buffer during electrophoresis.

The velocity of the electroosmotic flow can be written as

$$v_{eo} = \mu_{eo} E \tag{2.12-2}$$

where μ_{eo} is the electroosmotic mobility. It is defined as

$$\mu_{eo} = \frac{\varepsilon\zeta}{\eta} \tag{2.12-3}$$

where ε is the permittivity of the buffer, ζ is the zeta potential of the capillary wall, and η is the dynamic viscosity. Hence, the velocity v of an analyte in the capillary electrophoresis is

$$v = v_{ep} + v_{eo} = \left(\mu_{ep} + \mu_{eo}\right)E = \left(\frac{Q}{6\pi r} + \varepsilon\zeta\right)\frac{E}{\eta} \tag{2.12-4}$$

Without electroosmosis the negative ions should migrate to the anode and never reach the detector at the cathode end of the capillary. The detector is reached first by the cations, then by the neutral molecules, and finally by the anions. The charge-to-mass ratio of proteins also plays an important role.

The capillary electrophoresis is characterized by its efficiency, resolution, and reproducibility.

2.12.1.1 Efficiency

The separation efficiency or number of theoretical plates N in capillary electrophoresis is given by

$$N = \frac{\mu U}{2D} \tag{2.12-5}$$

where μ is the apparent mobility of an anolyte in the separation medium, U is the applied voltage, and D is the diffusion coefficient of the analyte. The theoretical plate in many separation processes is the hypothetical zone in which two phases, such as the liquid and vapor phases of a substance, establish equilibrium with each other. More theoretical plates increase the efficiency of separation process [10,11]. According to this equation, the efficiency of a separation is limited by diffusion and the strength of electric field. Application of very high voltage (more then 20–30 kV) may increase the Joule heating of the buffer in the capillary even breakdown the capillary.

The efficiency of capillary electrophoresis is much higher than that of HPLC. In addition, the electroosmotic flow-driven profile in CE is flat, whereas the pressure-driven flow in chromatography columns has laminar flow rounded profile (Figure 2.12-2). As a result, the electroosmotic flow does not significantly contribute to band broadening as in the pressure-driven chromatography.

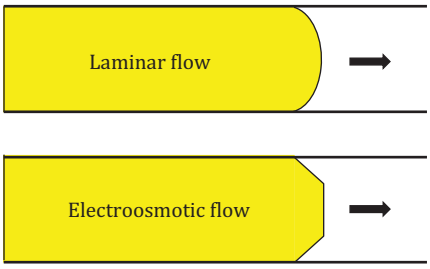


Figure 2.12-2: Profiles of laminar and electroosmotic flow.

Another advantage of capillary electrophoresis is that this method requires microliters of sample and reagents. Finally, the lifetime of a capillary is too long – it can be used for years.

2.12.1.2 Resolution

The resolution R_s of capillary electrophoresis can be denoted as

$$R_s = \frac{1}{4}\left(\frac{\Delta\mu_p\sqrt{N}}{\mu_p + \mu_0}\right) \tag{2.12-6}$$

According to this equation, maximum resolution should be reached when the electrophoretic and electroosmotic mobilities are similar in magnitude and opposite in sign.

The factors that may decrease the resolution of capillary electrophoresis are: diffusion, Joule heating, interactions between the analyte and the capillary wall, difference in height of the fluid reservoirs leading to siphoning, irregularities in the electric field, depletion of buffering capacity in the reservoirs, and electrodispersion (when a component to be resolved has higher conductivity than the background electrolyte) [12].

Best resolution of CE is obtained at the maximum field strength and insignificant Joule heating. Generally, capillaries of smaller inner diameter bear higher field strengths, due to improved heat dissipation, but cause greater difficulty in introducing buffer and sample into the capillary. They require greater pressure and longer times to force fluids through the capillary.

2.12.1.3 Reproducibility

The reproducibility of migration time in capillary electrophoresis is comparable with that of HPLC. It depends mainly on the electroosmotic flow, which, in turn, is affected by the capillary wall. The reproducibility of migration time of capillary zone electrophoresis (s. below), for example, is in the range of 0.2–2.0%.

2.12.2 Instrumentation

The CE instrument consists of a source vial, sample vial, capillary, destination vial, detector, high-voltage power supply, and recording device. The source vial, capillary, and destination vial are filled with a buffer. The capillary is flexible and made usually of fused silica. It is 50 cm long (range of 7–100 cm), and has about 375 μm outer diameter and 50 μm inner diameter (range of 20–200 μm). Teflon capillaries are also available.

The electroosmotic flow of these capillaries is probably a result of adsorption of electrically charged ions of the buffer onto the capillary wall. The electroosmotic

flow increases with the field strength and the pH value until all of the available silanol groups on the capillary wall are ionized and the charge density of the capillary wall is raised [13].

The outside of the capillary is coated with a thin layer of polyimide to get flexible. The inside of the capillary is coated with polyacrylamide, methylcellulose or other compounds. Thus, the adsorption of polyions on the capillary surface is avoided (Figure 2.12-3).

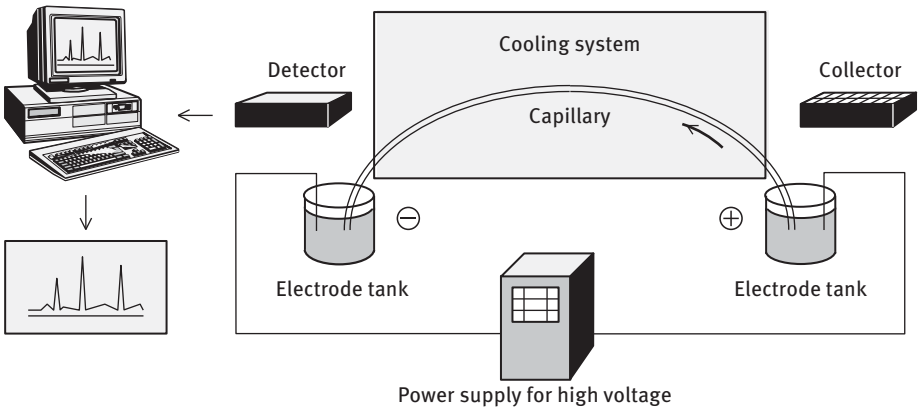


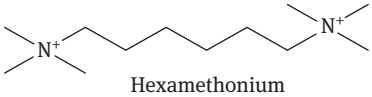
Figure 2.12-3: Capillary electrophoresis instrumentation.

The sample is introduced into the capillary by pressure, siphoning, or electrokinetically. When voltage is applied, the sample components separate from each other in the capillary and are detected at its outlet end. The results are presented as peaks in a pherogram.

2.12.2.1 Coating the capillaries

During capillary electrophoresis, noxious interactions appear between the proteins to be resolved and the capillary. For example, basic proteins, especially lipoproteins, adsorb onto the negatively charged internal wall of the fused silica capillary, which leads to impaired efficiency and poor resolution [14,15].

In addition to the direct interaction with the capillary wall, the proteins are moved by the electroosmotic flow, which also hinders the efficiency and resolution of capillary electrophoresis. Therefore, the control of electroosmotic flow is critical for achieving optimal separation of proteins. The electroosmotic flow can be slowed using ionic additives, such as Mg^{2+} and hexamethonium, which bind to the negatively charged silanolates and as a result lower the electric charge of the capillary.



Hexamethonium

Simpler strategy to slow the electroosmotic flow is to use electrolyte solutions with extreme pH values: if the pH value is strong acidic, the silanol dissociation is suppressed [16]; if the pH value is alkaline and higher than the protein isoelectric points (pI), both the proteins and the capillary wall have negative electric charges, which provokes electrostatic repulsion from each other [17].

Another strategy to slow the electroosmotic flow is to bind coating polymers on the capillary wall. First Hjerten [18,19] proposed covalently attachment of a neutral polymer, like polyacrylamide or methyl cellulose, on the inner capillary wall surface followed by a cross-linking reaction with formaldehyde.

There are two groups of coating polymers: permanent and dynamic [20].

Permanent capillary coating is a complex process. Prior to the coating process the capillary surface should be cleaned and activated by etching, leaching, dehydration, and silylation [21]. The etching can be done with sodium hydroxide; the leaching with hydrochloric acid. To improve the silylation reaction, all water has to be removed from the surface at 160 °C overnight.

Dynamic capillary coating is the simplest approach to deminish the electroosmotic flow. During it, polymers are adsorbed on capillaries *via* physical bonds. The dynamic capillary coating has advantages over the permanent capillary coating. For example, the dynamic coated capillaries can be regenerated [22].

The dynamic capillary coating can be performed using neutral or cationic polymers. Surfactants can also be used.

Neutral polymers [23] quench the protein binding to the silica wall in only 50–60%. The neutral polymer that shows higher inhibition (ca. 85%) is poly(N,N-**d**i**m**ethyl**a**crylamide) (poly(DMA)) because it is a hydrophobic polymer. The neutral polymers can be divided into two classes: hydrophilic and hydrophobic polymers (Figure 2.12-4).

Hydrophilic neutral polymers are: **l**inear **p**oly**a**crylamide (LPA) [24,25], **p**oly(**a**cryloyl**a**mino-**e**thoxy**e**thanol) (PAAEE) [26], **p**oly(**a**cryloyl**a**mino**p**ropanol) (PAAP) [27], celluloses and dextran [28,29,30], **p**oly(**v**inyl **a**lcohol) (PVA) [31,32,33], epoxy polymer [34,35,36], **p**oly(**e**thylene-**p**ropylene **g**lycol) (PEPG) [37,38], **p**oly**e**thylene **o**xide (PEO) [39,40], **p**oly-N-(**a**cryloyl**a**mino**e**thoxy)**e**thyl-(3-D-**g**lyco**p**yranoside (PAAEGP) [41], and **f**luoro**c**arbon (FC). Hydrophobic neutral polymers are: cellulose acetate [42] and highly cross-linked poly(styrene-divinylbenzene) layers [43].

To the group of cationic polymers belong: **p**oly**e**thylen**i**mine (PEI) [44,45,46], **p**oly**b**rene-**d**extran **s**ulfate-**p**oly**b**rene (PB-DS-PB), **p**oly(N-**h**ydroxy**e**thyl**a**crylamide) (PHEA) [47,48], **p**oly**b**rene (PB) [49,50], **p**oly-(**d**i**a**llyl**d**imethyl **a**mmonium **c**hloride) (PDADAC) [51,52], chitosan [53,54], PEO-**b**lock-**p**oly(**4**-**v**inyl**p**yridine) (PEO-b-P4VP) [55], **cat**ionized **h**ydroxy**e**thyl**c**ellulose (cat-HEC) [56], HEC-graft-poly

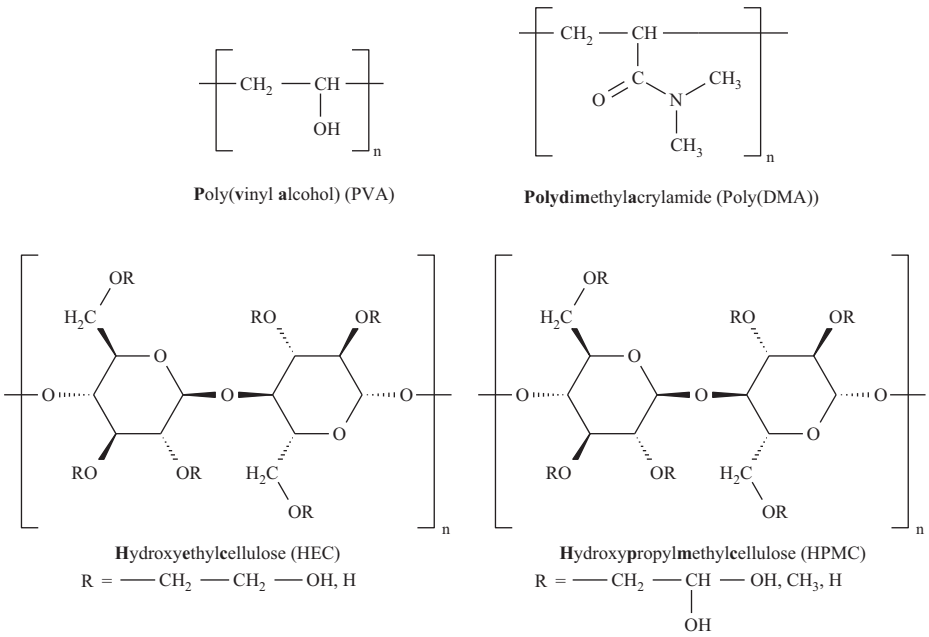
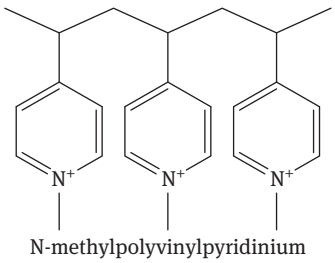


Figure 2.12-4: Chemical formulas of coating polymers.

(N,N-dimethylacrylamide) [57], **p**oly**a**rginine (PA) [58], **p**oly(**dim**ethyl**s**iloxane) (PDMS) [59,60], and more.

A novel cationic polymer is reported, consisting of a N-**me**thyl**p**oly**v**inyl**py**ridinium quaternary ion (PVPy-Me) [61]. N-methylpolyvinylpyridinium quaternary ion does not seem to adsorb low- to high-M_r proteins, even large size proteins as human albumin.



The cationic polymers, once adsorbed onto the silica surface, do not neutralize its negative charges, but induce an excess of positive charges, due to regions of the chains dangling out from the surface [62]. At acidic pH values (e.g., pH = 3.0) the protein

aspartic acid and glutamic acid residues will bear negligible negative charges, and lysine, histidine, and arginine residues will bear positive charges. As a result, the proteins will repel from the cationic polymers on the capillary wall surface.

A new simple and fast noncovalent coating method based on poly(l-**v**inyl**p**yrrolidone-**co**-2-**di**m**e**thyl**a**mino**e**thyl **m**eth**a**crylate) (poly(VP-co-DMAEMA)) copolymer was developed for CE [63]. Poly(l-vinylpyrrolidone-co-2-dimethylaminoethyl methacrylate) includes an "anchor part" (poly(DMAEMA)) and a "functional part" (poly(VP)). The anchor part is attached to the capillary surface *via* electrostatic interactions and hydrogen bonding, while the functional part acts as a non-biofouling interface. The copolymer is adsorbed onto the fused silica surface by hydrogen bonding and electrostatic interactions. The electroosmosis is almost totally suppressed over a wide pH range.

Surfactants that can be used as coating agents can be divided in two groups: nonionic and zwitterionic surfactants (Table 2.12-1). The nonionic (neutral) surfactants interact with the silica wall *via* hydrophobic interactions [64,65].

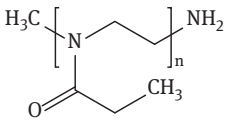
Beside the above showed coating polymers, fluorinated polymers have also been used as coating substances, for example **p**oly(**t**etra**f**luoro**e**thene) (PTFE, or Teflon) and **f**luoro**c**arbon (FC). They have considerable advantages over nonfluorinated polymers, for example, poly(dimethylsiloxane): do not swell in the presence of organic solvents and are optically transparent to lower wavelengths. Teflon is the most widely used polymer for capillary coating but its mechanical softness and absorptivity in the low-UV spectral region can be problematic [66,67].

In terms of long storage, after use the coated capillary must be rinsed with water for half an hour and then stored in water at 4 °C to prevent microbial growth. Drying the coated capillary is not recommended [68].

2.12.2.2 Sieving matrix in capillary electrophoresis

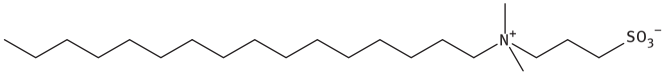
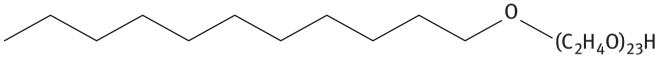
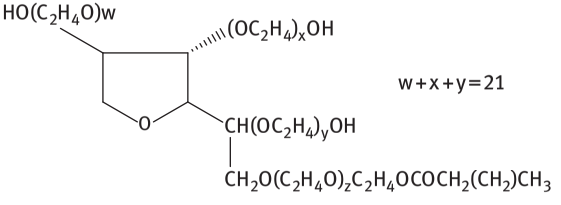
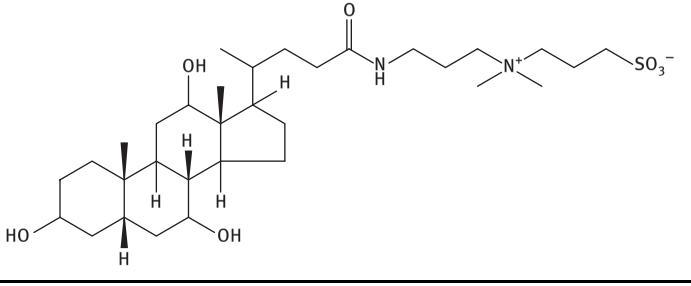
Generally, a sieving matrix in the capillary originates additional electroosmotic flow. Even the linear polyacrylamide (a no-charged gel) at concentrations less than 4 g/dl causes EOF when mixed with SDS [69,70].

A sieving matrix that was proposed for capillary electrophoresis is the polymer **p**oly(2-**e**thyl-2-**ox**azoline) (PEOX). It has a relative molecular mass M_r from 50,000 to 500,000 and can be used in concentrations of 6–12 g/dl for SDS capillary electrophoresis [71]. PEOX has good hydrolytic stability because the amide is highly substituted. It is synthesized by cationic ring-opening polymerization of 2-ethyl-2-oxazoline [72].



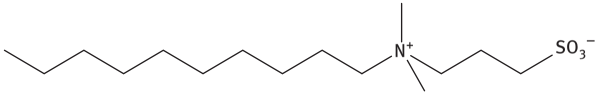
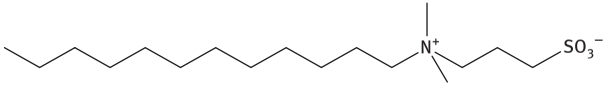
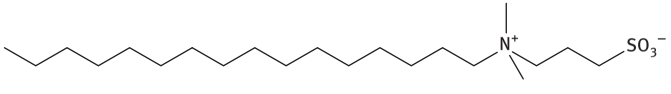
Poly(2-ethyl-2-oxazoline)

Table 2.12-1: Chemical formulas of nonionic and zwitterionic surfactants.

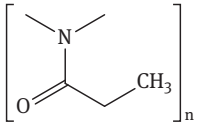
Detergent	Formula	CMC (mmol/l)	M_r
Nonionic surfactants			
Triton X-100 (polyethylene glycol tert-octylphenyl ether)		0.02–0.09	625
Brij 35 (polyoxyethylene lauryl ether)		0.05–0.1	1225
Tween 20 (polyoxyethelene sorbitanmonolaurate)		0.06	1228
Zwitterionic surfactants CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate)		6–10	614.9

(continued)

Table 2.12-1 (continued)

Detergent	Formula	CMC (mmol/l)	M_r
Caprylyl sulfobetaine SB-10 (N-decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate)		25–40	307.6
Lauryl sulfobetaine SB-12 (N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate)		2–4	335.6
Palmityl sulfobetaine SB-16 (N-hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate		0.01–0.06	391.6

PEOX is a homolog of **p**oly(N,N-**d**i**m**ethyl**p**ropion**a**mide) (PDMPA) which has the chemical formula



Poly(N,N-dimethylpropionamide)

Hydroxypropylcellulose was also used as a sieving matrix when LIF detection was applied [73].

Single cells were separated by CE first using pullulan [74] and later using **p**oly-**e**thylene **o**xide) (PEO) with M_r = 100,000. These polymers have low viscosities, which allows a hydrodynamic injection of the cells into a coated capillary. PEO is used in concentration of only 2.0 to 2.5 g/dl to achieve the optimal mesh size for protein separation [75].

2.12.3 Practice of capillary electrophoresis

The capillary electrophoresis is carried out in three steps: injection of sample into the capillary, separation, and detection of protein bands.

2.12.3.1 Injection

The injection of sample requires appropriate equipment and is carried out from the sample vial by electromigration, gravity or pressure [76].

To inject a sample in a capillary by electromigration, a sample applier for capillary electrophoresis is needed. If the sample is applied at the anode, no positive ions are allowed to be present in the sample – all its molecules must be neutral or in form of negative ions. If this is undesirable, the injection can be carried out by gravity.

Injection by gravity can be run when the sample vial and the corresponding capillary end are raised to a higher level than the other capillary end, whereby the sample is sucked by the siphon effect.

Injection by pressure is carried out when overpressure or a subpressure on the sample is applied. The injection by overpressure is a more controllable.

2.12.3.2 Separation

The migration of proteins is initiated by electric field that is applied between the source and destination vials and is supplied by a high-voltage power supply. All ions, positive or negative, are pulled through the capillary in the same direction by electroosmotic flow and are detected near its outlet end. The output of the detector is sent to a data output and handling device such as an integrator or computer.

During the capillary electrophoresis the electric voltage reaches up to 30 kV at very low electric current strength of 10 to 20 μA. The separation time is 10 to 20 min. The Joule heating is removed by a blower.

2.12.3.3 Detection

Capillary electrophoresis can be carried out with diverce detection devices. The majority devices use UV or UV-Vis absorbance. The capillaries used for UV detection must be transparent. For polyimide-coated capillaries, a segment of the coating is typically burned or scraped off to provide a bare window several millimeters long. The path length of the detection cell in capillary electrophoresis (50 μm) is far less than that of a traditional UV cell (10 mm).

Detection (UV, fluorescence) is across a window burnt into the polyimide coating of a silica capillary. Detection at the end of capillary can be achieved using electrochemical detectors or more usefully mass spectrometry. The mass spectrometry provides extremely high sensitivity of capillary electrophoresis for very small sample concentrations.

According to the Beer-Lambert law, to improve the sensitivity of a detector, the path length must be increased, though this results in a loss of resolution. The capillary tube itself can be expanded at the detection point. So a "bubble cell" is created with a longer path length. Another way of increasing the path length is a tubing to be added at the detection point (Figure 2.12-5). Both of these methods, however, will decrease the resolution of separation [77].

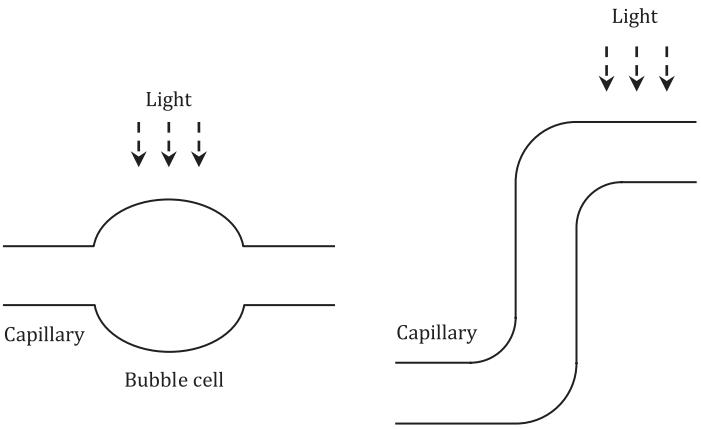


Figure 2.12-5: Ways for increasing the path length of a capillary.
Left – bubble cell; *Right* – additional tubing

A monochromatic light is transmitted through fiber optics to the detector and split there in signal and reference path. The focusing lenses are unnecessary and the

scattered light is reduced to a minimum. When using fused silica capillaries, a direct UV measurement can take place in the capillary, usually at 280 or 260 nm. The measurement is processed using computer program [78].

According to the type of detection, diverse techniques are known: ultra violet detection, laser-induced fluorescence detection, mass spectrometry detection, chemiluminescence detection, and whole-column imaging detection.

Ultra violet detection

The **U**ltra **v**iolet (UV) detection is the most common detection. It is based on the UV absorbance and is used for analyzing the hemoglobin variants. The frequently employed wavelengths are 210, 280, and 415 nm. Zhu *et al.* [79,80,81] has found that the maximal absorbance of the heme group is at 415 nm wavelength. This is in agreement with the results of Hempe *et al.* [82,83]. Yeung *et al.* [84] and Bolger *et al.* [85] pointed out that UV absorption of **h**emoglo**b**in (Hb) chains was greater at 210 nm than at longer wavelengths, and found that some buffer additives, such as urea, at high concentration introduced a high background absorbance below 210 nm. Therefore, 210 nm is the best wavelength for detection of globin chains.

Laser-induced fluorescence detection

Laser-**i**nduced **f**luorescence (LIF) is a method in which an atom or molecule is excited to a higher energy level by absorption of laser light followed by spontaneous emission of light [86,87,88]. Hb exhibits native fluorescence that relies on the fluorescence of aromatic amino acid residues [89]. This was firstly observed by Wong and Yeung [90] within single red blood cells by exciting them at 275 nm. The intrinsic fluorescence of Hb and its sub-attomole intracellular amount simplify the LIF detection, allowing analysis of Hb by capillary electrophoresis at single-cell level [91,92,93].

LIF detection has the advantage of several orders of magnitude higher sensitivity for low-abundance proteins and wider dynamic range for protein profiling compared with UV detection. However, it requires that the light beam be focused on the capillary, which can be difficult for many light sources.

Multi-color fluorescence detection can be achieved by including multiple dichroic mirrors and bandpass filters to separate the fluorescence emission, or by using a prism or grating to project spectrally resolved fluorescence emission onto a detector such as a **c**harge-**c**oupled **d**evice (CCD).

Mass spectrometry detection

Mass **s**pectrometry (MS) is a technique, which includes ionization of chemical species and sorting the ions based on their mass-to-charge ratio. So the masses of compounds within a sample are measured.

Capillary electrophoresis can be coupled with mass spectrometers or **s**urface **e**nhanced **R**aman **s**pectroscopy (SERS). In most systems, the capillary outlet is introduced into an ion source that utilizes **e**lectro**s**pray **i**onization (ESI). The resulting ions are analyzed by a mass spectrometer. The analysis is mostly done with specialized gel analysis software.

MS analysis is used to measure Hb variants, that is, M_r of various Hb chains, and amino acid sequences [94]. Prior to the MS analysis, a chromatographic separation step, such as gel electrophoresis [95], and HPLC [96] is often necessary to differentiate components of either the intact or the fragmented Hb. Compared to other separation techniques used in the Hb analysis, CE cuts down the analysis time from hours to minutes.

The first online capillary electrophoresis mass spectrometry analysis of Hb appeared in 1994 for identification of a tryptic digest of human Hb. It is based on detection using atmospheric pressure ionization mass spectrometry [97]. With the development of mass spectrometry techniques, more and more on-line CE-MS for the analyses of Hb's has emerged.

An on-line **c**apillary **i**so**e**lectric **f**ocusing **m**ass **s**pectrometry (CIEF-MS) utilizing gravity-induced flow with cathodic mobilization was employed by Tang *et al.* [98] for the analysis of Hb A, C, S, and F. The Hb variants were almost baseline-resolved with a pI difference of 0.05 pH units.

An improvement of CE-MS, namely **c**apillary **e**lectrophoresis **e**lectro**s**pray **i**onization **m**ass **s**pectrometry (CE-ESI-MS), was applied to analyze the α- and β-chains of glycated Hb [99] and the α- and β-chains of Hb S [100]. It was also employed for studying the toxic oligopeptides in *Amanita fungi* [101]. With this method, the toxins alpha-, beta- and gamma-amanitin, phalloidin, and phallacidin were separated.

Chemiluminescence detection

Chemi**l**uminescence (CL) detection is another method of capillary electrophoresis widely used in combination with chromatography, spectrometry, and immunoassay [102]. It is characterized by excellent sensitivity and selectivity, allowing high resolution and precise quantification [103].

The heme-containing hemoglobin molecules can induce an enhanced luminol-hydrogen peroxide chemiluminescence [104]. Tsukagoshi *et al.* [105] demonstrated a high-sensitive chemiluminescence detection of hemoglobin bands obtained by free zone CE in a basic carbonate buffer (pH = 10.0). Later the same authors [106] developed a CE-CL method using Cu^{2+} catalyst as a probe.

Whole-column imaging detection

Whole-**c**olumn **i**maging **d**etection (WCID) is a novel detection technology for capillary isoelectric focusing. It has been successfully employed for analysis of hemoglobins.

For WCID a short capillary (a few cm long) is used as a separation channel, which is connected with two pieces of capillary through two dialysis hollow fiber junctions. The dialysis hollow fiber junctions contact the electrolyte reservoirs and provide electric conduction and passage of small ions (such as protons and hydroxide ions), but confine large molecule (such as proteins) inside the capillary. When a high voltage is applied, the analytes in the separation channel will be focused at their pI values. Then the focused analyte bands are imaged with a **c**harge **c**oupled **d**evice (CCD) camera (Figure 2.12-6).

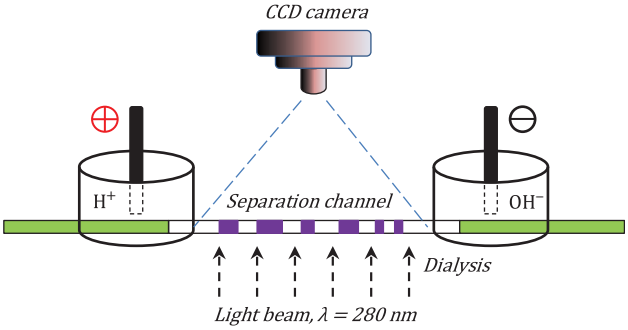


Figure 2.12-6: Principle of the whole-column imaging detection.

Compared to the conventional detection used for CIEF, WCID eliminates the mobilization process. As a result of the high resolution of CIEF, proteins with pI difference as low as 0.03 pH unit can be resolved. In addition, WCID provides a fast separation – usually in less than 5 min. WCID is also very suitable for multi-channel CE separations.

Wu and Pawliszyn [107] developed the UV-WCID technology for CIEF of met-HbA, deoxy-Hb, and oxy-Hb. Bienvenu *et al.* [108] used CIEF with a whole-column imaging detector for the common Hb variants A, F, S, and C.

2.12.4 Types of capillary electrophoresis

According to the main electrophoretic methods, three capillary electrophoresis methods are distinguished: capillary zone electrophoresis, capillary isotachophoresis, and capillary isoelectric focusing.

2.12.4.1 Capillary zone electrophoresis

For **c**apillary **z**one **e**lectrophoresis (CZE), capillaries with inner diameter of 40 μm have been used [109]. A high ratio of the inner capillary surface to the respective volume of the electrolyte inside influences positively the thermostating of the

migrating zones. From this point of view, the most advantageous separation capillaries are of a rectangular type where the electrolyte forms a thin flat layer between two cooled walls.

In capillary zone electrophoresis phosphate buffer is used. It can cover a broad range of pH value (2.55–11.43) due to the three dissociation constants of phosphoric acid and its ions (pK_1 = 2.0, pK_2 = 7.2, and pK_3 = 11.0). So, only one multivalent weak electrolyte can be used instead of a few independent weak electrolytes [110].

Capillary zone electrophoresis is characterized by very high resolution of proteins and peptides. In addition, it offers fast analysis times, minimal sample requirement, and high-sensitivity on-line detection in the lower UV range (200–214 nm) [111,112]. A subtype of CZE is the **c**apillary **i**on **a**nalysis (CIA), which is used to determine simple ion species in aqueous solutions.

2.12.4.2 Capillary isotachophoresis

The **c**apillary **i**so**t**acho**p**horesis (CITP) [113,114] needs two buffers in the gel used, which contain a leading and a trailing ion. The leading ion, for example, formate ion, must have higher mobility than the trailing anion, for example, taurinate ion. In the capillary isotachophoresis, capillaries with inner diameters of 0.8–0.2 mm are used.

A similar method to CITP named **f**ield-**a**mplified **s**ample **s**tacking (FASS) can be used for a rapid concentration of analytes in a narrow zone at the boundary between a low-conductivity sample and a higher-conductivity running buffer. With the help of this method, the main alkaloids from *Sophora flavescens Ait* were separated [115].

2.12.4.3 Capillary isoelectric focusing

In **c**apillary **i**so**e**lectric **f**ocusing (CIEF) the proteins are separated in a pH gradient, due to their different isoelectric points [116].

It is important for the capillary isoelectric focusing that the electroosmotic flow is equl to zero. This can be achieved by modification of the capillary surface or by means of a suitable pH value of the buffer [117]. In traditional fused silica capillaries, generally permanent or dynamic capillary coating is used. In the case of no coated capillaries, the separation medium viscosity has to be increased by adding glycerol [118] or methylcellulose [119].

The pH gradient is usually formed by carrier ampholytes mixtures present in the separation medium. To avoid the drift phenomenon, Mosher and Thormann [120] recommend maximizing electrolyte concentration to electric current density ratio. In order to limit the entrance of electrode solutions inside the separation channel, their viscosity can also be increased.

The capillary isoelectric focusing allows the separation of amphoteric compounds such as peptides or proteins in a pH gradient according to their pI [121,122,123]. It has been also described capillary affinity electrophoresis that is used for studying carbohydrates and glycoproteins [124].

2.12.5 Applications of capillary electrophoresis

The capillary electrophoresis is widely used in the clinical and forensic medicine [125,126]. Some of its applications are listed in Table 2.12-2.

Table 2.12-2: Substances, which are analyzed with the help of capillary electrophoresis.

Diagnostic indicator	Source	Disorder
Albumin	Urine	Renal failure
β_2-Macroglobulin	Serum	Cancer
C-reactive protein		Cancer, inflammation
Olygomonoclonal antibodies	Cerebrospinal fluid	Multiple sclerosis
Isoenzymes	Serum	
Aminotransferases		Myocardial infarction
Creatine kinase		Myocardial infarction
Alkaline phosphatase		Osteoporosis, hepatobiliary disorders
Fibrinolytic peptides	Urine	Intravascular coagulation
Hemoglobin A_{1c}	Erythrocytes	Diabetes
Amino acids	Serum	Amino acid inborn errors
Normetanephrine	Urine	Pheochromocytoma
Carbohydrates	Serum, urine	Diabetes
Oxalate	Urine	Renal stones

2.12.5.1 Clinical applications of capillary electrophoresis

Whereas the cellulose acetate and agarose electrophoresis methods allow for multiple samples to be analyzed, current CE instrumentation allows for only single-sample analysis. An instrument for **c**linical **c**apillary **e**lectrophoresis (cCE) must have the capability for rinsing, washing, thermostating, and easy replacement of the capillaries as well as for accurate applying of nanoliter volumes of sample. In addition, the cCE design must include the dilution of sample, automated buffer replenishment, and automated loading of multiple samples.

One of the advantages of capillary electrophoresis is that it is not restricted only to proteins. It can be used for separating diverse analytes including small molecules and organic and inorganic ions [127].

Serum protein analysis

Chen *et al.* [128] were the first to show that capillary electrophoresis is a viable alternative to agarose electrophoresis. Figure 2.12-7 shows the comparative profiles obtained with scanning densitometry of an agarose gel and direct analysis of a pooled

serum sample by capillary electrophoresis with absorbance at 214 nm. The five zones comprising the serum proteins are better represented by capillary electrophoresis analysis.

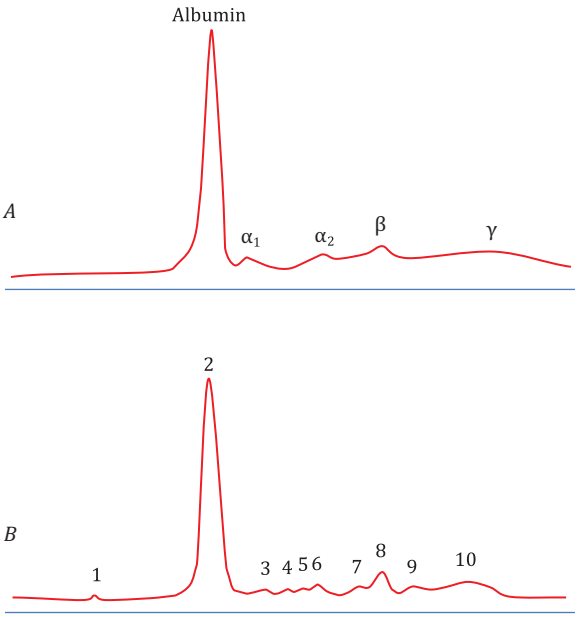


Figure 2.12-7: Serum protein analysis by agarose gel electrophoresis and capillary electrophoresis.
A – Separation of normal serum into five zones by agarose electrophoresis, stained and quantified by scanning densitometry
B – Zone capillary electrophoresis with identification of the individual components separated in TRIS-borate buffer, pH = 8.3, detection at 214 nm.
1. Prealbumin; 2. Albumin; 3. α_1-acidic glycoprotein; 4. α_1-antitrypsin; 5. β-lipoprotein; 6. Haptoglobin; 7. α_2-macroglobulin; 8. Transferrin; 9. Complement C3; 10. γ-globulin

Detection of prealbumin (transthyretin). Prealbumin transports thyroxine and triiodothyronine. Therefore, its concentration is an important indicator of nutritional status, inflammation, malignancy, liver cirrhosis, or Hodgkin disease. Prealbumin is a protein that is difficult to be resolved in most electrophoretic methods. It occurs in a relatively narrow concentration in "normal" serum (200–360 mg/l) and can be detected with capillary electrophoresis at 214 or 200 nm wave length because of its clear resolution from other serum components.

Lipoproteins. The serum lipoproteins transport lipids in the blood. They have spherical molecules composed of a hydrophobic core and a polar shell. The lipoproteins are subdivided in 4 or 5 main density classes: **h**igh **d**ensity **l**ipoproteins

(HDL), **l**ow **d**ensity **l**ipoproteins (LDL), **v**ery **l**ow **d**ensity **l**ipoproteins (VLDL), **i**ntermediate **d**ensity **l**ipoproteins (IDL), and chylomicrons (s. there). Low-density lipoproteins are the major cholesterol depot in the blood circulation. They carry cholesterol from the liver to tissue cells [129]. High-density lipoproteins carry cholesterol in the reverse direction – from the tissues, for example arterial intima, to the liver for excretion [130].

Schmitz *et al.* [131] reported the utility of isotachophoretic CE for separation of serum lipoproteins in the two major fractions: HDL and LDL. Tadey and Purdy [132] found that the presence of a detergent in the separation buffer increases the lipoprotein solubility and using CE separated the HDL and LDL apolipoproteins in less than 15 min.

The concentrations of LDL and HDL are highly important in clinical diagnostics. However, the adsorption of lipoproteins onto the fused silica capillary wall makes their separation difficult [133,134]. Attempts have been made to diminish the adsorption by dynamic or permanent coating of capillaries [135,136]. The dynamic coating is simple and fast, only the addition of methylglucamine or SDS or polymers such as **p**oly(**e**thylene**o**xide) (PEO) or hydroxypropylmethylcellulose into the solution is needed [137,138].

Classification of immunoglobulin subtypes. Klein and Jolliff [139] have shown that immunoglobulin subtypes (IgG, IgA, IgM, heavy chains, light chains κ and λ) can be identified by CE. In this method, serum samples are incubated with a solid phase to which specific antibodies are bound. During the mixing and incubation, the proteins that bind specifically to the bound antibodies, for example, antibodies for IgG, IgA, IgM, heavy chains, κ, and λ, are retained by the solid phase.

Classification of gammopathies. Gammopathies are typically subdivided in five distinct classes: polyclonal, monoclonal with normal heterogeneous immunoglobulins, monoclonal with decreased heterogeneous immunoglobulins, hypogammaglobulinemias with urinary Bence Jones proteins, and oligoclonal with multiple monoclonal bands. Distinct peak shapes characterize these diseases when CE analyzes the serum. They show a change in the κ/λ ratio, which can be determined with immuno-subtraction or specific κ- and λ-immunoassays.

Hemoglobins

Hemoglo**b**in (Hb) (M_r = 64,500) is the major protein in the **r**ed **b**lood **c**ells (RBC). It transports oxygen and carbon dioxide in the body [140]. Hb types are classified by their four globin chains (α, β, δ, and γ). Normal adult hemoglobin (HbA) is a noncovalent tetramer composed of two α-chains (each with 141 amino acid residues), two β-chains (each with 146 residues) ($\alpha_2\beta_2$), and a heme [141,142]. Mutations that change the amino acid sequences of hemoglobin chains cause

various pathological Hb types, for example HbS ($\alpha_2\beta^S{}_2$) – the hemoglobin in the sickle cell disease.

Traditionally hemoglobin chains are analyzed by cellulose acetate [143] or agarose gel electrophoresis [144,145] after sample extraction with acetone and denaturation with urea. Most clinical laboratories use in the beginning alkaline electrophoresis, then acidic electrophoresis for more precise identification, and at the end isoelectric focusing of hemoglobins. The combination of alkaline and acidic electrophoresis is used for identification of Hb variants. Both methods must be used because the separation of Hb variants with similar charge is insufficient with either method alone. For example, HbS cannot be differentiated from HbD or HbG; and HbC cannot be differentiated from HbE, HbC-Harlem, or HbO-Arab by alkaline electrophoresis alone [146].

Nowadays the capillary electrophoresis has important significance in the hemoglobin analysis [147,148,149] (Figure 2.12-8). It is characterized by high speed, low sample consumption, and high resolution. With its help, the hemoglobin structure [150] as well the Hb variants A_2, F, and A_{1c} [151] were studied.

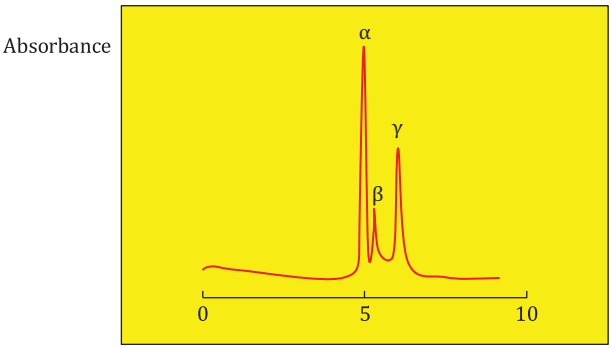


Figure 2.12-8: Separation of fetal α-, β-, and γ-globin chains.

The CZE methods for Hb separation use strongly acidic buffers (pH = 2.0–2.5) [152,153,154], and strongly alkaline buffers (pH = 11.8) [155]. The extreme acidic or alkaline buffers cause dissociation of Hb chains from the heme during electrophoresis [156,157]. Red blood cells were diluted (hemolyzed) in water and injected into a capillary. The separation was performed in a concentrated phosphate buffer at pH = 12.6 and 2.15. The common variants of the β-chains, such as β^S, β^C, and β^E, were also separated from each other. The intact Hb molecule was analyzed using the same sample but in a TRIS-arginine buffer with pH = 8.6.

Using CZE, Ong *et al.* [158] separated hemoglobins obtained from normal individuals and from patients with β-thalassemia in a high-pH phosphate buffer system (pH = 11.8) in less than 8 min.

With the help of capillary isoelectric focusing [159,160], hemoglobins A, F, S, and C were separated. Zhu *et al*. [161] demonstrated that using this method α-thalassemias can be identified. The computer-assisted CIEF is an excellent method for the clinical assessment of hemoglobinopathies and thalassemias because of its automatization, high resolution, and possibility of simultaneous quantification of Hb variants [162,163,164]. Laboratory diagnosis of congenital hemoglobinopathies and thalassemias requires both identification of abnormal Hb variants and quantification of pathological Hb variants [165,166,167].

Isoenzymes

The capillary electrophoresis can also be used for enzyme assays [168], for example of numerous isoenzymes such as alkaline phosphatase, creatine kinase, A and B forms of O-N-acetylglucosaminidase, P and S types of amylase, proteolytic isoenzymes, γ-glutamyltranspeptidase, kallikrein, renin, cathepsin B, and 5′-nucleotidase.

Immune complexes

Nielsen *et al*. [169] have shown that CE can be used for separation of immune complexes from unbound antibody and antigen. An ultratrace detection is possible using fluorescence-tagged monoclonal antibodies and **l**aser-**i**nduced **f**luorescence (LIF) detection [170]. This methods could allow for detection of cancer biomarkers in nanomolar even femtomolar range.

It has been demonstrated that **l**inear **p**oly**a**crylamide (LPA) coated capillaries are suitable for the capillary electrophoresis mass spectrometry analysis of subunits of IgG molecules [171]: heavy chains and light chains of the IgG molecules were separated on the LPA coated capillaries by more than three minutes, with peak width of about 45 s.

Single cell analysis

Jorgenson and coworkers [172] were the first to show the potential of CE for analysing cells differing in age, differentiation, subtype, and so on. Olefirowicz and Ewing [173,174] furthered these studies by using special capillaries for studying the cytoplasm of dopaminergic neurons.

Other applications of CE are the separations of human chorionic gonadotropin and interferon-β_1 proteoforms. For human chorionic gonadotropin, a highly glycosylated protein, the identification of over 20 glycoforms have been reported using **p**oly**v**inyl **a**lcohol (PVA) coated capillary [175,176]. For interferon-β_1 proteoforms, instead of using regular polyethylenimine coated capillary, a cross-linked polyethylenimine coating has been reported to be used on the quantitative CE-MS analysis [177].

Therapeutic drug monitoring

Over the years, capillary electrophoresis has been used for analyzing drugs in biological fluids by means of ultraviolet absorbance and **l**aser-**i**nduced **f**luorescence (LIF) detection. Zare *et al.* [178] measured the concentrations of methotrexate and its metabolites as low as 0.1–1 nmol/l. Prufionosa *et al.* [179] showed that the antihypertensive drug cicletanine could be monitored in the blood serum. Honda *et al.* [180] found that the oral cephalosporin antibiotic cefixime could be resolved from its five metabolites generated by the gastrointestinal flora. Zhang *et al.* [181] separated flavoxate, a smooth-muscle antispasmodic drug, as well as its metabolites in urine of patients in less than 9 min and with a detection limit of 200 pg/l. Naylor and coworkers resolved with CE haloperidol, a neuroleptic drug, from its metabolites [182].

Similarly, Lloyd *et al.* [183] showed that the antileukemic agent cytosine-O-D-arabinoside could be monitored in plasma by CE. Reinhoud *et al.* [184] demonstrated that the anthracycline antibiotics in the treatment of cancer doxirubicin and its epimer, epirubicin, could be separated by CZE.

Shihabi [185] showed that CE can be used to evaluate pentobarbital in serum. Later the same author and Oles [186] analyzed felbamate, an antiepileptic drug in blood serum in less than 5 min, whereas the corresponding 12-min HPLC assay required either extraction of the serum with chloroform or precipitation with acetonitrile. Also the utility of **m**icellar **e**lectrophoretic **c**apillary **c**hromatography (MECC) for determining the concentration of theophylline and other purines in human serum, saliva, and urine was demonstrated [187,188].

Drugs of abuse

CE is useful for determination of nonillicit drug abuse. Tagliaro *et al.* [189] with the help of CZE detected morphine, the main metabolite of heroin, and cocaine in the blood. Wernly *et al.* [190] found that other metabolites, such as morphine-3-glucuronide, could be detected by CZE in urine in less than 10 min.

With micellar electrophoretic capillary chromatography, tolbutamide, tolazamide, acetohexamide, chlorpropamide, glyburide, glipizide and more can be resolved using different concentrations of micelle-forming detergents.

2.12.6 Protocols

2.12.6.1 Capillary IEF of proteins

Materials and equipment

IEF markers
Ampholyte mixture, pH = 3.0 to 10.0
Phosphoric acid
Sodium hydroxide

50 µm i.d. coated (for unknown pI) or uncoated (for known pI) fused silica capillary
CE instrument
Power supply delivering voltage of 2,000 to 3,000 V and power of 6 W

Sodium borate buffer, 5 mmol/l, pH = 8.0

Sodium borate anhydrate	1.01 g (5 mmol/l)
Ajust pH to 8.0 using HCl.	
Deionized water to	1,000.00 ml

Sodium borate buffer, 50 mmol/l, pH = 8.0

Sodium borate anhydrate	10.06 g (50 mmol/l)
Ajust pH to 8.0 using HCl.	
Deionized water to	1,000.00 ml

Sodium borate buffer, 500 mmol/l, pH = 8.0

Sodium borate anhydrate	10.06 g (500 mmol/l)
Ajust pH to 8.0 using HCl.	
Deionized water to	100.00 ml

10 mmol/l phosphoric acid

85% H_3PO_4	2.3 ml
Deionized water to	100.0 ml

20 mmol/l sodium hydroxide

NaOH	4.0 g
Deionized water to	100.0 ml

Procedure

For separations of proteins with unknown pI

- Dilute a sample with 50 mmol/l sodium borate buffer to obtain a final concentration of 1 mg/ml.
- Fill a coated column with 500 mmol/l sodium borate buffer.
- Inject the sample.
- Separate the proteins at 10 kV and 25 °C for 30 min.
- Add ampholyte mixture to 0.5 ml protein sample to obtain a final concentration of 2.5 g/dl ampholytes. If desired, add IEF markers to a final concentration of 0.1 mg/ml to calibrate the column.
- Fill the capillary by pressurizing the reservoir.
- Fill the anode reservoir with 10 mmol/l phosphoric acid and the cathode reservoir with 20 mmol/l NaOH.
- Focus the sample at 10 kV constant voltage for 5 min.
- Wash the column after each run with 10 mmol/l phosphoric acid for 1 min.
- Store the column in deionized water at room temperature.

For separations of proteins with known pI

- Dilute the protein with a 5 mmol/l sodium borate buffer with pH value above the pI of the protein to create a final protein concentration of 1.0 mg/ml.
- Fill an uncoated capillary column with the 50 mmol/l sodium borate buffer.
- Inject the sample.
- Separate the proteins at 25 kV and 25 °C for 30 min.

2.12.7 Troubleshooting

Problem	Cause	Solution
Prior to electrophoresis		
The polyacrylamide gel does not polymerize or polymerizes too slowly.	The concentration of TMEDA or APS in the monomer solution is too low.	Check the recipe for gel casting.
	The APS solution is too old or improperly stored.	Use new APS solution.
	The room temperature is too low.	Cast gel at 20–25 °C.
Gas bubbles during gel polymerization.	The monomer solution contains air.	Degas the monomer solution.
During electrophoresis		
It does not flow or flows too little electric current.	One of the connectors has no or poor contact.	Check all connections.
Polymer **d**elivery **p**ump (PDP) is noisy and/or vibrating.	PDP is not pushed to back wall. Array locking lever is incorrectly positioned.	Restart the instrument. Push PDP against the back wall.
Bubble detect error message: *Polymer delivery pump*.	Air bubbles in *Polymer delivery pump*.	Run *Remove bubble wizard*.
Debris in *Polymer delivery pump* or upon removal of polymer pouch from *Polymer delivery pump*.	Shipping seal of polymer pouch has become delaminated; polyethylene remains on pouch fitment.	Prior to installation, remove the entire seal from the pouch fitment. Run *Bubble remove wizard*. Run *Wash the pump chamber and channels*.
Miscommunication between the data collection and instrument.	No communication between computer and software.	Click the refresh button on the dashboard screen.
Plate does not link.	The spatial or spectral calibration is not performed.	Run the spatial or spectral calibration and relink the plate.

(continued)

Problem	Cause	Solution
The instrument cannot perform spatial calibration with fill.	Conditioning reagent installed instead of polymer.	Replace conditioning reagent with polymer and fill array.

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2.13 Two-dimensional electrophoresis

The **two**-**d**imensional gel electrophoresis (2D electrophoresis, 2D PAGE) is a combination of two high-resolution electrophoretic methods: isoelectric focusing and SDS-PAGE. It was independently introduced 1975 by O'Farrell [1] and Klose [2], as IEF with carrier ampholytes in vertical round gels (1st dimension), then as SDS disc-electrophoresis in vertical slab gels in direction 90 degrees from the first dimension (2nd dimension). Today the 2D electrophoresis is preferably carried out in horizontal thin gels [3,4].

The two-dimensional electrophoresis is the separation technique with the highest resolution available today. It has an extremely high resolution – allows a separation of two protein polyions that differ one from another by only one charged amino acid residue. The 2D electrophoresis is capable of resolving over 3,000–4,000 protein spots in a single gel, detected by silver staining, autoradiography, or immunoblotting (Figure 2.13-1). Therefore, it is used for studying proteins of microorganisms [5,6], plants [7,8], or milk [9,10]. The clinical diagnostics is also an area, where the precise resolution of proteins is of great importance [11,12]. However, the high technical effort, the long analysis time, and the lack of reproduction still do not make the 2D electrophoresis a routine method.

Gel spots containing proteins of interest can be excised from a 2D gel and extracted for further analysis by **m**atrix-**a**ssisted **l**aser **d**esorption/**i**onization and **m**ass **s**pectometry (MALDI MS) [13,14,15].

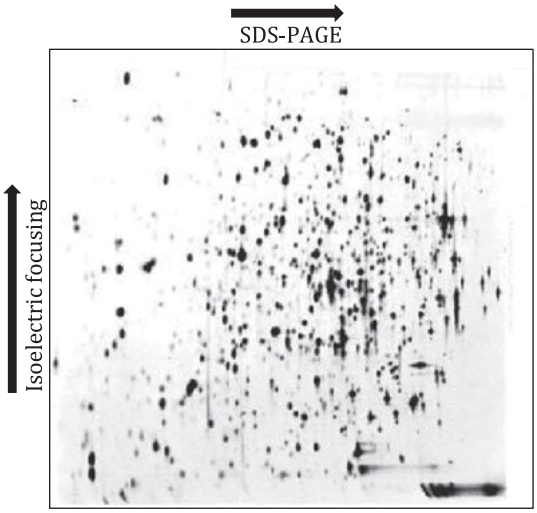

Figure 2.13-1: Protein spots obtained by 2D electrophoresis.

2.13.1 Theory of two-dimensional electrophoresis

The principle of two-dimensional electrophoresis was discovered 1969 [16,17]: In one-dimensional electrophoresis the proteins are resolved according to their charge or mass; in two-dimensional electrophoresis they are resolved according to both (Figure 2.13-2).

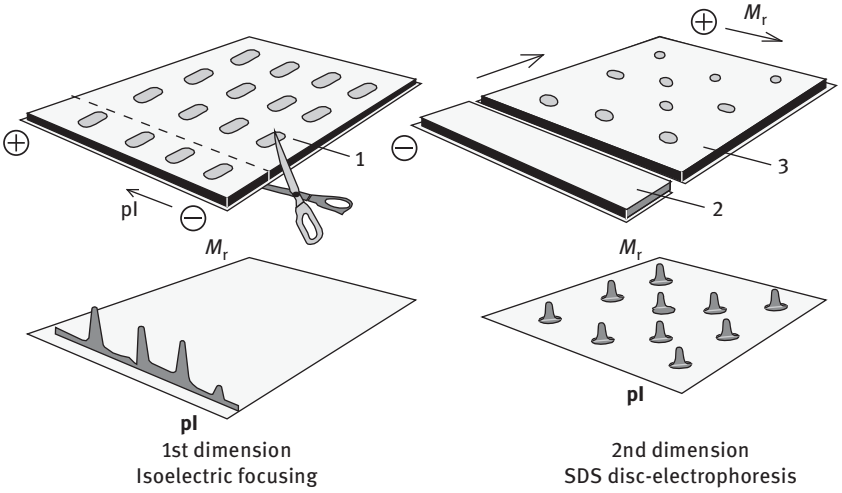

Figure 2.13-2: Principle of two-dimensional electrophoresis. The proteins are resolved in the first dimension by isoelectric focusing, and then in the second dimension by SDS disc-electrophoresis. 1. Focusing gel; 2. Contact gel; 3. SDS polyacrylamide gel

The proteins in the first direction (dimension) are resolved by isoelectric focusing according to their isoelectric points: The proteins, at all pH values other than their isoelectric points, are charged. If they are positively charged, they will be pulled toward the negative end of the gel; if they are negatively charged, they will be pulled to the positive end of the gel. The proteins are focused isoelectrically in a gel with 8–9 mol/l urea, or a nonionic detergent, and dithiothreitol. The urea or the nonionic detergent dissolves the quaternary structure of proteins, whereas dithiothreitol reduces and breaks down their disulfide bonds to give subunits (polypeptide chains). An improvement in the 2D electrophoresis is the isoelectric focusing in a gel with an immobilized pH gradient (IPG) [18]. The first dimension of 2D electrophoresis can also be carried out in hybrid IPG gels, which contain in addition carrier ampholytes [19].

Afterwards, the proteins are resolved in a second direction (dimension) by denaturing SDS disc-electrophoresis according to their masses: Prior to the SDS disc-electrophoresis, the gel strip with the proteins is treated with SDS. This unfolds (denatures) the proteins into long straight polyions and binds a number of SDS ions to them proportional to their mass. Since the SDS ions are negatively charged, all proteins will have approximately the same charge-to-mass ratio.

The 2D electrophoresis with carrier ampholytes is characterized by a shorter focusing run than the 2D electrophoresis in IPG gels, so it is still preferred. However, the 2D electrophoresis in IPG gels has higher reproducibility.

If the isoelectric focusing is performed by carrier ampholytes, the 2D electrophoresis is referred to as ISO-DALT system [20]: **Iso** for the pI separation by isoelectric focusing using carrier ampholytes; **Dalt** for the mass resolving by means of SDS disc-electrophoresis (Dalton is the old unit for mass, which has found no place in the SI system). If the isoelectric focusing is performed in an **i**mmobilized **p**H **g**radient (IPG) gel, the 2D electrophoresis is referred to as IPG-DALT system.

As a result of 2D electrophoresis, protein polyions form a two-dimensional pherogram of spots that looks like a geographical map. The position of each protein spot can be coordinated in the rectangular (Cartesian) system, where the increasing pI values are plotted on the abscissa axis, and the increasing molecular masses are plotted on the ordinate axis.

It is possible that 2D electrophoresis is carried out under non-denaturing conditions [21]. However, the resolution of this method is less than that under denaturing conditions. The native 2D electrophoresis has an important role in preserving the biochemical activity of separated enzymes.

2.13.2 Isoelectric focusing in the first dimension

Prior to isoelectric focusing in the first dimension, the protein sample must be appropriate prepared.

2.13.2.1 Sample preparation

Tissue or blood samples for 2D electrophoresis need to be processed and solubilized before applying onto an IEF gel. Solubilization requires lysis reagents, such as neutral detergents (e.g., CHAPS). Don't use Tween 20 and Triton X-100. The protein concentration in the sample should be about 10 mg/ml. In addition, 8.0–9.0 mol/l urea, 1.0 to 2.0 ml/dl Nonidet NP-40, 0.5 g/dl DTT, and 0.8–1.0 g/dl carrier ampholytes (if the first dimension gel contains carrier ampholytes) must be added to the sample. The protein sample to be separated by 2D PAGE should be prepared in a low-ionic strength buffer that maintains the native charges of the proteins to keep them soluble.

2.13.2.2 ISO-DALT and IPG-DALT

The gels for IEF with carrier ampholytes have T concentration of 4–5 g/dl and a degree of crosslinking C = 0.03, and contain 8–9 mol/l urea and 1–2 g/dl carrier ampholytes. They are placed on a coated with kerosene or silicone oil DC-200 cooling block. Their ends are covered with paper or gel strip electrodes: the anode strip contains 10 mmol/l glutamic acid or 0.8 mol/l phosphoric acid (54.3 ml of 85 g/dl phosphoric acid to 1,000.0 ml deionized water); the cathode strip contains 10 mmol/l lysine, or 0.8 mol/l ethylenediamine (53.7 ml ethylenediamine to 1,000.0 ml deionized water), and 10 ml/dl glycerol. Onto the gel, a silicone template is placed, into the slots of which 10 µl of samples each are applied.

For IPG-Dalt system, immobilized pH gradient of pH = 4.0–10.0, 4.0–7.0, or 7.0–10.0 are used. They can be prepared as follows (Table 2.13-1).

The gels are prepared using a gradient mixer and are polymerized at room temperature or 37 °C. Thereafter, the IPG gel is removed from the casting cassette, and washed 3 times for 20 min in deionized water, 1 time in 2 ml/dl glycerol, and finally dried at room temperature using a fan. At the end, the gel is put in a plastic wrap and stored at –20 °C.

When necessary, the dried IPG gel is removed from the freezer and left at room temperature for 10 min. Then from the IPG gel 4–5 mm wide gel strips are cut with a paper cutter and placed in a cassette with a rehydration solution. The rehydration solution contains usually 8.0–9.0 mol/l urea (480.5–540.5 g urea to 1,000.0 ml deionized water), 0.5 ml/dl Nonidet NP-40, and 10 mmol/l DTT (1.54 g DTT to 1,000.0 ml deionized water).

After the rehydration in 3–24 hours at room temperature, the swollen IPG gel strips are picked out from the rehydration cassette, and put between two filter papers to remove the excess of the rehydration solution on the strips so that urea should not crystallize on their surface. Then the strips are placed with their acidic ends to the anode on the coated with kerosene cooling block of a horizontal chamber. Thereafter, the IPG gel strips are covered with paper or gel electrode strips (Figure 2.13-3). At the end, the sample is applied onto the gel strips with the aid of a silicone application template.

Table 2.13-1: Preparing of solutions for 250 × 120 × 0.5 mm IPG gels (T = 4 g/dl, C = 0.03) with pH gradient of 4.0–10.0, 4.0–7.0, and 7.0–10.0.

	IPG gradient					
	pH = 4.0–10.0		pH = 4.0–7.0		pH = 7.0–10.0	
	Light solution	Heavy solution	Light solution	Heavy solution	Light solution	Heavy solution
Immobilines pK = 3.6, µl	–	588.0	161.0	308.0	48.0	289.0
pK = 4.6, µl	61.0	–	394.0	59.0	–	–
pK = 6.2, µl	27.0	243.0	81.0	240.0	–	–
pK = 7.0, µl	260.0	47.0	143.0	–	173.0	202.0
pK = 8.5, µl	84.0	178.0	–	–	187.0	187.0
pK = 9.3, µl	190.0	–	467.0	–	149.0	–
Monomer solution, ml (T = 50 g/dl, C = 0.03)	0.64	0.64	0.64	0.64	0.64	0.64
87% Glycerol, ml	0.3	2.0	0.3	2.0	0.3	2.0
10 g/dl TMEDA, µl	37.0	37.0	37.0	37.0	37.0	37.0
10 g/dl APS, µl	37.0	37.0	37.0	37.0	37.0	37.0
Deionized water to, ml	8.0	8.0	8.0	8.0	8.0	8.0

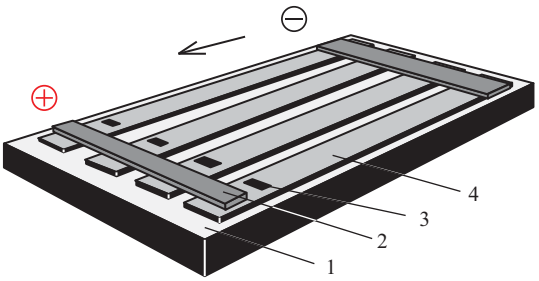

Figure 2.13-3: IEF in IPG gel strips.
1. Separation chamber; 2. Electrode strip; 3. Sample; 4. IPG gel strip

After isoelectric focusing, a IPG strip is equilibrated in a solution containing SDS, DTT, urea, glycerol and iodoacetamide, and placed onto a SDS gel. For horizontal setups, the hand-made or ready-made SDS-PAGE gel on a support film is placed onto the cooling plate of a horizontal electrophoresis system.

The equilibrated IPG gel strip is transferred onto the surface of the SDS gel to run SDS electrophoresis. For vertical setups, the equilibrated IPG gel strip is loaded on top of a vertical SDS polyacrylamide gel. After SDS electrophoresis, the polypeptides are stained either with Coomassie brilliant blue or silver nitrate, or are blotted onto an immobilizing membrane and detected using specific reagents such as lectins or antibodies.

The isoelectric focusing (the first dimension of 2D electrophoresis) is carried out at 10–15 °C, wherein a prefocusing is needed only at IEF with carrier ampholytes. At the isoelectric focusing in IPG gels, prefocusing is unnecessary as the pH gradient is already preformed. After isoelectric focusing, the gel with the carrier ampholytes or the IPG gel strips are stored in the freezer at −80 °C or in liquid nitrogen, or are used immediately for the 2nd dimension of 2D electrophoresis.

Instead of isoelectric focusing, **b**lue **n**ative **p**oly**a**crylamide **g**el **e**lectrophoresis (BN-PAGE) can be run. Using a combination between BN-PAGE (in the first dimension) and SDS-PAGE (in the second dimension), **m**ulti**p**rotein **c**omplexes (MPC) can be subdivided into individual constituents. The 2D BN-PAGE/SDS-PAGE electrophoresis begins with lysis of cells in a buffer containing 0.1 g/dl Triton X-100 that disrupts the membranes and solubilizes the membrane protein complexes. Later the lysates are dialyzed against BN-dialysis buffer to remove salts and small metabolites. Then, the multiprotein complexes are separated in *T*4–15 gradient BN-PAGE followed by SDS-PAGE (Figure 2.13-4). At the end the proteins are visualized by immunoblotting with antibodies against the MPC subunits. So the eukaryotic 19S, 20S, and 26S proteasomes were characterized [22].

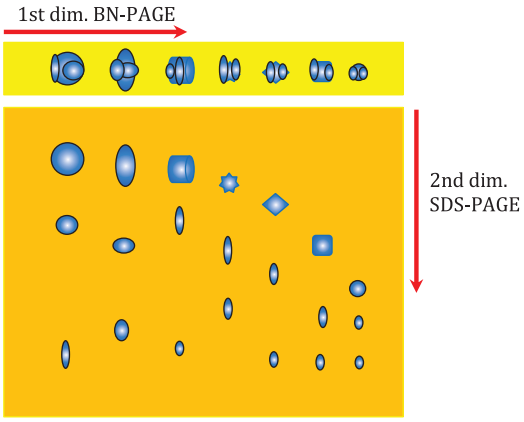

Figure 2.13-4: Schematic representation of two-dimensional BN-PAGE/SDS-PAGE.
The multiprotein complexes are separated under native conditions by gradient BN-PAGE in a first dimension. Then the multiprotein complexes are subjected to denatured linear SDS-PAGE in a second dimension.

2.13.3 SDS disc-electrophoresis in the second dimension

After isoelectric focusing in the first dimension, SDS electrophoresis is running in the second dimension of 2D electrophoresis. The focused proteins are solubilized in SDS solution and separated according to their molecular masses. First they are concentrated in the stacking gel and then are separated in a homogeneous or gradient resolving gel. This events can be fulfilled in the classical discontinuous buffer system of Ornstein [23], and Davis [24] with addition of SDS [25].

After the isoelectric focusing the IEF or IPG gel is removed from the cooling block of the electrophoretic chamber and cut in gel strips with scissors. The gel strips are soaked in a SDS-equilibration solution. The equilibration solution (Table 2.13-2) forms, on the one hand, SDS-protein complexes in the strip; and on the other hand, the carrier ampholytes in the gel (if the isoelectric focusing was carried out with carrier ampholytes) are washed.

Table 2.13-2: Composition of an equilibration solution.

Concentration	Preparation and Instruction
50 mmol/l TRIS-chloride buffer, pH = 6.8 1–2 g/dl SDS	Dissolve 6.06 g TRIS in 80 ml of deionized water and titrate with 4 mol/l HCl to pH = 6.8. Then fill with deionized water to 100 ml.
6.0 mol/l urea	Dissolve 36.04 g urea in 100 ml of deionized water.
1 g/dl 1,4-Dithiothreitol	Add before use.
0.26 mol/l 2-Iodoacetamide	Dissolve 4.81 mg 2-iodoacetamide in 100 ml deionized water.
0.1 mmol/l Na_2EDTA	Dissolve 3.0 mg/dl Na_2EDTA in 100 ml deionized water.
30/dl Glycerol 0.01 g/dl Bromophenol blue Na salt	

The SDS-electrophoresis is running at 10–15 °C. After 60 min at 30–80 mA, 200 V, and 6–16 W (for a standard gel of 250 × 120 × 0.5 mm), the gel strip is removed and SDS-electrophoresis is carried out at 30–80 mA, 800 V, and 15–40 W, depending on the buffer, for further 1.5–5 h, until the Bromophenol blue front reaches the anodic electrode strip (Figure 2.13-5).

2.13.3.1 SDS equilibration of the IEF gel strips for the second dimension

The equilibration of the gel strips takes place in test tubes. The carrier ampholytes containing IEF gel strips are equilibrated for 2 min, and the IPG gel strips are

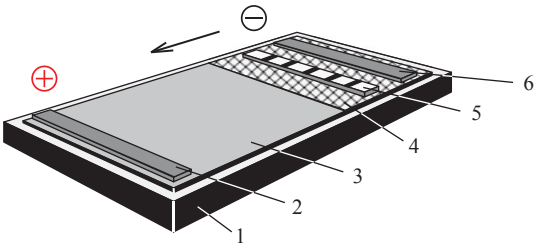

Figure 2.13-5: SDS-electrophoresis after placing an equilibrated gel strip on a SDS-stacking gel. 1. Separation chamber; 2. Anode strip; 3. Separating gel; 4. Stacking gel; 5. Gel strip with protein bands separated by IEF; 6. Cathode strip

equilibrated twice for 15 min each. Prior to the second IPG gel strips equilibration, 0.26 mol/l 2-iodoacetamide is added to the equilibration solution. 2-Iodoacetamide alkylates the protein SH groups and avoids streaking in the gel during silver staining. Thereafter, the equilibrated IEF or IPG gel strips are sandwiched for 1 min between two filter papers to suck the excess of the equilibration solution.

2.13.3.2 Gels for SDS disc-electrophoresis

For the second dimension of 2D electrophoresis, a flat SDS gradient gel or a flat SDS homogeneous gel [26,27] can be used. In both cases, every gel consists of a stacking and a resolving gel.

The stacking gel has usually T = 5 g/dl and C = 0.02–0.04, and contains 0.125 mol/l TRIS-chloride buffer, pH = 6.8. The SDS resolving gel contains usually gradient concentration of T = 10–15 g/dl and C = 0.02–0.04, and 0.375 mol/l TRIS-chloride buffer with pH = 8.8.

The gradient concentration gel is cast using a gradient mixer: A casting cassette is filled up to a 2/3 of its height with gradient resolving gel and then is overlaid with resolving buffer or deionized water. After the polymerization, the resolving buffer is aspirated and the remaining volume of the cassette is filled with the stacking gel solution (Table 2.13-3).

The casting cassette with the gel solutions is left for 60 min at room temperature. Then the equilibrated gel strip with the isoelectric focused proteins is placed on the stacking gel of the homogeneous or gradient SDS gel, previously laid down on the with kerosene overlaid cooling plate of an electrophoretic chamber. The equilibrated gel strip can be also inserted into a long slot in the SDS-gel (lay-in technique) or can be placed onto it (lay-on technique).

The lay-in technique is preferred after isoelectric focusing with carrier ampholytes. In this case, one should make sure that the edges of the IEF gel strip are exactly in the SDS gel slot, and the thickness of the IEF gel strip has the depth of the SDS gel.

Table 2.13-3: Production of monomer solutions for casting a *T*5 SDS stacking gel and a *T*12–15 SDS gradient gel in a casting cassette with internal dimensions of 250 × 120 × 0.5 mm.

	Stacking gel	Light resolving gel	Heavy resolving gel
	$T = 5$ g/dl, $C = 0.03$	$T = 12$ g/dl, $C = 0.02$	$T = 15$ g/dl, $C = 0.02$
Gel buffer	1.25 ml	1.50 ml	1.50 ml
Monomer solution ($T = 50$ g/dl, $C = 0.03$)	0.50 ml	1.44 ml	1.80 ml
SDS	0.01 g	0.01 g	0.01 g
87% Glycerol	1.50 ml	0.60 ml	1.20 ml
10 g/dl TMEDA	25.0 µl	30.0 µl	30.0 µl
10 g/dl APS	25.0 µl	30.0 µl	30.0 µl
Deionized water to	5.0 ml	6.0 ml	6.0 ml

The lay-on technique is used after an isoelectric focusing in IPG gel strips. Then the gel strip is laid down on the SDS stacking gel with its gel side down.

As electrode buffer, TRIS-glycinate buffer with pH = 8.3 is used. After electrophoresis, SDS is removed from the gel by incubating the gel in a solution of Triton X-100.

2D electrophoresis in small gels is a quick method to separate proteins. The isoelectric focusing time is usually 2 to 4 h at 500 V, because the IEF gels are much shorter (6 to 8 cm). Furthermore, less protein is required. Stacking gels are not required. The second-dimension gel is processed in the same way as a one-dimension minigel.

Lipoproteins that build biological membranes can be dispersed using detergents. The hydrophobic moiety of detergents competes with the lipids in lipoproteins and makes them water-soluble [28]. Beside the classical surfactants, **f**luorinated **a**mido-**s**ulfo**b**etaines (FASB) were also used for 2D electrophoresis [29]. They are characterized by a hydrophilic polar head, a hydrophobic and lipophilic tail, and an amido group as connector. The tail is in part fluorinated resulting in the modulation of its lipophilicity. Their effect on the **r**ed **b**lood **c**ell (RBC) membrane showed a specific solubilization depending on the length of the hydrophobic part.

2.13.4 Detection and evaluation of proteins in 2D pherograms

As a result of 2D electrophoresis, a gel with protein spots is obtained [30]. To visualize proteins in 2D gels, the proteins should be stained or labeled. For protein staining a variety of methods can be detected, but most commonly used among them are the silver and Coomassie brilliant blue staining. The choice of staining method is

determined by several factors: desired sensitivity, ease of use, expense, and type of imaging equipment available.

2.13.4.1 2D gel staining

The silver staining is 100 times more sensitive than the Coomassie staining [31]. Its sensitivity is 0.05–0.10 ng protein for mm^2 and correlates to the amount of protein in a given spot. The silver binds to cysteine groups within the proteins. However, the protein spots show different color, which makes the protein quantification difficult.

2.13.4.2 Autoradiography and fluorography

The autoradiography and fluorography are the most sensitive detection methods for 2D pherograms. The autoradiography is referred to as the emission of radioactively labeled proteins onto an X-ray film. The fluorography is created when a visible light is produced by interaction between a radioactive radiation and a scintillation substance, which blackens an X-ray film.

By autoradiography, two proteins can be compared. They should be labeled with different isotopes – the one protein with ^{3}H-amino acids, the other protein with ^{14}C-amino acids or ^{35}S-methionine. The protein marking is carried out usually in living cells. The two proteins are mixed and separated in the same gel. After 2D electrophoresis two different results can be obtained by different exposures of a film: a ^{3}H and a ^{14}C autoradiogram, which can be compared by overlaying one on the other.

For fluorography, 15 g/dl 2,5-diphenyloxazole (PPO) is used in **di**m**e**thyl **s**ulf-**o**xide (DMSO). To do this, after the 2D electrophoresis the gel is stored in a fixing solution for 1 h, in a destaining solution for 10 min, in DMSO overnight, in 15 g/dl PPO in DMSO for 3.5 hours, and in 5 g/dl glycine for 1 h. Then the gel is dried, wherein 10 wet sheets of filter paper, a wet cellophane membrane, the gel, a second wet cellophane membrane, and a porous polyethylene sheets are put on a perforated metal plate and covered with a rubber flap. All layers are compressed under vacuum, produced by a water-jet pump. The drying is carried out overnight at 50 °C.

An X-ray film is placed on the dried gel. The exposure of the film takes place at –70 °C for 2–3 days. Finally, the film is developed as usual: 6 min developing, 10 min fixing, and 10–20 min water cleaning.

2.13.4.3 2D gel image analysis

To collect data in digital form is a major factor in making 2D electrophoresis a practical method for collecting proteomics information. Digital gel imaging allows comparison of gels, transfer of information among research groups, and cataloging the immense amounts of data. Many types of imaging devices interface with software designed specifically to collect, interpret, and compare proteomics data.

Modern 2D gel analysis software can rapidly analyze spots in the gel. So gel images can be compared, protein spots and catalog data annotated, background subtracted, and noise filtered. With appropriate computer program, the entire analysis process from background correction to spot matching results takes minutes. In modern laboratories, even robots are used for isolation of protein spots from 2D gels.

2.13.5 Protocols

2.13.5.1 Two-dimensional gel electrophoresis using the O'Farrell system

Sample preparation

Materials and equipment

Buffer
Tissue sample
Homogenizer with a pestle
Centrifuge

Procedure

- Place the tissue sample in the homogenizer.
- Add 1.5 to 2.0 ml buffer per 100 mg tissue and homogenize using strokes with a pestle.
- Transfer an aliquot to a 200 µl centrifuge tube and centrifuge at 200,000 g for 1 h.
- Load the supernatant onto the first-dimension gel.

First-dimension gels (isoelectric focusing)

Materials and equipment

Acrylamide
Bisacrylamide
TMEDA
APS
Urea
Ampholytes, pH = 4.0 to 8.0
Nonidet P-40
NaOH
H_3PO_4
Protein samples
Casting cassette
Small vacuum flask

Electrofocusing unit
Power supply

Monomer solution, T = 30.8 g/dl, C = 0.03

Acrylamide	30.0 g
Bisacrylamide	0.8 g
Deionized water to	100.0 ml

Procedure

- Mix 8.25 g urea, 2.0 ml of the monomer solution, 0.75 ml ampholytes, pH = 4.0 to 8.0, 6.0 ml, and deionized water in a small vacuum flask.
- Place the flask in a warm water bath on a magnetic stirrer and stir until the urea is dissolved.
- Deaerate the solution by applying a vacuum for 2 to 3 min.
- Add 0.3 ml Nonidet P-40, swirl until dissolved, and pass through a filter.
- Add TMEDA and APS.
- Pipette the mixture into a casting cassette.
- Allow the monomer solution to polymerize for 1 h.
- Fill the one reservoir of the electrofocusing unit with 0.085 g/dl phosphoric acid, and the other reservoir with 0.02 mol/l NaOH.
- Prefocus the gel at 200 V for 1 h.
- Apply 10 to 30 µl protein samples onto the gel.
- Attach the electric leads to the power supply.
- Focus at constant voltage for 16 h.

Second-dimension gels (SDS electrophoresis)

A 10 to 20 g/dl gradient gel is recommended for resolution over a wide molecular-mass range.

Materials and equipment

First-dimension gel
Acrylamide
Bisacrylamide
SDS
TMEDA
APS
Equilibration buffer
Electrode buffer
Casting cassette
Electrophoresis cell
Power supply

Procedure

- Assemble glass plates to produce a casting cassette with 1.0 mm spacers.
- Prepare a monomer solution mixing buffer, 30 g/dl acrylamide/0.8 g/dl bisacrylamide, 10 g/dl SDS, 10 g/dl TMEDA, 10 g/dl APS, and deionized water (Table 2.13-4). Deaerate by applying vacuum for 5 min.
- Pour the monomer solution in the casting cassette to 5 mm below the top and overlay with deionized water.
- Allow the solution to polymerize for 1 h.
- Using a spatula, slide off a strip from the first-dimension gel and equilibrate it in the equilibration buffer.
- Place the equilibrated gel piece on the SDS gel.
- Mount the gel in an electrophoresis unit.
- Fill the tanks with tank buffer.
- Start cooling at 10 to 20 °C.
- Attach the electric leads to the power supply.
- Electrophorese at 15 to 20 mA/gel until the tracking dye reaches the opposite end of the gel.
- Turn off the power supply.
- Stain the gel and process it for immunoblotting or autoradiography.

Table 2.13-4: Solutions for second-dimension gels.

Stock solutions	**Final monomer concentration, g/dl**			
	7.5	*10*	*12.5*	*15*
Gel buffer	12.50 ml	12.50 ml	12.50 ml	12.50 ml
30 g/dl acrylamide/0.8 g/dl bisacrylamide	12.50 ml	16.70 ml	20.80 ml	25.10 ml
10 g/dl SDS	0.50 ml	0.50 ml	0.50 ml	0.50 ml
10 g/dl TMEDA	0.26 ml	0.26 ml	0.26 ml	0.26 ml
10 g/dl APS	0.25 ml	0.23 ml	0.21 ml	0.19 ml
Deionized water to	50.00 ml	50.00 ml	50.00 ml	50.00 ml

2.13.5.2 Rehydration of IPG strips

IPG strips are rehydrated either in a rehydration chamber or in a IEF system for 1D electrophoresis.

Materials and equipment
IPG strips
Rehydration chamber

Rehydration buffer

Urea	4.80 g (8.0 mol/l)
CHAPS	0.10 g (1.63 mmol/l)
DTT	0.02 g (13.0 mmol/l)
40 g/dl Ampholytes 4–7	0.125 ml (0.5 g/dl)
Bromophenol blue Na salt	0.001 g (0.15 mmol/l)
Deionized water to	10.00 ml

The rehydration buffer can be stored at –20 °C for 3 months.

Procedure
- Pipette the rehydration buffer in the appropriate volume into the rehydration chamber.
- Remove the protective film from an IPG strip.
- Dip the strip with the gel side down into the rehydration buffer and spread it bubble-free over the entire length of the strip.
- After 5–10 min, when the rehydration buffer has been completely absorbed, cover the IPG strip with 2 ml of silicone oil to prevent it from drying out, and left at room temperature for at least 6 hours (better overnight).

2.13.5.3 2D electrophoresis with immobilized pH gradients (IPG-Dalt)

Materials and equipment
Immobilines
40 g/dl Ampholytes, pH = 3.0–10.0
CHAPS
Acrylamide
Bisacrylamide
Urea
SDS
87% Glycerol
TMEDA
APS
Iodoacetamide
1,2-**Dit**hio**t**hreitol (DTT)
Amberlite MB-1 ion exchanger resin
Roller
Glass plates (260 x 125 x 4 mm)
0.5 mm thick U-frame (260 × 125 mm)

Rehydration cassette
Supporting PAG-film (260 × 125 mm)
Magnetic stirrer
Laboratory shaker
Horizontal electrophoresis apparatus
Power supply (3500 V)

Lysis buffer

Urea	30.0 g (9.5 mol/l)
Amberlite MB-1	0.5 g (1.0 g/dl)
Deionized water to	40.0 ml

Stir for 10 min and filter. Do not heat urea solution above 37 °C because of risk of protein carbamylation. Add

CHAPS	1.0 g (2 g/dl CHAPS)
Dithiothreitol	0.5 g (1 g/dl)
Pefabloc	50.0 mg (5 mmol/l)
Pharmalyte pH = 3.0–10.0	1.0 ml (0.8 g/dl)
Deionized water to	50.0 ml

Acrylamide/Bisacrylamide solution (30 g/dl T, 0.04 C)

Acrylamide	28.8 g
Bisacrylamide	1.2 g
Deionized water to	100.0 ml

Add 1 g of Amberlite MB-1, stir for 10 min and filter. Store the solution at 4 °C for mostly a week.

Ammonium persulfate solution

APS	0.4 g
Deionized water to	1.0 ml

Make the solution prior to gel casting.

Sample preparation

- Disrupt the sample in the lysis buffer by sonification in an ice bath and centrifuge at 42,000 g and 15 °C for 60 min.
- Homogenize in a liquid nitrogen cooled mortar, and suspend yeast cells, human or animal tissue in the lysis buffer so that the protein concentration will reach 5–10 mg/ml.
- Precipitate the proteins in the sample at –18 °C for 45 min.

- Centrifuge and wash the pellet with ice-cold acetone containing 0.07 g/dl 2-mercaptoethanol.
- Discard the supernatant and dry the pellet *in vacuo*.
- Apply 0.05–0.100 mg protein for analytical IPG isoelectric focusing onto a single IPG gel strip.

Procedure

- Wet a glass plate with a few drops of water.
- Place a support film for polyacrylamide gel on it, the hydrophilic side upward.
- Place the U-frame (0.5 mm thick) on the support film and a second glass plate on it to build a cassette.
- Clamp the cassette together and let it stay vertically.
- Close the outlet tubing of the mixing chamber and the valve between reservoir and mixing chamber.
- Pipette 12.0 ml of the acidic (heavy) solution into the mixing chamber of the gradient mixer. Add 0.08 ml of 10 g/dl TMEDA and 0.12 ml of 10 g/dl APS, and mix.
- Pipette 12.0 ml of the basic (light) solution into the reservoir of the gradient mixer. Add 0.08 ml of 10 g/dl TMEDA and 0.12 ml of 10 g/dl APS, and mix with a spatula.
- Switch on the magnetic stirrer.
- Open the valve connecting the chambers and then the pinchcock on the outlet tubing so that the solution begins to fill the cassette from above. Take care that the solutions in both chambers flow down equally fast.
- Let the density gradient solution polymerize at room temperature for 1 h.
- Open the cassette and remove the gel.
- Wash the IPG gel with deionized water in a glass tray for 1 h.
- Equilibrate the gel in 2 ml/dl glycerol for 30 min and dry it overnight at room temperature.
- Store the dried IPG gel in a sealed plastic bag at –20 °C for several months.
- Prior to IEF rehydrate the IPG gel strips to the original gel thickness of 0.5 mm.
- Place the rehydrated gel strips on the cooling block of an electrofocusing chamber.
- Apply the samples into the slots of a silicone template placed on the surface of the IPG gel strips.

  Proceed with the 2D electrophoresis as described above.

2.13.6 Troubleshooting

Problem	Cause	Solution
Prior to isoelectric focusing		
The polyacrylamide gel does not polymerize or is sticky.	The concentrations of acrylamide, BIS, TMEDA, or APS in the monomer solution are too low.	Increase the concentrations. Use 5 µl 10 g/dl TMEDA and 5 µl 10 g/dl APS solution per 1 ml monomer solution.
	The APS solution is too old or is stored improperly.	Use a new APS solution. Keep the APS solution in a refrigerator at 4–8 °C. Under these conditions, it is usable for a week.
	The concentration of the air oxygen in the monomer solution is too high.	Deaerate the monomer solution by a water-jet pump.
	The higher concentrations of carrier ampholytes (above 3 g/dl) decrease the gel polymerization.	Use lower concentrations of carrier ampholytes. Polymerize the gel in the absence of carrier ampholytes, dry and rehydrate it with carrier ampholytes containing solutions.
The gel upper edge is sticky and detaches from the support film.	The atmospheric oxygen has inhibited the polymerization of the gel edge.	Overlay the monomer solution with deionized water after casting.
The monomer solution has polymerized too fast.	The concentration of TMEDA or APS in the monomer solution was too high.	Check the recipe for the gel casting.
	The temperature in the lab was very high.	Cast gel at 20–25 °C.
There are air bubbles in the gel.	The glass plate that comes in contact with the monomer solution was dirty.	Wash the glass plate before use.
	The support film was unclean.	Do not touch the hydrophilic side of the support film with fingers.
	Air bubbles in the monomer solution formed by the gradient mixer.	Before casting the gel, clean and dry the outlet tubing of the gradient mixer. Remove the air bubbles in the casting cassette by vigorous tapping.

(continued)

Problem	Cause	Solution
The gel separates from the support film.	An incorrect support film was used.	Do not exchange the support films for polyacrylamide and agarose gels.
	The gel was cast on the wrong side of the support film.	Cast gel only on the hydrophilic side of the support film. Find the correct site using water drops.
	The monomer solution contained a high concentration of non-ionic detergent, such as Triton X-100 or Nonidet NP-40.	Use lower detergent concentration or polymerize the gel in absence of detergents.
The gel does not stick to the support film, but to the glass plate.	The glass plate was hydrophilic.	Clean the glass plate and treat it with Repel-silane.
	The gel was left too long in the casting cassette.	Take out the gel from the casting cassette not later than an hour after the polymerization.
There is liquid on the surface of the polyacrylamide gel.	Over time the polyacrylamide gel hydrolyzes in alkaline buffers.	Use polyacrylamide gels with alkaline buffers within 2 weeks. If possible, let the monomer solution polymerize in neutral or near-neutral buffers.
During electric focusing or SDS-electrophoresis		
It does not flow or flow too little electric current.	One of the connectors has no or poor contact.	Check all connections. The default setting for IPG gel is 5,000 V, 1.0 mA, and 5.0 W.
	There is poor contact between the electrodes and the electrode strips or gels.	Check the uniform laydown of the electrodes on the electrode strips or gels.
The electric current intensity increases during the IEF.	The electrode solutions are changed.	Connect the acidic solution to the anode, and the basic solution to the cathode.

(continued)

Problem	Cause	Solution
There is condensation water on the lid of the separation chamber.	The cooling is not sufficient.	Check the coolant temperature. The cooling block should be made of glass, metal or best of ceramic. Give kerosene or silicone oil DC-200 between the cooling block and support film.
	The electric power is too high.	The electric power should be maximal 2.5 W/ml gel.
There is water condensation on some parts of the gel.	Local overheating is due to air bubbles in the contact fluid.	Remove the air bubbles.
The gel "sweats" (is covered with drops of water).	There are no water-binding additives (glycerol or sucrose) in the gel.	Give in the monomer solution 20 g/dl glycerol or 10 g/dl sucrose. Add to the rehydration solution 20 g/dl glycerol.
The gel sparks and burns.	The support film was not fixed on the glass plate during the gel casting. Therefore, thin spots were formed in the gel.	Before gel casting, roll the support film on the glass plate of the casting cassette with a roller.
Urea in the IPG gels crystallizes.	The IEF temperature is too low.	Focus at 15 °C.
Iso-pH lines in the gel.	The concentration of APS is too high. Braking down of urea in the gel gives isocyanate.	Reduce the concentration of APS or use rehydratable gels. Use the urea gels immediately after casting.
After SDS-electrophoresis		
No bands in the gel.	The cathode and anode were changed.	Check the connection of electrodes to the power supply.
The protein bands in the gel are too weak.	The protein concentration in the sample was too low.	Concentrate the sample, or apply larger sample volumes.
The protein bands are blurred.	The protein-SDS-complexes were not formed.	The SDS concentration should not be lower than 1 g/dl in sample, and 0.1 g/dl in the gel.
	Proteolytic degradation of proteins.	Mix the sample with protease inhibitors (PMSF, EDTA, *etc*.).
	Diffusion after the separation.	Fix the proteins immediately after the power supply is turned off.

(continued)

Problem	Cause	Solution
The gel dissolves from the support film when stained.	The strong acids (TCA) contained in some solutions hydrolyze the bonding between the support film and gel.	Produce gels with a degree of crosslinking $C = 0.02$, instead of usually $C = 0.03$.
The support film rolls on.	The gel contracts.	Give into the final wash solution 5–10 g/dl glycerol to make the gel more elastic.

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2.14 Preparative electrophoresis of proteins

Using preparative electrophoresis, diverse proteins are isolated, for example, ATP-dependent enzymes, respiratory complexes, and more.

Three types of preparative electrophoresis are used: disc-electrophoresis, isoelectric focusing, and QPNC-PAGE.

2.14.1 Preparative disc-electrophoresis

The proteins after disc-electrophoresis can be eluted from the gel either during or after the electrophoresis.

2.14.1.1 Elution of proteins during electrophoresis

Elution of proteins from a gel during electrophoresis can be carried out, if the proteins leaving the resolving gel are removed by a buffer flowing to a collector. For this purpose a gel cassette having a narrow channel in its lower part can be used [1]. The channel is connected with a peristaltic pump. The pump pushes slowly a buffer through the narrow channel and transports the protein fractions to a collector (Figure 2.14-1).

Other construction for protein elution during electrophoresis also uses a gel casting cassette [2]: The cassette is divided by three vertical spacers (two outer and one inner spacer), which form two compartments: a large compartment for the preparative gel, and a small compartment for the detection gel (Figure 2.14-2). On both sides of the preparative polyacrylamide gel, polyethylene tubings are attached reaching its bottom. The detection gel is used to determine the protein positions. Both gels consist of a stacking gel (T = 4 g/dl, C = 0.03) and a resolving gel (T = 10 g/dl, C = 0.03).

After gel polymerization, the glass plates of the gel casting cassette are removed from the preparative gel and a dialysis membrane and both polyethylene tubings

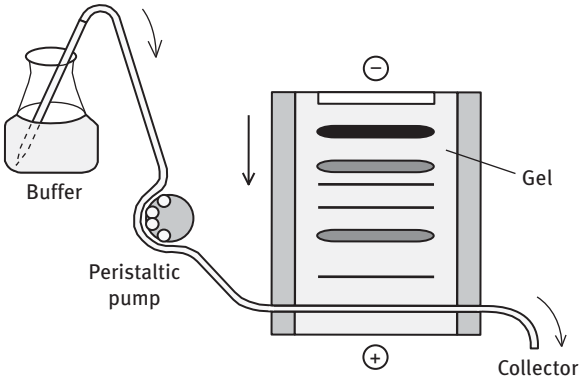

Figure 2.14-1: Scheme of a simple device for protein elution during electrophoresis.

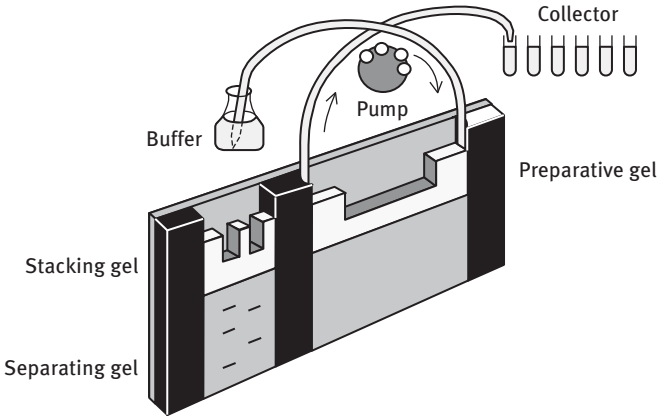

Figure 2.14-2: Cassette for elution of proteins during electrophoresis.
The cassette has two sections: a large section for the preparative gel, and a small section for the detection gel. The lower end of the preparative gel is wrapped in a dialysis membrane. With the help of a 2-channel peristaltic pump, the protein fractions are transported from the dialysis membrane to a collector.

are put around its bottom. Thereafter the glass plates are placed back onto the gel, and the tubings are connected to a peristaltic pump. The inlet tubing is connected to a buffer tank; the outlet tubing is connected to a fraction collector. The eluting buffer is pumped through the tubings, whereas the slow buffer flow leads to smaller collected volumes with high protein concentration; and the fast buffer flow leads to larger collected volumes with low protein concentration.

The electrophoresis can be carried out on polyacrylamide gels containing the SDS buffer system of Laemmli [3] or Schägger–Jagow [4], or in native systems [5,6,7]. The protein yield reaches 89–99%.

2.14.1.2 Elution of proteins after electrophoresis

Usually the location of protein bands in the gel has to be situated prior to preparative electrophoresis. Then the bands must be excised and the proteins eluted. In the case of colored proteins, is easy to find the bands. Otherwise, the protein bands should be detected in the gel.

The protein-staining procedures use organic solvent, acids, or other harsh additives that have to be avoided in preparative electrophoresis [8]. One alternative is to stain a reference lane with proteins or to use prestained standards. A mild staining procedure is the negative zinc-imidazole method [9,10,11]. During it, only the gel background but not the protein bands become turbid. Other alternatives for protein detection in unstained gels are: UV absorbance densitometry [12,13], fluorescence imaging [14,15,16], or phosphorescence at low temperatures [17]. An elegant way to identify enzymes in a gel are in-gel assays for enzyme activities [18,19,20].

The elution of proteins after electrophoresis can be performed by diffusion, dissolving the gel, or electroelution.

Elution by diffusion

Elution by diffusion in a buffer is the simplest method to release proteins from a gel [21]. The solution can be freed from the rests of the gel by filtration or centrifugation. Monomers, TMEDA, and APS, which diffuse together with the proteins, can be removed using ion exchange chromatography [22,23].

For elution by diffusion, buffers without detergent should be used [24]. Only mild reagents, as Triton X-100, can be added [25]. The elution by diffusion is time-consuming and incomplete, as proteins still remain in the gel – the yields are too low [26].

Elution by dissolving the gel

Elution by dissolving (depolymerizing) the gel is a complicated method leading often to chemical modification of proteins. To obtain proteins from gels with the standard cross-linker methylene **bis**acrylamide (BIS), harsh conditions are needed. For example, if a polyacrylamide gel is dissolved by incubation in 30 ml/dl hydrogen peroxide (H_2O_2) at 50 °C [27], irreversible damages of all proteins take place. Another alternative is to use special cross-linker, for example, N,N'-diallyltartardiamide. This gel is solubilized in 2 g/dl periodic acid (H_5IO_6), which is suited for elution of high molecular mass proteins [28]. Unfortunately, the usage of periodic acid is not adequate for most protein complexes [29,30,31].

Extraction of proteins from gels that contain ethylene diacrylate as cross-linker is carried out by adding strong bases like 1 mol/l NaOH [32,33,34]. Milder dissolving conditions are needed for gels with cleavable disulfide cross-linkers. The cleavable bonds are not stable under alkaline conditions [35].

Electroelution

The electroelution of proteins from a preparative gel is made in electric field, which drives the proteins to migrate out from the gel to be trapped in a dialysis bag. This technique is characterized by a high yield. With the help of electroelution, insulin, myoglobin, and bovine serum albumin are eluted from the gel up to 95%.

A standard slab gel electrophoresis device for vertical electrophoresis can be used for electroelution. A support gel is cast and overlaid with gel pieces, which contain the proteins of interest. A small volume of buffer is poured in and the chamber is sealed by a dialysis membrane. The elution is carried out in direction opposite to the electrophoresis direction [36,37,38].

The electroelution of resolved proteins can be carried out in 0.05 mol/l ammonium hydrogen carbonate (NH_4HCO_3) buffer with pH = 7.4 at room temperature [39], or in a buffer system [40]. Good results are obtained in a 0.05 mol/l TRIS-chloride buffer with pH = 7.4 [41].

A few types of electroelution are known: vertical-type elution, horizontal-type elution, bridge-type elution, discontinuous conductivity gradient elution, and steady-state stacking elution [42] (Figure 2.14-3).

Vertical-type elution

In its simplest variant, an apparatus for vertical-type elution consists of a tube with buffer, sieve, a dialysis bag, and a tank with buffer [43]. The elution is carried out in the following steps: the dialysis bag is attached on the sieved tube, which is filled with buffer, and placed into a tank with the same buffer (Figure 2.14-3*a*). The polyacrylamide gel is cut into small pieces and put into the tube. Thereafter, electric current is applied, which causes the proteins to penetrate through the sieve into the dialysis bag and stay there. The dialysis membrane can be omitted [44] using a layer of hydroxylapatite, which captures the protein. The bound proteins can be eluted by phosphate buffer with other pH value. Finally the protein eluate can be concentrated and lyophilized.

The main problem of the vertical-type elution is that air bubbles are easily trapped beneath the dialysis membrane when the column is placed into the buffer.

Horizontal-type elution

For horizontal-type elution, a flat-bed electrophoresis tank and a special column are needed [45,46] (Figure 2.14-3*b*). Both ends of the column are sealed with semipermeable membranes to form an elution chamber. The chamber is divided by a large-pore membrane into two compartments. The gel pieces are placed in the large division. After applying an electric field, the proteins migrate into the small division and are retained there by a small-pore membrane.

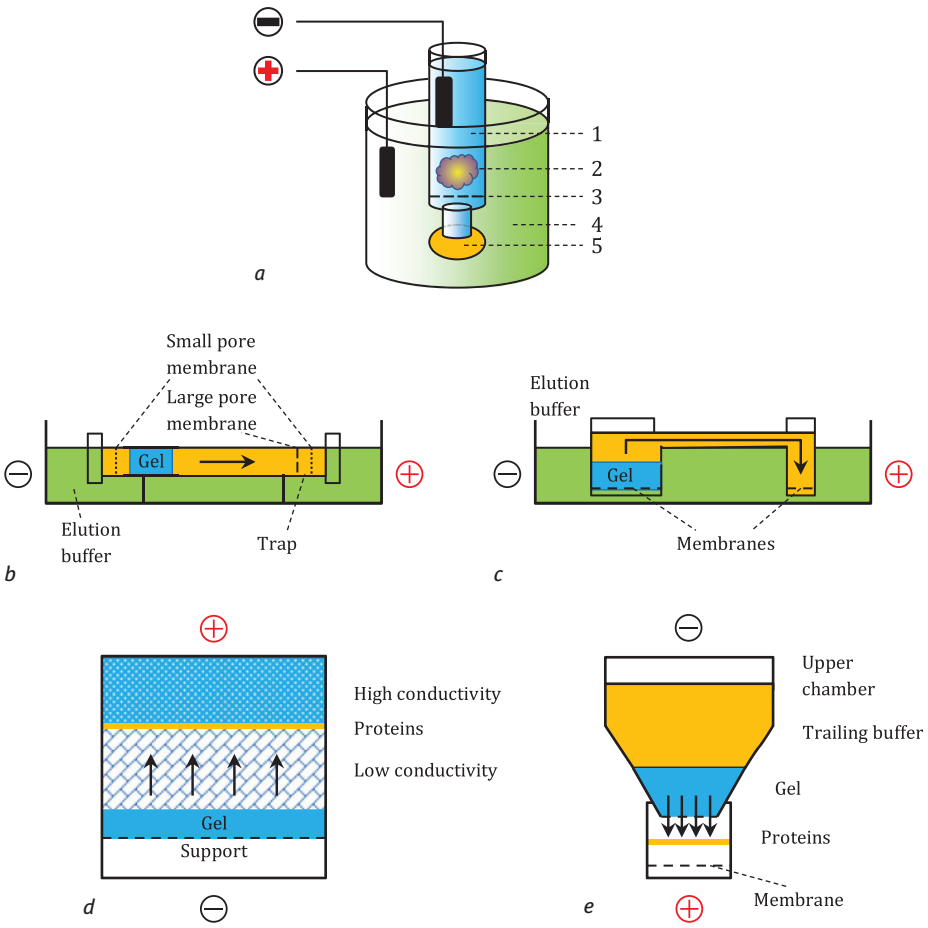

Figure 2.14-3: Different types of electroelution of proteins. The proteins are collected on a small-pore membrane that supports the gel.
a) Vertical-type elution: 1. Tube with buffer; 2. Gel; 3. Sieve; 4. Tank with buffer; 5. Dialysis bag.
b) Horizontal-type elution: Small-pore and large-pore membranes are used to collect the eluted proteins.
c) Bridge-type elution: The electric field drives proteins out from the bigger chamber through a bridge into the smaller chamber.
d) Discontinuous conductivity gradient elution: The trapping process is based on the migration of proteins in a high conductivity layer.
e) Steady-state elution: Isotachophoresis is expanded to elute proteins in a funnel-shaped device.

Bridge-type elution

The construction for bridge-type elution consists of two separate horizontal chambers, which are connected by a bridge [47,48] (Fig. 2.14-3*c*). Both chambers are sealed by dialysis membranes. The bigger chamber contains the gel material with proteins, and

the smaller chamber collects the eluate. The proteins migrate electrophoretically out of the bigger chamber, pass the bridge, and are captured in the smaller chamber.

Discontinuous conductivity gradient elution

Instead of using a dialysis membrane, a combination of two solutions can retain the proteins. A low-conductivity glycerol layer is surrounding the gel (Figure 2.14-3*d*). A second high-conductivity glycerol layer is set upon the first one [49,50,51]. Proteins in the low-conductivity zone migrate rapidly out of the gel. When they reach the upper zone, their migration slows down because of the highly concentrated solution. So no membrane is needed what is an advantage. However, the high concentrated layer can cause a salting-out effect, which diminishes the yield.

Steady-state stacking elution

The steady-state stacking (isotachophoresis) [52,53] can be expanded [54] and the separated proteins can be electroeluted in funnel shaped device [55,56] (Figure 2.14-3*e*). Standard electrophoresis equipment can also be applied for steady-state stacking elution, if gel pieces are embedded in a new stacking gel overlaid with a glycerol layer [57]. As a result, proteins leaving the gel pieces are concentrated and released into the glycerol solution. The steady-state stacking elution can be combined with a stabilizing medium like Sephadex G-25 [58,59], or cellulose [60].

2.14.2 Preparative isoelectric focusing

Preparative isoelectric focusing can be performed with carrier ampholytes or in immobilized pH gradients.

2.14.2.1 Preparative IEF with carrier ampholytes in granulated gels

The preparative isoelectric focusing using carrier ampholytes is carried out in horizontal granulated gels [61,62] in the following steps:

- Producing a granulated gel
- Introducing a sample into the granulated gel
- Isoelectric focusing
- Elution of proteins

Producing a granulated gel

For preparative IEF, dextran products Sephadex G-75, G-200, or best Ultrodex can be used [63].

Prior to preparing the gel, Sephadex G-75 and G-200 should be washed with deionized water. First, the gel absorbs water and swells, and then the water flows

through the swollen gel and is extracted using a water-jet pump. When the difference between the conductivities of water before and after washing has reached a minimum, the washing process can be stopped. If Ultrodex is used, washing is omitted.

The preparation of a horizontal granulated layer involves 3 steps: 1. Mixing the washed gel with carrier ampholytes; 2. Inserting electrode strips into the mold and casting the mixture of gel and carrier ampholytes; and 3. Drying partially the mixture of gel and carrier ampholytes (Figure 2.14-4).

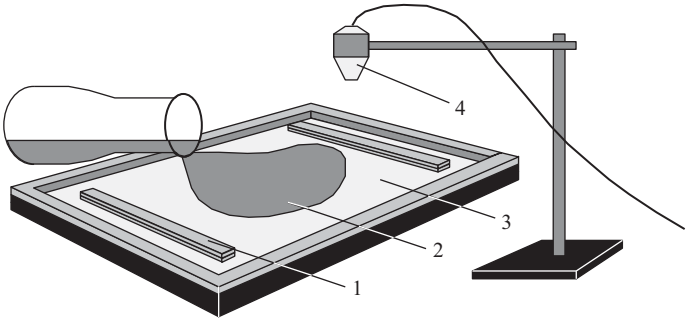

Figure 2.14-4: Producing a horizontal layer of granulated gel for preparative IEF with carrier ampholytes.
The granulated gel is mixed with carrier ampholytes. Then electrode strips (1) are inserted, the mixture (2) is cast into a mold (3), and the gel layer is partially dried with cold air using a hair dryer (4).

Mixing the washed gel with carrier ampholytes. For preparative isoelectric focusing of 10 mg protein using 2 g/dl carrier ampholytes, the volume of the partially dried gel layer

$$V_g = V_o \frac{m_p}{10} \tag{2.14-1}$$

where V_0 is the standard volume of 1 ml, and m_p, in mg, is the mass of protein to be separated. Because the mass of the swollen gel is reduced at 65% when dried, the volume of the swollen gel must be 100/65 = 1.5 times greater than the volume of the partially dried gel.

The volume of carrier ampholytes solution V_{CA}, which must be added to the gel, is given by the equation

$$V_{CA} = V_g \frac{c_{CA}}{c_{SL}} \tag{2.14-2}$$

where c_{CA} is the final concentration of carrier ampholytes (usually 2 g/dl) after partial drying of the gel – carrier ampholytes mixture, and c_{SL} is the concentration of carrier ampholytes stock solution (usually 40 g/dl). The mixture should be cast into a mold (a glass plate with a silicone frame).

Inserting electrode strips into the mold and casting the mixture of gel and carrier ampholytes. Filter paper strips, 5–7 mm wide and as long as the mold, are impregnated with 1 g/dl carrier ampholytes and placed onto the cathode and anode side of the mold. Then, the gel – carrier ampholytes mixture is cast inside.

Drying partially the mixture of gel and carrier ampholytes. The gel – carrier ampholytes mixture is dried with cold air using a hair dryer from about 70 cm height to obtain 65% of its original mass. This process takes place in about 2 h. During it, the mixture mass must be weighted, with the mold, from time to time until its final mass

$$m = m_g + 0.65m_o \tag{2.14-3}$$

where m_g is the mass of the empty mold with electrode strips, and m_0 is the mass of the gel – carrier ampholytes mixture prior to the partial drying.

Introducing a sample into a granulated gel

The salt concentration in the sample must be, as in analytical isoelectric focusing, as low as possible; the concentration of carrier ampholytes in gels is usually 2 g/dl.

The sample can be uniformly distributed in the gel layer, or introduced as a zone in the gel layer. In the first case, the sample is added when the gel is mixed with the carrier ampholytes. In the second case, the gel layer, containing the dissolved ampholines, is cast in a rectangular frame, and a part of it is taken off using a spatula (at least 2 cm away from the electrode strips) forming a well. The gel part is transferred into a Becher glass where is mixed with the sample. Then the mixture is poured back into the well (Figure 2.14-5).

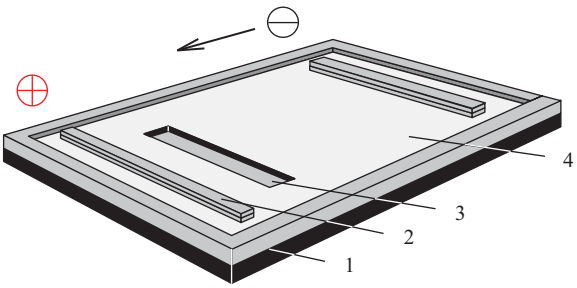

Figure 2.14-5: Application of a sample as a zone in a granulated gel layer.
1. Glass plate with a silicone frame; 2. Electrode strip; 3. Well in the gel layer; 4. Granulated gel

Isoelectric focusing

Before starting the preparative isoelectric focusing, moist filter paper strips are placed on both electrodes. The anode filter paper strip is impregnated with 0.5 mol/l H_3PO_4,

the cathode filter paper strip is impregnated with 0.5 mol/l NaOH. Thereafter, the mold with the granulated gel layer is placed onto the cooling plate of an electrophoresis unit. Between the glass plate of the mold and the cooling plate of the electrophoresis unit, 1–2 ml of water is applied to improve the heat exchange. Then the safety lid of the separation unit is placed on and the electrophoresis unit is connected to the power supply. The conditions for the preparative isoelectric focusing are given in Table 2.14-1:

Table 2.14-1: Conditions for preparative IEF in 20 cm horizontal granulated gel layer containing 2 g/dl carrier ampholytes.

	***P*, W**	***I*, mA**	***U*, V**	***Time*, h**
Prefocusing	Increase the electric power until voltage is 300 V.	Maximum	Maximum	2
Focusing	Increase the electric power until voltage is 600 V.	Maximum	Maximum	16–20

As mentioned, the general problem of biological samples subjected to IEF is the high concentration of salts, which can destroy or constrict the pH gradient and thus decrease the resolution of IEF [64]. Various techniques have been used to remove the salts from the sample, for example, dialysis [65,66], or desalting by columns [67].

Based on the newly established electrophoretic method called **d**ivergent **f**low IEF (DF IEF), a DF IEF instrument was proposed which operates without carrier ampholytes [68]. In DF IEF, the proteins are separated, desalted and concentrated in one step. For example, the proteins in a yeast lysate sample, collected at the DF IEF output and subjected to gel IEF, contained proteins gradually covering the pI range from 3.7 to 8.5. The highest number of proteins has been found in fractions with pI values around 5.3 as proved by polyacrylamide gel IEF with Coomassie brilliant blue staining. During DF IEF, the proteins have been concentrated up to 16.8-fold.

Elution of proteins

After preparative electrofocusing, the visualization of protein bands in the mold is performed placing a filter paper on the granulated gel, staining the protein blot, then placing it under the mold, and finally taking out the gel segment with the desired protein. Next, the protein can be extracted from the gel segment and analyzed.

So collected, proteins must be freed from the accompanying carrier ampholytes. For this purpose, different methods are used to separate the low mass carrier ampholytes ($M_r \approx 800$) from high mass proteins: dialysis, gel filtration, and ultrafiltration. Finally, the proteins are lyophilized and frozen.

2.14.2.2 Preparative IEF in immobilized pH gradients

Preparative isoelectric focusing can also be carried out in immobilized pH gradients [69,70,71]. Two types of this technique are known: preparative IEF in thick IPG gels, and channel focusing.

Preparative IEF in thick IPG gels

The preparative IEF can be carried out in 5 mm thick IPG gels [72]. The gels are prepared in the same way as thin IPG gels, but in cassettes with appropriate thickness. For the sample application, a 2–3 mm deep sample slot is formed during the gel casting. It should be located at least 15 mm away from the anode or cathode gel end, and at least 5 mm away from the left and right gel margin.

After the preparative IEF in a thick IPG gel, a gel strip is cut off from the gel and stained with Coomassie brilliant blue R-250. So the positions of the separated proteins are determined.

The proteins bind more strongly to IPG gels than to other media, so they have to be electrophoretically eluted. To do this, strips with the separated proteins of interest are cut off from the IPG gel and transferred into a granulated gel. The process is fulfilled in the following way:

Agarose with low electroosmosis in concentration of 0.8 g/dl is suspended in a buffer, for example, 0.1 mol/l TRIS-glycinate buffer (pH = 9.1), and after dissolving by heating is cast in a mold with a 5 mm thick silicone frame. Then two bands are excised from the agarose gel, so that two wells are formed. The IPG gel strip with the proteins to be eluted is inserted in the first well; the second well is filled with granulated gel swollen in the same elution buffer. Thereafter, the proteins are transferred electrophoretically out of the IPG gel strip through the agarose gel in the granulated gel. Finally the granulated gel is placed into an elution column and the proteins are eluted with elution buffer (Figure 2.14-6).

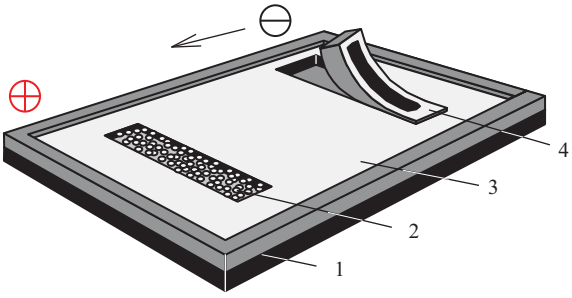

Figure 2.14-6: Electrophoretic elution of proteins by preparative IEF in a thick IPG gel.
1. Glass plate with a silicone frame; 2. Granulated gel that collects the proteins; 3. Agarose gel; 4. IPG gel strip with focused proteins

Channel focusing

In the channel focusing [73,74], the proteins are separated by preparative electrofocusing in an IPG gel, and then are transferred in a granulated gel. This method comprises the following steps:

- Casting a preparative IPG gel
- Analytical IEF in the margins of the IPG gel
- Digging channels and running a preparative isoelectric focusing
- Elution of proteins (Figure 2.14-7)

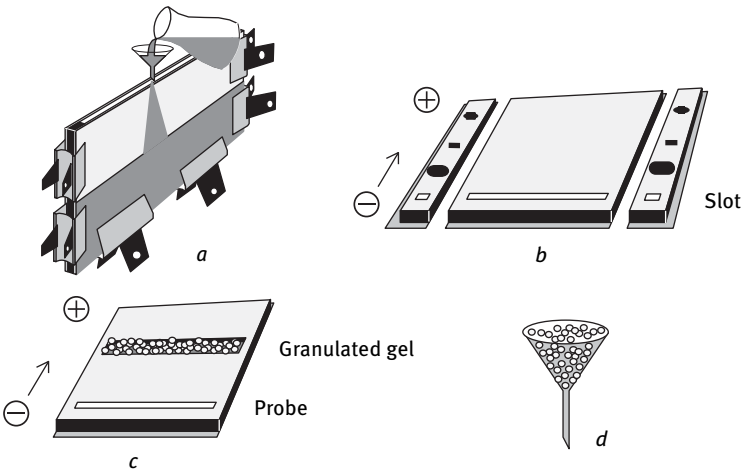

Figure 2.14-7: Channel focusing.
a) Casting an IPG gel; *b*) Cutting margins of the IPG gel and running analytical IEF to determine the position of proteins of interest; *c*) Making a well (channel), filling it with granulated gel, and carrying out preparative focusing; *d*) Extracting and eluting proteins from the granulated gel

Casting a preparative IPG gel. An IPG gel for channel focusing is cast as an IPG gel for analytical IEF. Two short slots are made in the gel margins, for analytical electrophoresis of proteins of interest, and a long slot is made in the medium of the gel, for application of sample.

Analytical IEF in the margins of the IPG gel. Margin strips containing the application slots are cut from the dried IPG gel and rehydrated. Then a small portion of the sample is undergone analytical electrofocusing and the obtained protein bands are stained to determine the protein position.

Digging channels and running preparative isoelectric focusing. The remaining gel is rehydrated as the margin strips and is placed onto a glass plate next to the strips with the stained protein bands. Using a spatula, a channel is cut into the gel, against the desired protein. The channel is filled with a swollen granulated gel, which will collect the protein at its migration. If necessary, more channels can be cut for different proteins.

The swollen granulated gel is prepared in the following way: a granulated gel is suspended in a test tube with excess of water and is swollen for 15 min. Then the supernatant water is decanted and the swollen gel is cast into the channel using a syringe with a thick needle.

The preparative isoelectric focusing in an IPG gel is carried out under the same conditions as the analytical IEF in IPG gels.

Elution of proteins. After the preparative electrofocusing in an IPG gel, the content of the channel is taken up with a spatula and is transferred to an elution column. The proteins are eluted, frozen, or lyophilized, as the proteins separated in the preparative IEF with carrier ampholytes. The gel is cleaned with a buffer from the rest of proteins, washed and dried. It can be used for next channel IPG focusing.

2.14.2.3 Recycling isoelectric focusing

The **r**ecycling **i**so**e**lectric **f**ocusing (RIEF) according to Bier *et al.* [75,76] is based on the principle of continuous recirculation of a separation medium in a special apparatus. It consists of an IEF unit, a multi-channel peristaltic pump, pH monitor, and a thermostat. The pH value, the conductivity, the temperature, and the optical density of the protein fractions are measured and controlled with a computer program.

The IEF unit has a width of 3 cm and a total volume of 25 ml. It is composed of 12 Plexiglas columns, each of them having an inlet tubing above and outlet tubing below the recycling buffer. Each column is separated from the neighboring column by a 10 μm pore size membrane. To the first and twelfth column, electrode solutions are connected, which are separated from the adjacent columns by ion exchange membranes. The multi-channel peristaltic pump transports the electrode solutions, the mixture of sample, and the carrier ampholytes, divided in 10 fractions. The thermostat has also 12 channels: 10 for the mixture of sample and carrier ampholytes, and 2 for the electrode solutions (Figure 2.14-8).

The IEF unit is filled with carrier ampholytes and the sample, which are then recycled through the pump, pH monitor, and thermostat. When a pH gradient between the columns is formed, the proteins migrate through the membranes until they reach their isoelectric points. The Joule heating, which is produced during the recycled isoelectric focusing, is removed by the thermostat.

The recycled isoelectric focusing has been used for purification of monoclonal and polyclonal immunoglobulins [77].

2.14.3 QPNC-PAGE

QPNC-PAGE (**q**uantitative **p**reparative **n**ative **c**ontinuous **p**oly**a**crylamide **g**el **e**lectrophoresis) is a variant of polyacrylamide gel zone electrophoresis. It is used to

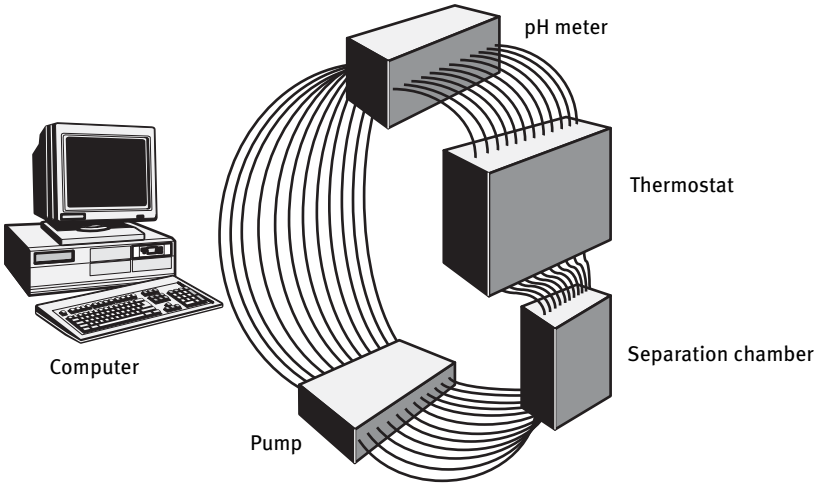

Figure 2.14-8: Setup of recycling isoelectric focusing apparatus.

isolate metalloproteins in biological samples and to resolve properly and improperly folded metal cofactor-containing proteins or protein isoforms [78]. QPNC-PAGE is based on the **t**ime of **pol**ymerization of **a**cryl**a**mide (tpolAA).

The time of polymerization of a gel may affect the peak-elution times of separated metalloproteins in a pherogram due to the compression of the gel and its pores on proteins. In order to insure maximum reproducibility in the gel pore size and to obtain a fully polymerized large pore gel for a PAGE, the polyacrylamide gel is polymerized for 69 h at room temperature (tpolAA = 69 h). The heat generated by the polymerization process is dissipated constantly. As a result, the prepared gel is homogeneous, stable, hydrophilic, electrically neutral, free of monomers or radicals, and does not bind proteins [79].

The buffer used for electrophoresis contains 20 mmol/l TRIS-HCl and 1 mmol/l NaN_3, and has pH = 10.0. Under these conditions, most proteins in the sample are charged negatively and migrate from the cathode to the anode in an electric field. The proteins are eluted continuously by a physiological buffer and isolated in different fractions.

A gel of T = 4 g/dl and C = 0.267 is non-sieving, the interactions of the gel with the biopolyions are negligible low, and proteins migrate only according to their free mobility [80]. Therefore, this gel is optimal for electrophoresis of proteins with M_r = 200,000.

QPNC-PAGE is used for isolating native or improperly folded metalloproteins, for example, **c**opper **c**haperone for **s**uperoxide dismutase (CCS), **s**uper**o**xide **d**ismutase (SOD), prions, transport proteins, amyloids, and metalloenzymes, which are present in brain blood or other samples in Alzheimer's disease or amyotrophic lateral sclerosis [81]. CCS or SOD molecules control the concentrations of essential

metal ions (e.g., Cu^{+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Mo^{2+}, Pd^{2+}, Co^{2+}, Mn^{2+}, Pt^{2+}, Cr^{3+}, Cd^{2+}, and more) in organisms and thus balance prooxidative and antioxidative processes in the cytoplasm [82]. They can be identified and quantified by **i**nductively **c**oupled **p**lasma **m**ass **s**pectrometry (ICP-MS). During the electrophoresis the metalloproteins are not dissociated into apoproteins and metal cofactors.

2.14.4 Protocols

2.14.4.1 Visualizing and elution of proteins in a granulated gel

Materials and equipment

Mold
Granulated gel with separated proteins
Filter paper
Tri**c**hloro**a**cetic acid (TCA)
Coomassie brilliant blue R-250
Methanol
Acetic acid
Glass plates
Hair dryer
Elution buffer

Procedure

- Place a filter paper sheet for 60 s on the granulated gel so that no air bubbles are trapped (Figure 2.14-9).
- Remove the filter paper sheet, place it on a glass plate, and dry out with hair dryer.
- Fix the transferred proteins in 10 g/dl trichloroacetic acid for 5 min.
- Stain in 0.1 g/dl Coomassie brilliant blue R-250 in methanol – acetic acid – water (3:1:6, *V:V:V*) for 5 min.
- Destain the background in methanol – acetic acid – water (3:1:6, *V:V:V*).
- Place the moist filter paper sheet under the mold to determine the location of the protein fractions in the granulated gel.
- Using a spatula take out those segments of the granulated gel, which contain the desired proteins.
- Transfer the segments of the granulated gel into polyethylene or glass tubes closed below by a sieve or glass wool.
- Rinse the segments of the granulated gel with elution buffer. During this process, the granulated gel remains retained in the tube while the buffer, together with the proteins, is collected in test tubes.
- Analyze the proteins.

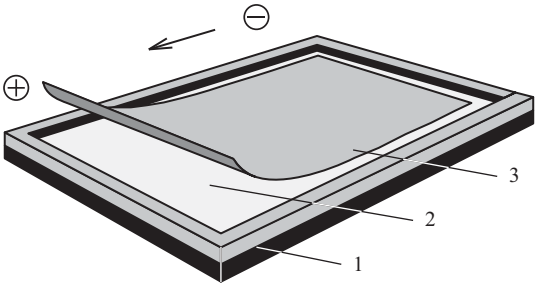

Figure 2.14-9: Placing a filter paper sheet onto a granulated gel to visualize the protein positions. 1. Glass plate with a silicone frame; 2. Granulated gel; 3. Filter paper sheet.

2.14.4.2 QPNC-PAGE

Materials and equipment

TRIS
HCl
Acrylamide
BIS
TMEDA
Ammonium **p**eroxydi**s**ulfate (APS)
Sodium azide
Buffer recirculation pump

Stock solutions

200 mmol/l TRIS-HCl, 10 mmol/l NaN_3, pH = 10.0
200 mmol/l TRIS-HCl, 10 mmol/l NaN_3, pH = 8.0
Acrylamide/BIS, T = 40 g/dl, C = 0.027.
10 g/dl **A**mmonium **p**eroxydi**s**ulfate (APS).
Prepare freshly.

Electrophoresis buffer

20 mmol/l TRIS-HCl, 1 mmol/l NaN_3, pH = 10.0. Keep at 4 °C.

Monomer solution, T = 4 g/dl, C = 0.027

Acrylamide/BIS	10.0 ml
10 g/dl TMEDA	0.5 ml
10 g/dl APS	0.5 ml
Add prior to gel casting.	
Electrophoresis buffer to	100.0 ml

Elution buffer

20 mmol/l TRIS-HCl, 1 mmol/l NaN_3, pH = 8.0. Keep at 4 °C.

Procedure

- Pipette the monomer solution to a level of 40 mm in a graduated glass column with an inner diameter of 28 mm. Add APS last. The total time of polymerization is 69 hr at room temperature.
- Mix 3.0 ml of a sample with 0.3 ml glycerol.
- Apply the mixture carefully under the upper electrophoresis buffer and run electrophoresis at 4 °C.
- Elute the separated proteins continuously by the eluent in a special elution chamber and transport them to a fraction collector.

2.14.5 Troubleshooting

Problem	Cause	Solution
Prior to electrophoresis		
The monomers do not polymerize or polymerize to slow.	The monomer solution is too old.	The monomer solution should be used within one month.
	The concentration of TMEDA or APS in the monomer solution is too low.	Increase the concentration of TMEDA or APS. Use 5 µl 10 g/dl TMEDA, and 5 µl 10 g/dl APS per 1 ml monomer solution.
	The APS solution is too old or stored improperly.	Use a new APS solution.
	The polymerization temperature is too low.	Cast gel at 20–25 °C.
	The concentration of air oxygen in the monomer solution is too high.	Degas the monomer solution using a water-jet pump.
The monomer solution polymerizes too quickly.	The concentration of TMEDA or APS in the monomer solution is too high.	Check the recipe for gel casting. Reduce the concentration of TMEDA or APS.
	The polymerization temperature is too high.	Cast gel at 20–25 °C.
Polyacrylamide gels are too soft and sticky.	The concentration of acrylamide or BIS is too low.	Increase the concentration of acrylamide or BIS.
	The monomer solution was superstored.	Store the monomer solution in the refrigerator for maximum one month.
	The concentration of TMEDA or APS in the monomer solution was too low.	Use 5 µl 10 g/dl TMEDA and 5 µl 10 g/dl APS solution per 1 ml monomer solution.
	There was too much oxygen in the monomer solution.	Degas the monomer solution with a water-jet pump.

(continued)

Problem	Cause	Solution
Air bubbles in the polyacrylamide gel.	The support film was unclean.	Do not touch the hydrophilic side of the support film with fingers.
	The glass plate that comes in touch with the monomer solution was dirty.	Wash the glass plate with detergent or ethanol before use.
The gel separates from the support film.	An incorrect support film was used.	Do not exchange the support film for polyacrylamide and agarose gels.
	The gel was poured onto the wrong side of the support film.	Cast monomer solution onto the hydrophilic side of the support film; check first the site with water drops.
	The support film was stored incorrectly or too long.	Store the support film dry and in the dark at room temperature.
During electrophoresis		
It does not flow or flows too little electric current.	The contact with the power supply is poor.	Check all connections.
	The contact between the electrode strips (gels) and the separation gel is poor.	Check the horizontal position of the electrodes; weight up with a glass plate.
The gel “evaporates” and the lid of the separation chamber is covered with condensed water.	The voltage is too high. The cooling is insufficient.	Reduce the voltage. Check the coolant temperature. The cooling blocks should be made of glass, metal, or best of ceramic. Give kerosene or silicone oil DC 200 between the cooling block and support film.
The gel sparks and burns.	Poor cooling of the gel.	Give kerosene or silicone oil DC 200 between the support film and cooling plate.
Sparking along the edges of the gel.	The electrode strips have been extended over the gel edges.	Cut the electrode strips shorter than the gel width.
The electric current intensity increases during IEF.	The electrode solutions were exchanged.	Apply the basic electrode solution on the anode gel end, and the acidic electrode solution on the cathode gel end.

(continued)

Problem	Cause	Solution
After electrophoresis		
No protein bands in the gel.	The cathode and anode were exchanged. The proteins have left the gel.	Check the connections to power supply. Monitor the dye front.
The protein bands form tails.	The sample contained too much protein. The sample contained undissolved particles.	Apply a smaller sample volume or dilute the sample. Dissolve the particles in the sample using ultra-sonic device.
There are precipitates on the place where the sample was applied.	The proteins were not resolved totally. The protein or salt concentration in the sample was too high.	Use higher urea concentration (9 mol/l). Add non-ionic detergent (Nonidet NP-40) to the sample and into the gel. Dilute the sample and desalt.

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2.15 Microchip electrophoresis of proteins

The **m**icrofluidic chip (microchip) **e**lectrophoresis (ME) resembles a miniaturized capillary electrophoresis in a planar minidevice, which has a higher degree of automation and carries out quick analyses. Sample application and separation are run in narrow channels, which are made from a glass, silicone or polymer material by using photolithographic processes. The channels are arranged in the form of a cross and are connected with four reservoirs. The shorter channel is dedicated to sample injection, whereas the longer one represents the separating channel [1]. The channels are filled with buffers and samples, and are collecting the wastes. They have diameters of about 50 µm, volumes of 10–50 µl, and size of 1 to 10 cm.

Since the pioneer works of Harrison, Manz *et al.* [2,3], the microchips are of considerable interest owing to their portability, small sample consumption, minimal reagent consumption, and high speed of electrophoresis [4,5,6]. They are used in clinical and forensic diagnostics [7], proteomics [8], pharmaceutical analysis, environmental monitoring, and more.

The principle of microchip electrophoresis is the same principle of conventional electrophoresis. Here a tiny portion of sample is injected at the intersection of the channels, then is punched by switching the voltage, and is sent to the obstacle course for separation (Figure 2.15-1).

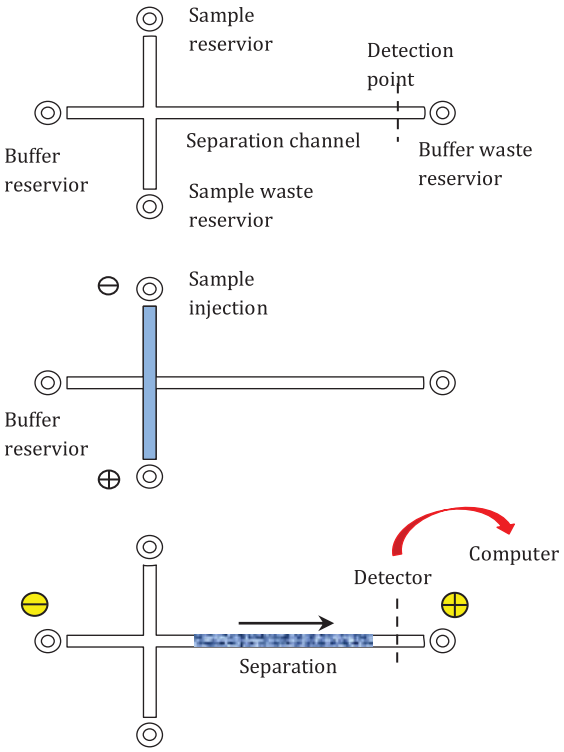


Figure 2.15-1: Events in microchip electrophoresis.

After filling with buffer, the injection of a sample into the microchip is achieved by electrokinetic mode: The sample is first located into the sample reservoir, and then a suitable voltage is applied between the sample load reservoir and sample waste reservoir. The electrophoretic mobilities of analytes during microchip electrophoresis are often combined to the electroosmotic flow, driven by the surface charges of the channel. This leads to displacement of the sample through the intersection of the two channels. Then voltage is given to run electrophoresis.

In order to decrease the electroosmotic flow on polymeric microchips, many surface modification methods have been investigated [9]: coating with inorganic anions [10] or polyelectrolytes [11,12,13], addition of surfactants to the resolving buffer [14], covalent modifications, and more. In all cases, the added compounds change the ζ-potential [15,16]. Such surfactants are: Brij-35, Tween 20, **c**etyl**t**rimethyl**a**mmonium

bromide (CTAB) [17], **s**odium **d**odecyl **s**ulfate (SDS) [18], **di**dodecyldimethyl**a**mmonium **b**romide (DDAB), 1,2-**d**i-**l**auroyl-*sn*-**p**hosphatidyl**c**holine (DLPC), Triton X-100, and palmityl sulfobetaine. They all have a strong influence on the electroosmotic flow [19].

2.15.1 Advantages and disadvantages of microchip electrophoresis

The microchip electrophoresis has many advantages toward the conventional electrophoresis [20,21,22]:

- Low reagent volumes consumption.
- Small sample volume.
- Faster analysis time.
- Compactness of the system.
- Mass production [23].

However, it has also some disadvantages:

- Physical and chemical effects (capillary forces, surface roughness, and chemical interactions of construction materials) can make the processes more complex than in the conventional electrophoresis.
- Difficulties with the detection processes.
- The microchips do not offer convenient methods for collection of the sample bands.

2.15.2 Microchip materials

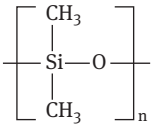
Early microchips were made from glass [24,25,26], silicon [27,28,29], and quartz [30,31,32]. The glass microfluidic chips were mainly fabricated using standard photolithographic techniques [33]. However, their application was limited because of high cost, harmfulness, and complicated fabrication procedures [34]. Nowadays the microchips are made of rigid polymers [35,36,37]. They can be fabricated using *in situ* polymerization [38], laser ablation [39], imprinting [40], injection molding [41,42], hot embossing [43,44,45], and so on.

The most popular polymers for preparing microchips are PDMS, PMMA, and PC.

2.15.2.1 PDMS

Poly**di**m**e**thyl**s**iloxane (PDMS) is the most appropriate polymeric material for microchip fabrication. It has many advantages over other polymer materials: PDMS is non-toxic, inert, optically transparent, flexible, and non-flammable [46,47,48].

PDMS belongs to the group of polymeric organosilicon compounds that are referred to as silicones [49,50]. It has the chemical formula $CH_3[Si(CH_3)_2O]_nSi(CH_3)_3$, where n is the number of the repeating monomer $[Si(CH_3)_2O]$ units [51].



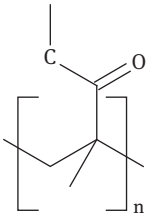
PDMS

Industrial synthesis of PDMS begins from dimethyldichlorosilane and water under UV radiation. The UV radiation generates free radicals that initiate polymerization and release of HCl. For medical applications, the chlorine atoms in the silane precursor were replaced with acetate groups. In this case, the polymerization produces CH_3COOH, which is less aggressive than HCl. When n has a low value, the polymer is manufactured as a thin pourable liquid; when n has a high value, a thick rubbery semi-solid is produced. PDMS microchips can be cast in molds or prosess using soft lithography [52,53].

However, PDMS has some disadvantages. The most prominent among them is its hydrophobicity [54,55]. It hinders the introducing of aqueous solutions into the microchannels. To decrease the hydrophobicity of PDMS, oxygen plasma [56], or UV/ozone [57] treatment can be used. During oxygen plasma treatment, surface silanol groups (–SiOH) are added to the siloxane backbone, which makes the polymer hydrophilic and transparent down to 280 nm [58]. For this process atmospheric air plasma and argon plasma are needed. The oxidized surface is stable in air for about 30 min [59].

2.15.2.2 PMMA

Poly(**m**ethyl **m**eth**a**crylate) (PMMA), known also as acrylic glass as well as Plexiglas, Crylux, Acrylite, Lucite, and Perspex, was developed in 1928 in different laboratories by many chemists, such as William Chalmers, Otto Röhm, and Walter Bauer. It is a transparent thermoplastic used often in sheet form as alternative to glass [60,61,62]. PMMA is one of the most common polymeric substrates for fabrication of microchips, which are applied for protein separation [63]. Chemically, PMMA is the synthetic polymer of methyl methacrylate.



PMMA

PMMA is an ideal polymer for fabrication of microfluidic chips because of low price, optical transparency, and good mechanical properties [64,65]. It is the least hydrophobic polymer among the plastic materials, which generates limited electroosmosis in the microchannels during electrophoresis [66].

The PMMA surface can be modified by aminolysis, reduction, and photoactivation (Figure 2.15-2) [67].

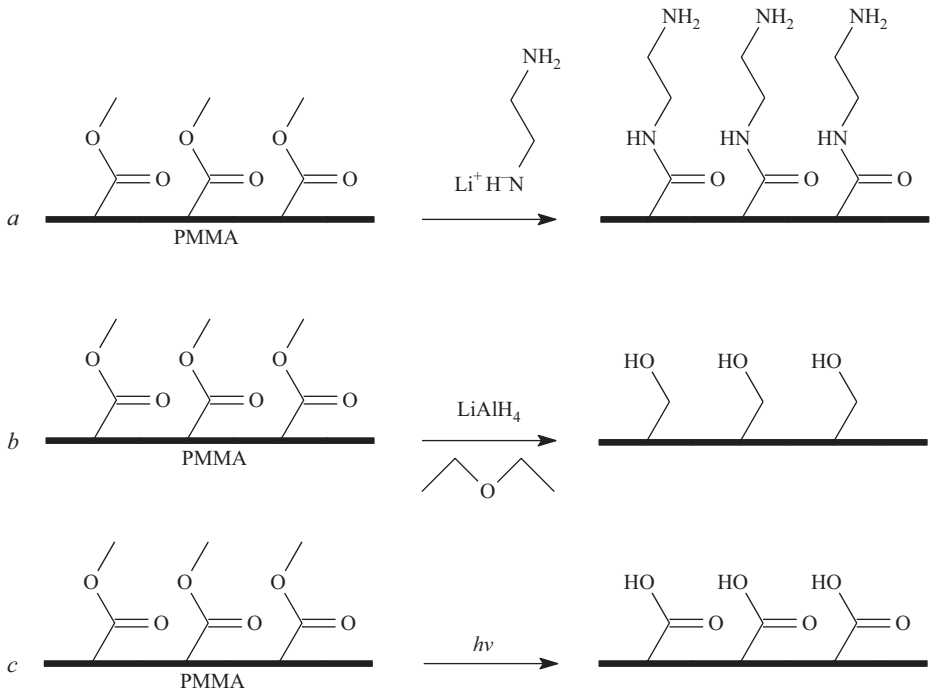


Figure 2.15-2: Modification of PMMA surface using aminolysis (*a*), reduction (*b*), and photoactivation (*c*).

To aminolyze a PMMA surface, Henry *et al.* [68] produced *n*-lithiodiaminoethane and *n*-lithiodiaminopropane by mixing ethylenediamine, propylenediamine, and *n*-butyl lithium. *n*-Lithiodiaminoethane and *n*-lithiodiaminopropane were then cast on isopropyl-alcohol-cleaned PMMA surface. After a short period of time, the reaction was quenched with deionized water.

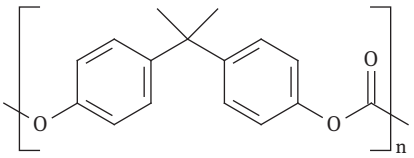
The pendant ester groups of PMMA can also be reduced or oxidized. Cheng *et al.* [69] immersed PMMA sheets into a lithium aluminum hydride diethyl ether solution for 24 h to reduce the ester groups to hydroxyl groups. To convert the pendant ester groups of PMMA to carboxyl groups, a 254 nm UV lamp or a pulsed UV excimer laser (KrF, 248 nm) was employed [70]. Additionally, Zangmeister and Tarlov [71] used UV/O_3 to produce hydroxyl groups on the channel surface of a PMMA microdevice. Then 3-**m**ethacryloxy**p**ropyl-**t**rimethoxy**s**ilane (MPTS) was covalently linked through silanization reaction.

Wang *et al.* [72] reported a method by which a solution containing the monomer methylmethacrylate, a "modifier" (methacrylic acid, 2-sulfoethylmethacrylate, or 2-aminoethylmethacrylate), and a photoinitiator was subjected to 365 nm UV radiation. The dense prepolymer solution was poured into a cartridge with a silicon template fixed inside. Then the cartridge was exposed to UV radiation again to fully cure the prepolymer. Finaly a blank PMMA substrate of the same composition was thermally bonded to the patterned one.

Later, a surface-reactive acrylic polymer, **p**oly(**g**lycidyl **m**eth**a**crylate-co-**MMA**) (PGMAMMA), was synthesized for the fabrication of microchips [73]. Its surface can be easily modified, using a variety of chemical procedures, to control the electroosmotic flow.

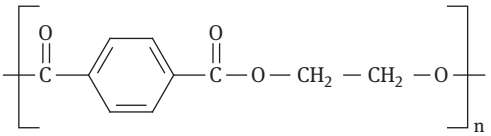
2.15.2.3 PC

Poly**c**arbonates (PC) are a group of thermoplastic polymers containing carbonate groups [74,75,76]. They are strong, tough, robust, molded and thermoformed materials, and some grades are optically transparent. Polycarbonates are pliable and resistant to chemicals. Products made from polycarbonate contain the precursor monomer **b**is**p**henol **A** (BPA).



PC

Other polymers used for preparation of microchips are: **p**oly**e**thylene **t**erephthalate **g**lycol (PETG), **p**oly**e**thylene **t**erephthalate (PET) [77,78], **p**oly**s**tyrene (PS) [79,80,81], Mylar [82], **p**oly**i**mide (PI) [83,84], **c**yclic **o**lefin **c**opolymer (COC), and more.



PETG

2.15.3 Microchip fabrication

The microchip fabrication includes a few processes, as fabrication of channel and cover plates, wall coating, and bonding the plates.

2.15.3.1 Fabrication of channel plate

The channel plate can be fabricated by lithography, hot embossing, room-temperature imprinting, injection molding, laser ablation, *in situ* polymerization, solvent etching, and so on.

PDMS microsystems are fabricated by soft lithography, rapid prototyping, and replica molding [85]. A **c**omputer-**a**ided **d**esign (CAD) program is employed for the design of the microchips. The CAD-generated patterns are printed on a transparency that is used as a photomask in UV-photolithography to generate a master. In this process, a thin layer of negative photoresist is spin-coated onto a silicon wafer and is exposed to UV light through a photomask. The resulting relief structure serves as a master for fabricating PDMS molds, into which a liquid PDMS prepolymer is poured.

The templates for the fabrication of PMMA microchips can be made of silicon, stainless steel, and nickel. Silicon and stainless steel template are fabricated by the combination of photolithography and wet chemical etching (Figure 2.15-3).

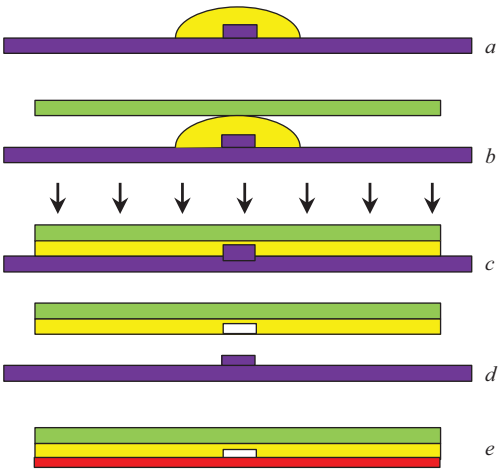


Figure 2.15-3: Scheme of the microfabrication process.
a – Casting methyl methacrylate prepolymer molding solution on a PDMS template; *b* – Sandwiching between PMMA plate and PDMS template; *c* – Exposing the sandwich mold to UV light; *d* – Demolding the PMMA channel plate; *e* – Covering the cover sheet and thermal sealing to form the complete microchip

Another method for fabrication of microchips is the *in situ* polymerization of methyl methacrylate. It takes place in molds with the aids of UV light [86] and heat [87]. To define the dimension of the PMMA channel plates, a rigid rectangle-shaped frame with a rectangular cavity is sandwiched between glass plate and a silicon or stainless-steel template to form a mold. Prepolymerized methyl methacrylate molding

solution is injected into the rigid mold. The solution in the mold is exposed to UV light (365 nm lamp) within 4–6 h or heated in a water bath within 11–12 h. Finally, the formed channel is removed from the mold by sonicating in water bath.

Brister *et al*. [88] developed a method for fabricating PMMA channel plate by solvent etching. It is a mixture of acetone and ethanol (1:1, *V:V*).

Chen *et al*. [89] have developed a simple method for fabricating fiber-based PMMA microchip: Methyl methacrylate molding solution containing UV initiator was sandwiched between a PMMA cover plate and a PMMA base plate bearing glycerol-permeated fiberglass bundles and exposed to UV light. During the UV-initiated polymerization, the fiberglass bundles formed fiberglass-packed microchannels. When the glycerol in the fiberglass bundles was flushed away with water, the obtained porous fiberglass-packed channels could be employed to perform electrophoresis separation. So the fiber-based microchips were fabricated without the need for expensive lithography-based techniques of microchip production. To do the electrodes, a piece of copper wire (10 cm long, 150 μm diameter) was inserted into a 3 cm long fused-silica capillary (320 μm id × 435 μm od) and a 2 mm opening was left in the capillary for the subsequent filling of the graphite-epoxy composite. The other end of the capillary was sealed together with copper wire by hot-melt adhesive. The graphite-epoxy composite was subsequently packed into the capillary by pressing the opening end of the capillary into a sample of the composite. The composite was then allowed to cure at room temperature for 3 h.

2.15.3.2 Fabrication of cover plate

The cover plate of PMMA microdevices can be fabricated by injection molding [90,91]: The PMMA pellets are melted and injected under pressure into a mold cavity containing an inserted master. The masters are usually made from nickel or silicon and are employed to define the structure of the microchips. Finally, the cavity is cooled to allow the channel plate to demold from the mold.

Laser ablation is also employed to fabricate PMMA microdevices [92,93]. In this process, a beam of high-energy laser breakes bonds in polymer molecules. The laser scriber consists of an eximer laser and an XY-table on which the PMMA plate is mounted. The table moves in the *x* and *y* directions while a focused laser beam scannes. The fabrication of the microfluidic chips uses software.

Rapid fabrication of PMMA microfluidic chips has been performed based on the *in situ* surface polymerization of methyl methacrylate [94]. Methyl methacrylate containing 2,2′-azo-bis-isobutyronitrile was allowed to prepolymerize in a water bath to form a viscous solution that was mixed with methyl methacrylate containing a redox initiation couple of benzoyl peroxide and N,N-dimethyl-aniline. Then the viscous solution was sandwiched between a silicon template and a PMMA plate. The polymerization was complete within 50 min under ambient temperature.

2.15.3.3 Wall coating

An important aspect of microfluidic chips is the nature of the channel surface. For separation of proteins, hydrophilic channels are necessary. However, the polymers used for the fabrication of microchips are hydrophobic. Several strategies, as oxidation, physisorption, and covalent grafting, have been used to make the hydrophobic surface hydrophil and so to limit the protein adsorption on the wall of the channels during electrophoresis.

The wall coating can be dynamic or permanent.

Dynamic wall coating

Dynamic wall coating happens when hydrophilic substances in the buffers coat the wall of the michrochip channels during electrophoresis. It is considered as an effective and easier way to reduce protein adsorption on the microchannels. For example, SDS has been used for a dynamic coating in denaturing electrophoresis of proteins on PMMA microchips [95]. Also, cationic starch derivatives can play a role in the dynamic coating of PMMA microchips.

To decrease the electroosmotic flow, neutral polymers, such as poly(dimethylacrylamide) [96], poly(hydroxyethylacrylamide) [97], **h**ydroxy**e**thyl**c**ellulose (HEC) [98], and **h**ydroxy**p**ropyl**m**ethyl**c**ellulose (HPMC) [99] were used. Many of them can serve simultaneously as both dynamic coating and sieving matrix. Cationic detergents such as **c**etyl**t**rimethyl**a**mmonium **b**romide (CTAB) [100], didode-cyldimethylammonium bromide [101]; and polycations such as spermine [102], putrescine [103], polyarginine [104], polyethyleneimine [105], and chitosan [106] were also employed. In addition, the anionic polymer dextran sulfate was used [107].

Diverse methods for dynamic wall coating are known: for glass microchips, PDMS microchips, and PMMA microchips.

Glass has good optical properties and a low fluorescence background. Therefore, it is a popular material for the preparation of microchips. For glass microchips, PDMS is used simultaneously as a wall coating material and a sieving matrix. Its presence reduces the electroosmosis to $0.5 \bullet 10^{-9}$ $m^2/(sV)$ [108]. SDS on glass microchips allows the separation of lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase *b*, β-galactosidase, and myosin in 40 s.

The surface of PDMS is hydrophobic. To make the channel surface hydrophilic, ionic detergents were added into the electrophoresis buffers, for example, Brij 35. Brij 35 (0.01 g/dl) was applied for the separation of proteins at pH = 7.0 [109,110]. It reduces the electroosmosis too.

Dynamic coatings of the channel surface of PMMA microchips was made by dissolving positively or negatively charged surfactants, or hydrophilic neutral polymers in the electrophoresis buffers [111,112,113]. A dynamic coating process using 2 g/dl **h**ydroxy**e**thyl **c**ellulose (HEC) for the surface modification of PMMA microfluidic chips was developed [114]. Mohamadi *et al.* [115] have described a dynamic

coating method using methylcellulose and the nonionic detergent Polysorbate 20, which suppresses protein adsorption onto PMMA channel wall. So nondenaturing microchip electrophoresis of protein samples with relative molecular masses ranging from 20,000 to 100,000 were completed in 100 s.

Permanent wall coating

Permanent wall coating happens after hydrophilic substances react and coat the wall of the michrochip channels. Diverse methods for permanent wall coating are used for glass microchips, PDMS microchips, PMMA microchips, and polycarbonate microchips.

The classical wall coating of glass microchips proposed by Hjerten was applied with some modifications [116,117,118]. In the first step, after flushing channels with NaOH, they are filled with γ-methacryloxypropyltrimethoxysilane in diluted acetic acid and acetonitrile for 1 h. In the second step, an aqueous solution of acrylamide with ammonium persulfate and TMEDA is pumped into the channel and polymerized at room temperature. The channels are then flushed with water and dried by vacuum.

The PDMS channel walls have a hydrophobic surface, which adsorbs hydrophobic proteins but repel hydrophilic proteins. Therefore, several techniques were applied to make it more hydrophilic, for example, oxygen plasma [119,120] and ultraviolet light [121] treating. Besides, poly(2-acrylamido-2-methylpropanesulfonate), which polymerizes from vinyl sulfonic acid, acrylic acid, 2-acrylamido-2-methylpropanesulfonic acid, 4-styrenesulfonic acid, and stearyl methacrylate, using Ce(IV) as a catalyst, was incorporated in the microchip wall [122]. Microchip walls were also coated with a phospholipid bilayer made from dinitrophenyl conjugate lipids [123]. Silanization of the plasma oxidized PDMS surface with di(trimethoxysilane) was used, too [124]. Additionally, the surface of PDMS microchannels could be made hydrophilic by using UV/O_3 plasma cleaner prior to the grafting of a silane monolayer to yield a functional layer that could be used for the covalent attachment of the poly(acrylamide) chains [125]. Once again, this acrylamide coating was adapted from the previous work of Hjerten [126].

One of the earliest methods for modification of the PMMA surface was proposed by Wichterle [127]. His principle consists in reesterification of PMMA with polyfunctional hydroxyl compounds, such as ethylene glycol, glycerol, mannitol, saccharose, and so on. First the PMMA surface was covered with ethylene glycol or another polyalcohol; then it was treated with hot sulfuric acid to perform reesterification and replace methanol rests in PMMA with ethylene glycol rests. After neutralization with sodium hydrogen carbonate, a hydrophilic transparent layer is formed on the surface of PMMA. Later the method was modified [128]: To avoid dissolving of polymer in hot sulfuric acid, the polymer was hydrolyzed first with hot sulfuric acid containing sodium or potassium hydrogen sulfate; then the hydrolyzed PMMA was esterified with glycerol.

A covalent hydrophilization of channels was introduced by Soper *et al.* [129,130] who imparted amine or octadecyl groups to the surface of PMMA microchannel *via* aminolysis and reactions with n-octadecylisocyanate. The electroosmotic flow in the aminated PMMA microchannels was significally reduced and enzymes could be immobilized onto the amine-terminated PMMA surfaces. Using atom-transfer radical polymerization, **p**oly**e**thylene **g**lycol (PEG) was grafted to the surface of PMMA channels, which also reduced the electoosmotic flow and the adsorption of proteins on the PMMA surface [131]. Kitagawa *et al.* [132] have developed a one-step covalent immobilization of **p**oly(**e**thylene**i**mine) (PEI) onto PMMA substrates to achieve an efficient separation of basic proteins in microchip electrophoresis. The PEI-treated PMMA microchip showed the anodic electroosmotic flow and reduced the surface adsorption of cationic proteins.

Polycarbonate is a thermoplastic material useful for microchip coating. Its aromatic rings offer a way to sulfonate the microchip surface. After cleaning with isopropyl alcohol, the polycarbonate surface is modified by reaction with sulfur trioxide (SO_3) at 50 °C for 10 min. Then it is rinsed with water and dried in a stream of nitrogen. The electroosmotic mobility of the polycarbonate surface at pH = 4.0 was about $23 \bullet 10^{-9}$ $m^2/(sV)$, and, after sulfonation, it achieved a value of $43 \bullet 10^{-9}$ $m^2/(sV)$. At pH = 8.0–10.0, the electroosmotic mobility of sulfonated polycarbonate was about $70 \bullet 10^{-9}$ $m^2/(sV)$ [133,134].

2.15.3.4 Bonding the plates

The microchannels have to be closed without changing their physical parameters, or altering their dimension. A variety of bonding techniques have been developed: thermal, solvent, polymerization, microwave bonding, and room-temperature imprinting.

Thermal bonding

The thermal bonding techniques allow the formation of PMMA microchannels with uniform surfaces composed of the same materials. The channel and cover plates are assembled and heated to 105 °C in a convection oven and pressed together using a bonding device [135]. However, variance of the pressure and temperature may cause microchannel deformation and affect the reproducibility. In an attempt to overcome these limitations, hot-press bonding conducted in a vacuum [136] or in a hot water bath has been developed. The bonding processes take approximately 1 h to yield bonding pressure of 130–150 kPa. Kelly and Woolley [137] have demonstrated that PMMA substrates can be bonded together to form microfluidic chips by clamping a blank piece to a channel plate and heating the assembly in a boiling water bath.

Solvent bonding

Solvent bonding utilizing 2,4-pentadione has also been reported for PMMA microchips [138]. Klank *et al.* [139] employed a plasma-enhanced ethanol bonding for such purpose. Woolley *et al.* [140,141] developed a solvent bonding technique for sealing the channel and cover plates of PMMA microchip. Prior to device sealing, the channels in an embossed PMMA piece were filled with a heated liquid (paraffin wax) that forms a solid layer at room temperature. This material prevented the bonding solvent from filling the channels. Once the sealing step was complete, it was melted and removed. Lin el al. [142] sealed PMMA microfluidic chips utilizing a solvent composed of ethanol and 1,2-dichloroethane at room temperature.

Polymerization bonding

The polymerization is a method for bonding PMMA microchips at temperatures below the glass transition temperature of PMMA [143]: Methyl methacrylate containing initiators was allowed to prepolymerize in an 85 °C water bath for 8 min to produce a bonding solution. Prior to bonding, the cover plate was coated with a thin layer of the bonding solution and was bonded to the channel plate at 95 °C for 20 min. Because the coating solution contained both low molecular mass PMMA and methyl methacrylate on the PMMA channel plate, a temperature below 105 °C was used. Then the bonding device was put in a 95 °C convection oven when the monomer methyl methacrylate polymerized to realize the final bonding.

Microwave bonding

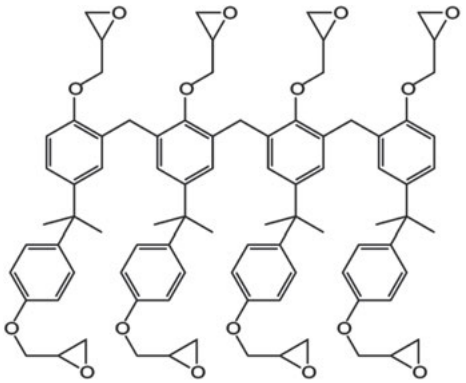
PMMA is transparent to microwave. This can be used for microwave bonding. Lei *et al.* [144] have proposed microwave-based technique for low-temperature bonding of PMMA microchips. Yussuf *et al.* [145] have reported bonding of PMMA microfluidic chips using microwave energy and conductive polyaniline. The absorbed energy was converted into heat, which realized microwave bonding of both PMMA plates.

Room-temperature imprinting

Xu *et al.* [146] have fabricated PMMA microchips by room-temperature imprinting: The PMMA plate was placed on a silicon template and the whole assembly was sandwiched between two polished aluminum plates. A hydraulic press was employed at room temperature. After the pressure was released, the open channels on the plastic substrate were sealed with a layer of PDMS film. This approach obviated the necessity of heating the plastic substrate during the stamping process.

Later Woolley *et al.* [147] developed a method for rapid prototyping of PMMA microfluidic chips using solvent imprinting and bonding. The patterned SU-8 photoresist on a glass slide was employed as a template for solvent imprinting. SU-8 is

an epoxy-based negative photoresist. A piece of PMMA plate was exposed to **ac**eto-**n**itrile (ACN) for 2 min. Then the SU-8 patterned glass slide was pressed into one solvent-wetted surface for 6–60 min.



SU-8

2.15.4 Zone electrophoresis on microchip

Zone electrophoresis is one of the most employed modes for protein analysis in microchips. Separations of proteins are based on differences in their electrophoretic mobility, which generally correlate to their charge-to-mass ratio.

2.15.4.1 Micro free-flow zone electrophoresis

The first device for **micro**-**f**ree-**f**low **e**lectrophoresis (μFFE) was developed by Raymond *et al.* in 1994 [148]. It was constructed in silicon and incorporated a separation region, inlet and outlet channels, electrode beds, and microchannels, which acted as a membrane separating the electrodes from the separation channels. Using this device, the authors separated three rhodamine-B isothiocyanate-labeled amino acids: lysine, glutamine, and glutamic acid. Later Raymond *et al.* [149] separated high molecular mass compounds with the same microchip design, namely a mixture of human serum albumin, bradykinin, and ribonudease A at electric field strength of 100 V/cm and a residence time of 62 s. They also demonstrated that the microchip was capable to separate diluted rat plasma and tryptic digests of bovine cytochrome *c* and melittin.

To work with larger electric field, the silicon substrate was replaced by glass [150]. In an all-glass microchip with a large separation channel in an electric field of 285 V/cm, Kobayashi *et al.* separated cytochrome *c* and myoglobin [151]. They implemented parallel shallow side banks (20 μm) in-between the separation channel (30 μm) and the electrode beds (30 μm).

Fonslow and Bowser [152] presented an FFE microchip fabricated from two glass plates. This device contained closed electrode side channels, where gas bubbles were flushed out by a pressure-driven flow passing the integrated gold electrodes. The channels were separated by connecting side channel arrays. Due to the side channel arrays and its electric resistance, 50% of the applied voltage was utilized across the separation region in the device.

Later, a device for microchip free-flow electrophoresis on glass substrate using laser-printing toner as structural material was proposed [153]: It can be manufactured in only 1 h. The separation channel is 8 μm deep and has an internal volume of 1.42 μl. The Joule heating dissipation in the separation channel was found to be very efficient up to an electric current density of 8.83 mA/mm^2 that corresponds to power dissipation per unit volume of running electrolyte of 172 mW/μl. The electrophoresis was run at a maximum voltage and electric current of 500 V and 100 mA, respectively. The pumping of the running buffer and the sample solutions through the separation channel was performed by connecting the outlet reservoir of the microchip to the air entrance of a small air compressor working as a vacuum pump (Figure 2.15-4). The flow rate of the solutions can be controlled by a valve that adjusts the pressure reducing in the outlet reservoir.

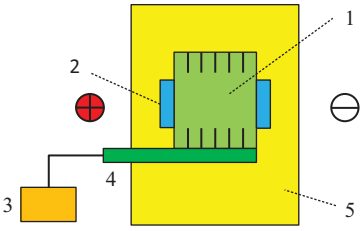


Figure 2.15-4: Diagram of the μFFE chip.
1. Separation channel; 2. Side reservoirs; 3. Air compressor; 4. Outlet reservoir; 5. Glass plate

Kohlheyer *et al.* [154] presented a FFE glass microchip with photopolymerized acrylamide membranes. It was fabricated by using two wafers of Borofloat glass, one containing a 15 μm high separation chamber as well as inlet and outlets. The glass wafers were bonded directly by third glass wafer bonding without an intermediate silicon layer as proposed by Fonslow and Bowser [155]. The electrodes were placed in open reservoirs.

Fonslow *et al.* [156] developed a FFE microchip for FFZE with higher voltages. They separated fluorescein, rhodamine 110, and rhodamine 123 at a linear flow velocity of 5 mm/s and electric field strength up to 58.6 V/mm.

μFFE could be used for online quality control of medical applications – it allowed analytical and preparative separation of small volume samples [157,158]. This device reduced Joule heating and included an online detection system.

Various modes of μFFE, such as micro free-flow zone electrophoresis, micro free-flow isotachophoresis, and micro free-flow isoelectric focusing, have been demonstrated.

2.15.4.2 Affinity- and immunoelectrophoresis on microchips

Affinity- and immunoelectrophoresis on microchips are used for separation of polyions that take place in affinity interaction during electrophoresis. Most common among them are the immune processes [159]. For example, immunoelectrophoresis on microchips has allowed the fast quantitation of inflammatory cytokines of the cerebrospinal fluid of patients presenting a head trauma [160]. Reservoir was silanized to exhibit amino terminal group in order to obtain an affinity area between the Fab fragments and the fluorescently tagged analytes. Then a low-pH buffer was added to dissociate the antibody-antigen complex and to run the electrophoresis.

Phillips and Wellner [161] developed a microchip-based capillary affinity electrophoresis for separation of antigens. They immobilized antibodies on a glass filter and inserted it into the injection port of a microchip. Then the antigens in the sample were captured, fluorescently labelled, eluted, and separated by capillary electrophoresis. Using this method, six chemokines in the cerebrospinal fluid in a concentration of 10 pg/ml were separated within 10 min.

Affinity microchips with mass spectrometry analysis

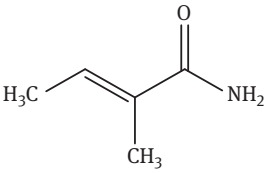
The first affinity-based protein microarrays for protein analysis, also referred to as **s**urface-**e**nhanced **l**aser **d**esorption/**i**onization (SELDI) microchips, were introduced in 1998 by Ciphergen [162]. SELDI is a method in **m**ass **s**pectrometry (MS), which is used with **t**ime-**o**f-**f**light (TOF) mass spectrometers to detect proteins in tissue samples, blood, urine, or other clinical samples. It is a variation of **m**atrix-**a**ssisted **l**aser **d**esorption/**i**onization (MALDI) [163,164]. The limitation of SELDI technology includes problems with detection sensitivity and the highly complex nature of the results obtained [165].

In MALDI, the sample is mixed with a matrix material and applied to a metal plate before irradiation by a laser [166], whereas in SELDI, proteins of interest in a sample become bound to a surface before MS analysis. Feuerstein *et al.* [167] reported the development and application of specially derivatized carrier materials (cellulose, silica, poly(glycidyl methacrylate/divinylbenzene) particles, and diamond powder) for fast and direct MALDI-TOF-MS protein profiling to evaluate human serum samples.

2.15.4.3 Microchip-based Phos-tag electrophoresis

Han *et al.* synthesized a linear copolymer of the acrylamide-pendent Phos-tag and dimethylacrylamide as a separation medium. They used it to form a microchip for phosphate affinity electrophoresis [168,169,170]. This method is useful for rapid

quantitative analysis of phosphorylated and dephosphorylated peptides after kinase and phosphatase reactions, respectively.



Dimethylacrylamide

2.15.5 Isotachophoresis on microchip

Iso**t**acho**p**horesis (ITP) is used to separate and concentrate samples by placing them between a leading and a trailing ions. The leading ion is chosen to have higher mobility than any ions (of the same-polarity charge) in the sample. The trailing ion is chosen to have a lower mobility than any ions in the sample. After applying an electric field, equilibrium is reached, in which each velocity of ions in the system has the same value. As a result, the analytes concentrate (stack) between the leading and trailing ions. The concentration of ions in the sample arises and is a consequence of the concentration of leading ion.

ITP has been used extensively in free-flow electrophoresis [171] and μFFE [172]. It has been successfully coupled with a number of analytical techniques, such as zone electrophoresis [173,174], isoelectric focusing [175], and more. ITP of proteins was demonstrated in a PDMS channel with T-junctions [176]. The dispersion of protein zones as they pass a T-junction during electrophoresis is due to the deformation of electric field lines as the electric current passes the open channel [177].

2.15.5.1 Disc-electrophoresis on microchip

Lin *et al*. [178] have integrated isotachophoresis preconcentration with zone gel electrophoresis of carbonic anhydrase, ovalbumin, BSA, and conalbumin in microchips. Each channel of the microchip was designed with a long sample injection channel to increase the sample loading and to allow stacking the sample into a narrow zone. As a result, the ITP system decreased the detectable concentration by 40-fold.

2.15.5.2 Micro free-flow isotachophoresis

Micro **f**ree-**f**low **i**so**t**acho**p**horesis (μFFITP) is a method for preconcentrating small sample volumes. Mathematical modeling has been performed for optimization of FFITP [179], but to date the validity of these models with regard to μFFITP has not been confirmed experimentally.

Janasek *et al.* first demonstrated μFFITP by focusing fluorescein, acetylsalicylic acid, and eosin G [180]. The applied electric field strength was 525 V/cm with a residence time in the chamber of 50 s. With a second μFFITP device using electrostatic induction, a maximum electric field of 180 V/cm was achieved in the separation chamber.

2.15.6 Isoelectric focusing on microchip

In isoelectric focusing (IEF), the charged proteins are separated according to their pI in a pH gradient generated inside the microchannels by an ampholyte mixture in an electric field. The proteins stop their migration in the separation channel once they reach the pH zone corresponding to their pI. Next mobilization of the focused sample zones [181,182] is necessary to perform an in-channel detection of proteins. To avoid mobilization step, **w**hole-**c**olumn **i**maging (WCI) method may be employed. WCI detection is carried out using an epifluorescence microscope with a xenon lamp [183], a 100-W mercury lamp [184,185], or LED [186,187,188].

2.15.6.1 Micro free-flow isoelectric focusing

Micro **f**ree-**f**low **i**soelectric **f**ocusing (μFFIEF) is a free-flow electrophoresis where the proteins are separated according to their isoelectric points (pI). A pH gradient is established within the separation chamber as the electric field is applied. Charged analytes migrate through the pH gradient until they reach a pH zone equal to their pI. Any deviation from this position causes the molecule to become charged and refocus. This focusing limits the effects of diffusional brand broadening in IEF. Using a device previously reported for μFFE [189], Xu *et al.* first introduced μFFIEF and focused fluorescent dyes and labeled angiotensins I and II [190]. Later Lu *et al.* separated subcellular organelles at low voltages but required long focusing time of about 6 min [191]. Song *et al.* presented a μFFIEF device in which no external electric field was applied [192]. The device created diffusion potential by placing the sample between two high ionic-strength buffers. Using this device they separated a mixture of peptides and proteins. Kohlheyer *et al.* used their device for μFFIEF to separate fluorescent markers of different pI [193]. Later separation resolution of analytes with pI differences of 0.4 pH units was achieved [194]. The separation occurred within 2.5 s at electric field strength of 200 V/cm.

Albrecht and Jensen [195] presented a μFFIEF device with a functionalized gel within the separation channel. In a subsequent paper, they [196] presented the first micro free-flow device in which inline back-to-back separations were performed. The first μFFIEF separation occurred using a steep pH gradient to achieve a low-resolution separation. Separated analytes flowed from one of three outlets into one of three identical separation chambers operating in parallel. In the second

separation, a shallow pH gradient was used to achieve high-resolution IEF separations. Different proteins were separated using this device.

Rapid free-flow IEF was achieved in a microfluidic device by separating the electrodes from the focusing region with porous buffer regions [197].

2.15.7 2D electrophoresis on microchip

Li *et al.* [198] combined IEF with SDS-electrophoresis on a plastic microfluidic network. A complete 2D protein separation was achieved in less than 10 min using a planar microchip with dimensions of 2 × 3 cm. Later a miniaturized instrument capable of performing 2D electrophoresis was described [199]. The device consists of a compartment for a first-dimensional IEF polyacrylamide gel, which is connected to a second-dimensional polyacrylamide gel. It is able to focus and separate a mixture of proteins in approximately 1 h, excluding the time required for the staining procedure. The focused samples are automatically transferred from the IEF gel to the polyacrylamide gel by electromigration. Separated protein spots can be excised from stained gels, digested with trypsin, and identified by mass spectrometry.

Shadpour and Soper [200] carried out 2D electrophoretic separation of proteins using an embossed PMMA microchip. The separation in the first dimension was based on size using SDS electrophoresis; for the second dimension, a fast **m**icellar **e**lectro**k**inetic **c**hromatography (MEKC) was used, which sorted the proteins according to the differences in their interaction with SDS. Also, microfluidic two-dimensional separation of proteins combining temperature gradient focusing and SDS polyacrylamide gel electrophoresis was described [201].

2.15.8 Protein separation on microchip

Protein microchip technology is mostly available in the form of printed glass slides, bioaffinity surfaces, and **t**issue **m**icro**a**rray (TMA)-based techniques. The glass slide-based microchips are simple and have low cost. Bioaffinity surface-based protein microchip techniques are applicable to minute amounts of starting material (<1 pg), but the instrumentation is expensive, especially the mass spectrometers. Tissue microarrays are useful for parallel testing of antibodies. Protein microarrays have been successfully implemented for serum tumor marker profiling.

Protein studies in biomedical research and clinical diagnostics are usually based on protein-protein interactions, most frequently using antibodies that are attached to solid glass surfaces. This approach allows simultaneous interrogation of a few hundred proteins on a single microchip.

The protein electrophoresis in microchips, similar to the common gel electrophoresis, is carried out in three steps: concentrating the samples, running the electrophoresis, and detecting the separated protens.

2.15.8.1 Concentrating the protein samples prior to microchip electrophoresis

A concentration technique is the porous filtering [202]: A 355-nm laser beam was used to excite a photoinitiator in a monomer/solvent solution, leading to polymerization and phase separation of a thin membrane that was covalently bonded to the acrylate-functionalized silica channel surface. The irradiated region was defined by shaping the adjustable slit with cylindrical and spherical optics. The resulting zwitterionic membranes were 50 μm thick. Upon application of voltage, linear electrophoretic concentration of charged proteins is achieved at the membrane surface because buffer ions can pass through the membrane while proteins larger than the molecular mass cutoff of the membrane (>5700) are retained.

The protein samples can be concentrated using a porous silica membrane located between adjacent microchannels which allowed the passage of buffer ions but excluded larger migrating molecules [203]. So fluorescein isothiocyanate-labeled proteins (trypsin inhibitor, carbonic anhydrase, alcohol dehydrogenase, bovine serum albumin, galactosidase and myosin) were concentrated. Fluorescein-labeled ovalbumin could be detected at initial concentrations of 100 fmol/l. For this purpose a combination of field-amplified injection and preconcentration at a membrane was used prior to zone electrophoresis in coated channels.

Wang *et al.* [204] have developed a microfluidic sample concentration device based on the electrokinetic trapping mechanism enabled by nanofluidic filters. The device can be fabricated using the standard microfabrication techniques. Flat nanofluidic channels filled with buffer solution are used as an ion-selective membrane to generate an ion-depletion region for electrokinetic trapping. The field in the nanofluidic channel is used to generate the ion-depletion region and to extend the charge layer that traps the biomolecules. The tangential field in the microfluidic channel, on the anodic side, is used to generate electroosmotic flow to bring the molecules into the trapped region from the reservoir. The trapping and collection can be maintained for several hours and concentration factors as high as 106–108 for bovine serum albumin and green fluorescent protein were achieved.

The group of Hasselbrink [205] developed a glass/PDMS microfluidic system for protein concentration. PDMS and glass are both negatively charged at neutral pH, and therefore, a nanochannel is formed between the PDMS thin-walled section and glass substrate. The electric field applied through the whole channel network creates electroosmotic flow, which dominates over electrophoresis. Negatively charged proteins in the anode side microchannel are transported by higher electroosmotic flow through the microchannel toward the nanochannel; they are preferentially

excluded and thus accumulate near the micro/nanochannel junction. The concentration for bovine serum albumin and ovalbumin were achieved in 30 min.

2.15.8.2 Running the protein electrophoresis

Various injection procedures have been proposed in the literature, such as pinched or gating modes [206]. These injection modes are technically different, and provide alternative routes to prevent from sample zone dispersion by tuning the electric potential value in the reservoirs. The double T intersection design is often preferred, because it drastically improves the control over the injected sample volume. Chiem and Harrison [207] have used the double T intersection to perform injection to obtain impressive immunoassay on microchip electrophoresis device.

The surface of a protein molecule is not homogeneous (Figure 2.15-5). It may have hydrophobic, hydrophilic, cationic, and anionic patches, according to the amino acid residues in the polypeptide chain. When the hydrophobic patches on proteins attach to a hydrophobic surface, the ordered water molecules are released, which results in an entropy increase, a thermodynamically favorable process. The magnitude of the electric charges depends on pH value, ionic strength, and temperature.

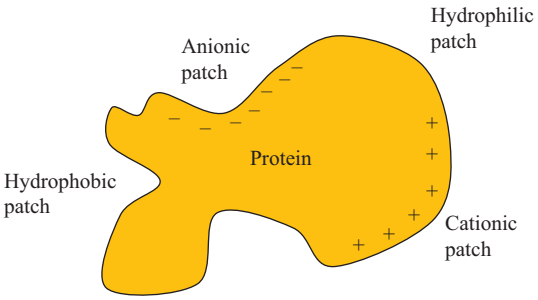
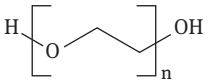


Figure 2.15-5: Scheme of a protein molecule with heterogeneous surface.

Poly**e**thylene **g**lycol (PEG, also known as **p**oly**e**thylene **o**xide, PEO) is well known to be protein-resistant materials. Iki and Yeung [208] passivated the silanol groups on the inner surface of a fused-silica capillary with high-molecular PEG, and employed the capillary to electrophoretically separate four basic proteins. To determine protein masses, He and Yeung [209] used PEG as a sieving matrix.



Polyethylene glycol

Beside polyethylene glycol, **p**oly(**v**inyl **a**lcohol) (PVA) should also be considered as an alternative to coat microchannels. PVA is highly hydrophilic. As a result it suppresses the electroosmosis and improves the protein separation in glass microchips [210]. This coating also reduces the adsorption of fluorescent compounds in electrophoretic procedures.

Lu and Liu [211] have developed a cross-linked polyacrylamide derivatization method. This protocol proposes an efficient route to cover the cavities for the silicium-based surface and thus improves lifetime and stability of surface properties. A one-step cationic polymer grafting process for PMMA microchips has been published by Kitagawa *et al.* [212]. They reported that poly(ethyleneimine)-treated PMMA microchip was able to improve the electrophoretic separation of cationic proteins in microchips. Avidin and ribonudease *A* were separated with a resolution much better than that attained with the unmodified PMMA microchip. Wang *et al.* [213] have grafted methyl-PEG on chitosan-modified PDMS microchip. This surface treatment offers a drastic decrease of hemoglobin and albumin adsorption onto the inner walls of PDMS microchannels.

Size-based protein separations that use SDS to impart the proteins with a constant charge-to-size ratio are an established electrophoresis technique on gel, capillary, and microchip platforms [214,215]. SDS, however, is incompatible with mass spectrometry as it suppresses signal from the analyte in MALDI (s. there) and **e**lectro**s**pray **i**onization **m**ass **s**pectrometry (ESI-MS) [216]. To eliminate this problem, an **a**cid-**l**abile **s**urfactant (ALS) has been used to replace SDS because it degrades at low pH value, and therefore does not interfere with down-stream mass spectrometry analysis [217,218,219]. The separations were performed in less than 3 min, compared with the 8 h separations reported for gel electrophoresis [220,221].

2.15.8.3 Detecting the proteins

There are different methods for detecting proteins:

Zuborova *et al.* [222] have performed electrophoresis of cytochrome *c*, avidin, conalbumin, human hemoglobin, and trypsin inhibitor on a PMMA microchips with conductivity detection. Separations were carried out at pH = 2.9. Olvecka *et al.* [223] have separated proteins cytochrome *c*, avidin, bovine albumin, apo-transferrin, human hemoglobin, insulin, and more by an on-line combination of zone electrophoresis with isotachophoresis on a PMMA microchip with integrated conductivity detection.

Trypsin-encapsulated sol-gel was fabricated onto a PMMA microchip by Sakai-Kato *et al.* [224] to form an on-chip bioreactor for tryptic digestion, separation, and detection. Trypsin-immobilized silica sol-gel made from alkoxysilane was fabricated in a

sample reservoir of the microchip. This PMMA microreactor was applicable to digestion of protein and separation of digest fragments. Huang *et al.* [225] have proposed an enzymatic microreactor by modifying a PMMA microchip with zeolite nanoparticles. Trypsin was subsequently immobilized in the PMMA microchannel *via* sol-gel entrapment. The proposed microreactor provides an efficient digestion of cytochrome *c* and bovine serum albumin in a very short reaction time of less than 5 s.

Ping *et al.* [226] have determined lipoproteins by PMMA microchip electrophoresis with light-emitting diode confocal fluorescence detection. The peak shape of lipoproteins was greatly improved, as the lipoprotein adsorption on a PMMA microchip was dramatically reduced due to electrostatic repulsion.

Hauser *et al.* [227] used PMMA microchip electrophoresis for the direct determination of peptides, proteins, Ig, and DNA. It was also possible to separate the products of the enzymatic digestion of human serum albumin digested by pepsin.

Staining the proteins

The binding between dyes and proteins can be covalent or non-covalent.

Covalent binding

Covalent binding can be realized between the protein molecules and the amine or other reactive groups (carboxylic or thiol groups) of the dyes. Gottschlich *et al.* [228] have developed a microchip where naphthalene-2,3-dicarboxaldehyde was used for labeling the insulin chains A and B, produced after a disulfide-reducing.

Non-covalent binding

Non-covalent binding of proteins is performed with fluorogenic reagents. For efficient on-chip labeling and detection during continuous microchip operation, the binding should occur rapidly and the association should be favored. For low background signal and high sensitivity, the labeling reagent must have low fluorescence in the unbound state and a high fluorescence enhancement when bound to protein.

Bousse *et al.* [229] have developed a glass microchip for non-covalent fluorescent labeling method. Denatured protein-SDS complexes bound the fluorescent dye when the separation begins. The SYPRO Orange and the Agilent dye exhibited a fluorescent enhancement upon binding to SDS micelles or SDS-protein complexes. At the end of the separation channel, an intersection was used to dilute the SDS below its critical micelle concentration before the detection point.

PMMA microchip electrophoresis of proteins labeled either off- or on-chip with the "chameleon" dye 503 was presented [230]. A simple dynamic coating using the cationic surfactant **c**etyl**t**rimethyl**a**mmonium **b**romide (CTAB, cetrimonium bromide $[(C_{16}H_{33})N(CH_3)_3]Br$) prevented non-specific adsorption of protein and dye to the

channel walls. The labeling reactions for both off- and on-chip labeling proceeded at room temperature without requiring heating steps.

Fluorescence detection

Among the detection methods, fluorescence detection is the most widely used optical method for protein detection in microchip electrophoresis due to its exquisite sensitivity. A common fluorescence wavelength detection approach using integrated optical fibers was presented by Lee *et al.* [231]. They fabricated a glass/PDMS hybrid microchip with two sets of integrated optical fibers facing each other. The two channels was excited by a separate laser source, enabling two different wavelengths to be produced (488 and 632.8 nm). The optical fibers were connected to two modules equipped with appropriate band-pass filters to exclude excitation light.

Advances in differential fluorescent dye labeling brought a real breakthrough to protein labeling and analysis, especially with the cyanine dyes Cy3 and Cy5 [232,233] (Figure 2.15-6). Cy3 dye fluoresces greenish yellow (~ 550 nm excitation, ~ 570 nm emission), while Cy5 dye fluoresces in the red region (~ 650 nm excitation, 670 nm emission). Cy3 can be detected by various fluorometers, imagers, and microscopes with standard filters for tetramethylrhodamine. Due to its high molar extinction coefficient, this dye is easily detectable by naked eye on electrophoresis gels, and in solution. Cy5 became a popular replacement for far red fluorescent dyes because of its high extinction coefficient (as small as 1 nmol can be detected in gel electrophoresis by naked eye) and its fluorophore emission maximum in the red region, where many detectors have maximum sensitivity.

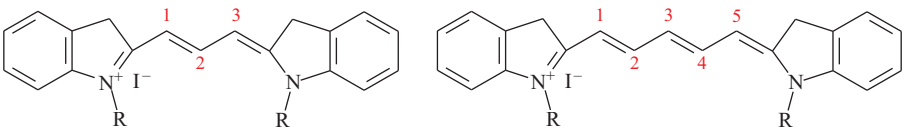


Figure 2.15-6: Chemical formulas of the dyes Cy3 (left) and Cy5 (right).

The protein labeling with Cy3 and Cy5 needs no radioactivity, provides accurate quantification, and offers the option to compare many samples on the surface of a single microchip. The microchips are interrogated by fluorescence scanners and cameras, which can differentiate several fluorescent dyes emitting at different wavelengths. The protein microchips accommodate a few hundred μm-sized protein spots on a one square centimeter surface simplifying the method and making possible fast, accurate, and large-scale measurements [234].

Laser-induced fluorescence detection

Laser-**i**nduced **f**luorescence (LIF) detection is important method used for protein detection after electrophoresis in microchips [235,236]. According to it, the laser is focused on very small protein volumes to obtain high irradiation.

Shen *et al.* [237] have developed a microfluidic system for parallel electrophoresis of biomolecules. The microfluidic array layout consists of two common reservoirs coupled to four separation channels connected to sample injection channel on the soda-lime glass substrate. A CCD camera using a line-focused laser beam was used to monitor electrophoretic separations with laser-induced fluorescence detection, allowing direct plotting of pherograms without transformation and reconstruction by additional software.

A microfluidic chip with line-focused laser detection was developed [238] for the quantitation of insulin secreted from islets of Langerhans. Online assays were performed by electrophoretically sampling anti-insulin antibody, and insulin from separate reservoirs, which allowed the analytes to be mixed as they traveled through a 4-cm reaction channel.

Lamp-based fluorescence detection

The lamp-based fluorescence detection methods form the second largest group of optical detection techniques, following LIF detection, for microchip separations. They use microscope-based detector setups using xenon or mercury lamps. For this purpose, epifluorescence microscopes was combined with **p**hoto**m**ultiplier **t**ube (PMT) detection [239].

Vieillard *et al.* [240] have reported a method combining microfluidic chip with integrated optics. A channel with optical waveguides was prepared on a soda-lime glass plate on which the microfluidic system was fabricated. The wave guiding optics was used to excite the fluorescence in the microchannels. The separation and detection of β-lactoglobulin A and carbonic anhydrase II was obtained in less than 1 min.

Chemiluminescence detection

Another detection method used in microfluidic electrophoresis is **c**hemi**l**uminescence (CL) detection. Ren *et al.* [241] have described a new strategy to afford chemiluminescence detection of heme proteins on microchips. The detection principle was based on the catalytic effects of the heme proteins on the chemiluminescence reaction of luminol-H_2O_2 enhanced by pora-iodophenol. Cytochrome *c*, myoglobin, and horse radish peroxidase were well separated within 10 min on a glass microchip.

Mass spectrometry detection

The possibility to integrate a microfluidic chip separation with **m**ass **s**pectrometry (MS) detection has been studied by many researchers [242,243]. However, until now

no report deals with real connection between electrophoretic separation of proteins and mass spectrometry detection. This is probably one of the future developments that should be considered.

MALDI mass spectrometry

A PMMA microfluidic-based gel protein recovery system, in which fluid is manipulated to transport protein from a PAGE gel piece to a collection reservoir *via* a microfluidic channel has been developed [244]. The protein sample was mobilized out of the gel into a low electroosmosis microfluidic channel under the influence of an electric field. Simultaneously, hydrostatic pressure from the filled buffer reservoirs was used to direct the protein sample to a third reservoir, through a field-free channel connected to the electrophoresis channel. Using this novel process of protein transport from a gel sample, proteins from Coomassie-stained gels have been transferred into solution in 15–30 min, with good sample recovery, using a run buffer containing an anionic surfactant. A variety of small- and medium-sized proteins were successfully recovered and detected using both electrospray and **m**atrix **a**ssisted **l**aser **d**esorption/**i**onization **m**ass **s**pectrometry (MALDI MS) over gel loads of 0.1–10 pg.

The sample for MALDI is uniformly mixed in a large quantity of matrix. The matrix absorbs the ultraviolet light (nitrogen laser light, wavelength 337 nm) and converts it to heat energy. A small part of the matrix heats rapidly and is vaporized, together with the sample (Figure 2.15-7).

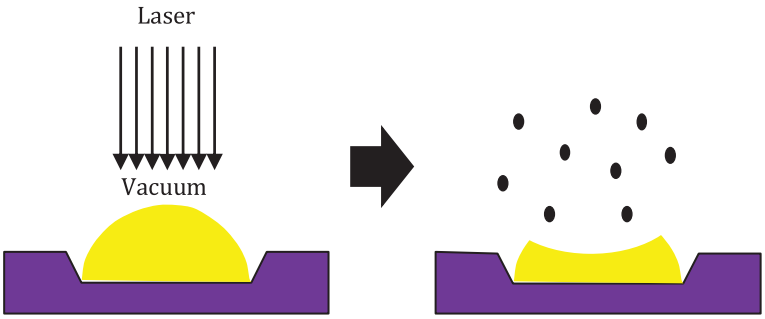


Figure 2.15-7: A small part of the matrix heats rapidly and is vaporized, together with the sample. *Left* – before irradiation; *Right* – after irradiation

The gel separated proteins can be digested *in situ* by proteases and identified by MALDI MS [245]. Then, structural information of intact proteins in gas phase is obtained [246,247,248]. Post-translational modifications of proteins can also be researched [249].

2.15.9 Microchips in clinical diagnostics

Analysis of proteins in body fluids by microfluidic chips is difficult due to the complexity of the method. Therefore, few cases of microfluidic analysis of proteins from blood, urine, cerebrospinal fluid, saliva, tears, and more were described. For example, four human serum proteins (IgG, transferrin, α_1-antitrypsin, and albumin) in artificial serum were resolved on microchip, in less than 60 s [250]. In this work, a post-column labeling of proteins with 2-toluidinonaphthalene-6-sulfonate was developed. However, analysis of proteins present in a normal human serum was not achieved, since 2-toluidinonaphthalene-6-sulfonate preferentially bind to albumin rather than to globulins.

Giordano *et al.* [251] developed a method for dynamic labeling of proteins with NanoOrange in microchip zone electrophoresis with SDS of serum albumin. Later the group of Chan [252] proposed a method for quantifying urinary albumin, a marker of microalbuminuria, which is a risk factor of cardiovascular diseases. Unlike immunoassay, microchip electrophoresis could detect both immunoreactive and nonimmunoreactive forms of albumin, and albumin could be quantified without any discemable interference from other urinary proteins.

Protein kinases are enzymes that catalyze the phosphorylation of proteins by transferring the γ-phosphate group from ATP onto the hydroxyl group of a serine, threonine, or tyrosine residue. They are known to play a role in signal transduction, and are believed to be involved in diseases like cancer. Protein kinase *A* has been analyzed on microchip by Cohen *et al.* [253] using the substrate Kemptide (described by Kemp *et al.* [254]), a heptapeptide containing residues of Leu-Arg-Arg-Ala-Ser-Leu-Gly. In this reaction, the γ-phosphate from ATP is transferred to the serine of the Kemptide, which had been previously labeled with fluorescein at the terminal leucine.

MALDI-based techniques were successfully used with the goal of identifying proteins in connection with HER-2/neu positive, aggressive type breast cancer [255]. Tumor cells were collected by **l**aser-**c**aptured **m**icrodissection (LCM) from HER-2/neu positive and negative tumors. Fifty to seventy thousand cells were used from one sample to separate and isolate proteins of interest using two-dimension electrophoresis. Twenty-one overexpressed proteins were found in the HER-2/neu positive tumors. These proteins are considered to play an important role in different metabolic processes including stress reactions, as well as antioxidative and detoxifying processes.

The examination of serum proteins in metastatic tumors or viral infections are important areas for the application of protein microarrays. For example, protein microarray system could simultaneously determine the level of two viral antigens (HBsAg, and HBeAg) and seven antiviral protein antibodies (HBsAb, HBcAb, HBeAb, HCVAb, HDVAb, HEVAb, HGVAb) of human hepatitis viruses in human sera within 20 min. The detection limit of this technique was 0.1 ng/ml for HBsAg. The results were confirmed by ELISA [256].

Data from SELDI-MS and two-dimension electrophoresis have been used to compare protein expression in pharynx normal epithelium and squamous tumor tissues. Three potential biomarkers were identified from pharyngeal cancer as calgranulin *A*, calgranulin *B*, and calgizzarin. They play a role in the Ca^{2+} transport process, cell growth and motility, as well as cell cycle control and cell differentiation [257].

2.15.9.1 Analysis of endotoxins

The endotoxins are bacterial **l**ipo**p**oly**s**accharides (LPS) of the outer membrane of Gram-negative bacteria with complex amphiphilic structure. They are composed of three parts: a glycophospholipid region (termed lipid *A*) [258,259]; a core oligosaccharide (called rough-oligosaccharide, consisting of two or three 2-keto-3-deoxy-octonic acids and 8–12 monosaccharides); and a hetero-polysaccharide (*O*-polysaccharide, or *O*-specific chain with different numbers of repeating oligosaccharide units) [260,261]. The absence of the *O*-specific region (due to mutation) is characteristic for a bacterial "rough" strain, which produces rough-type endotoxins [262,263]. The total molecular masses of the endotoxins vary between 10,000 and 20,000 [264]. As surface antigens, they are virulence factors that play an essential role in the interactions between pathogens and host cells.

The endotoxins released into the bloodstream by division or lysis of the bacteria [265] strongly influence the mammalian immune system resulting in numerous biological responses (e.g., thrombocytolysis, blood coagulation, changes in white blood cell counts, fever, hypotension, shock, and death [266]).

A microchip electrophoresis method for detection and quantitative analysis of endotoxins extracted from Gram-negative bacteria [267] provides a fast and quantitative differentiation of smooth and rough endotoxins based on the solubilization and complexation of the lipopolysaccharides with dodecylsulfate, and then with a fluorescent dye. The electrophoresis of the complexes was followed by laser-induced fluorescence detection.

2.15.10 Troubleshooting

Problem	Cause	Solution
Fluid is not wicking through channels.	Debris is present.	Restart process with 10 min filtered NaOH incubation, or rinse three times with methanol.
Bubbles in the gel after removing the microchip from the UV oven.	Bubbles are not completely removed from momomeric solution.	Sonicate the acrylamide-BIS solution until no bubbles are present.
	Extra air introduced by centrifugation or pipetting.	Do not centrifuge. Pipette with the tip in the middle of liquid when removing or depositing fluid.
Gel is not polymerized.	Missing photoinitiator.	Add photoinitiator.

(continued)

Problem	Cause	Solution
	Too much air oxygen in the monomeric solution.	Degas the monomeric solution.
Protein not loading under applied electric field.	Electrodes are not touching the bottom of the wells. Electrodes are damaged. Wrong electrodes.	Press down and tape to hold. Use longer electrodes. Change electrodes. Confirm that the electrodes are placed into the corresponding wells.
Grayscale is still saturating.	Fluorescence of protein plug is too bright.	Go to a lower exposure time. Use a lower concentration of protein.

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2.16 Blotting of proteins

Electrophoretically separated proteins can be visualized directly in the gel or indirectly after blotting onto a membrane. The proteins on a blot membrane are immobilized and cannot diffuse or be washed out. They are accessible to reagents or high-molecular-mass ligands such as lectins or antibodies, antigens, lectins, DNA, and RNA.

The direct visualization of proteins on gel can be made by unspecific (universal) dyes like Coomassie brilliant blue or silver, whereas the indirect visualization on blot membrane can be made by specific dyes for glycoproteins or lipoproteins, autoradiography, or by immunochemical methods. The immunochemical methods are performed by antibodies or hybridization probes that have a joined enzyme. The enzymatic activity is visualized by incubation with proper reactive, rendering either a colored product on the blot membrane or a chemiluminiscent reaction which is showed by photographic film. With the help of blotting, it is possible to gain N-terminal or even internal amino acid sequence information of proteins [1].

Today different blotting methods of proteins are known: Western blotting, Far-Western blotting, Southwestern blotting, Northwestern blotting, Eastern blotting, and more (Table 2.16-1).

Table 2.16-1: Blotting methods.

Blotting methods	Goals
Western blotting	Proteins
Far-Western blotting	Protein clusters
Southwestern blotting	DNA-binding proteins
Northwestern blotting	RNA binding proteins
Eastern blotting	Posttranslational modifications

2.16.1 Theory of protein blotting

Although blotting can be carried out in an electric field, it is not an electrophoresis method because it does not lead to separation of charged particles.

Protein samples for blotting can be taken from whole tissue or cell culture. Solid tissues are first broken down mechanically using a blender (for larger volumes), or a homogenizer (for smaller volumes), or by sonication. Often cold temperature is needed to avoid protein denaturing. Protease and phosphatase inhibitors may be added to prevent the digestion of sample by its own enzymes.

Protein samples are loaded into wells in the gel or *via* application templates onto the gel. One lane is usually reserved for protein markers of defined molecular masses, which can be prestained to be visible. Proteins can also be separated by two-dimensional (2D) electrophoresis.

Blotting is carried out in three steps: transfer of proteins on a blot membrane, blocking the free binding sites on the blot membrane, and detection of the blotted proteins (Figure 2.16-1). The unoccupied binding sites on the blot membrane should be blocked with substances, which do not participate in the detection reaction. The blotted proteins are detected with high molecular mass ligands (probes), or with unspecific dyes.

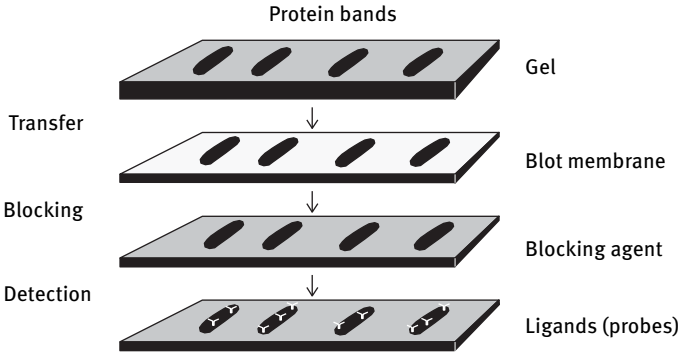

Figure 2.16-1: Steps of blotting.

2.16.2 Blot membranes

For blotting, special blot membranes are used: nitrocellulose, polyvinilydene difluoride membrane, nylon, diazobenzyloxymethylcellulose, diazophenylthioethercellulose, or activated glass fiber paper:

- **N**itro**c**ellulose membrane (NC) is the most commonly used blot membrane [2,3]. Typically its pores are 0.2 or 0.45 µm wide. The smaller the pores, the greater is the binding capacity of the blot membrane. The nitrocellulose membrane is used for adsorption of glycoproteins and lipoproteins [4], and for preparative purposes, since the proteins can be eluted out again [5]. The mechanical stability of NC membranes is limited, so non-woven fiber reinforced NC membranes were produced.
- **P**oly**v**inylidene **dif**luoride membrane (PVDF) has high binding capacity and is mechanically stable [6,7]. It is thicker and more resistant than the nitrocellulose membrane. Besides, it allows easier stripping than the nitrocellulose membrane. Another difference is that, unlike nitrocellulose membrane, PVDF membrane must be soaked in 95 ml/dl ethanol, isopropanol, or methanol prior to use.
- Nylon has good mechanical stability [8]. It binds the proteins electrostatically by hydrophobic interactions. This causes a strong background when staining the proteins.
- **D**iazo**b**enzyloxy**m**ethyl (DBM) paper [9] and **d**iazo**p**henyl**t**hioether (DPT) paper [10] bind proteins electrostatically and covalently. Today they are replaced by nylon.
- Activated glass fiber paper [11] is used when the blotted proteins must be sequenced.

2.16.3 Transfer of proteins

The effectiveness of protein transfer from the gel onto a membrane can be checked by staining the membrane with Ponceau S or Coomassie brilliant blue. Ponceau S is preferred for this event, because it is water soluble, has enough sensitivity and can be easier destained from the membrane background [12].

The most widespread transfer is the electotransfer. Another transfer process is the capillary transfer. It is also used for transferring of nucleic acids (s. there).

2.16.3.1 Electrotransfer of proteins

The electrotransfer (electroblotting) was patented in 1987 by William Littlehales [13]. It uses electric current to pull proteins from the gel onto a PVDF or nitrocellulose membrane. The electrotransfer is the fastest way of blotting – in half an hour to 24 hours. It can be made with polyacrylamide or agarose gels.

The film-supported gels are problematic for electrotransfer, since the film must be removed after electrophoresis [14]. Meanwhile, there are net-supported gels available [15,16], which are suitable for electroblotting.

The electroblotting has two variants: tank blotting and semidry blotting.

Tank blotting of proteins

To carry out tank blotting [17], the gel with the electrophoretically separated proteins and the blot membrane are placed vertically between filter papers and set down in a tank filled up with buffer. Thereafter, the gel is blotted between two electrodes (Figure 2.16-2). The buffer should be cooled so that the blot sandwich is not heated. The transfer continues usually overnight.

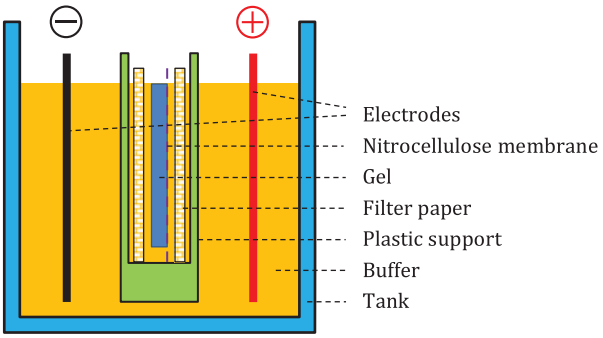

Figure 2.16-2: Tank blotting.
The blot sandwich is constructed of a pad, buffer-saturated sheet of filter paper, gel, membrane, another buffer-saturated sheet of filter paper, and another pad. It is located in a plastic cassette. The entire assembly is placed in a tank containing transfer buffer. The transfer is carried out at a voltage of 100 V for 1 to 2 h or at 15 V overnight.

The tank blotting can be performed in continuous buffer systems. The tank blotting of SDS gels is carried out, for example, in TRIS-glycinate buffer with pH = 8.3 containing SDS and methanol. The continuous Na-borate buffer with pH = 9.2 [18] is recommended for blotting of glycoproteins, because the boric acid forms complex compounds with the alcohol groups of the sugar residues, thereby increasing the number of their negative charges.

Semidry blotting of proteins

The semidry blotting is very popular [19,20]. The gel and the blot membrane are placed between filter papers soaked with transfer buffer and the resulting blot sandwich is inserted between horizontal graphite plates of a blotter. Blotters with glassy carbon plates are also available, which are more solid than the graphite plates.

Graphite and glassy carbon electrodes have a high electric conductivity. They create a homogenous electric field, which has high field strength since the distance between the plates is small. As a result, the blotting is carried out quickly and without cooling.

The semidry blotting can be performed, similarly to electrophoresis, in a continuous buffer or in a disc-buffer system.

When the semidry blotting is carried out in a *continuous buffer*, the same buffer is to be used on both sides of the blot membrane. Under these conditions, the blotted bands on the membrane are not enough sharp.

Many buffers can be used for semidry blotting in continuous buffers. The buffer preferred is that buffer, which has been used for the electrophoresis. The transfer buffer should contain 0.05 to 0.06 g/dl SDS and 20 ml/dl methanol. SDS ensures that hydrophobic proteins will be transferred easily, especially proteins that are focused at their pI values, when they have no charges. Methanol prevents the gel swelling during the transfer. If the nativity or the enzymes activity of proteins should be retained, the transfer buffer should not contain methanol. Methanol also changes the agarose structure.

An often used buffer for blotting is the TRIS-glycinate buffer (Table 2.16-2).

Table 2.16-2: TRIS-glycinate buffer with pH = 8.3.

Component	Production	Concentration
TRIS	5.81 g	48.0 mmol/l
Glycine	2.93 g	39.0 mmol/l
SDS	0.58 g	2.0 mmol/l
Methanol	200.00 ml	5.0 mol/l
Sodium azide	0.10 g	1.5 mmol/l
Deionized water to	1,000.00 ml	–

The *disc-semidry blotting* is carried out in a blotter according to the following scheme (Figure 2.16-3):

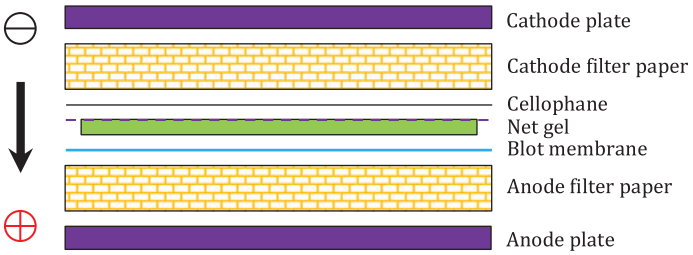

Figure 2.16-3: Disc-semidry blotting.

On the lower (anode) plate of the blotter, a thick filter (anode) paper soaked with the anode buffer is placed, and over it a blot membrane, a net gel (with the gel side down), a cellophane membrane, another filter paper soaked with anode buffer, and finally a thick filter paper soaked with the cathode buffer. The resulting blot sandwich is rolled with a photo roller to remove the air bubbles between the layers. Then the upper (cathode) plate of the blotter is placed on and is weighted with 1 kg. The gas bubbles formed during the transfer leave the blot sandwich. The power supply is turned on to start the blotting.

During semidry blotting in disc-buffer systems, the electrophoretically separated proteins are overtaken by a moving ionic boundary. Therefore, the protein bands are sharp on the blot membrane. We recommend the discontinuous TRIS-formate-taurinate buffer system for semidry blotting [21] (Table 2.16-3). It can be used for native proteins (after disc-electrophoresis or isoelectric focusing) or denatured proteins (after SDS electrophoresis).

Transfer conditions. The transfer takes place usually at an electric current density of 0.8–1.0 mA/cm^2 of blot surface. The electric current density can be increased up to 1.5 mA/cm^2, but it should not be forgotten that the Joule heating will also increase. The transfer duration depends on the gel thickness. We propose that a 0.5 mm thick gel is blotted for 60 min. This means that a 0.25 mm thick gel should be blotted for 30 min, and a 1.0 mm thick gel for 120 min. The transfer time also depends on the ionic strength of the buffer – the blotting time is shorter in a dilute buffer. The gel concentration also plays a significant role – gels of lower concentrations are blotted faster than gels of higher concentrations. At last, the blotting of native and focusing gels is faster, because the native proteins have globular shape, and the electrofocusing gels have wide pores.

Table 2.16-3: TRIS-formate-taurinate buffer system for disk-semidry blotting.

Anode buffer: TRIS-formate buffer, pH = 7.8, *I* = 0.06 mol/l		
TRIS	9.36 g	0.077 mol/l
99 g/dl formic acid	2.29 ml	0.018 mol/l
(SDS	0.58 g	2.0 mmol/l)
Methanol	200.00 ml	5.0 mol/l
Sodium azide	0.10 g	1.5 mmol/l
Deionized water to	1,000.00 ml	–
Cathode buffer: TRIS-taurinate buffer, pH = 8.5, *I* = 0.01 mol/l		
TRIS	4.20 g	0.035 mol/l
Taurine	6.02 g	0.048 mol/l
(SDS	0.58 g/l	2.0 mmol/l)
Methanol	200.00 ml	5.0 mol/l
Sodium azide	0.10 g	0.0015 mol/l
Deionized water to	1,000.00 ml	–

2.16.3.2 Capillary transfer

The electrophoretically separated proteins can be transferred from a gel onto a blot membrane also by capillary transfer (blotting), used mainly for nucleic acids (s. there).

The capillary transfer was performed by Southern (Southern blotting) in 1975 [22], when he transferred DNA fragments onto a nitrocellulose membrane. In this procedure [23], a blot membrane is placed onto the gel with the resolved bands, and the blot membrane is covered with a stack of dry papers. The paper stack acts with capillary force on the gel, and sucks its buffer, together with the resolved bands onto the blot membrane. The transfer needs a long time, usually overnight.

After the protein transfer is complete, the blot membrane should be blocked and the proteins detected.

2.16.4 Blocking

To cover the free binding sites on the blot membrane, suitable macromolecular substances (blocking reagents) are used. They should not react with the transferred proteins or with the detection reagents.

Usually 3–5 g/dl **b**ovine **s**erum **a**lbumin (BSA) is used as a blocking reagent. Other blocking reagents are: 5 g/dl skimmed milk powder, 3 g/dl fish gelatin, 0.05–0.1 g/dl Tween 20 or Triton X-100, 1.0 g/dl casein, or 10 g/dl fetal calf serum.

They block the blot membrane fastest at 37 °C. When blocking PVDF membranes, liquid fish gelatin is preferred [24].

2.16.5 Detection

After transfer and blocking, the blotted proteins can be detected by dyes or high molecular mass ligands (probes), for example, antibodies, antigens, lectins, or nucleic acids.

2.16.5.1 Detection by dyes

Non-specific staining of proteins on blot membranes can be carried out with the anionic dyes as Ponceau S, Amido black 10B, Coomassie brilliant blue R-250, India ink, Fast green FCF, with chelates, and metals (s. also *Qualitative evaluation of protein pherograms*).

The staining with Ponceau S [25,26,27] is easy to be carried out. The staining with Amido black 10B (Naphtol blue black) is most prevalent [28,29,30]. The staining with Coomassie brilliant blue R-250 [31,32,33] has limited use for blot membranes, since it binds strongly to most membranes and cannot be completely freed from them.

Simple to use is the India ink staining of proteins blotted on nitrocellulose membrane [34,35]. It is appropriate for enzyme-linked immunoelectrotransfer blot technique. The India ink staining is more sensitive than the stainings with Coomassie brilliant blue, Amido black 10B, and Fast green FCF and its sensibility is almost as high as the silver staining methods. The transferred proteins appear as black bands on a gray background.

India ink, also named Chinese ink, is a black or colored ink once widely used for writing and printing and now more commonly used for drawing and outlining. It is composed of fine soot, combined with water to form a liquid. The carbon molecules are in colloidal suspension. If a binder is used, India ink may be waterproof or non-waterproof.

Fast green FCF is used in a concentration of 0.1 g/dl dissolved in 1 ml/dl acetic acid [36,37]. The destaining of the membrane background is carried out in deionized water for 5 min.

Chelates can also serve as a dye for blotted proteins. Such ability has, for example, the purple colored chelate ferrozine/ferrous ion [38], which binds to membrane proteins (Figure 2.16-4). Reversibility of the chelate staining can be carried out by incubating the stained blot membrane in a neutral or basic solution in the presence of 0.02 mol/l EDTA. EDTA ions combine with the ferrous ions and destroy the chelate complex.

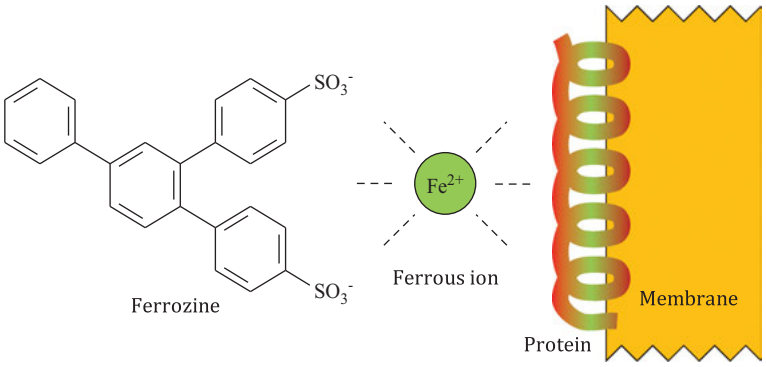

Figure 2.16-4: Formation of a chelate between ferrozine and a ferrous ion.

The metal (colloidal gold, silver) staining of membranes provides the highest sensitivity but has long incubation and troublesome preparation. Copper iodide staining is also sensitive but needs complicated steps to prepare the necessary reagents [39].

2.16.5.2 Detection by probes

A few methods of detection by probes are known: colorimetric, chemiluminescent, radioactive, and fluorescent detection.

Colorimetric detection

During colorimetric detection the blot membrane is probed for the protein of interest with an antibody, linked to an enzyme (for example peroxidase). The enzyme drives a colorimetric reaction converting an appropriate substrate into a colored product. Next, the blot is washed away from the soluble dye and the protein concentration is evaluated through densitometry according to the intensity of the bound stain. In the enzyme blotting, native enzymes are blotted and detected using specific coupled color reactions [40,41].

A cheaper but less sensitive approach utilizes a 4-chloronaphthol stain with 1 g/dl hydrogen peroxide. The reaction of peroxide radicals with 4-chloronaphthol produces a dark purple stain that can be photographed without using specialized photographic film.

Another method of secondary antibody detection utilizes a **n**ear-**i**nfra**r**ed (NIR) fluorophore-linked antibody. Proteins can be accurately quantified because the signal generated by the different amounts of proteins on the membranes is measured in a static state, as compared to chemiluminescence, in which light is measured in a dynamic state [42].

Two colorimetric detection methods are known: with two-steps and one-step incubation.

Two steps incubation

The two steps detection is carried out using two types of antibodies: primary and secondary.

After blocking, the blot membrane is incubated in a solution of *primary antibody* (between 0.5 and 5 μg/ml) under gentle agitation. Typically, the solution is composed of buffered saline with a low detergent concentration, and sometimes with BSA or powdered milk. The antibody solution and the membrane are incubated together for 30 min to overnight. Then the blot membrane is rinsed up to remove the unbound primary antibody and incubated with a *secondary antibody* against the primary antibody. The secondary antibody is usually linked to biotin or to an enzyme such as **h**orse**r**adish **p**eroxidase (HRP) or **al**kaline **p**hosphatase (ALP). In Table 2.16-4, substrates of horseradish peroxidase are given.

Table 2.16-4: Substrates of horseradish peroxidase.

Substrates	Reaction/Detection	Comments
4CN	Oxidized products form purple precipitate.	Not very sensitive; fades rapidly upon light.
DAB/$NiCl_2$	Forms dark brown precipitate.	More sensitive than 4CN but is potentially carcinogenic.
TMB	Forms dark purple stain.	Stable, less toxic than DAB/$NiCl_2$; can be used with membranes.
BCIP/NBT	BCIP hydrolysis produces indigo precipitate after oxidation with NBT; dark blue-gray stain results.	Sensitive and reliable ALP-precipitating substrates; phosphate inhibits ALP activity.

Abbreviations: 4CN = 4-**c**hloro-1-**n**apthol; DAB = 3,3′- **dia**mino**b**enzidine tetrahydrochloride; TMB = 3,3′,5,5′-**t**etra**m**ethyl**b**enzidine; BCIP = 5-**b**romo-4-**c**hloro-3-**i**ndolyl **p**hosphate; NBT = **n**itro **b**lue **t**etrazolium

Most commonly, a horseradish peroxidase-linked secondary antibody cleaves a chemiluminescent agent, and the reaction product produces luminescence according to the protein concentration. A sensitive photographic film is placed against the membrane to obtain an image of the antibodies bound to the blot membrane (Figure 2.16-5).

One step incubation

First the probing process was performed in two steps, which need primary and secondary antibodies. However, there has been an interest in developing one-step probing systems that would allow the process to occur faster. Such a method requires a probe antibody, which recognizes both the protein of interest and contains a detectable label. The primary probe is incubated with the membrane in a manner

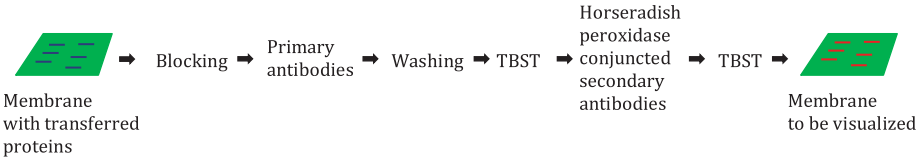

Figure 2.16-5: Detection in a buffer containing horseradish peroxidase.
TBST = **T**RIS-**b**uffered **s**aline with **T**ween 20

similar to that for the primary antibody in a two-step process, and then is ready for direct detection after a series of wash steps.

A detection of alkaline phosphatase using an electrochemical biosensor in a single-step approach was developed [43]. It is based on the detection of phenol produced by an ALP enzymatic reaction. Experimental results are compared to those obtained by spectrophotometric measurements in bovine serum. Excellent linearity between the biosensor outputs and the ALP concentrations exists.

Chemiluminescence detection

Chemiluminescence detection needs the incubation of a Western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is detected by **c**harge-**c**oupled **d**evice (CCD) cameras that capture a digital image of the Western blot or by a photographic film. The use of a film for Western blot detection is slowly disappearing because of non-linearity of the image. The image is analyzed by densitometry that evaluates the protein staining. Nowadays software allows molecular mass analysis (Figure 2.16-6).

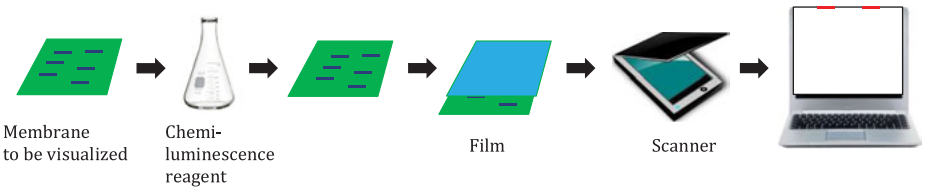

Figure 2.16-6: Chemiluminescence detection.

Radioactive detection

Radioactive detection does not require enzyme substrates but labeled proteins. After placing an X-ray film on the Western blot, dark regions corresponding to the protein bands of interest are created. The importance of this method is declining due to its hazardous radiation, therefore it is now rarely used.

Fluorescent detection

The fluorescence-labeled probes are excited by light and the emission is detected by a photosensor such as a CCD camera equipped with appropriate filters. It captures a digital image of the Western blot and allows further data analysis such as molecular mass analysis and quantitative blot analysis. Fluorescence is considered to be one of the best methods for quantification, but is less sensitive than chemiluminescence [44].

2.16.5.3 Immunoblotting

In immunoblotting [45,46], the antigens (proteins) are transferred to the blot membrane where they form precipitation bands with specific immunoglobulins. The precipitation bands can be visualized, if the proteins are labeled, for example, with ^{125}I [47].

The immunoblotting with peroxidase-coupled secondary antibodies is a standard method for immunological identification of proteins [48,49]: After electrophoresis, transfer and blocking of the free binding sites on the blot membranes, the protein blot is soaked in a solution containing mono-specific rabbit antibodies against a protein. Washing in a buffer removes the unbound antibodies, while the immune precipitates remain. The protein blot is dipped into another buffer with peroxidase-coupled secondary antibodies to the rabbit antibodies. As a result, additional immunoprecipitates containing the enzyme-linked antibodies are formed. Finally, the immunoprecipitates are made visible with the help of a suitable color reaction, in particular with the tetrazolium blue method according Taketa [50]. The rest of other protein bands remain invisible. The comparison between the non-specifically stained gel and the immunoblot makes the identification of the proteins precisely (Figure 2.16-7).

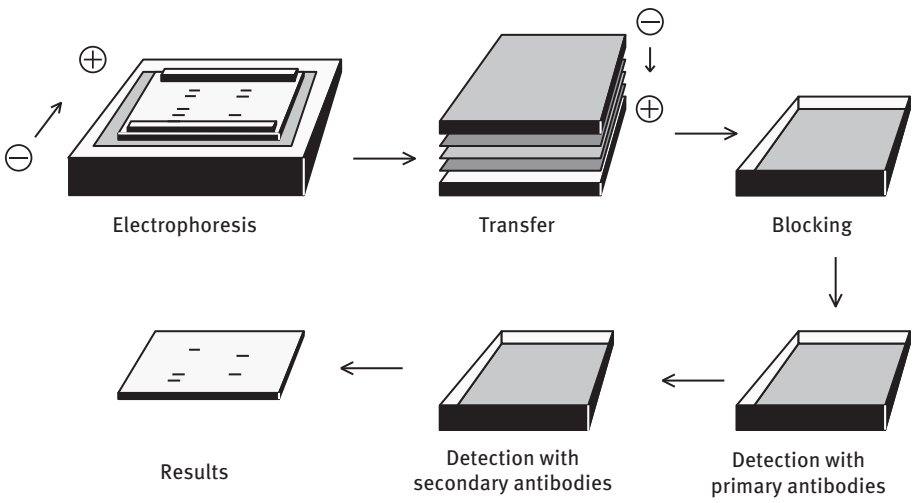

Figure 2.16-7: Immunoblotting with enzyme-coupled secondary antibodies.

The immunoblotting can also be carried out using the biotin-avidin system. Biotin (vitamin H) has been discovered as a growth substance for the yeast. Avidin is a glycoprotein isolated from the egg white. It is strongly connected to biotin. The complex cannot be hydrolyzed by peptide hydrolases (proteases).

The biotin-avidin system is coupled with an enzyme, for example, the peroxidase [51], and with appropriate antibodies. After the transfer, certain proteins on the blot membrane react with the coupled antibodies. The bound enzyme (peroxidase or alkaline phosphatase) catalyzes the conversion of corresponding substrates into a product that can be detected by a color reaction (Figure 2.16-8).

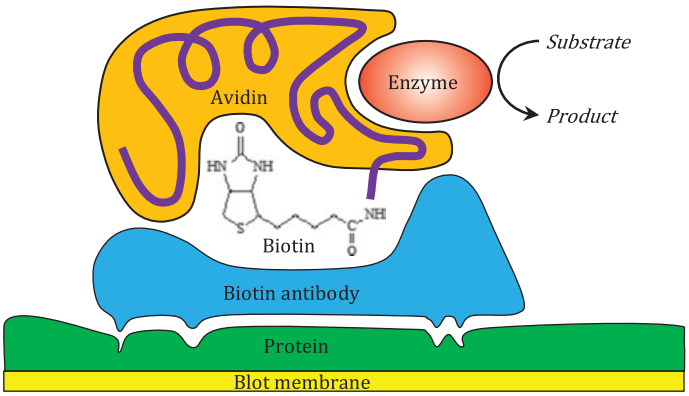

Figure 2.16-8: Detection of blotted proteins by the biotin-avidin system.

Very sensitive is also the evidence with secondary antibodies coupled to colloidal gold [52] (gold-coupled secondary antibodies). In this case, the detection sensitivity can be further increased with silver to reach detection limit of 100 pg [53].

2.16.5.4 Lectinblotting

For detection of glycoproteins, lectin probes are used [54,55]. The visualization is carried out *via* glycoprotein aldehyde groups or avidin-biotin methods [56]. With the help of lectinblotting, the carbohydrate-containing meprins were separated [57].

2.16.6 Making blot membranes transparent

After staining, the nitrocellulose blot membrane can be made transparent without the color intensity of the stained bands to be changed [58]. For this purpose, the blot membrane is to be impregnated with a monomer solution, which has the same refractive index as the nitrocellulose. Thereafter, the monomer is polymerized with the aid of a photo-initiator and UV irradiation.

The monomer solution consists usually of 2 g/dl benzoin methyl ether in **t**ri**m**ethylol**p**ropane **t**ri**m**eth**a**crylate (TMPTMA). This solution, if held in a dark bottle, can be used at room temperature for 2 weeks, and at 4–8 °C longer. The transparency is achieved by the following procedure:

- A few drops of the monomer solution with a photo-initiator are pipetted on the blot membrane and a second PVC film is placed on;
- The air bubbles between the blot membrane and the PVC film are removed with a photo roller;
- Both sides of the resultant sandwich are irradiated for 15 s each with UV light.

2.16.7 Blotting techniques

Different blotting techniques are used: Western, Far-Western, Southwestern, Northwestern, and Eastern blotting, also blotting after SDS electrophoresis in a net gel.

2.16.7.1 Western blotting

Western **b**lotting (WB, protein immunoblot) is a technique for transferring native or denatured proteins, separated by gel electrophoresis, onto a nitrocellulose or PVDF membrane where they bind to specific antibodies. It is used in molecular biology, immunogenetics, and other disciplines (Figure 2.16-9).

Figure 2.16-9: Western blot of separated proteins. Coomassie brilliant blue staining.

Western blotting originates from the laboratory of George Stark at Stanford University [59] and the laboratory of Harry Towbin at the Friedrich Miescher Institute [60]. Its name was given by Neal Burnette [61] as a nod to Southern blotting, developed earlier by Edwin Southern [62].

Several factors lead to increased background during Western blotting. The most common reason of high background of Western blot membrane is its inappropriate

washing. An improved protocol of Western blotting was published that shortens the washing time [63]. This method uses a washing buffer containing 0.5 ml/dl Tween 20. The buffer for the reaction with the antibody also contains a low concentration of Tween 20 (0.02–0.005 ml/dl), while standard protocols use 0.1 ml/dl [64].

The HIV test contains a Western blotting procedure to detect anti-HIV antibody in human serum samples. Proteins from HIV-infected cells are electrophoretically separated and blotted onto a blot membrane. Then, the serum to be tested is incubated with primary antibodies and the free antibodies are washed away. Finally a secondary anti-human antibody linked to an enzyme signal is added.

The Western blotting is used as a definitive test for bovine spongiform encephalopathy (BSE, commonly referred to as mad cow disease). It can also be used for diagnosing some forms of Lyme disease (Lyme borreliosis), hepatitis B infection, and HSV-2 infection caused by herpes type 2.

An improved protocol for quantitative immunodetection by Western blotting is described [65]. Based on the use of fluorescence-labeled secondary antibodies combined with fluorescent staining of all proteins on the membrane, it allows for simultaneous visualization of the specific immunoreactive protein and the total protein. This **f**luorescence-**b**ased **W**estern **b**lotting technique (FB-WB) may be used as a routine test for quantitation of biomarkers in clinical samples.

2.16.7.2 Far-Western blotting

Far-**W**estern **b**lotting (FWB) was derived from the standard Western blotting to detect protein-protein interactions *in vitro*. Proteins in a cell lysate containing prey proteins are separated by SDS or native PAGE, and transferred onto a membrane, as in the standard Western blotting. The proteins on the membrane are denatured and renatured, and the membrane is blocked and probed, usually with purified bait proteins. The bait proteins are detected on the membrane where the the bait proteins and the prey protein form together complexes [66]. Antibody probe is then used to detect the presence of these complexes.

There are many other ways to generate probes for Far-Western blots. The protein probe may be labeled *in vitro* with ^{125}I [67] or enzymatically with ^{32}P [68]. Biotin-labeled probes may be detected with streptavidin-biotin detection schemes [69,70,71]. If an antibody to the interacting protein is available, then an unlabeled protein probe can be bound to the blots as usual and then detected by Western analysis (Figure 2.16-10).

Compared with other biochemical assays, Far-Western blotting allows prey proteins to be endogenously expressed without purification. Unlike most methods using cell lysates (e.g., co-**i**mmuno**p**recipitation (co-IP)) or living cells (e.g., **f**luorescent **r**esonance **e**nergy **t**ransfer (FRET)), Far-Western blotting determines whether two proteins bind to each other directly. In cases where they bind to each other

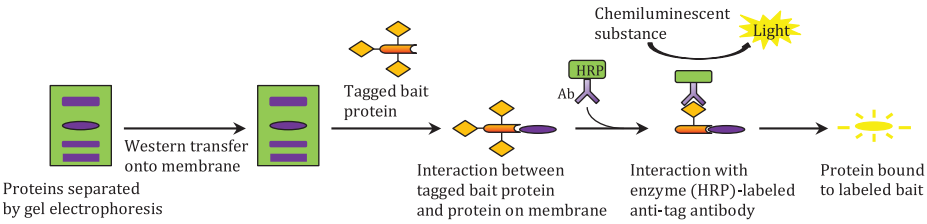

Figure 2.16-10: Steps of Far-Western blotting.
HRP – **h**orse**r**adish **p**eroxidase; Ab – **a**nti**b**ody

indirectly, Far-Western blotting allows the examination of candidate proteins that form complexes between them.

2.16.7.3 Southwestern blotting

South**w**estern **b**lotting (SWB) is based on Southern blotting (for DNA) and Western blotting (for proteins). It is used to identify and characterize DNA-binding proteins by their ability to bind to specific oligonucleotide probes. The Southwestern blotting was first described by B. Bowen, J. Steinberg, and colleagues in 1980 [72,73].

Proteins are separated on a polyacrylamide gel containing SDS, renatured by removing SDS in the presence of urea, and blotted onto nitrocellulose membrane by diffusion. The genomic DNA region of interest is digested by restriction enzymes. They produce fragments of different sizes, which are end-labeled and allowed to bind to the separated proteins. The specifically bound DNA is eluted from the protein-DNA complexes and analyzed by polyacrylamide gel electrophoresis.

2.16.7.4 Northwestern blotting

North**w**estern **b**lotting (NWB) is a hybrid of Western and Northern blotting. It is used to detect interactions between proteins that are immobilized on a nitrocellulose membrane, and RNA. It is used to study the gene expression. The RNA binding proteins tend to bind to different kinds of RNA through either known domains or unknown amino acid sequences of proteins.

After separating by electrophoresis on agarose or polyacrylamide gel, RNA-binding proteins are transferred for 30–45 min onto a nitrocellulose membrane [74]. Then the blot is soaked in a blocking solution, such as TRIS-HCl (pH = 7.5), containing Mg acetate, DDT, Triton X-100, non-fat milk or bovine serum albumin [75]. Once the blocking substances have blocked the free places on the membrane, a specific competitor RNA is applied and the blot is incubated at room temperature, typically one hour [76]. During this time, the competitor RNA binds to the RNA binding proteins on the membrane. After the incubation is complete, the blot is

washed at least 3 times for 5 min each, in order to remove the unbound RNA. Common wash solutions are **p**hosphate **b**uffered **s**aline (PBS) buffer or a Tween 20 solution [77]. At the end the blot is developed by *X*-rays or similar autoradiography methods [78] (Figure 2.16-11).

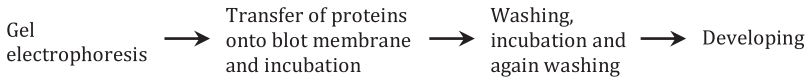

Figure 2.16-11: Steps of Northwestern blotting.

The location and concentration of the RNA binding protein on the blot can help researchers determine the size and concentration of the RNA binding protein of interest [79]. When the approximate size of the protein is known, the sample can also be run chromatographically to separate it by size. In addition, the isolated protein can be digested by trypsin and analyzed by mass spectrometry [80,81].

An advantage of Northwestern blotting is that the specific proteins that bind to RNA can be detected and their molecular masses can be assessed [82]. Once the molecular mass is known, it allows for further purification through other methods as chromatography [83]. A disadvantage of the Northwestern blotting is that some RNA-proteins with poor RNA binding properties may not be detected.

2.16.7.5 Eastern blotting

Most proteins that are synthesized on mRNA undergo modifications before becoming functional in cells. These modifications are known as **p**ost-**t**ranslational **m**odifications (PTM). Post-translational protein modifications are: phosphorylation, acetylation, acylation, alkylation, nitroalkylation, arginylation, biotinylation, formylation, geranylgeranylation, glutamylation, glycosylation, hydroxylation, isoprenylation, lipoylation, methylation, selenation, succinylation, sulfation, transglutamination, ubiquitination, and more [84,85]. They occur at the N-terminus of the polypeptide chain and have as a result secretory proteins in prokaryotes and eukaryotes, and proteins that are incorporated in various cellular and organelle membranes such as lysosomes, chloroplast, mitochondria, and plasma membrane.

Eastern **b**lotting (EB) is a biochemical technique used to analyze protein post-translational modifications leading to synthesis of lipoproteins, phosphoproteins, and glycoproteins. It is similar to lectin blotting (i.e., detection of carbohydrate epitopes on proteins) [86]. Other applications of Eastern blotting include detection of protein modifications in the bacterial species *Ehrlichia muris* and ***I**xodes **o**vatus **E**hrlichia* (IOE) [87]. To detect protein modifications, Cholera toxin B subunit (which binds to gangliosides), Concanavalin A (which detects mannose-containing glycans), and nitrophospho molybdate-methyl green (which detects phosphoproteins) were used.

2.16.7.6 Blotting after SDS electrophoresis in net gels

After SDS electrophoresis, proteins are often transferred from a gel onto a blot membrane and are detected there specifically or non-specifically. Thin film supported gels can be used for the diffusion blotting, but not for electroblotting. The hurdle is the support film which is impermeable for electric current. Electroblotting can be performed with SDS gels on a net support because the net allows free movement of all ions, including the SDS-protein complexes. Good results are obtained in a TRIS-taurinate buffer with pH = 8.3 (continuous blotting), or in a TRIS-formate-taurinate buffer system at pH = 7.4–8.3 (discontinuous blotting) [88] (Figure 2.16-12).

Figure 2.16-12: Semidry blotting of proteins in a TRIS-formate-taurinate buffer system after SDS disc-electrophoresis in a *T*13-SDS net gel.
The blotting was carried out at constant electric current of 1 mA/cm^2 for 30 min. Amido black staining.

Proteins can also be blotted after 2D electrophoresis. They can be detected by probes or dyes.

2.16.8 Blotting of high-molecular-mass proteins from agarose gels

Gel electrophoresis and immunoblots are useful tools for characterizing proteins. However, using these methods for very large proteins (M_r > 500 kDa) is difficult, since large porosity polyacrylamide gels are mechanically unstable. In addition, transfer efficiency of such proteins is low, and this makes Western blotting more challenging.

Cardiac and skeletal muscles contain very large proteins. The biggest is titin [89], also described as connectin [90], with a maximum subunit size over $M_r = 4 \bullet 10^6$ [91]. Another large muscle protein is nebulin with varying sized isoforms ($600–900 \bullet 10^3$) that may determine the thin filament length [92]. To overcome above obstacles, a 1 g/dl **v**ertical SDS **a**garose **g**el **e**lectrophoresis (VAGE) system has been developed [93]. So the electroblotting of very large proteins was nearly 100% efficient.

2.16.9 Protocols

2.16.9.1 Electroblotting

Materials and equipment

TRIS
Glycine
Boric acid
SDS
Methanol
Sodium chloride
PVDF or nitrocellulose membrane
Film remover
Photo roller
Coomassie brilliant blue or India ink
Semidry blotting apparatus

TRIS-glycine transfer buffer, pH = 8.5

TRIS	3.0 g (0.025 mol/l)
Glycine	14.4 g (0.19 mol/l)
SDS	1.0 g (0.003 mol/l)
Methanol	200.0 ml
Deionized water to	1,000.0 ml

Stir and degas for 20 min. Prepare freshly.

TRIS-borate transfer buffer, pH = 8.5

TRIS	6.0 g (0.05 mol/l)
Boric acid	3.1 g (0.05 mmol/l)
Methanol	100.0 ml
Deionized water to	1,000.0 ml

Stir and degas for 20 min. Prepare freshly.

Sodium chloride solution

Sodium chloride	9.0 g (0.15 mol/l)
Deionized water to	1,000.0 ml

Procedure

- Wet a PVDF membrane in methanol. Nitrocellulose membranes are soaked with transfer buffer without prewetting in methanol.
- Wash the PVDF membrane twice in deionized water.
- Soak the PVDF membrane in the transfer buffer.
- Remove the gel from the support film by means of a film remover: Place the gel onto the cylindrical surface of the film-remover, gel side up, and tighten it with the help of a clamp. Pull the stainless-steel wire between the gel and the

support film toward your body so that the gel is released from its plastic support (Figure 2.16-13).

- Transfer the gel into the semidry blotting apparatus: The lower, anodic graphite electrode is soaked with water and covered with six layers of filter paper soaked in transfer buffer and a nitrocellulose or polyvinylidene difluoride (PVDF) sheet also soaked in the transfer buffer.
- Place the gel onto the immunoblot membrane and cover it with another six layers of filter paper soaked in transfer buffer.
- Squeeze out air bubbles with the help of a photo roller or a glass pipette.
- Cover the blotting "sandwich" with the water-saturated cathodic graphite electrode.
- Perform the electrophoretic transfer at 0.8 mA per cm^2 gel surface for about 1 h.
- Wash the membrane with 0.9 g/dl sodium chloride (3x5 min) and let it dry at room temperature. The dried blot membrane can be stored in a sealed plastic bag at 4 °C for several weeks.
- The blot membrane should be stained either unspecifically with Coomassie brilliant blue or India ink, or specifically with antibodies or lectins.

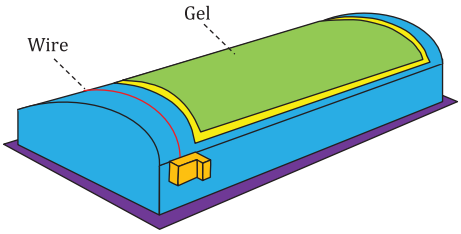

Figure 2.16-13: Removing the gel from the support film using a film remover.

2.16.9.2 Capillary blotting

Materials and equipment

Nitrocellulose paper (pore size 0.20–0.45 µm)
Transfer buffer
Transfer membrane: 0.45 µm nitrocellulose, PVDF, neutral or positively charged nylon
Methanol
Filter paper
Glass plates
A weight of 500-1,000 g

Blotting buffer (pH = 8.3)

TRIS	2.42 g (0.02 mol/l)
Glycine	11.26 g (0.15 mol/l)

Methanol	200.00 ml
Deionized water to	1,000.00 ml

Store at 4 °C.

Staining solution

Amido black 10B	0.1 g
Methanol	40.0 ml
Acetic acid	10.0 ml
Deionized water to	100.0 ml

Destaning solution

Methanol	40.0 ml
Acetic acid	10.0 ml
Deionized water to	100.0 ml

Equilibration buffer (pH = 7.0)

TRIS-HCl	1.00 mol/l
NaCl	1.43 ml (5.0 mol/l)
$Na_2EDTA \cdot H_2O$	3.72 g (0.1 mol/l)
Dithiothreitol	0.14 ml (0.1 mol/l)
Urea	34.34 g
Deionized water to	100.00 ml

Procedure

- Equilibrate a gel with separated proteins in equilibration buffer for 30 min with shaking.
- Place a 25 × 20 cm glass plate and six layers of chromatographic 3 mm filter paper on it.
- Place two trays with blotting buffer on either side of the platform.
- Dip the two ends of the paper in buffer and allow the papers to wet completely.
- Place the equilibrated gel with proteins on the wetted filter paper.
- Wet a piece of nitrocellulose membrane of the same size as the gel that is to be blotted.
- Wet the gel with blotting buffer and place on it the wetted nitrocellulose membrane, avoiding air bubbles trap in between. For this purpose, lower first the middle portion of the membrane on the gel and then the rest of it.
- Place six layers of chromatographic 3 mm filter paper onto the gel and place over another glass plate.
- Place a weight of approximately 500 g over the set up and leave the set for 1–2 days. The buffer moves from the bottom layers of the filter paper upward through the gel to the nitrocellulose membrane. During this movement proteins are transferred from the gel to the nitrocellulose membrane by capillary action.
- Recover the nitrocellulose membrane, and press it between filter papers.

- Immerse the protein blot in the Amido black solution for 10 min with shaking.
- Destain in the destaining solution.
- Dry the blot between sheets of filter paper.

2.16.9.3 Semidry transfer of proteins

Materials and equipment

Transfer buffer
Transfer membrane: 0.45 μm nitrocellulose, PVDF, neutral or positively charged nylon
Methanol
Acetic acid
Coomassie brilliant blue G-250
Filter paper
Semidry transfer unit
Power supply

Procedure

- Soak a stack of chromatography papers with electrode buffer and place half on the lower electrode of a semidry blotter (the cathode).
- Wet a PVDF membrane with methanol and incubate it in electrode buffer until the membrane is submerged in the buffer.
  If nitrocellulose membranes are used instead of PVDF membranes, do not use methanol because it dissolves nitrocellulose.
- Place a gel with separated proteins on top of the chromatography papers and cover it with the PVDF membrane.
- Put on top the remaining half of the chromatography papers stack soaked with electrode buffer.
- Place the anode.
- Place on the anode a 5 kg load to avoid expansion of the gel during protein transfer.
- Set the voltage to 15 V, limit the electric current to 0.4 mA per cm^2 of gel area, and electroblot at room temperature for 16–24 h.
- Stain the wet PVDF membranes in a mixture of 25 ml/dl methanol, 10 ml/dl acetic acid, and 0.02 g/dl Coomassie brilliant blue G-250 for 5 min.
- Destain twice with 25 ml/dl methanol and 10 ml/dl acetic acid for 10 min.
- Rinse in deionized water and let the PVDF membrane dry.

2.16.9.4 Ponceau S staining of proteins on membrane

Materials and equipment
Ponceau S
Acetic acid
Rocking platform

Procedure
- Rinse the membrane with deionized water after proteins have been transferred.
- Stain with 0.2 g/dl Ponceau S in 1 ml/dl acetic acid for 5 min.
- Destain several times in 5 ml/dl acetic acid.
- Photograph the membrane.
- Wash with deionized water.

2.16.9.5 Amido black staining of proteins on membrane

Materials and equipment
Amido black 10B
Acetic acid
Rocking platform

Staining solution

Amido black 10B	0.1 g
Acetic acid	2.0 ml
Deionized water to	100.0 ml

Stir for 30–60 min and filter.

Destaining solution

Methanol	20.0 ml
Acetic acid	10.0 ml
Deionized water to	100.0 ml

Procedure
- Soak the blot membrane in water. PVDF membranes have to be prewetted with methanol.
- Stain the membrane in the staining solution for 5 min on the rocking platform.
- Destain the membrane with the destaining solution (3x5 min) on the rocking platform.
- Dry the stained membrane at room temperature. If the intensity of the stain diminishes, rewet the membrane with water.

2.16.9.6 Coomassie brilliant blue staining of proteins on membrane

Materials and equipment

Coomassie brilliant blue R-250
Methanol
Acetic acid

Staining solution

Coomassie brilliant blue R-250	0.1 g
Methanol	40.0 ml
Acetic acid	10.0 ml
Deionized water to	100.0 ml

Stir for 30–60 min and filter.

Destaining solution

Methanol	50.0 ml
Acetic acid	10.0 ml
Deionized water to	100.0 ml

Procedure

- Rinse the membrane with deionized water after proteins have been transferred.
- Saturate the membrane with methanol for a few seconds.
- Stain in the staining solution for 5 min.
- Destain several times in the destaining solution.
- Wash with deionized water.

2.16.9.7 India ink staining of proteins on membrane

Materials and equipment

TRIS-**b**uffered **s**aline with **T**ween 20 (TBST)
India ink
Acetic acid
Tween 20
Rocking platform

TBST buffer, pH = 8.0

TRIS	0.24 g (0.02 mol/l)

Adjust pH to 8.0 with HCl.

Sodium chloride	0.82 g (0.14 mol/l)
Potassium chloride	0.02 g (0.003 mol/l)
Tween 20	0.05 ml
Deionized water to	100.00 ml

Staining solution

India ink	0.1 ml
Acetic acid	1.0 ml
TBST solution to	100.0 ml

Stir for 60 min and filter.

Procedure

- Soak the blot membrane in deionized water after proteins have been transferred. *PVDF membranes have to be prewetted in methanol.*
- Incubate the membrane in TBST buffer in a glass tray on a rocking platform for 60 min.
- Stain in the staining solution for 2 h or overnight.
- Destain the membrane several times in TBST buffer.
- Wash the membrane 2 × 2 min with deionized water.
- Dry the membrane for long-term storage.

2.16.9.8 Fast green staining of proteins on membrane

Materials and equipment

Fast green FCF
Acetic acid

Staining solution

Fast green FCF	0.1 g
Acetic acid	1.0 ml
Deionized water to	100.0 ml

Procedure

- Stain the blot membrane in the staining solution for 5 min.
- Destain in deionized water for 5 min.
- Dry the blot membrane.
  Thereafter, the blot membrane may be blocked and an immunological or a lectin proof can be used.

2.16.9.9 Visualization with luminescent substrates

Materials and equipment

Substrate buffer: 0.05 mol/l TRIS-HCl, pH = 7.5 (for **h**orse**r**adish **p**eroxidase, HRP)
or
Dioxetane phosphate substrate buffer (for **al**kaline **p**hosphatase, ALP)

TRIS-**b**uffered **s**aline (TBS)
Nitro block (for ALP only)
Luminescent visualization solution
Plastic wrap

Procedure

- Equilibrate twice a membrane with separated enzymes in 50 ml substrate buffer in 15 min washes.
  For ALP reactions using nitrocellulose or PVDF membranes: Incubate 5 min in 50 ml freshly prepared 5 ml/dl Nitro-Block in dioxetane phosphate substrate buffer, followed by 5 min in 50 ml substrate buffer alone.
- Transfer the membrane into 50 ml luminescent visualization solution.
- Soak for 30 s (for HRP) or for 5 min (for ALP).
- Remove the membrane, drain, and place face down on a sheet of a clear plastic wrap.
- Fold the plastic wrap onto the membrane to form a liquid-tight enclosure.
- In a darkroom, place the membrane face down onto a film. Expose the film for a few s to several h.
- If desired, wash the membrane twice in 50 ml TBS for 15 min.
- Process the chromogenic development.

2.16.9.10 Detecting proteins by immunoblotting

Materials and equipment
TBST (**T**RIS-**b**uffered **s**aline with **T**ween 20)
Methanol
SDS
Recombinant protein or unlabeled translated protein for probe
5 g/dl non-fat instant dry milk in TBST
Primary antibody specific for protein probe
Alkaline **p**hosphatase (ALP) conjugated secondary antibody against Ig of species from which specific antibody was obtained
0.10 mol/l EDTA, pH = 8.0
Developing solution
PVDF (**P**oly**v**inylidene **dif**luoride) membrane
Paper sheets
Semidry transfer equipment

Semidry transfer buffer

TRIS	0.58 g (48 mmol/l)
Glycine	0.29 g (39 mmol/l)
Methanol	20.00 ml
SDS	0.10 g
Deionized water to	100.00 ml

Adjust volume to 1 liter with deionized water. Store at room temperature.

Procedure

- Transfer the proteins with the transfer buffer and block the blot.
- Incubate the blot in 5 ml/dl non-fat milk in TBST at room temperature for 1 h, on an orbital shaker.
- Wash three times in TBST for 10 min by agitating on an orbital shaker.
- Dilute the ALP-conjugated secondary antibody in 5 ml/dl milk in TBST.
- Incubate the blot for 1 h.
- Wash the blot in TBST 6 times for 5 min each, with agitation.
- Rinse the blot in alkaline phosphatase buffer.
- Incubate the blot in developing solution for 1 to 15 min.
- Rinse the blot with 100 ml deionized water.
- Wash the blot with 0.10 mol/l EDTA, pH = 8.0, for 5 min to stop the development reaction.
- Rinse with deionized water, dry, and photograph.

2.16.9.11 Immunoprobing with directly conjugated secondary antibody

Materials and equipment

Blocking buffer
Primary antibody specific for protein of interest
TBST (**T**RIS **b**uffered **s**aline with **T**ween 20) for nitrocellulose or PVDF membranes or TBS (**T**RIS **b**uffered **s**aline) for nylon membranes
Secondary antibody: anti-Ig conjugated with **h**orse**r**adish **p**eroxidase (HRP) or **al**kaline **p**hosphatase (ALP)
Heat-sealable plastic bag

Procedure

- Place a membrane with transferred proteins in a heat-sealable plastic bag with 5 ml blocking buffer.
- Seal the bag and incubate at room temperature for 30 to 60 min with agitation on an orbital shaker.
  If the membrane is to be stripped and reprobed, the blocking buffer must contain sein (for ALP systems) or nonfat dry milk.
- Dilute primary antibody in 5 ml blocking buffer.

Both primary and secondary antibody solutions can be used at least twice. Storage more than 2 days at 4 °C is not recommended.

- Open the bag and pour out the blocking buffer.
- Replace with diluted primary antibody and incubate at room temperature for 30 to 60 min with agitation.
- Remove the membrane from the plastic bag with gloved hand.
- Wash four times, 10 min each, with 200 ml TBST (for nitrocellulose or PVDF membrane) or TBS (for nylon membrane) by agitating.
- Dilute the secondary antibody in the blocking buffer.
- Place the membrane in a new heat-sealable plastic bag, add the diluted secondary antibody, and incubate at room temperature for 30 to 60 min with agitation.
- Remove the membrane from the bag and wash.
- Develop according to appropriate visualization protocol.

2.16.9.12 Immunoprobing with avidin-biotin coupling to secondary antibody

Materials and equipment

Nitrocellulose or PVDF membrane
Blocking buffer
TBST
TBS
Avidin
Biotinylated HRP or ALP
Primary antibody
Biotinylated secondary antibody
Sealable plastic bags

Procedure

- Place the membrane with the transferred proteins in a heat-sealable plastic bag with blocking buffer.
- Seal the bag and incubate on a shaker or rocking platform. For nitrocellulose or PVDF membranes, incubate at room temperature for 30 to 60 min. For nylon membrane, incubate at 37 °C for 2 h.
- Prepare primary antibody in 5 ml TBST (for nitrocellulose or PVDF membrane) or TBS (for nylon membrane).
- Open the bag, remove the blocking buffer, and replace with primary antibody solution.
- Incubate at room temperature for 30 min with gentle rocking.
- Remove the membrane from the plastic bag and place it in a plastic box.
- Wash the membrane three times in TBST (for nitrocellulose or PVDF membrane) or TBS (for nylon membrane) for 15 min.

- Dilute two drops of biotinylated secondary antibody in 50–100 ml of TBST (for nitrocellulose or PVDF membrane) or TBS (for nylon membrane).
- Transfer the membrane into a plastic bag containing the secondary antibody solution.
- Incubate at room temperature with slow rocking for 30 min, and then wash.
- While the membrane is being incubated with the secondary antibody, prepare avidin-biotin-HRP or -ALP complex. Dilute two drops of avidin solution and two drops of biotinylated HRP or ALP in 10 ml TBST (for nitrocellulose or PVDF membrane) or TBS (for nylon membrane).
- Incubate at room temperature for 30 min, then dilute with TBST or TBS to 50 ml.
- Wash and transfer the membrane into the avidin-biotin-enzyme solution.
- Incubate at room temperature for 30 min with slow rocking.
- Wash 2–3 times for 30 min each.
- Develop according to an appropriate visualization protocol.

2.16.9.13 Peroxidase protocol

Materials and equipment

Na_2HPO_4
KH_2PO_4
NaCl
Tween 20
H_2O_2
Blot membrane

Hydrogen phosphate buffer, pH = 7.4

Na_2HPO_4	0.14 g (0.01 mol/l)
KH_2PO_4	0.02 g (0.002 mol/l)
NaCl	0.80 g (0.14 mol/l)
KCl	0.02 g (0.003 mol/l)
Tween 20	1.00 ml
Deionized water to	100.00 ml

Staining buffer, pH = 7.4

Hydrogen phosphate buffer	10.00 ml
Diaminobenzidine	0.10 g
H_2O_2	0.03 ml
Deionized water to	100.00 ml

Procedure

- Wash the membrane in the hydrogen phosphate buffer for 10 min.
- Incubate with monoclonal antibodies or sera diluted 1:100 in the hydrogen phosphate buffer at room temperature for 1 h.

- Wash in the hydrogen phosphate buffer three times.
- Incubate the membrane with peroxidase-conjugated secondary antibody diluted 1:1,000 in the hydrogen phosphate buffer for 1 h.
- Wash the membrane three times in the hydrogen phosphate buffer.
- Stain in the staining buffer.

2.16.9.14 Far-Western blotting

Materials and equipment

SDS sample buffer
Phosphate-**b**uffered **s**aline (PBS), pH = 7.9
Ponceau S staining solution
Blocking buffer I: 0.05 ml/dl Tween 20 in PBS. Prepare fresh.
Blocking buffer II: 1 g/dl **b**ovine **s**erum **a**lbumin (BSA; fraction V) in PBS. Prepare fresh.
cDNA encoding protein of interest cloned into an *in vitro* expression vector
In vitro transcription/translation kit
10 mCi/ml ^{35}S-methionine (1,000 Ci/mmol)
Probe purification buffer
Probe dilution buffer
Poly**v**inyl**di**f**l**uoridine (PVDF) or nitrocellulose membrane for protein transfer
Microfiltration centrifuge columns

Procedure

- Suspend protein samples to be analyzed in SDS sample buffer.
- Separate the samples in SDS polyacrylamide gel.
- Transfer the proteins from the gel to a PVDF or nitrocellulose membrane by semidry electroblotting.
- Stain the membrane in fresh Ponceau S solution for 5 min.
- Wash the membrane several times in deionized water until the proteins are clearly visible.
- Destain in deionized water until the red staining fades.
- Block the blot in blocking buffer I at room temperature for 2 h with agitation.
- Decant and add blocking buffer II.
- Incubate.
- Decant blocking buffer II and rinse the membrane briefly in PBS.
- Prepare a radiolabeled *in vitro* translated probe of the protein of interest using ^{35}S methionine, according to manufacturer's procedures.
- After translation, dilute the probe with buffer, and purify by microcentrifuging at 10,000 g and room temperature for 15–30 min. Save aliquots of the purified probe for analysis by SDS-PAGE, and for scintillation counting.

- Preincubate the blot in probe dilution buffer at room temperature for 10 min by agitating.
- Dilute the translated probe with probe dilution buffer.
- Add the probe to the membrane and incubate at room temperature for 2 h by agitating.
- Transfer the membrane to a plastic dish.
- Wash the membrane with PBS at room temperature for 5 min four times.
- Air dry the membrane and expose to X-ray film (autoradiography) or phosphor imager screen.

2.16.9.15 Northwestern blotting

Materials and equipment

Nitrocellulose membrane
TRIS
HCl
Mg acetate
1,4-**Dit**hio**t**hreitol (DTT)
Bovine **s**erum **a**lbumin (BSA)
Triton X-100

Renaturation buffer (pH = 7.5)

TRIS	1.2 g (0.1 mol/l)
Adjust pH to 7.5 with HCl.	
NP-40	0.1 ml
Deionized water to	100.0 ml

Blocking buffer (pH = 7.5)

TRIS	0.12 g (0.01 mol/l)
Deionized water	80.00 ml
Add HCl to pH = 7.5.	
Mg acetate	0.07 g (5 mmol/l)
1,4-Dithiothreitol	0.03 ml (2 mmol/l)
Bovine serum albumin	5.00 g
Triton X-100	0.01 ml
Deionized water to	100.00 ml

Procedure

- Separate recombinant proteins in SDS polyacrylamide gel with $T = 12$ g/dl.
- Transfer them onto a PVDF membrane.
- Renature the proteins on the membrane in the renaturation buffer overnight at 4 °C.

- Wash the blot 4 times in deionized water for 15 min.
- Incubate the membrane in the blocking buffer at room temperature for 5 min.
- Hybridize the blot overnight in the blocking buffer (without BSA) at 4 °C in the presence of labeled mRNAs for 1 h.
- Wash the blot 3 times in the blocking buffer (without BSA) for 5 min.
- Autoradiograph to obtain a signal.

2.16.9.16 Lectinblotting

Materials and equipment

Chemicals for gel electrophoresis
Transfer buffer for protein blotting
Nitrocellulose or PVDF membrane
Filter papers
Biotinylated lectins and HRP-labeled extravidin or ALP-labeled extravidin
Lectins, anti-lectin primary antibodies and HRP-labeled second antibodies
Chromogenic or luminescent visualization regent for detecting tagged lectin or antibody
TRIS-**b**uffered **s**aline (TBS) or **p**hosphate-**b**uffered **s**aline (PBS)
TBS-**T**ween (TBST) or **PBS**-**T**ween (PBST)
Bovine **s**erum **a**lbumin (BSA)
Reagents and equipment for SDS-PAGE, immunoblotting and immunodetection
Image analyzer for chemiluminescence

Washing buffer

Tween-20	0.5 ml
TBS to	100.0 ml

Blocking buffer

BSA or skim milk	3.0 g
TBST (or PBST) to	100.0 ml

Procedure

- Prepare transfer buffer and degas it for 1 h.
- Wet the nitrocellulose membrane thoroughly in methanol.
- Wet filter papers and the membrane in the transfer buffer.
- Place the membrane onto the gel with separated glycoprotein bands so that no air bubbles appear between gel and membrane.
- Place 5 wetted pieces of filter papers onto the membrane and make sure that there are no bubbles.
- Connect the lead to the power source and transfer for 1 h.

- After transferring the proteins from the gel onto the membrane, remove the membrane and rinse briefly in TBS.
- Treat the membrane with blocking buffer at room temperature for 1 h under agitation.
- Remove the blocking buffer and incubate the membrane in biotinylated lectin, in a concentration of 1 μg/ml in the blocking buffer at room temperature for 1–2 h under agitation.
- Wash the blots with TBST at least 4 times, 5 min each.
- Add Extravidin-HRP or Extravidin-ALP in the blocking buffer at room temperature for 1 h under agitation.
- Wash the blots with TBST at least 4 times, 5 min each, and rinse twice with TBS.
- Perform the chemiluminescence detection of peroxidase activity.
- Incubate the blots at room temperature for 1 min without agitation.
- Detect the luminescence with an automatic developer or manually by electronic imaging systems.

2.16.10 Troubleshooting

Problem	Cause	Solution
Prior to blotting		
Bubbles between the filter papers.	The blot sandwich was not assembled under a buffer.	The air bubbles must be removed with a photo roller after formation of the blot sandwich.
During blotting		
No electric current or too small electric current.	One of the connectors had no or poor contact.	Check all connections.
The electric power is too high.	The electric current flows around the blot sandwich.	Cut filter papers and blot membrane according to the gel size.
The voltage increases during blotting.	Gas bubbles between the electrode plates and filter papers.	Weight the cathode plate with 1 kg to push the gas bubbles laterally.
The blotter becomes hot during blotting.	The electric current intensity is too high.	Blot at 0.8–1.0 mA/cm^2.
After blotting		
No transfer onto the blot membrane.	The electrodes were installed incorrectly.	In a basic buffer blot in anode direction; and in an acidic buffer blot in cathode direction.

(continued)

Problem	Cause	Solution
The transfer is incomplete.	The transfer time was too short.	Extend the transfer time, if the gel is thick or concentrated.
	The electric current passed around the blot sandwich.	Cut the blot membrane and filter papers according to the gel size.
The transfer is unregularly.	The electric current flow was irregular because the electrode plates were dry.	Wet the electrode plates with deionized water before blotting.
The blotting results are badly.	Bubbles in the blot sandwich.	Prior to blotting roll the blot sandwich with a roller to push out air bubbles.
	The polyacrylamide gel swelled during blotting.	Add 20 ml/dl methanol to the transfer buffer.
The proteins have left the gel, but are not found on the blot membrane.	The low molecular peptides have been washed during the detection procedure.	When using a nylon membrane, the fixative must contain glutardialdehyde.
Strong background on the blot membrane.	Blocking was ineffective.	Block longer or use higher temperature (37 °C).
	Cross-reactions with the blocking agent.	Use a different blocking agent, for example, fish gelatin or skim milk.

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2.17 Dielectrophoresis

The term ***die****lectro****p****horesis* (DEP) was first adopted by Pohl [1]. It contains the Greek word *phorein* (being carried) and means that particles are carried as a result of their dielectric properties. Dielectrophoresis is based on the fact that an inhomogeneous electric field polarizes all particles, and the appeared *dipoles* can be either attracted or repulsed in it. If the particles move in the direction of an increasing electric field, the dielectrophoresis is referred to as **p**ositive DEP (pDEP); if the particles move in the direction of a decreasing electric field, it is referred to as **n**egative DEP (nDEP).

Many articles are dedicated on the theory, technology, and applications of dielectrophoresis. Prominent among them is the review article by Ronald Pethig [2].

Dielectrophoresis, initially used to characterize the dielectric properties of cells and other bioparticles, is now used even in therapy.

2.17.1 Theory of dielectrophoresis

The theory of dielectrophoresis is based on the inhomogeneous electric field acting on dielectric spheres, according to Maxwell [3,4] and Hatfield [5], and creating dipole moments in them [6]. Pohl and Pethig [7,8] directed their efforts to characterize the dielectrophoretic separation of biological cells and bacteria. Later, the influence of the electric double-layer on DEP behavior was examined too [9,10,11].

2.17.1.1 Dielectrophoretic force

According to most scientists [12,13], the time-average DEP force F_{DEP}, which acts on a spherical particle, is

$$F_{DEP} = 2\pi\varepsilon r^3 F_{CM} \nabla E^2 \tag{2.17-1}$$

where ε is the permittivity of the surrounding medium (equal to the product of the relative permittivity ε_r and the (di)electric constant ε_0), r is the particle radius, F_{CM} is the Clausius–Mossotti factor [14,15] related to the polarizability of the particle, ∇ represents the gradient operator, and E is the electric field. Although Clausius [16] and Mossotti [17] did not derive this factor, it is named after them in recognition of their early studies on the permittivity.

This expression is used for the dielectrophoretic behavior of particles such as erythrocytes (as oblate spheroids) or thin tubes (as prolate ellipsoids), such as tobacco mosaic viruses. As seen, F_{DEP} depends on the square of the applied electric field strength, which indicates that this process can be observed using either *dc* or *ac* field.

Most particles, especially biological ones, are not homogeneous. Bacteria and cells have the so-called multishell structure [18]. For example, erythrocytes, which have about 7 μm diameter, can be represented as a cytoplasm (first shell) surrounded by a thin spherical membrane (second shell). Other cells, which have nuclei, such as leukocytes, have a three-shell model (the first shell is the nucleus; the second shell is the cytoplasm; and the third shell is the plasma membrane). Later the multishell model was extended to describe nonspherical shells [19] and to account for the dielectric anisotropy of the plasma membrane [20].

For the permittivity of a particle ε_p, which is constructed of a homogeneous core surrounded by a shell, the dielectric response can be obtained from the combination of the properties of the core and shell [21]:

$$\varepsilon_p = \varepsilon_2 \frac{\left(\frac{r_2}{r_1}\right)^3 + 2\frac{\varepsilon_1 - \varepsilon_2}{\varepsilon_1 + 2\varepsilon_2}}{\left(\frac{r_2}{r_1}\right)^3 - \frac{\varepsilon_1 - \varepsilon_2}{\varepsilon_1 + 2\varepsilon_2}} \tag{2.17-2}$$

In accordance with this equation, the permittivity of a cell (e.g., human erythrocyte) is given by the expression

$$\varepsilon_{cell} = \varepsilon_{mem} \frac{\left(\frac{r_{mem}}{r_{cyt}}\right)^3 + 2\frac{\varepsilon_{cyt} - \varepsilon_{mem}}{\varepsilon_{cyt} + 2\varepsilon_{mem}}}{\left(\frac{r_{mem}}{r_{cyt}}\right)^3 - \frac{\varepsilon_{cyt} - \varepsilon_{mem}}{\varepsilon_{cyt} + 2\varepsilon_{mem}}} \tag{2.17-3}$$

where *cyt* is the index for the cytoplasm, *mem* is the index for the plasma membrane, r_{cyt} is the radius of the cytoplasm, and r_{mem} is the radius of the plasma membrane.

2.17.2 DEP technology

The advances of DEP technology include fabrication of microelectrodes, introduction of silicone polymers, fabrication of microfluidics devices for DEP, and developing "funnel" electrodes for DEP.

The *microelectrodes* were fabricated by photolithography and metallic vapor. They have interdigitated and castellated geometry (Figure 2.17-1) and use modest voltage [22]. Their thickness is ~70 nm, and the dimension defining the planar geometry ranges from 10 to 120 μm, 5 to 10 times the diameter of the particles to be analyzed. In older techniques (Figure 2.17-1*a*), the particles were directed into flow paths [23].

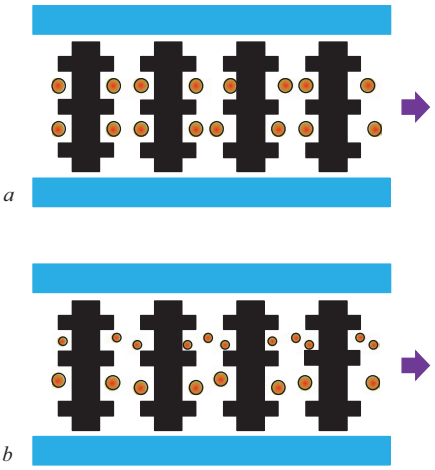

Figure 2.17-1: Microelectrodes.
(*a*) Particles focused into narrow bands of flow in an interdigitated and castellated electrode system.
(*b*) A modified interdigitated and castellated design for separating particles according to their size into separate fluid flow streams.

Later Yasukawa *et al.* [24] modified the method to separate particles according to their size (Figure 2.17-1*b*); by coupling acoustic waves into an interdigitated microelectrode system, particles can first be concentrated then focused into flow channels. These electrodes can be used for both positive and negative DEP of cells [25].

The *introduction of silicone polymers* was a new DEP trend. Cheng *et al.* [26] described a technique they termed molecular DEP, in which a cusp-shaped silica nanocolloid, functionalized with a DNA probe, was used to create a local field gradient for detecting picomolar single-stranded DNA within 1 min.

The *microfluidic* devices for DEP were worked by film techniques, photo and electron beam lithography, laser ablation, **c**omplementary **m**etal-**o**xide-**s**emiconductor (CMOS) technology [27,28], scanning force DEP [29], and assembling the nanoparticles in two or more differing surfaces [30].

Besides photographic recording through a microscope, the DEP particles at electrodes can be measured by monitoring changes in the optical scattering of light beams through particle suspensions [31], by computerized image analysis of particle motion [32], by changes of the impedance of the electrodes [33,34], and by fluorescence spectroscopy of submicron particles [35] onto a quartz crystal [36].

The *"funnel" electrodes* were developed by Fiedler *et al.* [37] (Figure 2.17-2). With their help the particles are guided by angled electrodes to a small exit gap, at which the particles are concentrated prior to be introduced into the device. Angled electrodes are employed in a DEP microchip design for filtering and sorting of bioparticles [38,39,40].

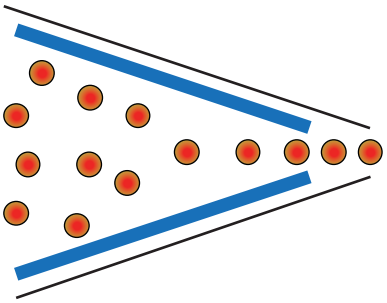

Figure 2.17-2: DEP funnel electrode design for concentrating particles in a flowing aqueous suspension.

Schnelle *et al.* [41] described simple device consisting of two strip electrodes mounted on the top and bottom of a microchannel. As a result, particles exhibiting negative dielectrophoresis are repulsed from both electrodes and introduced into the central stream of the fluid flow. Later Demierre *et al.* [42] fabricated a microfluidic device, which combines the concept of insulator-based "electrodeless" DEP with multiple frequencies to achieve focusing and continuous separation of dielectric particles flowing through a channel. A simple method for producing microwell DEP traps has been described by Fatoyinbo *et al.* [43], who involved drilling holes through a laminate consisting of 20 aluminum layers and 19 epoxy layers. The use of curved microchannels for focusing particles into defined flow streams has also been investigated [44].

2.17.3 Applications of dielectrophoresis

Dielectrophoresis has industrial and biomedical applications. It is applied in medical diagnostics, particle filtration, biosensors, nanoassembly, microfluidics, cell therapeutics, and more. Dielectrophoresis has made possible the separation of cancer cells [45], proteins [46,47], DNA [48,49,50], chromosomes [51], red and white blood cells [52,53,54], stem cells [55,56,57], neurons [58,59], pancreatic β-cells [60], bacteria and yeast [61,62,63], and viruses [64,65]. Dielectrophoresis can also be used for the research and practice of drug discovery and deliver [66], and for detecting apoptosis after drug induction measuring the changes in the electrophysiological properties of the cells [67].

The electrodeless DEP trap is capable of trapping DNA. It has been used to enhance the concentration of immobilized DNA in the vicinity of sensors in high ionic strength buffers required for DNA hybridization [68]. Thus, the DNA concentration and the DNA hybridization kinetics can be tenfold enhanced, as a result of which the sensitivity limit goes down to 10 pmol for a sensor platform. Positive DEP has been demonstrated for DNA in an optical manipulator using micron-sized electrodes created by laser illumination of hydrogenated amorphous silicon [69].

The work of Markx *et al*. [70] is an example of how DEP can be used in stem cell research. Stem cells are immature cells characterized by the ability to differentiate into one or more different derivatives with specialized function.

A cytoplasmic **m**embrane (m) acts as a capacitor because it is constructed like a thin dielectric situated between two conductors (the outer and inner electrolytes). For a cell of radius r suspended in an electrolyte of conductivity σ, the membrane capacitance C_m can be determined from a measurement of the DEP cross-over frequency f using the relationship

$$C_m = \frac{\sqrt{2}}{2\pi r f}\sigma \tag{2.17-4}$$

This equation shows that the high resistance value of the cell membrane has not been impaired due to damage or onset of cell death. For a cell with radius r, the membrane capacitance of a cell will be less than that of a cell having a complex cell surface associated with the microvilli, blebs, membrane folds, or ruffles. This will influence the value of frequency f, which has important implications for applying DEP to characterize and isolate target cells from other cells [71,72,73].

The most common methods used to characterize cell populations for research or translational applications include flow cytometry [**f**luorescence **a**ctivated **c**ell **s**orting (FACS)] or magnetic bead-coupled cell separation. These methods depend on the existence of specific cell-surface antigens and high-affinity probes to these antigens. Irreversible attachment of these probes to target cells has the potential to influence cell behavior. DEP operates on the intrinsic (di)electric properties of cells, and is so potentially capable of sorting cells without the need for engineered labels or tags.

2.17.4 Dielectrophoresis field-flow fractionation

Dielectro**p**horesis **f**ield-**f**low **f**ractionation (DEP-FFF) was introduced by Davis and Giddings [74]. In it, the dielectrophoresis forces, in combination with a drag flow, separate different particles [75,76,77]. The particles are injected into a carrier flow that passes through a separation chamber, where the DEP force is applied perpendicularly to the flow. By means of diffusion, steric factors, hydrodynamic, and other effects, particles with a diameter bigger than 1 μm attain different positions away from the chamber wall. Particles that move further away from the chamber wall reach higher positions in the liquid flowing through the chamber and are eluted from the chamber more quickly.

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2.18 Qualitative evaluation of protein pherograms

The protein concentration in a solution can be determined using the classical biuret reaction that leads to formation of cupric-peptide complexes. In 1951, Oliver Lowry (1910–1996) and colleagues adapted the biuret reaction arguing that the cupric-peptide complexes reduce the Folin-Ciocalteu's phenol reagent [1] changing its color from yellow to blue. Lowry's method for protein determination (Figure 2.18-1) is the most highly citied method in the scientific literature – over a quarter of million times [2]. It can detect about 10 pg protein in 1 ml solution. Lowry's method is excellent for many purposes, but cannot be used for qualitative evaluation of protein pherograms.

The protein concentration in a solution can be measured by Bradford protein assay [3]. It is realized with **C**oomassie **b**rilliant **b**lue (CBB) G-250 in acidic solutions.

CBB G-250 binds mainly to the basic side chains of the proteins, and less to their aromatic side chains (Figure 2.18-2). The protonated CBB G-250 absorbs at 465 nm, which is shifted to 595 nm when bound to proteins.

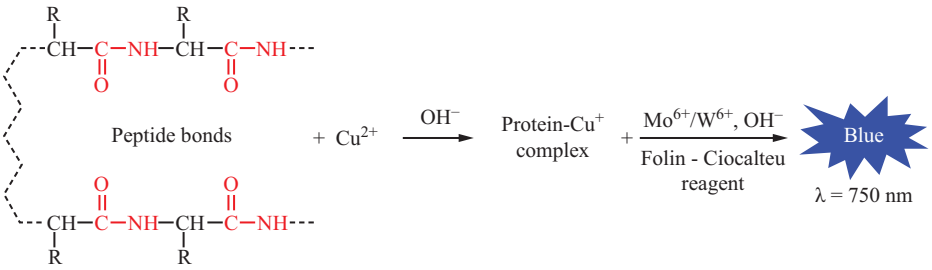


Figure 2.18-1: Lowry reaction scheme.

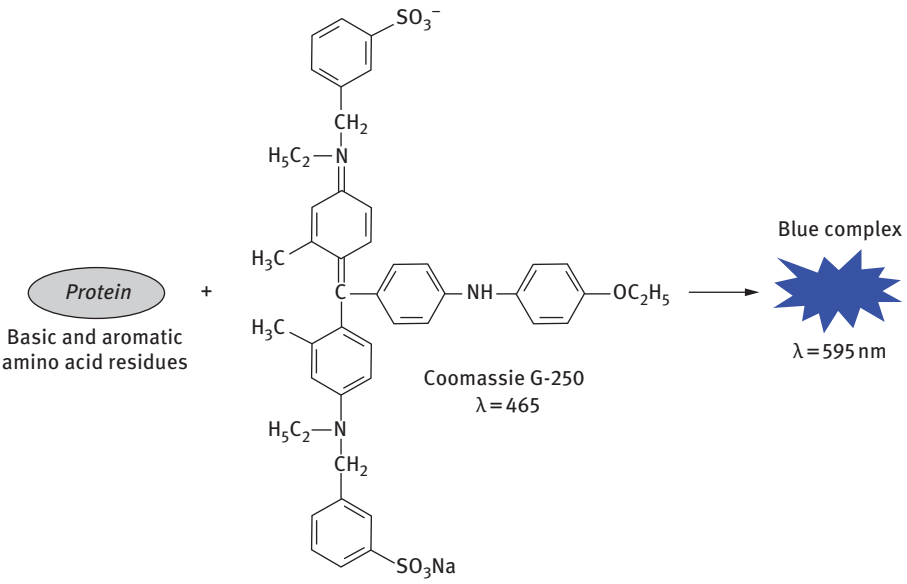


Figure 2.18-2: Bradford reaction.

Most proteins are colorless in nature. Therefore, they are usually stained after electrophoresis. The nonspecific staining involves three steps: fixing, staining, and destaining, and ends with drying of the gel.

2.18.1 Fixing of proteins

The fixing causes denaturation and precipitation of proteins. For this purpose, chemical substances are used that bind together individual protein molecules, such as methanol, ethanol, acetic acid, trichloroacetic acid, 5-sulfosalicylic acid, or glutardialdehyde.

Mixture of alcohol, acetic acid, and water. The volume ratio between the constituents of this mixture is most frequently 3:1:6. The alcohol, methanol or ethanol, dehydrates the proteins, while acetic acid diminishes the pH value of the solution. Thus, on the one hand, the hydration shell of proteins is damaged; on the other hand, most of proteins are put at or near their pI points. As a result, the protein solubility decreases and they precipitate. Two mixtures are used usually: methanol – acetic acid – water (3:1:6, *V:V:V*) or ethanol – acetic acid – water (4:1:5, *V:V:V*).

Trichloroacetic or (and) 5-sulfosalicylic acid. **Tri**c**h**loro**a**cetic acid (TCA) in a concertation of 10–20 g/dl settles all proteins with relative molecular masses above 5,000. It makes, however, the polyacrylamide gels of higher polyacrylamide concentrations (T > 15 g/dl) turbid. TCA can also be used with methanol and water (1:3:6, *V:V:V*). 5-Sulfosalicylic acid in a concentration of 2–5 g/dl also precipitates most proteins, except serum α_1-acidic glycoprotein. Better results are obtained when using a mixture of 10 g/dl trichloroacetic acid and 5 g/dl 5-sulphosalicylic acid.

Glutardialdehyde. Glutardialdehyde, in a concentration of 0.5–1.0 ml/dl, is used for crosslinking of oligopeptides or small protein molecules (M_r = 1,000–10,000). Its aldehyde groups react with the amino groups of adjacent proteins and form an insoluble network (Figure2.18-3). This reaction takes place at pH = 6.0–7.0. Therefore, it should be carried out in an appropriate buffer, for example, in hydrogen phosphate buffer.

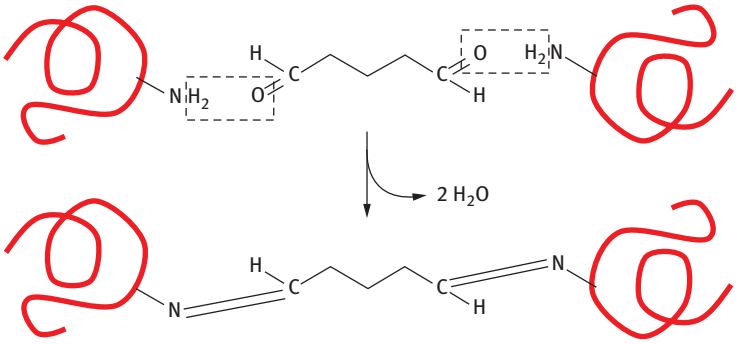


Figure 2.18-3: Glutardialdehyde as a cross linker of proteins.

2.18.2 Staining of proteins

The proteins in a gel can be found (visualized) using anionic dye staining, counterionic dye staining, metal staining (silver, chelates), autoradiography, fluorography (SIPRO Ruby staining), immunoprobing, or other methods.

When the background of the separation medium is destained, it becomes transparent, so that only the stained bands are seen. In principle, the destaining can be done in the solution where the dye has been dissolved, for example, in the mixture of ethanol – acetic acid – water (4:1:5, *V:V:V*). Too long destaining can lead to disappearance of the color of the weak protein bands.

The comparison between all protein staining methods is shown on Table 2.18-1.

Table 2.18-1: Comparison of protein staining methods.

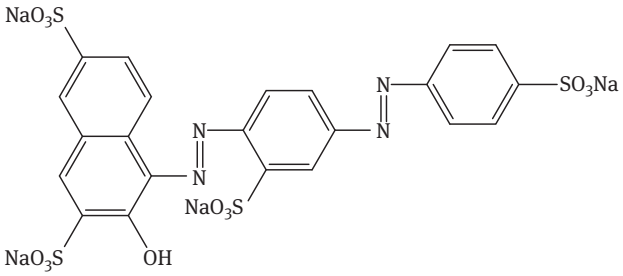
Method	Sensitivity	Advantages	Disadvantages	Imaging
Anionic dyes (Ponceau S, Amido black 10B, Coomassie brilliant blue R-250)	100–1,000 ng	Inexpensive, rapid	Low sensitivity, shrink membrane	Photography with epiillumination or reflectance densitometry
Stain-free (SFX)	2–28 ng	Rapid – no additional staining or destaining required	Special gels and imaging equipment required	Gel Doc™ EZ system
Fluorescence	2–8 ng	Sensitive, mass spectrometry-compatible	Fluorescence detection system required	Fluorescence visualization with UV, LED epiillumination, or laser scanning
Colloidal gold (enhanced)	0.1–1 ng	Very sensitive, rapid; optional enhancement increases sensitivity	Expensive	Photography with epiillumination or reflectance densitometry

2.18.2.1 Anionic dye staining of proteins

To the anionic dye staining methods belongs the staining with Ponceau S, Amido black 10B, Coomassie brilliant blue R-250, Fast green FCF, India ink, and more.

Ponceau S staining of proteins

The staining with Ponceau S, M_r = 760.58, can be easily performed [4, 5, 6]. It is used after electrophoresis on cellulose acetate membranes [7].

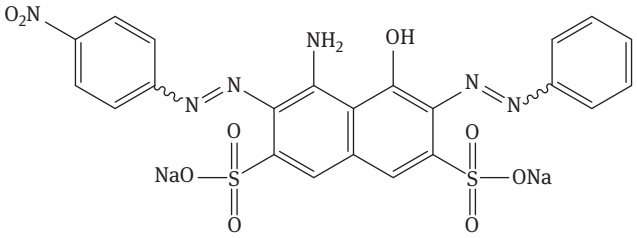


Ponceau S

The fixing and staining is done in 0.3 g/dl Ponceau S in 3 g/dl trichloroacetic acid; the destaining in 0.5 g/dl trichloroacetic acid; and the drying at room temperature.

Amido black staining of proteins

Amido black 10B (Naphthol blue black), M_r = 616.50, is a dye, which has been used since a long time for staining of proteins. The staining with it is most prevalent for agarose and polyacrylamide gels [8, 9], but also for nitrocellulose blot membranes [10].



Amido black 10B

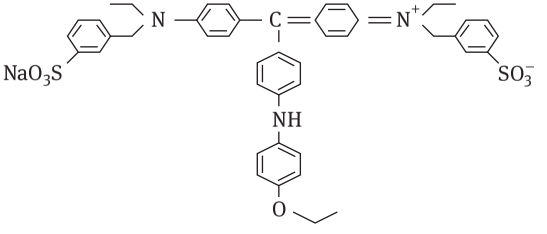
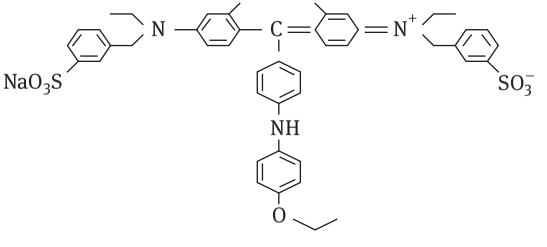
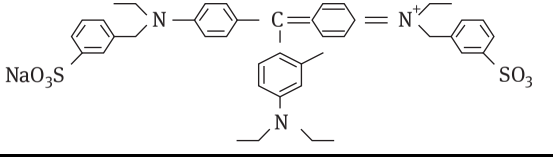
Prior to staining, the agarose gel should be dried after fixation. Then it should be placed in the staining solution. Thus, the proteins are stained faster, and the gel absorbs less color that results in clear background.

Coomassie brilliant blue staining of proteins

Originally the Coomassie dyes were used for staining silk and wool. In the 60s they were introduced to color electrophoretically separated proteins – first Coomassie brilliant blue R-250 [11, 12, 13], then Coomassie brilliant blue G-250 [14] and Coomassie violet R-200 [15, 16, 17] (Table 2.18-2).

Coomassie **b**rilliant **b**lue (CBB) R-250 is the most popular protein stain. It was developed as an acid wool dye. CBB R-250 is soluble in methanol. In acidic solutions it

Table 2.18-2: The main Coomassie dyes used in the electrophoresis.

Dyes	Chemical formulas	Properties
Coomassie brilliant blue R-250 (Acid blue 83), sodium salt		M_r = 825.97 λ = 560 nm Soluble in methanol, and ethanol Sensitivity 10–50 ng/mm^2
Coomassie brilliant blue G-250 (Acid blue 90), sodium salt		M_r = 854.03 λ = 580 nm Soluble in hot water, methanol, and ethanol Sensitivity 15–50 ng/mm^2
Coomassie violet R-200 (Acid violet 17), sodium salt		M_r = 761.92 Soluble in water, methanol, and ethanol Sensitivity 50–100 ng/mm^2

binds to the amino groups of proteins, contained in lysine, arginine, and histidine residues. The high intensity of Coomassie brilliant blue R-250 can be explained with the secondary binding between the dye molecules. The proteins are detected as blue bands on a clear background.

The staining with Coomassie brilliant blue R-250 [18, 19, 20] has a limited use in the blot membranes, since it binds strongly to most membranes and cannot be completely freed from them.

There are two staining methods with Coomassie dyes: conventional and colloidal staining. In the conventional staining, organic solvents and organic acids are used; in the colloidal staining ammonium sulfate and phosphoric acid (or sulfuric acid) are used.

Conventional Coomassie brilliant blue staining of proteins

Compared to other staining procedures, the conventional Coomassie staining is relatively fast and less complicated method. However, it needs acetic acid in the staining and destaining solutions, which have unpleasant odor. Coomassie staining methods are used mainly for polyacrylamide gels. Their sensitivity reach 100 ng protein/band.

First Fazekas de St. Groth *et al.* described a procedure for staining proteins on a cellulose acetate sheet using Coomassie brilliant blue R-250 [21]. They suggested that the negatively charged sulfonate groups of CBB R-250 bind electrostatically to the positively charged protonated amino groups of proteins, and the positively charged quaternary ammonium groups of CCB R-250 bind to the negatively charged carboxyl groups of proteins. In this process, hydrophobic interactions, van der Waals forces, and hydrogen bonding take part, too. Later, Meyer and Lambert [22], and Chrambach *et al.* [23] employed CBB R-250 for staining of proteins in polyacrylamide gels.

Usually CBB R-250 is dissolved in two solutions: a mixture of methanol – acetic acid – water, and a solution of **t**ri**c**hloro**a**cetic acid (TCA). In the first case, the gel background remains bluish; in the second case, it remains clear. Later, methanol, a compound with toxic properties, was replaced by ethanol. The change to ethanol not only yielded gels with lower background staining but also allowed to omit the destaining step [24].

Compared to the previously employed azo-dyes staining methods, Coomassie staining methods are much simpler. They have good sensitivity, however, are less sensitive than the silver staining [25]. The CBB staining methods can be used for native, SDS, and IEF polyacrylamide gels, which contain nonionic detergent [26,27].

Colloidal Coomassie brilliant blue staining of proteins

Chrambach *et al.* [28] and Diezel *et al.* [29] replaced CBB R-250 by colloidal **C**oomassie **b**rilliant **b**lue G-250 (CBB G-250) dissolved in 12.5 g/dl TCA, and so reduced the gel background. Neuhoff *et al.* [30,31] employed the same staining principle including ammonium sulphate and phosphoric acid into the protocol. Their modification did not

only alleviate the problem of background staining but also enabled the detection of very weak spots. Neuhoff dye ("blue silver") recipe contains 2 g/dl H_3PO_4, 10 g/dl $(NH_4)_2SO_4$, 20 ml/dl methanol, and 0.1 g/dl Coomassie brilliant blue G-250. The sensitivity of this method is more than 30 ng protein/band (0.7 ng protein/mm^2). However, the CBB G-250 staining needs a very long time [32].

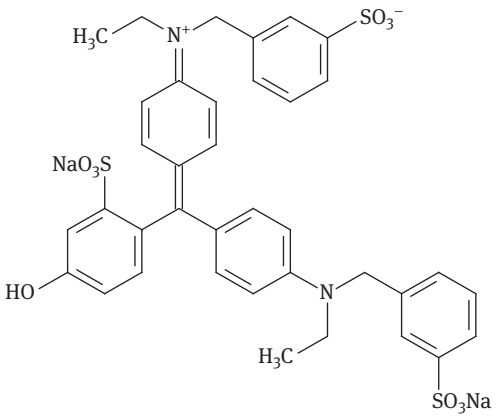
Various CBB-G-250-based methods for staining of proteins separated by 2D gel electrophoresis were compared with regard to sensitivity and resolution. Kang [33], Candiano [34], and Wang [35] suggested a different protocol to overcome the problem of the limit detection of CBB G-250 staining. A modified Kang's CBB G-250 staining protocol includes phosphoric acid in a concentration of 8 g/dl instead of the original 2 g/dl concentration [36]. So protein amounts as low as 2 ng and about 2300 spots in a gel can be detected.

A fixation-free and fast protein-staining method for SDS polyacrylamide gel electrophoresis using Coomassie blue was described [37]. The protocol comprises staining and quick washing steps, which can be completed in 0.5 h. It has a sensitivity of 10 ng, comparable with that of conventional Coomassie Brilliant Blue G staining with phosphoric acid in the staining solution. In addition, the dye stain does not contain any amount of acid and methanol, such as phosphoric acid. Considering the speed, simplicity, and low cost, the dye stain may be of more practical value than other dye-based protein stains in routine proteomic research.

For **m**ass **s**pectrometry (MS) analysis, it is important that the staining methods used do not interfere with the MS methods and the detection limit be in the nanogram range of protein [38,39,40]. Among the visible dyes available, colloidal CBB G-250 is appropriate for this purpose [41].

Fast green staining of proteins

Fast green FCF is a dye used for protein staining in native PAGE, SDS-PAGE, and IEF. It stains linear over a wider range of protein concentrations than Brilliant Blue R.



Fast green FCF

After electrophoresis, the proteins in the gel are fixed. Then the gel is stained with 0.1 g/dl Fast Green FCF in 30 ml/dl ethanol in 10 ml/dl acetic acid for 2 hr. Next the gel is destained with either 30 ml/dl ethanol in 10 ml/dl acetic acid, or with 7 ml/dl acetic acid. The stained gel can be scanned at 625 nm. The sensitivity is much lower than that of Coomassie staining [42].

India ink staining of proteins

The staining with carbon (India ink) [43,44] is more sensitive than the Coomassie staining and comes closely to the sensitivity of silver staining. However, it is used mostly for staining of blot membranes (s. there).

2.18.2.2 Counterionic dye staining of proteins

The counterionic dye staining method employs two oppositely charged dyes (a negatively charged acidic dye and a positively charged basic dye) to form an ion-pair complex [45]. The ion-pair complex compensates the charges of each dye producing a colloidal form in the staining solution. During the counterionic dye staining, an equilibrium exists between the ion-pair complexes and the free forms of dyes. The majority of the dye molecules form ion-pair complexes while small amounts of the free dye molecules penetrate into the gel matrix and stain proteins through charge interactions.

A counterionic dye staining method is the EV-ZC staining [46]. It uses the basic dye **e**thyl **v**iolet (EV), and the acidic dye **z**in**c**on (ZC). They stain proteins by two different charge interactions, enhancing the staining effect of each other. The detection limit of this method is 4–8 ng of protein in about 60 min with quick washing step.

The EV-ZC staining is comparable to the sensitivity of the colloidal **C**oomassie **b**rilliant **b**lue (CBB) G-250 stain. However, the linear dynamic ranges of the amount of proteins stained with EV-ZC is greater than that of colloidal Coomassie staining since the slopes of band intensity in colloidal staining are much steeper than those of EV-ZC staining. Due to its sensitivity and speed, EV-ZC staining may be more practical than any other dye-based stains for routine laboratory purposes.

2.18.2.3 Metal staining of proteins

To the metal staining of proteins belong the staining with silver [47] and chelate dye staining (zinc-imidazole staining, copper [48,49], iron [50], or gold [51,52,53]).

Silver staining of proteins

Coomassie staining is easier and more rapid than silver staining, but silver staining is considerably more sensitive [54]. However, silver staining is less reproducible and requires extremely pure chemicals [55].

Silver staining was developed in the 14th century for coloring glass surface. Hundreds of years later, Camillo Golgi perfected it for studying the nervous system [56]. Kerenyi and Gallyas introduced silver staining for proteins in gels [57]. However, its vast application to protein detection after electrophoresis was established by Merril, Switzer and colleagues, who used formaldehyde to reduce silver ions into silver atoms under alkaline conditions [58,59]. Later silver staining was applied to analyze proteins in human **c**erebro**s**pinal **f**luid (CSF) [60] showing that it was some 100 times more sensitive than Coomassie staining and its detection limit was 1 to 0.1 ng protein per mm^2 of band aria (50 ng protein in a band).

The main disadvantage of silver staining is the poor correlation between staining intensity and protein concentration, because this mehod is not an end-point staining method like the less sensitive Coomassie or SYPRO Ruby staining where densitometric or/and software-based determination of protein amounts is possible [61]. Besides, there are proteins that react little or do not react with the silver ions [62], as staining depends on the complexation of Ag^+ ions with glutamate, aspartate, and cysteine residuals [63]. As a result, different proteins cannot be compared quantitatively using silver staining neither with the **t**ime-**b**ased **a**nalysis (TBA) method nor with conventional analysis [64,65].

Taking into account the dynamic range of the silver staining, a time-based silver staining method for analyzing protein samples in polyacrylamide gels is introduced [66]. Instead of the end-point image analysis, gray intensities of time series images of a developing gel are determined and times until a threshold gray value is reached are calculated. These times are used to calculate a new grayscale image, which can be analyzed using software. Concerning an 8-bit grayscale image (256 gray tones), short times are converted into low color numbers within the palette (black = 0, white = 255), and longer times into higher gray values. Regions where the gray threshold is not reached within the observation period are set to “white”. The gels are photographed during development [67]. This procedure provides information about the absorbance for each pixel in the protein spots on the gel. The maximum rate of change correlates with the amount of protein. Regarding this, it has been suggested that quantification can be improved by recording the gel image at several times during development [68,69].

The mechanisms of the silver staining of proteins are similar to those of the photographic process [70]: The gel matrix with the separated proteins is saturated with Ag^+ ions. They bind preferentially to basic amino acid residues of proteins. The proteins become visible when the Ag^+ ions are reduced to elementary Ag. Then the stained proteins develop the typical brownish-grey-black color [71,72]. Different silver-staining protocols use different fixation solutions (ethanol or methanol as well as different acids), and different reducing reagents.

The silver staining comprises the following steps: fixing, sensitizing, developing and stopping. Finally, the gel is dried.

The *fixing* is not different from that of the Coomassie staining. It causes precipitation of proteins by denaturation. For this purpose, usually weak acids, for example, acetic acid and alcohol, are used. The power value of pH prevents the proton dissociation from proteins.

During the *sensitizing* (impregnating), silver ions are bound to the precipitated proteins and form there silver nuclei. The silver nuclei catalyze the reduction of other silver ions by reducing reagents (most formaldehyde) to black metallic silver.

Developing (visualizing) requires a strong reducing agent, such as formaldehyde, whereby the silver ions are reduced to metallic silver (Figure 2.18-4). The reaction takes place in the immediate vicinity of the silver nuclei much faster than in the gel, since the silver nuclei catalyze the reduction and cause a transformation of the protein bands into dark brown to black bands on a slightly yellowish to colorless background. The developing takes place at higher pH values, for example, in a sodium carbonate solution.

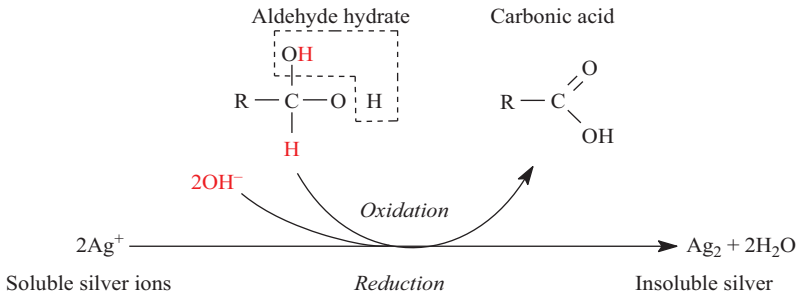


Figure 2.18-4: Mechanism of silver staining.

There are two methods of sensitizing and developing. In the first method, the sensitizing is carried out in an alkaline silver diamine solution [73], while the development proceeds at a low pH value, for example, in a citric acid solution. In the second method, the sensitizing is carried out in a neutral silver nitrate solution, while the development proceeds at a higher pH value, for example in a sodium carbonate solution [74,75].

After the developing, the reduction should be *stopped*. Otherwise, all the silver ions will be reduced to metallic silver in the gel, which will obtain a black color. In principle, the stopping is caused by a strong decrease of the pH value.

The *drying* of the colored pherogram is similar to that after the Coomassie staining. It is fulfilled usually under a hair dryer. If the polyacrylamide concentration

is high, the wet gel should be covered, free of air bubbles, with water-swollen cellophane.

Although many other advanced staining techniques such as fluorescence 2D **di**fference **g**el **e**lectrophoresis (DIGE) or Deep purple staining exist in the field of protein research [76,77], many labs still prefer the classical silver-staining method [78,79] to detect even small amounts of proteins (down to 5 ng, [80]) in polyacrylamide gels.

Classic silver staining of proteins according to Merril *et al.*

The classic silver staining according to Merril *et al.* [81] is used for protein bands obtained in SDS, 2D and IEF electrophoresis, especially after electrophoresis in IPG gels. All steps should be carried out on a shaker. This method is 50 times more sensitive than the Coomassie staining and is the hitherto the most sensitive staining method for 2D electrophoresis. Its disadvantage is the difficult quantification and the too dark gel background. The sensitivity of Merril's method is 0.05 to 0.1 ng protein/mm^2.

Rapid silver staining of proteins

Silver staining is a time consuming method. Therefore, many similar methods are obtained that economize time. Here belong the methods of Ansorge [82], the polychromatic silver staining according to Hempelmann and Kaminsky [83], the methods of Blum *et al.* [84], Patestos *et al.* [85], Nesterenko *et al.* [86], and others. Silver staining can also be used for agarose gels, as established by Willoughby and Lambert [87].

Chelate dye staining of proteins

Metal chelates form other class of stains. They represent organometallic complexes that bind to proteins [88]. The metal chelates can be easily prepared, are stable at room temperature, and can be reused more times. They are formed at acidic pH and can be eluted, if the pH value is increased to 7.0–10.0. Metal chelates can be used to detect proteins on nitrocellulose, PVDF, and nylon membranes as well as in polyacrylamide gels. They do not modify proteins, and are compatible with immunoblotting, lectin blotting, and mass spectrometry [89,90,91].

Zinc-imidazole staining of proteins

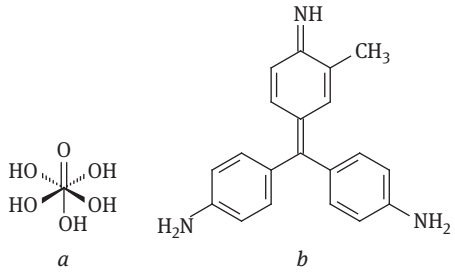
The negative zinc-imidazole staining of proteins is based on the in-gel reaction of imidazole with zinc ions. As a result, a homogeneous white precipitation of metal chelate is formed in the gel background, whereas the protein bands remain unstained. The sensitivity of this method is as good as that of silver staining [92].

Similarly, a zinc-imidazole staining method was proposed for **p**oly(**e**thylene **g**lycol) (PEG) linked proteins separated in SDS polyacrylamide gels [93,94,95].

Nonreacted (free) PEG was almost undetected. After electrophoresis, the gels were rinsed with deionized water and then incubated under gentle agitation in the imidazole-SDS solution. Then, the gels were soaked in zinc sulfate solution. The PEGylated protein bands remained transparent. Both protein-bound and free poly (ethylene glycol) [96] in polyacrylamide gels could be visualized with barium iodine (BaI_2) staining [97], whereas the protein component, either free or conjugated, was stained with Coomassie brilliant blue R-250.

2.18.2.4 Staining of glycoproteins

The staining method used to detect polysaccharides such as glycoproteins, glycolipids, mucins, and glycogen is the **p**eriodic **a**cid – **S**chiff (PAS) test [98]. Periodic acid oxidizes the vicinal diols in these sugars, usually breaking up the bond between two adjacent carbon atoms not involved in the glycosidic linkage between the monosaccharide units that are parts of the long polysaccharides, creating a pair of aldehydes of the broken monosaccharide ring. The aldehydes react with the Schiff reagent to give a purple-magenta color. The Schiff reagent is a reaction product of fuchsine and sodium hydrogen sulfite.



Chemical formulas of periodic acid (*a*) and fuchsine (*b*).

A new variant of PAS test is the staining test of Hart *et al.* [99]. It uses the dye Pro-Q Emerald 488, which reacts with periodic acid-oxidized carbohydrate groups, generating a bright green-fluorescent signal on glycoproteins. This dye permits detection of less than 5–18 ng of glycoprotein per band, depending on the nature and the degree of protein glycosylation, making it 8–16-fold more sensitive than the standard colorimetric periodic acid – Schiff test using acidic fuchsin. The green-fluorescent signal from Pro-Q Emerald 488 dye may be visualized using charge-coupled device/xenon arc lamp-based imaging systems or 470–488 nm laser-based gel scanners. Thereafter the total protein profiles may be evaluated using SYPRO Ruby protein staining (s. there).

2.18.2.5 Staining of lipoproteins

Staining with Sudan Black B. This method is commonly used for agarose gels where the lipoprotein electrophoresis is carried out most often. After electrophoresis, the gel is dried at 60 °C. Then it is stained in freshly prepared staining solution (Sudan black and sodium chloride in ethanol). If a precipitate is formed, ethanol must be added until the precipitate dissolves. Next, the gel is destained in a solution of NaCl in ethanol. After washing in deionized water the gel is dried at 60 °C.

In addition to the non-specific detection methods described up to here, there are many detection methods for specific active proteins that have been separated by electrophoresis.

2.18.2.6 Staining of enzymes

Enzymes may be detected directly in the gel or indirectly by color reactions. In the direct detecting, the gel is placed with the electrophoretically separated enzymes in a solution with specific substrates coupled to a diazo dye. In the indirect detecting, the gel is covered with an additional carrier, for example chromatography paper, cellulose acetate film, or agarose gel, which are impregnated with specific substrates.

Specific reactions have been developed for the detection of the following enzymes: alkaline phosphatase [100,101], alcohol dehydrogenase [102,103], amylase [104,105], cellulase [106], deoxyribonuclease [107], glucosidase [108], glycosyl transferase [109,110], catalase [111,112], creatine kinase [113], lactate dehydrogenase [114], malate dehydrogenase [115], peroxidase [116,117], phosphogluco mutase [118], peptide hydrolases [119,120], acid phosphatase [121,122], trypsin [123], and more.

Detecting esterase isoenzymes. The method is suitable for native and IEF polyacrylamide gels. Its sensitivity is higher than the Coomassie staining. In a hydrogen phosphate buffer with pH = 7.0, the isoenzymes hydrolyze α-naphthyl acetate liberating α-naphthol, which couples to Fast Blue RR salt (a diazonium salt) forming a diazo dye complex. The reaction is stopped and the reddish-yellow background is destained in methanol-acetic acid-deionized water until it becomes clear.

2.18.3 Autoradiography of proteins

To be proven with autoradiography and fluorography, the proteins must be labeled with radioactive isotopes. After electrophoresis, the radioactive emission, which is emanated by the protein bands, is made visible by blackening on X-ray films.

Autoradiography is the most sensitive detection method for proteins. With the aid of it, protein traces of 10^{-6}–10^{-5}% of the total protein mass can be detected and quantified. In autoradiography, X-ray film is used to visualize radioactive molecules that have been electrophoresed on agarose or polyacrylamide gels, or hybridized (e.g., immunoblots). A photon of light or a β-particle or γ-ray released from a radioactive

molecule “activates” silver bromide crystals on the film emulsion. This renders them capable of being reduced through the developing process to form grains of silver. The silver grains on the film form the image (Table 2.18-3).

Table 2.18-3: Isotope detections and their sensitivities.

Isotope	Sensitivity
^{32}P	50
^{125}I	100
^{14}C	400
^{35}S	400
^{3}H	8,000

The choice of film is critical for autoradiography. Double-coated films contain two emulsion layers on either side of a polyester support and are most commonly used for autoradiography [124]. They are ideal for detecting high-energy β-particles emitted by ^{32}P and ^{125}I, since they can penetrate the polyester support and expose both emulsion layers. These films are normally used with calcium tungstate ($CaWO_4$) in intensifying screens.

Single-coated films, containing one emulsion layer, are optimized for direct-exposure techniques with medium-energy radioisotopes (e.g., ^{14}C, ^{35}S, and ^{33}P, but not ^{3}H). The majority of β-particles emitted by these isotopes cannot pass through the polyester support of double-coated films, and therefore the emulsion layer on the other side of the film is useless. Even though direct exposure with single-coated films gives better clarity for medium-energy isotopes; single-coated films often require longer exposure times. Fluorography, therefore, is often used to enhance sensitivity. The blue-light-sensitive double-coated X-Omat AR film is generally used for fluorography with 2,5-diphenyloxazole, which emits at 388 nm, or sodium salicylate, which emits at 420 nm.

The separated proteins should be first fixed. Then the polyacrylamide gel containing radiolabeled proteins should be dried to prevent its sticking to the film. Next, the dried gel should be covered (exposure) with an X-ray film (Figure 2.18-5).

The autoradiography can be performed directly or indirectly.

For the *direct autoradiography*, the proteins are marked with the isotopes ^{14}C, ^{35}S, ^{32}P, or ^{125}I [125] and after electrophoresis the resolving gel is brought into contact with an X-ray film for 24 h. The blackening on the film is made by the direct irradiation of the radioactively labeled proteins in the gel.

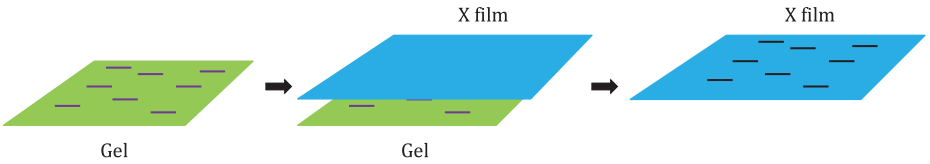


Figure 2.18-5: Autoradiography.
A gel with radiolabeled proteins or nucleic acids is dried and overlaid with a photographic film in a cassette to prevent light entering. After hours to days, the film is developed and as a result dark lines appear where the radioactive molecules were present.

In the *indirect autoradiography*, the radioactive radiation is amplified with the aid of a calcium tungstate screen. Therefore, it is several times more sensitive than the direct autoradiography. After electrophoresis, a "sandwich" is built on, which consists of the separation gel, an X-ray film, and the intensifying screen, and is incubated at –70 °C. The radioactive radiation emitted from the gel, hits the film and produces darkness on it. The part of the radiation that penetrates the film hits the screen and excites there emission of light quanta. They take place back on the film and enhance the blackening.

In autoradiography, the resulting blackening can be excised and weighed. Thus, the concentrations of electrophoretically separated proteins can be determined.

2.18.4 Fluorography of proteins

Fluorescent staining is more sensitive than Coomassie staining, and often as sensitive as silver staining.

Fluorescence occurs when a molecule absorbs light with a certain wavelength and releases this energy by emitting photons. The common adsorptive staining methods (Coomassie and silver stainings) do not provide the requisite dynamic range, being linear over only a 10- to 40-fold difference in protein concentration [126], and suffer from being requiring harsh chemicals. Fluorescence detection technologies provide linear signal response over a much wider signal range.

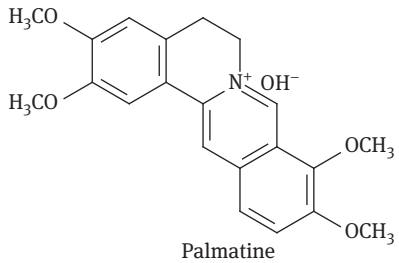
Numerous fluorescent reactions have been reported, the most of which being either fluorescent or fluorogenic derivatives of primary amines [127,128]. Fluorescent modification of proteins can be divided into two categories: covalent and noncovalent. In covalent methods, the proteins are derivatized with a dye prior to electrophoresis [129,130]. The main advantages of covalent derivatization methods are the elimination of staining and destaining of gels and the ability to image and capture the protein separation pattern immediately after electrophoresis. In noncovalent methods, the proteins are first separated by SDS-PAGE followed by staining with dyes that bind to SDS-protein complexes, for example, SYPRO dyes [131,132,133].

Most commonly utilized fluorogenic reagents are **o**-**p**hthaldi**a**ldehyde (OPA) and **n**aphthalene **d**icarbox**a**ldehyde (NDA) [134]. OPA is used in the presence of a thiol, for example, **β**-**m**ercapto**e**thanol (BME) [135], 3-mercapto-1-propanol [136], **d**i-**t**hio**t**hreitol (DTT) [137], ethanethiol [138], or **N**-**a**cetyl-L-**c**ysteine (NAC) [139]. The primary limitation of the OPA/BME reaction is the UV excitation (335 nm) required for detection of the resulting isoindole products. However, the UV lasers are generally more expensive and less stable than the visible lasers. Moreover, the quantum yield of the isoindole products varies with weak molar extinction.

The fluorescence-labeling of Cys residues presents a more attractive alternative than labeling of Lys residues. Cys residues are highly reactive, their frequency of occurrence in a protein is lower than most other residues, and they are not cleaved by commonly available proteases. Analysis of the SWISS-PROT database demonstrates that 88% of all proteins, 92% of yeast proteins, and 89% of human proteins have at least a single Cys residue.

In the *fluorography*, the gel with the electrophoretically separated proteins, which have been previously labeled with ^{3}H, ^{14}C or ^{35}S, are impregnated with scintillating substances such as 2,5-diphenyloxazole [140] or sodium salicylate [141]. After the gel is dried, it is let to act on X-ray film at −70 °C. In the interaction between the radioactive radiation and the scintillating substance visible light is produced that blackens the film. It is also possible to treat the sample, prior to electrophoresis, with a fluorescent marker [142,143], which makes the bands visible under UV light after the electrophoresis.

A fast and sensitive protein fluorescent detection method for SDS-PAGE using the natural product palmatine was described [144,145]. Palmatine is an alkaloid found in various plants exhibiting a broad spectrum of antibiotic activity in humans. It appears to bind to the detergent coat surrounding proteins in SDS gel, because SDS ions act as counterions having negatively charged groups [146]. This method does not involve a destaining step. Fixing and staining of proteins take about 30 min. As little as 2 ng of protein can be detected. The sensitivity is comparable to colloidal Coomassie staining. Stained proteins can be photographed using a UV transilluminator. The method can be compared with the of SYPRO Ruby staining.



Palmatine

2.18.4.1 SYPRO Ruby staining of proteins

SYPRO Ruby staining was first introduced in 2000 by Berggren *et al.* [147]. Its detection sensitivity for proteins separated by 1D and 2D polyacrylamide gel electrophoresis is better than any of the currently used protein stainings and as sensitive as the best silver staining procedures available [148].

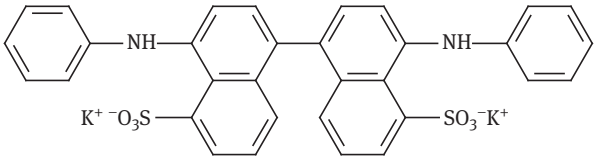
SYPRO Ruby stain is a fluorescent ruthenium-based metal chelate dye, which interacts with basic amino acid residues (of lysine, arginine, or histidine). Its staining is superior because of the broad linear dynamic range of quantization, good reproducibility, and ability to identify low amounts of protein [149,150]. In addition, the SYPRO Ruby staining needs only three steps: fixation, staining, and destaining. Only the high cost for SYPRO Ruby stain limits its application in most laboratories.

The SYPRO Ruby staining of proteins in nondenaturing gels is generally less sensitive than the staining of proteins in SDS gels. Therefore, it is better, if native gels are soaked in 0.05 g/dl SDS for 30 min and then stained with SYPRO Ruby stain diluted in 7.5 ml/dl acetic acid.

SYPRO Ruby stain is compatible with **m**atrix-**a**ssisted **l**aser **d**esorption/**i**onization (MALDI) mass spectrometry. MALDI mass spectrometry is a highly versatile and sensitive analytical technique, which is carried out through soft ionizing biomolecules such as peptides and proteins. It requires little sample preparation, and can be automated using robotic liquid-handling systems. MALDI mass spectrometry is utilized in the search for biomarkers, for example prostate cancer biomarker [151].

A fluorescent dye with detection close to that of SYPRO Ruby stain is **D**eep **P**urple (DP). It consists of the naturally fluorescent compound, epicocconone, derived from the fungus *Epicoccum nigrum* [152]. Deep Purple can detect picograms of protein [153], however is poor in comparison to SYPRO Ruby stain in 2D electrophoresis analysis [154,155]. Another well-known disadvantage to the use of Deep Purple is its photoinstability – its signals are reduced by half after six minutes of light exposure, while three-fold more time is needed for the signals of SYPRO Ruby stain [156]. Additionally, there is no difference in its cost relative to SYPRO Ruby stain [157].

Another fluorescent dye is BisANS (4,4′-Di**a**nilino-1,1′-bi**n**aphthyl-5,5′-di**s**ulfonic acid dipotassium salt). It is an amphipathic compound with two sulfonate groups: aromatic naphthalene and phenyl moieties. BisANS binds to high affinity to hydrophobic regions of protein surrounded by positively charged amino acid residues [158,159,160]. This leads to the formation of stable complexes between it and protein chains. Therefore, it is used to probe hydrophobic sites of proteins and to study protein-substrate interaction and protein conformational changes. Furthermore, BisANS was applied to visualize proteins in SDS-PAGE with the enhancement of KCl or $BaCl_2$, which gives much less nonspecific background fluorescence. As a result the fluorescence of this dye increases dramatically. It approaches the sensitivity of Coomassie blue staining [161].



BisANS

Similar simple and sensitive fluorescent staining method for detection of proteins in SDS-PAGE is the **i**mproved **B**isANS (IB) staining [162]. Using it, as low as 1 ng of protein band can be detected briefly by 30 min washing followed by 15 min staining without the aiding of stop or destaining step. The sensitivity of IB staining is similar to that of SYPRO Ruby staining.

Another very sensitive fluorescent staining of protein in SDS polyacrylamide gels was described [163]. In this method, salicylaldehyde azine was introduced as a sensitive fluorescence-based dye for detecting proteins both in 1D and 2D polyacrylamide electrophoresis gels. Down to 0.2 ng of single protein band could be detected within 1 h, which is similar to that of glutaraldehyde-silver stain, but approximately four times higher than that of SYPRO Ruby fluorescent stain. According to the same authors, hydrogen bonding and hydrophobic forces contributed the interaction between salicylaldehyde azine and protein.

2.18.5 Double isotope detection

The double isotope detection is a combination of fluorography and autoradiography. It is of interest, if the stains are to be compared with each other by two samples after two-dimensional electrophoresis.

One of the two samples is labeled with weak β-emitters such as ^{3}H, the other emitted with an isotope, the energy-rich β-emitters, such as ^{14}C or ^{35}S. Then the two samples are mixed and a two-dimensional electrophoresis is run.

First, the 2D-pherogram is made visible by fluorography, wherein the proteins of the two samples cause blackening on the X-ray film. Then, an opaque layer is put between the gel and an X-ray film. Thus, only the high-energy β-rays can penetrate the layer and expose the film. In this way, the 2D pherogram of the second sample is obtained autoradiographically, without the proteins of the first sample appear on the film as spots. The comparison between the fluorogram and the autoradiogram helps to explain the protein composition of both samples.

2.18.6 Phosphor imaging

Phosphor imaging screens can be used as an alternative to the film for recording and quantifying autoradiographic images [164]. They can detect radioisotopes such

as ^{32}P, ^{125}I, ^{14}C, ^{35}S, and ^{3}H. Phosphor imaging screens have some advantages over the film: the linear dynamic ranges are 5 orders of magnitude, compared to 1.5 orders of magnitude for the film; the exposure times are 10 to 250 times faster than that with the film; the quantification is much easier and faster, and the phosphor screens can be reused indefinitely.

Phosphor imaging screens are composed of crystals of $BaFBr{\cdot}Eu^{2+}$. When the screen is exposed to ionizing radiation, such as α-, β-, or γ-radiation, or wavelengths of light shorter than 380 nm, the electrons from Eu^{2+} are excited and then trapped in the BaFBr complex. This results in the oxidation of Eu^{2+} to Eu^{3+}, which forms the latent image on the screen. After exposure, the latent image is released by scanning the screen with a laser (633 nm). During scanning, Eu^{3+} reverts back to Eu^{2+}, releasing a photon at 390 nm. The luminescence can be collected and measured in relation to the position of the scanning laser beam. The result is a representation of the latent image on the storage phosphor imaging plates. The image can be viewed on a video monitor and analyzing with the aid of appropriate software.

2.18.7 Destaining of gel background

Generally, the destaining of gel background is carried out in the solution where the stain was dissolved.

Cellulose acetate membranes, stained with Ponceau S, are destained in 0.5 g/dl TCA.

Agarose gels, stained with Amido black 10B, are destained in 10 ml/dl acetic acid.

Polyacrylamide gels, stained with Coomassie brilliant blue R-250, are destained in a mixture of methanol (ethanol)-acetic acid-water (3:1:6, *V:V:V*). The last destaining solution should contain 5 ml/dl glycerol.

Polyacrylamide gels, stained with colloidal Coomassie brilliant blue G-250, are destained in 0.1 mol/l TRIS-hydrogen phosphate buffer with pH = 6.5.

Agarose gels, stained for glycoproteins with periodic acid and Schiff reagent, are destained in 7 g/dl acetic acid.

Agarose gels, stained for lipoproteins with Sudan black B, are destained in a mixture of ethanol and 2 g/dl NaCl (55:45, *V:V*).

Polyacrylamide gels, stained with silver, are destained in deionized water.

Polyacrylamide gels, stained with SYPRO Ruby stain, are destained by incubating the gel overnight in 0.1 ml/dl Tween 20 or in 7.5 ml/dl acetic acid

2.18.8 Drying of gels

Gel drying can be carried out in air, under a hair fan, or in a drying room. If the polyacrylamide concentration is higher than 10 g/dl, prior to drying the wet gels

can be covered with a cellophane membrane. The gradient polyacrylamide gels are placed, with the support film down on a glass plate and are covered free of air bubbles with a water-swollen cellophane membrane, wherein the protruding margins of the membrane are folded onto the underside of the glass plate. Upon drying, the cellophane membrane adheres firmly to the gel surface.

The ultrathin polyacrylamide gels and the agarose gels usually do not require to be covered with a cellophane membrane.

The dried gels can be stored as documents.

2.18.9 Documentation of protein pherograms

The documentation of a protein pherogram is carried out by photography.

Gel photography of Coomassie- or silver-stained gels. Any good **s**ingle-**l**ens **r**eflex (SLR) camera attached to a copy stand will give good results. Kodak T-Max 400 film is a fine-grained panchromatic half-tone film that provides extremely high resolution and works well for 35 mm gel photography. For contrast enhancement of Coomassie blue-stained gels, photographing of gels through a deep-yellow to yellow-orange filter is recommended.

Silver-stained gels are photographed with a blue-green filter. A simple test for the effectiveness of a filter is to place the gel on a light box and observe the gel through the filter. Increased contrast of the bands should be obvious. Kodak X-Omat duplicating film is a useful alternative because it yields black banding in spite of the coloration caused by silver staining.

The instant films from Polaroid are ideal for fast, high-quality photographs of gels for laboratory notebooks and publications. Type 55 and 665 positive/negative films provide not only a high-quality print but also a fine-grain medium-format negative that can be used to produce multiple photographs of the gel in any size. Aperture settings of f11 or higher should be used for sharp photographs. The shutter speed and stop are also necessary to optimize results.

Gel photography of fluorescently stained gels. Photographing and archiving the gel is essential to obtain high sensitivity. The camera's integrating effect can make bands visible that are not visible to the eye. The fluorescently labeled gel should be placed directly on a standard 300-nm UV transilluminator or a blue-light transilluminator. PhastGels (Amersham Pharmacia Biotech) have a polyester supporting material (Gelbond) that not only is highly autofluorescent, but also binds the SYPRO Orange and Red protein gel stains, producing additional background fluorescence. This plastic support should be removed before trying to visualize bands.

Charge-**c**oupled **d**evice (CCD) cameras provide good sensitivity. A Polaroid 667 black-and-white print film and a photographic filter for SYPRO protein gel stain can

be used to obtain highest sensitivity. Do not use standard ethidium bromide filters, as they will block much of the light and lead to lower sensitivity. The use of different film types may require longer exposure times or different filters. Exposure time should also vary with the intensity of the illumination source.

2.18.10 Protocols

2.18.10.1 Ponceau staining of cellulose acetate membranes

Materals and equipment
Ponceau S
Tri**c**hloro**a**cetic acid (TCA)

Procedure
- Fix and stain a CA membrane with separated proteins in 0.3 g/dl Ponceau S in 3 g/dl TCA for 5 min.
- Destain 3 times in 0.5 g/dl TCA.
- Dry at room temperature.

2.18.10.2 Amido black staining of proteins in agarose gels

Materals and equipment
Amido black 10B
Acetic acid
Hair dryer

Staining solution

Amido black 10B	0.1 g
Methanol	20.0 ml
Acetic acid	10.0 ml
Deionized water to	100.0 ml

Procedure
- Fix an agarose gel with separated protein bands in 10 ml/dl acetic acid for 10 min.
- Dry the gel in oven or under a hair dryer.
- Stain the protein bands in the staining solution for 10 min.
- Destain the gel background in 10 ml/dl acetic acid until the background is clear.
- Dry the gel at room temperature, or under a hair dryer.

2.18.10.3 Coomassie brilliant blue staining of proteins in polyacrylamide gels

Materals and equipment
Trichloro**a**cetic acid (TCA)
Coomassie brilliant blue R-250
Acetic acid
Methanol or ethanol

Fixing solution

Ethanol	50.0 ml
Acetic acid	10.0 ml
Deionized water to	100.0 ml

Solution A

Coomassie brilliant blue R-250	0.2 g
Methanol or ethanol	60.0 ml
Deionized water to	100.0 ml

Solution B

Acetic acid	20.0 ml
Deionized water to	100.0 ml

Procedure

- Fix a polyacrylamide gel with separated protein bands in the fixing solution for 20 min. Gels that contain non-ionic detergents (Nonidet NP-40, Triton X-100) are to be fixed with 30 ml/dl isopropanol.
- Rinse the gel with tap water.
- Stain the protein bands with a mixture of solution A and Solution B (1:1, *V:V*) for 20 min.
- Destain the gel several times in 10 ml/dl acetic acid or methanol (ethanol)-acetic acid-water (3:1:6, *V:V:V*). The last destaining solution should contain 5 ml/dl glycerol. The fixation, staining, and destaining can be accelerated, if the temperature is increased to 60 °C.
- Place the polyacrylamide gel onto a glass plate, with its support film down.
- Cover the gel with a cellophane membrane swollen in water.
- Remove the air bubbles between the gel and the cellophane membrane using a roller. Place the edges of the cellophane membrane around the glass plate.
- Dry at room temperature.

2.18.10.4 Colloidal Coomassie staining

Materals and equipment
Glutardialdehyde

Coomassie brilliant blue G-250
Ammonium sulfate [$(NH_4)_2SO_4$]
Phosphoric acid (H_3PO_4)
Methanol or ethanol

Purification of Coomassie brilliant blue G-250
Dissolve 4 g of Coomassie brilliant blue G-250 in 250 ml of 10 ml/dl acetic acid and heat up to 60 °C. After cooling, filter or centrifuge the solution and use the sediment.

Preparing the staining solution
Add 10.0 g of ammonium sulfate to 98 ml of 2 g/dl H_3PO_4 until it dissolves completely. Mix then with 2 ml of 5 g/dl Coomassie brilliant blue G-250 solution. Do not filter.

Procedure
- Fix a polyacrylamide gel with separated protein bands in 10 g/dl TCA for 1 h.
- Stain the proteins in the staining solution overnight.
- Rinse the gel in 0.1 mol/l TRIS-hydrogen phosphate buffer with pH = 6.5 for 2 min, and in 25 ml/dl methanol for 1 min.
- Stabilize the protein-dye complexes in 20 g/dl ammonium sulfate.

2.18.10.5 Silver staining according to Merril *et al.* (modified)

Use reagent-grade chemicals. Work with gloves.

Materials and equipment
Fixing solution
Silver nitrate solution
Developing solution

Solutions
Fixing solution (methanol-acetic acid-water, 30:1:6, *V:V:V*)
DTT reagent (0.004 g/dl D,L-dithiothreitol)
Oxidizer (0.1 g/dl potassium dichromate, 0.46 ml/dl 65 g/dl nitric acid)
Silver nitrate-reagent (0.2 g/dl silver nitrate)
Developer (3 g/dl sodium carbonate, 0.12 ml/dl 37 g/dl formaldehyde)

Procedure
- Fix a polyacrylamide gel with separated proteins in the fixing solution for 1 h.
- Wash in methanol-acetic acid-water (2:1:7, *V:V:V*) for 1 h.
- Wash in 1 ml/dl acetic acid for 30 min.
- Reduce in the DTT reagent for 30 min.

- Oxidize in the oxidizer for 5 min.
- Wash twice in deionized water for 2 min.
- Incubate in the silver nitrate-reagent for 30 min.
- Wash twice in deionized water for 20 s.
- Develop in the developer for 5 min.
- Stop in 1 ml/dl acetic acid for 5 min.
- Wash twice in deionized water for 30 min.

2.18.10.6 Rapid silver staining

This protocol is rapid but is not enough sensitive in detecting very small protein bands.

Materials and equipment

Formaldehyde fixing solution
$AgNO_3$
Sodium thiosulfate ($Na_2S_2O_3$)
Citric acid
Dialysis membrane
Glass plates

Procedure

- Place a polyacrylamide gel with separated proteins in a plastic container, add formaldehyde fixing solution, and agitate on a shaker at room temperature for 10 min.
- Wash the gel twice with deionized water for 5 min.
- Soak the gel with 0.2 g/l $Na_2S_2O_3$ for 1 min.
- Wash the gel twice in deionized water for 20 s.
- Soak the gel in 0.1 g/dl $AgNO_3$ for 10 min.
- Wash the gel with deionized water.
- Wash with fresh 0.2 g/dl sodium thiosulfate developing solution and agitate until the band intensities are adequate.
- Add 2.3 mol/l citric acid and agitate for 10 min.
- Wash the gel in deionized water, agitating for 10 min.
- Sandwich the gel between two pieces of wet dialysis membrane on a glass plate and dry overnight at room temperature.

2.18.10.7 Nonammoniacal silver staining

Materials and equipment

1,4-**di**thio**t**hreitol (DTT)
Silver nitrate (store in a brown bottle)
Na_2CO_3
Citric acid

Procedure

- Place a polyacrylamide gel with resolved proteins into a glass or polyethylene container filled with fixing solution. Agitate on a shaker at room temperature for 30 min.
- Immerse the gel in the destaining solution, and agitate for another 30 min.
- Cover the gel with 10 g/dl glutardialdehyde, and agitate for 10 min.
- Wash the gel in running water for 2 h.
- Soak the gel in 5 µg/ml DTT for 30 min.
- Add 0.1 g/dl silver nitrate and agitate for 30 min.
- Wash the gel quickly with deionized water.
- Soak the gel in 0.03 g/dl Na_2CO_3 and agitate until desired level of staining is achieved.
- Stop the staining by adding of 2.3 mol/l citric acid and agitating for 10 min. The carbonate and citric acid solutions bring together neutral pH value.
- Wash the gel with deionized water.
- Photograph the gel.

2.18.10.8 Staining of glycoproteins

Materials and equipment

Tri**c**hloro**a**cetic acid (TCA)
Periodic acid
Acetic acid
Schiff reagent

Fixing solution

12.5 g/dl TCA

Staining solution

1 g/dl periodic acid

Washing solution

15 ml/dl acetic acid

Schiff reagent

- Dissolve 5 g of basic fuchsine in 900 ml of boiling deionized water.
- Cool to 50 °C and add 100 ml of 1 mol/l HCl.
- Cool to 25 °C and dissolve 10 g of $K_2S_2O_5$.
- Shake for 3 min and incubate in the dark at room temperature for 24 h.
- Add 5 g of fine activated charcoal and shake for 3 min.
- Filter and store at 4 °C in a dark bottle.

Destaining solution
7 g/dl acetic acid

Procedure
- Fix the glycoprotein bands in the fixing solution for 10 min.
- Stain in the staining solution for 2 h.
- Wash 4 times 15 min each in the washing solution.
- Incubate with Schiff reagent in the refrigerator at 4 °C for 2 h.
- Destain overnight in the destaining solution until colorless background. The glycoprotein bands become red-purple (magenta).
- Dry on the air.

2.18.10.9 Staining of lipoproteins with Sudan black B

Materials and equipment
Sudan back B
Ethanol
Sodium chloride

Sudan black solution

Sudan black B	2.0 g
Ethanol	100.0 ml

Sudan black staining mixture

Sudan black solution	10.0 ml
2 g/dl NaCl	75.0 ml
Ethanol	90.0 ml

Prepare fresh. If precipitates are formed, add ethanol until the precipitates dissolve.

Destaining solution

2 g/dl NaCl	55.0 ml
Ethanol to	100.0 ml

Procedure
- Dry a gel with separated lipoproteins at 60 °C.
- Stain in the freshly prepared Sudan black staining solution for 15 min.
- Destain in the destaining solution for 15 min.
- Wash in deionized water for several seconds.
- Dry the gel at 60 °C.

2.18.10.10 Detecting esterase isoenzymes
All procedures for detecting esterase isoenzymes are performed at room temperature on a shaker. The method is suitable for native and IEF polyacrylamide gels. The sensitivity of this method is higher than the Coomassie staining.

Materials and equipment

Hydrogen phosphate buffer, I = 0.1 mol/l, pH = 7.0

Na_2HPO_4	20.6 g
NaH_2PO_4	4.4 g
Deionized water to	1,000.0 ml

α-Naphthyl acetate solution

α-Naphthyl acetate	0.06 g
Acetone to	5.00 ml

Fast blue salt solution

Fast blue salt (Fast blue B)	0.1 mg
Deionized water to	3.0 ml

Destaining solution

Methanol	20.0 ml
Acetic acid	10.0 ml
Deionized water to	100.0 ml

Procedure

- Stain a gel with esterase isoenzymes in a mixture of Fast blue salt solution and hydrogen phosphate buffer (1:1, *V:V*).
- Wait until full development of purple red esterase bands. This takes about 10 min.
- Stop and destain in destaining solution until the reddish-yellow background is clear.
- Dry at air.

2.18.10.11 Preparing gels for autoradiography of proteins

Remember that some of the solutions coming in contact with the gel are radioactive.

Materials and equipment

Glass dish
Filter paper sheets
Rotary shaker
UV-transparent plastic wrap
Gel dryer with vacuum pump
Fluorescent glow-in-the-dark ink
X-ray film
Film cassette

Fixing solution

Acetic acid	10.0 ml
Ethanol	40.0 ml
87% Glycerol	3.0 ml
Deionized water to	100.0 ml

Procedure

- After electrophoresis make a notch in the upper right-hand corner of the polyacrylamide gel for orientation.
- Place the gel in the glass dish full with the fixing solution.
- Place the dish onto a shaker and rotate until all Bromophenol blue in the sample buffer (if used) has disappeared.
- Rinse the gel with deionized water for a few minutes. Drain.
- Place a sheet of chromatographic filter paper over the gel.
- Cover the dried gel with plastic wrap to prevent it from sticking to the film and contaminating the cassette with radioactive materials.
- Place the plastic wrap with the gel in the film cassette in a dark room illuminated with a safelight.
- Use the fluorescent glow-in-the-dark ink to mark the gel.
- Place an X-ray film onto the gel, and close the film cassette (Figure 2.18-6).
- Expose the film for desired length of time and at appropriate temperature. The sensitivity of the film can be improved using flours or intensifying screens (Table 2.18-4).

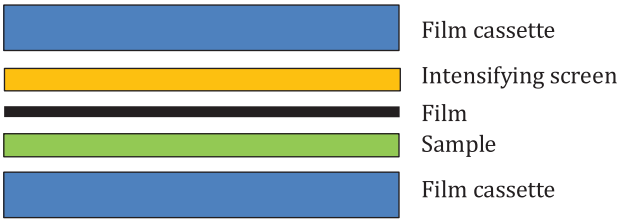


Figure 2.18-6: Autoradiography setup.

Table 2.18-4: Films and exposure temperatures for autoradiography.

Isotope	Film	Exposure temperature
^{3}H	Double-coated	−70 °C
^{35}S, ^{14}C, ^{32}P	Single-coated	Room temperature
^{35}S, ^{14}C, ^{32}P	Double-coated	−70 °C
^{32}P, ^{125}I	Double-coated	−70 °C

Time of exposure depends on the strength of the radioactivity in the gel.
- After exposure, return cassette to the dark room.
- Remove the film for developing.
- Immerse the film in 20 °C developer for 5 min, and wash in running water at room temperature for 1 min.
- Immerse the film in 20 °C fixer for 5 min, and wash in running water for 15 min.
- Hang the film to dry.

2.18.10.12 SYPRO Ruby staining of proteins

Materials and equipment

SYPRO Ruby protein stain (ready to use solution).
Acetic acid
Ethanol
Tween 20
Gel dryer

Fixing solution

Acetic acid	10.0 ml
Ethanol	40.0 ml
Deionized water to	100.0 ml

Destaining solution

Tween 20	0.1 ml
Deionized water to	100.0 ml

Procedure
- Fix a polyacrylamide gel with separated proteins in the fixing solution for 20 min.
- Place the gel into a plastic dish containing fluorescent staining solution. Cover the gel with aluminum foil to protect from light.
- Agitate gently at room temperature for 10 to 60 min.
- Stain 1D and 2D gels for a minimum of three hours. Stain IEF gels overnight.
  The staining time depends on the thickness of the gel and gel concentration.
- Destain the gel background by incubating overnight in the destaining solution to minimize background fluorescence.
- Incubate the gel in a 2 ml/dl glycerol for 30 min.
- Dry the gel using a gel dryer.
  Gels may be dried between cellophane membrane sheets in the dark to prevent photobleaching.

2.18.10.13 Visualization with luminescent substrates

Materials and equipment

TRIS-**b**uffered **s**aline (TBS)
Substrate buffer
50 mmol/l TRIS-HCl, pH = 7.6 (for **h**orse**r**adish **p**eroxidase, HRP) or dioxetane phosphate substrate buffer (for **al**kaline **p**hosphatase, ALP)
5 ml/dl Nitro-Block (only for ALP)
Luminescent visualization solution
Plastic wrap

TRIS-buffered saline

TRIS	6.05 g
NaCl	8.76 g
Deionized water to	800.00 ml
Adjust pH to 7.6 with 1 mol/l HCl.	
Deionized water to	1,000.00 ml

Procedure

- Equilibrate the membrane in substrate buffer two times for 15 min each.
- Incubate the nitrocellulose or PVDF membrane for ALP reactions in freshly prepared 5 ml/dl Nitro-Block in dioxetane phosphate substrate buffer for 5 min, then in substrate buffer for 5 min.
- Transfer the membrane into luminescent visualization solution.
- Soak for HRP reaction for 30 s, and for ALP reaction for 5 min.
- Remove the membrane, drain, and place face down on a sheet of clear plastic wrap.
- Fold the wrap back onto the membrane to form a liquid-tight enclosure.
- Place the membrane face down onto a film in a darkroom.
- Expose the film for a few seconds to several hours.
- Wash the membrane in TBS two times for 15 min each and process for chromatogenic development.

2.18.11 Troubleshooting

Problem	Cause	Solution
The protein bands are slightly colored.	The sensitivity of the staining method is low. Detection sensitivity of the peroxidase reaction is low.	Stain more time. Use another staining method, for example silver staining. Use another peroxidase method, for example tetrazolium or immunogold method.

(continued)

Problem	Cause	Solution
The Coomassie color has insufficient intensity.	SDS was not completely removed from the proteins.	Clean the gels more time to remove SDS, and color longer time.
	The alcohol concentration in the destaining solution was too high.	Reduce the concentration of ethanol or methanol in the destaining solution, or use a colloidal staining method.
The gel background is blue after Coomassie staining.	The basic immobilines in IPG gels bind Coomassie.	Use a Coomassie staining solution with a concentration of less than 0.5 g/dl. Apply a colloidal or silver staining method.
Colloidal gold protein stain has low sensitivity.	The incubation time was insufficient.	Increase the incubation time for detection of low-level signals. Overnight incubation is possible, although background staining can increase.
	The stain was exhausted, as evidenced by loss of the dark burgundy color.	Discard the reagent.
	Buffer salt contamination has occurred; the solution is light blue instead of dark burgundy.	Discard the reagent.
Colloidal gold staining causes a high background.	The blocking step was insufficient or was omitted.	Block with 0.3 ml/dl Tween 20 in TBS using 3 washes of 20 min each.
	Contamination occurred during electrophoresis or transfer.	Replace or thoroughly clean contaminated fiber pads, if a tank blotter was used.
	Excessive amount of protein was loaded on the gel or too much SDS was used in the transfer buffer. Proteins can pass through the membrane without binding and recirculate through a tank blotting system.	Reduce the amount of protein on the gel or SDS in the transfer buffer. Add a second sheet of membrane to bind excess protein.
	The colloidal gold stain solution was contaminated.	Use a clean container to store the solutions.
	The stain can be contaminated with buffer salts, which react with the gold solution causing nonspecific precipitation of the reagent onto the membrane.	If the solution is no longer dark burgundy but light blue, discard it.

(continued)

Problem	Cause	Solution
	The development step was too long. Overnight development may slightly increase sensitivity but may also increase background.	Reduce the development step to 1–2 h.
Low sensitivity with anionic dyes.	Anionic dye stains do not detect bands with protein below 100 ng.	Use more sensitive stains such as colloidal gold stain or fluorescent stain. Increase the sample load.
High background after staining with anionic dyes.	Destaining was insufficient.	Increase the number and duration of washes with the destaining solution.
	The dye solution was too concentrated.	Prepare new solution.
Low sensitivity of fluorescent blot stains.	Proteins have low hydrophobicity. SDS is stripped off them during transfer, resulting in little retention of the SYPRO dye on proteins.	Decrease SDS concentration.

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2.19 Quantitative evaluation of a protein pherogram

The presence or absence of a polyionic band can be detected with the naked eye. However, it is difficult or impossible to determine the differences between the stain intensities (concentrations) of the bands. This can be done by densitometry or by scanning the pherograms.

2.19.1 Densitometry

Densitometry means quantitative measurement of the absorbance (optical density) of materials when light passes through them [1]. The maximum and minimum density that can be produced by a material is called D_{max} and D_{min}, respectively. The difference between them is referred to *density range* [2]. It is related to the exposure range (*dynamic range*) of light intensity represented by the Hurter–Driffield curve [3,4].

Densitometry is carried out on dry gels and gel photos. As a result, a densitogram is obtained. The densitogram is a curve that represents the bands obtained by cellulose acetate, agarose gel or polyacrylamide gel electrophoresis (Figure 2.19-1).

Film images obtained by autoradiographic methods can also be quantified by densitometry. The amount of light absorption is proportional to the amount of radioactivity in the gel. Most densitometers are available with software that facilitates calculations and allows the user to define the region of the film to be measured.

Densitometers are also offered that measure the light reflected by samples. They are useful when the sample medium is opaque.

2.19.1.1 Theory of densitometry

When a light beam (radiant flux) of a certain wavelength and intensity I_0, in cd, irradiates a substance, a part of it is absorbed, resulting a reduction of its intensity into I, in cd. According to the Beer–Lambert law [5,6], the natural logarithm of the ratio between the irradiated to transmitted light is referred as absorbance A (**o**ptical **d**ensity, OD):

$$A = ln\frac{I_o}{I} \tag{2.19-1}$$

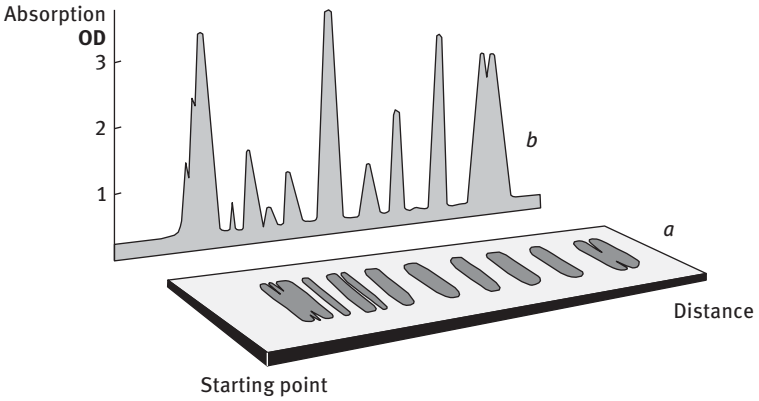

Figure 2.19-1: Densitometry.
a) Pherogram; *b*) Densitogram.

Using the Beer–Lambert law, only the absorption (extinction) of dilute solutions can be measured. However, the electrophoretic bands are protein precipitates, which are bound to a dye, or are transformed in autoradiographic spots. Therefore, the absorption is not linear but hyperbolic or sigmoidal.

In the densitometry, white light or laser light is used. The white light must be adjusted, with the aid of a suitable filter, because the dyes used for protein staining have different absorption maxima (Figure 2.19-2). On the contrary, the laser beam has a fixed wavelength, an extremely narrow width and only one spectral line. For example, the wavelength of the helium-neon laser is 632.5 nm. With white light is possible to obtain linear absorption measurement up to 2.5 OD, with a laser up to 4.0 OD.

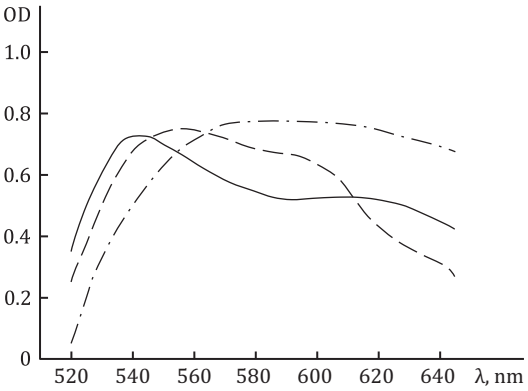

Figure 2.19-2: Absorption spectra of different dyes.
— Coomassie brilliant blue R-250; — — Coomassie brilliant blue G-250; — ·— Amido black 10B (Naphtol blue black)

Depending on whether the measurement is performed at the surface or inside of the separating medium, reflecting or transmitting densitometry is discriminated.

Baseline and band integration

The baseline of a densitogram defines its background. Two methods are used to define the baseline (Figure 2.19-3): In the first method, the baseline is drawn as a horizontal line through the lowest point of a densitogram (horizontal baseline). This method is used for pherograms with homogeneous background. In the second method, the baseline connects each band minimum (manual baseline). This method is used at unevenly colored background. The first method has some advantages, but the manual baseline brings more precise results, as it excludes the influence of the background.

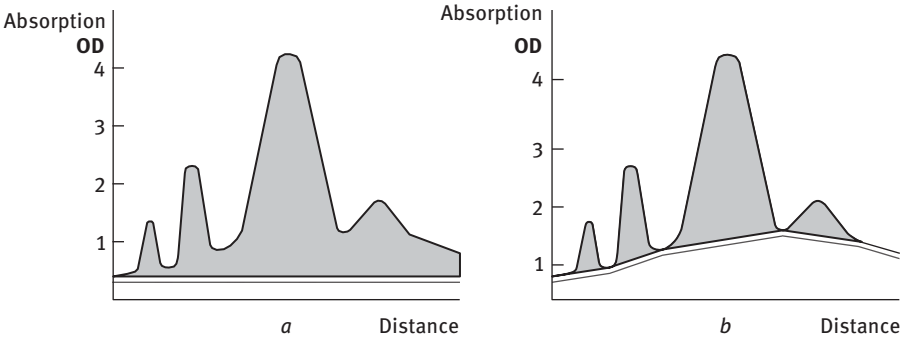

Figure 2.19-3: Different baselines. *a*) Horizontal baseline; *b*) Manual baseline.
– – – Baseline

The band integration (band analysis) can be made using two methods (Figure 2.19-4). According to the first method, the band minima are connected by vertical lines with the baseline, and the circumscribed area is measured. According to the second method, called Gaussian integration, the bands are represented as Gaussian surfaces. This method is much more accurate, but requires a computer with an appropriate software.

Evaluation of a densitogram

Evaluating a densitogram, absolute or relative concentrations can be determined. In the first case, calibration curves are required; in the second case, a relationship between the total concentration and the band surface is needed.

The absolute concentration of a protein can be calculated from its calibration curve. However, the relative concentration is often more important than the absolute

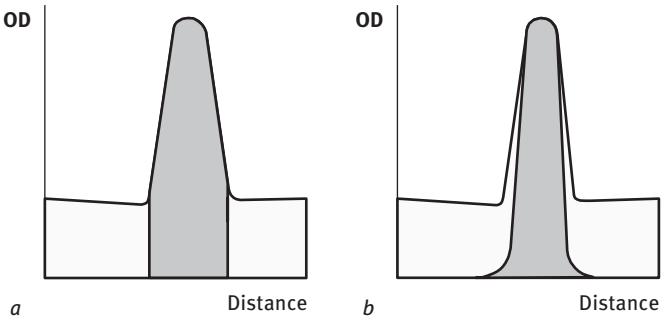

Figure 2.19-4: Integration of a band area.
a) Vertical integration; *b*) Gaussian integration

concentration. In this case, the pherogram is densitometered and the ratio between the band surface and the total bands surface of the protein is calculated. In regions of low protein concentrations, a linear relationship exists between the concentration and the corresponding band area.

Two-dimensional densitometry

There are similarities and differences between one-dimensional and two-dimensional densitometries.

In the one-dimensional densitometry, the bands of a pherogram are scanned only in one direction, the light beam scanning the general pherogram. In the two-dimensional densitometry, the spots of a pherogram are scanned in two directions, the information is translated into digital signals and processed in a computer [7,8]. In the one-dimensional densitometry, a mountain-shaped curve is obtained on one base-line; in the two-dimensional densitometry, a mountain-shaped curve is obtained on a base surface. So, the two-dimensional densitogram looks like a map (Figure 2.19-5).

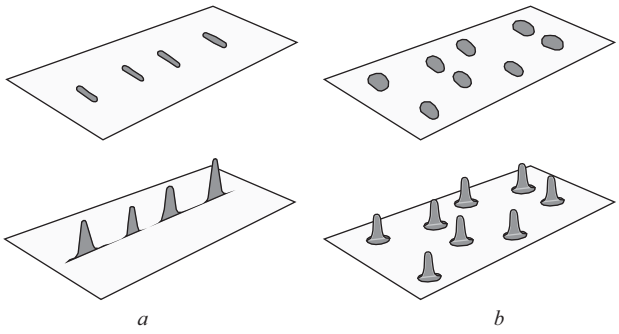

Figure 2.19-5: One-dimensional (*a*) and two-dimensional (*b*) scanning of protein bands.

In the one-dimensional densitometry, the evaluation of protein concentrations uses the proportionality between the protein concentration and the band surface; in the two-dimensional densitometry, the protein concentration is calculated from the proportionality between the protein concentration and the band volume. Therefore, appropriate computer software is needed for the two-dimensional densitometry, which takes into account the base line and the spot volumes [9,10].

Densitometry errors

The evaluation of a pherogram depends on the quality of separation. If the bands are bent or badly stained, or the pherogram has a strong background, the evaluation is implausible. The gradient gels possess after staining changing background, which is due to the gradient of their polyacrylamide concentration. The above mentioned situations can lead to densitometry errors.

Most often, densitometry errors occur, if the bands are overestimated or underestimated. When densitometry is overestimated, additional spots are detected that are not protein bands. This may be dirt particles, fingerprints, and so on. When densitometry is undervaluated, the protein bands are not complete detected. In this case, several parallel scans should be made until all bands are detected (Figure 2.19-6).

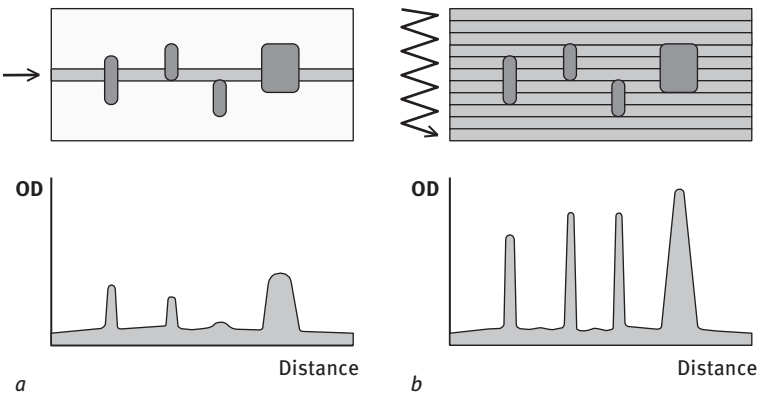

Figure 2.19-6: Densitometry errors at incomplete detection of protein bands.
a) The light beam is too narrow to detect all bands; *b*) Multiple parallel scans detect all bands.

2.19.1.2 Densitometers

The densitometry of electrophoretic results is done with the help of densitometers, which produce densitograms.

The densitometer measures the absorbance of a transparent material or a reflecting surface. It works usually in a transmission mode, since gels, blot membranes, or autoradiograms are usually transparent. It this case, the densitometer determines the optical density of a sample placed between a light source and a photoelectric cell [11].

A high-resolution densitometer can scan and analyze the bands using computer and appropriate software. So the molecular masses and isoelectric points of proteins can also be determined. A densitometer can also serve for gene studying, and quantifying compounds such as radiolabeled DNA.

Resolution of a densitometer

The resolution of a densitometer depends on the width of light beam and the depth of measurement, and is characterized by its increment.

Width of light beam. The width of light beam has a crucial importance for the resolution of a densitometer when the bands lie close together. The wider the light beam, the more the measured absorption differs from the band reality on the gel (Figure 2.19-7). The light beam, however, should not be below a certain minimum width, as this should reduce its intensity. Therefore, a compromise has to be taken in the choice of beam width. A white light beam should not be thinner than 100 µm, a laser beam should not be thinner than 50 µm.

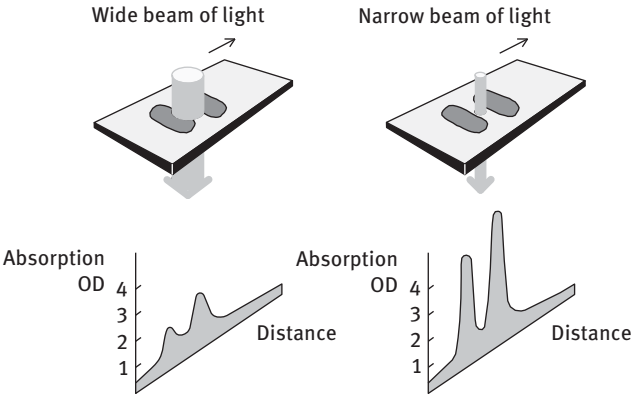

Figure 2.19-7: Measuring the neighboring bands with a wide and a narrow light beam.

Depth of measurement. The beams from a white light source are focused with the help of optical lenses onto a focal point in the gel. Before and behind the focal point, they are not focused, which leads to a loss of information, if the gels are thick.

The laser beams are parallel. Therefore, a laser densitometer has greater resolution than a densitometer with conventional light source. A laser beam can be used for examination of gels up to 3 mm thick without loss of resolution. For thin and ultrathin gels, the depth of measurement is not very important.

Increment of densitometer. The increment of a densitometer is referred to as the distance between two points of measurement in a densitogram. It should be smaller than the width of the light beam. If it is greater, loss of resolution is observed.

Microdensitometer

On the market, microdensitometers are also available. They are optical instruments for measuring optical densities in microscopic domains [12,13]. An alternative version to microdensitometers is the ultra-thin expanded laser beam microdensitometer [14,15]. It can illuminate simultaneously a few quadrate centimeters area with an ultra-thin height. Its advantages are: increased data collection speed, increased depth of focus, and superior signal to noise ratios [16].

2.19.2 Scanning

Using a standard scanner and appropriate software, the resolved bands in an electrophoresis gel can be quickly quantified.

The image produced by a scanner must be an accurate representation of both the band positions and the band intensities. The spatial accuracy of a scanner can be tested by scanning a piece of paper containing grid lines, and measuring the distance between the lines in the scanned image. The resolution of a standard scanner with 300 dpi is equal to 1/300 inch.

To convert gel images to data, the scanner must be able to extract the image intensity. In order to test, if a scanner can extract the image intensity, a photographic gray scale image of known optical density is to be scanned in gray scale mode and the pixel values of each region is to be extracted. If the results show that the scanner has a linear intensity response to the scanned image, it can be used as a densitometer.

The challenges for an automatic software-based analysis of 2D-electrophoresis gels include:

- weak spots (noises)
- incompletely separated (overlapping) spots
- undetected spots, which lead to missing values [17]
- mismatched spots
- errors in quantification, for example, several distinct spots may be detected as a single spot, and thus can be excluded from quantification
- differences in software algorithms

A few software programs for scanning pherograms are offered. One of them is the software program UN-SCAN-IT (Silk Scientific, Inc., Orem, USA). This program turns a scanner into a gel densitometer and allows automatically analyzing of gel electrophoresis images. It can also quantify Western blots, agarose gels, PCR gels, thin-layer chromatography, and so on. In addition to the gel analysis features, UN-SCAN-IT can save the data in ASCII and clipboard format, calibrate the image intensity to normalize results, export gel data to other spreadsheet, data analysis, and graphics programs, and digitize the graphs.

2.19.3 Troubleshooting

Problem	Cause	Solution
Overvaluation of a densitogram.	Spots were detected that are not bands, but dirt particles, fingerprints, or others.	Handle with gloves. Use clear solutions.
Undervaluation of a densitogram.	The measuring beam was too narrow. Therefore, the broad protein bands are not fully evaluated.	Make many parallel scans to evaluate all protein bands.

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2.20 Precast gels for protein electrophoresis. Rehydratable gels

The production of precast (ready-made) gels is a trend in the electrophoresis development. Agarose and polyacrylamide are used mostly for precast gels.

2.20.1 Precast agarose gels

The precast agarose gels can be used for months, if stored at 4 °C. They should not be frozen because they shrink. Their dimensions are different, but their thickness is usually 0.5 mm. They are most commonly used in the clinical diagnostics.

2.20.1.1 Precast agarose gels for zone electrophoresis of proteins

The precast agarose gels for zone electrophoresis of proteins are used for separation of serum, cerebrospinal fluid, and urine proteins. Additionally, they can be used for separation of hemoglobins and isoenzymes of creatine kinase, lactate dehydrogenase, and alkaline phosphatase.

An agarose gel kit contains precast agarose gels on support film, electrode buffer (the electrode buffer of ELPHO, Nuremberg is free of barbituric acid), fixing, staining, and destaining solutions, application templates, and filter papers. The dimensions of the gels are usually 51 × 82, or 100 × 80 mm, and their concentration is usually 0.8–1.0 g/dl. The samples are applied in the slots of application templates.

2.20.1.2 Precast agarose gels for immunofixation and affinity electrophoresis

The precast gels for immunofixation of proteins are used for analyzing serum proteins, immunoglobulins G, A, and M, and their light chains (κ and λ).

The affinity electrophoresis of proteins can be performed in precast lectin gels. They are used for separation of isoenzymes of **al**kaline **p**hosphatase (ALP).

A kit of ELPHO, Nuremberg for separation of alkaline phosphatase isoenzymes contains agarose gels on support film, electrode buffer, substrate solutions for enzymatic detection of ALP-isoenzymes, sample buffer, application templates, and filter papers. The buffer in the precast agarose gels and the electrode buffer are non-toxic and contain no barbituric acid.

2.20.1.3 Rehydratable agarose gels

Dry agarose gels cannot be usually rehydrated, although this cannot be excluded [1]. A method was described [2] for preparation of rehydratable agarose gels containing an uncharged linear polyacrylamide. They can be rehydrated in 9 mol/l urea or carrier ampholytes for 60 min, and used in isoelectric focusing. In these gels, proteins with a relative molecular mass M_r = 970,000 can be separated by isoelectric focusing. Then the focused proteins can be quantitatively transferred onto nitrocellulose membrane in less than 10 min, where the blotted proteins can form immune precipitates and be stained.

2.20.2 Precast polyacrylamide gels

Since the early 1990's, several companies offer a wide variety of precast polyacrylamide gels on the market. Most of them contain the Laemmli buffer system at different *T*-concentration (in g/dl).

The precast polyacrylamide gels on a support film or a net are usually 0.5 mm thick. They are covered with a thin polyester film and are packed in polyethylene envelopes. The precast polyacrylamide gels can be stored for months at 4 °C, regardless of whether they are intended for zone electrophoresis, disc-electrophoresis, SDS electrophoresis, or isoelectric focusing.

A precast polyacrylamide gel for disc-electrophoresis consists of a stacking gel and a resolving gel, or contains no stacking gel (Figure 2.20-1). The resolving gel can have polyacrylamide concentration (*T*) from 8 to 18 g/dl or even more.

2.20.2.1 Precast polyacrylamide gels for disc-electrophoresis of native proteins

The precast polyacrylamide gels for native disc-electrophoresis of proteins are supplied with paper strip electrodes (ELPHO, Nuremberg). They are cast on a film or a net, in sizes of 51 × 82, 100 × 80, 125 × 125, or 250 × 125 mm, and have *T*-concentration of 9, 11, 13, 15, or 17 g/dl.

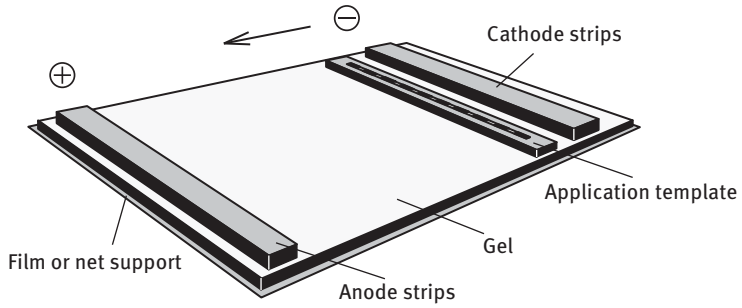

Figure 2.20-1: A homogeneous precast gel without stacking gel.

2.20.2.2 Precast polyacrylamide gels for SDS disc-electrophoresis of proteins

The precast gels for SDS disc-electrophoresis of proteins from ELPHO, Nuremberg are 0.5 mm thick. They are cast on film or net [3], and covered with polyester film. Prior to electrophoresis, the cover film should be removed so that an application template can be placed onto the gel. The net supported SDS gels are suitable for electroblotting. All gels are available in different sizes: 51 × 82, 100 × 80, 125 × 125, and 250 × 125 mm.

Pharmacia Biotech, Uppsala (Amersham Pharmacia Biotech) offers homogeneous and gradient gels for SDS electrophoresis. The *T*-concentration of the homogeneous gels is 7.5, 12.5 or 15 g/dl; the *T*-concentration of the gradient gel is 8–18 or 12–14 g/dl. The gradient gels of T = 8–18 g/dl are available as *ExcelGels*. They are 0.5 mm thick, contain a TRIS-acetate-TRICINEate buffer system [4] and consist of a stacking and a resolving parts. The electrode contact with the gel is transmitted by gel electrode strips, which contain buffer.

The SDS precast polyacrylamide gels hydrolyze over time, therefore they are unstable. The shelf life of a gel containing the Laemmli buffer (pH = 8.8) is about 3–4 months. On contrary, the SDS precast gels, when cast in neutral buffers, are more stable.

The relationship between the relative molecular masses M_r of native and SDS-proteins and the *T*-concentration of precast polyacrylamide gels is shown on Figure 2.20-2 and Table 2.20-1.

2.20.2.3 Precast polyacrylamide gels for isoelectric focusing with carrier ampholytes

The precast polyacrylamide gels for isoelectric focusing with carrier ampholytes are 0.15–0.30–0.50 mm thick. Their polyacrylamide concentration T is 5 g/dl and the crosslinking degree C is 0.03. They are cast on a support film or a net. The net gels are suitable for blotting.

The precast polyacrylamide gels for isoelectric focusing with carrier ampholytes are used to form linear pH gradients in the pH ranges of 3.0–10.0, 4.0–6.0, 5.0–8.0, 7.0–9.0 (Figure 2.20-3) and more. They are covered with a thin polyester film,

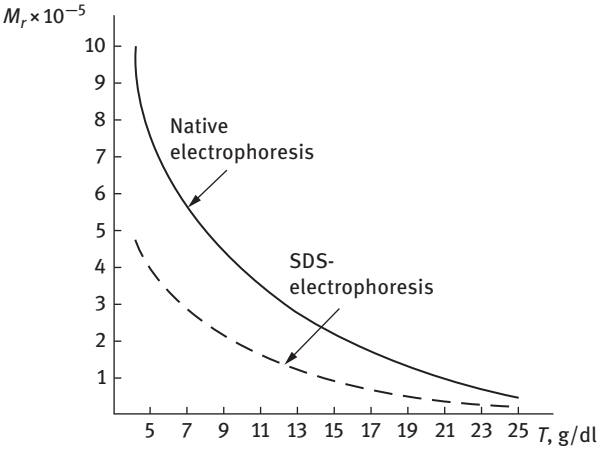

Figure 2.20-2: Relationship between the relative molecular masses M_r of proteins and the total polyacrylamide concentration T of precast polyacrylamide gels.

Table 2.20-1: Polyacrylamide concentrations used for resolving proteins with different molecular masses.

Polyacrylamide T, g/dl	M_r in native gel electrophoresis	M_r in SDS gel electrophoresis
9	$350–600 \times 10^3$	$150–300 \times 10^3$
11	$300–450 \times 10^3$	$120–250 \times 10^3$
13	$200–400 \times 10^3$	$100–200 \times 10^3$
15	$150–300 \times 10^3$	$50–150 \times 10^3$
17	$100–250 \times 10^3$	$5–100 \times 10^3$

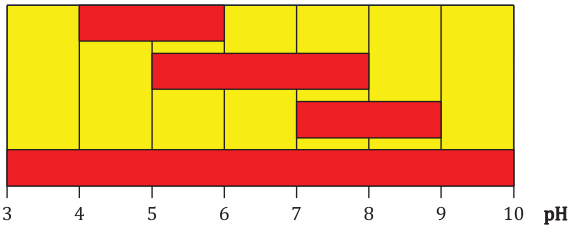

Figure 2.20-3: pH gradients, which can be obtained with precast gels.

which is loosely connected to the gel. It must be removed before electrofocusing to allow applying a template onto the gel. The dimensions of the gels are: 51 × 82, 100 × 80, 125 × 125, and 250 × 125 mm. Additionally, the gels are supplied with paper electrode strips, which contain an anode and a cathode solution.

2.20.2.4 Precast polyacrylamide gels for isoelectric focusing with immobilines

The immobiline precast gels of ELPHO, Nuremberg are 0.5 mm thick. They are cast on a film or a net, and are supplied with paper electrode strips containing an anode and a cathode solution. The immobiline precast gels are suitable for pH ranges of 4.0–10.0, 4.0–7.0, 7.0–10.0, and 5.0–6.0, and are available in the following dimensions: 51 × 82, 100 × 80, 125 × 125, and 250 × 125 mm.

2.20.2.5 Rehydratable polyacrylamide gels

On the market, CleanGels are available. They represent rehydratable homogeneous polyacrylamide gels with a stacking gel of T = 5 g/dl, and a resolving gel of T = 10 g/dl. CleanGels can be rehydrated in native or SDS buffers. At –20 °C they can be stored almost indefinitely.

Pharmacia Biotech (Amersham Pharmacia Biotech) offers rehydratable immobiline DryPlates, which are to be rehydrated in deionized water. There are immobiline DryPlates containing the following pH gradients: 4.0–7.0, 4.2–4.9, 4.5–5.4, 5.0–6.0, and 5.6–6.6. The same company offers also immobiline DryStrips for pH gradients 3.0–10.0, 3.0–10.5, and 4.0–7.0.

2.20.3 Protocols

2.20.3.1 Casting agarose gels for submarine electrophoresis

Agarose gels for submarine zone electrophoresis are 5-10 mm thick. They are cast in trays (Figure 2.20-4). To form wells in the gel, a comb is fixed in the agarose solution before gelling.

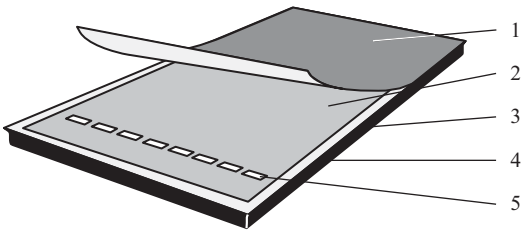

Figure 2.20-4: Agarose gel for submarine electrophoresis.
1. Flexible cover; 2. Agarose gel; 3. Casing tray; 4. Margins of the casting tray; 5. Wells

Materials and equipment

TRIS-**b**orate-**E**DTA (TBE) buffer
Agarose
Casting tray (*It should be made of material, which is transparent to the **u**ltra**v**iolet (UV) light. So the movement of nucleic acids can be seen during electrophoresis, if the*

gel contains a bound to nucleic acids dye that fluoresces under UV light. In general, this is ethidium bromide.)
Ethanol
Comb

Procedure

- Rinse and dry the gel casting tray (with 95% ethanol, if available).
- Place the casting tray on a level surface.
- Prepare 1 g/dl agarose in TBE buffer.
- Melt the agarose solution.
- Pour agarose into the casting tray.
- Adjust the comb to rest evenly in the agarose solution.
- Allow the solution to solidify (about 30 min); then remove the comb.

2.20.3.2 Casting agarose gels on a support film

Agarose gels on support film can be cast by capillary or cassette technique.

Capillary technique

Materials and equipment

Agarose
Support film
Glass plates
Repel-silane
Silicone spacers
Clamps
Syringe or pipette

Procedure

The capillary technique is used for casting thin (0.5 mm) agarose gels. It is carried out in the following steps (Figure 2.20-5):

- Place some drops of water onto a glass plate. Lay a support film on it and roll over so that any air bubbles are removed.
- Hydrophobilize a second glass plate with a repellent solution (Repel-silane) and mount 0.5 mm silicone spacers on its longer sides.
- Fix the glass plates together with clamps to make a cassette.
- Heat the cassette to 60 °C in a drying oven.
  To avoid bubble formation when adding agarose, the temperature should not exceed this limit.
- Place the sandwich onto a flat surface.

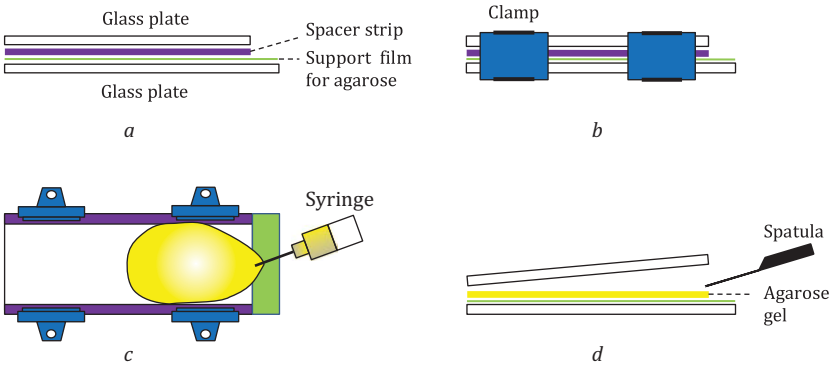

Figure 2.20-5: Steps (*a, b, c, d*) of agarose gel casting by capillary technique.

- Introduce the agarose solution into the casting cassette with a preheated syringe or pipette. A casting cassette of internal dimensions of 240 × 110 × 0.5 mm needs approximately 15 ml agarose solution. The solution is drawn into the cassette *via* capillary action.
- Let the cast solution at room temperature for 30 min, and then harden it at 4 °C for additional 30 min.
- Disassemble the cassette and remove the gel from the cassette. Use the gel or cover it with a polyethylene film and store in a refrigerator at 4 °C for weeks.

Cassette technique

Materials and equipment

Agarose
Support film
Glass plates
0.5 mm U-shaped gasket
Repel-silane
Clamps

Procedure

The casting of thin (0.5 mm) agarose gels is carried out in the following steps (Figure 2.20-6):

- Weigh agarose and dissolve it in a buffer. Heat the resulting suspension at near-boiling temperature on a laboratory heating plate at slow rotation of a magnetic stirrer until the agarose is completely dissolved.
- Degas the hot agarose solution.

- Place a support film with its hydrophobic side down onto a glass plate, using water as an adhesive agent, and roll the support film with a roller to remove the air bubbles between the film and glass.
- Place a 0.5 mm thick U-shaped gasket on the margins of the support film.
- Smear a second glass plate of same dimensions with a repelling solution (Repel-silane) to make it hydrophobic.
- Set the glass plates together in vertical position and fix them with clamps to build a cassette.
- Cast the agarose solution into the cassette with a syringe or a pipette, heated to 75 °C.
- After 1 hour, remove the clamps from the cassette and free the gel between the glass plates. It is already usable, but its mechanical strength improves, if it is covered with a polyethylene film and stored overnight in the refrigerator at 4–8 °C. At 4 °C, it can be stored for several weeks. The gel should not be frozen.

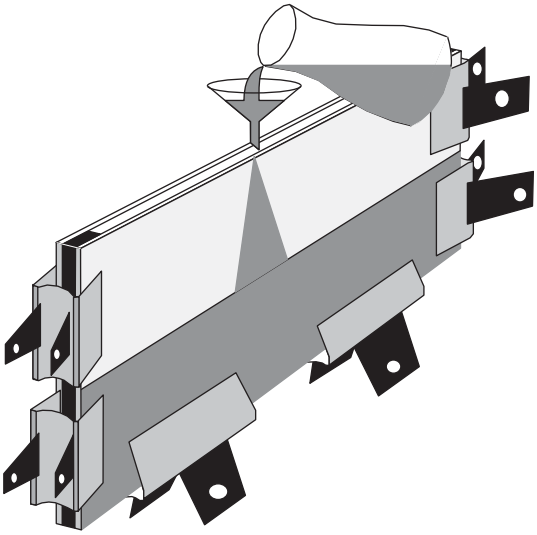

Figure 2.20-6: Casting of agarose gel in a preheated cassette.

2.20.3.3 Rehydration of a rehydratable polyacrylamide gel

Materials and equipment

Buffer
Repel-silane
Spacers
Clamps
Urea
Triton X-100 or Nonidet NP-40

1,4-**Di**thio**t**hreitol (DTT)
Glycerol, or sorbitol (sucrose)

Procedure

- Take out a rehydratable gel from the refrigerator and let it for 10 min at room temperature.
- Lay the gel on a glass plate with its support film down.
- Roll the gel carefully over with a photo roller.
- Coat another (upper) glass plate with Repel-silane to protect adhesion between it and the gel.
- Build a rehydration cassette with the aid of spacers and clamps.
- Introduce a rehydrating solution into the cassette with a syringe or pipette. It can contain 8 mol/l urea, non-ionic detergents (Triton X-100 or Nonidet NP-40 in a concentration of 0.5–2 ml/dl), 10 mmol/l 1,4-dithiothreitol (DTT), and 20 ml/dl glycerol, or 10 g/dl sorbitol (sucrose).
  The rehydration takes place only a few minutes for ultra-thin gels (0.05–0.10 mm), or 1–2 h for thin gels (0.5 mm) until the gel fills up the cassette. If the rehydrating solution contains additional chemicals, for example, urea or non-ionic detergents, the rehydration must be extended overnight [5,6]. Gel strips can also be rehydrated.
- Open the cassette after rehydration and take the gel out. If the rehydration solution is not completely absorbed by the gel, the gel surface must be dried with a filter paper.

2.20.4 Troubleshooting

Problem	Cause	Solution
The monomer solution does not polymerize or polyacrylamide gel is soft and sticky.	The concentration of acrylamide or BIS in the monomer solution was too low.	Check the recipe before the gel casting.
	The concentration of TMEDA or APS in the monomer solution was too low.	Use 5 µl 10 g/dl TMEDA and 5 µl 10 g/dl APS per 1 ml of monomer solution.
	The APS solution was too old or is stored improperly.	Use a fresh APS solution. Its maximum storage time in the refrigerator is a week.
	The temperature was too low.	Polymerize the gel at room temperature.
	The concentration of the air-oxygen in the monomer solution was too high.	Deaerate the monomer solution with a water-jet pump.

(continued)

Problem	Cause	Solution
The gel surface edge is sticky and detaches from the support film.	The atmospheric oxygen has inhibited the polymerization of the gel surface edge.	Overlay the monomer solution with deionized water after gel casting.
The gel polymerizes too rapidly.	The concentration of TMEDA or APS in the monomer solution was too high.	Check the recipe for gel casting.
	The room temperature was too high.	Cast gel at room temperature.
Air bubbles in the polyacrylamide or agarose gels.	In the monomer solution, air was dissolved.	Deaerate the monomer solution.
	The glass plate that came into contact with the monomer solution was dirty.	Wash the glass plate before use.
	The support film was unclean.	Do not touch the hydrophilic side of the support film with fingers.
	The agarose solidified on casting because the glass plates were too cold.	The temperature of the glass plates should be 60–70 °C during the agarose casting.
The consistency of the agarose gel is too soft.	The agarose concentration was too low.	Check the recipe.
	The gelling time was too short.	The agarose gelling must continue at least 60 min at room temperature or better overnight.
	The urea changed the agarose structure.	Use higher agarose concentration and prolong the gelling time.
The gel separates from the support film.	An incorrect support film was used.	Do not exchange the support films for polyacrylamide and agarose gels.
	The gel was cast onto the wrong side of a support film.	Cast gel only on the hydrophilic side of a support film. Check the side previously with water.
	The support film was stored improperly or too long.	Keep the support films at room temperature and in the dark.
The gel does not stick to the support film, but to the glass plate.	The glass plate was hydrophilic.	Clean the glass plate and coat it with Repel-silane.
	The gel was too long in the casting cassette.	Take the gel from the casting cassette not later than an hour after the polymerization.

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Problems 2. Electrophoresis of proteins

2.1 How can be prepared a monomer solution of acrylamide and BIS with a total monomer concentration T = 50 g/dl and a degree of crosslinking C = 0.03?

2.2 The "persistent" function of Kohlrausch F_k is equal to 0.8. What should be the concentration of the trailing electrolyte c_{HB}, if the concentration of the leading electrolyte c_{HA} is equal to 0.10 mol/l?

2.3 Explain the principle of disc-electrophoresis.

2.4 What will be the concentration of protein H_nP, if the concentration of the strong leading acid c_{HA} = 0.10 mol/l and the Kohlrausch function F_k = 0.02?

2.5 Calculate the electrophoretic mobility of negatively charged albumin μ_{alb}, if it has run the distance d of 38 mm for one hour at voltage U of 250 V between the electrodes, located from each other at a distance l of 10 cm.

2.6 Are the specific conductivity of the monobasic strong acids HA and HB equal, if they have same concentrations?

2.7 At what pH value borate, taurinate, and glycinate ion will follow certain proteins, if the slowest of them has a velocity of $-13 \bullet 10^{-9}$ $m^2/(sV)$ and is running in a solution with an ionic strength I of 01 mol/l at 25 °C? At 25 °C the absolute mobilities of these ions are equal to $-33.63 \bullet 10^{-9}$, $-34.11 \bullet 10^{-9}$, and $38.45 \bullet 10^{-9}$ $m^2/(sV)$, respectively. The pK values of the corresponding acids at the same temperature and I = 0.1 mol/l are 9.02, 8.84, and 9.56.

2.8 Three bases are available: imidazole (pK = 6.99), TRIS (pK = 8.07), and morpholine (pK = 8.60); and three acids: acetic acid (pK = 4.76), ACES (pK = 6.84), and glycine (pK = 9.78). Which buffer would have highest buffer capacity β at pH = 7.0, if the buffer concentrations are equal?

2.9 At what pH value the dissociation degrees of TRIS and glycine in a TRIS-glycinate buffer would be equal, if the temperature is 25 °C and the ionic strength is 0.06 mol/l? At this temperature and ionic strength the dissociation constants of TRIS-ion pK_{HTRIS^+} and of glycine pK_{HG} are equal to 8.27 and 9.58, respectively.

2.10 Prepare a TRIS-glycinate buffer with pH = 8.30 and an ionic strength of 0.10 mol/l at 25 °C. The pK values of TRIS-ion pK_{HTRIS^+} and glycine pK_{HG} at 25 °C are 8.07 and 9.78, respectively.

2.11 What is a peptide bond?
- A A covalent bond
- B A weak bond
- C An ionic bond
- D A hydrophobic bond

2.12 Which are the bonds between the amino acid residues in a polypeptide chain?
A Ester B O-glycoside C Peptide D N-glycoside

2.13 Which bonds are most important for the alpha-helix structure of polypeptide chains?
- A Hydrogen bonds, which are parallel to the axis of the polypeptide chain
- B Disulfide bonds
- C Hydrogen bonds that are chaotically scattered along a α-helix
- D Hydrogen bonds that are perpendicular to the axis of the polypeptide chain

2.14 Which bonds or interactions stabilize the secondary structure of proteins?
- A Covalent bonds
- B Hydrophobic interactions between distant chemical groups
- C Electrostatic interactions between chemical groups
- D Hydrogen bonds between neighboring peptide groups

2.15 What is typical for globular proteins?
- A They do not have own electric charge
- B They have a hydrate shell
- C They have a zero electric charge at the physiological pH value
- D They are not dissolved in aqueous solutions

2.16 Which amino acid forms disulfide bridges in proteins?
A Methionine B Serine C Cysteine D Threonine

2.17 Which statement is true?
- A A protein is most stable in its isoelectric point
- B The isoelectric points of all animal proteins are in the pH range 5.5–7.0
- C The isoelectric point of a protein is different in aqueous solutions with equal ionic strength
- D The isoelectric point of a protein is the pH value at which the total electric charge of the protein equals zero

2.18 The isoelectric point pI (pH(I)) of pepsin (an enzyme in gastric juice) is approximately equal to 1. Which amino acids are presented in it?
A Tryptophan and tyrosine
B Serine and alanine
C Aspartic and glutamic acid
D Lysine and arginine

2.19 The electrophoretic separation of serum proteins is carried out at pH > 8.6 when the proteins are charged:
A Positively
B Negatively
C Some of them are positively charged, and other are negatively charged
D They have no charge

2.20 The total charge of a protein depends on
A Structure of the electrophoresis gel
B Intensity of the electric field
C pH value of the buffer
D Concentration of the electrophoresis gel

2.21 What happens in agarose gel electrophoresis?
A Proteins separate from each other according to their electric charges
B The separation of DNA fragments does not depend on the concentration of agarose
C Proteins move with same velocity in buffers of different pH values
G Proteins are separated according to their molecular masses

2.22 Which bond does not break during nonreductive denaturation of proteins?
A Hydrophobic B Hydrogen C Electrostatic D Disulfide

2.23 Which method is not used for protein separation?
A Isoelectric focusing
B Affinity chromatography
C X-ray analysis
D Disc-electrophoresis

2.24 Western blotting is a method for testing the presence of
A DNA B RNA C Protein D None of the above

3 Electrophoresis of nucleic acids

The nucleic acids electrophoresis as well as the protein electrophoresis can take place in continuous or discontinuous buffer systems. The electrophoresis in continuous buffers is referred to as zone electrophoresis; the electrophoresis in discontinuous buffer systems is referred to as disc-electrophoresis.

The nucleic acids consist of mononucleotide residues, whose sugar-phosphate backbone carries negative charges [1]. They are separated in weak acidic, neutral, or weak alkaline buffers with pH range of 6.0 to 10.0. Therefore, the nucleic acids behave themselves as polyanions (nucleates) and move in an electric field to the anode.

If the pH value of a buffer is too low, the bases cytosine and adenine are protonated and have positive electric charges; if the pH value of a buffer is too high, the bases thymine and guanine are deprotonated and have negative electric charges. In both cases, the additional electric charges cause denaturation of nucleic acids and change their mobility.

The electrophoresis of large DNA or RNA is usually carried out on agarose gel. Double-stranded DNA fragments naturally look like long rods, whose migration through a gel is a function of their size and gyration [2,3]. Circular DNA, such as in plasmids, however, may be resolved in multiple bands, which depend on whether it is relaxed or supercoiled. Single-stranded DNA or RNA tends to fold up into structures with complex shapes, which migrate through the gel in a complicated manner because of their tertiary structure. Therefore, agents that disrupt the hydrogen bonds, such as NaOH or formamide, are used to denature the nucleic acids and cause them resemble long rods. In addition, the characterization of nucleic acids or their fragments may be performed through ligand interactions in affinity electrophoresis.

3.01 Buffers for electrophoresis of nucleic acids

For DNA electrophoresis, buffers with pH values in the range of 7.0–9.0 are best suited. They should contain electrolytes whose pK_c values are close to the desired

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pH value. The pK_c values are functions of the ionic strength and temperature of the buffer. A buffer will have high capacity when the basic and acidic forms of its electrolytes have high concentration.

Buffers that contain ions of high charge magnitude (+ 2, + 3, −3, *etc*.) yield high ionic strength without providing high buffer capacity. In this situation, the gel conducts much electric current at relatively low electrolyte concentrations. When a buffer contains ions that move quickly through the gel, it depletes fast.

In Table 3-1, most commonly used buffers for electrophoresis of nucleates are given.

Table 3-1: Most commonly used buffers for nucleate electrophoresis.

Buffer's name	Description	Composition	
TRIS-**d**i**h**ydrogen **p**hosphate-**E**DTA (TDHPE) buffer pH = 8.0	High ionic-strength buffer for agarose gels; not recommended for preparative agarose gel electrophoresis.	**For 1l 10x buffer** TRIS $NaH_2PO_4 \cdot 2H_2O$ $Na_2EDTA\ 2H_2O$ Deionized water to	 43.60 g 46.80 g 3.76 g 1,000.00 ml
Glycinate buffer pH = 8.0	Low ionic-strength buffer for agarose gel electrophoresis. Good for preparative gels. Can also be used for analytical gels.	**For 1l 10x buffer** Glycine 1 mol/l NaOH 0.5 mol/l EDTA Deionized water to	 150.0 g 150.0 ml 40.0 ml 1,000.0 ml
TRIS-**a**cetate-**E**DTA (TAE) buffer pH = 8.0	Used in analytical and preparative agarose gels when DNA is purified by glass beads. Sometimes used in pulse-field electrophoresis.	**For 1l 50x buffer** TRIS Glacial acetic acid 0.5 mol/l EDTA Deionized water to	 242.0 g 57.1 ml 100.0 ml 1,000.0 ml
TRIS-**b**orate-**E**DTA (TBE) buffer pH = 8.3	Low ionic-strength buffer. It can be used for both preparative and analytical agarose gels. Most commonly used in polyacrylamide gels. Rarely used in sequencing electrophoresis.	**For 1l 5x buffer** TRIS Boric acid 0.5 mol/l EDTA *Adjust pH to 8.3 by HCl* Deionized water to	 54.0 g 27.5 g 20.0 ml 1,000.0 ml

The buffers must be diluted to 1x prior to use in electrophoresis. Higher concentrations bring poor results due to excessive heat generation. Borate ion in the TRIS-borate-EDTA buffer inhibits many enzymes and, as a result, DNA keeps its integrity. This makes the TBE buffer very popular.

TRIS-**a**cetate-**E**DTA (TAE) buffer is used as a running buffer in denaturing agarose gel electrophoresis, and more. Compared to TBE buffer, it possesses advantages in subsequent enzymatic applications of DNA. For example, if a DNA sample

is going to be used in a cloning experiment, the step that follows its running on an agarose gel is to ligate (covalently link) it to a cloning vector (most likely a plasmid). DNA sample from TAE buffer is suitable for this purpose, while DNA from TBE buffer is not suitable.

Usually the amount of DNA or RNA to be analyzed is too little. Therefore, DNA must be amplified with the help of the polymerase chain reaction.

3.02 Polymerase-chain reaction

Polymerase **c**hain **r**eaction (PCR) is the most widely used technique in molecular biology to amplify *in vitro* DNA segments generating many copies of them [4,5]. It mimics the DNA replication, but confines it to specific DNA sequences of interest. The polymerase chain reaction requires about 500-fold less DNA than Southern blotting and is less time consuming.

PCR applications include DNA cloning for sequencing, gene cloning, gene manipulation, functional analysis of genes, diagnosis of hereditary diseases, amplification of ancient DNA, DNA profiling, and nucleic acid tests for diagnosis of infectious diseases. The polymerase chain reaction was developed in 1983 by Kary Mullis [6,7], who was awarded the Nobel Prize in Chemistry in 1993.

Also **q**uantitative **PCR** (qPCR) methods are known that allow the estimation of the amount of a given sequence present in a sample. They are techniques, which are applied to determine quantitatively the levels of gene expression. The quantitative PCR measures the accumulation of DNA product after each round of PCR amplification.

There are two PCR methods for simultaneous detection and quantification of DNA. The first method uses fluorescent dyes that are retained nonspecifically in between the double DNA strands. The second method uses probes that are fluorescently labeled. An interesting combination is the **r**everse **t**ranscription and **qPCR** (RT-qPCR). With the help of this technique, mRNA is converted to cDNA, which is further quantified using qPCR. This method lowers the possibility of error at the end point of PCR [8], increasing chances for detection of genes associated with genetic diseases and cancer [9].

3.02.1 Principle

The polymerase chain reaction is carried out in the following sequence: First, the DNA molecule in the sample is denatured with heat giving two DNA strands. Then, two DNA primers are added to occupy the 3′ and 5′ ends of the two DNA strands. Finaly, bacterial thermostable Taq polymerase catalyzes the elongation of each

primer adding four **d**eoxy**n**ucleoside **t**riphos**p**hates (dNTPs). In this way, a new DNA chain is synthesized that is complementary to the DNA strands.

3.02.2 Components

A basic PCR setup requires several components [10]:

- A *buffer* providing a suitable pH value and ions necessary for the optimum activity and stability of the DNA polymerase. The following buffer is often used: 10–50 mmol/l TRIS-HCl of pH = 8.3–9.0 containing up to 50 mmol/l KCl and 0.5–5 mmol/l $MgCl_2$. The buffer may also contain gelatin, **b**ovine **s**erum **a**lbumin (BSA), nonionic detergents (e.g., DMSO (**dim**ethyl**s**ulf**o**xide)), and so on. Mg^{2+} is the most common, but Mn^{2+} can also be used. However, it can cause errors during DNA synthesis [11]. Typically, monovalent cation is K^+.
- A *DNA template* that contains the DNA target region to be amplified.
- Two *DNA primers* that are complementary to the 3′ ends of each of the sense and anti-sense strands of the DNA target. They are often custom-made in a laboratory or purchased from biochemical suppliers. The primers are short synthetic oligonucleotides constructed from 14 to 40 mononucleotides. They participate in the synthesis of DNA at each cycle of PCR. The optimal concentration of the primers in the amplification reaction is 0.1–1 µmol/l.
- *Deoxynucleoside (deoxynucleotide) triphosphates* dATP, dGTP, dTTP, and dCTP. They take place in the synthesis of new DNA strands with the help of the DNA polymerase, which align them on the DNA matrix according to the principle of complementarity. The four dTTPs should be present at equimolar concentrations in the reaction mixture. Their optimal concentration is 20 to 200 µmol/l.
- *DNA polymerase*, an enzyme that catalyzes the synthesis of new DNA strands. It binds to a double-stranded region of DNA and elongates using the primers. Heat-resistant Taq polymerase is especially common, as it remains intact during the high-temperature DNA denaturation process. The Taq polymerase was first isolated by Chien *et al*. from the microorganism ***T****hermophylus* ***aq****uaticus* (Taq) [12]. It is a thermostable enzyme that retains its activity at 94 °C for 45 to 60 min; its optimal temperature is 72 °C.

3.02.3 Procedure

PCR can be carried out with samples of a volume of 10–200 µl in small thin-walled reaction tubes of 0.2–0.5 ml in a thermal cycler. The thermal cycler heats and cools the reaction tubes 20–40 times to achieve the temperatures required for each step

of the reaction. Modern thermal cyclers permit both heating and cooling by reversing the electric current.

Every PCR cycle consists of several temperature steps. The temperatures used in each cycle depend on the enzyme used for DNA synthesis, the concentration of bivalent ions, dNTPs in the reaction, and the melting temperature of the primers [13]. The cycle steps are as follows:

- *Initialization:* This step is used for DNA polymerases that require heat activation [14]. It consists of heating the reaction chamber to a temperature of 94–96–98 °C for 1–10 min, if thermostable polymerases are used.
- *Denaturation:* During this step, the reaction chamber is heated to 94–98 °C for 20–30 s. This causes DNA denaturation (melting) of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules (Figure 3-1).

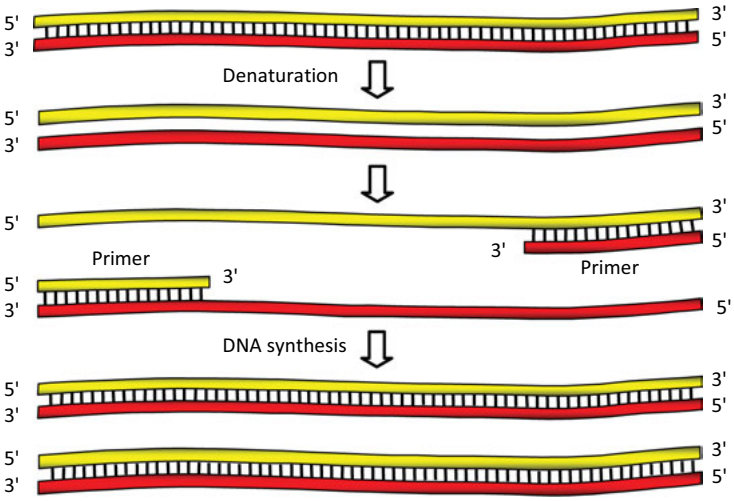

Figure 3-1: Scheme of the polymerase chain reaction (PCR).

The strands of double DNA are separated by heating (denaturation). Then, primers are joined complementarily to their ends. The DNA polymerase extends the primers in direction 5′ → 3′ and synthesizes two new chains, which are complementary to the template chains. After multiple cycles of heating and cooling, many copies of the DNA segment are produced. As a result, multiple increase of DNA of interest takes place.

- *Annealing:* In this step, the reaction temperature is lowered to 50–65 °C for 20–40 s. At this temperature the two primers are annealing to each of the single-stranded DNA templates at the 3′ end of each strand.

- *Elongation:* The temperature for this step depends on the DNA polymerase used. The optimum activity temperature for the thermostable DNA polymerase of Taq polymerase (from the name of the microorganism ***T****hermophylus* ***aq****uaticus*) is approximately 75–80 °C [15,16]; therefore, a temperature of 72 °C is commonly used. In this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding free dNTP from the reaction mixture in the 5′ to 3′ direction. The 5′-phosphate group of dNTP bind to the 3′-hydroxyl group at the end of each DNA strand. As a rule of thumb, most DNA polymerases polymerize a thousand bases per minute. Under optimal conditions, at each elongation step the number of DNA target sequences is doubled. The original template strands plus all newly generated strands become template strands for the next round of elongation, leading to exponential amplification of the specific DNA target region.
- *Final elongation:* This step is performed at 70–74 °C for 5–15 min after the last PCR cycle to ensure that any remaining single-stranded DNA is elongated.
- *Final hold:* In this step, the reaction chamber is cooled to 4–15 °C for an indefinite time.

In automating the process, the time for one cycle is a few minutes. For 20 cycles, the amount of starting DNA can be increased 10^6 times and for 30 cycles 10^9 times. Most PCR methods amplify DNA fragments of between 0.1 and 10 **k**ilo **b**ase **p**airs (kbp), although some techniques allow for amplification of fragments up to 40 kbp [17]. These DNA copies are sufficient in quantity to be used in hybridization techniques.

To check whether PCR successfully generated the anticipated DNA target region (amplicon), agarose gel electrophoresis may be employed. Then the size of PCR product can be determined by comparison with a DNA ladder – a molecular mass marker, which contains DNA fragment of known size.

3.02.4 Stages

The PCR process can be divided into three stages based on the reaction progress:

- *Exponential amplification:* The amount of product is doubled at every cycle. After 30 cycles, a single copy of DNA can be multiplied up to 1,000,000,000 copies.
- *Leveling off stage:* The reaction slows as the DNA polymerase loses activity and as reagents such as dNTPs and primers are consumed.
- *Plateau:* No more products accumulate due to exhaustion of reagents and enzyme.

3.02.5 Advantages

PCR has a number of advantages. It is highly sensitive with the potential to produce rapidly millions of DNA copies of a specific product for sequencing, cloning, and analysis. qRT-PCR shares the same advantages as PCR, plus the quantification of the synthesized product. Therefore, it is used to analyze alterations of gene expression in microbes, tumors, or other diseases [18].

The PCR technique can help identify the sequence of previously unknown viruses related to already known ones and thus can give a better understanding of the virus diseases. If the procedure can be simplified and nonradiometric detection systems can be developed, PCR will assume a prominent place in the clinical laboratory [19].

3.02.6 Limitations

A major limitation of PCR is that prior information about the target sequence is necessary in order to generate primers that will allow its selective amplification. Another limitation of PCR is that even the smallest amount of contaminating DNA can be amplified, resulting in misleading or ambiguous results. To minimize contamination, investigators should use separate rooms for reagent preparation, PCR, and analysis of product.

Like all enzymes, DNA polymerases are prone to error, which in turn causes mutations in the PCR fragments that are generated [20].

3.02.7 Applications

PCR can be used for analyzing extremely small amounts of samples. It may also be applied in the analysis of ancient DNA that is tens of thousands of years old. The PCR techniques have been successfully used on animals, such as a 40,000-year-old mammoth, and also on men, ranging from the Egyptian mummies to the Russian tsar relatives.

In forensics and DNA paternity testing, PCR is used to amplify and examine highly variable DNA regions. These are regions that vary in length from individual to individual and fall into two categories: (1) **v**ariable **n**umber of **t**andem **r**epeats (VNTR) and (2) **s**hort **t**andem **r**epeats (STR). A VNTR is a region that is variably composed of a 15–70 base pair sequence that is repeated 5–100 times. An STR is similar to a VNTR except that the repeated unit is two to four nucleotides in length. By examining several different VNTR or STR from the same individual, investigators obtain a unique DNA profile for an individual.

Medical applications

PCR has been applied to a large number of medical procedures:

- PCR is used for genetic testing. DNA samples for prenatal investigation can be obtained by amniocentesis and chorionic villus sampling, or fetal cells circulating in the mother's bloodstream can be used.
- PCR permits early diagnosis of malignant diseases such as leukemia and lymphomas. PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells. Its sensitivity is 10,000-fold higher than that of other methods [21]. PCR can be used to quantify single cells, as well as recognize DNA, mRNA, and protein confirmations and their combinations [22].
- PCR can also be used as a sensitive test for tissue typing, vital to organ transplantation. There is a proposal to replace the antibody-based tests for blood type with PCR-based tests [23].

PCR allows for rapid and highly specific diagnosis of infectious diseases, including those caused by bacteria or viruses:

- PCR can help to detect viral DNA. The primers used must be specific to the targeted sequences in the viral DNA. The high sensitivity of PCR permits virus detection soon after infection and before the onset of disease [24].
- The **h**uman **i**mmunodeficiency **v**irus (HIV) is a difficult target to find. The earliest tests for HIV infection relied on the presence of antibodies to the virus circulating in the bloodstream. However, antibodies don't appear until many weeks after infection. PCR tests can detect as little as one viral genome among the DNA of over 50,000 host cells [25].
- Some microorganisms, such as *Mycobacterium tuberculosis*, grow slow in the laboratory. PCR-based tests allow detection of small numbers of such organisms.

Forensic applications

The PCR-based methods have found widespread application in forensics. They can discriminate any one person from the entire population of the world. Minute samples of DNA can be isolated from a crime scene and compared to that from the suspects:

- Forensic DNA typing is an effective way of identifying or exonerating criminal suspects due to analysis of criminal evidence discovered. The human genome has many repetitive regions (up to 40% of human DNA) that can be found within gene sequences or in noncoding regions of the genome [26]. A single human hair with attached hair follicle has enough DNA to conduct the analysis. Similarly, a few sperm, skin samples from under the fingernails, or a small amount of blood can provide enough DNA for conclusive analysis.

- DNA fingerprinting can help in paternity testing, where an individual DNA from unidentified human can be compared with that from possible parents, siblings, or children. The actual biological father of a newborn can also be confirmed.
- PCR can also be used to determine the sex of not only ancient specimens but also of suspects in crimes [27].

Research applications

PCR has been applied to many areas of molecular genetics:

- Known segments of DNA can easily be produced with the help of PCR.
- PCR is used for the study of gene expression.
- Using PCR, deletions, insertions, translocations, or inversions can be analyzed.
- The ability of PCR to amplify loci from individual sperm [28] has enhanced the task of genetic mapping by studying chromosomal crossovers after meiosis.
- PCR generates probes for Southern or Northern blotting hybridization.
- The PCR methods are widely performed to detect the presence of a viral infection before its first symptoms.
- Although DNA breaks down over time, PCR has been successfully applied to clone genes more than 40,000 years old.

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3.1 Agarose gel electrophoresis of nucleic acids

Nucleic acid electrophoresis is used to separate DNA or RNA fragments. They are set upon a gel, where an electric field induces them (nucleic acids are negatively charged due to their sugar-phosphate backbone) to migrate toward the anode. Longer nucleates migrate more slowly because they experience more resistance within the gel; smaller fragments migrate faster.

3.1.1 Theory of agarose gel electrophoresis of nucleic acids

Compared to proteins, the nucleic acids are more difficult to handle. Every mononucleotide residue contains a negatively charged phosphate group. Therefore all nucleates have a constant ratio of charge-to-mass. As a result, nucleates move toward the positively charged anode during electrophoresis. In absence of gel matrix, this

movement is independent from DNA molecular mass and no separation by size takes place [1]. The free solution mobility of DNA varies as the logarithm of the negative charge [2]. Besides, hydrodynamic interaction between the different parts of DNA are cut off by streaming counterions moving in the opposite direction, so no mechanism can explain the dependence of the DNA velocity on DNA length larger than 10 nm [3]. This event makes the electrophoretic separation of DNA different from other analytical processes, such as sedimentation or diffusion where hydrodynamic interactions play significant role.

3.1.1.1 Electrophoretic behavior of nucleic acids in gel

DNA nucleates migrate different in gel, if they have circular or linear conformation. In circular conformation, they can be either supercoiled or open circled. The open circular nucleates have a larger radius. The assuming of supercoiled or open circular form depends on the conditions under which DNA nucleates were isolated. A nick in one of the two DNA strands is sufficient for its supercoiled form to change in an open circle form.

There are three types of nucleate migrating through agarose and polyacrylamide gels: as through a sieve (Ogston sieving), as reptiles, and as rigid rods [4,5,6].

Ogston sieving

The electrophoresis separation of DNA takes place in a gel matrix. The widely accepted mechanism for this event is Ogston sieving model [7]. It treats the polymer matrix as a sieve consisting of randomly distributed network of connected pores. The migration through the gel pores is determined by the DNA thermal radius. Quite small nucleates behave themselves as if no gel is available. However, if the thermal radius of DNA nucleates corresponds to an average pore size in the gel, the more likely that it would pass through the pore. The medium-sized nucleates clash with the gel pores and penetrate inside, if their radius is smaller.

According to Ogston theory

$$\mu = \mu_o e^{-K_R T} \tag{3.1-1}$$

where μ and μ_0 are the mobilities of a nucleate in a gel and in a buffer, respectively; and K_R and T are the retardation coefficient and the concentration of the gel, respectively. The coefficient K_R depends on the nucleate radius.

Reptation

The Ogston model breaks down for large nucleates, which are greater than the gel pores. Under the influence of an electric field, these nucleates expand their conformation and enter the gel pores first with one end (chair end), and then crawl their rest (trailing end). De Gennes compared this movement with meandering of a reptile

and developed the *reptation theory* of separating DNA nucleates [8], also called theory of *crawling hike* [9,10].

The reptation model of nucleate migration takes into account that the mobility of nucleates depends in a much greater extent on the free ends of the nucleate instead of the middle segment of the nucleate. According to this model, a very long DNA nucleate receives in electric field a U-form shape and meanders to the positive electrode simultaneously with both ends. Once in a U-form shape, the nucleate winds around a gel pore, which significantly reduces its velocity [11]. Then it begins to move again when one of its ends moves forward through the pore.

The relationship between the mobility μ of a nucleic chain with a length N, and the electric field strength E is described by the following equation [12]:

$$\mu = K\left(\frac{1}{N} + bE^2\right) \tag{3.1-2}$$

where K is a constant that describes the charge and friction force of the nucleate, and b represents the pore structure of the gel.

Real-time fluorescence microscopy of stained nucleates showed more subtle dynamics during electrophoresis, with DNA showing alternately stretching in the direction of the applied field and then contracting into a ball, or becoming hooked into a U-shape when it gets caught on the polymer fibers [13,14]. This observation may be termed the *caterpillar model* [15]. Other model proposes that DNA gets entangled with the polymer matrix, and the larger the nucleate, the more likely it is to become entangled and its movement impeded [16]. Beyond a certain size, the DNA nucleate cannot be separated [17].

Migration as rigid rods

Increasing the field strength, larger nucleates lose slowly their spiral conformation, enlarge their length, and form rigid rod-shaped structures. As a result, they move faster through the gel pores toward the positive pole at a rate that is independent on their molecular size. So the retardant properties of the gel are reduced. Nucleates greater than 30,000 bp can be separated only at lower voltage and prolonged time.

3.1.1.2 Factors affecting migration of nucleic acids

There are many factors, which influence the electrophoretic migration of nucleates in agarose gel: ionic strength of the buffer used, size and conformation of DNA nucleates, gel concentration, concentration of ethidium bromide, if used during electrophoresis, strength of electric field, and more [18].

Size of DNA

Gels sieve DNA nucleates by their size, whereby smaller nucleates move faster, and on the contrary. Double-stranded DNA migrates slowly with velocity that is approximately inversely proportional to the logarithm of the number of base pairs. This relationship breaks down when DNA nucleates are very large and the agarose gel electrophoresis cannot separate them.

Conformation of DNA

The conformation of the DNA nucleate can significantly affect the movement of DNA. For example, supercoiled DNA usually moves faster than relaxed DNA because it is more compact. In a plasmid, multiple forms of DNA may be present [19]. The plasmid electrophoresis normally shows a main band, which is the supercoiled form, and minor bands that depict other DNA forms. The rate at which the various forms move can change using different electrophoresis conditions, for example, linear DNA may run faster or slower than supercoiled DNA depending on conditions; and the mobility of larger circular DNA may be more affected than linear DNA by the pore size of the gel [20].

Gel concentration

The concentration of the agarose gel determines the pore size of the gel, which affects the migration of DNA. Increasing the agarose concentration reduces the migration velocity and improves separation of smaller DNA molecules, while lowering the agarose concentration permits large DNA molecules to be separated.

For standard agarose gel electrophoresis, 0.7 g/dl agarose gel gives good separation and resolution of large 5,000–10,000 bp DNA fragments, while 2 g/dl agarose gel gives good resolution for small 200–1,000 bp DNA fragments. Up to 3 g/dl agarose can be used for separating small DNA fragments but polyacrylamide gel would be more appropriate for their resolution. High concentrations of agarose gel requires longer run times (sometimes days) and is often brittle. Low concentration agarose gels (0.1–0.2 g/dl) are fragile and may break. Concentration of 1 g/dl gel is common for many applications. The limit of resolution for standard agarose gel electrophoresis is about 750 kb. It can be overcome by pulsed-field gel electrophoresis, when alternating orthogonal electric fields are applied over the gel.

Concentration of ethidium bromide

Ethidium bromide affects also the DNA movement in a gel. When intercalated, the DNA nucleate can change its electric charge, mass, as well as superhelicity. The presence of ethidium bromide can convert a nucleate from negatively supercoiled into relaxed, even into positively coiled superhelix [21]. Circular DNA are more strongly affected by ethidium bromide than linear DNA.

Strength of electric field

The resolution of nucleates in a gel depends on field strength [22]. In weak electric fields, the migration velocity of DNA is proportional to the voltage applied, that is, the higher the voltage, the faster is the DNA. In standard gel electrophoresis, for optimal resolution of DNA larger than 2 kb, 5 to 8 V/cm are recommended [23]. The voltage is limited because it heats the agarose gel and may melt it, if the electrophoresis is run in a gel containing low-melting agarose.

In an electric field that is periodically reversed, the mobility of DNA nucleates may drop significantly [24]. This phenomenon, used in pulsed-field gel electrophoresis, can result in band inversion whereby larger DNA nucleates move faster than smaller ones. This method is named **f**ield-**i**nversion **g**el **e**lectrophoresis (FIGE).

3.1.1.3 Determining DNA masses

The molecular mass of a DNA fragment, obtained with the catalytic help of endonucleases, can be determined using electrophoresis in agarose or polyacrylamide gels. The agarose gels are suitable for DNA fragments containing 100–60,000 bp; the polyacrylamide gels are suitable for DNA fragments containing 10–1,000 bp. A single-chain DNA migrates slower than the double-chain DNA of the same length, if their length is greater than 100 mononucleotides. This event can be explained by the random structure of the single-chain DNA [25].

For determining the molecular mass of a DNA nucleate in agarose gels, the linear relationship between the logarithm of its mobility μ and the agarose concentration c is used:

$$\log\mu = \log\mu_0 - K_R c \tag{3.1-3}$$

where μ_0 is its electrophoretic mobility in an agarose gel of concentration of 0 g/dl, and the slope K_R is the retardation coefficient. The magnitude of K_R depends on the ionic strength of the buffer and the properties of the gel. This equation becomes invalid when the agarose concentration is over 0.9 g/dl [26].

DNA nucleates of different molecular masses are to be separated on agarose gels of different concentrations (Table 3.1-1).

To obtain a half-logarithmic plot for estimating the size of unknown linear DNA nucleates, following steps are to be fulfilled:

1. Measure the distance between the point where the DNA markers were applied and the outermost point, which has been reached by the dye (front).
2. Determine the distance between the point of application and the DNA marker bands.
3. Give the relative mobility (the ratio between the DNA marker distance and the front distance) of each band on the x-axis.

4. Register the common logarithm of each marker length, in bp, on the *y*-axis against the relative mobility of each marker, wherein 100 bp = 2.00, 1,000 bp = 3.00, and so on.
5. Bind the marker points by a straight line.
6. Obtain the length of the unknown DNA nucleate giving the value of its relative mobility on the *x*-axis, drawing up a straight line parallel to the *y*-axis until crossing the plot, and draw a line perpendicular to the *y*-axis.
7. Find the exact length of the unknown DNA nucleate, in bp, using a linear regression to calculate the slope (m) and the segment (c) in the linear equation

$$y = mx + c \tag{3.1-4}$$

Table 3.1-1: Agarose concentrations suitable for electrophoretic separation of DNA fragments.

Agarose gel, g/dl	Linear DNA fragments, kb
2.0	0.1–3
1.5	0.2–4
1.2	0.4–6
0.9	0.5–7
0.7	0.8–10
0.6	1.0–20
0.3	5.0–60

If, for example, $m = -2.16$, the relative mobility (x) of the unknown nucleate is 0.54, and $c = 4.62$, the equation is transformed to give

$$y = -2.16 \bullet 0.54 + 4.62 = 3.45,$$

which corresponds to a molecular length of 2,818 bp (Figure 3.1-1).

3.1.2 Native agarose gel electrophoresis of nucleic acids

Native agarose gel electrophoresis of nucleic acids is an analytical technique for separation of DNA or RNA fragments by size. Nucleates move in an electric field to the anode because their phosphoric residues are deprotonated and, as a result, negatively charged. Longer DNA nucleates migrate more slowly because they undergo more resistance within the gel; smaller fragments migrate faster.

The native agarose gel electrophoresis is most commonly used for analysis of nucleic acids and PCR products [27,28]. It is carried out for separation of double-stranded

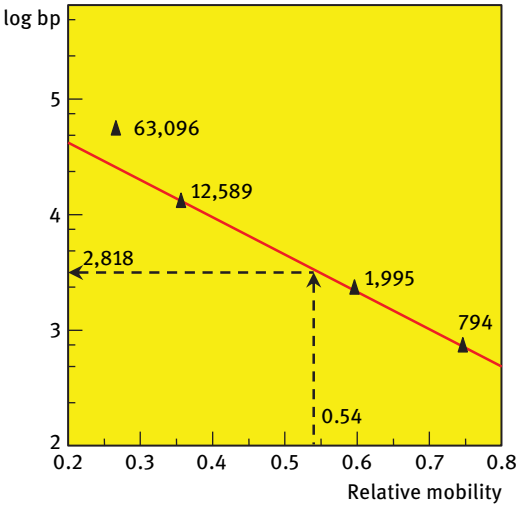

Figure 3.1-1: Determining the *bp* length of an unknown DNA nucleate, based on a plot of the relative mobility against the common logarithm of DNA markers length.

nucleates of a length from 500 to 20,000 bp, hence of particles with radii larger than 5–10 nm [29]. However, the agarose gel electrophoresis has lower resolving power for DNA than PAAG electrophoresis.

The nucleic acids to be separated can be prepared in several ways. In the case of large DNA nucleates, they are cut into smaller fragments using a DNA restriction endonuclease. In PCR-amplified samples, enzymes presented in the sample might affect the separation of nucleates. Therefore, these enzymes must be removed before electrophoresis. Then the samples with the nucleic acids are loaded into slots and a voltage is applied across the gel for a given time.

Xylene cyanol or Bromophenol blue is added often to DNA samples to build a moving front. These dyes run with the same speed as DNA fragments that are 5000 bp and 300 bp in length, respectively. Other less-frequently used progress markers are Cresol Red and Orange G, which run as fast as 125 bp DNA and 50 bp DNA, respectively.

The electrophoretic separations of nucleic acids are carried out in pH range from 6 to 10. If the pH value is too low, the bases cytosine and adenine bind protons and are charged positively; if the pH value is too high, the bases thymine and guanine split protons and are charged negatively. In both cases, the additional electric charges cause denaturing of nucleic acids and change their mobility.

The DNA is commonly visualized after reacting with ethidium bromide and viewed under UV light. Days are also available, such as SYBR Green, GelRed, Methylene blue,

and Crystal violet. If the separated DNA fragments are needed for further experiment, gel slices with them can be cut off for appropriate manipulations.

The measurement and analysis of the resolved nucleates are mostly carried out using specialized software.

Agarose gel zone electrophoresis is used in the following techniques: submarine electrophoresis of nucleic acids, restriction fragment length polymorphism, variable number of tandem repeat, DNA profiling, and more.

3.1.2.1 Submarine electrophoresis of nucleic acids

Agarose gel electrophoresis is commonly run horizontally in a submarine mode whereby the slab gel is completely submerged in a buffer [30]. The submarine electrophoresis is a standard method for nucleic acid analyzing. Using it, DNA and RNA fragments can be separated and identified, and **r**estriction fragment length polymorphism analysis [31] can be performed. The buffer used in the agarose gel is the same as the running buffer in the electrophoresis tanks.

The submarine electrophoresis is carried out in an electrophoresis unit consisting of two buffer tanks. They are connected with each other by a flat bridge, for example 145 × 145 mm. On the bridge, an agarose gel cast on a support film is laid down [32] (Figure 3.1-2).

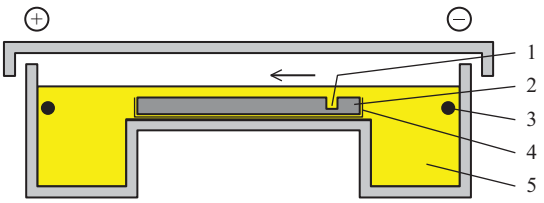

Figure 3.1-2: Unit for submarine electrophoresis.
1. Well for sample; 2. Agarose gel; 3. Electrode wire; 4. Gel dish; 5. Electrode buffer

In practice, 0.8 g/dl agarose gels are used. Highly concentrated agarose gels (2–4 g/dl) are applied, for example, for analysis of PCR products. The higher ionic strength increases the resolution [33], but generates a lot of heat. This may destroy the helix structure of DNA.

Buffers for submarine electrophoresis

For submarine electrophoresis, **T**RIS-**b**orate-**E**DTA (TBE), **T**RIS-**a**cetate-**E**DTA (TAE), or **T**RIS-**h**ydrogen phosphate-**E**DTA (THE) buffers are used. They have pH values of 7.7 to 9.0 (Table 3.1-2). The TRIS-borate-EDTA and TRIS-hydrogen phosphate-EDTA buffer have higher buffer capacity. The TRIS-acetate-EDTA buffer has a lower buffer capacity, so it needs to be circulated during electrophoresis from one buffer tank to

Table 3.1-2: Buffers for submarine electrophoresis.

Buffer for DNA electrophoresis	Description	Solution	
TRIS-**b**orate-**E**DTA (TBE) buffer, pH = 8.3	Most common buffer used for both analytical and preparative agarose gels.	**5x buffer**	
		TRIS	54.5 g
		Boric acid	27.5 g
		0.5 mol/l EDTA	20.0 ml
		Deionized water to	1,000.0 ml
TRIS-**a**cetate-**E**DTA (TAE) buffer, pH = 8.0	It is appropriate for analytical and preparative agarose gels. Sometimes it is used in pulsed-field electrophoresis.	**50x buffer**	
		TRIS	242.0 g
		Glacial acetic acid	57.1 ml
		0.5 mol/l EDTA	100.0 ml
		Deionized water to	1,000.0 ml

another. All buffers can be stored at room temperature as 10-fold concentrated solutions because they are protected by EDTA against molds.

The most common buffer for submarine zone electrophoresis is the **T**RIS-**b**orate-**E**DTA buffer (TBE), which was introduced by Peacock and Dingman [34]. It has a pH value of 8.3 and TRIS concentration of 50–100 mmol/l. Michov has established that the TRIS-borate buffer contains a complex compound formed by a condensation reaction between boric acid and TRIS [35]. The compound, which was referred to as *TRIS-boric acid*, has a zwitterionic structure. Its existence in the TRIS-borate buffer was reproved 30 years later [36].

The DNA fragments migrate more slowly in TRIS-borate buffers than in TRIS-acetate or TRIS-hydrogen phosphate buffers [37]. This can be explained by the higher electroosmosis due to in a reaction between boric acid and alcohol groups of the agarose [38].

According to Michov, another buffer, which can be used for nucleic acid separation with high resolution, is the **T**RIS-**t**aurinate-**E**DTA buffer (TTE) [39]. Its recipe is as follows:

TRIS-taurinate-EDTA buffer, pH = 8.5, *I* = 0.10 mol/l	
TRIS	34.16 g
Taurine	48.93 g
$Na_2EDTA \cdot 2H_2O$	0.37 g
Deionized water to	1,000.00 ml

A new **s**odium **th**reonine (STh) medium for electrophoresis of supercoiled DNA on 1 g/dl agarose gel containing low-ionic-strength **T**RIS-**b**orate-**E**DTA (TBE) or **T**RIS–**a**cetate–**E**DTA (TAE) buffers was described [40]. This medium provided better

resolution, less heat generation, and prevention of changes among linear, covalently closed circular, and open circular DNA in the range of 2–10 kilobase pairs.

Casing agarose gels for submarine electrophoresis

Nowadays, the classic submarine electrophoresis in 3–4-mm-thick agarose gels is replaced by submarine electrophoresis in thin gels [41,42]. The casting of these gels is carried out in the following steps:

- A support film for agarose gel is rolled over a horizontal glass plate having a few drops of water, and stuck around with a tape strip so that a trough is formed.
- The agarose required is weighed, suspended in a buffer, and heated in a boiling water bath or microwave oven until dissolved.
- The agarose solution is cooled to 70 °C, and 0.5 µg/ml ethidium bromide is added. Thus, the nucleic acids can be detected immediately after electrophoresis.
- The edges of the trough are sealed with the agarose solution using a Pasteur pipette, and the remaining solution is cast inside.
- A comb is inserted into the agarose solution, 1 cm from the edges of the trough, so that wells in the agarose gel are built.
- The agarose solution is left at room temperature, and after one hour the gel can be used. The agarose gel gets a stronger structure, if left overnight in a refrigerator.

Sample preparation

Nucleates are dissolved in a sample buffer. It contains the same electrode buffer, 20 g/dl glycerol, or 10 g/dl of sorbitol, and 0.025 g/dl Xylene cyanol or Bromophenol blue. Glycerol or sorbitol maintains high viscosity of the samples, so they can be applied under the electrode buffer. It is recommended to apply 5–10 µg nucleic acids into a sample well. The lower detection limit for an ethidium bromide treated DNA is 5 ng [43].

Electrophoretic conditions

The agarose gel is placed on the bridge of an electrophoretic unit. Thereafter, the unit is filled with an electrode buffer until the gel is at least 1 mm under the buffer superficial. The sample with the nucleic acids is applied under the buffer into the gel wells. If the agarose gel contains ethidium bromide, the buffer must also contain ethidium bromide in the same concentration, usually 0.5 µg/ml. Prior to electrophoresis, the power supply is switched on whereby the electric field strength should not exceed 500–1,000 V/m.

3.1.2.2 Restriction fragment length polymorphism

Restriction **f**ragment **l**ength **p**olymorphism (RFLP) is a laboratory technique by which differences between samples of homologous DNA molecules can be illustrated. It begins with fragmenting a sample of double-helical DNA by restriction enzymes (restrictases) in sequences of lengths of four to six pairs of bases, in a process known as restriction digest. Most DNA sequences that are recognized by the restrictases are palindromes.

Restrictases are endonucleases, which catalyze the cleavage of the phosphate bonds within both strands of DNA. They require Mg^{2+} for activity and generate a 5′-phosphate and a 3′-hydroxyl group at the point of cleavage. The restriction enzymes cut at specific sequences of bases called recognition sites.

Restriction enzymes are produced by many species of bacteria including blue-green algae. They have three-letter nominations that are associated to the bacteria from which they are isolated. The first (main) letter coincides with the generic name. The second and third letters are small. They show the bacteria type. Roman numbers show the order of invitation. For example, *AluI* comes from ***A****rthrobacter* ***lu****teus*. Often, after the first three letters and before the Roman number, another letter shows the strain. For example, *EcoRI* and *EcoRII* are the first restriction enzymes isolated from *Escherichia coli R*. Until now, over 3,000 restriction enzymes have been discovered.

The size of a restriction fragment can vary (Figure 3.1-3). Here, a small segment of the genome is being detected by a DNA probe (thicker red line). In allele *A*, the genome is cleaved by a restriction enzyme at three sites (1, 2, and 3), but only fragment 1 will be detected by the probe. In allele *a*, restriction site 2 has been lost by a mutation, so the probe will detect the larger fused fragment between sites 1 to 3.

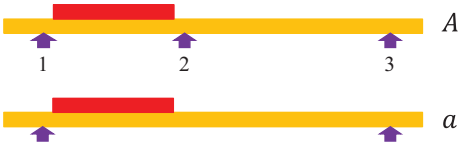

Figure 3.1-3: Schematic diagram of restriction fragment length polymorphism by cleavage site loss.

The restriction fragments are then separated by length using agarose gel electrophoresis, and transferred onto a membrane (Southern blotting). Then hybridization between them and labeled DNA probe determines their length. *Hybridization* is a process of recombination of complementary chains of different origins (between different DNA molecules, DNA and RNA, or different RNA molecules). The test DNA strand is referred to as a *matrix*, and the DNA or RNA probe of known nucleotide sequence and size is referred to as a the *probe*. The probes are labeled either with an isotope (^{32}P, ^{35}S, or ^{3}H) or with fluorescent ligands. A probe will hybridize with a

matrix, if the two chains are complementary to each other and can form a sufficient number of hydrogen bonds.

Restriction fragment length polymorphism is a useful method for genome mapping and genetic diseases analyzing. It plays an important role in the paternity testing, forensics, and DNA fingerprinting.

3.1.2.3 Variable number of tandem repeat

A **v**ariable **n**umber **t**andem **r**epeat (VNTR) is a tandem repeat in a genome constructed of short nucleotide sequence. VNTR exist on many chromosomes, and often show variations in length (number of repeats) among individuals. Each variant of them is an inherited allele, allowing to be used for personal or parental identification.

VNTR are a source of RFLP genetic markers used in linkage analysis (mapping) of diploid genomes. When removed from surrounding DNA by PCR amplifying, their size can be determined by gel electrophoresis or Southern blotting, where they build bands unique to each individual.

Let us explain the topic with an example: The probe and restriction enzyme could detect a region of the genome that includes a VNTR segment (the boxes on Figure 3.1-4). Allele *a* has four repeats in the VNTR, and the probe detects a longer fragment between the two restriction sites. In allele *b*, there are only two repeats in the VNTR, and the probe detects a shorter fragment between the same restriction sites. Insertions, deletions, translocations, and inversions of genes can also lead to restriction fragment length polymorphism.

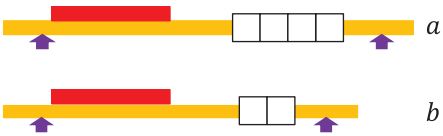

Figure 3.1-4: Schematic diagram of restriction fragment length polymorphism by variable number tandem repeat length variation.

VNTR analysis is being used to study genetic diversity, and to distinguish strains of bacterial pathogens. In this context, such assays are usually called **m**ultiple **l**oci **V**NTR **a**nalysis (MLVA).

3.1.2.4 DNA profiling

DNA profiling (DNA fingerprinting, DNA testing, or DNA typing) is a process of determining an individual's DNA characteristics, which is as unique to individuals as the fingerprints. DNA profiling was developed in 1984 by Sir Alec Jeffreys while

working in the Department of Genetics at the University of Leicester [44,45,46]. It is most commonly used as a forensic technique to identify an unidentified person, to place a person at a crime scene, or to eliminate a person from consideration [47]. DNA profiling is also used to help clarify paternity [48] and in genealogical or medical research. It is also used in the study of animal and floral populations and in the fields of zoology, botany, and agriculture [49].

DNA profiling uses repetitive sequences that are highly variable, called **v**ariable **n**umber **t**andem **r**epeats (VNTR). The VNTR are similar between closely related individuals, but unrelated individuals are extremely unlikely to have the same VNTR (Figure 3.1-5).

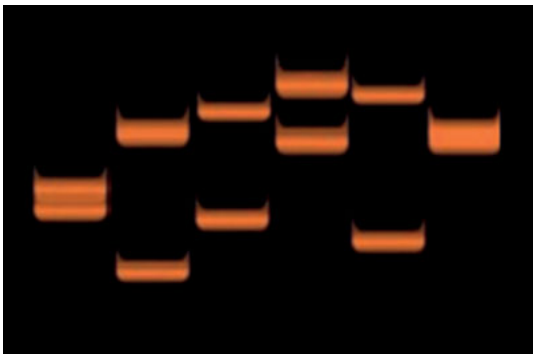

Figure 3.1-5: Variations of VNTR allele lengths in six individuals.

The DNA testing is performed by collecting buccal cells on the inside of a cheek using a swab. The swab has wooden or plastic stick handle with cotton on synthetic tip. It rubs the inside of a cheek to collect as many buccal cells as possible. When this is not available, a sample of drop of blood, saliva, sperm, vaginal lubrication, hair, or other appropriate fluid or tissue from personal items (e.g. a toothbrush, razor) can be used. A minimum amount of the biological material is sufficient for forensic research, as DNA in the sample can be amplified. This is achieved by the polymerase chain reaction (s. there).

Prior to PCR, DNA is restricted by restriction enzymes. Next, gel electrophoresis, blotting on a nitrocellulose membrane (Southern blotting), hybridization with isotopically labeled probes, and more are performed.

For paternity DNA profiling, samples obtained from the mother, child, and possible fathers are analyzed. The child's DNA is a composite of its parent DNAs. Therefore, comparison of DNA fragmentation patterns obtained from the mother and child will give a partial match. Electrophoretic bands in the child's DNA fingerprint that are not present in the mother's DNA must be contributed by the father (Figure 3.1-6).

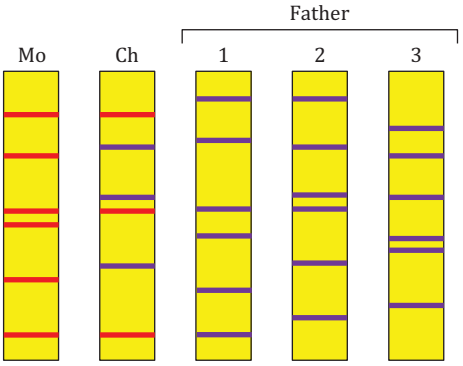

Figure 3.1-6: Example of DNA profiling in order to determine the father of a child.
Child's DNA sample (Ch) should contain a mixture of different size DNA bands of both parents: mother (Mo) and suspected father (Fa). In this case, man #2 is the father.

DNA profiling may also be used to determine evolutionary relationships among organisms, that is, between the genes of 16S rRNA and recA in microorganisms [50].

Paternity or maternity testing for child

The genetic material of an individual is derived from the genetic material of both parents in equal amounts. It is known as the nuclear DNA or genome of the individual, because it is found in the nucleus. Beside the nuclear DNA in the nucleus, the mitochondria in the cells also have their own genetic material termed the "mitochondrial DNA." Mitochondrial DNA comes only from the mother, without any shuffling. Comparison of the mitochondrial genome is much easier than that based on the nuclear genome. However, testing the mitochondrial genome can prove only if two individuals are related by common descent through maternal lines. It could not be used to test for paternity.

In testing the paternity of a male child, comparison of the Y chromosome can be used, since it is passed directly from father to son.

Prenatal paternity testing for unborn child

Paternity testing can also be performed while the woman is still pregnant [51]. For this purpose, a procedure called amniocentesis is performed. Amniocentesis supplies placental tissue in either a transcervical or a transabdominal manner, or amniotic fluid by inserting a needle through the pregnant mother's abdominal wall.

Interpretation of DNA test results

A paternity or maternity test report lists the genetic profiles of each tested party, displaying the allele sizes of the different markers tested. It also lists the

relationship **i**ndex (RI) for each marker – a statistical measure of how powerful a match is at a particular locus, which signifies relatedness. The RI for each marker are multiplied with each other to produce the **c**ombined **p**aternity **i**ndex (CPI), which is the genetic odds that the tested man is more likely to be the biological father. It is generally accepted by most courts and government agencies that a CRI of 100 and a probability of 99% or higher is strong proof that the tested man is the biological father. On the other end of the spectrum, the probability is always 0% when the tested man is excluded as the biological father of the tested child.

Forensic medicine

The recombinant DNA technologies have great importance for forensic medicine. Let us illustrate this by the following example: A girl was found dead and raped. A police doctor collected sperm from the vagina and blood under the girl's nails. The sperm and blood were supposed to be from the rapist and murderer. DNA from the samples was amplified by PCR. Meanwhile, the police detained three men who were with the victim in the night of the murder. Their DNA was compared with the victim's DNA and with the sperm DNA from the vagina. The DNA profiling proved that the DNA from the second suspect and sperm DNA had the same profile (Figure 3.1-7).

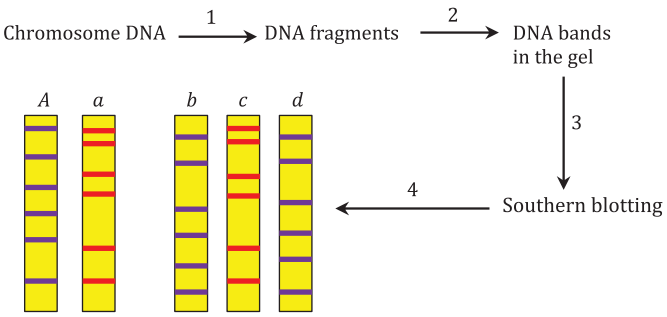

Figure 3.1-7: Comparative analysis of victim's DNA and DNA from three suspected men. The test proves that the murder is the suspected men c.
1 – DNA restriction with restriction enzymes; 2 – Gel-electrophoresis of DNA fragments;
3 – Southern blotting; 4 – Hybridization with radioactive sample, and autoradiography
A – Victim's DNA; *a* –DNA isolated from victim's body; *b*, *c*, *d* – DNA from suspicious men

There are many criminal cases that used the DNA profiling:

- In 1986, Richard Buckland was exonerated, despite having admitted to the rape and murder of a teenager. Later, Colin Pitchfork was identified as the perpetrator of the same murder using the same techniques that had cleared Buckland [52].

- In 1994, the claim that Anna Anderson was Grand Duchess Anastasia Nikolaevna of Russia was tested after her death using samples of her tissue that had been stored at Virginia hospital following a medical procedure. The tissue was tested using DNA fingerprinting, and showed that she bore no relation to the Romanovs [53].
- In 2004, DNA testing shed new light into the mysterious 1912 disappearance of Bobby Dunbar, a four-year-old boy who vanished during a fishing trip. He was found eight months later, but a woman claimed that the boy was her son, Bruce Anderson. The courts disbelieved her claim and boy was raised and spent his life as Bobby Dunbar. Later, DNA tests on Dunbar's son and nephew revealed the two were not related, thus establishing that the boy found in 1912 was not Bobby Dunbar, whose real fate remains unknown [54].

3.1.3 Denaturing agarose gel electrophoresis of nucleic acids

The separation of DNA strands each from other is most often achieved by thermal denaturing (melting). Double-stranded DNA that contains more G-C base pairs (with three hydrogen bonds between the bases) requires a higher temperature than double-stranded DNA containing predominantly pairs of A-T bases (with two hydrogen bonds between the bases). Higher temperatures are also needed when DNA chains are very long and, as a result, contain more hydrogen bonds. Denaturation is facilitated by reduced salt concentration or in the presence of urea that breaks down the hydrogen bonds.

Denaturing agarose gel electrophoresis is used for single-stranded DNA or RNA, which consist of 500 to 20,000 mononucleotides, for example for **m**essenger **RNA** (mRNA). RNA samples are most commonly separated on agarose gels containing formaldehyde as a denaturing agent to save the RNA secondary structure [55]. Another method for separation of high-molecular-mass RNA such as ribosomal RNAs and their precursors by agarose-formaldehyde gel electrophoresis was described [56]. It minimizes errors and can be used for subsequent analysis of RNA by Northern blotting. RNA stained with **et**hidium **br**omide (EtBr) can be viewed under UV light and also used for blotting [57].

3.1.4 Pulsed-field gel electrophoresis of nucleic acids

Agarose gel electrophoresis is the method of choice regarding DNA fragments in size of between 1 and 23 kbp. However, it cannot separate DNA nucleates in length of more than 30 kbp, because they are moving together through the gel. In 1984, David Schwartz and Charles Cantor resolved larger DNA nucleates by agarose gel

electrophoresis in an electric field with changing direction (pulsed electric field) [58]. Originally, the electric field was pulsed in one direction; later, it was pulsed in different directions. Now this method is known as **p**ulsed-**f**ield **g**el (zone) **e**lectrophoresis (PFGE).

3.1.4.1 Theory of pulsed-field gel electrophoresis of nucleic acids

The principle of pulsed-field electrophoresis is that the voltage is alternatively switched on and off. The pulses are so arranged that two steps in straight ahead direction are followed by one step back. As a result, the long DNA nucleates migrate in the gel as a reptile, which forms in the pauses their globular configuration again. Depending on nucleates length, the next pulse finds the nucleate either completely folded or still refolded. For folding and unfolding, larger nucleates need longer time compared to smaller nucleates [59]. The same happens when pulses come from other directions.

While smaller nucleates have enough time to fold and unfold, so that they migrate quickly through the gel, greater nucleates need more time to fold and unfold and move slowly. The time required for repairing the snake conformation of nucleates is called *viscoelastic relaxation time*. It depends on the molecular mass: larger nucleates require longer viscoelastic relaxation time, and on the contrary. As a result, the resolution of pulsed-field gel electrophoresis is higher than that of the submarine electrophoresis.

Geometry of the pulsed-field

Pulsed-field gel electrophoresis requires a special separation chamber, where the polarity, the angle of separation direction, and the pulse rate are changing alternatively by microprocessor controlled point electrodes. Depending on this, following types of pulsed-field gel electrophoresis are known: gel electrophoresis in contour-clamped homogeneous electric fields; field-inversion gel electrophoresis; transverse alternating field gel electrophoresis; and rotating gel electrophoresis in a constant electric field [60] (Figure 3.1-8).

3.1.4.2 Sample preparation for pulsed-field gel electrophoresis

In the conventional methods, a double-stranded DNA molecule is significantly sheared or broken. As a result, DNA nucleates of maximum length of 200 kbp are obtained. This is not a problem, if plasmid or bacteriophage DNA, smaller than 200 kbp, must be resolved. If genomic DNA is cut by restriction endonucleases in fragments of less than 200 kbp, the broken nucleates cause a weak background in subsequent Southern blots.

If, however, the DNA fragments are larger than 200 kbp, the samples for pulsed-field gel electrophoresis must be prepared *in situ*: The cells are pipetted in a liquid

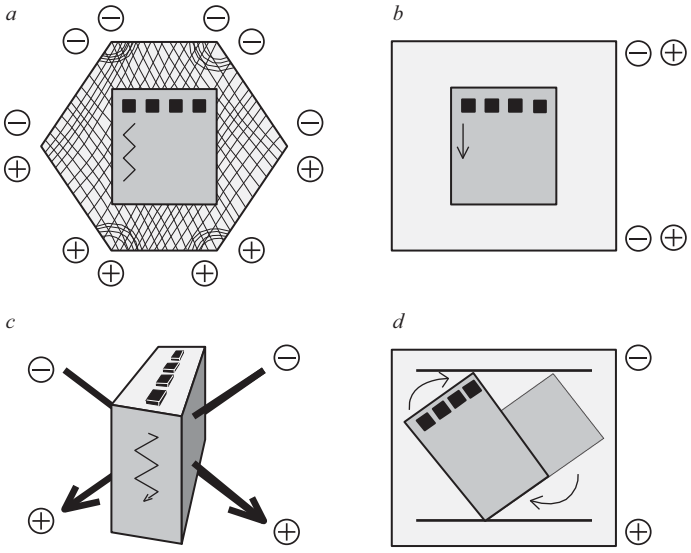

Figure 3.1-8: Types of pulsed-field gel electrophoresis. The electric field changes alternately from the one to another electrode.
a – Gel electrophoresis in a contour-clamped homogeneous electric field and the electric field lines, generated by hexagonally arranged point electrodes; *b* – Field-inversion gel electrophoresis; *c* – Transverse alternating field gel electrophoresis; *d* – Rotating gel electrophoresis in a constant electric field

agarose of a low-melting point and agarose is hardened in a little block. Then, the block is placed into a slot of the agarose resolving gel. Afterward, the DNA nucleates run during the electrophoresis, whereas the cell membranes remain in the agarose block.

3.1.4.3 Running pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis is similar to the standard gel electrophoresis. However, the voltage is periodically switched on and off in three directions: one through the central axis of the gel, and two in directions located at an angle of 60 degrees on both sides. The pulse times are equal for each direction. For extremely large DNA nucleates (up to 2 Mbp), the pulse times can be increased for each direction. For instance, at field strengths of 2–10 V/cm, the pulse time can vary from 5 to 1,000 s. As a result, pulsed-field gel electrophoresis takes between 10 h and several days depending on the size of DNA fragments (Figure 3.1-9).

In contrast to conventional gels, which are not affected by temperature fluctuations, pulsed-field gels must be held at lower temperatures (4-15 °C) to achieve good separation.

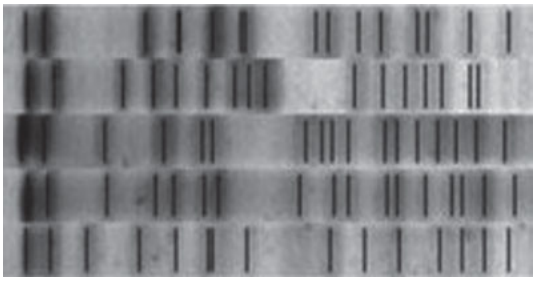

Figure 3.1-9: Analysis of Escherichia coli DNA by pulsed-field gel electrophoresis.

The pulsed-field electrophoresis allows separation of linear double-stranded DNA nucleates of 20 kbp up to 10 Mbp, for example of whole chromosomes [61]. This is important for mapping of human genome, which comprises about 3,000 Mbp.

Pulsed-field gel electrophoresis of nucleic acids is used in the following techniques: CHEF gel electrophoresis, field-inversion gel electrophoresis, transverse alternating field gel electrophoresis, and rotating gel electrophoresis.

3.1.4.4 CHEF gel electrophoresis

Contour-clamped **h**omogeneous **e**lectric **f**ields (CHEF) gel electrophoresis [62,63] is a type of pulsed-field gel electrophoresis that enables the resolution of DNA fragments with lengths from 10 kilobases up to 9 megabases.

In CHEF gel electrophoresis, two electric fields form an angle of 120° each to other using point electrodes in a hexagonal arrangement. An electric pulse continues from 1 s up to 90 min, depending on the length of DNA nucleates to be separated. Longer nucleates need larger pulse times; smaller nucleates need shorter pulse times. A separation can persist up to several days.

3.1.4.5 Field-inversion gel electrophoresis

In the **f**ield-**i**nversion **g**el **e**lectrophoresis (FIGE), the electric field is reversed in certain pulse frequencies [64,65]. Beside a gel electrophoresis unit and a power supply, a device is needed for periodic inversion of the electric field direction over the electrophoresis. The four electrode pulsed-field gel electrophoresis is reduced to a standard submarine gel electrophoresis with only two electrodes. This configuration generates a uniform electric field across the gel, making the lane-to-lane comparison easy.

3.1.4.6 Transverse alternating field gel electrophoresis

In **t**ransverse **a**lternating **f**ield **g**el **e**lectrophoresis (TAFGE) [66,67], two electrodes are located on each side of a vertical agarose gel. The gel is located in a large Plexiglas tank. The electrode wires stretch across the Plexiglas tank.

The electric field is reversed after certain time, as a result of which the direction of DNA migration is changed alternatively. With the help of this technique, DNA nucleates that distinguish in size from 200 up to 7 million base pairs are separated. Resolution of very large molecules requires several days electrophoresis. The velocity of identical nucleates does not vary from lane to lane, and there is no distortion in the paths of DNA. The transverse alternating field gel electrophoresis has been used in the human chromosomes research.

3.1.4.7 Rotating gel electrophoresis

Rotating **g**el **e**lectrophoresis (RGE) is a technique for separating large DNA fragments [68,69,70]. It requires periodic changing of the orientation of the electric field by setting the gel on a rotating platform within a constant homogeneous electric field. The DNA nucleates of different sizes are separated by adjusting the frequency of rotation.

3.1.4.8 Mapping the human genome

Due to pulsed-field gel electrophoresis, genome mapping is carried out [71,72,73]. This was made for mammalian genomes that can be split by restriction endonucleases, as well as for bacterial chromosomes that can be separated in undigested form. Pulsed-field gel electrophoresis is also used for karyotyping, and analysis of chromosomes.

An example for mapping is the genome of *Theileria parva*, a unicellular parasite [74]. Transmitted by ticks in Africa, this parasite causes the east coast fever – a cattle disease. Due to the recombination, an antigenic polymorphism is developed, which helps the parasite to escape the host immune system. Using pulsed-field electrophoresis, the 10 Mbp genome of *Theileria parva* was separated into its fragments.

STR analysis

The unrelated people have different numbers of short DNA sequences. The **s**hort **t**andem **r**epeats (STRs) consist usually of four bases. Each STR is polymorphic, but the number of alleles is very small. Typically each STR allele is shared by 5–20% of individuals. In the STR analysis, their loci (locations on a chromosome) are targeted with sequence-specific primers and amplified using PCR. Then the resulted DNA fragments are separated by electrophoresis. The STR analysis is looking at multiple STR loci simultaneously. The more STR regions that are tested in an individual, the more discriminating the test becomes.

AmpFLP

Amplified **f**ragment **l**ength **p**olymorphism (AmpFLP) [75] is faster than RFLP analysis. It relies on **v**ariable **n**umber **t**andem **r**epeat (VNTR) polymorphisms to distinguish various alleles, which are separated on a gel in the presence of an allelic ladder.

Bands can be visualized by silver staining. AmpFLP analysis can be automated, and allows for easy creation of phylogenetic trees based on comparing individual samples of DNA.

DNA family relationship analysis

All the cells forming the body contain the same DNA – half from the father and half from the mother. The DNA of an individual is the same in all somatic (nonreproductive) cells. As a result, every cell type can be used for testing the family relationship: cells from the cheeks, blood, or other biological samples. In a routine DNA paternity test, the markers used are **s**hort **t**andem **r**epeats (STRs). These repeats are short DNA pieces that characterize the individuals.

Each marker is assigned with a **p**aternity **i**ndex (PI). It is a statistical measure of how powerfully a match at a particular marker indicates paternity. The PI of each marker is multiplied with each other to generate the **c**ombined **p**aternity **i**ndex (CPI), which indicates the overall probability of an individual being the biological father of the tested child relative to a randomly selected man from the entire population of the same race.

Y-chromosome analysis

For analysis of Y-chromosome in forensics, paternity, and genealogical DNA testing, the **s**hort **t**andem **r**epeat (STR) on the **Y**-chromosome (Y-STR) analysis is used. The Y chromosome is found only in males. The Y male sex-determining chromosome, as it is inherited only from the father, is almost identical along the patrilineal line. Y-STR analysis can help in the identification of paternally related males. It was performed to determine if Thomas Jefferson had sired a son with his slave Sally Hemings [76].

Mitochondrial analysis

Mitochondrial **DNA** (mtDNA) is maternally inherited. Therefore, directly linked maternal relatives can be used as match references. Scientists amplify the HV1 and HV2 regions of the mtDNA, and then sequence each region to compare single-nucleotide differences. Heteroplasmy and poly-C differences may throw off straight sequence comparisons. mtDNA can be obtained from the same materials as nuclear DNA: hair shafts, bones, or teeth. Using mtDNA analysis, it was determined that Anna Anderson was not Anastasia Romanov – the Russian princess she had claimed to be.

3.1.5 Elution of DNA from agarose gels

In order to isolate nucleic acids from agarose gels, several methods are available. They include electroelution; absorption on positively charged **di**ethyl**a**mino**e**thyl

(DEAE) paper, on resin, or on finely dispersed glass suspensions; digestion with agaroses; and melting of agarose.

After DNA samples are run in an agarose gel, UV light is shone on the gel in order to illuminate all the ethidium bromide-stained DNA. The desired band in the gel slice is removed with a cover slip or razor blade. In general, DNA nucleates that contain less than 1,000 bp can be eluted. If DNA nucleates are larger, they can be obtained more difficult; if they exceed 20 kbp, the elution yield is decreased to 20% of the DNA amount.

Next a pocket of Parafilm wax paper is created and the agarose fragment is placed inside. The agarose fragment is compressed with fingers into the corner of the pocket to liquefy the gel, and the liquid droplets are pipetted into a small tube. Afterward, the ethidium bromide stain is removed with the help of butanol extraction, and, at the end, phenol/chloroform extraction of the cleaned DNA fragment is carried out.

Instead of extracting DNA from an agarose gel, the gel can be previously melted. For this purpose, agarose with a low-melting point is needed, which solidifies at 30 °C and melts at 65 °C. Agarose with a low-melting point is produced from standard agarose after introducing hydroxyethyl groups in the agarose polysaccharide chain.

The elution from a hydroxyethyl agarose gel can be performed in the following way [77]: After electrophoresis, the desired bands are excised from the gel and dissolved in a fivefold volume of 20 mmol/l TRIS-chloride buffer, pH = 8.0 with 1 mmol/l EDTA. Then, the gel is melted at 65 °C and extracted with an equal volume of phenol. The aqueous phase is retaken by centrifugation at room temperature, treated with phenol-chloroform solution, and finally extracted with chloroform. The DNA is precipitated with methanol.

3.1.5.1 Dialysis

The gel fragment is placed in a dialysis tube. It is permeable to fluids but impermeable to DNA nucleates, thus preventing DNA from passing through the membrane when soaked in a buffer. An electric field is established around the dialysis tube so that DNA is removed from the gel but remains in the tube. The tube solution can then be pipetted out and will contain the DNA.

3.1.6 Visualization of nucleic acids

DNA and RNA themselves are not visible within an agarose gel. They can be visualized using dyes that bind to DNA or RNA. The most common dye for this purpose is **et**hidium **br**omide (EtBr) [78]. After intercalating into the major groove of DNA, it fluoresces reddish-orange under UV light. So any band containing more than 20 ng DNA becomes visible. The gel can then be photographed with a digital or Polaroid camera – the images are usually in black and white (Figure 3.1-10).

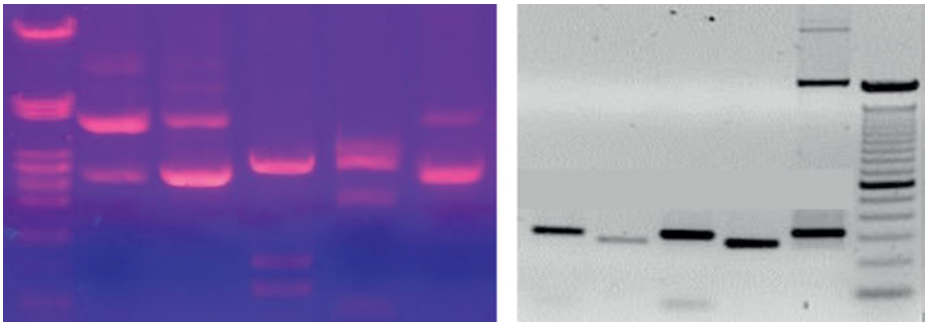

Figure 3.1-10: Two pherograms after DNA gel electrophoresis. Ethidium bromide staining and digital photo.
Left – under UV light; *Right* – photographs with digital camera

Ethidium bromide is a mutagen. Therefore, safer alternatives of DNA staining are available, such as GelRed [79]. It binds to the minor groove of DNA. Another safer dye for DNA is the asymmetrical cyanine SYBR Green I [80]. It is more expensive, but 25 times more sensitive than EtBr. Another dye is GelGreen. It is structurally related to Acridinium orange and consists of two acridinium subunits that are bridged by a linear spacer [81].

Visualization can also be achieved by transferring DNA to a nitrocellulose membrane followed by exposure to a hybridization probe. This process is termed "Southern blotting" (s. there).

Gel electrophoresis research often takes advantage of software-based image analysis tools.

3.1.7 Protocols

3.1.7.1 Preparation of high-molecular-mass DNA sample and size markers

Materials
Sample to be prepared (Table 3.1-3)
1 g/dl agarose
Lysis buffer
Storage buffer
400 mmol/l **p**henyl**m**ethyl**s**ulfonyl **f**luoride (PMSF) in ethanol
10 mmol/l TRIS-HCl, pH = 8.0
Restriction enzyme and buffer
Block molds or petri plates

Table 3.1-3: Preparation of high-molecular-mass DNA samples and size markers.

Starting material	Preparation
Bacteria and phage particles	Resuspend bacteria or phage particles at a concentration calculated to yield desired amount of DNA per lane; e.g., 5×10^8 *E. coli* per ml will yield ~100 ng DNA in a lane.
Tissue culture cells	Wash cells several times in a medium containing no serum, as serum may inhibit proteinase *K*.
Yeast	Remove the cell walls from *Saccharomyces cerevisiae* before starting agarose electrophoresis.
Nuclei	Isolate DNA from nuclei. Approximately 1 µg DNA is contained in 10^5 mammalian nuclei.
Lambda ladders for size markers	Start with a concentrated stock of phage λ particles. Try several dilutions of phage stock to see which works best. The second incubation in lysis buffer should be done at 25 °C rather than 37 °C; the cohesive ends of the molecules will anneal, giving multimers of varying lengths. Run electrophoresis at maximum 25 °C.

- Prepare block molds by sealing one end with tape.
- Suspend sample at room temperature in water or buffer or medium at twice the desired concentration.
- Add an equal volume of 1 g/dl agarose, melted and cooled to 50 °C.
- Mix and aliquot into block mold. Let solidify on ice.
- Remove tape from mold and carefully push hardened blocks into a 50 ml conical tube containing 20 vol lysis buffer.
- Incubate overnight at 50 °C with gentle agitation.
- Pour off lysis buffer, add fresh lysis buffer, and incubate overnight at 37 °C.
- Pour off lysis buffer, add 20 vol storage buffer, and store at 4 °C.
- To digest samples with restriction enzymes, wash three times, 1 h each at room temperature, in > 10 vol storage buffer containing 1 mmol/l PMSF (added immediately before use).
  PMSF should be handled in a fume hood.
- Wash sample three times, 30 min each at room temperature, in 10 vol of 10 mmol/l TRIS-HCl, pH = 8.0.
- Place in a 1.5 ml microcentrifuge tube and remove excess liquid.
- Add an amount of 3x restriction buffer (containing the restriction enzyme) equal to half the volume of the block.
- Incubate at appropriate temperature.

3.1.7.2 Agarose gel electrophoresis of nucleic acids

DNA fragments of 0.2 to 30 kb are well resolved using this method.

Materials and equipment

TRIS-**a**cetate-**E**DTA (TAE) buffer or **T**RIS-**b**orate-**E**DTA (TBE) buffer

Electrophoresis-grade agarose
DNA sample
DNA molecular mass markers
Ethidium **br**omide (EtBr)
Front dye (0.25 g/dl Bromophenol blue or 0.25 g/dl Xylene cyanol)
55 °C water bath
Gel casting tray
Comb
Gel box
Horizontal gel electrophoresis apparatus
DC power supply

TAE buffer, 10x, pH = 8.0

TRIS	48.4 g
Acetic acid	11.4 ml
0.5 mol/l EDTA	20.0 ml
Deionized water to	1,000.0 ml

Procedure

Preparing the gel

– Add electrophoresis-grade agarose to TAE or TBE buffer (Table 3.1-4) into a 500 ml flask.
The concentration of agarose in a gel will depend on the sizes of DNA fragments to be separated (ranging between 0.5 and 2 g/dl).

Table 3.1-4: Agarose concentrations for separating DNA fragments of various sizes.

Agarose (g/dl)	Effective resolution of linear DNA fragments (kb)
0.5	1–30
0.7	0.8–12
1.0	0.5–10
1.2	0.4–7
1.5	0.2–3

– Melt agarose in a microwave or hot-water bath until the solution becomes clear.
– Cool to 55 °C in water bath.
– Add ethidium bromide to a concentration of 0.5 µg/ml.
Ethidium bromide is a suspected carcinogen. Gloves should always be worn when handling gels containing EtBr.
– Pour the agarose solution into the gel casting tray.
– Insert the comb into the agarose solution.

- Allow the gel to harden at room temperature.
- Remove the comb to obtain sample wells and place the gel into the gel box.

Loading the sample

- Add TAE buffer so that 1–2 mm of the buffer covers the gel, making sure that no air is trapped in the wells.
- Add loading dye to the DNA samples to be separated (0.25 g/dl Bromphenol blue, or 0.25 g/dl Xylene cyanol).

  Loading dye helps to track how far the DNA fragment have traveled. Bromophenol blue co-migrates with 0.5 kb DNA fragments, while Xylene cyanol co-migrates with 5 kb DNA fragments.
- Pipette with a micropipettor 10–20 µl of each DNA sample mixture and DNA ladder standards into the gel wells.

Running the electrophoresis

DNA will move to the positive electrode (red) and away from the negative electrode (black).

- Remove the lid of the electrophoresis apparatus.
- Place the gel box into the electrophoresis apparatus. The cathode (black lead) should be closer the wells than the anode (red lead).
- Place the lid onto the gel box.
- Attach the leads of the gel box to the power supply.
- Switch on the power supply to about 100 V – it should not exceed 5 V/cm between electrodes.
- Let the electrophoresis run until the front dye approaches the end of the gel.
- Turn off the power supply when Bromophenol blue or Xylene cyanol has migrated sufficient distance.
- Remove the lid of the electrophoresis chamber.
- Remove the tray and gel.

Staining the DNA bands

- Expose the gel to UV light. DNA bands should show up as orange fluorescent bands.
- If necessary, photograph DNA by placing the gel on a UV light source. Allow the gel to dehydrate and reserve it.

3.1.7.3 Isolation of DNA fragments using low-melting agarose gels

Fragments > 1,000 bp can be separated on low-melting agarose gels and purified by phenol extraction. Typical yield is 70%.

Materials and equipment
Low-melting agarose
TE (**T**RIS-**E**DTA) buffer, pH = 8.0
Buffered phenol

Procedure
- Digest DNA in a sample and run electrophoresis on a 1 g/dl low-melting agarose gel.
- Excise target band with a scalpel and melt at 65 °C. Add TE buffer, pH = 8.0, to decrease agarose concentration to < 0.4 g/dl.
- Add an equal volume of buffered phenol, mix 5 to 10 min, and centrifuge at 15,800 g and room temperature for 10 min. Set aside aqueous phase and reextract phenol phase and interface with an equal volume of TE buffer, pH = 8.0. Repeat, if a large interface still appears.
- Combine aqueous phases and ethanol precipitate.
- Resuspend in appropriate buffer.
- Add 10 to 20 vol Elutip low-salt solution and proceed with purification, maintaining all solutions at 37 °C.
- Precipitate with ethanol and quantitate.

3.1.7.4 Field-inversion gel electrophoresis

Field-inversion gel electrophoresis is used for resolving DNA nucleates of 10 to above 2000 kb. To avoid DNA shearing, use wide-bore pipette tips and do not vortex.

Materials and equipment
Agarose
TBE (**T**RIS-**b**orate-**E**DTA) buffer
DNA samples in an agarose gel or a solution
Peristaltic pump
Programmable switching device
DC power supply

Procedure
- Prepare a 1 g/dl agarose gel for a horizontal gel apparatus using TBE buffer. To reduce heat, make the gel as thick as necessary for the samples. Ethidium bromide is recommended for fragments smaller than 100 kb.
- Insert the samples that have been prepared in agarose blocks into wells. Place the gel into the box, cover it with 2 to 3 mm buffer, and apply samples.
- Adjust the peristaltic pump to 5 to 10 ml/min for a minigel or 20 to 50 ml/min for a large gel. Place the gel into the buffer tank.

- Connect a programmable switching device to constant voltage DC power supply and to the apparatus.
- Set the switching device for an appropriate switching regime. Most commonly, the ratio of forward to reverse time is 3:1.
- Start power, allow Bromophenol blue to migrate 1 cm, and switch the device and peristaltic pump.
- After the electrophoresis run, stain and photograph the gel. The gel may be Southern blotted.

3.1.7.5 Electroelution of nucleic acids from agarose gels

The elution of DNA is effective when DNA ranges from 50 to 20,000 bp and is in large mass (more than 500 ng). It yields 80–90% from DNA fragments more than 1 kb, and 50–60% from smaller DNA fragments.

Materials and equipment

TRIS-**a**cetate-**E**DTA (TAE) buffer, pH = 8.3
High-salt and low-salt solutions
2.5 mol/l NaCl
Dialysis membrane tubing (11.5 mm diameter, M_r = 3,500)
Column

TRIS-EDTA (TE) buffer, pH = 8.0

TRIS	0.12 g (10 mmol/l)
EDTA	0.03 g (1 mmol/l)
Deionized water	80.00 ml
Bring with HCl to pH = 8.0.	
Deionized water to	100.00 ml

Procedure

- Digest 0.1 to 25 µg DNA, run electrophoresis in a preparative agarose gel, stain, and photograph.
- Rinse dialysis tubing with TAE buffer for 5 min.
- Excise the target DNA band with a scalpel, slide it into the tubing, fill with TAE buffer and seal.
- Place the tubing in a horizontal electrophoresis cell, fill with TAE buffer until dialysis bag is covered, and set constant voltage of 2 V/cm between the electrodes.
- Electroelute 30 to 45 min for 50–500 bp fragments, 2 h for 500–2,000 bp fragments, and 4 h for 2,000–4,000 bp fragments. Larger DNA fragments electroelute overnight at 1 V/cm.
- Reverse the polarity of electrodes and run electrophoresis at 100 V for 30 s to free the rest of DNA.

- Open the tubing and collect the eluted DNA.
- Wash the bag with TAE buffer, and add to the eluted DNA.
- Place 2 ml of high-salt solution in a syringe, attach to a column, and push solution through the column. Repeat with a 5 ml of low-salt solution.
- Load DNA in 0.2 mol/l NaCl and bring onto a column at 20 drops/min. Wash the column with 5 ml of low-salt solution at 40 drops/min.
- Elute DNA with 400 µl of high-salt solution at 20 drops/min. Repeat to increase yield.
- Add 2.5 ml of ethanol and precipitate at –20 °C.
- Pellet DNA, wash in 70 m/dl ethanol, and dry.
- Resuspend in TE buffer with pH = 8.0, and quantitate.

3.1.8 Troubleshooting

Problem	Cause	Solution
Prior to electrophoresis		
Air bubbles in the agarose gel.	The support film or glass plate that came into contact with the agarose solution was dirty.	Do not touch the hydrophilic side of the support film or the glass plate with fingers. Wash the glass plate with dishwashing detergent before use.
	The agarose solution or glass plates were cold – the agarose solution gelled during casting.	Preheat the agarose solution and the glass plate at 70 °C.
The consistency of the agarose gel is insufficient.	The agarose concentration was low.	Check the recipe. Keep agarose dry and out of a refrigerator because it is hygroscopic.
The agarose gel separates from the support film.	An incorrect support film was used. The gel was cast onto the wrong side of the support film.	Do not exchange support films for agarose and polyacrylamide gels. Cast gel only onto the hydrophilic side of the support film. Check the film side before casting with water drops.
During electrophoresis		
It does not flow or flows little electric current.	One of the connectors has no or poor contact.	Check all connections.
Bands broaden or disappear at top of the gel.	The temperature is too high.	Decrease temperature or voltage.

(continued)

Problem	Cause	Solution
Less DNA than expected enters the gel matrix.	Incomplete lysis of microbial cells.	Check the pH value of buffers used.
Smeared bands.	Prolonged nuclease activity.	Keep the samples in 0.5 mol/l EDTA at 4 °C. Handle the samples using sterile instruments and reagents; do not use metal spatulas, as their ions help at cleaving DNA.
	Incorrect temperature.	Monitor temperature during electrophoresis.
	An inappropriate electric field.	Add buffer, if necessary.
The samples do not run straight.	Broken electrodes.	Repair electrodes.
After electrophoresis		
Faint or no bands on the gel.	Insufficient quantity of DNA on the gel.	Increase the amount of DNA; don't exceed 50 ng/band.
	DNA was degraded.	Avoid nuclease contamination.
	Improper light source was used for visualization of ethidium bromide-stained DNA.	Use a short wavelength (254 nm) light.
	The anode and cathode were reversed.	Check the connections with the power supply.
	The nucleates have left the gel.	Monitor the moving of dye front.
The DNA separation is bad.	Wrong agarose concentration.	Higher agarose concentration will help to resolve smaller DNA molecules, while lower agarose concentration will help to resolve larger DNA molecules.
The bands are fuzzy.	Incorrect buffer solutions.	Check the pH in the gel and electrode buffers.
	Excess salt in the samples.	Check the salt concentrations in the gel and electrode buffers.

(continued)

Problem	Cause	Solution
DNA bands are smeared.	DNA was degraded.	Avoid nuclease contamination
	Too much DNA was loaded on the gel.	Decrease the DNA concentration.
	Too much salt in the DNA sample.	Remove excess salts using ethanol precipitation.
	Small DNA nucleates diffused during staining.	Add ethidium bromide during electrophoresis.
The nucleate bands build tails.	The concentration of nucleic acids in the sample was too high.	Apply small sample volumes or dilute the sample.
	Diffusion after the separation.	Shorten the time between the end of electrophoresis and detection procedure.
The samples from the adjacent tracks ran into each other.	The sample volumes were too much.	Concentrate the samples and apply smaller volumes.
	The application template was not tight on the gel.	Press the application template lightly on the gel to push away air bubbles between the template and gel.
The DNA fragments are arranged in a curved manner – "smiling" effect.	The temperature in the central part of the gel was higher than in the gel margins.	Reduce the voltage. Apply sample at some distance from the gel margins.

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3.2 Native polyacrylamide gel electrophoresis of nucleic acids

If small double-stranded DNA fragments are to be resolved on agarose gels, 2 g/dl agarose gels should be cast. However, because of their high concentration, these agarose gels are opalescent and as a result the sensitivity of the ethidium bromide proving is reduced. In addition, the zone electrophoresis on agarose gels is running several hours. Therefore, low-molecular mass DNA fragments are separated in native polyacrylamide gels from where they can be easily extracted.

The native polyacrylamide gel electrophoresis is used for separation of double-stranded DNA with length of 15–300 bp fragments, for example PCR fragments, and single-stranded DNA with the same length. It takes place in continuous or discontinuous buffer systems [1,2]. The electrophoresis should be run at pH range of 6.0 to 10.0. If the pH value is too low, the bases cytosine and adenine protonate and become positively charged; if the pH value is too high, the bases thymine and guanine deprotonate and become negatively charged. In both cases, the additional electric charges cause denaturation of nucleic acids and change their mobility.

3.2.1 Zone polyacrylamide gel electrophoresis of native nucleic acids

The zone polyacrylamide gel electrophoresis is the simplest form of polyacrylamide gel electrophoresis of nucleic acids.

3.2.1.1 Theory of sieving migration

According to Ogston [3], the electrophoretic migration of nucleic acids through a gel can be compared with the migration of aimlessly particles through a sieve (Ogston sieving). The more the radius of nucleate gyration approximates the size of a gel pore, the more likely is that the nucleate will pass through the pore. The smaller particles behave themselves as if no gel is available, so they cannot be separated in this way.

Double-stranded DNA fragments behave as long rods, so their migration through a gel is relative to their size or, for cyclic DNA fragments, their radius of gyration. However, cyclic DNA such as plasmids may show multiple bands, because the speed of migration may depend on whether DNA fragment is relaxed or supercoiled. Single-stranded DNA or RNA tends to fold up into molecules with complex shapes and migrate through the gel in a complicated manner based on their tertiary structure. Therefore, agents such as sodium hydroxide or formamide that disrupt the hydrogen bonds are used to denature the nucleic acids and cause them to behave as long rods.

3.2.1.2 Practice of zone polyacrylamide gel electrophoresis of native nucleic acids

The native polyacrylamide gel electrophoresis of nucleic acids is a zone electrophoresis carried out in a buffer. It is characterized by continues strength of the electric field in the gel during the electrophoresis.

With the help of the native zone electrophoresis on polyacrylamide gel, DNA nucleates, oligonucleotides, RNA nucleates, and viroids are resolved according to their molecular masses. DNA fragments may be separated, if they differ from each other with 6 bp in a length of 500 to 1,200 bp (M_r up to 800,000).

The native polyacrylamide gel electrophoresis gives good resolution of short DNA fragments, such as 20 to 60 bp double-stranded DNA [4]. RNA and single-stranded DNA, as well as RNA, are better to be run and visualized on polyacrylamide gels containing denaturing agents such as urea.

Preparation of polyacrylamide gels. Polyacrylamide gels for zone electrophoresis of nucleic acids are cast in the same way as any other polyacrylamide gels: A support film is placed, with its hydrophobic side down, onto a glass plate covered with drops of water, and rolled over by a roller. Then it is framed with a U-shaped spacer and covered with another glass plate. The resulting casting cassette is clamped together and placed upright. A buffer, for example TRIS-borate-EDTA buffer, is mixed with a monomer solution and APS. The mixture is cast into the cassette and covered with some deionized water to prevent air oxygen to diffuse into the mixture.

If the monomer concentration T is 50 g/dl, polyacrylamide gels for zone electrophoresis can be prepared as follows (Table 3.2-1):

After one-hour polymerization at room temperature, the polyacrylamide gel can be removed from the cassette. To do this, the cassette is opened and the cover glass

Table 3.2-1: Producing polyacrylamide gels.

Reagents	Total monomer concentration *T*, g/dl				
	3.5	**5.0**	**8.0**	**12.0**	**20.0**
TRIS-borate-EDTA buffer, 10x	10.00 ml	10.00 ml	10.00 ml	10.00 ml	10.00 ml
Monomer solution (T = 50 g/dl, C = 0.03)	7.00 ml	10.00 ml	16.00 ml	24.00 ml	40.00 ml
10 g/dl TMEDA	0.46 ml	0.46 ml	0.46 ml	0.46 ml	0.46 ml
10 g/dl APS	0.97 ml	0.68 ml	0.43 ml	0.38 ml	0.17 ml
Deionized water to	100.00 ml	100.00 ml	100.00 ml	100.00 ml	100.00 ml

plate is raised using a spatula. Thereafter, the spacer is removed and the gel is covered with a hydrophobic polyester film, or packed in a wrap.

Electrophoretic separation conditions. Prior to electrophoresis, the thermostat is set to 10 °C and the cooling plate of a separation unit is coated with 0.5–1 ml of kerosene or silicone oil DC-200 as a contact fluid. The gel, with its support film down, is laid down on the colding plate, and its cover film is rolled over gently to remove air bubbles between the cooling plate and the support film.

The cover film is removed from the gel, and paper electrode strips impregnated with the electrode buffer are placed onto the cathode and anode ends of the polyacrylamide gel. Before the cathode strip, at a distance of 1 cm, an application template is placed down and lightly pressed against the gel. Then 0.1 mg/ml DNA containing 0.025 g/dl of Xylene cyanol or Bromophenol blue Na salt is applied in the template slots.

The electrophoresis is performed at a constant electric current of 50 mA, limiting voltage of 500 V, and limiting power of 25 W. It is terminated after the dye front has run at least two-thirds of the gel.

The relationship between the polyacrylamide concentration and the mass of nucleic acids to be resolved can be seen in Table 3.2-2. It shows that there are differences between the behavior of native and denatured DNA nucleates during the electrophoresis.

As a rule, the native double-stranded DNA nucleates are moving faster than the denatured (single-stranded) DNA with same masses. Moreover, the smaller DNA fragments move together with Bromophenol blue, while the larger DNA fragments follow xylene cyanol [5]. This can be explained by the structures of Xylene cyanol and Bromophenol blue (Figure 3.2-1): The molecular mass of Xylene cyanol Na salt (M_r = 538.61) represents approximately three-fourth of the molecular mass of Bromophenol blue Na salt (M_r = 691.94), but Xylene cyanol includes four additional methyl groups, which diminishes its hydrophilicity. Therefore, Xylene cyanol moves slower than Bromophenol blue.

Table 3.2-2: Polyacrylamide concentrations used for different sizes (lengths) of DNA nucleates.

Polyacrylamide concentration *T*, in g/dl	Double-stranded DNA, in bp, moving with Xylene cyanol	Double-stranded DNA, in bp, moving with Bromophenol blue	Polyacrylamide concentration *T*, in g/dl	Single-stranded DNA, in b, moving with Xylene cyanol	Single-stranded DNA, in b, moving with Bromophenol blue
3.5	460	100	5.0	130	35
5.0	260	65	6.0	106	26
8.0	160	45	8.0	76	19
12.0	70	20	10.0	55	12
15.0	60	15	20.0	28	8
20.0	45	12			

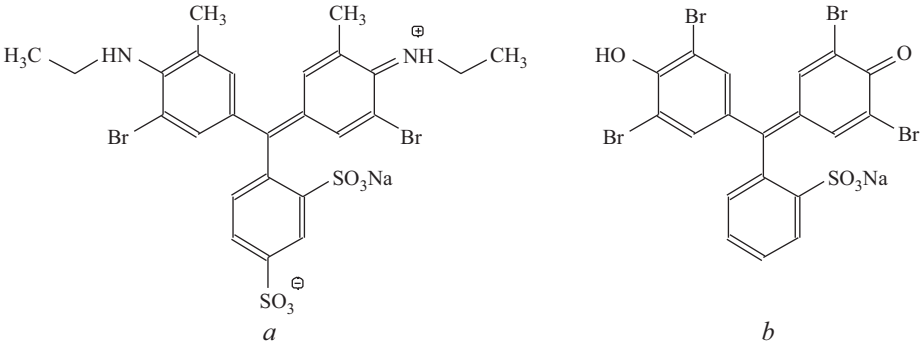

Figure 3.2-1: Chemical structures of the sodium salts of Xylene cyanol (*a*) and Bromophenol blue (*b*).

After run, the nucleic acid bands can be stained with ethidium bromide. If the DNA concentration is too low, a silver staining can be applied. If this is insufficient, good results can be received by radioactive labeling.

Zone polyacrylamide gel electrophoresis of native oligonucleotides

The oligonucleotide synthesis [6,7] has reached a purity of over 99%, so that additional cleaning is not necessary in most cases. A complete cleaning of oligonucleotide solutions is possible only with the help of a polyacrylamide gel electrophoresis. Applying it, not only low-molecular mass but also high-molecular mass substances can be removed.

The polyacrylamide gel electrophoresis of oligonucleotides is carried out in a polyacrylamide gel of T = 20 g/dl and C = 0.05. After electrophoresis, the gel with the nucleic acid bands is treated with ethidium bromide. Then it is placed on a thin-layer chromatography plate with fluorescent indicator and illuminated with UV light from above. The DNA bands absorb the light and throw "shadows" on the bright fluorescent background. Another possibility is the DNA bands to be stained with silver.

3.2.2 Disc-electrophoresis of native nucleic acids

The disc-electrophoresis is run in a polyacrylamide gel. It presents a combination between an isotachophoresis, which is carried out in a stacking and a resolving gel. The nucleic acids are concentrated in the stacking gel according to the principle of isotachophoresis, and are then separated from each other in the resolving gel according to the principle of zone electrophoresis.

The native disc-electrophoresis is used for separation of short DNA segments such as 20 to 60 bp double-stranded DNA [8], DNA [9,10], and RNA [11]. It gives sharper bands than the continuous electrophoresis [12] in only a TRIS-borate-EDTA buffer [13,14]. Same gels are widely used in DNA foot printing, **e**lectrophoretic **m**obility **s**hift **a**ssay (EMSA), and other DNA-protein interaction techniques. However, the native disc-electrophoresis requires more complicated preparation than the zone electrophoresis.

3.2.2.1 Theory of disc-electrophoresis of native nucleic acids

DNA has negative charge on its phosphate backbone, therefore it moves during electrophoresis toward the positively charged anode. The migration of DNA nucleates in solution, in the absence of a gel, is independent of molecular mass during electrophoresis, that is, no separation by size happens [15,16]. During a native disc-electrophoresis in a gel, however, nucleic acids migrate as long as their mobilities are higher than the mobility of the trailing ion and are concentrated in the ionic boundary between the leading and the trailing ion. In the resolving gel, however, the mobilities of the nucleate polyions are getting slower and they are overtaken by the ionic border. Thereafter, the electrophoresis runs as a zone electrophoresis. If the concentration of the leading ion is low, nucleates separate faster from each other; however, the resolution is poor. When the concentration of the leading ion is higher, nucleates run slower but the resolution of electrophoresis is higher [17].

The gel matrix is, therefore, responsible for the separation of DNA by size during electrophoresis, although the precise mechanism of separation is not entirely clear. The widely accepted explanation is the Ogston model, which treats the polymer matrix as a sieve consisting of randomly distributed network of interconnected pores [18]: A random coil DNA moves through the connected pores large enough to accommodate its passage, but the movement of larger molecules is impeded and slowed down, and so nucleates of different sizes can be separated.

The Ogston model however breaks down for large molecules moving through pores significantly smaller than their size. For DNA molecules of size greater than 1 kb, a reptation model is most commonly used. This model assumes that DNA can crawl in a snakelike fashion (reptation) through the pores as an elongated molecule. At higher electric field strength, this turns into a biased reptation model, whereby the leading end of the molecule becomes strongly biased in the forward direction and pulls the rest of the molecule along. In the fully biased mode, the mobility reaches a saturation point and DNA beyond a certain size cannot be separated.

Real-time fluorescence microscopy of stained molecules showed more subtle dynamics during electrophoresis, with DNA showing considerable elasticity as it is alternately stretching in the direction of the applied field and then contracting into a ball, or becoming hooked into a U-shape when it gets caught on the polymer fibers

[19,20]. This observation may be termed the "caterpillar" model [21]. Other model proposes that DNA gets entangled with the polymer matrix, and the larger the molecule, the more likely it is to become entangled and its movement impeded [22].

Factors affecting migration of nucleic acids

A number of factors affect the migration of nucleic acids: ionic strength of the buffer, gel concentration that defines the dimension of gel pores, electric field strength, and concentration of ethidium bromide, if used during electrophoresis [23].

Ionic strength of buffer

The mobility of nucleic acids decreases when the buffer ionic strength increases [24]. This can be explained with the concept of the geometric and electrokinetic radius [25]. When the ionic strength increases, the electrokinetic radius of a nucleate, a_{pi}, grows and the ζ-potential decreases. The reverse takes place when the ionic strength decreases – then the electrokinetic radius decreases and the ζ-potential increases.

Gel concentration

The gel concentration determines the size of the gel pores, which in turn affects the migration of DNA. Increasing gel concentration reduces the migration speed and improves the separation of smaller DNA nucleates, while lowering gel concentration permits large DNA nucleates to be separated.

Double-stranded DNA nucleates move at a rate that is approximately inversely proportional to the logarithm of the number of base pairs. This relationship however breaks down with very large DNA fragments. The limit of resolution depends on gel composition, and the mobility of larger circular DNA may be more strongly affected by the pore size of the gel than linear DNA [26].

Electric field strength

In increasing electric field strength, the mobility of high-molecular-mass DNA fragments increases and the effective range of separation decreases. For optimal resolution of DNA > 2 kb, 5 to 8 V/cm of gel is recommended. Voltage is limited by the fact that it heats the gel and may cause DNA to melt.

Concentration of ethidium bromide

If ethidium bromide is present in the gel during electrophoresis, circular DNA is more strongly affected by ethidium bromide concentration than linear DNA. Ethidium bromide, which intercalates into circular DNA, can change the charge, length, as well as the superhelicity of the DNA molecule. Increasing ethidium bromide concentration

can change DNA from a negatively supercoiled through a fully relaxed form to positively coiled superhelix [27].

3.2.2.2 Running the electrophoresis of native nucleic acids

The nucleic acid to be separated can be prepared in several ways before running the electrophoresis. In the case of large DNA molecules, DNA is cut into smaller fragments using a DNA restriction endonuclease. In PCR amplified samples, the enzymes, which are present in the sample, are to be removed before analysis. Once the nucleic acid is properly prepared, the samples are applied into the wells of the gel or into slots and a voltage is applied across the gel for a given time.

The DNA fragments of different lengths are visualized using a fluorescent dye specific for DNA, such as ethidium bromide. Under UV light, such gels show bands corresponding to nucleic acids with different molecular masses.

After the electrophoresis, DNA nucleates can be eluted. Their bands can be cut out with a scalpel or razor blade from the gel and transferred into a centrifuge tube containing 200 µl of sterile deionized water. After several hours or overnight extraction at 80 °C, the suspension is centrifuged for 5 min in a microcentrifuge at high rotation. The supernatant is filled with sterile deionized water to 300 µl, and diluted with 30 µl of 3 mol/l sodium acetate with pH = 4.8, and 750 µl of ethanol. After 30 min at −70 °C, the precipitated DNA is centrifuged and washed with 1 ml of 70 ml/dl ethanol. Then DNA is dried briefly in vacuum and dissolved in a small volume of sterile TRIS-borate-EDTA or TRIS-taurinate-EDTA buffer. The measurement and analysis are done with specialized gel analysis software.

3.2.3 Disc-electrophoresis of double-stranded PCR products

Nucleates of greater length cannot be separated by native polyacrylamide gel electrophoresis. Therefore, they must be broken down by restriction endonucleases, which can be multiply in the **p**olymerase **c**hain **r**eaction (PCR) [28]. The products of the polymerase chain reaction can be later separated in horizontal polyacrylamide gels using, for example, disc-electrophoresis in a TRIS-formate-borate buffer system [29], or TRIS-formate-taurinate buffer system [30].

The TRIS-formate-taurinate buffer system consists of a TRIS-formate buffer as leading buffer (the anode buffer), and a TRIS-taurinate buffer as a trailing buffer (the cathode buffer). The TRIS-formate buffer is contained in the polyacrylamide gel and the TRIS-taurinate buffer is contained in the anode strip. During the electrophoresis, a moving ionic boundary is formed between the formate ion in the gel and the taurinate ion in the cathode strip, which concentrates the PCR products and overtakes them later (Figure 3.2-2).

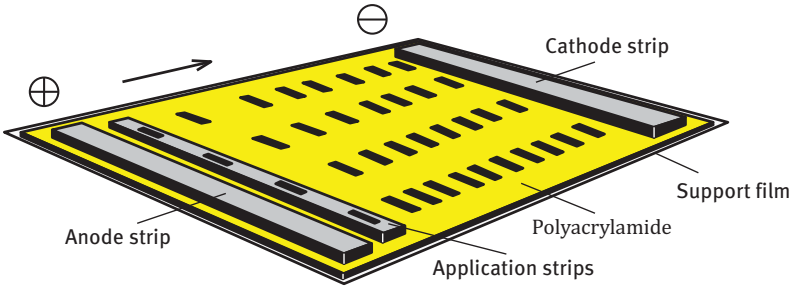

Figure 3.2-2: Disc-electrophoresis of PCR products.

Table 3.2-3: Recipes for a TRIS-formate-taurinate buffer system for disc-electrophoresis of nucleic acids.

	Anode buffer, pH = 7.4	**Cathode buffer pH = 8.3**	**Monomer solution *T* = 50 g/dl, *C* = 0.03**
TRIS	16.32 g	2.23 g	–
99 ml/dl Formic acid	4.57 ml	–	–
Taurine	–	6.02 g	–
Acrylamide	–	–	48.50 g
BIS	–	–	1.50 g
87% Glycerol	10.00 ml	10.00 ml	–
$Na_2EDTA \cdot 2H_2O$	0.45 g	0.22 g	–
NaN_3	0.06 g	0.03 g	–
Deionized water to	100.00 ml	100.00 ml	100.00 ml

The recipes for the TRIS-formate-taurinate buffer system and the preparation of polyacrylamide gels for disc-electrophoresis of nucleic acids are presented in Table 3.2-3 and Table 3.2-4, and some results are shown in Figure 3.2-3.

After the electrophoresis, the PCR products can be detected by various methods. The most common of them is the silver staining [31], which is more sensitive than the ethidium bromide method [32]. The PCR products can also be labeled with fluorescent substances [33,34]. Labeled PCR products can be visualized by the chemiluminescence digoxigenin system [35].

Purification of PCR using buffer dilution series are given on Figure 3.2-4 [36]. More diluted buffer cuts off more and more of the low-molecular mass bands.

A few research groups have made progress toward constructing gel electrophoresis systems [37]. As early as 1972, Maurer and Dati investigated the design and operation of a microslab gel electrophoresis apparatus for separation of human serum proteins [38]. In their system, a vertical polyacrylamide gel was cast between standard glass microscope slides with injection wells formed using

Table 3.2-4: Recipes for polyacrylamide gels containing TRIS-formate-taurinate buffer system for disc-electrophoresis of nucleic acids.

	T = 5 g/dl pH = 7.4	*T* = 7 g/dl pH = 7.4	*T* = 9 g/dl pH = 7.4	*T* = 11 g/dl pH = 7.4	*T* = 13 g/dl pH = 7.4
Anode buffer, ml	16.67	16.67	16.67	16.67	16.67
Monomer solution, ml	10.00	14.00	18.00	22.00	26.00
87% glycerol, ml	20.00	20.00	20.00	20.00	20.00
10 g/dl TMEDA, ml	0.46	0.46	0.46	0.46	0.46
10 g/dl APS, ml	0.68	0.49	0.38	0.31	0.26
Deionized water to, ml	100.00	100.00	100.00	100.00	100.00

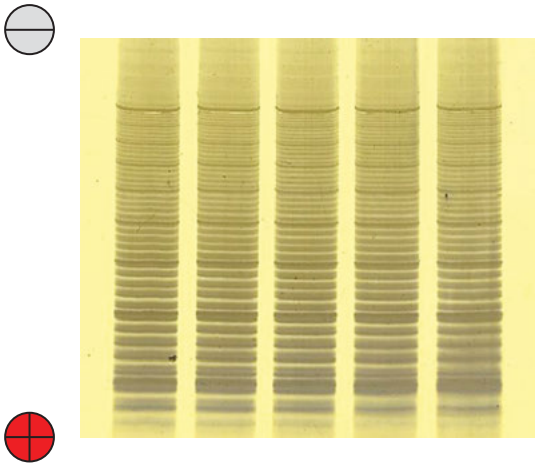

Figure 3.2-3: Disc-electrophoresis of 20 to 100 bp DNA fragments in a *T7*-polyacrylamide gel. Silver staining.

a specially constructed Teflon comb. A 1–5 µl sample was loaded into each lane, and electrophoresis was performed at 60–100 V (3 mA) for 100 min at room temperature. After the separation was completed, the gel was carefully removed from the casting mold and poststained to allow visualization of the separated bands.

In the late 1990s, Guttman and coworkers revisited the use of a microslab gel format in a series of applications involving both protein and DNA [39,40,41] analysis. Their separation platform consisted of a glass gel cartridge incorporating 15 ml plastic buffer tank at both ends. A forced air cooling system was also included in order to counteract the effects of Joule heating during the separation run. A gel matrix was cast inside the cartridge with **T**RIS-**b**orate-**E**DTA (TBE) buffer containing 50 nmol/l of ethidium bromide to enable visualization of the separated bands. A membrane-mediated

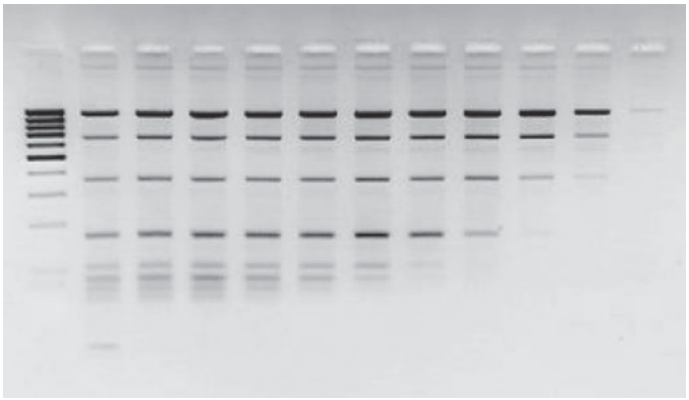

Figure 3.2-4: Bands of PCR fragments using buffer dilutions.
Lane 1: 100 bp DNA ladder; *Lane 2*: DNA ladder (21 base primer, 50, 65, 79, 100, 164, 359, 645, and 982 bp fragment) amplified by DNA polymerase; *Lane 3*: Dilution with buffer; *Lanes 4–12*: Dilution with 1–9 volumes of deionized water

sample loading technique was used in order to avoid problems associated with the traditional process of using a comb to define injection wells. This technique also allowed automated gel loading to be performed by robotically spotting 0.2–0.5 µl samples using a 32- or 96-tab membrane loader [42]. Electrophoresis was performed at 750 V and 25 °C for 25 min. A significant advance was the incorporation of real-time detection of the migrating bands using a scanning LIF/avalanche photodiode apparatus. Subsequent work resulted in development of a simplified system employing a microscope slide-based platform coupled with a conventional microarray scanner for fluorescence detection that allowed coseparation of multiple-dye-labeled samples [43].

Another construction of a simple gel electrophoresis apparatus for size-selective separations of DNA fragments in both polyacrylamide and agarose gels is described [44]. It employs a microslab gel format with a novel gel casting technique that eliminates the need for delicate combs to define sample loading wells. The microslab gel format allows rapid separations to be performed at low voltages using microliter sample volumes. Real-time fluorescence detection of the migrating DNA fragments is accomplished using a digital microscope that connects to any PC with a USB interface. The microscope is adaptable for this application by replacing its white light source with a blue **l**ight-**e**mitting **d**iode (LED) and using an appropriate emission filter.

3.2.4 Electrophoretic mobility shift assay

The **e**lectrophoretic **m**obility **s**hift **a**ssay (EMSA), referred also as mobility shift electrophoresis, band shift assay, gel mobility shift assay, or gel retardation assay, is

based on methods described by Garner and Revzin [45], and Fried and Crothers [46,47]. It is an affinity electrophoresis used to study protein-DNA or protein-RNA interactions. During this method, a protein or mixture of proteins is binding to a given DNA or RNA sequence.

EMSA is one of the most sensitive methods for studying the DNA-binding properties of a protein. It is used to deduce the binding parameters and relative affinities of a protein for one or more DNA sites or for comparing the affinities of different proteins for the same sites.

Proteins require specific physiological ionic strength and physiological pH value to recognize specific sequences of DNA. Besides, DNA must be native and double-stranded to interact with them. Therefore, electrophoretic mobility shift assay is carried out in a buffer.

EMSA is often performed simultaneously with DNase footprinting, primer extension, and promoter-probe experiments when studying transcription initiation, DNA replication, DNA repair, or RNA processing and maturation. It is carried out in 15 to 20 cm polyacrylamide or agarose gels for 1.5–2 h [48]. The control DNA probe (without protein) corresponds to unbound DNA or RNA.

If a double-stranded DNA fragment contains a detection sequence for some protein, for example for a transcription factor, the protein is bound to this DNA fragment during electrophoresis. The resulting DNA-protein complexes migrate slower through the gel and, as a result, displace from the DNA band – *band shift* (Figure 3.2-5). When the DNA fragment binds more than one protein polyion, the mobility of the complex reduces further.

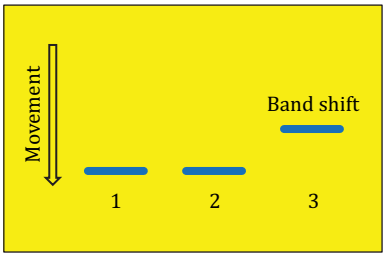

Figure 3.2-5: Scheme of the electrophoretic mobility shift assay.
Lane 1 contains only genetic material. *Lane 2* contains protein and DNA fragments, which do not interact with each other. *Lane 3* contains protein and DNA fragments that have reacted with each other to build a slower-moving complex.

To visualize the reaction, the nucleic acid fragments in the gel are usually labeled radioactively, fluorescently, or bound to biotin. While the isotopically ^{32}P-labeled DNA has less or no effect at the protein binding affinity, the non-isotopic labels including fluorophores or biotin can alter the affinity and the stoichiometry of the protein-DNA interaction. When using a biotin label, streptavidin, conjugated to an enzyme, such as horseradish peroxidase, is used.

If the starting concentrations of the protein and its DNA probe, as well as the stoichiometry of the complex, are known, the apparent affinity between the protein

and nucleic acid sequence can be determined [49]. If the protein concentration is not known but the complex stoichiometry is known, the protein concentration can be determined by increasing the concentration of DNA probe until all the protein is bound. Then the number of protein molecules bound to the DNA fragment can be calculated.

3.2.4.1 Supershift assay

The electrophoretic mobility shift assay is also useful for studying higher-order complexes containing several proteins, referred to as *supershift assay* [50]. The supershift assay can be used to identify a protein present in a protein-nucleic acid complex.

The supershift assay is carried out using a third lane loaded with a radiolabeled nucleic acid, a protein mixture, and an antibody for a specific protein. If an extra retardation is observed, this is due to the formation of a larger complex including the antibody. By this approach, at least one protein of the complex can be directly identified.

3.2.5 Clinical application

Using the native disc-electrophoresis, recombination frequencies in a semen sample can be determined. For example, some loci have been investigated [51]: **r**estriction **f**ragment **l**ength **p**olymorphism (RFLP) in the **p**ara**t**hyroid **h**ormone (PTH) gene, and in the **Gγ**-globin gene (Gγ, HBG2), both on chromosome 11; and the **l**ow-**d**ensity **l**ipoprotein **r**eceptor (LDLr) on chromosome 19.

Electrophoretic mobility shift assays seem well suited to detect many types of genetic differences, including single-base substitutions and larger sequence alterations such as insertions, deletions, and inversions, and could be used for the efficient screening of samples for sequence alterations [52]. It was applied, for example, to study the MUC1 promoter in breast cancer cells [53]. The MUC1 gene encodes the DF3 protein, which represents a high-molecular mass glycoprotein. With the help of this method, it has been found where proteins bind to it. Membrane proteins can also be identified by the electrophoretic mobility shift assay using charged detergents.

3.2.6 Protocols

3.2.6.1 DNA electrophoresis on agarose gels

Materials and equipment

TRIS-**b**orate-**E**DTA (TBE) buffer, pH = 8.0
DNA samples

DNA markers
10 µg/ml ethidium bromide
Thin filter paper
Electrophoresis cell with gel bridge
DC power supply
Longwave UV lamp

Procedure

- Blot the upper surface of an agarose gel near the cathode with a thin filter paper.
- Lay an application template onto the gel near the cathode.
- Press the template carefully against the gel to remove the air bubbles.
- Pipette 5 µl each of the DNA samples and DNA markers into the slots of the application template and wait for 5 min to diffuse into the gel.
- Blot the remained sample volumes with a thin filter paper and remove it together with the application template.
- Hang the gel with the gel side below on the gel bridge.
- Full the tanks of the electrophoresis cell with TBE buffer and put the bridge with the gel into the electrophoresis cell to obtain direct contact between gel and electrode buffer.
- Close the electrophoresis cell with its lid and switch on the power supply.
- Run the electrophoresis at a constant current and room temperature according to the following electrophoresis program until the tracking dye reaches the anode.
- Soak the gel after the electrophoresis in 0.5 µg/ml ethidium bromide at room temperature for 45 min and dry it.
- Visualize the DNA bands under a UV lamp as shadows.

Gel size (mm)	Current (mA)	Voltage (V)
51 × 82	10	80
260 × 125	78	120

3.2.6.2 Separation of small DNA fragments by polyacrylamide gel zone electrophoresis

Materials and equipment

10x **T**RIS-**b**orate-**E**DTA (TBE) buffer, pH = 8.0
TRIS-**E**DTA (TE) buffer, pH = 7.5
Monomer solution (T = 30 g/dl, C = 0.03)

10 g/dl TMEDA
10 g/dl APS
5x loading buffer
DNA samples
DNA markers
10 µg/ml ethidium bromide
Electrophoresis cell
Glass plates, spacers, and combs
DC power supply
Longwave UV transilluminator

Gel solution, T = 5 g/dl

10x TBE buffer	6.00 ml
Monomer solution	10.00 ml
10 g/dl TMEDA	0.34 ml
10 g/dl APS	0.25 ml
Deionized water	44.00 ml

Procedure

- Place spacers between two glass plates to construct casting cassette.
- Prepare monomer solution with desired polyacrylamide concentration.
- Add TMEDA and APS to the monomer solution and cast the mixture into the cassette.
- Insert a comb into the casting solution, not allowing air bubbles to become trapped under its teeth.
- Let the solution polymerize for 60 min.
- Fill the lower tank of the electrophoresis tank with 1x TBE buffer.
- Place the gel into the lower tank.
- Clamp the plate to the top of the electrophoresis tank and fill the upper tank with 1x TBE so that the wells are covered.
- Remove the comb from the gel.
- Add buffer to DNA samples and molecular-mass markers and load them into the wells of the gel.
- Run electrophoresis at 5 V/cm.
- Turn off the power supply. Apart the gel plates so that the gel remains attached to one plate.
- Submerge gel and plate for 5 to 10 min in 0.5 µg/ml ethidium bromide.
- Wrap the gel and plate with plastic wrap, invert, place onto a UV transilluminator, and photograph.
- Cut out the desired DNA band with a scalpel or razor blade. Crush the gel fragment into fine pieces by pushing it through a disposable syringe.

- Collect pieces in a tube and add 2 vol of elution buffer. Incubate at room temperature with shaking for 4 h (for fragments <300 bp) to overnight (for fragments > 750 bp).
- Pellet gel fragments at room temperature for 10 min in a tabletop centrifuge. Transfer the supernatant to a clean tube.
- Rinse the gel pieces with elution buffer, recentrifuge, and pool the two supernatants.
- Precipitate DNA using 2 vol of ethanol. Pellet DNA by centrifuging at 12,000 g. Dissolve in TE buffer, pH = 7.5.

3.2.6.3 DNA electrophoresis in TRIS-taurinate buffer at 2 pH values according to Michov

Materials and equipment

TRIS
Taurine
Acrylamide
BIS
87% Glycerin
Bromo**p**henol **b**lue (BPB) Na salt
TMEDA
Ammonium **p**eroxydi**s**ulfate (APS)
Casting cassette

Buffer 1 (Anode buffer), pH = 8.6, I = 2•0.20 mol/l

TRIS	14.54 g (1.2 mol/l)
Taurine	13.01 g (1.04 mol/l)
Deionized water to	100.00 ml

Buffer 2 (Cathode buffer), pH = 7.6, I = 2•0.02 mol/l

TRIS	7.27 g (0.06 mol/l)
Taurine	13.01 g (0.10 mol/l)
Deionized water to	1,000.00 ml

Monomer solution, T = 50 g/dl, C = 0.03

Acrylamide	48.5 g
BIS	1.5 g
Deionized water to	100.0 ml

The solutions should be prepared according to Table 3.2-5.

Table 3.2-5: Preparing the stacking and resolving gels for DNA electrophoresis in TRIS-taurinate buffer at 2 pH values according to Michov.

	Stack. gel T = 6 g/dl,	**Resolv. gel T = 9 g/dl**	**Resolv. gel T = 11 g/dl**	**Resolv. gel T = 13 g/dl**	**Resolv. gel T = 15 g/dl**	**Resolv. gel T = 17 g/dl**
	pH = 7.6, I = 0.02 mol/l			**pH = 8.6, I = 0.20 mol/l**		
Buffer 1, ml	–	50.00	50.00	50.00	50.00	50.00
Buffer 2, ml	50.00	–	–	–	–	–
Monom. solution, ml	12.00	18.00	22.00	26.00	30.00	34.00
87% Glycerin, ml	30.00	10.00	10.00	10.00	10.00	10.00
10 ml/dl TMEDA, ml	0.45	0.45	0.45	0.45	0.45	0.45
10 g/dl APS, ml	0.36	0.20	0.16	0.14	0.12	0.11
Deion. water, ml, to	100.00	100.00	100.00	100.00	100.00	100.00

Procedure

- Pour the resolving solution into the casting cassette.
- Overlay the resolving solution with deionized water.
- After the resolving solution has gelled, pour out the deionized water and overlay it with the stacking solution.
- Allow the stacking solution to gel.
- Run the electrophoresis at 20 mA for 120 min.

Use paper electrode strips with anode and cathode buffer, respectively.

3.2.6.4 Separation of PCR products by polyacrylamide gel disc-electrophoresis

Materials and equipment

TRIS-formate buffer, pH = 8.3
TRIS-taurinate buffer, pH = 7.4
Monomer solution (T = 30 g/dl, C = 0.03)
10 g/dl TMEDA
10 g/dl APS
DNA samples
DNA markers
Gel electrophoresis cell
Glass plates, spacers, and combs
DC power supply
SYBR Green EMSA nucleic acid gel stain

Procedure

- Put 0.5–1.0 ml of contact fluid, for example, kerosene or silicone oil DC 200, on the cooling plate of an electrophoretic chamber.
- Place above a film-supported gel, together with the cover film.
- Roll gently on the cover film with a photo roller so that the contact fluid is evenly distributed between the cooling plate and support film.
- Remove the cover film and place onto the gel ends paper electrode strips according to their polarity.
- Lay an application template (preferable of silicone) in front of the cathode strip.
- Apply 5–8 µl of nucleic acid samples with a concentration of 10 mg/ml in the slots of the application template.
- Place the electrodes on the corresponding paper strips.
- Run the disc-electrophoresis at constant electric current and at a temperature of 10 °C until the dye front reaches the opposite strip (Table 3.2-6).
- Stain DNA bands with ethidium bromide or SYBR Green EMSA nucleic acid gel stain.

Table 3.2-6: Electrophoresis programs for disc-electrophoresis of nucleic acids in 0.5 mm polyacrylamide gels with TRIS-formate-taurinate buffer system.

Gel sizes (mm)	Electric current (mA)	Limiting voltage (V)	Limiting power (W)	Separation time (min)
82 × 57	20	300	6	~ 80
125 × 125	40	400	16	~ 120
125 × 260	80	400	32	~ 120

3.2.6.5 Electrophoretic mobility shift assay

Materials and equipment

TRIS-**b**orate-**E**DTA buffer (TBE)
10,000x Concentrate in dimethylsulfoxide
Tri**c**hloro**a**cetic acid (TCA)
7 ml/dl Acetic acid
10 ml/dl Methanol
DNA sample
DNA ladder
Orange G
SYBR Green EMSA nucleic acid gel stain
SYPRO Ruby EMSA protein gel stain
Staining tray

0.5x TBE running buffer

TRIS	0.54 g (44.5 mmol/l)
Boric acid	0.28 g (44.5 mmol/l)
EDTA (free acid)	0.03 g (1 mmol/l)
Deionized water to	100.00 ml

Procedure

- Peel the tape from the bottom of casting cassette.
- Pull the comb from the gel.
- Seat the gel on the bottom of a cell and lock into place with the gel tension wedge.
- Fill the upper buffer chamber with TBE running buffer. Buffer level should exceed the level of the wells.
- Apply DNA samples and DNA ladder
- Fill the lower buffer chamber at the gap near locking mechanism also with TBE running buffer.

- Run electrophoresis at 100 V for 90 min.
- When the Orange G dye front reaches the bottom, shut off the power.

Gel staining

- Warm the SYBR Green EMSA gel stain concentrate to room temperature.
- Dilute 5 µl of 10,000x SYBR green EMSA gel stain concentrate in 50 ml TBE buffer and pour into gel staining tray.
- Separate the two plates attempting to leave the gel on the bottom slotted plate.
- Flip over and transfer the gel into the staining tray.
- Incubate for 20 min on orbital shaker, protected from light.
  Don't use a glass container – glass adsorbs the dye.
- Place the gel in the staining tray.
- Add SYPRO Ruby EMSA protein gel stain solution with TCA.
- Incubate for 3 h on orbital shaker at 50 rpm, protected from light.
- Wash the gel in deionized water for 10 s.
- Destain the gel in 10 ml/dl methanol and 7 ml/dl acetic acid for 60 min.
- Wash the gel in deionized water for 10 s.
- Take a gel picture using 300 nm transilluminator.

3.2.6.6 Supershift assay

Materials and equipment

TRIS
TRIS-HCl
Acrylamide
BIS
Glycine
TMEDA
APS
EDTA
Dithio**t**hreitol (DTT)
NaCl
Glycerol
Labeled oligonucleotide probe with ^{32}P-ATP by polynucleotide kinase
Antibody

10x TRIS-glycine-EDTA (TGE) buffer

TRIS	30.3 g (0.25 mol/l)
Glycine	142.0 g (1.89 mol/l)
EDTA	37.2 g (0.13 mol/l)
Deionized water to	1,000.0 ml

Binding buffer, 5x, pH = 7.6

TRIS-HCl	0.61 g (50 mmol/l)
EDTA	0.07 g (2.5 mmol/l)
Adjust with HCl to pH = 7.6.	
1,4-Dithiothreitol	0.08 ml (5 mmol/l)
NaCl	1.46 g (250 mmol/l)
Glycerol	50.00 ml
Deionized water to	100.00 ml
Store at –20 °C.	

Monomer solution, T = 30 g/dl, C = 0.03

Acrylamide	29.0 g
BIS	1.0 g
Deionized water	100.0 ml

Resolving gel, T = 5 g/dl

10x TGE	6.00 ml
Monomer solution	10.00 ml
Glycerol	3.00 ml
10 g/dl TMEDA	0.60 ml
10 g/dl APS	0.45 ml
Deionized water	40.00 ml

Procedure

- Prepare whole cell extract.
  Make sure solution does not contain DTT.
- Add 4 ml 5x binding buffer, 2.5 ml nuclear extract, and deionized water into a microfuge tube.
- Mix the contents of the tube.
- Add the antibody to the mixture and incubate at room temperature for 15 min.
- Add the ^{33}P-labeled probe, mix and incubate at room temperature for 15 min.
- Add 2 µl of loading dye, load total amount of the sample on 4 g/dl 0.25x TBE gel, which has been prerun in 0.25x TBE buffer for at least 20 min.
  Run the dye separately in the first and last lanes of the gel.
- Load the mixture into each lane of the polyacrylamide gel.
- Run electorophoresis in TBE buffer at 100–130 V for 3 h.
- Dry the gel and perform autoradiography to visualize bands.

3.2.6.7 Electroelution of small DNA fragments from polyacrylamide gel

Materials and equipment

TBE buffer, pH = 8.0
3 mol/l Sodium acetate, pH = 5.2

Ethanol
Samples with DNA fragments
Dialysis bag
Horizontal gel electrophoresis cell

Procedure

- Run gel electrophoresis of DNA.
- Cut out the desired DNA band with a scalpel or razor blade.
- Transfer the gel piece in an appropriate dialysis bag and add enough TBE buffer.
- Place the bag in a horizontal gel electrophoresis cell containing TBE buffer and run electrophoresis at 4 V/cm for 2 h, if DNA < 300 bp, or for 6 h, if DNA are longer.
- Reverse the polarity of the electrophoresis cell for 1 min to free the bound DNA.
- Rinse the gel piece and the inside of the dialysis bag to obtain all DNA.
- Precipitate DNA molecules with 3 mol/l sodium acetate, pH = 5.2, and ethanol.

3.2.7 Troubleshooting

Problem	Causes	Solution
Prior to electrophoresis		
Air bubbles during casting the polyacrylamide gel.	The support film was unclean. The glass plate that comes in contact with the monomer solution was dirty.	Do not touch the hydrophilic side of the support film with fingers. Wash the glass plate with dishwashing detergent or ethanol before use.
Air bubbles during the polymerization of monomer solution.	The atmospheric oxygen in the monomer solution inhibited the gel polymerization.	Degas the monomer solution before casting.
The monomer solution does not polymerize or polymerizes to slow.	The concentration of TMEDA or APS in the monomer solution was too low. The APS solution was too old or stored improperly. The room temperature was too low.	Check the composition of the monomer solution. Increase the concentration of TMEDA or APS. Use new APS solution. Store the APS solution in a refrigerator. Cast gel at 20–25 °C.

(continued)

Problem	Causes	Solution
The polyacrylamide gel is too soft and sticky.	The concentration of acrylamide or BIS was too low.	Increase the concentration of acrylamide or BIS.
	The acrylamide, BIS or APS solution was too old.	In refrigerated room the maximum storage for acrylamide and BIS solution is four weeks, and for APS solution is a week.
	The concentration of TMEDA or APS in the monomer solution was too low.	Use 5 µl of 10 g/dl TMEDA and 5 µl of 10 g/dl APS solution per 1 ml monomer solution.
	There was too much oxygen in the monomer solution.	Degas the monomer solution with a water-jet pump.
The gel upper end is sticky, swells when washing, and detaches from the support film.	The atmospheric oxygen has inhibited the polymerization of the gel upper end.	Overlay the monomer solution with deionized water after casting.
The monomer solution polymerizes too quickly.	The concentration of TMEDA or APS was too high in the monomer solution.	Check the recipe of the monomer solution. Reduce the concentrations of TMEDA or APS.
	The room temperature was extremely high.	Cast gel at 20–25 °C.
The gel separates from support film.	An improper support film was used.	Do not exchange the support film for polyacrylamide and agarose gels.
	The gel was cast onto the wrong side of the support film.	Cast gel only on the hydrophilic side of the support film, first checking the side with water drops.
	The support film was stored incorrectly or too long.	Store the support film at room temperature, dry, and in the dark.
	The glass plate that comes into contact with the monomer solution was unclean.	Wash the glass plate with dishwashing detergent or ethanol before use.
The gel does not adhere to the support film, but to the glass plate.	The glass plate was too hydrophilic.	Treat the glass plate with Repelsilane.
	The gel concentration was too low.	The polyacrylamide concentration should be at least 4 g/dl at $C = 0.03$.
	The gel was left too long in the casting cassette.	Take the gel from the cassette 1 h after the start of polymerization.

(continued)

Problem	Causes	Solution
During electrophoresis		
It does not flow or flows too little electric current.	One of the connectors has no or poor contact.	Check all connections.
	Poor contact between the electrode strips and the gel.	Check the uniform lay down of the electrodes on the gel and possibly complain with a glass plate.
The gel "sweats" – is covered with water drops.	There are no water-binding additives in the gel.	Add 20 g/dl glycerol or 10 g/dl sucrose to the monomer solution.
The gel "evaporates" – the separation chamber lid is covered with condensed water.	The voltage is too high. The cooling is insufficient.	Reduce the voltage. Check the coolant temperature. The cooling block should be made of glass, metal, or best of ceramic.
The gel sparks and burns.	There are thin spots in the polyacrylamide gel – the support film was not fixed on the glass plate during the gel casting.	Before gel casting, roll the support film on the casting cassette glass plate to get an even contact.
The gel sparks along the gel edges.	The electric current flows under the support film.	Use kerosene or silicone DC 200 as contact liquid between the cooling plate and support film.
After electrophoresis		
No nucleic acid bands in the gel.	The cathode and anode were exchanged.	Check the connections to the power supply.
	The nucleic acids have left the gel.	Monitor the passage of dye front.
	The mass of the nucleic acid was too large – the nucleic acid could not enter the gel.	Reduce the total concentration T of polyacrylamide gel or use agarose gel.
The nucleic acid bands are very weak.	The concentration of nucleic acids or the sample volume was too small.	Concentrate the sample, apply greater volume, and use more sensitive detection method.
The nucleic acid bands form tails.	The sample contained too much nucleic acid.	Apply smaller sample volume or dilute the sample.

(continued)

Problem	Causes	Solution
The samples from adjacent tracks have run into each other.	The sample volume was too large. The application template was not tight on the gel.	Concentrate the samples and apply smaller volumes. Press lightly the application template onto the gel to push out air bubbles between it and gel.
The DNA fragments are arranged in a curved manner – "smiling" effect.	The temperature in the central part of the gel was higher than that of the gel margins.	Reduce the voltage. Apply samples at a distance from the gel margins.
Extraneous bands of lower intensity and irregular spacing.	Impure DNA fragment.	Repurify DNA fragment prior to labeling.

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3.3 Denaturing polyacrylamide gel disc-electrophoresis of nucleic acids

The denaturing polyacrylamide gels are preferred, compared to the denaturing agarose gels, because the electrophoresis there separates better nucleates. They are used for resolution of short (< 500 nucleotide) fragments of single-stranded DNA or RNA. Such gels can resolve nucleate fragments differing by only one mononucleotide in length. They are used also for analyzing mRNA by using S_1 nuclease, ribonuclease, or primer extension. In addition, the denaturing polyacrylamide gels are useful for purification of radioactive single-stranded probes and large-scale purification of synthetic oligonucleotides.

The denaturing polyacrylamide gel disc-electrophoresis is used for sequencing the human genome [1,2]. Subsequent projects [3,4] help the researchers analyze genome structure and function, and human evolution [5,6,7]. This creates possibilities for treating the diseases.

3.3.1 Theory of the denaturing polyacrylamide gel electrophoresis of nucleic acids

The denaturing electrophoresis of DNA is carried out on polyacrylamide gels containing usually 8 mol/l urea. Urea [$CO(NH_2)_2$, M_r = 60.06] breaks the hydrogen bonds between the base pairs of nucleic acid strands and the van der Waals forces, and causes the strands to separate from each other. Heating the samples to at least 60 °C further promotes denaturation [8,9,10].

3.3.2 DNA sequencing

The DNA electrophoresis is run in discontinuous buffer systems [11]. The leading buffer is located in the polyacrylamide gel; the trailing butter is located in the cathode tank; the counterion is the same in all parts of the buffer system. The sequencing is possible, if the nucleic acid fragments contain up to 1,000 nucleotides. The electrophoresis of large DNA single strands can take several days.

Two DNA sequencing methods are known. In Maxam–Gilbert sequencing method, called chemical mode, base-specific cleavage reactions are used to cut DNA into fragments. In Sanger sequencing method, called dideoxy method, base-specific dideoxynucleotides are used to cancel a DNA polymerizing reaction.

3.3.2.1 Maxam–Gilbert sequencing

Maxam–Gilbert (chemical) sequencing of DNA is based on nucleobase-specific partial chemical modification of DNA and subsequent cleavage of the DNA backbone at sites adjacent to the modified nucleotides [12].

A DNA nucleate is labeled at its 3′ or 5′ end. In the rule, a λ-^{32}P-ATP is linked at the 5′ end with the help of the enzyme *polynucleotide kinase*. Then the labeled strand is cut in parts with the help of a *restriction endonuclease* and the obtained fragments are separated in an agarose or a polyacrylamide gel. Labeling at the 3′ end can be achieved with terminal *transferase* and an α-^{32}P-ATP followed by alkaline hydrolysis to remove all but the first adenylic acid residue, or by "filling in" the complementary strand of a 5′-single-stranded protruding end created by digestion with the restriction endonuclease. The labeled fragments are then fractionated by gel electrophoresis and eluted from the gel.

Chemical treatment generates breaks at a small proportion of one or two of the four nucleotide bases in each of four reactions (G, A + G, C, C + T). For example, the purines (A + G) are depurinated using formic acid at pH = 2.0; guanine (and, to some extent, adenine) is methylated by dimethyl sulfate at pH = 8.0, and the pyrimidines (C + T) are hydrolyzed using hydrazine in 1.5 mol/l NaCl.

Thereafter, the DNA sample is incubated in 1 mol/l piperidine at 90 °C, where phosphate sugar backbones carrying modified bases are destroyed. In this way, a random population of fragments is obtained, whose different lengths correspond to the specific nucleotide position. The fragments in the four reactions are electrophoresed side by side in denaturing gels (Figure 3.3-1).

To visualize the separated bands, the gel is exposed to X-ray film for autoradiography, yielding a series of dark bands each showing the location of identical radiolabeled DNA nucleates. From the presence or absence of certain fragments the sequence may be inferred. An automated Maxam–Gilbert sequencing protocol was developed by Boland *et al.* [13].

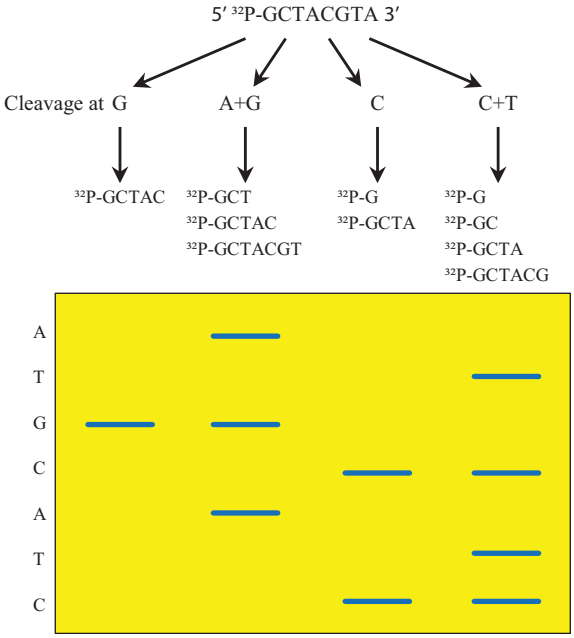

Figure 3.3-1: Maxam–Gilbert sequencing reaction.
Cleaving a tagged segment of DNA at different points yields tagged fragments of different size. The fragments are then separated by gel electrophoresis.

3.3.2.2 Sanger sequencing

Sanger sequencing (chain termination) is a method of DNA sequencing, based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during DNA replication [14,15]. It is the most widely used sequencing method.

The normal DNA nucleotides wear no OH-group at 2′-position of ribose, while the OH-group at 3′ position is coupled to the next nucleotide. The **di**deoxy**n**ucleoside **trip**hosphates (ddNTP): ddATP, ddTTP, ddGTP, or ddCTP have no OH-groups neither at their 2′- nor at their 3′-carbon atoms. So they lack a 3′-OH group required for the formation of a phosphodiester bond between two nucleotides, causing DNA polymerase to cease extension of DNA when a modified ddNTP is incorporated.

The DNA sample is divided into four separate reactions, containing all four of the standard deoxynucleotides (dATP, dGTP, dCTP, and dTTP) and the DNA polymerase. The nucleotides are labeled radioactively or with fluorescent substances whereby a fluorescent substance binds to the 5′ end of the sequencing primer. To each reaction, only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP) is added. The concentration of each dideoxynucleotide is approximately 100-fold lower than the concentration of the corresponding deoxynucleotide (e.g., 0.005 mmol/l ddATP to 0.5 mmol/l dATP). This is necessary for enough DNA nucleotides to be produced.

If a specific oligonucleotide primer and the four dNTP are present in a mixture, the DNA polymerase synthesizes a 5′→3′ nucleate, which is complementary to the DNA template. When a ddNTP is added to the growing nucleotide chain, the polymerase stops the polynucleotide chain elongation and DNA polymerization breaks off. The result is a fragment whose length corresponds to the position of a particular mononucleotide in the DNA template. Thereafter the DNA fragments are resolved by denaturing polyacrylamide gel electrophoresis and the DNA sequence is read out by a computer [16]. A fixed laser beam penetrates through the polyacrylamide gel and reaches a photocell, which is located below the resolving track. If a band hits the laser beam, the fluorescent DNA fragment emits light signals [17]. Thus, the migrating bands of each separating track are registered in their order, that is the sequence of the separated DNA, in a computer (Figure 3.3-2).

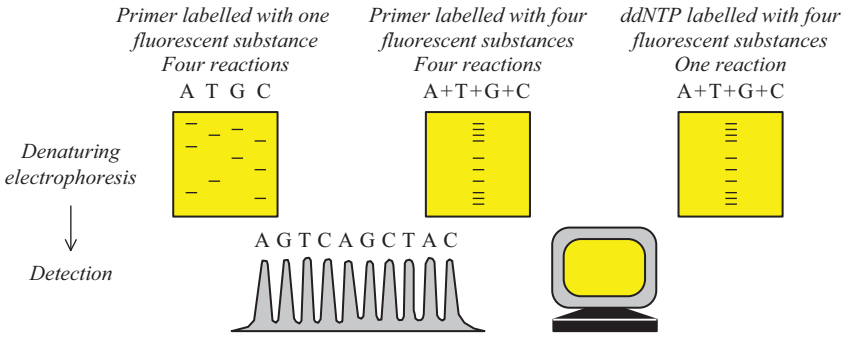

Figure 3.3-2: Principle of DNA sequencing according to Sanger.

Automated DNA-sequencing instruments (DNA sequencers) can sequence up to 384 DNA samples in a single batch. Batch runs may occur up to 24 times a day. DNA sequencers detect and record dye fluorescence.

To investigate RNA nucleates and viroids, bidirectional electrophoresis [18] is used: RNA fragments and viroids are separated under native conditions in a polyacrylamide gel at 15 °C. Thereafter a denaturing electrophoresis is performed in the same gel in the reverse direction. RNA nucleates denature in 4 mol/l urea and at 50 °C, and do not lose their mobility and leave. The viroids migrate no longer in the denaturing gel and remain in the gel.

3.3.3 Single-strand conformation polymorphism

Single-**s**trand **c**onformation **p**olymorphism (SSCP) analysis is based on the conformational difference of single-stranded nucleotide sequences of identical length

induced by differences in the sequences under certain experimental conditions. This property allows sequences to be distinguished using gel electrophoresis, which separates fragments according to their different conformations [19].

If DNA fragments are denatured by heat or formamide and are rapidly cooled, intramolecular base pairs are formed and single strands obtain stable conformation. They get different three-dimensional conformations because the two DNA strands differ in their base sequences, with the exception of the repeat sequence. The difference in shape between the two single DNA strands with different sequences can cause that they migrate differently in an electrophoresis gel, even though the number of nucleotides is the same. The different structures of a DNA strand have different mobilities in a native polyacrylamide gel, so they can be seen as two bands [20] (Figure 3.3-3).

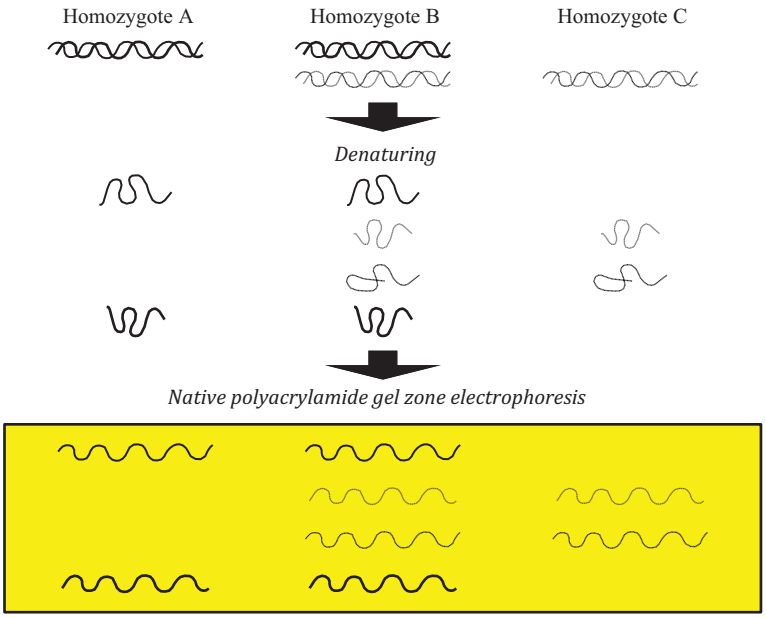

Figure 3.3-3: Scheme of SSCP analysis.

SSCP analysis is carried out on 30–40 cm gels, and the electrophoresis continues often more than 10 h. The sensitivity of detection of sequence variations is very high [21] and the usage of appropriate gels and conditions can further increase it [22].

Single-strand conformation polymorphism analysis is used in molecular biology, for example in genotyping to detect homozygous individuals of different allelic states, as well as heterozygous individuals [23]. Sequence variations such as small deletions or insertions and even single-base substitutions can change the DNA conformation and therefore can result in a band shift in PAGE. For example, SSCP is

widely used in virology to detect variations in different strains of a virus, if both strains have undergone changes due to mutation, which will cause the two particles to assume different conformations [24].

Single-stranded DNA weakly binds ethidium bromide. Therefore, PCR is needed to amplify 100–500 bp genomic DNA or cDNA prior to the SSCP analysis. This can be made with the help of reverse transcription on an mRNA.

Using SSCP method, somatic mutations in cancer cells and inherited diseases can be analyzed [25].

3.3.4 DNase footprinting assay

DNase footprinting assay [26,27] uses the fact that a protein bound to DNA will protect DNA from enzymatic cleavage. With the help of this technique, a protein-binding site on a DNA molecule can be located.

First, the enzyme **d**eoxyribo**n**ucle**ase** (DNase) cuts a radioactively end-labeled DNA fragments. Then the DNA fragments are resolved by gel electrophoresis. The DNA fragments, which are smaller, will move faster in the gel than the longer fragments. The gel is then exposed to a special photographic film. When a protein binds DNA, the binding site of DNA is protected from enzymatic cleavage. This protection will result in a clear area on the gel, which is referred to as "footprint." By varying the concentration of the DNA-binding protein, the minimum concentration of protein can be estimated at which a footprint is observed. If DNA is marked at its ends, a marked and an unmarked single strand will be obtained (Figure 3.3-4). The unlabeled strand is not visible on the autoradiogram.

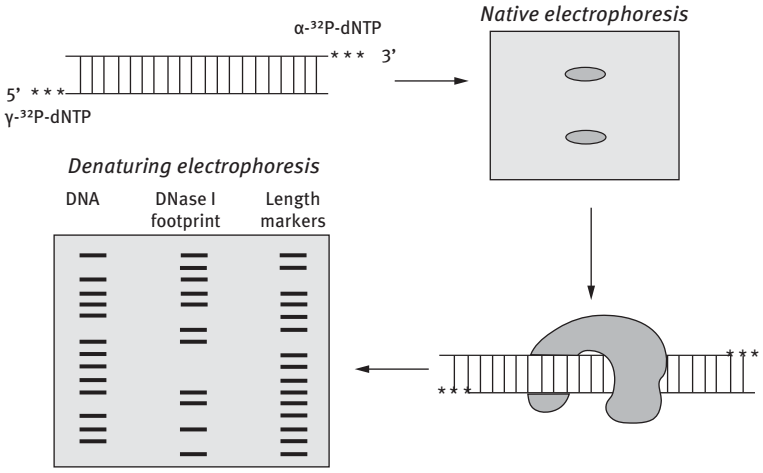

Figure 3.3-4: Principle of DNase footprinting assay.

Using DNase footprinting assay, the transcriptional control of MUC1 gene was investigated [28]. This gene encodes DF3 glycoprotein that is highly expressed in human breast cancer cells.

For analysis of RNA-protein interactions, similar "toeprinting" assay is used [29,30,31]. In this method, primer extension reaction is used to examine the binding of protein to RNA, from its 3′ (toe) to the other 5′ (heel) side.

3.3.5 Nuclease protection assay

The nuclease protection assay is a technique used to identify RNA molecules of known sequence in a heterogeneous RNA sample [32,33]. RNA is first mixed with DNA or RNA probes, usually about 200–500 nucleotides in length that are complementary to the sequence of interest. They form hybridized double-stranded DNA-RNA or RNA-RNA hybrids. The mixture is exposed to ribonucleases that cleave the rest of probe and single-stranded RNA to individual mononucleotides but have no activity against double-stranded RNA-RNA or DNA-RNA hybrids. Then they are analyzed by electrophoresis through a denaturing polyacrylamide gel, and are detected autoradiographically (Figure 3.3-5).

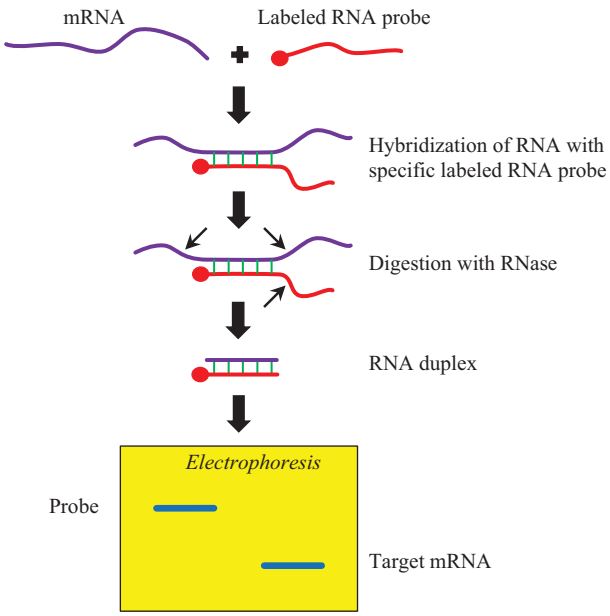

Figure 3.3-5: Principle of the nuclease protection assay: mRNA and probes, which are not complementary to each other, are degraded by nucleases. Only hybrids between mRNA and their probes remain to be separated by denaturing electrophoresis.

Nuclease protection assay is used to detect the presence of double-stranded RNA and to map introns and 5′ and 3′ ends of transcribed gene regions. It is the method of choice for determining **m**essenger **RNA** (mRNA) amount. With its help, a **c**yclic **DNA** (cDNA) from the plant *Arabidopsis thaliana* was isolated [34].

The nuclease protection assay has a higher signal-to-noise ratio compared to the Northern or slot-blotting. So, rapid and quantitative hybridization can be achieved at low probe concentrations. Furthermore, specific information on transcription initiation or termination can be obtained depending on what region of the mRNA is covered by the probe.

If the probe is labeled radioactively, the gel must be dried after electrophoresis to be prepared for autoradiography. The bands can be cut out from the dried polyacrylamide gel, the gel can be dissolved in H_2O_2, and the radioactivity can be determined using a scintillation counter.

3.3.5.1 S_1 nuclease protection assay

In order to find the 5′ end of an mRNA, S_1 nuclease protection assay is used. During it, labeled DNA probes hybridize with mRNA sample. Then the single-stranded DNA probe out of the hybrids is destroyed with the S_1 nuclease and the hybrids are run on a denaturing polyacrylamide gel together with size markers differing in length by only one mononucleotide. So, the distance between the end of the probes and the 5′ end of the mRNA can be determined (Figure 3.3-6).

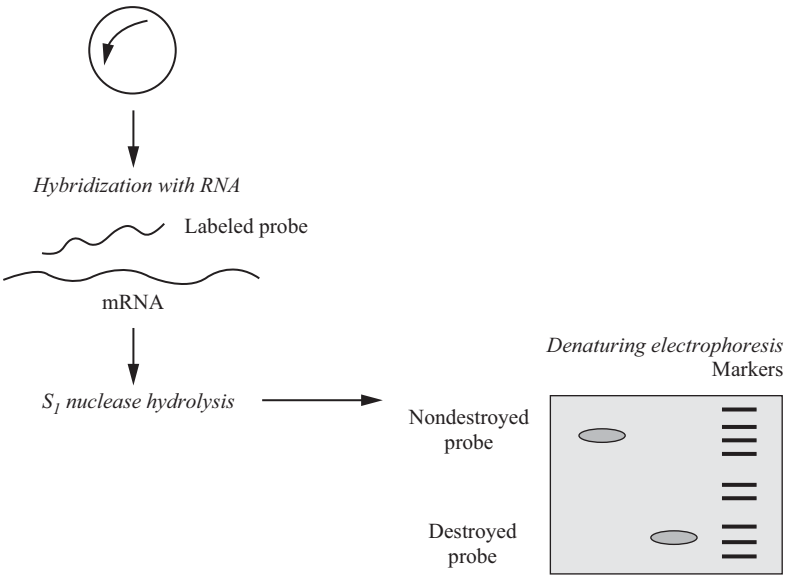

Figure 3.3-6: Principle of S_1 nuclease protection assay.

The probes are prepared by cloning the gene of interest under the control of any of the following promoters: SP6, T7, or T3. These promoters are recognized by DNA-dependent RNA polymerases from bacteriophages. The probes produced are radioactive as they are composed of radioactive nucleosidetriphosphates.

With S_1 nuclease protection assay, the characterization of the promoter region of mouse gene δ/YY1 has been described [35], which is responsible for a transcription factor.

3.3.6 RNA separation

Separation of fragmented RNA or micro RNA also finds place on polyacrylamide gels containing urea [36]. An RNA ladder is often run alongside the samples to show the size of fragments obtained. The ribosomal subunits can also act as size marker. Since the large ribosomal subunit is 28 S (approximately 5 kb) and the small ribosomal subunit is 18 S (approximately 2 kb), two prominent bands appear in the gel [37].

A method for resolving RNA fragments [38] is based on the migration of RNA fragments on a polycationic polyacrylamide gel, made by incorporating positively charged immobilines into the neutral polyacrylamide backbone. The separation is carried out in a 0–10 mmol/l immobiline (pK = 10.3) gradient under denaturing conditions (6 mol/l urea). In the 100–1,000 bp length, sharper bands of RNA are obtained, in comparison with the conventional electrophoresis. Good separations of single-stranded RNA of 21–23 mononucleotides in length, which appear to regulate the gene expression, are also obtained.

3.3.7 Primer extension assay

The RNA polymerases can polymerize mononucleotides without a primer when a specific promoter sequence is present. In contrast, DNA polymerases and reverse-transcriptases need a free 3′-OH end of a DNA or RNA primer whose bases are paired with a matrix, for example, mRNA.

In the **p**rimer **e**xtension **a**ssay (PEA), the transcription start site for a gene is determined experimentally by identifying the 5′ end of the encoded **m**essenger **RNA** (mRNA). The protocol begins with a primer, usually a synthetic oligonucleotide of about 20 residues that is complementary to an mRNA sequence ~50–150 nucleotides downstream of the anticipated 5′ end. The primer is 5′ end-labeled using γ-^{32}P-ATP and T4 polynucleotide kinase and is annealed to the specific mRNA molecules within an RNA sample.

Reverse **t**ranscriptase (RT), deoxyribonucleoside triphosphates, and appropriate buffer components are added to the primer-mRNA hybrids to catalyze elongation of

the primer to the 5′ end of the mRNA. So the 5′ end of mRNA is reached and the distance to the transcription start site can be determined. The resulting radiolabeled **c**omplementary **DNA** (cDNA) products are analyzed by denaturing polyacrylamide gel electrophoresis, followed by autoradiography.

The sizes of the bands detected on the gel, as compared to an adjacent sequencing ladder or molecular mass standards, provide a measure of the distance from the 5′ end of the synthetic oligonucleotide to the beginning of the mRNA transcript. In theory, the 3′ end of the cDNA will coincide with the 5′ end of the mRNA. Thus, the size of the radiolabeled cDNA should represent the distance from the labeled 5′ end of the primer to the 5′ end of the mRNA (i.e., the 3′ end of the cDNA). If the labeled cDNA products are within the resolution range of the gel, the transcription start site can be determined with an accuracy of plus or minus one nucleotide.

A disadvantage of the primer extension protocol is that it can be difficult to find a primer that works for a new gene. Moreover, background bands caused by premature termination of reverse transcription (because of the RNA secondary structure) often appear, which makes difficult to find the start site location. Because of these and other limitations, other methods, such as RNase protection assay, are recommended.

3.3.7.1 Primer annealing and reverse transcription

Reverse transcriptases lack RNase activity but allow extension reactions to be performed at elevated temperatures. So the problems associated with RNA secondary structure are reduced. For example, **m**oloney **m**urine **l**eukemia **v**irus (MMLV) reverse transcriptase expressed in *Escherichia coli* can catalyze extension reactions. Other reverse transcriptases, including avian reverse transcriptase, can also catalyze extension reactions.

cDNA products shorter than 50 bp are undesirable because reverse transcriptase reactions sometimes terminate or pause after extending a very short distance, resulting in strong background bands that are 10–20 bp longer than the primer. Additionally, reverse transcriptase will sometimes synthesize a copy of the primer, leading to the so-called “primer dimer” artifact. If cDNA synthesized has at least 50 nucleotides (e.g., a 20-nucleotide primer plus a 30-nucleotide extension), it will be usually well separated from the majority of these background band.

cDNA products longer than 150 bp can often be obtained, but the efficiency of reverse transcriptase extension decreases with distance because of pausing and premature termination. Furthermore, the longer the extension product, the more difficult it will be to determine a size, because of the decreased resolution of large fragments on a denaturing polyacrylamide sequencing gel.

3.3.8 Protocols

3.3.8.1 Chemical sequencing using ^{32}P-labeled DNA

Materials

^{32}P-DNA to be sequenced
3 mol/l sodium acetate, pH = 5.0
Isopropanol
70 ml/dl ethanol prepared with sterile deionized water
5 mol/l NaCl
Dimethyl **s**ulfate (DMS)
Dimethyl sulfate reaction buffer
Formic acid
Hydrazine
DMS stop buffer
Ethanol, prechilled to –20 °C
10 ml/dl piperidine, freshly prepared in sterile deionized water
Formamide loading buffer
90 °C water bath or oven

Procedure

- Precipitate ^{32}P-DNA with 10 vol of 3 mol/l sodium acetate, pH = 5.0, and 1 vol of isopropanol.
- Distribute 10 µl ^{32}P-DNA into each of four 1.5 ml snap-top microcentrifuge tubes labeled G, G + A, T + C, and C.
- Resuspend the salt-free DNA pellet in 40 µl sterile deionized water. Count the ^{32}P-DNA using Cerenkov counting by placing the entire tube into a 20 ml scintillation vial, on the ^{14}C channel. Multiply the result by 4 to obtain the counts per min.
- Add DMS reaction buffer, 5 mol/l NaCl, and deionized water as shown in Table 3.3-1(A).
- Add DMS, formic acid, and hydrazine as shown in Table 3.3-1(B). Close the tubes and mix.
- Incubate at 25 °C for the times indicated, using a stopwatch.
- Add appropriate stop buffer plus prechilled (–20 °C) ethanol as indicated in Table 3.3-1(C), and immerse in a dry ice/ethanol bath for 5 min. Microcentrifuge at 15,000 g for 5 min, discard supernatants, and rinse twice with 70 ml/dl ethanol.
- Resuspend each pellet in 200 µl sterile deionized water and reprecipitate with 20 µl of 3 mol/l sodium acetate and 500 µl of ethanol, immersing in a dry ice/ethanol bath for 5 min before centrifuging.
- Rinse twice with 1 ml of 70 ml/dl ethanol and dry pellets.

Table 3.3-1: Chemical sequencing reactions.

Procedure	Reaction specificity			
	G	G + A	T + C	C
A. Preparing DNA samples				
DNA	10 µl	10 µl	10 µl	10 µl
DMS reaction buffer	200 µl	–	–	–
5 mol/l NaCl	–	–	–	5 µl
Deionized water	–	–	10 µl	5 µl
B. Carrying out base-specific modification reactions				
DMS	1 µl	–	–	–
Formic acid	–	25 µl	–	–
Hydrazine	–	–	30 µl	30 µl
Time at 25 °C	4 min	5 min	8 min	8 min
C. Stopping reactions				
DMS stop buffer	50 µl	–	–	–
Hydrazine stop buffer	–	200 µl	200 µl	200 µl
Ethanol	750 µl	750 µl	750 µl	750 µl

- Resuspend in 70 µl of 10 ml/dl piperidine and transfer to 1.5 ml screw-top microcentrifuge tube.
- Cap the tubes and incubate at 90 °C for 30 min.
- Microcentrifuge briefly to collect condensate and evaporate to dryness.
- Resuspend in 30 µl sterile deionized water, transfer into new tubes, and dry pellet. Repeat using 20 µl sterile deionized water.
- Resuspend dried samples in 10 µl formamide loading buffer and vortex to dissolve any material adhering to the sides of the tube. Heat at 90 °C for 2-3 min, place on ice, and load on a preheated 6, 8, or 12 g/dl sequencing gel. Load related lanes (G, G + A, T + C, C).
- Electrophorese at 40 to 45 V/cm for 1.5 to 4.5 h. Dry the gel and autoradiograph on X-ray film with an intensifying screen at –70 °C for 1 to 4 days.

3.3.8.2 Sanger sequencing reactions using *Taq* DNA polymerase

Materials and equipment

10x *Taq* polymerase buffer (0.5 mol/l TRIS-HCl, pH = 9.0)
Taq Sanger mixtures
Taq DNA polymerase

dNTP chase
50 to 75 °C water bath

Procedure

- Anneal primer to template in 10x *Taq* polymerase buffer.
- Add 3 µl of *Taq* Sanger mixtures A, C, G, and T to the bottom of labeled A, C, G, and T tubes, respectively.
  Keep tubes closed to prevent evaporation.
- Before use, dilute *Taq* DNA polymerase to 2.5 U/µl in 1x *Taq* polymerase buffer, and keep on ice.
- Add
  2 µl 10 mCi/ml α-^{35}S-dATP and
  1 µl diluted *Taq* DNA polymerase
  to the annealed primer and template (total 13 µl) and mix by pipetting up and down.
- Add 2.5 µl of primer/template mixture to each tube containing *Taq* Sanger mix.
- Incubate at 50 to 75 °C for 10 min.
- Add 1.0 µl dNTP chase to each reaction, mix, and incubate at 50 to 75 °C for 10 min.
- Electrophorese.

3.3.8.3 Purification of oligonucleotides using denaturing polyacrylamide gel electrophoresis

Materials

10x **T**RIS-**b**orate-**E**DTA (TBE) buffer, pH = 8.0
TRIS-**E**DTA (TE) buffer, pH = 7.5
Monomer solution (40 g/dl acrylamide, 2 g/dl bisacrylamide)
Urea
10 g/dl TMEDA
10 g/dl APS
Oligonucleotides
Casting cassette (glass plates, spacers, and combs) for casting of 200 × 160 × 1.6 mm gels
Electrophoresis unit
DC power supply
Hand-held short-wave (254 nm) UV lamp

Polyacrylamide gel (T = 10 g/dl, C = 0.03) with 7 mol/l urea

10x TBE buffer	6.00 ml
Monomer solution	60.00 ml
Urea	52.97 g (7.0 mol/l)
Deionized water	60.00 ml

Procedure

- Assemble a casting cassette using two glass plates, spacers, and clamps.
- Dissolve urea in the monomer solution in a 60 °C water bath. Stir for 20 min to complete mixing.
- Add 0.40 ml of 10 g/dl TMEDA and 0.30 ml of 10 g/dl APS, and mix.
- Pour the solution in the casting cassette.
- Insert a comb so that air bubbles are avoided under the comb teeth.
- Allow the solution to polymerize for 60 min.
- Remove the comb and bottom spacer.
- Fill the lower tank of the electrophoresis apparatus with TBE buffer.
- Place the gel into the apparatus and clamp the gel plate to it.
- Fill the upper tank with 1x TBE so that the wells are covered.
- Prerun the gel using the DC power supply at 20 to 40 V/cm (constant voltage) for 30 min.
- Add urea loading buffer to an oligonucleotide pellet and resuspend it by heating at 90 °C for 5 min.
- Apply the samples into the wells.
- Electrophorese at 20 to 40 V/cm (constant voltage). Avoid temperatures over 65 °C.
- Turn off the power supply, and remove the gel plates.
- Cover the gel with a plastic wrap and invert the plate onto a TLC plate with a fluorescent indicator.
- Visualize the bands exposing briefly the gel with a short-wave (254-nm) hand-held UV lamp. The bands will appear as black shadows on a green background. Outline the desired band using a marking pen.
- Cut out the band of interest with a scalpel or razor blade. Crush the gel into fine particles by forcing through a small-bore (5 ml) syringe. Place the crushed gel into a 15 ml centrifuge tube.
- Add 3 ml of TE buffer for every 0.5 ml gel and freeze at −80 °C for 30 min. Thaw in a hot-water bath (~ 50 °C) and let soak at 90 °C for 5 min. Elute on a rotary shaker overnight at room temperature.
- Centrifuge at 1,000 g at room temperature for 2 min. Remove the supernatant, using a syringe, and pass through 0.2 µm filter to remove any remaining polyacrylamide fragments.
- Concentrate against 1 vol n-butanol. Remove the upper butanol layer and repeat until the volume of the lower, aqueous layer is convenient for precipitation.
- Precipitate using 1/10 vol of 3.0 mol/l sodium acetate, pH = 5.2, and 2 vol of ethanol (for DNA) or 3 vol (for RNA).
- Chill at −20 °C for 20 min and centrifuge at 12,000 g and 4 °C for 10 min.

3.3.9 Troubleshooting

Problem	Cause	Solution
Prior to electrophoresis		
Air bubbles in the polyacrylamide gel.	The support film was unclean.	Do not touch the hydrophilic side of the support film with fingers.
	The glass plate that comes in contact with the monomer solution was dirty.	Wash the glass plate with detergent or ethanol before use.
	The atmospheric oxygen in the monomer solution inhibits the gel polymerization.	Degas the monomer solution before casting it.
The monomer solution does not polymerize or polymerizes to slow.	The concentration of TMEDA or APS in the monomer solution is too low.	Check the composition of the monomer solution. Increase the concentration of TMEDA or APS.
	The APS solution is old or was stored improperly.	Use a new APS solution. Store the APS solution in the refrigerator.
	The room temperature is too low.	Cast gels at 20–25 °C.
The polyacrylamide gel is too soft and sticky.	The concentration of acrylamide or BIS is too low.	Increase the concentration of acrylamide or BIS.
	The concentration of TMEDA or APS in the monomer solution is too low.	Use 5 µl of 10 g/dl TMEDA and 5 µl of 10 g/dl APS per 1 ml of the monomer solution.
	The APS solution is old.	The maximum storage for an APS solution is a week in the refrigerator.
	There is too much oxygen in the monomer solution.	Degas the monomer solution using a water-jet pump.
The upper gel edge is sticky, swells at washing, and dissolves from the support film.	The atmospheric oxygen has inhibited the polymerization of the upper gel edge.	After casting, overlay the monomer solution with deionized water.
The monomer solution polymerizes too fast.	The concentration of TMEDA or APS in the monomer solution is too high.	Reduce the concentrations of TMEDA or APS.
	The temperature is too high.	Cast the gel at 20–25 °C.

(continued)

Problem	Cause	Solution
The gel separates from the support film.	An improper support film was used.	Do not exchange the support films for polyacrylamide and agarose gels.
	The gel was cast onto the wrong side of the support film.	Cast the gel only on the hydrophilic side of the support film. Check first the site with water drops.
	The support film was stored incorrectly or too long.	Store the support film in the dark at room temperature.
The gel does not adhere to the support film, but to the glass plate.	The glass plate was too hydrophilic.	Treat the glass plates of the casting cassette with Repel-silane.
	The gel concentration was too low.	The polyacrylamide concentration T should be at least 4 g/dl at $C = 0.03$.
	The gel was left too long in the casting cassette.	Remove the gel from the casting cassette 1 h after the start of polymerization.
During electrophoresis		
Too little or no electric current flows.	One of the connectors has no or poor contact.	Check all connections.
	The contact between the electrodes and the electrode strips is poor.	Check the contact between the electrodes and the electrode strips; eventually weight them down with a glass plate.
The gel “sweats” – is covered with water drops.	The monomer solution contains no water-binding additives.	Add 20 g/dl glycerol or 10 g/dl sucrose to the monomer solution.
The gel “evaporates” – the lid of the electrophoresis unit is covered with condensed water.	The voltage is too high.	Reduce the voltage.
	The cooling is insufficient.	Check the temperature. The cooling block should be made of glass, metal, or best of ceramic.
The gel sparks and burns.	The polyacrylamide gel contains thin spots because the support film was not fixed on the glass plate.	Prior to casting roll the support film on a wet glass plate to get an even contact between the film and plate.

(continued)

Problem	Cause	Solution
The gel sparks along the support film edges.	The electric current flows under the support film.	Use kerosene or silicone DC 200 as a contact liquid between the cooling plate and support film.
	The cooling is insufficient.	Check the temperature. The cooling block should be made of glass, metal, or best ceramic.
After electrophoresis		
No nucleic acid bands are in the gel.	The cathode and anode were exchanged. The nucleic acids had left the gel. The mass of nucleic acid was too large; therefore, the nucleic acids could not migrate into the gel.	Check the connection to the power supply. Use a dye to monitor the front movement. Diminish the total polyacrylamide concentration *T* or use agarose gel.
The nucleic acid bands are weak.	The concentration of the nucleic acids or the sample volume was too small.	Concentrate the sample, or apply more volume.
The nucleic acid bands form tails.	The sample contained too much nucleic acid.	Apply smaller sample volume or dilute the sample.
The samples from adjacent tracks have run into each other.	The sample volumes were too large. The application template was not tight on the gel.	Concentrate the samples and apply less volume. Press gently the application template onto the gel to push out air bubbles.
High background and smeared bands on TBE-urea gels.	The oligonucleotides are not fully denatured. A sample volume was too large.	Prior to loading heat the samples at 70 °C for 3 min. Use less sample volume.
Bands intensity is too low on top of the gel.	Ratio of ddNTP to dNTP is too high.	Decrease ddNTP/dNTP ratio by lowering ddNTP in the mix.
Bands intensity is too low on bottom of the gel.	Ratio of ddNTP to dNTP is too low.	Increase ddNTP/dNTP ratio by increasing ddNTP in the mix.
Bands are diffuse or fuzzy.	The gel did not polymerize enough.	Let the gel polymerize longer.
High background in all lanes.	Impure template DNA.	Make new template DNA. For double-stranded templates, treat with RNase A, extract with phenol, and precipitate with ethanol.

(continued)

Problem	Cause	Solution
Blank autoradiogram.	Inactive polymerase. Incorrect or defective primer or template. Incorrect exposure procedure.	Replace the defective primer, template or reagents.
The autoradiogram is too light.	Old label. Not enough primer. Not enough template. Low enzyme activity. Incorrect development of autoradiogram.	Use new label. Check the primer concentration. Check the template concentration. Use ~ 0.5 pmol template DNA per reaction. Use fresh enzyme. Reexpose the gel. If necessary, change developing reagents.

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3.4 Temperature and denaturing gradient gel electrophoresis of nucleic acids

Temperature gradient gel electrophoresis and denaturing gradient gel electrophoresis use either a temperature or other agent to denature the sample as it moves through a polyacrylamide gel. They can be applied to investigate DNA and RNA, and (less commonly) proteins.

3.4.1 Temperature gradient gel electrophoresis of nucleic acids

Temperature **g**radient **g**el **e**lectrophoresis (TGGE) relies on temperature dependent changes in structure to separate nucleic acids. It was first described by Thatcher and Hodson [1] and by Wartell [2].

3.4.1.1 Theory of temperature gradient gel electrophoresis

DNA has a negative electric charge in neutral buffers. When an electric field is applied, it begins to move through a polyacrylamide gel to the positive electrode at a speed inversely proportional to the DNA length: shorter DNA travel faster, and on the contrary.

At room temperature, DNA exists stable in a double-stranded form. When the temperature is increased, the DNA strands begin to separate from each other (DNA melts) and the speed at which they move through the gel decreases [3,4]. The melting depends on the DNA sequence: GC base pairs are more stable than AT base pairs, because the bases guanine and cytosine are bound by three hydrogen bonds, and the bases adenine and thymine are bound by two hydrogen bonds.

If a temperature gradient is applied perpendicular to the electrophoresis direction, continuous conformational transitions in the nucleic acids are observed that depend on the enlargement of their volumes (Figure 3.4-1). As a result, their mobility slows down.

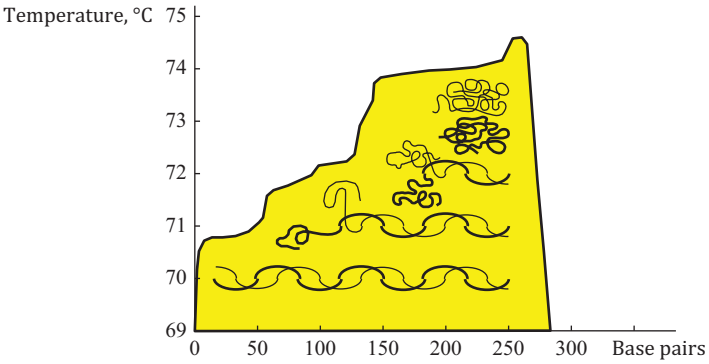

Figure 3.4-1: Conformational transitions of DNA double helix at temperature of 70–75 °C.

When comparing TGGE in commercially available gels (CleanGels) with TGGE in self-cast gels on a special TGGE device [5,6,7], a superior resolution of PCR amplificates on CleanGels are demonstrated. The main advantages of the CleanGels include ready-to-go and easy handling of a nontoxic, prewashed, dried polyacrylamide gel on a carrier film. In addition, the buffer system may be varied.

3.4.1.2 Running temperature gradient gel electrophoresis

To separate nucleic acids by TGGE, the following steps are undertaken:

- *Preparing and casting gels*. It is important that the buffer system chosen remains stable within the context of increasing temperature. For this purpose, urea is utilized for gel preparation; however, researchers need to be aware that the amount of urea used will affect the overall temperature required to separate DNA.
- *Electrophoresis*. The gel is loaded, the sample is applied on the gel according to the type of gel to be run, the voltage is adjusted, and the sample is left to run. Depending on the type of TGGE to be run, either perpendicular or parallel, varying amounts of sample need to be prepared and loaded. A larger volume of a sample is used with perpendicular TGGE, while a smaller volume of a sample is used with parallel TGGE.
- *Staining*. After the electrophoresis, the gel is stained to visualize the results. Silver staining has proven to be the most effective tool.
- *Elution of DNA*. After the silver staining, DNA can be eluted from the gel for PCR amplification.

The results of the temperature gradient gel electrophoresis depend on the ratio between the DNA mobility in the gel, which is a function of base number, and the number of base pairs.

3.4.1.3 Devices for temperature gradient gel electrophoresis

An apparatus for creating a linear temperature gradient consists of a horizontal metal plate, which is heated at one end by a thermostat and cooled at the other end by another thermostat. The generated temperature gradient may be perpendicular or parallel to the electrophoresis direction (Figure 3.4-2).

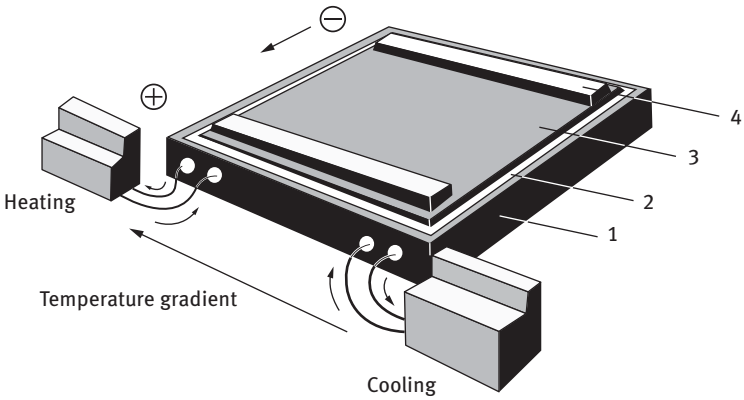

Figure 3.4-2: Device for temperature gradient gel electrophoresis of nucleic acids. The temperature gradient is perpendicular to the electrophoretic direction.
1. Metal plate isolated with Teflon; 2. Support film; 3. Polyacrylamide gel; 4. Electrode strip

3.4.1.4 Applications of temperature gradient gel electrophoresis

Temperature gradient gel electrophoresis is an approach for analyzing structural transitions of nucleic acids [8]. By creating upon an electric field a linear temperature gradient generated by cold- and hot-water circuits, homo- and hetero-DNA strand duplexes separate from each other according to their thermal stability [9,10].

Using the temperature gradient gel electrophoresis, diverse objects can be examined, for example viroid conformations, mutations in mtDNA, p53 mutation in pancreatic juice, and B-cells in lymphoma.

Viroid conformations

The viroids contain circular single-stranded RNA. For example, the potato spindle tuber viroid consists of 359 ribomononucleotide residues [11,12]. When temperature is increased, hydrogen bonds between the bases in RNA are destroyed, the viroid

RNA ring is opened, and it loses speed in an electric field (Figure 3.4-3). Due to the decreasing mobility, a simple method for diagnosis of this disease has been developed [13].

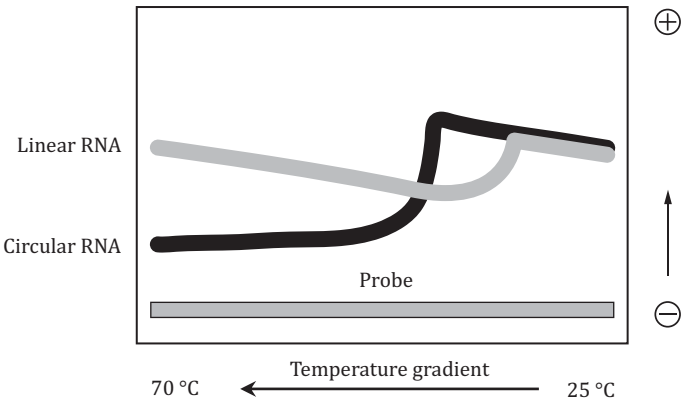

Figure 3.4-3: Separation of linear RNA from circular RNA using temperature gradient gel electrophoresis.

Mutations in mtDNA

Mitochondrial **DNA** (mtDNA) represents a small portion of DNA in the eukaryotic cells. In humans, its 16,569 base pairs encode for 37 genes [14]. In most species, including humans, mitochondrial DNA is inherited solely from the mother [15].

According to an investigation by Wong *et al.* [16], TGGE can be utilized to examine mtDNA of an individual. The same authors determined two novel mutations in the mitochondrial genome of a 21-year-old woman.

p53 mutation in pancreatic juice

Tumor **p**rotein **53** (p53), known also as cellular tumor antigen p53, phosphoprotein p53, tumor suppressor p53, or **t**ransformation-**r**elated **p**rotein **53** (TRP53), has obtained the name p53 because of its apparent molecular mass (M_r = 53,000) established by SDS-PAGE. However, the actual relative molecular mass of the full-length p53 protein (p53α), calculated on the sum of masses of the amino acid residues, is only 43,700. This difference is due to the high number of proline residues in the protein, which slow its migration in SDS-PAGE, thus making it appear heavier than it is actually [17].

The tumor protein p53 is encoded by homologous genes in various organisms, such as *TP53* (in humans). It is a tumor suppressor in the multicellular organisms [18]. Therefore, p53 has been described as "the guardian of the genome," because it prevents genome mutation [19]. *TP53* (*Italics* are used to denote the *TP53* gene name

and distinguish it from the protein it encodes) is classified as the tumor suppressor gene [20,21,22]. The human *TP53* gene encodes at least 15 protein isoforms, ranging in size from M_r = 3,500 to 43,700. All of them are called p53 isoforms.

The *TP53* gene is the most frequently mutated gene (> 50%) in human cancer, indicating that it plays a crucial role in preventing cancer formation. Because mutations of p53 has been extensively found in pancreatic carcinomas [23], the researchers were attempting to determine, if the mutation itself can be linked to the development of pancreatic cancer.

Detection of p53 gene mutations in pancreatic juice samples from patients with chronic pancreatitis was established by temperature gradient gel electrophoresis and single strand conformation polymorphism for exons 5–8 [24].

B-cell lymphoma

TGGE technique can be run also in precast 0.5 mm polyacrylamide gels. With the help of it, patients with B-cell lymphoma were treated with high-dosage radiochemotherapy followed by autologous transplantation for continuous presence of clonal (tumor-specific) rearrangements. Combining the PCR sensitivity with the TGGE specificity, it was possible to examine a great number of patients with B-cell neoplasias [25].

3.4.1.5 Temperature gradient focusing

Methods such as **i**so**e**lectric **f**ocusing (IEF) [26,27] and **e**lectric **f**ield **g**radient **f**ocusing (EFGF) [28,29,30] have been successfully implemented in microcolumn formats. The **t**emperature **g**radient **f**ocusing (TGF) [31] is a technique for simultaneous concentrating and separating of ionic compounds. It is a field gradient method based upon balancing the electrophoretic velocity of the analyte against the bulk solution flow.

Theory of temperature gradient focusing

Let us consider a **b**ack**g**round **e**lectrolyte (BGE) consisting of the monoprotic acid HA, which dissociates according to the process

$$HA \rightleftarrows H^+ + A^-$$

Its dissociation constant (a dimensionless magnitude)

$$K_a = \frac{[H^+][A^-]}{[HA]} \tag{3.4-1}$$

where $[H^+]$, $[A^-]$, and $[HA]$ are the equilibrium concentrations of H^+, A^-, and HA, respectively, and its ionic strength

$$I = \frac{1}{2}\sum_i c_i z_i^2 \approx \frac{1}{2}([H^+] + [A^-]) \tag{3.4-2}$$

where c_i and z_i are the concentration and charge number of the ith ion, respectively.

Using the dissociation constant from eq. (3.4-1), the last equation transforms to give

$$I \approx \frac{1}{2}\left(-K_a + \sqrt{{K_a}^2 + 4K_a c_a}\right) \tag{3.4-3}$$

where c_a is the acid concentration.

The dissociation constant of an electrolyte depends on the ionic strength of the solution [32], which in turn depends on the temperature, according to the van't Hoff equation [33,34]

$$\frac{d}{dT}\ln K_a = \frac{\Delta H^0}{RT^2} \tag{3.4-4}$$

where T is the absolute temperature, in K; ΔH^0 is the standard molar enthalpy, in J/mol; and R is the molar gas constant, equal to 8.314 46 J/(mol K).

To cover the pH range between 3 and 11, a combination of a weak acid and a weak base should be used. The ionic strength can be determined by a binary weak acid–weak base quadratic equation:

$$I \approx \frac{1}{2}([A^-] + [BH^+]) \approx \frac{\frac{K_a}{K_b}(c_a + c_b) - \sqrt{\frac{K_a}{K_b}\left(4c_a c_b + \frac{K_a}{K_b}(c_a - c_b)^2\right)}}{2\left(\frac{K_a}{K_b} - 1\right)} \tag{3.4-5}$$

where c_b is the base concentration, and K_b is the base association constant.

This equation shows that the temperature dependence of the ionic strength is determined by the ratio K_a/K_b, or the difference between pK_a and pK_b (Rilbe [35]). Therefore, a background electrolyte in the temperature gradient focusing requires a weak acid–weak base pair for which pK_a and pK_b have different temperature dependencies. For example, pairing 10 mmol/l acid with a pK_a of 7.0 and 7.6 at 20 °C and 80 °C, respectively, with 10 mmol/l base with a nontemperature-dependent pK_a of 7.0 would yield a change in the ionic strength from 5.0 to 3.3 mmol/l over 60 °C, or a relative change of 33% [36].

Running temperature gradient focusing

Temperature gradient focusing can be conducted at a wide range of pH values. Buffer suitability for it is assessed experimentally by concentrating and separating a pair of fluorescent analytes. One analyte is held at constant concentration for use, while the other dye is of varied concentration. Focusing and separation by

temperature gradient focusing was successfully conducted quantitatively in background electrolyte solutions of pH from 3.0 to 10.5 (Table 3.4-1).

Table 3.4-1: Background electrolytes for temperature gradient focusing.

Acid	Base	pH
1 mmol/l sulfuric acid	–	2.8
2 mmol/l phosphoric acid	–	3.0
1 mol/l MES	–	3.2
10 mmol/l benzoic acid	10 mmol/l 3-fluoroaniline	3.8
10 mmol/l acetic acid	10 mmol/l aniline	4.6
10 mmol/l acetic acid	20 mmol/l TRIS	4.7
0.5 mol/l TAPS	–	5.3
10 mmol/l phosphoric acid	15 mmol/l sodium hydroxide	7.0
1 mol/l boric acid	0.5 mol/l TRIS	7.5
30 mmol/l boric acid	1 mmol/l ethanolamine	7.9
10 mmol/l boric acid	10 mmol/l TRIS	8.3
1 mol/l boric acid	1 mol/l TRIS	8.5
10 mmol/l boric acid	10 mmol/l ammonium hydroxide	9.4
30 mmol/l boric acid	30 mmol/l ethanolamine	10.0
30 mmol/l boric acid	60 mmol/l ethanolamine	10.5
–	5 mmol/l arginine	11.0
0.35 mol/l TAPS	0.35 mol/l sodium hydroxide	11.3
–	0.5 mol/l TRIS	11.9
–	2 mmol/l triethylamine	12.0
–	3 mmol/l ethanolamine	12.1
–	0.5 mol/l morpholine	12.3

Acidic solutions have been used for resolution of nucleotides and nucleosides [37], chloride determinations in biological samples [38], and analyses of pharmaceutical amines [39]. Alkaline solutions have been used in sugar and carbohydrate analyses [40,41], as well as for protein mixtures [42]. It is anticipated that by expanding the working pH range of temperature gradient focusing, the flexibility of the technique will be increased.

Instrumentation of temperature gradient focusing

The temperature gradient focusing is run in appropriate apparatuses. The scanning can be performed as follows [43]: A 5 mm wide optical window was burned into a 3-cm long capillary prior to enclosure between two polycarbonate sheets. The sandwich assembly was then placed in a hydraulic press at 500 kg, heated to 200 °C, and then cooled to 120 °C. The device was placed in the temperature

gradient-focusing apparatus (Figure 3.4-4) consisting of two copper anchor blocks: one maintained at an elevated temperature *via* a thermoelectric heater, and the other maintained at 20 °C by thermostatic cooler, with the two anchors separated by a 2–5 mm long gap. The cold end of the capillary was inserted into a 360-μm diameter hole drilled into a polypropylene analyte reservoir, whereas the heated end was inserted through a Teflon-backed silicone septum into a polypropylene block connected by 0.3 cm outside diameter nylon tubing to a pressure controller mounted on a vertical translation stage to provide precisely controlled bulk hydrodynamic flow.

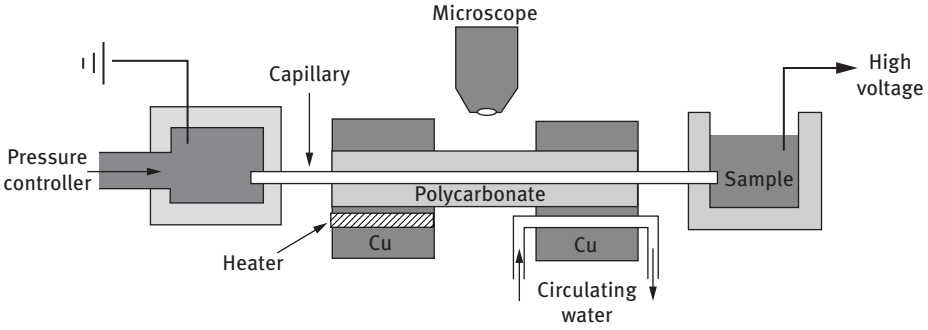

Figure 3.4-4: Scheme of scanning temperature gradient focusing apparatus.
A capillary is mounted in two copper blocks, one heated and one cooled by circulating water. Bulk hydrodynamic flow is controlled through a pressure controller coupled to the capillary outlet through a sealed and grounded polypropylene block. Detection is performed by fluorescence microscopy.

Applications of temperature gradient focusing

Temperature gradient focusing experiments were performed on a fluorescent microscope equipped with a long-working distance 10x objective, Hg arc lamp, color camera, and appropriate fluorescence filter sets. Electrophoretic data were evaluated with appropriate software [44].

Temperature gradient focusing has been successfully applied to wide variety of species, including small dye molecules, amino acids, DNA, and proteins [45,46].

3.4.2 Denaturing gradient gel electrophoresis of nucleic acids

Denaturing **g**radient **g**el **e**lectrophoresis (DGGE) was invented by Fischer and Lerman [47,48]. It is a molecular fingerprinting method for separating PCR-generated DNA products (or RNA) on gels that contain a denaturing agent [49]. Under these circumstances, DNA melts stepwise in strands, whose migration slows dramatically through

the gel. This makes it possible to discern differences in DNA sequences. Sequence differences in DNA fragments of the same length cause them to reach different positions in the gel. Placing two DNA samples side by side on denaturing gradient gels, it is possible to analyze DNA fragments, as small as 200–700 base pairs, and to detect mutations.

3.4.2.1 Applications of denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis finds diverse applications, for example in microbiology. DGGE of small ribosomal subunit coding genes was described by Muyzer [50], and has become a widely used technique. Besides, PCR amplification of DNA extracted from mixed microbial communities with PCR primers for 16S rRNA gene fragments of bacteria, and 18S rRNA gene fragments of eukaryotes results in mixtures of PCR products. Because all amplicons have the same length, they cannot be separated from each other by agarose gel electrophoresis. However, sequence variations between different microbial rRNA results in different denaturation properties of DNA molecules. This method is often referred to as community fingerprinting.

Studies have also shown that DGGE of functional genes can provide information about microbial function and phylogeny simultaneously. For instance, Tabatabaei *et al.* [51] applied DGGE to reveal the microbial pattern during the anaerobic fermentation of **p**alm **o**il **m**ill **e**ffluent (POME).

3.4.3 Protocols

3.4.3.1 Separation of nucleic acids by TGGE

Materials and equipment

Buffer
Silver staining

Procedure

- Pour gels.
  The gel buffer must remain stable within the increasing temperature.
- Place the sample on the gel parallel or perpendicular.
  Depending on which type of TGGE is to be run, either perpendicular or parallel, load varying volumes of sample. A larger volume of sample is used at perpendicular TGGE, while a smaller volume of sample is used at parallel TGGE.
- Adjust the voltage and run the electrophoresis.
- After the electrophoresis, stain the DNA bands. Silver staining has proven to be the most effective.
- Elute the stained DNA from gel and multiply it by PCR.

3.4.3.2 Denaturing gradient gel electrophoresis

Materials and equipment

Urea
Formamide
Glycerol
10 g/dl TMEDA
10 g/dl APS (*Make fresh*.)
Ethidium **b**romide (EB)
16 × 16 cm glass plate
16 × 14 cm glass plate
Spacers
Plate clamps
Pouring stand
Well comb
Gradient mixer
Electrophoresis system
Power supply

50x TAE buffer, pH = 7.4

TRIS	2.42 g (20.0 mmol/l)
Sodium acetate	0.08 g (10.0 mmol/l)
Adjust with acetic acid to pH = 7.4.	
Na_2EDTA	0.02 g (0.5 mmol/l)
Deionized water to	100.00 ml

Monomer solution, T = 4 g/dl, C = 0.025

Acrylamide/BIS (39:1)	10.00 g
Urea	42.00 g
Formamide	40.00 ml
10 g/dl TMEDA	0.02 ml
10 g/dl APS	0.02 ml
0.5x TAE buffer to	100.00 ml

EB-staining solution

10 mg/ml EB	0.05 ml
Deionized water to	500.00 ml

Stir for two hours and filter twice. Distribute in 1 ml aliquots. Store at −20 °C.

Procedure

- Place a 1 mm spacer along the edges of a glass plate.
- Place the second glass plate onto the first one.
- Place the entire assembly into the pouring stand.
- Make the denaturing solutions according to Table 3.4-2.

Table 3.4-2: DGGE gel composition.

Reagent	Concentration	Denaturant concentration					
		20 g/dl	30 g/dl	40 g/dl	50 g/dl	60 g/dl	70 g/dl
50x TAE buffer, ml	1x	0.30	0.30	0.30	0.30	0.30	0.30
40 g/dl AA*/BIS (37:1), ml	8 g/dl	3.00	3.00	3.00	3.00	3.00	3.00
Urea, g	Variable	1.30	1.90	2.50	3.10	3.70	4.40
Formamide, g	Variable	1.20	1.80	2.40	3.00	3.60	4.20
Glycerol, ml	2 ml/dl	0.30	0.30	0.30	0.30	0.30	0.30
10 g/dl TMEDA, ml	0.30 g/dl	0.05	0.05	0.05	0.05	0.05	0.05
10 g/dl APS, ml	0.40 g/dl	0.08	0.08	0.08	0.08	0.08	0.08
Deionized water, ml	Variable	9.50	8.50	7.50	6.50	5.50	4.50

AA* = acrylamide

- Use two solutions of 15 ml each: a *low* denaturant concentration solution, and a *high* denaturant concentration solution. For example, if you wish to make a 40–55 g/dl gradient, you should make a 40 g/dl (low) solution and a 55 g/dl (high) solution based on the recipes in Table 3.4-2.
- Add TMEDA and APS into each solution and swirl gently to mix.
- Pour the high-concentration mixture in the mixing cylinder of the gradient mixer.
- Fill the reservoir cylinder with the low-concentration mixture and switch on the magnetic stirrer.
- Open the tubing between the cylinders, and open the tubing that leads into the cassette and fill it slowly from above.
- Add 1x TAE buffer upon the gel solution to form a layer approximately 5 mm thick. This layer will help the top boundary of the gel to be smooth.
- Let the gel polymerize for about 1 h.
- Remove the TEA buffer with a syringe.
- Prepare the stacking solution, pour it on top of the gradient gel and insert the comb in the stacking gel.
- Let the stacking solution polymerize for another hour.

Running electrophoresis

- Remove the comb from gel.
- Preheat the TAE buffer to 65 °C and fill the buffer chamber with it.
- Load approximately 40–50 µl of PCR product containing tracking dye into each well.
- Place the lid on the electrophoresis unit.
- Reset the temperature to 60 °C and prerun the electrophoresis at 20 V for 10 min, then run at 200 V for 5 h (1,000 Vh).

DNA staining

- Disassemble the gel cassette.
- Put the gel in a tray with ethidium bromide solution and stain for 15 min.
- Destain the gel in deionized water for 5 min.
- Analyze the gel on an UV-transilluminator.

3.4.4 Troubleshooting

Problem	Cause	Solution
Prior to electrophoresis		
Polyacrylamide gels are too soft and sticky.	The concentration of acrylamide or BIS was too low.	Increase the concentration of acrylamide or BIS.
	The monomer solution or APS solution was overlaid.	Store the monomer solution in the refrigerator maximum for four weeks, and the APS solution for a week.
	The concentration of TMEDA or APS in the monomer solution was too low.	Apply 5 µl of 10 g/dl TMEDA and 5 µl of 10 g/dl APS solution per 1 ml of monomer solution.
The gel upper edge is sticky, swells at washing, and detaches from the support film.	The atmospheric oxygen has inhibited the polymerization of monomer solution.	After casting, overlay the monomer solution with deionized water.
The monomer solution polymerizes too fast.	The concentration of TMEDA or APS in the monomer solution is too high.	Check the recipe for gel casting. Reduce the concentrations of TMEDA or APS.
	The polymerization temperature was too high.	Cast gel at 20–25 °C.
Bubbles in the polyacrylamide gel.	The support film was unclean.	Do not touch the hydrophilic side of the support film with fingers.
	The glass plate that comes in contact with the monomer solution was dirty.	Wash the glass plate with dishwashing detergent or ethanol before use.
	The atmospheric oxygen in the monomer solution inhibited the polymerization.	Degas the monomer solution before casting.
The gel separates from the support film.	An incorrect support film was used.	Do not exchange the support films for polyacrylamide and agarose gels.
	The gel was cast onto the wrong side of the support film.	Cast gel only on the hydrophilic side of the support film. Check the site with water drops.

(continued)

Problem	Cause	Solution
The gel does not adhere to the support film, but to the glass plate.	The glass plate was too hydrophilic. The gel concentration is too low.	Treat the glass plate with Repel-silane. The polyacrylamide concentration T should be at least 4 g/dl at $C = 0.03$.
	The gel was left too long in the casting cassette.	Take the gel from the cassette 1 h after the polymerization started.
During electrophoresis		
There is too little or no electric current.	One of the connectors has no or poor contact. There is poor contact between the electrodes and electrode strips.	Check all connections. Weight the electrodes with a glass plate.
The gel "sweats" – is covered with water drops during the electrophoresis.	The monomer solution has no water-binding additives.	Add 20 g/dl glycerol or 10 g/dl sucrose to the monomer solution.
The gel "evaporates" – the separation chamber lid is covered with condensed water.	The voltage is too high.	Reduce the voltage.
The gel sparks and burns.	Thin spots in the polyacrylamide gel – the support film was not fixed on the glass plate during the gel casting.	Roll the support film with a roller on the bottom glass plates of the casting cassette to get an even contact between them.
The gel sparks along the edges of the support film.	The electrode strips extend over the gel edges out. The electric current flows under the support film.	Cut the electrode strips shorter than the gel width. Use kerosene or silicone DC-200 as a contact liquid between the cooling plate and support film.
After electrophoresis		
There are no nucleic acid bands in the gel.	The cathode and anode were exchanged. The mass of nucleic acid was too large; therefore the nucleic acid could not enter the gel.	Check the connection to the power supply. Diminish the total concentration T of the polyacrylamide gel or use an agarose gel.
The nucleic acid bands are very weak.	The concentration of the nucleic acids was too low or the sample volume was too small.	Concentrate the sample, apply greater volume, or use more sensitive staining methods.
The nucleic acid bands form tails.	The sample contained too much nucleic acid.	Apply smaller sample volume or dilute the sample.

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3.5 Capillary electrophoresis of nucleic acids

The capillary electrophoresis (CE) is very effective for DNA sequencing, fragment analysis, and DNA typing [1]. In CE methods, nucleates are separated according to their electrophoretic mobility and noncovalent interactions with the phase in the capillaries. Capillary electrophoresis has many advantages over slab gel electrophoresis in terms of speed, resolution, sensitivity, and data handling.

3.5.1 Theory of capillary electrophoresis

In free solutions, DNA nucleates have a constant size-to-charge ratio, and, as a result, their electrophoretic mobilities are independent of size. In an entangled polymer matrix, however, the friction coefficient of DNA nucleates increases with increasing DNA chain length, which results in decreases of their electrophoretic mobilities (Figure 3.5-1).

Formaldehyde and formamide denature RNA at a concentration of 2.2 mol/l [2,3], but they are highly toxic and carcinogenic substances [4,5]. In contrast, urea is a low-toxicity substance, but concentrations above 4 mol/l are required to interrupt the formation of intramolecular hydrogen bond in RNA [6]. However, high concentrations of urea cause a loss in the physical strength of agarose.

A strong denaturant to cleave intramolecular hydrogen bonds in RNA is required for RNA size separation in a small sample volume (<10 nl). Sumitomo *et al.* [7] have found that carboxylic acids (e.g., acetic acid) were strong denaturants for RNA, and the RNA separation performance was dramatically improved by capillary electrophoresis with a sieving matrix containing acetic acid. The same authors have established that the denaturing ability of 2.0 mol/l acetic acid was stronger than

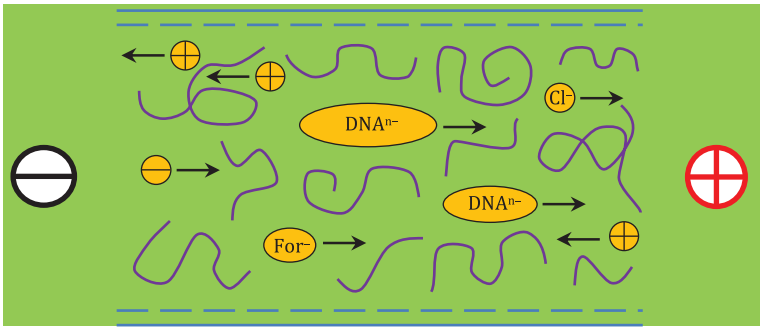

Figure 3.5-1: DNA migrates through entangled strands of a polymer twisting and tumbling through the gel. Small ions such as chloride (Cl^-) and formate (For^-) have higher electrophoretic mobility.

that of either 2.5 mol/l formaldehyde or 7.0 mol/l urea by estimating DNA melting temperature.

3.5.2 Instrumentation for capillary electrophoresis

The capillary electrophoresis instrument contains capillaries, coating polymers, and gels. It requires also a suitable sample injection module, a detector, adequate temperature control, and protection from high voltages used.

3.5.2.1 Capillaries

The capillaries are thin walled, which allows for dissipation of the Joule heating during electrophoresis. This minimizes convective effects that result in band broadening. The fused-silica capillary is coated on the outside with a polyimide layer that eliminates oxidation of the fused-silica glass. The polyimide sheathing is burned from a small portion of the capillary to expose a clear section of the silica. This section is placed in the light path of a UV or fluorescence detector and becomes the on-column flow cell. As DNA migrates through the capillary, it passes through the detector light and is measured.

3.5.2.2 Coating polymers

Usually, a coated capillary is utilized to eliminate the charge effects that are contributed by the native silica surface. With cellulose-derived polymers or some specially modified acrylamides, however, uncoated capillaries may be used, because of the strong interaction of the polymer with the inner surface of the bare fused-silica capillary, forming its own coating.

The separation of both ssDNA and dsDNA by CE uses a coated capillary and an uncharged sieving matrix. Separation matrices include a cross-linked polyacrylamide gel, non-cross-linked linear polyacrylamide, or a flowable polymer such as **h**ydroxy**p**ropyl **m**ethyl **c**ellulose (HPMC), **h**ydroxy**e**thyl**c**ellulose (HEC), or **p**oly**e**thylene **o**xide (PEO).

The synthesis and characterization of a novel, hydrophilic, replaceable, self-coating polymer matrix, **p**oly-N-**h**ydroxy**e**thyl**a**crylamide (PHEA), for application in DNA sequencing by CE is reported [8].

The hydrophilicity of N-**h**ydroxy**e**thyl**a**crylamide (HEA) is compared to those of **a**cryl**am**ide (AAM) and N,N-**di**m**e**thyl**a**cetamide (DMA) (Figure 3.5-2).

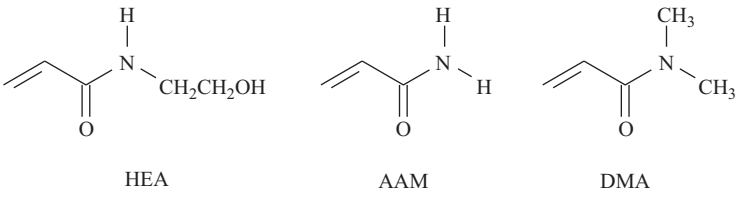

Figure 3.5-2: Chemical structure of HEA, AAM, and DMA.

The controlled radical polymerizations, especially the **a**tom **t**ransfer **r**adical **p**olymerization (ATRP), opened a new route to synthesize polymers with well-defined structures [9]. Carlmark and Malmstrom [10] grafted **m**ethyl **a**crylate (MA) onto cellulose fibers with controlled molecular mass by using ATRP.

Using ATRP, **h**ydroxy**e**thyl**c**ellulose-**g**raft-**p**oly**a**cryla**m**ide (HEC-g-PAM) for dsDNA separation by CE was presented [11]. The separation performance of HEC-g-PAM, which has the same graft density and different graft length, has been investigated in **T**RIS-**b**orate-**E**DTA (TBE) buffers. The results showed that dsDNA fragments between 72 and 1,353 bp were achieved at 150 V/cm with a 30-cm effective fused-silica capillary length.

An ideal separation medium for DNA separations using CE should possess the following properties: high sieving ability, dynamic coating ability, and relatively low viscosity. The separation medium with these specifications can be used for automation of CE and for further enhancing of its performance [12]. Homopolymers, such as **l**inear **p**oly**a**crylamide (LPA) and its derivatives [13,14,15,16], **p**oly**v**inyl**p**yrrolidone (PVP) [17], **p**oly**e**thylene **o**xide (PEO) [18], cellulose and its derivatives [19,20,21], are known.

It was proved that optimized linear polyacrylamide for capillary electrophoresis-sequencing applications can replace the cross-linked acrylamide gels that are used in slab gels. LPA is hydrophilic and forms strongly entangled networks that are excellent for high-quality DNA separations, and long sequencing read lengths

(up to 1,300 bases) can be obtained with highly pine DNA-sequencing samples [22]. LPA forms physical entanglements between chains of the network used to sieve the DNA as opposed to the chemical cross-links that form pores in the gels. Doherty *et al.* [23,24] showed that by adding very small amounts of cross-linking monomers into the polymer (the polymer solution still flowed) to form LPA "nanogels," sequencing performance could be improved compared with LPA synthesized in the absence of any cross-linker.

LPA can also be used as a polymer wall coating for channel surfaces by using the method developed by Hjerten [25]. This method creates covalently linked polymer chains on the surface of the channels to form the coating. The synthesis of the polymer coating is performed within the capillary and requires up to 10 h completing.

However LPA does not bind well to the silica surface to suppress electroosmotic flow in CE. Therefore, the use of LPA solution needs precoated capillaries. Another disadvantage of LPA is that additional high-pressure injecting equipment or manual injection is generally needed to fill capillary and replace solution because high-molecular-mass LPA solution is very viscous, which makes it difficult to achieve automatic replacement of sieving matrices.

In addition to linear polyacrylamide, the acrylamide derivative **p**oly(N,N-**d**i**m**ethyl**a**crylamide) (PDMA) has also been demonstrated as a high-quality polymer for sequencing DNA [26,27,28]. Sequencing matrices using pDMA generally have lower viscosities than LPA matrices and are also able to form coatings on the capillary surfaces, eliminating the need to perform chemical reactions at the capillary surface prior to polymer matrix loading and DNA fragment separation. Dynamic coatings are alternatives to covalent coatings because these coatings can be applied relatively quickly (45 min or less) and are extremely stable [29].

New copolymers have better alternatives to homopolymers, such as the following:

- copolymers of **p**oly-N,N-**d**i**m**ethyl**a**crylamide (PDMA) and **p**oly-N,N-**die**thyl**a**crylamide (PDEA) [30,31], LPA/PDMA [32,33], hydroxylated PDMA [34]
- graft copolymers of LPA-g-**p**oly(**N**-**i**so**p**ropyl**a**cryla**m**ide) (PNIPAM) [35], PNIPAM-g-PEO [36,37], PDMA-g-PMMA [38], **p**oly**a**cryla**m**ide (PAM)g-PDMA [39,40]
- polymer surfactants of **p**oly**p**ropylene **o**xide (PPO)-PEO [41,42,43], n-dodecane-PEO-n-dodecane [44], n-alkyl polyoxyethylene ethers [45], and so on.

Graft copolymers could form micelles with less number of chains compared with block copolymers. Even unimolecular micelles could often be formed by intramolecular association. Graft copolymer solutions should also have higher sieving ability than linear polymer solutions, since grafted points could prevent the polymer chains from sliding away from each other and thus could form relatively more stable pore sizes.

3.5.2.3 Gels

The frictional resistance of a gel can vary with its chemical composition, molecular mass, and concentration. Two types of CE gels are employed in DNA separations: cross-linked and non-cross-linked gels.

Cross-linked gels are polymerized inside the capillary and can be reused for 30 to 100 separations. They are usually covalently bound to the capillary surface. They are not removed from the capillary between runs. These gels are preferred when larger DNA molecules must be resolved – for example, for fragment analysis and DNA sequencing.

Non-cross-linked (flowable) gels are viscous hydrophilic polymer solutions that can be pumped into the capillary. The flowable polymer has the advantage that it can be expelled from the capillary by pressure. These capillaries have lifetimes of several hundred injections, being replaced for loss of the surface coating or mechanical breakage. The selection of the appropriate matrix can significantly affect the quality of the separation. Flowable polymers can be used for oligonucleotides and dsDNA fragment analysis.

Double-**s**tranded **DNA** (dsDNA) fragments of various sizes (e.g., 5 and 50 bp) have the same charge-to-mass ratio. Therefore, separation based on charge-to-mass ratio is not effective. Consequently, the separation can be based only on size, which requires a sieving matrix. Linear polymers, such as low- or zero-cross-linked polyacrylamide, polyethylene glycol, and methylcellulose derivatives [46], act as effective molecular sieves and allow for high-resolution separations within a narrow DNA size range.

3.5.3 Running capillary electrophoresis

Capillary electrophoresis can be run when a fused-silica capillary, filled with gel or liquid, joins two buffer tanks, and is located in a high voltage (10 to 30 kV). The resulting electric field drives the nucleic acids along the capillary. The capillaries are generally 20 to 50 cm long, have 50 to 100 μm internal diameter, and are coated from inside. The coating is important because the capillary wall becomes charged at high voltages, producing irreproducible migration shifts. Wall effects can affect the peak resolution [47].

Capillary electrophoresis of nucleic acids is carried out in three steps: injection, separation, and detection of the separated nucleic acids.

3.5.3.1 Injection

DNA samples are injected electrokinetically or by pressure into the sample tube and then into the capillary using a voltage of 5–15 kV for a few seconds. Buffer ions, such as Cl^-, move faster in an electric field than DNA. Therefore, the samples must be diluted with formamide or water [48] to reduce the ionic strength.

Formamide denatures the DNA. A disadvantage of formamide is its tendency to hydrolize over time giving formic acid and ammonia. Formic acid splits the positively charged hydrogen ion and the negatively charged formate ion. Formate ion competes with DNA for injection and reduces the method sensitivity. Forensic laboratories use 5 s injections at 15 kV, although longer injection times will enhance the method sensitivity [49].

DNA analysis by CE is performed in polymer buffers. These buffers can be easily pumped into a capillary prior to a separation and pumped out at its conclusion. A typical buffer for forensic DNA separation contains 4 g/dl **p**oly**d**i**m**ethyl **a**crylamide (PDMA), buffered to pH = 8.0 [50]. This polymer also coats the capillary wall.

3.5.3.2 Separation

The separation is done with nanogram mass of DNA in a small-diameter (50–100 μm) quartz capillary under a high voltage in a few minutes up to an hour [51,52,53]. In the presence of appropriate standards with known electrophoretic mobility, the physical properties of DNA can be examined [54]. The separation is performed in TBE buffer with alkaline pH. Urea is often included as a denaturant when analyzing ssDNA (e.g., synthetic oligonucleotides).

To the polymer and buffer in the capillaries, denaturants, such as urea and pyrolidinone, are added to keep the DNA single stranded. **S**ingle-**s**tranded **DNA** (ssDNA) is preferred because it interacts more efficiently with the sieving polymer, and its size is proportional to its length. Denaturants also prevent the complementary sequences in ssDNA molecules from binding to each other. This can create loops and hairpins. Elevated temperatures can minimize the formation of such structures; therefore, temperature must be carefully controlled, as it strongly influences DNA mobility and the calculated allele size [55]. Temperature affects also separations by altering the viscosity of the polymer solution.

To avoid problems with temperature and the building of secondary structure, DNA separations are performed with an internal sizing standard. Under these circumstances, if temperature or electric current fluctuates, the resulting shift in mobility will affect both the sizing ladder and sample. Two different methods can then be used to estimate allele size: a point-to-point method known as local Southern and a regression fit method known as global Southern. Both methods produce highly precise results [56]. However, the allele size estimate determined by these methods is a relative size. To find the true allele size, the result must be compared to allelic ladders. Because the analysis of the samples can take a day or more, size estimates may change. Therefore, the allelic ladder must be run a few times.

Separation times range from 10 to 45 min at 1 to 10 kV.

3.5.3.3 Detection

Capillary electrophoresis uses laser-induced fluorescence to detect DNA. In many CE systems, a 488-nm argon ion laser is focused onto a window burned in the polyimide coating of the capillary, providing efficient access to the sample.

The laser universally excites dyes added to the 5′-end of each DNA fragment. Once excited, the dyes emit fluorescent light at different wavelengths. The emitted light is separated into its constituent wavelengths and captured by a photoarray detector. Each dye has a characteristic emission spectrum.

However, the dye spectra overlap, making direct analysis of mixtures impossible. A set of four equations, known as a matrix, is used to determine the contribution of a given dye at a particular wavelength range (or virtual filter set). A computer is used to separate the individual contributions from the dyes at each data point along the pherogram.

DNA fragments can be detected also in the UV spectrum (at 260 nm) in the presence or absence of ethidium bromide. Sensitivity can be increased by at least two orders of magnitude by using fluorescence detection. Since DNA possesses no native fluorescence, intercalating dyes such as cyanine derivatives [57] or rhodamine derivatives must be covalently attached to the DNA prior to electrophoresis or added to the electrophoresis buffer. These intercalating dyes can also improve resolution and sharpening of the bands by physically disrupting the DNA structure. The selection of specific dyes is dictated by their excitation and emission spectra and by their compatibility with individual detection systems. Specific examples of intercalating dyes include Thiazole orange, YOPRO-1, YOYO-1, and Sybr Green (molecular probes).

The advantage of splitting the probe into two parts is that only the ligated oligonucleotides, but not the unbound probe oligonucleotides, are amplified. If the probes were not split in this way, the primer sequences at either end would cause the probes to be amplified regardless of their hybridization to the template DNA, and the amplification product would not be dependent on the number of target sites present in the sample DNA. Each complete probe has a unique length, so that its resulting amplicons can be separated and identified by electrophoresis. Comparing the peak pattern obtained on a given sample with that obtained on various reference samples, the relative quantity of each amplicon can be determined.

Most laboratories use peak height as the primary measurement of sensitivity. However, the analyst should understand that the signal-to-noise ratio, not the absolute intensity of a peak, determines sensitivity. An instrument showing low signal with minimal noise is just as sensitive as an instrument showing larger peak heights with higher amounts of noise. Different instruments will not always have similar sensitivities, and therefore laboratory results must be validated on each system. Unfortunately, as instruments age, peak intensity can drop due to dying lasers, dusty optics, or other problems. To keep track of this effect, the signal-to-noise ratio of the internal standard must be monitored.

3.5.4 Pulsed-field capillary electrophoresis

Another type of capillary electrophoresis is **p**ulsed-**f**ield **c**apillary **e**lectrophoresis (PFCE) [58]. It can be used not only for resolution of DNA chains containing above 1 kbp [59,60], but also for resolution of DNA chains into the Mbp range. Pulsed-field capillary electrophoresis can be performed in a gel or in an ultradilute polymer solution.

There is no theory for pulsed-field capillary electrophoresis in ultradilute polymer solutions. However, it is believed that DNA interacts mechanically with the ultradilute polymer solutions and the polymer leads to a transient entity, which has lower mobility than random-coil DNA.

Visualization experiments in 0.01 g/dl DNA has a size-dependent reorientation time, which depends on its length and on the electric field strength. At constant field, a DNA fragment collides with **h**ydroxy**e**thyl **c**ellulose (HEC), becomes elongated, and then collapses back to the random coil configuration. Long-chain DNA fragments are more stretched than short-chain fragments. As a result, they migrate together. PFCE in ultradilute polymer solutions containing buffer is used to separate long-chain dsDNA of below 10 kbp and greater than 1.5 Mbp in less than 4 min.

The capillary electrophoresis of double-stranded nucleic acids can be performed in cross-linked gels or entangled linear polymer solutions [61,62,63]. A permanent cross-linked polymer network or an entangled linear polymer network is needed to allow separation by sieving and reptation. Additionally, Barron and coworkers [64,65] demonstrated that dsDNA can be separated in buffers containing linear polymers, such as hydroxyethyl cellulose in low concentrations. The electrophoresis in low-viscosity polymer solution is fast. For example, using pulsed-field electrophoresis in an ultradilute polymer solution, separations of 200 kbp- to 1.6 Mbp-length nucleic acids in about 13 min were reported [66,67].

3.5.5 Applications of capillary electrophoresis

Schwartz *et al.* [68] demonstrated that 0.5 g/dl hydroxypropyl-methyl-cellulose having M_r = 90,000 allowed for adequate separation in the 72–1,353 bp range and a 3.0 bp peak capacity in the 100–200 bp size range. With this approach, Del Principe *et al.* [69] showed that CE is an effective tool for genomic analysis. They indicated that CE analysis is suitable for carrier detection and prenatal diagnosis of X-linked recessive disorders.

An important type of mutation comprises small exon deletions and duplications, which cannot be detected by SSCP or sequencing. Such deletions/insertions can be searched for using **m**ultiplex **l**igation-dependent **p**robe **a**mplification (MLPA) [70].

In MLPA, not the nucleic acids analyzed but probes added to the samples are amplified and quantified. This is a multiplex polymerase chain reaction that permits multiple targets to be amplified with only a single primer pair. Each probe consists of two oligonucleotides, which recognize adjacent target sites on the DNA. One probe oligonucleotide contains the sequence recognized by the forward primer; the other contains the sequence recognized by the reverse primer. Only when both probe oligonucleotides are hybridized to their respective targets, can they be ligated into a complete probe. All ligated probes have identical terminal sequences, permitting simultaneous PCR amplification using a single primer pair. Each probe generates an amplicon of unique size (130–480 bp), and probe target sequences are rather small (50–70 nucleotides).

CE systems with four- and five-color **l**aser-**i**nduced **f**luorescence (LIF) detection systems are used routinely for capillary DNA sequencing and genotyping (DNA fingerprinting) applications [71,72].

In its modern inception, the genome sequencing involves fragmenting the genome into small single-stranded pieces, followed by amplification of the fragments by polymerase chain reaction. Adopting the Sanger method, each DNA fragment is irreversibly terminated with the incorporation of a fluorescently labeled dideoxy chain-terminating nucleotide. So a DNA ladder of fragments is produced that differs in length by one base and bears a base-specific fluorescent label at the terminal base. Amplified base ladders are then separated by **c**apillary **a**rray **e**lectrophoresis (CAE) with automated detection of the fluorescently labeled ssDNA fragments, which provides an ordered sequence of the fragments. It is carried out in devices accommodating 96 capillaries or more to analyze many samples simultaneously. So, capillary array electrophoresis is nowadays the dominant technique for DNA sequencing [73]. These sequence reads are then computer assembled into overlapping or **contig**uous **s**equences (contigs), which resemble the full genomic sequence once fully assembled [74].

Optimized CE and fluorescence-labeled PCR appear to be the methods of choice in Huntington disease and sickle cell anemia diagnostics, often combined with Southern blotting.

Huntington **d**isease (HD) is a neurodegenerative genetic disorder that affects muscle coordination and leads to mental decline and behavioral symptoms. With the time, the uncoordinated body movements become more apparent and the mental abilities decline into dementia [75]. In 1993, a research group, using genetic linkage analysis [76], isolated the causal gene at 4p16.3 [77], so founding the first autosomal disease locus.

Sickle **c**ell **d**isease (SCD), also known as **s**ickle **c**ell **a**nemia (SCA), is a group of genetically blood disorders. It is characterized by a hemoglobin abnormality. This leads to a sickle-like erythrocytes under certain circumstances [78]. Temperature changes, dehydration, high altitude, and more may develop pain attacks (sickle cell crises), anemia, bacterial infections, and stroke [79]. Sickle cell disease occurs when a

person inherits two abnormal hemoglobin genes, one from each parent. A person with a single abnormal gene does not have symptoms and is referred to as a carrier [80].

The analysis of double-stranded DNA fragments, such as those found in polymerase chain reaction products and DNA restriction digests [81,82], has led to the development of a complete map of the human genome.

DNA crude product. By means of capillary electrophoresis, impurities can be proved better and faster than by HPLC. This is the case, for example, in the separation of crude DNA (Figure 3.5-3). The additional peak in the pherogram cannot be detected by the HPLC method. Besides, DNA appears in the capillary electrophoresis after 6 min, while it is being eluted after 28 min when using HPLC [83].

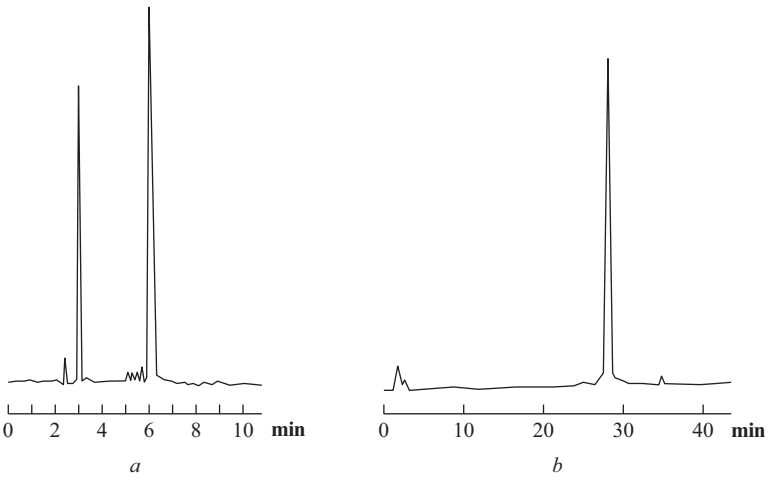

Figure 3.5-3: Capillary electrophoresis (*a*) and HPLC (*b*) of a DNA crude product.
The separation takes place in a TRIS-borate buffer with pH = 8.4 at a voltage of 20 kV. Detection at 254 nm.

CE has also been shown to be of use for analysis of larger DNA fragments. Heiger *et al.* [84] separated DNA fragments of 75 to 12,216 bp in a single run with a polyacrylamide matrix, and Strege and Lagu [85] separated fragments of DNA using 0.5 g/dl high-viscosity methylcellulose. Agarose solutions have also been applied to the CE separation of DNA fragments. Separations of large kilobase- and megabase-sized DNA has not yet been shown with the standard CE format. However, Guszczynski and Chrambach, using a constant voltage, 1-mm glass tubes, and linear polyacrylamide, separated the chromosomes of *Schizosaccharomycea pombe* (3-5 Mb) in 2 h [86].

3.5.6 Protocols

3.5.6.1 Quantitative PCR analysis

Quantitative PCR analysis can be used in conjunction with CE separation to amplify and quantitate DNA target sequence by coinjecting an intercalating dye with the samples or by using a covalently modified, fluorescently labeled oligonucleotide primer.

Materials and equipment

TBE buffer
Separating gel
65 cm 100 µm i.d. coated capillary
Standard 174 DNA ladder (in a concentration of 10 µg/ml and stored at –20 °C)
CE instrument with fluorescent detection

Procedure

- Prepare gel buffer mixture and add intercalating dye. Store at 4 °C up to 30 days.
- Reverse standard polarity of CE instrument electrodes.
- Rinse with buffer at high pressure for 10 min, if the capillary is new.
- Place standard DNA ladder in inlet position and load at low pressure for 10 s.
- Electrophorese at 9.4 kV and 25 °C for 30 min.
  Replace inlet gel reservoir after 30 injections.

3.5.6.2 Separation of oligonucleotides

DNA oligonucleotides are analyzed in the presence of urea to keep them in single-stranded configuration.

Materials and equipment

ssDNA oligonucleotide
TRIS-borate (TB) buffer
Separation gel solution
Poly$(A)_{40\text{-}60}$ sizing standard
60 cm 100 µm i.d. coated capillary
CE device

Procedure

- Reverse the standard polarity of CE device electrodes.
- Rinse a capillary with deionized water for 5 min.
- Fill the capillary with separation gel solution using a 20-min pressure rinse.
- Equilibrate the capillary in a running buffer increasing the voltage from 0 to 8 kV over 20 min and holding 8 kV for 10 min.

- Dissolve the poly$(A)_{40\text{-}60}$ sizing standard in deionized water to a concentration of 100 µg/ml, and inject at 7.5 kV for 10 s.
- Electrophorese at 8 kV and 30 °C for 40 min.
- Dissolve samples in deionized water to concentrations of 10 µg/ml.
- Load the sample solutions onto the autosampler, and inject them at 7.5 kV for 10 s.
- Electrophorese at 8 kV and 30 °C for 40 min.

3.5.7 Troubleshooting

Problem	Cause	Solution
During electrophoresis		
Air bubbles are introduced in the system.	If the capillary moves in its holder or buffer volumes become low, air bubbles may be introduced into the system.	Check, if the capillary moves in its holder or the buffer volumes become low.
Spikes are observed during the electrophoresis.	The system is not clean and free of buffer crystals. Therefore, high voltages can seek alternative pathways to ground. Listen during the run for sounds of sparking between the sample block and the autosampler.	Clean all vials used for capillary electrophoresis with deionized water and alcohol, and dry.
No signal or decreased signal.	Sample was prepared with water instead of formamide. High salt concentration. Salt inhibits injection of larger fragments. Degraded or improperly stored formamide solution. Expired or incorrectly stored reagents.	Prepare the sample with formamide Inject the majority of salt with the first injection. Use fresh or properly stored formamide solution. Use fresh reagents.
Size-standard peaks are not migrating.	Degraded polymer. Incorrect oven temperature. Low ionic buffer strength. Incorrect capillary length or run module. Variation in ambient temperature causes faster or slower migration rates.	Use fresh polymer. Check the temperature. Prepare fresh buffer. Specify correct capillary length or run module. Ensure ambient temperature is stable.
Electric current is too high.	Decomposition of urea in the polymer. Incorrect buffer formulation.	Use fresh polymer. Use correctly prepared buffer.

(continued)

Problem	Cause	Solution
After electrophoresis		
The results are inconsistent.	The voltage was not enough high.	Examine the voltage during the run.
Loss of resolution.	Incomplete replacement of polymer between runs.	Check the polymer delivery system for leaks, looking for residue in and around the polymer block area.
	Sample or reagent is contaminated.	Use fresh samples and reagents.
	High salt concentration. Salt inhibits injection of larger fragments.	Reinject the sample.
	Samples are degraded because they have been sitting in the instrument more than 24 h.	Run samples as soon as possible after preparation.
Poor resolution.	High sample concentration.	Dilute the sample before adding to formamide solution.
	Wrong capillary array or polymer used.	Use appropriate capillary array or polymer.
	Insufficient heat denaturation.	Make sure that samples are heated at 95 °C for 3 to 5 min, and then placed on ice for 2 to 3 min before loading.
Broad, lagging peaks.	Old or clogged capillary array.	Replace the capillary array or flush the capillary array with polymer.
Tailing peaks.	Degraded or improperly stored formamide solution.	Use fresh, properly stored formamide solution.
Extra peaks.	Degraded PCR products.	Repeat PCR.
	Sample is not denatured.	Make sure that samples are heated at 95 °C for 3 to 5 min, and then immediately placed on ice for 2 to 3 min before loading.
	Sample or reagent contamination.	Use fresh sample or reagent.
	Contamination with exogenous DNA.	Use appropriate techniques to avoid introducing foreign DNA.
	Renaturation of denatured samples.	Load the sample immediately after denaturation.
Missing size-standard peaks.	Incorrect concentration of size standard in sample loading reagent.	Increase the concentration of size standard added to subsequent runs.
	The injection time was too short.	Prolong the injection time.
	High salt concentration.	Salt injects smaller fragments and inhibits injection of larger fragments, so the majority of salt may have been injected in the first injection. Reinject the sample.

(continued)

Problem	Cause	Solution
Noise peaks are detected as size-standard peaks.	Contaminated sample.	Prepare new sample.
Amplified DNA concentration is lower than expected.	Amplification cycle setting is low.	Add three to five cycles.
	Low $MgCl_2$ concentration.	Increase the $MgCl_2$ concentration.
	Low affinity of the primer to the template.	Decrease the annealing temperature 2 to 3 °C at a time.
	Low sample concentration.	Increase sample concentration.
	Inhibitors in template.	Purify template.
	Thermal cycler malfunction.	Refer to the thermal cycler user guide for information.
	PCR reagents are contaminated or expired.	Use fresh PCR reagents.
	Degraded primers.	Store unused primers at –20 °C.
PCR inhibition.	Sample contains PCR inhibitors: hemoglobin, heparin, polysaccharides.	Dilute the sample before amplification to reduce the concentration of PCR inhibitors.
Noisy baseline.	Degraded or incorrectly stored formamide can cause low signal and degraded products.	Use fresh, properly stored formamide.
	Capillary is contaminated.	Perform a water wash.
	High salt concentration.	Salt injects smaller fragments and inhibits injection of larger fragments, so the majority of salt may have been injected in the first injection. Reinject the sample.
Smaller size-standard fragments are not labeled.	Size-standard peak and primer peak are in the same read region.	Remove the size-standard peak that overlaps with the primer peak.

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3.6 Microchip electrophoresis of nucleic acids

Since its introduction in the early 1990s by Manz *et al.* [1] and Harrison *et al.* [2], (**m**icro)chip **e**lectrophoresis (ME) (microfluidic electrophoresis, lab-on-a-chip) has become an important method for analyzing DNA fragments because of speed, miniaturization, small reagent volumes, high resolution, and automation [3,4,5]. Allowing for more rapid separations and smaller device footprints [6,7], ME is one of the most powerful analytical tools in recent years [8]. However, the sensitivity of microchip electrophoresis is still rather limited due to low sample loading.

The separation of DNA by microchip electrophoresis is achieved in a high-molecular-mass linear acrylamide-based polymer or a cellulose-based polymer. It is achieved by the mobility difference of DNA nucleates: small DNA molecules are able to pass through pores within the gels and elute first, whereas larger molecules are retarded by the gel and elute later. As a result, DNA can be sized, sequenced, and genotyped [9].

To separate PCR products, the polymer chains must be entangled, which increases the solution viscosity [10]. However, the increase in viscosity can require higher pressures and longer times to load a microchannel.

3.6.1 Theory of microchip electrophoresis of nucleic acids

Under influence of an electric field, the negatively charged nucleates migrate through a buffer with velocity v, in m/s, which can be expressed by the product of the electrophoretic mobility μ and the electric field strength E [11,12]:

$$v = \mu E \tag{3.6-1}$$

Since E is equal to the ratio between the voltage U and the length l between the electrodes, eq. (3.06-1) can be transformed to give

$$v = \mu \frac{U}{l} \tag{3.6-2}$$

As the length between the electrodes is a constant, the last equation shows that only the voltage controls the velocity of nucleates. An increase in voltage increases the

velocity of nucleate fragments and reduces the migration time. However, higher voltages increase the Joule heating too, which may lead to broader peaks, nonreproducible results, even boiling of the gel buffer. These effects can decrease the resolving power of the method.

The separation of DNA nucleates with different chain lengths in a gel of a given pore size is a function of the molecule size and the applied electric field [13].

Isotachophoresis (ITP) of nucleates is a method that permits loading of a large volume of a sample between a leading and a trailing electrolytes, and fractions it into narrow bands [14]. Its theory is based on the **K**ohlrausch **r**egulating **f**unction (KRF) [15]. ITP gel electrophoresis on a microchip has been used to analyze dsDNA. Wainright *et al.* [16] developed a buffer system where the separation buffer contained the leading electrolyte and a microfluidic chip with several arms where samples and the terminating electrolyte were injected. So an enrichment factor of 40 was achieved for PCR products. Xu *et al.* [17] developed another buffer system to perform electrokinetic injection for preconcentration and separation of DNA in a single microchannel with an enrichment factor of 100 for step-ladder DNA.

Electro**o**smotic **f**low (EOF) is substantially reduced in a **p**oly**e**thylene **o**xide (PEO) matrix due to dynamic coating [18]. However, at pH values higher than 7.0, the PEO coating is unstable [19]. **P**oly**v**inyl**p**yrrolidone (PVP) in a concentration of 0.5 g/dl also reduces the EOF and the adsorption of DNA fragments in the microchip.

3.6.2 Construction of a microchip for electrophoresis of nucleic acids

Usually a chip for electrophoresis of nucleic acids consists of separation channel, injection channel, and reservoirs. The separation channel can be 85 mm long, 50 µm wide and 20 µm deep, begins with a buffer inlet reservoir and ends with a buffer outlet reservoir (buffer waste). The injection channel can be 8.0 mm long and represent a double-T channel with a 100 µm offset, beginning with sample inlet and ending with sample outlet (sample waste). All reservoirs can have 2.0 mm diameter and can be 1 mm deep (Figure 3.6-1) [20].

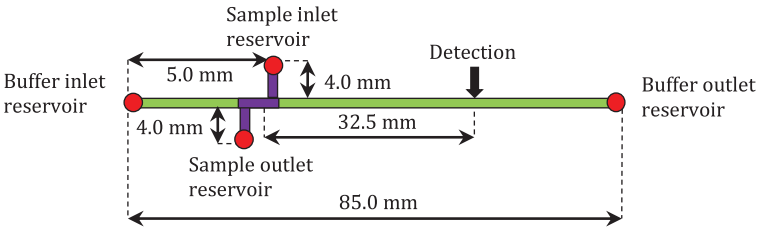

Figure 3.6-1: Schematic diagram of a microfluidic chip.

Electrokinetic injection is likely to suffer from injection bias [21]. A pressure introduction method for microchip ITP-zone electrophoresis was demonstrated by Bodor *et al.* [22] using a relatively complicated control unit. Santiago and coworkers [23] developed a 1D no dispersive model and used a high concentration of leading electrolytes to maximize the achievable enrichment factor as well as to suppress EOF. However, such buffer conditions are not compatible with gel electrophoresis separation.

An integrated **ITP**-**g**el **e**lectrophoresis (ITP-GE) device on a plastic substrate, in which 50 µl of samples could be hydrodynamically or electrokinetically injected and enriched by ITP into narrow bands and then subsequently introduced for separation and detection, was demonstrated [24]. This microchip design rendered a simple introduction scheme for creating sandwiched stacking buffer system and flexibilities in choosing separation and stacking buffers independently. Compared to conventional microchip gel electrophoresis, the sensitivity of microchip ITP-GE was estimated to increase by one to two orders of magnitude based on the dilution factor of the injected sample and the S/N ratio detected from the pherogram. Moreover, ITP stacking leads to an enhancement for analytes with lower concentrations compared to those with higher concentrations. Therefore, a reduction in the detection dynamic range for ITP-GE was gained. So ITP-GE could lead to two- to four-fold of reduction in the signal dynamic range for two PCR products in a mixture.

Fast DNA fragment separations have been reported in glass [25] and plastic [26,27] microfluidic devices.

3.6.3 Fabrication of a microchip for DNA electrophoresis

All microchips are fabricated by hot embossing method with a master fabricated on soda-lime glass substrates using photolithography techniques. After transferring the pattern to **p**oly**m**ethyl**m**eth**a**crylate (PMMA) substrates, reservoirs are created by drilling, and the PMMA substrate is sealed with a piece of **p**oly**e**thylene (PE) film [28] (Figure 3.6-2). Before experiments, the microchip is rinsed with 1 mol/l NaOH and then with deionized water.

The sequencing chip has a four-layer construction, consisting of three 100 mm diameter glass wafers (on which device elements are microfabricated) and a **p**oly**d**i**m**ethyl**s**iloxane (PDMS) membrane. Reaction chambers and capillary electrophoresis channels are etched between the top two glass wafers, which are thermally bonded. Three-dimensional channel interconnections and microvalves are formed by the PDMS and bottom manifold glass wafer.

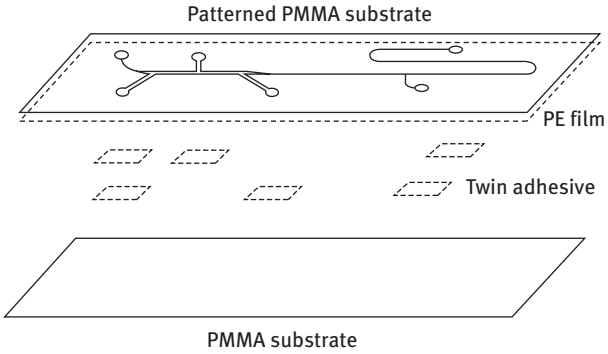

Figure 3.6-2: Bonding steps with **p**oly**e**thylene (PE) film.
A piece of PE film was attached to the patterned PMMA chip, and then several pieces of twin adhesives were attached to the reservoirs on the other side of the PE film. Finally, a blank PMMA substrate was attached on the PE film to support the substrate.

3.6.3.1 Polymers

The polymer solutions used as DNA sieving matrices define the quality of the separation [29,30]. Among them, the N-alkoxyalkylacrylamide polymers **p**oly(**N**-**m**ethoxy**e**thyl**a**crylamide) (pNMEA) and **p**oly(**N**-**e**thoxy**e**thyl**a**crylamide) (pNEEA) were investigated [31]. pNMEA showed a small increase in viscosity upon heating to 50 °C, but successfully sequencing. pNEEA was shown to exhibit a dramatic increase in viscosity above 36 °C, which, however, had poor sequencing results. The polymers pNMEA and pNEEA were synthesized *via* free-radical polymerization from the monomers **N**-**m**ethoxy**e**thyl**a**crylamide (NMEA) and **N**-**e**thoxy**e**thyl**a**crylamide (NEEA), respectively (Figure 3.6-3). The concentrations of the monomers are presented by their mass parts in the total monomer mix; for example, NMEA90-NEEA10 means 90% NMEA monomer and 10% NEEA monomer in the total mix.

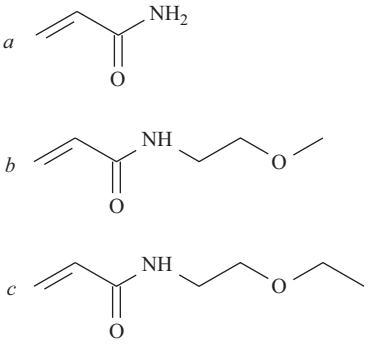

Figure 3.6-3: Monomer structures.
(*a*) Acrylamide; (*b*) N-methoxyethylacrylamide (NMEA); (*c*) N-ethoxyethylacrylamide (NEEA)

To produce the polymers, NMEA and NEEA monomers were dissolved in water at 1 g/dl total monomer concentration in a jacketed flask connected to a recirculating water bath of 25 °C. The solution was bubbled with nitrogen for 45 min prior to being initiated with 0.5 µl of 10 g/dl APS and 1 µl of 10 ml/dl TMEDA. After 4 h the solution was poured onto cut-off cellulose acetate dialysis membranes with M_r = 100,000 and dialyzed against deionized water for 10 days with frequent water changes. Thereafter, the solution was frozen and lyophilized [32]. According to the same authors, the N-alkoxyalkylacrylamide polymers can be used for PCR product and **r**estriction **f**ragment **l**ength **p**olymorphism (RFLP) sizing. At 25 °C, they can provide improved DNA separations compared with linear polyacrylamide in terms of reduced separation time and increased separation efficiency, particularly for larger DNA fragments. However, the DNA separation efficiency at 50 °C decreases significantly with increasing hydrophobic character of the polymers, and no separations are possible in solutions with a temperature below 50 °C.

3.6.4 Running microchip electrophoresis of nucleic acids

Various preconcentration techniques were developed and applied for microchip electrophoresis to increase the sensitivity, including high salt stacking [33], **f**ield-**a**mplified **s**ample **s**tacking (FASS) [34,35,36], sweeping [37], base stacking [38], porous membrane stacking [39], and **t**ransient **i**so**t**acho**p**horesis (TITP) [40,41,42].

Xu *et al.* [43] performed electrokinetic injection for transient isotachophoresis preconcentration. Santiago and coworkers [44] used high concentrations of leading electrolytes to maximize the achievable enrichment factor as well as to suppress the electroosmotic fluid in order to minimize dispersion effects. Wainright *et al.* [45] developed a buffer system, in which the separation buffer contained the leading electrolyte and the samples and trailing electrolyte were electrokinetically injected.

Kang *et al.* [46] proposed a TRIS-borate-EDTA buffer (pH = 8.3) with 0.5 µg/ml ethidium bromide for the ME run. The buffer waste was filled hydrodynamically by applying a vacuum. The dynamic coating matrix was made by dissolving 0.5 g/dl of M_r = 1,000,000 **p**oly**v**inyl**p**yrrolidone (PVP), and the sieving matrix was made by dissolving 0.5 g/dl of M_r = 8,000,000 **p**oly**e**thylene **o**xide (PEO). The DNA sample was injected by an electrokinetic injection into the injection region by applying a voltage of 480 V.

Another PCR buffer (pH = 5.5) was composed of 10 mmol/l TRIS-HCl, 50 mmol/l KCl, and 2.5 mmol/l $MgCl_2$ [47]. The leading buffer (pH = 7.2) contained 15 mmol/l HCl, 36 mmol/l imidazole, 1.8 g/dl **h**ydroxy**p**ropyl**c**ellulose (HPC), and 0.48 g/dl 2-**h**ydroxy**e**thyl**c**ellulose (HEC); the trailing buffer (pH = 7.8) contained 40 mmol/l HEPES and 160 mmol/l imidazole. Three separation buffers were used for DNA analysis: buffer A was the same buffer as the leading buffer; buffer B was

composed of 2 g/dl **h**ydroxy**p**ropyl**m**ethyl**c**ellulose (HPMC) in 1 x TBE (**T**RIS-**b**orate-**E**DTA) (pH = 8.4); buffer C contained 2.5 g/dl HPC and 1.5 g/dl HEC in a solution composed of 15 mmol/l HCl and 36 mmol/l imidazole (pH = 7.4). All the separation buffers were prepared by heating at 75 °C for 1.5 h, followed by overnight stirring at room temperature and then filtered by 0.45 μm cellulose acetate membranes. The concentration of DNA fragments was estimated by UV absorbance measured at 260 nm.

Woolley *et al.* [48] described ultrahigh-speed DNA fragment separations, using etched microchannels in glass having a length of only 3.5 cm. DNA fragments from 70 to 1,000 bp were separated in 120 s, 10 times faster than in conventional instruments. High-speed sizing of PCR-amplified alleles was also demonstrated. Fast DNA fragment separations were reported in glass [49] and plastic [50,51] microfluidic devices.

Xu *et al.* [52] enhanced DNA separation by poly(methyl methacrylate) microchip using low-viscosity solutions and some polyhydroxy compounds.

3.6.5 Applications of DNA microchip electrophoresis

DNA chips are used to purify products from unwanted primers and dimers in a polymerase chain reaction [53]. A side channel perpendicular to the separation channel was used to capture the 200-bp fragment during the separation of a 100-bp double-stranded (ds) DNA ladder [54]. Electric fields between microelectrodes ensured high-fidelity extraction of the targeted fragment [55].

Lin *et al.* [56] separated dsDNA by a PMMA electrophoresis microchip using polymer solutions containing gold nanoparticles. Later, Xu *et al.* [57,58] have developed a convenient single-step quantitation technique for separating specific dsDNA fragments in PCR products based on PMMA electrophoresis microchip with UV or fluorescence detection.

Fixe *et al.* [59] developed a method for direct immobilization of modified DNA oligonucleotides (aminated or thiolated) onto a PMMA substrate. Using the methyl esters present on nonmodified PMMA, they established covalent bonds between the electron donor of a DNA probe and the C-terminal ester of the PMMA substrate. Sassi *et al.* [60] have performed fast, parallel separations of alleles of the D1S80 locus in a multichannel plastic chip.

The analysis of single-base mutations in genomic DNA was carried out using a PMMA chip for detection of the allele-specific ligation detection reaction products using a universal array platform. Sung *et al.* [61] have used PMMA microchip electrophoresis for analysis of PCR products. The sequence flanking the gene was amplified by betaine-PCR and the amplified products were desalted and analyzed by microchips fabricated on PMMA substrates. Lee *et al.* [62] have developed a hot-embossed PMMA-PCR chip for amplifying a human cancer tumor-suppressing DNA

sequence. Liu *et al.* [63] have reported a method based on PMMA microchips for separation of multiplex PCR products.

A fast diagnosis by microchip electrophoresis was evaluated, using **p**rogrammed **f**ield **s**trength **g**radients (PFSG) in a conventional glass double-T microfluidic chip [64]. Compared to ME that used a constantly applied electric field, the ME-PFSG achieved 15-fold faster analysis time during the separation of 100 bp DNA ladder. ME-PFSG allows for ultrafast separation and enhanced resolving power for target DNA fragments. These results are based on electric field strength gradients that use an ME separation step in a sieving gel matrix polyethylene oxide. ME-PFSG technique was also applied for fast analysis of the PCR products, 591 and 1191 bp DNA fragments from the 18S rRNA of *Babesia gibsoni* and *Babesia caballi*.

Highly sensitive and rapid analysis of the methylated p16 gene in plasma and tissue DNA in cancer patients was realized by Zhou *et al.* [65] using a PMMA microchip.

Hashimoto *et al.* [66] have performed PCR, ligase detection reaction, and hybridization assays using flow-through PMMA microchip for the detection of low-abundant DNA point mutations.

Sanger method achieves read lengths of approximately 800 bp. The long-read lengths display significant advantages over other sequencing methods especially in terms of repetitive regions of the genome. Short-read sequences are typical for cancer genomes or for regions of chromosomes that exhibit structural variation [67]. In ME, the Sanger sequencing steps (thermal cycling, sample purification, and capillary electrophoresis) are integrated. This technology generates long and accurate sequence reads, while obviating many of the shortcomings of the conventional Sanger method.

An electroactuation-based MC device that leverages multifrequency actuation of samples and reagents droplets for chip-based nucleic acid extraction and real-time reverse transcription (qRT) PCR (qRT-PCR) amplification from clinical samples have been implemented [68]. This prototype micro-device combines chemical lysis with electric field-assisted isolation of nucleic acid in a four-channel parallel processing scheme. Furthermore, a four-channel parallel qRT-PCR amplification and detection assay is integrated to deliver the N-acryloyl-tris(hydroxymethyl) aminomethane (NAT) chip. The NAT chip combines dielectrophoresis and electrostatic actuation methods with resistive micro-heaters and temperature sensors to perform chip-based integrated NAT. The two-chip modules have been validated using different panels of clinical samples and their performance compared with standard platforms. Compared to the standard methods, this study reduces the sample and reagent volumes 10-fold.

3.6.6 Troubleshooting

Problem	Cause	Solution
The gel is not polymerized.	Use photoinitiation.	Restart the fabrication process.
Fluid is not wicking through channels.	Debris is present.	Filter the flushes with deionized water. If persists, incubate the debris with filtered NaOH for 10 min, or use methanol.
Bubbles in the gel after removing the chip from the UV oven.	Bubbles are not completely removed from the monomer solutions.	Sonicate the monomer solution until no bubbles are present.
	Extra air bubbles introduced by centrifugation or pipetting.	Carefully pipette when removing or deposing fluid.
Cloudy monomer solution after sonication.	Waste is mixing with the monomer solution.	Clean vacuum tube with bleach and water, or alcohol. Vacuum dry.

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3.7 Blotting of nucleic acids

Blotting of nucleic acid is the widespread technique for hybridization studies. It is used to understand the nucleic acid sequences, gene expression, gene organization, *etc*. The identification of abnormal genes in the genomic DNA has become important in the clinical research and genetic counseling. Blotting technique can be used to identify infectious agents present in the sample, and inherited diseases.

However, there are also disadvantages of blotting and hybridization techniques: The processes are complex, cumbersome, and time consuming. Blotting techniques also give information about the presence of DNA or RNA but do not give information about the gene regulation and gene interactions.

There are different nucleic acid blotting methods: Southern blotting, Northern blotting, reverse Northern blotting, Middle Eastern blotting, and more (Table 3.7-1). Different membranes are used for blotting of nucleic acids (Table 3.7-2.)

Table 3.7-1: Blotting methods for nucleates.

Blotting methods	Goals
Southern blotting	DNA
Northern blotting	RNA
Reverse Northern blotting	RNA
Middle Eastern blotting	polyA RNA

Table 3.7-2: Properties of membranes used for immobilization of nucleic acids.

	Nitrocellulose	Supported nitrocellulose	Uncharged nylon	Positively charged nylon	Activated paper
Application	ssDNA, RNA	ssDNA, RNA	ssDNA, dsDNA, DNA	ssDNA, dsDNA, RNA	ssDNA, RNA
Binding capacity (µg nucleic acid/cm^2)	80–100	80–100	400–600	400–600	2–40
Tensile strength	Poor	Good	Good	Good	Good
Mode of nucleic acid attachment	Noncovalent	Noncovalent	Covalent	Covalent	Covalent
Suitability for reprobing	Poor (fragile)	Poor (loss of signal)	Good	Good	Good

3.7.1 Blotting principles

As with proteins, nucleic acid blotting runs in three steps:

1. Transfer of nucleate bands onto a blot membrane. In principle, same blot membranes are used, as in Western blotting, made of nitrocellulose, Polyvinilydene difluoride, nylon, and other materials.
2. Blocking the free (unoccupied) binding sites of the blot membrane by a blocking agent.
3. Detection of the blotted nucleates by specific high-molecular ligands (probes), such as antibodies or DNA, staining, or autoradiography.

3.7.1.1 Transfer

The transfer of electrophoretically separated nucleates onto a membrane is necessary because nucleates in gel cannot react wholly with their specific probes. There are a few transfer methods for nucleic acids. Most commonly used are the capillary and vacuum transfers; less used are the diffusion transfer and electrotransfer. Polyacrylamide gels are blotted with a transfer buffer of low ionic strength.

Capillary transfer

The capillary transfer (blotting) is preferred because it acts gently on nucleic acids. It was performed by Southern (Southern blotting) in 1975 [1]. He transferred electrophoretically separated DNA fragments onto a nitrocellulose membrane and hybridized them with a radioactively labeled DNA probe. The blotting of RNA, known as Northern blotting (s. below), is also based on this technique [2,3].

In the capillary transfer [4], a buffer is sucked up into a stack of dry papers, which are placed on a blot membrane covering the gel with resolved bands. Usually a glass

plate is placed onto the paper stack together with extra ballast on it (Figure 3.7-1). In principle, the transfer is carried overnight. The paper stack acts with its capillary force on the gel and sucks the gel buffer upstairs, and the resolved nucleates stop on the blot membrane.

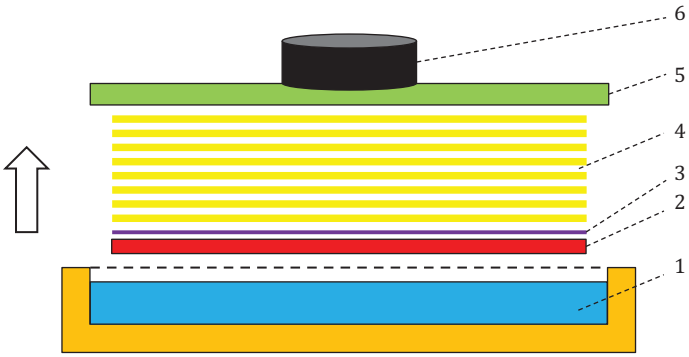

Figure 3.7-1: Capillary blotting.
1. Buffer; 2. Gel; 3. Blot membrane; 4. Filter papers; 5. Glass plate; 6. Weight

Vacuum transfer

The vacuum transfer (blotting) [5,6] requires a vacuum blotter, which makes a negative pressure (incorrect named vacuum) of 200–400 Pa (20–40 cm of water) using an adjustable pump (Figure 3.7-2). For this purpose, a water jet pump can be used. It should be made carefully, since the gel can be crushed. The transfer of nucleic acids using the vacuum blotting takes place in 30–40 min.

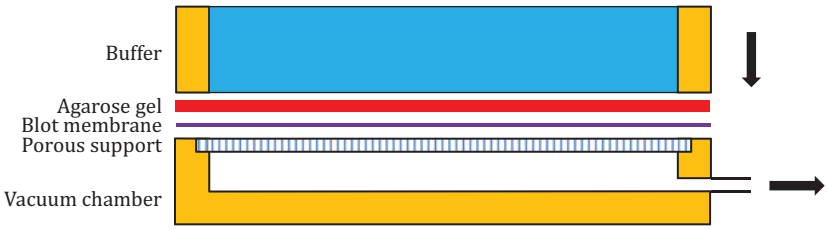

Figure 3.7-2: Vacuum blotting.

Diffusion transfer

In the diffusion transfer (blotting) [7], the blot membrane is placed on the gel surface and the nucleate transfer is carried out by diffusion (Figure 3.7-3). The blotting takes a long time – up to 36–48 h. The gel may also be blotted between two blot

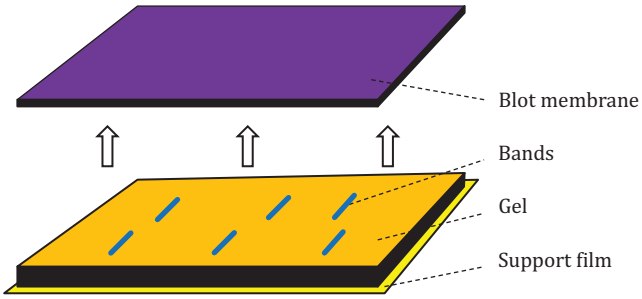

Figure 3.7-3: Diffusion blotting.

membranes, if it is not cast on a support film. The transfer can be accelerated by increasing the temperature what is referred to as thermotransfer (thermoblotting). In practice, this method is not used more as it takes too much time.

Electrotransfer

The electrophoretic transfer (electrotransfer, electroblotting) of nucleic acids is fulfilled when the gel, containing electrophoretically separated DNA or RNA, is clamped to a blot membrane in a cassette placed in an electric field between two plate electrodes. It is faster than the capillary transfer and takes place in a few minutes to 24 h. To electrotransfer bands from film-supported gels, the gel must be removed from the film [8,9]. Meanwhile, net-supported gels [10,11] are available, which are suitable for electroblotting.

The electrotransfer is applied in two versions: tank transfer (blotting) and semidry transfer (blotting).

The *tank transfer* [12] of nucleates is preferred to the semidry transfer. According to it, the net-supported gel with the separated nucleates and the blot membrane are applied between filter papers soaked with buffer. The blot sandwich is then introduced into a grid cassette and placed vertically between two platinum electrodes wires located in a tank with buffer. The buffer should be cooled so that the blot sandwich is not heated. The transfer is carried out in a few hours to overnight.

The *semidry transfer* of nucleic acids has significant limitations in reliability. Therefore, it has been optimized, giving now fast and good results [13,14].

In the semidry transfer, the electrodes are placed directly in contact with the gel-nitrocellulose membrane sandwich. The polyacrylamide gels must be equilibrated in a transfer buffer, to leave buffer salts and detergents. The nitrocellulose membranes and filter papers are wetted scarce (“semidry”). Using a platinum-coated titanium plate as an anode and a stainless-steel plate as a cathode, the transfer is carried in a horizontal location without a buffer tank or gel cassettes. Because of the direct contact, there is a minimum volume of transfer buffer required.

Simultaneous transfer

The simultaneous transfer (simultaneous blotting) can be used during vertical electrophoresis on polyacrylamide gels. During this procedure, the nucleic acids run from the lower gel end onto a horizontal membrane that moves slowly. Changing the speed of the membrane, the distance between the DNA and RNA bands can be programed.

3.7.1.2 Blocking

The unoccupied binding sites in the blot membrane should be blocked with certain substances in order not to participate in the subsequent detection reactions. To cover them, high-molecular blocking reagents are used, which are not taking place in the detection reactions. One of them is the Denhardt's buffer [15]. It contains 10 to 50 g/ml heterogeneous DNA with 0.02 g/dl bovine serum albumin, 0.02 g/dl ficoll, 0.02 g/dl polyvinyl pyrrolidone, 1 mmol/l Na_2EDTA, and 50 mmol/l NaCl in a 10 mmol/l TRIS-chloride buffer with pH = 7.0.

3.7.1.3 Detection by probes, dyes, and autoradiography

After the transfer and blocking, the nucleate bands can be bound with specific high-molecular ligands (probes) or dyes. For example, radiolabeled DNA or RNA probes can be hybridized with complementary DNA or RNA. To avoid radioactivity, the probes can be labeled only with the biotin-avidin system or digoxigenin.

If radioactivity is incorporated in the probes, the position of the blotted nucleates on the membrane can be determined by autoradiography: The blot membrane is placed in contact with photographic film and is exposed (hours to weeks) in the dark. The film is developed and the position of nucleates is revealed by the exposed parts of the negative. If required, the radioactive spots in the gel can be cut off, eluted, and scintillated.

Nucleic acids can be also stained, with silver. This process is similar to the silver staining of proteins (s. there).

Making blot membranes transparent

The nitrocellulose blot membrane can be made transparent, without the stained bands to change their intensity. For this purpose, it should be soaked with 2 g/dl benzoin in **t**ri**m**ethylol**p**ropane **t**ri**m**eth**a**crylate (TMPTMA). This solution has the same refractive index as the nitrocellulose and polymerizes with the aid of a photo initiator under UV irradiation.

3.7.2 Southern blotting

Southern blotting is created by the British biologist Edwin Southern [16]. Later, in reference to Southern's name, other blotting methods were named (i.e., Western

blotting, Northern blotting, Eastern blotting, Southwestern blotting, *etc.*), because they employ similar principles, but different electrophoretically resolved polyions.

Southern blotting is an analytical technique used to detect DNA sequence. After a gel electrophoresis, the separated DNA bands are transferred onto a membrane to become visible by hybridization with specific probes. The method is fulfilled in the following way:

- Using restriction endonucleases, high-molecular-mass DNA strands are cut into smaller fragments.
- The DNA fragments are electrophoresed in an agarose gel to be separated.
- The gel is placed into an alkaline solution to denature the double-stranded DNA.
- A nitrocellulose membrane is placed on the gel and a stack of filter papers and a weight is placed above to ensure a good contact between gel and membrane. As a result of originated capillary forces, the buffer moves from the gel into the filter papers and transfers the DNA bands from the gel onto the membrane. The transferred DNA bands bind to the membrane due to their negative charges and the positive charges of the membrane.
- The membrane is baked in a vacuum or regular oven at 80 °C for 2 h, or exposed to ultraviolet radiation to permanently attach the transferred DNA to the membrane.
- Then the membrane is exposed to a hybridization probe. The DNA probe is labeled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye. In some cases, RNA can be used for the hybridization probe rather than DNA.
- After hybridization, the excess probe is washed from the membrane, and the pattern of hybridization is visualized on X-ray film by autoradiography, if a radioactive or fluorescent probe is used, or by color reaction on the membrane, if a chromogenic method is used.

Hybridization of the probe to a specific DNA fragment on the membrane indicates that the DNA fragment contains a complementary DNA sequence. So Southern blots performed with restriction enzyme-digested genomic DNA may be used to determine the number of sequences (e.g., gene copies) in a genome. A probe that hybridizes only to a single DNA segment produces a single band, whereas multiple bands are observed when the probe hybridizes to several similar sequences.

Sequences that hybridize with the probe are further analyzed to obtain the full-length sequence of the targeted gene. Southern blotting can also be used to identify methylated sites in genes.

An example of native agarose gel zone electrophoresis of DNA, combined with Southern blotting, is the fractionation of the human cDNA clone, which expresses α-glucosidase (GAA) [17]. In patients where this enzyme is absent, the lysosomes are filling themselves with 1,4-glucose polymers, which causes muscle and liver damage.

Southern blotting also allows detection of larger gene alterations such as deletions, insertions, or rearrangements. Hereditary motor and sensory neuropathy type I, for example, is characterized by progressive distal muscle weakness and wasting between the ages of 10 and 30 years. Usually the disorder is a result of a duplication of 1.5 Mb of genomic DNA on the chromosome 17 short arm, including the gene for the **p**eripheral **m**yelin **p**rotein **22** (PMP22) [18].

Besides, Southern blotting can be used for separation of long stretches of repetitive sequences. For example, the fragile X syndrome characterized by mental retardation predominantly in males is caused by extensive $(CGG)_n$ expansions in the 5′-untranslated region of the FMR-1 gene. While healthy persons have < 50 copies of this triplet repeat, 50–200 units render this sequence unstable and likely to undergo further expansion during (female) meiosis. Sizes exceeding 200 triplets lead, in most cases, to the clinical phenotype of fragile X syndrome [19]. Stretches of 200–1,000 CGG repeats require conventional Southern blotting hybridization for the diagnosis in these patients [20].

3.7.3 Northern blotting

Northern blotting is a method, which is used to study RNA sequences with labeled RNA probes. It was developed by Alwine, Kemp, and Stark in 1977 at Stanford University [21]. The name of this technique comes from the similarity between it and the Southern blotting, developed by Edwin Southern. The difference between two methods is that in Northern blotting RNA, rather than DNA, is analyzed (Figure 3.7-4).

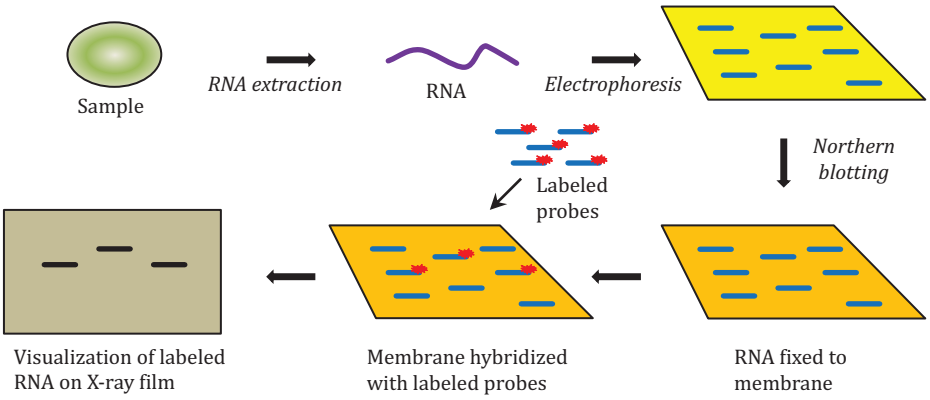

Figure 3.7-4: RNA detection by Northern blotting.

The procedure of Northern blotting starts with the extraction of total RNA from a homogenized tissue sample or from cells. From the total RNA, mRNA can be isolated

using oligo-(dT)-cellulose chromatography to maintain only RNAs with poly(A) tails [22,23]. Then electrophoresis in an agarose gel is carried out during which RNA are resolved by size. Since the agarose gels are fragile and the probes cannot enter them, the RNA samples are transferred onto a nylon membrane using a capillary or vacuum transfer system. For the Northern blotting, the nylon membrane is most effective since it is positive charged while RNA carries negative charges. The transfer buffer contains formamide because it lowers the annealing temperature of the probe-RNA interaction preventing RNA to degrade by high temperatures [24].

After RNA is transferred on a membrane, it is immobilized through covalent linkage to the membrane by UV light or heat. Then a labeled probe is hybridized to the bound RNA. At last the membrane is washed so that only the complexes between RNA and the probe remain, and the hybrid signals are detected by an X-ray film and are quantified by densitometry.

The probes for Northern blotting are composed of nucleic acids with a complementary sequence to RNA of interest. They can be DNA, RNA, or oligonucleotides with a minimum of 25 complementary bases to the target sequence [25]. The probes are labeled either with radioactive isotopes (^{32}P) or with chemiluminescence in which **al**kaline **p**hosphatase (ALP) or **h**orse**r**adish **p**eroxidase (HRP) break down chemiluminescent substrates producing an emission of light [26].

The chemiluminescent labeling can occur in two ways: either the probe is attached to the enzyme or the probe is labeled with biotin for which a ligand (avidin or streptavidin) is attached to the enzyme (e.g., HRP). X-ray film can detect both the radioactive and chemiluminescent signals, and many researchers prefer the chemiluminescent signals because they are faster, more sensitive, and reduce the health hazards that go along with radioactive labels [27].

Analysis of gene expression can be done by several different methods including **r**everse **t**ranscription **p**olymerase **c**hain **r**eaction (RT-PCR), RNase protection assays, microarrays, RNA-Seq, **s**erial **a**nalysis of **g**ene **e**xpression (SAGE), as well as Northern blotting [28]. The advantage that microarrays have over Northern blots is that thousands of genes can be visualized at a time, while Northern blotting is usually looking at one or a small number of genes [29,30].

A problem in Northern blotting is the sample degradation by RNases, which can be avoided by sterilization of glassware and the use of RNase inhibitors such as DEPC (**di**ethyl**p**yro**c**arbonate) [31]. The chemicals used in Northern blotting can be a risk to the researcher, since formaldehyde, radioactive material, ethidium bromide, DEPC, and UV light are harmful under certain exposures [32]. Compared to RT-PCR, Northern blotting has a low sensitivity, but its high specificity reduces false-positive results. The advantages of Northern blotting include the detection of RNA size and the observation of alternate splice products. The RNA bands on the membranes can be stored and reprobed for years after blotting.

With Northern blotting, it is possible to determine the gene expression rates during differentiation and morphogenesis, as well as in abnormal or diseased conditions

[33]. It allows observing the gene expression in tissues, organs, developmental stages, pathogen infection, and over the course of treatment [34,35]. The technique has been used to show overexpression of oncogenes and downregulation of tumor-suppressor genes in cancerous cells when compared to normal cells, as well as the gene expression in the rejection of transplanted organs. Except this, with the Northern blotting is possible to determine which region of the RNA is missing [36].

In Internet, the *BlotBase* exists, which is a database publishing Northern blots of human and mouse genes in different tissue types [37].

3.7.3.1 Reverse Northern blotting

The reverse Northern blotting is a variant of Northern blotting, in which DNA, rather than RNA, is first immobilized on a blot membrane and then is detected with labeled RNA probes.

The reverse Northern blotting is used to determine the levels of gene expression in particular tissues. In comparison to Northern blotting, it is able to probe a large number of transcripts at once [38]. It uses **s**uppression **s**ubtractive **h**ybridization (SSH) libraries to isolate differentially expressed transcripts and to create bacterial clones containing inserts for these sequences. They are probed by sample RNA.

The expression levels of particular RNA sequences in a tissue can be quantified by increase or decrease in fluorescent or radioactive signal over a control treatment. Dots, which appear darker versus a control sample, signify transcripts, which are overexpressed in the sample, and lighter dots indicate that a transcript is downregulated.

The membranes for reverse Northern blotting are prehybridized in Denhardt's solution with SSC (**s**aline-**s**odium **c**itrate) buffer. A 20x stock SSC buffer consists of 3 mol/l sodium chloride (NaCl) and 0.3 mol/l trisodium citrate ($Na_3C_6H_5O_7$) adjusted to pH = 7.0 with HCl. Then the labeled cDNA probes are denatured at 100 °C and added to the prehybridization solution. The membrane is incubated with the probes for at least 15 h at 65 °C, washed and exposed [39].

Northern blot is limited by its ability to probe only with one mRNA at a time, while q-PCR (quantitative PCR, also known as real-time PCR) requires transcripts to be long enough to generate primers for the sequence and probes can be costly. The reverse Northern blotting is used for confirming hits from **d**igital **d**roplet **PCR** (DD-PCR), a form of digital polymerase chain reaction. In this case, the membrane must be coated with amplified DD-PCR products, which have been cloned into vectors, sequenced and reamplified [40].

3.7.4 Middle-Eastern blotting

Middle Eastern blotting was described in 1984. It combines a blotting of polyA RNA, resolved in an agarose gel and its linkage with DNA probes [41].

3.7.5 Protocols

3.7.5.1 Southern blotting by downward capillary transfer

Downward capillary transfer is rapid and complete transfer.

Materials and equipment

TRIS-**b**orate-**E**DTA (TBE) buffer
Nondenaturing or denaturing polyacrylamide gel
Chromatographically clean filter paper

Procedure

- Run electrophoresis of DNA samples in a nondenaturing or denaturing polyacrylamide gel.
- Soak two pads in TBE buffer and remove air pockets by squeezing.
- Cut pieces of chromatographically clean filter paper of the same size as the gel and soak them also in TBE buffer for 15 min.
- Wet a nylon membrane with the dimensions of the gel in water for 5 min.
- Make a 2-3 cm stack of paper towels in a glass dish (Figure 3.7-5) and wet with transfer buffer.
- Wet the membrane and place it onto the filter paper, followed by the gel.
- Soak pieces of the same paper with transfer buffer and place on top.
- Place two pieces of the soaked paper onto the top and in a glass dish containing transfer buffer. Cover the sandwich with a glass plate to reduce evaporation, and transfer for 1 h.
- Remove the paper towels and filter paper. Mark with a pencil the positions of the wells on the membrane. Indicate up-down and back-front orientations by marking or cutting off one corner of the membrane.
- Place on a sheet of chromatographically clean filter paper, and dry at room temperature for 30 min.
- For nylon membrane, wrap in UV-transparent plastic wrap, place DNA-side-down on a UV transilluminator or UV light box, and irradiate. For nitrocellulose membrane, place between two sheets of filter paper and bake under vacuum at 80 °C for 2 h.
- Store between sheets of chromatographically clean filter paper or in a desiccator at room temperature.

3.7.5.2 Southern blotting onto a nylon membrane in an alkaline buffer

DNA binds covalently to a positively charged nylon membrane when transferred with alkaline buffer.

Materials and equipment

Saline-**s**odium **c**itrate (SSC) buffer, 20x

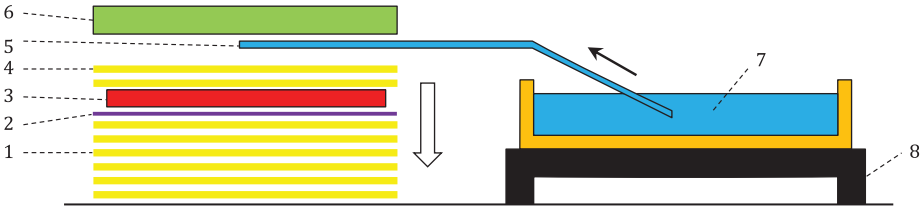

Figure 3.7-5: Setup for downward capillary transfer.
1. Paper towels; 2. Blot membrane; 3. Gel; 4. Paper sheets; 5. Tubing; 6. Glass plate; 7. Transfer buffer; 8. Support

Nylon membrane
0.4 mol/l NaOH for charged nylon membrane
0.25 mol/l NaOH with 1.5 mol/l NaCl for uncharged nylon membrane
Chromatographically clean filter paper
Platform shaker

Saline-sodium citrate (SSC) buffer, 20x

Trisodium citrate	7.74 g (0.3 mol/l)
Adjust with HCl to pH = 7.0.	
Sodium chloride	17.53 g (3.0 mol/l)
Deionized water to	100.00 ml

Procedure

- Digest DNA with restriction enzyme(s).
- Run electrophoresis in a nondenaturing or denaturing polyacrylamide gel or in an agarose gel together with DNA size markers.
- After electrophoresis, place the gel in a glass dish at room temperature and treat it as follows:
  Rinse with deionized water.
  Wash in 0.25 mol/l HCl for 30 min, shaking slowly on the platform shaker.
  Rinse with deionized water.
  Wash twice in denaturation solution.
  Rinse with deionized water.
  Wash twice in neutralization solution.
- Rinse a charged nylon membrane with deionized water and wash it in 0.4 mol/l NaOH for 20 min on a platform shaker. Use 0.25 mol/l NaOH with 1.5 mol/l NaCl for uncharged nylon membrane.
- Place a sponge, larger than the gel, in a glass or plastic dish and fill the dish with 20x SSC buffer. Cut three pieces of chromatographically clean filter paper of the same size as the sponge, wet them with 20x SSC buffer, and place on the sponge (Figure 3.7-6*a*).

- Otherwise, place a chromatographically clean filter paper wick in a dish with buffer (Figure 3.7-6b).
- Place the gel on pieces of chromatographically clean filter paper.
- Wet a nylon membrane by floating on water and place it onto the gel. Remove bubbles under the blot membrane by rolling gently a glass pipette over it.
- Flood the membrane surface with 20x SSC buffer, and place onto it five sheets of chromatographically clean filter paper and an 4 cm stack of paper towels, all cut to the same size as the membrane.
- Cover the sandwich with a glass plate and a 200–400 g weight, and leave overnight.
- Transfer for 2 h.
- Remove the paper towels and filter paper. Mark the membrane with pencil to indicate the position of wells. Indicate up-down and back-front orientations of the membrane.
- Rinse in 2x SSC buffer, place the charged membrane on chromatographically clean filter paper, and allow to air dry. For the uncharged membrane, immobilize DNA by UV-cross-linking

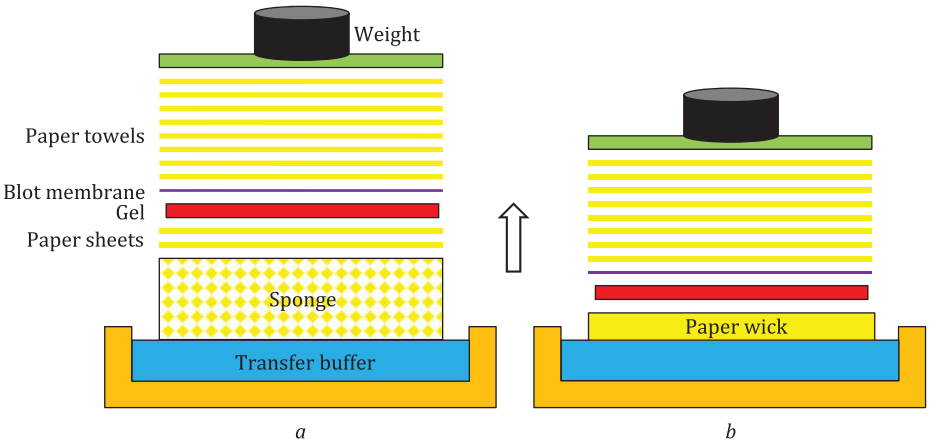

Figure 3.7-6: Setups for upward capillary transfer.
(*a*) Sponge method; (*b*) Wick method

3.7.5.3 Southern blotting with high salt buffer

Materials and equipment

Saline-**s**odium **c**itrate (SSC) buffer, 20x
0.25 mol/l HCl
Sponge
Chromatographically clean filter paper

Nylon or nitrocellulose membrane
254 nm UV transilluminator or UV light box

Denaturation solution

NaOH	2.00 g (0.5 mol/l)
NaCl	8.77 g (1.5 mol/l)
Deionized water to	100.00 ml

Neutralization buffer, pH = 7.0

TRIS	6.06 g (0.5 mol/l)
Add HCl until pH = 7.0.	
NaCl	8.77 g (1.5 mol/l)
Deionized water to	100.00 ml

Procedure

- Digest DNA with restriction enzyme(s), run electrophoresis in an agarose gel with DNA size markers, stain with ethidium bromide, and photograph.
- Place the gel in a clean glass dish and treat it at room temperature as follows:
  Rinse with deionized water.
  Wash 30 min in 0.25 mol/l HCl.
  Rinse with deionized water.
  Wash twice in denaturation solution.
  Rinse with deionized water.
  Wash twice in a neutralization solution.
- Place a sponge, larger than the gel, into a glass or plastic dish and fill the dish with 20x SSC buffer. Cut three pieces of chromatographically clean filter paper, wet them with 20x SSC buffer, and place on the sponge (Figure 3.7-6*a*).
- Otherwise, place a filter paper wick into a glass or plastic dish with a buffer (Figure 3.7-6*b*).
- Place the gel on chromatographically clean filter paper.
- Wet a nylon membrane in water for 5 min. For a nitrocellulose membrane, submerge in water, and then replace with 20x SSC and leave for 10 min.
- Place the membrane on gel. Remove bubbles from membrane by rolling gently a glass pipette over it.
- Flood the membrane surface with 20x SSC buffer, and place onto it five sheets of chromatographically clean filter paper and an 4 cm stack of paper towels, all cut to the same size as the membrane.
- Cover the sandwich with a glass plate and a 200–400 g mass, and leave overnight.
- Remove the paper towels and filter paper. Mark the membrane with pencil to indicate the position of the wells. Indicate up-down and back-front orientations of the membrane.

- Rinse in 2x SSC buffer, place on a sheet of chromatographically clean filter paper, and dry at 80 °C for 30 min.
- Place charged membrane on chromatographically clean filter paper, and allow to air dry. For uncharged membrane, immobilize DNA by UV-cross-linking.

3.7.5.4 Electroblotting from a polyacrylamide gel onto a nylon membrane

Materials and equipment

TRIS-**b**orate-**E**DTA (TBE) electrophoresis buffer
Saline-**s**odium **c**itrate (SSC) buffer
0.4 mol/l NaOH (for nondenaturing gels)
Chromatographically clean filter paper
Electroblotting cell with cooling coil and pads

Procedure

- Run electrophoresis of DNA samples in a polyacrylamide gel, stain and photograph it (if nondenaturing).
- Soak the gel in TBE for 20 min.
- Cut pieces of chromatographically clean filter paper to the same size as the gel.
- Wet a piece of nylon membrane with dimensions of the gel in water for 5 min.
- Cover the gel with a dry piece of chromatographically clean filter paper and lift the gel off peeling away the filter paper.
- Place one of the saturated pads onto a panel and cover it with soaked filter paper, carefully removing trapped bubbles by rolling gently a glass pipette over the surface.
- Flood the filter paper carrying the gel with TBE buffer and place on top of the stack the gel side-up. Flood the gel with TBE buffer and place the prewetted membrane on top. Flood the surface with 0.5x TBE buffer and place sheets of saturated filter paper on top, followed by the second saturated pad.
- Transfer at 30 V (~ 125 mA) for 4 h using precooled at 4 °C buffer, and a recirculating bath.
- Mark wells and orientation of the membrane after disassembly.
- If the gel is nondenaturing, denature the membrane for 10 min by placing DNA side-up on three pieces of chromatographically clean filter paper soaked in 0.4 mol/l NaOH.
- Rinse in SSC buffer, place onto a sheet of filter paper, and allow drying completely. Immobilize the DNA and store the membrane.
- For nylon membrane, place DNA side down on a UV transilluminator or UV light box, and irradiate. For nitrocellulose, place between two sheets of chromatographically clean filter paper and bake under vacuum at 80 °C for 2 h.
- Store between sheets of chromatographically clean filter paper or in a desiccator at room temperature.

3.7.6 Troubleshooting

Problem	Cause	Solution
Prior to blotting		
Air bubbles between the filter papers of blot sandwich.	The blot sandwich was not assembled under a buffer.	Roll the blot sandwich with a photo roller under a buffer to push out air bubbles.
No DNA precipitate.	DNA concentration is low.	Increase the DNA concentration, or use PCR method.
No DNA pellet after digestion.	Insufficient centrifugation.	Microcentrifuge at maximum speed and 4 °C for 15 min.
	Inadequate salt concentration.	Use 4 mol/l NaCl.
The nitrocellulose membrane is prewet unevenly.	The membrane is old or contaminated.	Use new nitrocellulose membrane.
The nitrocellulose membrane is getting fragile after baking.	The membrane is overbaked.	Check the oven temperature.
DNA floats, does not spread.	DNA is not digested.	Redigest the sample.
DNA spreads unevenly.	Excess salt in the pellet.	Redigest the sample.
	Inadequate enzyme activity.	Use fresh enzyme.
Uneven DNA separation.	Salt concentration is wrong.	Prepare fresh TBE buffer.
DNA dissipates out of well.	Residual ethanol in pellet.	Redigest the sample.
During blotting		
It does not flow or flows too little electric current.	One of the connectors has no or has poor contact.	Check all connections.
Low signal.	Insufficient probe concentration.	Relabel or use a different probe.
	Excessive washing.	Repeat transfer and wash at lower stringency.
	Inadequate DNA transfer.	Stain the gel after transfer to check.
	DNA is undigested.	Check the DNA digestion.
Specks and splotches.	Contaminated membrane.	Use new membrane.
	Hybridization solution is not equilibrated.	Warm the solution at room temperature for 1 h.
After blotting		
There is no transfer on the blot membrane.	The direction of blotting was wrong.	Blot toward the anode in an alkaline buffer.
The transfer is incomplete.	The transfer time was too short.	Extend the blotting time.

(continued)

Problem	Cause	Solution
The blotting results are looking badly.	Air bubbles in the blot sandwich.	Prior to blotting roll over the blot sandwich with a roller to push out air bubbles.
	The polyacrylamide gel was swollen during blotting.	Add 20 ml/dl methanol to the transfer buffer.
After blotting		
Loss of protein bands.	Low-molecular mass proteins (M_r < 20,000) may be lost during the post transfer washes.	Use glutardialdehyde fixation and a smaller pore size nitrocellulose membrane (0.2 µm).
The background of the blot membrane is too intensive.	The blocking was ineffective.	Block longer, use higher temperature (37 °C).
	Cross-reactions occurred with the blocking agent.	Use other blocking agents.
Low sensitivity of hybridization.	Low concentration of RNA.	Increase the RNA concentration up to 30 mg in each line. Use poly(A) RNA instead of total RNA; 10 mg of poly(A) RNA is ≈ 300–350 mg of total RNA (3–5%). Use optimal hybridization temperature. Use a freshly synthesized probe. Increase exposure time.
High background.	The probe contains repetitive DNA.	Use new probe of DNA.
	The hybridization solution is dried off on filter.	Wash the filter at low salt concentration and high temperature.
Cross-hybridization to rRNA sequences.	rRNA makes up ~ 80% of total RNA.	rRNA can nonspecifically bind to complementary sequence. Probe trapping by rRNA can be reduced by labeling only sequence complementary to mRNA.
Aberrant bands.	DNA digestion is incomplete. DNA contains contaminant (e.g., plasmid).	Redigest DNA with more enzyme. Homogenize new tissue.

(continued)

Problem	Cause	Solution
More bands after RNA hybridization instead of one expected.	Cross-hybridization.	The probe concentration was too high.
	The hybridization temperature was too low.	Increase the hybridization temperature up to 52 °C.
Specks and splotches.	Contaminated membrane.	Use new membrane.
	Probe not evenly mixed.	Mix hybridization solution thoroughly.
	Bubbles in hybridization solution.	Avoid bubbles, mix during hybridization.

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3.8 Surface electrophoresis

A new DNA electrophoresis, called **s**urface **e**lectrophoresis (SE), was emerged by Han and Craighead [1]; it involves electrostatic interactions between nucleates and surfaces [2,3].

Surface electrophoresis is carried out in a buffer on the surface of a flat silicon wafer. The separation of DNA nucleates is a result between their moving, from one side, and the friction of the surface and buffer, from other side. It arises from the different number of contacts due to the conformational differences between the adsorbed DNA chains. The friction can be controlled by coating the silicon surface with silane monolayer film, which permits the separation of different DNA sizes [4].

Electrophoresis on a flat silicon substrate is an effective method for separation of DNA of different configurations, for example, linear, supercoiled, and relaxed, or DNA of different length, for example, supercoiled DNA ladder [5]. Compared to the existing methods, which use topological constraints, surface electrophoresis has advantages, such as simple device fabrication, low electric field strength (< 10 V/cm), and a broad molecular size separation in the range of at least five orders of magnitude [6]. Furthermore, unlike other methods, the resolution of this technique is independent from the bp number, which is especially useful for separation of DNA chains of large size, for example, genomic DNA. However, the resolution of surface electrophoresis is poor, because it is strongly diffusion related.

3.8.1 Theory of surface electrophoresis

The theory of surface electrophoresis is still in development. Nevertheless, the following thoughts can be taken into account:

A single DNA chain on a surface is composed of a loop, a train, and a tail. Its length correlates with the ionic strength. Higher ionic strength leads to a smaller flexible DNA chain. The greater flexibility of the chain increases the train ratio (the ratio of the segment number in the train to the total chain segment number), which in turn increases the friction between DNA and the surface. Therefore, the mobility is getting smaller.

According to Li *et al.* [7], when migrating on a surface, a DNA chain experiences at least three different forces: driving force, buffer friction force, and surface friction force (Figure 3.8-1).

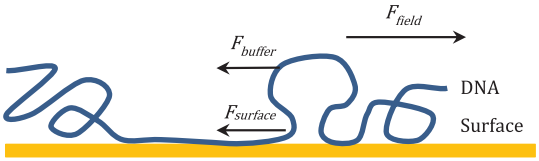

Figure 3.8-1: Forces experienced by a single DNA chain migrating in buffer on a surface under electric field.
F_{field} – driving force applied by the electric field; F_{buffer} – friction force created by the buffer; $F_{surface}$ – friction force created by the surface

Let us define E as the electric field strength, Q as the electric charge per bp, N_{bp} as the total number of bp, f_b as the friction coefficient per bp between the DNA chain and the buffer, v as the velocity of the DNA chain, f_s as the friction coefficient per bp between the DNA chain and the surface, ε as the interaction strength between one bp and the surface, and α as the train ratio. Then, the moving force

$$F_{field} = EQN_{bp} \tag{3.8-1}$$

the buffer friction force

$$F_{buffer} = f_b v N_{bp} \tag{3.8-2}$$

and the surface friction force

$$F_{surface} = f_s \varepsilon \alpha N_{bp} \tag{3.8-3}$$

If these three forces are in balance, then

$$EqN_{bp} = f_b v N_{bp} + f_s \varepsilon \alpha N_{bp} \tag{3.8-4}$$

Taking into account that the mobility

$$\mu \equiv \frac{v}{E} \tag{3.8-5}$$

from eq. (3.8-4) follows that

$$\mu = \mu_0 \left(1 - \frac{f_s \varepsilon \alpha}{QE}\right) \tag{3.8-6}$$

where

$$\mu_0 = \frac{Q}{f_b} \tag{3.8-7}$$

μ_0 is the free-buffer mobility when α equals zero. Since the mobility should be larger than zero, the threshold in the electric field strength is

$$E_{min} = \frac{f_s \varepsilon \alpha}{Q} \tag{3.8-8}$$

According to eq. (3.8-6), when $\alpha = 0$, that is, when there is no adsorption of DNA on the surface, μ is the free-buffer mobility. When $\alpha = 1$, that is, when there is fully adsorption of DNA on the surface, μ has a fixed value. Therefore, the length-dependent mobility is a function of α, which is a function of the chain length and has a value between 0 and 1.

Also according to eq. (3.8-6), μ increases with the electric field strength and would approach the free-buffer mobility when the electric field strength overwhelms the friction force of the surface.

3.8.2 Devices for surface electrophoresis

A construction of a device for surface electrophoresis, according to above-cited Li *et al.*, is given schematically on Figure 3.8-2.

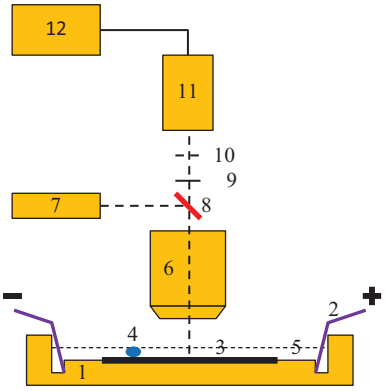

Figure 3.08-2: Surface electrophoresis.
Device for surface electrophoresis.
1. Separation cell; 2. Pt electrode; 3. Silicon substrate; 4. DNA droplet; 5. TBE buffer; 6. Lens; 7. Laser source; 8. Dichroic mirror; 9. Bandpass filter; 10. Pinhole; 11. PMT; 12. Computer.

Lee and Kuo [8] fabricated a gel-free microchannel electrophoresis device for separation of DNA fragments ranging from 3.5 to 21.2 kbp; DNA mobility on the glass substrate was higher than that on the silicon substrate. Another gel-free microchannel device made up of polydimethyl siloxane was fabricated for surface electrophoresis of double-stranded DNA [9].

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3.9 Evaluation of nucleic acid pherograms

The nucleic acids are colorless by nature. Therefore, they must be made visible after electrophoresis. This can be realized by counterion dye or silver staining, fluorescent substances, autoradiography, or protein probes. The results can be documented or evaluated quantitatively by densitometry or photography using a Gel Doc system. In addition, the bands with nucleic acids can be detected using absorption spectroscopy.

3.9.1 Counterion dye staining of nucleic acids

The counterion dye staining is applicable to nucleic acids after electrophoresis in agarose and polyacrylamide gels [1,2]. It is due to the interaction between negatively charged phosphate groups on DNA and cationic dyes, which intercalate between the base pairs of DNA. The small amount of dye used contributes to the improvement of the counterion dye staining.

The main counterion dyes for staining DNA on gels are **M**ethylene **b**lue (MB) [3,4], **B**rilliant cresyl **b**lue (BB) [5], **C**ristal **v**iolet (CV) [6], **N**ile **b**lue (NB) [7,8], and **I**odine **b**lue (IB) [9]. Methyl orange, a negative charged dye, is often used, because it reduces the background staining by couterion dyes (Figure 3.9-1). The couterion dye staining is simple, but its long destaining step and sensitivity is inferior to ethidium bromide staining.

The staining of DNA with 0.001 g/dl Crystal violet was used for agarose gels and could detect as little as 16 ng of DNA without destaining procedure. This

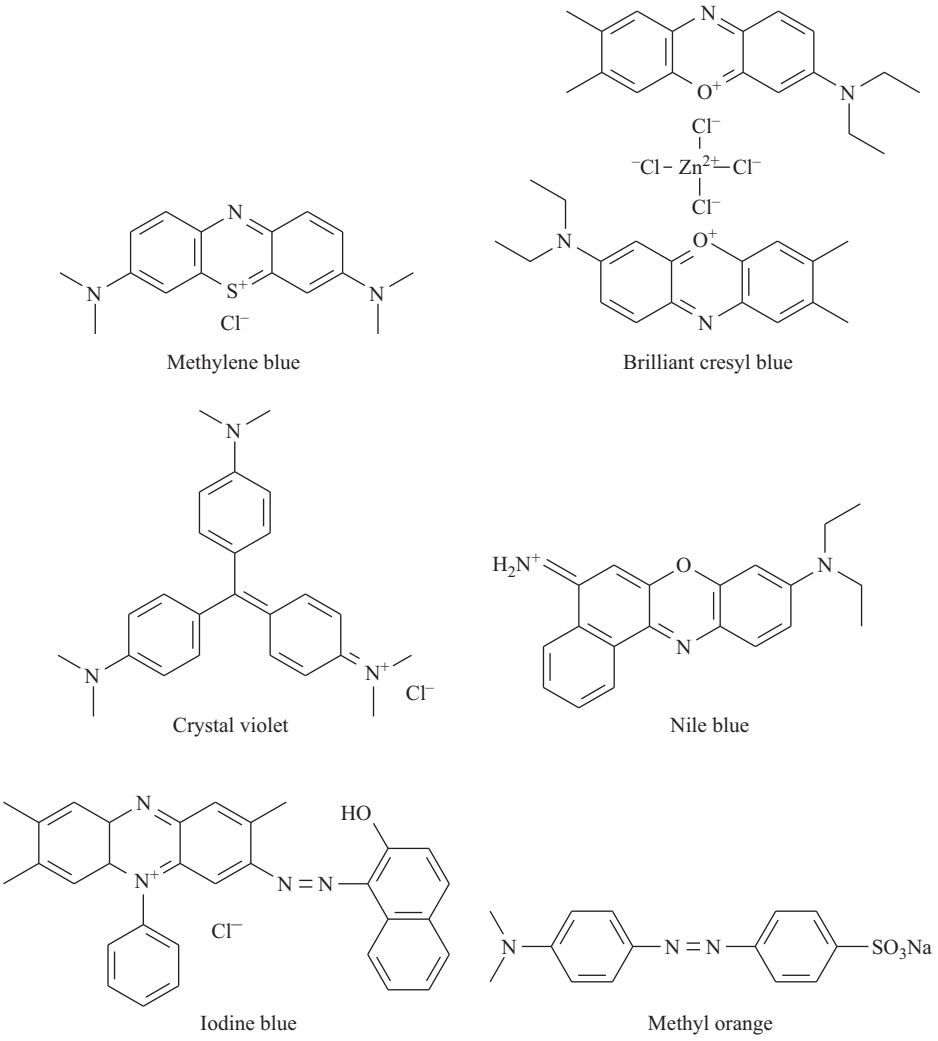


Figure 3.9-1: Chemical structures of the main counterion dyes.
Methyl orange, a negatively charged dye, reduces the background staining by the counterion dyes.

detection limit is four times lower than that of ethidium bromide. When the staining procedure is fulfilled with mixed solution of 0.0025 g/dl Crystal violet and 0.0005 g/dl Methyl orange, the sensitivity is increased by twofold and 8 ng of DNA can be detected within 30 min [10]. Crystal violet can be removed from the stained DNA band by destaining with 50 ml/dl ethanol for 2 h. This does not affect any sequential DNA manipulations, such as PCR, ligation, or transformation of plasmids.

Another sensitive staining method for DNA in agarose and polyacrylamide gels using a mixture of Indoine blue and Methyl orange was described [11]. Indoine blue is the main dye which has a positively charged group and six aromatic rings; Methyl orange is a dye which has a negatively charged group. The staining mixture has pH = 4.7, consists of 0.008 g/dl Indoine blue and 0.002 g/dl Methyl orange, and in addition of 10 ml/dl ethanol and 0.2 mol/l sodium acetate. It can detect 5–10 ng of DNA. The staining is fast and does not need a destaining of the gel background.

3.9.2 Silver staining of nucleic acids

Silver staining of proteins, which uses formaldehyde to reduce silver ions into silver atoms under alkaline conditions, was introduced by Switzer *et al.* [12,13,14]. Later, it was applied for nucleic acids in agarose gels and especially in polyacrylamide gels [15,16,17]. Silver staining enables the detection of 0.03 ng DNA or RNA per mm^2 [18,19], which corresponds to less than 50 pg nucleic acids per band. If the procedure is performed correctly, the bands with nucleic acids in a polyacrylamide gel are red-brown to dark brown on a light yellow to colorless background.

A rapid method for silver staining of PCR products separated on polyacrylamide gels was reported [20]. The number of steps was reduced compared to conventional protocols, thus achieving detection of PCR products in 7 min. The sensitivity was significantly improved – minimum of 0.097 ng/µl of DNA amount could be detected in denaturing polyacrylamide gels.

Silver staining has the same sensitivity as the radioisotopic methods. It provides higher sensitivity than ethidium bromide, however, and requires multiple steps, preparation, and handling of prior-to-use solutions, and the toxic chemical formaldehyde [21,22]. Besides, the sensitivity, color uniformity, and storage time of the stained bands are not always reproducible.

Silver staining of nucleic acids is similar to the silver staining of proteins and, therefore, comprises the same steps: fixing, sensitizing, developing, stopping, and drying.

Fixing causes precipitation of nucleic acids by denaturation. For this purpose, weak acids, such as acetic acid, are used, with or without alcohol, which lower the pH value of the solution and so prevent the proton dissociation from nucleic acids. As a result, the nucleic acids lose their negative charges and precipitate.

During *sensitizing* (impregnation), the precipitated nucleic acids bind silver ions. This process is performed usually in a neutral solution of silver nitrate and allows the building of silver nuclei.

Developing (visualizing) requires a strong reducing agent, such as formaldehyde, which reduces the silver ions to metallic silver. The reaction takes place much faster in the vicinity of silver nuclei than in the gel, since the silver nuclei catalyze it. So the nucleic bands transform in dark brown to black bands on a slightly yellowish to

colorless background. The developing takes place at higher pH values, for example, in a sodium carbonate solution [23,24].

After developing, the reduction must be quickly *stopped*; otherwise all the silver ions will be reduced to metallic silver, resulting in a black background. The stopping is caused in principle by a change of the pH value.

Drying is carried out in the air under a hair dryer or in a dryer. If the polyacrylamide concentration is high, the wet gel should be placed with the support film down on a glass plate, and should be covered free of air bubbles with a water-swollen cellophane membrane. The protruding edges of the cellophane membrane should be folded over onto the underside of the glass plate and the gel should be dried. The ultrathin polyacrylamide gels and agarose gels do not need cellophane covering.

Conventional procedures of silver staining involve several steps, which take about 40 min to 2 h in total. To accelerate the DNA staining, fixing was omitted and protocols were developed, which comprises only four steps (impregnating, rinsing, developing, and stopping), for example, in the method of Qu *et al.* [25].

Even quicker is the method of Han *et al.* [26]. According to it, nitric acid and ethanol in the impregnation step eliminate the need for prior treatment of polyacrylamide gels with a fixing solution and following rinse with water. The procedure can be completed within 10 min. The sensitivity is significantly improved by the silver-ion sensitizer **E**riochrome **b**lack **T** (EBT), which reduces the gel background staining and increases the sensitivity of the method [27]. The gel is first incubated in impregnating solution (0.1 g/dl $AgNO_3$ and 1 g/dl HNO_3) for 5 min at room temperature. Next, the impregnating solution is reclaimed, and the gel is quickly rinsed with deionized water for several seconds and developed at room temperature in 2 g/dl NaOH and 0.04 g/dl Na_2CO_3 with 2 ml 0.0025 g/dl Eriochrome black T and 1.5 ml 37 g/dl HCHO/l. When the gel color turns to yellow and all the bands of DNA fragments appear distinctly, the developing solution is discarded. Finally, the gel is rinsed in 2.5 g/dl ampicillin twice for several seconds each and can be photographed or stored for a long time. The minimum detectable mass of DNA using this method is 0.11 ng and 1.75 ng of DNA mass in denaturing and non-denaturing polyacrylamide gel, respectively.

Compared with the conventional silver staining methods, the method of Han *et al.* saves time and displays high sensitivity (Table 3.9-1).

3.9.3 Fluorescence methods for detecting nucleic acids

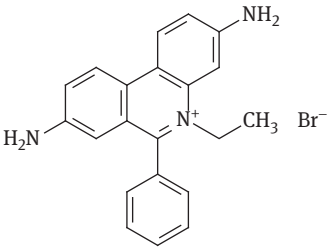
The localization of nucleic acids in gels is most performed by fluorescence detection methods. For this purpose, ethidium bromide as well as SYTO 13, TOTO-1, YOYO-1, GelRed, and more are used.

Table 3.9-1: Procedures and comparison of diverse methods for DNA silver staining.

	Method of Bassam *et al.* [28]	**Method of Carlos *et al.* [29]**	**Method of Qu *et al.* [30]**	**Method of Ji *et al.* [31]**	**Method of Han *et al.* [32]**
Fixing	10 ml/dl ethanol and 0.5 ml/dl acetic acid for 3 min	10 ml/dl acetic acid for 20 min	–	–	–
Rinsing	H_2O for 2 min, twice	H_2O for 2 min, thrice	–	–	–
Impregnating	10 ml/dl ethanol and, 0.5 ml/dl acetic acid, and 0.2 g/dl $AgNO_3$ for 5 min	0.1 g/dl $AgNO_3$ and 1.5 ml 37 g/dl HCHO/l for 30 min	25 ml/dl ethanol, 1 g/dl HNO_3 and 0.2 g/dl $AgNO_3$ for 2–5 min	0.1 g/dl $AgNO_3$ for 10–15 min	1 g/dl HNO_3 and 0.1 g/dl $AgNO_3$ for 5 min
Rinsing	H_2O for 2 min, twice	H_2O for 20 s, optional	H_2O for 2 min	H_2O for 5 s, twice	H_2O for 5 s, twice
Developing	3 g/dl NaOH and 0.1 g/dl HCHO for 5 min	3 g/dl Na_2CO_3, 1.5 ml 37 g/dl HCHO and 2 mg/l $Na_2S_2O_3$ for 2–5 min	3 g/dl Na_2CO_3 and 0.2 g/dl HCHO for 2–5 min	2 g/dl NaOH, 0.04 g/dl Na_2CO_3 and 2 ml 37 g/dl HCHO for 5–6 min	2 g/dl NaOH, 0.04 g/dl Na_2CO_3, 2 ml 0.0025 g/dl EBT and 1.5 ml 37 g/dl HCHO for 5 min
Stopping	10 ml/dl ethanol and 0.5 ml/dl acetic acid for 5 min, then in H_2O for 10 min	10 ml/dl acetic acid for 5 min	10 ml/dl acetic acid for 2–5 min	2 g/dl NaOH for 5 s	2.5 g/dl amp for 5 s, then H_2O
Sensitivity denat. DNA	3.50 ng	0.44 ng	0.44 ng	0.44 ng	0.11 ng
Sensitivity native DNA	14 ng	14 ng	3.5 ng	3.5 ng	1.75 ng
Background	lightest	dark	light	golden yellow	golden yellow
Used time	~40 min	~60 min	~20 min	~20 min	~10 min

3.9.3.1 Fluorescence detection of nucleic acids with ethidium bromide

Ethidium **b**romide (EB) is the most popular and commonly used reagent to detect nucleic acids (DNA and RNA). Its molecule has four flat atom rings, which intercalate between adjacent purine and pyrimidine rings of DNA or RNA.



Ethidium bromide

In intercalated state, nucleates absorb ultraviolet (UV) light (electromagnetic radiation with a wavelength from 10 to 400 nm) and emit it again in the visible range (560 nm). As a result, the complexes nucleic acid-ethidium bromide are seen in agarose gels as orange-red fluorescent bands on a dark mostly nonfluorescent background [33] (Figure 3.9-2). The DNA mass should be 1 ng or more per band.

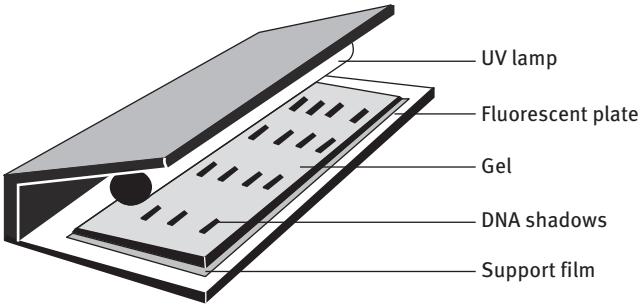


Figure 3.9-2: Visualizing the separated nucleic acids by ethidium bromide under UV light.

At a wavelength of 254 nm, DNA and RNA suffer considerable damages: breaks, cross-links, and base substitutions. For this reason, it is reasonable to irradiate nucleates at a greater wavelength. At 302 nm, ethidium bromide emits an orange-red light with less energy than at 254 nm, which damages less DNA and RNA. Therefore, DNA and RNA, when "colored" with ethidium bromide, are usually irradiated at 302 nm.

The shorter the DNA fragments, the weaker they become "stained" with ethidium bromide. Since the larger fragments provide more binding sites for ethidium bromide, they are stronger "colored". In contrast to the radioactive labeling, all polyion ends of the DNA fragments are affected equally.

In preparations of DNA with minimal RNA contamination or in DNA samples having an unusually high guanine and cytosine (GC) content, ethidium bromide offers an alternative to the more popular Hoechst 33258 DNA assay [34,35,36]. For this assay, a fluorimeter capable of an excitation wavelength of 302 or 546 nm is required.

Ethidium bromide can be added prior to or after electrophoresis. If ethidium bromide is added to the sample prior to electrophoresis, it combines with the double chain nucleates and its positive charges neutralize partially the negative charges of DNA. As a result, the DNA-ethidium bromide complexes move by about 15% slower than the "uncolored" DNA. If ethidium bromide is added after the electrophoresis, the agarose gel after a submarine zone electrophoresis must be kept at room temperature for 45 min in a TRIS-borate-EDTA buffer containing 0.5 μg/ml ethidium bromide.

Ethidium ion is positively charged, and will migrate in the opposite direction from the DNA. Such gels will have an area of high background where the ethidium ion has not yet migrated out of the gel.

The ethidium bromide staining is most used in agarose gels since the staining in polyacrylamide gels is weaker. Besides, the polyacrylamide gels must be disassembled, if they are cast onto a glass plate, since the glass is opaque to UV radiation.

The "colored" with ethidium bromide nucleates in agarose gels are usually photographed under UV light. In order to achieve good results, the film must be optimally exposed. If the exposure time is too short, the band contours are too weak; on the contrary, under too much light the image is too bright and the details of the pherograms are barely visible.

Ethidium bromide is a mutagen that requires care when being handled and discarded, as well as the UV irradiation may cause damage to both the DNA sample and experimenters [37,38,39]. To avoid the strong mutagenic effect of ethidium bromide, a fluorescent method using **b**er**b**erine/**M**ordant Yellow **3R** (BB/M3R) has been published [40].

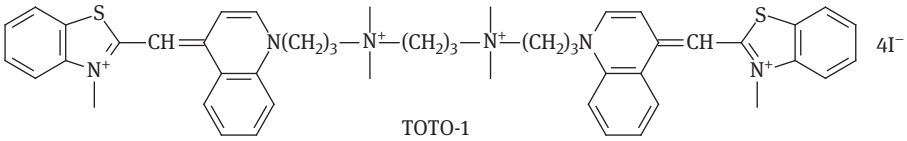
3.9.3.2 Fluorescence detection of nucleic acids with other dyes

In addition to ethidium bromide, three fluorescent substances (SYTO 13, TOTO-1, and YOYO-1) are used for staining of DNA and RNA [41,42]. TOTO-1 and YOYO-1 represent dimers of the compounds of TO-PRO-1 (Benzothiazolium-4-quinolinolium) and YO-PRO-1 (benzoxazolium-4-quinolinolium). Except high sensitivity, SYTO 13, TOTO-1, and YOYO-1 dissociate difficultly from nucleic acids. Therefore, DNA can be "colored" with them prior to electrophoresis.

SYTO 13. SYTO 13 is a cell-permeable fluorescent dye, which has high fluorescent yield when bound to DNA or RNA. It allows the detection of more microparticles as compared with a light scatter. **M**icro**p**articles (MP) are small membrane-bound vesicles, which contain DNA and RNA including ribosomal RNA, messenger RNA, and microRNA. They are released from activated or dying cells. Microparticles can display potent biological activities that impact on inflammation and thrombosis.

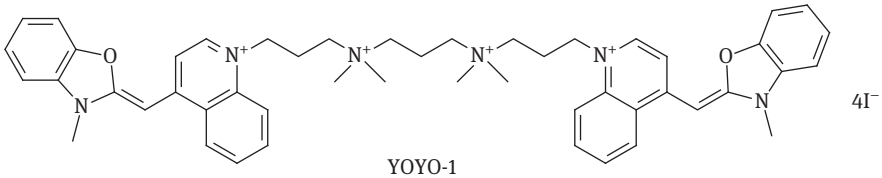
They can show increased numbers in diseases such as atherosclerosis, sepsis, and rheumatoid arthritis [43,44,45]. Their small size, however, limits their detection using flow cytometry with either light scatter or staining for surface markers. Improved detection of microparticles was demonstrated using flow cytometry after staining with propidium iodide [46]. So the use of SYTO 13 eliminates the need for fixation of nucleic acids.

TOTO-1. TOTO-1 iodide (quinolinium, 1-1-[1,3-propanediylbis[(dimethyliminio)-3,1-propanediyl]]bis[4-[(3-methyl-2(3H)-benzothiazolylidene)methyl]]-tetraiodide 143413-84-7) is a cell-impermeant high-affinity nucleic acid stain based upon a symmetric cyanine dye dimer that is nonfluorescent in the absence of nucleic acids but exhibits excitation (emission maxima at 514/533 nm) when bound to nucleic acids.



TOTO-1

YOYO-1. YOYO-1 (absorption: λ_{max} = 458 nm, emission: λ_{max} = 564 nm) is a green fluorescent dye used in DNA staining. It is a tetracationic homodimer of Oxazole yellow (hence YOYO), typically available as tetraiodide salt, which belongs to the family of monomethine cyanine dyes. In a buffer, the free YOYO-1 dye has very low fluorescence quantum yield, however the intensity of fluorescence increases 3200 times upon binding through bis-intercalation to double-stranded DNA (absorption: λ_{max} = 489 nm, emission: λ_{max} = 509 nm) [47].



YOYO-1

GelRed. Recently, it was found that the nucleic acid dye GelRed™ was the most sensitive and safest dye to use with UV light excitation, and both GelGreen™ and Diamond™ dyes were also sensitive using blue light excitation [48].

3.9.4 Autoradiography of nucleic acids

Autoradiography is the most sensitive detection method for nucleic acids. It requires that nucleic acids are labeled with isotopes prior to electrophoresis. After electrophoresis, the agarose or polyacrylamide gel must be dried prior to be brought into contact with an X-ray film for 24 h. Under these conditions, a photon of light, or a β-particle, or γ-ray released from a radioactive molecule "activates" silver

bromide crystals on the film emulsion. This renders them capable of being reduced through the developing process to form silver metal (a grain). The silver grains on the film form blackening on the X-film (Figure 3.9-3). The film is scanned by a special detector, which transmits the information into a computer. So a relative quantitative evaluation of the radioactivity in the sample is obtained.

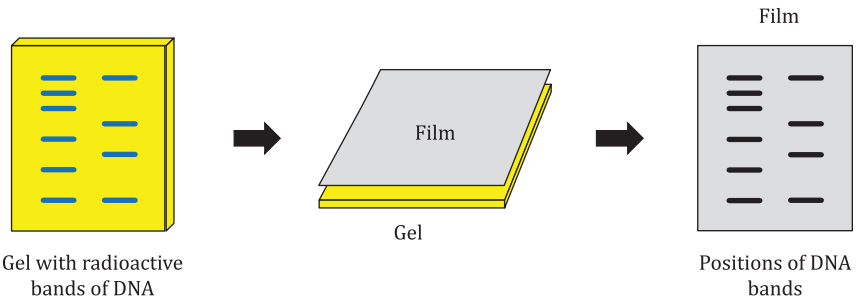


Figure 3.9-3: Autoradiogram of electrophoretically separated deoxynucleates.

The choice of film is critical for autoradiography. Single-coated films, containing one emulsion layer, are optimized for direct-exposure techniques with medium-energy radioisotopes (e.g., ^{14}C, ^{35}S, and ^{33}P, but not ^{3}H). The majority of β-particles emitted by these isotopes cannot pass through the polyester support of double-coated films, and therefore the emulsion layer on the other film side is useless. The direct exposure with single-coated films gives, however, better clarity for medium-energy isotopes. Because single-coated films often require longer exposure times, fluorography is often used to enhance sensitivity.

Double-coated films contain two emulsion layers on either side of a polyester support and are most commonly used for autoradiography. They are ideal for detecting high-energy β-particles emitted by ^{32}P and ^{125}I, since they can penetrate the polyester support and expose both emulsion layers. These films are normally used with calcium tungstate ($CaWO_4$) in intensifying screens at reduced temperature (–70 °C). They are highly sensitive to the blue light emitted by these screens.

The use of X-ray films for autoradiography suffers from two drawbacks: lack of sensitivity and a limited linear range over which the image density reflects the amount of radioactivity. The lack of sensitivity can be overcome by fluorography or by the use of intensifying screens, both enhancing radioactive signals. The exposure within a linear range requires careful controls and is important to ensure that the film can attain strong radioactive signals. Sensitivity and linear ranges of measurement can be greatly extended by using a phosphor imaging system. The phosphor imaging systems make the autoradiography much faster and easier to quantify radioactive samples.

To enhance radioactive signals, solid-state scintillation is employed to convert the energy released by radioactive molecules to visible light. This is accomplished in several ways. In fluorography, organic scintillants are incorporated into the sample to increase the energy emitted from low-energy β-particles (e.g., ^{3}H, ^{14}C, and ^{35}S). Another method uses high-density fluorescent intensifying screens, which are placed next to the sample and are used to capture the excess energy of γ-rays (e.g., from ^{125}I) and high-energy β-particles (e.g., from ^{32}P).

In autoradiography, three isotopes: ^{32}P, ^{33}P, and ^{35}S, are commonly used. The isotopes ^{32}P and ^{33}P can be incorporated into the α-phosphate residues at the 5′ end of DNA or RNA strands. The sulfur atom is similar to oxygen atom, because it is situated under the oxygen atom in the periodic table of the elements, so it has almost the same chemical characteristics. Therefore, oxygen atoms in α-phosphate residues of nucleoside triphosphates can be replaced with ^{35}S atoms and be incorporated by the DNA or RNA polymerases in the nucleotide chains.

The isotopes ^{32}P, ^{33}P, and ^{35}S release a high-energy electron named a β-particle (Table 3.9-2). ^{32}P has the highest energy and is the most dangerous isotope. The isotope ^{33}P radiates much weaker and, therefore, can be proved worse. However, it is not as harmful as the isotope ^{32}P. The energy of the isotope ^{35}S is less than that of the ^{33}P isotope.

Table 3.9-2: Characteristics of isotopes used in autoradiography.

Isotope	Strength of ß-radiation, MeV	Half-life, days	Maximum specific activity, Ci/mmol
^{32}P	1.71	14.3	6,000
^{33}P	0.249	25.4	3,000
^{35}S	0.167	87.4	1,500

The nucleic acids can be labeled with isotopes in different ways: The ^{32}P- or ^{33}P-labeled γ-phosphate of dATP can be bound to the 5′ end of a polynucleotide strand. Otherwise, the 3′ end of a polynucleotide strand can be labeled with ^{32}P, ^{33}P, or ^{35}S using a terminal transferase and α-labeled dNTP. In labeling by random priming, short oligonucleotides with random sequences of 6–9 nucleotides are used. They are extended with the help of DNA polymerase by α-labeled dNTP.

DNA may also be labeled by nick translation, random priming, or transcription. In the nick translation, DNase I introduces single strands into a double-stranded DNA. If the DNA polymerase 1 is present, it binds to the breaks and builds α-labeled dNTP.

3.9.5 Labeling nucleic acids with proteins

The nucleic acids can be labeled (coupled) with enzymes. Typically **h**orse**r**adish **p**eroxidase (HRP) and **al**kaline **p**hosphatase (ALP) are used, which are often referred to as reporter enzymes. The labeling may be indirect or direct (Figure 3.9-4).

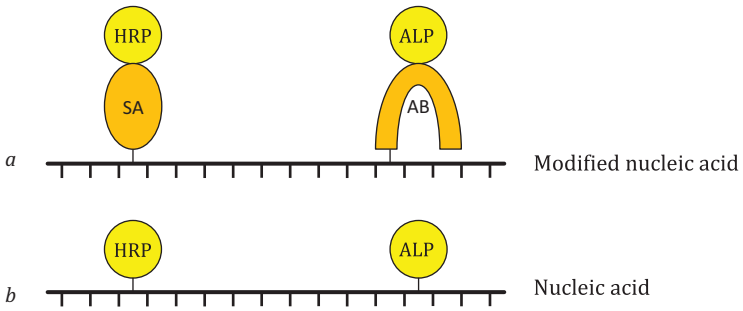


Figure 3.9-4: Labeling of nucleic acids with proteins.
In most cases, horseradish peroxidase (HRP) or alkaline phosphatase (ALP) are coupled to the nucleic acids indirectly (*a*) or directly (*b*).
SA – streptavidin; AB – antibody

The enzymes horseradish peroxidase and alkaline phosphatase can be detected by colorimetric or chemiluminescent substances. In the chemical luminescence, a chemical reaction produces visible light, which can last for several hours. The light can be recorded on an X-ray film, similarly as in autoradiography.

3.9.5.1 Indirect DNA-protein coupling

The indirect coupling of nucleic acids is based on the incorporation of a modified nucleotide in DNA or RNA. This is possible, if the nucleic acids are copied by DNA or RNA polymerases, if the 3′ end is modified, or if an artificial oligonucleotide is produced. In these cases, special proteins can be bound to the nucleotide chain, which can fix HRP or ALP.

For chemical modifications of the nucleic acids, three substances are used: biotin, fluorescein, and digoxigenin.

If nucleic acids are labeled with biotin, they can bind the bacterial protein streptavidin. For this purpose, biotin must be at the end of the nucleic acid; only then biotin can bind the large streptavidin molecule. So, the steric hindrance is avoided and the streptavidin molecule can come undisturbed closely to the biotin ligand. The biotin nucleotides can be constructed by 7, 14, or 21 atoms. Streptavidin conjugates with the modified nucleic acid alone or bound to a reporter enzyme.

3.9.5.2 Direct DNA-protein coupling

DNA can also be coupled directly covalently to the reporter enzymes HRP or ALP [49]. This, however, changes the DNA mobility. Therefore, a protein should be coupled directly to a nucleic acid only if it is used as a probe during hybridization of nucleic acids – in Southern or Northern blotting.

The advantage of the direct DNA-protein coupling, in contrast to the indirect method, is that it is made quickly, because the nucleotides should not be labeled with streptavidin or antibodies. However, the hybridization must be carried out carefully not to destroy the activity of the conjugated enzyme.

3.9.6 Absorption spectroscopy of nucleic acids

Absorption of the sample is measured at several wavelengths to calculate the concentration of nucleic acids. Absorbance at 260 and 280 nm can be used as an indicator of purity of DNA or RNA. Proteins have peak absorption at 280 nm. Absorbance at 325 nm indicates particles in the solution or dirty cuvettes. Contaminants containing peptide bond or aromatic moieties, such as protein and phenol, absorb at 230 nm.

A single-beam **u**ltra**v**iolet-to-**vis**ible (UV-VIS) range spectrophotometer possesses a cuvette for the sample solution that is compared to a reference cuvette containing the blank. A double-beam spectrophotometer simplifies the measurements, as it compares automatically the cuvette holding the sample solution to the reference cuvette containing the blank. The double-beam spectrophotometer scans at various wavelengths and reports the results automatically.

Some companies offer different screens for different isotopes. They vary in the protective coating on the screen, which is optimized for low- or high-energy β-particles or γ-rays. More recently, screens have also been developed that measure chemiluminescence.

3.9.7 Protocols

3.9.7.1 Staining of nucleic acids with Crystal violet and Methyl orange

Materials and equipment

TRIS
Boric acid
Acetic acid
1.0 g/dl Agarose slab gels
Crystal violet
Methyl orange
Na_2EDTA

Ficoll
Xylene cyanol Na salt
Bromophenol blue Na salt

TRIS-borate-EDTA (TBE) buffer, pH = 8.3

TRIS	0.59 g (49.0 mmol/l)
Boric acid	0.30 g (49.0 mmol/l)
Na_2EDTA	0.42 g (12.5 mmol/l)
Deionized water to	100.00 ml

or

TRIS-acetate-EDTA (TAE) buffer, pH = 8.3

TRIS	0.48 g (40.0 mmol/l)
Add acetic acid to pH = 8.3.	
Na_2EDTA	0.03 g (1.0 mmol/l)
Deionized water to	100.00 ml

Staining solution

Crystal violet	2.0 mg
Methyl orange	1.0 mg
Deionized water to	100.0 ml

Prepare freshly before use.

Procedure

- Dilute DNA with the sample buffer (TBE or TAE buffer) and load.
- Run electrophoresis at a constant voltage of 100 V.
- Stain the gel in the staining solution for 30 min.
- Destain in 1.0 mg/dl Methyl orange for 5 min.
- Wash in deionized water for 1 min.
- Dry the gel on a filter paper using a gel dryer.

3.9.7.2 Fast and sensitive silver staining of DNA bands according to Han *et al.*

Silver staining consumes time. Therefore, many methods for rapid staining of nucleic acids are proposed, for example the method of Han et al. [50].

Materials and equipment

Polyacrylamide slab gels
HNO_3
$AgNO_3$
NaOH
Na_2CO_3
Eriochrome **b**lack **T** (EBT)

HCHO
Ampicillin

Procedure

- Impregnate the gel in a mixture of 0.1 g/dl $AgNO_3$ and 1 g/dl HNO_3 at room temperature for 5 min.
- Rinse with deionized water for 5 s, twice.
- Develop in a mixture of 2 g/dl NaOH and 0.04 g/dl Na_2CO_3, containing 2 ml 0.0025 g/dl EBT and 1.5 ml 37 g/dl HCHO at room temperature for about 5 min until the gel color turned to yellow and all the bands of DNA fragments have appeared distinctly.
- Stop in 2.5 g/dl ampicillin for 5 s, twice.
- Rinse with deionized water.
- Dry in the air at room temperature.

3.9.7.3 Detection of DNA and RNA using ethidium bromide in gel

Materials and equipment
Polyacrylamide gel
DNA or RNA
Ethidium bromide

Ethidium bromide is a mutagen. Handle only with gloves.

Tris-Borate-EDTA (TBE) buffer, 10x, pH = 8.3

TRIS	1.08 g (0.089 mol/l)
Boric acid	0.55 g (0.089 mol/l)
Na_2EDTA	0.07 g (0.002 mol/l)
Deionized water to	100.00 ml

Instead TBE, TRIS-acetate-EDTA (TAE) buffer can be used.

Ethidium bromide stock

Ethidium bromide	1.0 g
Deionized water to	100.0 ml

The solution is stable for one to two months at room temperature in the dark.

Buffer preparation, pH = 8.3

TBE buffer, 10x	10.00 ml
Ethidium bromide stock	0.05 ml
Deionized water to	100.00 ml

Gel preparation

- Dissolve and heat agarose in buffer.
- Allow gel solution to cool to 60–70 °C.

- Add ethidium bromide stock to 0.5 µg/ml final concentration in the gel solution.
- Pour gel solution into a cassette to gel.

Procedure

- After electrophoresis run, place the gel into a plastic wrap on a UV light box. Bands will appear bright orange on a faint orange background.
  This method will detect approximately 5 ng of DNA. Destaining in water or 1 mmol/l $MgSO_4$ may be required to achieve full sensitivity.

3.9.7.4 Detection of nucleic acids using absorption spectroscopy

Materials and equipment

TRIS-NaCl-EDTA buffer
DNA sample
Matched quartz semi-micro spectrophotometer cuvettes (1 cm path length)
Single- or dual-beam spectrophotometer (ultraviolet to visible)

TRIS-NaCl-EDTA (TNE) buffer, pH = 7.4

TRIS	0.61 g (0.05 mol/l)
Add HCl to pH = 7.4.	
NaCl	0.64 g (0.1 mol/l)
Na_2EDTA	0.003 g (0.1 mmol/l)
Deionized water to	100.00 ml

Procedure

- Pipette 1.0 ml of the TNE buffer into a quartz cuvette.
- Place the cuvette in a single- or dual-beam spectrophotometer, set at 325 nm, and zero the instrument. Use this blank solution as the reference in double-beam instruments. For single-beam spectrophotometers, remove blank cuvette and insert cuvette containing DNA sample or standard suspended in the same solution as the blank. Repeat this process at 280, 260, and 230 nm.
- Determine the concentration (c) of DNA present using the A_{260} reading after the following equations:

$$c_{Single-stranded\ DNA}(pmol/\mu l) = \frac{A_{260}}{10 \bullet S}$$

$$c_{Single-stranded\ DNA}(\mu g/ml) = \frac{A_{260}}{0.027}$$

$$c_{Double-stranded\ DNA}(pmol/\mu l) = \frac{A_{260}}{13.2 \bullet S}$$

$$c_{Double-stranded\ DNA}(\mu g/ml) = \frac{A_{260}}{0.020}$$

$$c_{Single-stranded\ RNA}(\mu g/ml) = \frac{A_{260}}{0.025}$$

where S is the size of DNA in kilobases. Using these calculations, A_{260} of 1.0 indicates 50 pg/ml double-stranded DNA, 37 pg/ml single-stranded DNA, or 40 pg/ml single-stranded RNA.

- Use the A_{260}/A_{280} ratio and readings at A_{230} and A_{325} to estimate the purity of the nucleic acid sample.
  Ratios of 1.8 to 1.9 and 1.9 to 2.0 indicate highly purified preparations of DNA and RNA, respectively. Absorbance at 230 nm reflects contamination of the sample by phenol or urea, whereas absorbance at 325 nm suggests contamination by particles and dirty cuvettes. Light scatter at 325 nm can be magnified fivefold at 260 nm.

3.9.7.5 Autoradiography of radiolabeled DNA in gels and blots

Materials and equipment

Dried gel or membrane (e.g., after immunoblotting)
Developer and replenisher, 20 °C
Fixer and replenisher, 20 °C
Film or paper cassette with particle-board supports and metal binder clips
Plastic wrap
X-ray film
Trays for film processing solutions
Clips for hanging film

Procedure

- Place the sample (dried gel or membrane) in the film cassette in a darkroom illuminated with a safelight. Cover the sample with a plastic wrap to prevent it from sticking to the film and contaminating the cassette with radioactivity (Figure 3.9-5).
- Place a sheet of X-ray film on top of the sample, and then put the sample in a film cassette.
- Expose the film at appropriate temperature for desired length of time. The sensitivity of the film can be improved using flours or intensifying screens (Table 3.9-3). Time of exposure depends on the strength of the radioactivity in the sample and can be determined empirically by making exposures for different lengths of time. A Geiger counter can be used to detect the relative amount of radioactivity in a sample.

- After exposure, return the cassette to the darkroom and remove the film for developing.
- Immerse the film in 20 °C developer for 5 min, and then wash in running water for 1 min.
- Immerse the film in 20 °C fixer for 5 min, and then wash in running water for 15 min.
- Hang the film to dry.

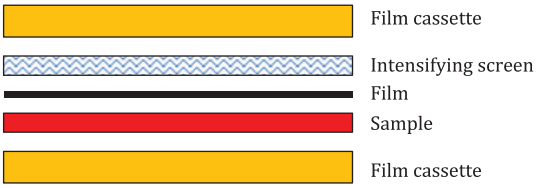


Figure 3.9-5: Autoradiography setup: intensifying screen, film, and sample in a film cassette.

Table 3.9-3: Film choice and exposure temperature for autoradiography.

Isotope	Enhancement method	Film	Exposure temperature
^{3}H	Fluorography	Double coated	–70 °C
^{35}S, ^{14}C, ^{32}P	None	Single coated	Room temperature
^{35}S, ^{14}C, ^{32}P	Fluorography	Double coated	–70 °C
^{32}P, ^{125}I	Intensifying screens	Double coated	–70 °C

3.9.8 Troubleshooting

Problem	Cause	Solution
The bands of nucleic acids are weakly stained.	The sensitivity of the silver staining was low.	Check the recipe.
Intensive background.	Impure reagents were used.	Use reagents p.a.
	The water quality was poor.	Use only deionized water.
Overvaluation of pherogram.	Spots were detected that are not bands but dirty particles, fingerprints, or other.	Handle with gloves. Use clean solutions.
Undervaluation of pherogram.	The measuring beam of densitometer was too narrow. Therefore, the broad nucleic acid bands are not full evaluated.	Do several parallel scans to detect all nucleic acid bands.

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3.10 Precast gels for nucleic acid electrophoresis

The precast gels for nucleic acid electrophoresis are as important as the precast gels for protein electrophoresis. They can contain agarose or polyacrylamide.

3.10.1 Precast agarose gels

The precast agarose gels on film or net for native zone or denaturing zone electrophoresis of nucleic acids are in principle 0.5 mm thick. They are available in different dimensions: 51 × 82, 80 × 100, 125 × 125, 125 × 200, 125 × 250 mm, or more. The concentration of the agarose is 0.60, 0.80, 1.00 g/dl, or more. The precast agarose gels contain buffers for resolving nucleic acids.

3.10.2 Precast polyacrylamide gels

The precast polyacrylamide gels on film or net for native or denaturing disc-electrophoresis of nucleic acids are in principle also 0.5 mm thick (ELPHO, Nuremberg [1]). They are supplied together with paper strips. The gels and paper strips contain buffers. The gel and anode strip contains **T**RIS-**f**ormate-**E**DTA buffer (TFE); the cathode strip contains **T**RIS-**t**aurinate-**E**DTA buffer (TTE).

The polyacrylamide concentration T of the precast gels is 5, 7, 9, 11, or 13 g/dl, and their dimensions correspond to the dimensions of the precast agarose gels. Film-supported gels are used for electrophoresis and analytical diffusion blotting, while net-supported gels are suitable for electroblotting.

The precast polyacrylamide gels are located between two polyester films. The cover film is loosely bound to the gel. It must be removed before an application template is applied on the gel. The bottom film is chemically bound to the gel. The lower film of a net-supported gel, which is loosely bound to the gel, should be removed after electrophoresis for electroblotting.

The precast polyacrylamide gels should be stored at 4–8 °C in the refrigerator.

BIO-RAD, Hercules, Ca. [2] offers precast gels for nucleic acid electrophoresis in small gels: minigels and midigels. The electrophoresis in small gels is run faster than

in larger gels. They are thin enough and have narrower wells and, as a result, require smaller amounts of DNA for application. The protocol for running is similar to that for larger gels. The vertical polyacrylamide gels for the chamber *Mini-Protean II* contain TRIS-borate-EDTA buffer. They are suitable for electrophoresis of nucleic acids from 50 to 2,000 base pairs. The dimensions of the gels are 80 × 100 × 1 mm.

3.10.3 Protocols

3.10.3.1 Casting agarose mini- and midi-gels

Materials and equipment
Appropriate buffer
Agarose
Casting cassette for vertical agarose mini- or midi gels

Procedure
- Fulfill the casting cassette (e.g., 80 × 50 × 2 mm) with agarose solution (e.g., 10 ml).
- Place a comb into the solution.
- After gelling, remove carefully the comb from the gel and fill the wells with the same buffer.

3.10.4 Troubleshooting

Problem	Cause	Solution
Monomer solution does not polymerize or polyacrylamide gel becomes soft and sticky.	The concentration of acrylamide or BIS in the monomer solution was too low.	Check the recipe of gel casting.
	The concentration of TMEDA or APS in the monomer solution was too low.	Use 5 µl of 10 g/dl TMEDA and 5 µl of 10 g/dl APS per 1 ml of monomer solution.
	The APS solution was too old or was stored improperly.	Use a new APS solution. The durability of an APS solution in the refrigerator is a week.
	The temperature was too low.	Polymerize the monomer solution at 20–25 °C.
	The concentration of air oxygen in the monomer solution was too high.	Deair the monomer solution with a water-jet pump.
The gel upper edge is sticky and detaches from the support film.	The atmospheric oxygen has inhibited the polymerization of the gel upper edge.	Overlay the monomer solution with deionized water after casting.

(continued)

Problem	Cause	Solution
The gel polymerizes too rapidly.	The concentration of TMEDA or APS is too high.	Check the recipe of gel casting.
	The room temperature is too high.	Let the monomer solution polymerize at 20–25 °C.
Air bubbles in the polyacrylamide or agarose gel.	Air dissolved in the monomer solution during gel polymerization.	Deair the monomer solution.
	The glass plate that came into contact with the monomer solution was dirty.	Wash the glass plate before use.
	The support film was unclean.	Do not touch the hydrophilic side of the support film with fingers.
	The glass plates were too cold – the agarose solidified at casting.	The temperature of the glass plates should be 60–70 °C during casting.
The consistency of the agarose or polyacrylamide gel is insufficient.	The agarose concentration is too low.	Check the recipe.
	The gelling time of agarose was too short.	The gelling of agarose must continue at least 60 min at room temperature or better overnight.
	Urea has damaged the agarose structure.	In the presence of urea, use higher agarose concentration, e.g., 2 g/dl, and let the monomer solution to gel longer.
The gel separates from the support film.	An incorrect support film was used.	Do not exchange the support films for polyacrylamide and agarose gels.
	The gel was cast onto the wrong side of the adhesive film.	Cast gel on the hydrophilic side of the support film. Check previously the hydrophilic side with water drops.
	The support film was stored improperly or too long.	Keep the support films at room temperature in the dark.
The gel does not stick to the support film, but to the glass plate.	The glass plate was too hydrophilic.	Clean the glass plate and coat with Repel-silane.
	The gel was left too long in the casting cassette.	Take the polyacrylamide gel from the casting cassette not later than 1 h after the polymerization.

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Problems 3. Electrophoresis of nucleic acids

3.1 How many base pairs have a double-helical DNA with M_r of $2 \bullet 10^6$ and $2 \bullet 10^7$?

3.2 How many electric charges have a single-stranded RNA with M_r of $4 \bullet 10^4$ and $4 \bullet 10^5$?

3.3 Why DNA, which consists of AT pairs, melts at 70 °C, and why DNA, which consists of GC pairs, melts at 90 °C?

3.4 Which equation expresses the relationship between the mobility of DNA and agarose concentration?

3.5 Why the migration of DNA fragments diminishes in a gel containing ethidium bromide?

3.6 What is PCR?

3.7 Calculate the electrophoretic mobility μ of DNA, which has run a distance d of 127 mm in an electric field E with a distance between the electrodes l of 20 cm at voltage U of 250 V in one hour electrophoresis.

3.8 At what pH value the effective mobility of TRIS ion $(HTRIS^+)$ will be equal to $5 \bullet 10^{-9}$ $m^2/(s\ V)$, if the temperature is 25 °C and the ionic strength is 0.01 mol/l? At these temperature and ionic strength, the mobility μ and the dissociation constant of TRIS-ion pK_c are equal to $24.06 \bullet 10^{-9}$ $m^2/(sV)$, and 8.16, respectively.

3.9 Make a TRIS-acetate-EDTA buffer with ionic strength 0.04 mol/l and pH = 8.0, which contains 0.001 mol/l EDTA. pK_c of TRIS-ion $(HTRIS^+)$ is 8.24, and pK_c of acetic acid is 4.59. The M_r values of TRIS, acetic acid (99%, d = 1.049), and $Na_2EDTA \cdot 2H_2O$ are 124.14, 60.05, and 372.24, respectively.

3.10 Calculate the composition of a TRIS-hydrogen phosphate-EDTA buffer at 25 °C, pH = 7.0, ionic strength I of 0.10 mol/l, and Na_2EDTA concentration of 0.001 mol/l. The pK value of TRIS-ion $(HTRIS^+)$ is 8.07, and the pK value of dihydrogen phosphate ion $(H_2PO_4^-)$ is 7.20 at 25 °C. $M_r(Na_2EDTA \cdot 2H_2O)$ is 372.24.

3.11 Which bases contain pyrimidine?
- A Adenine and guanine
- B Cytosine and guanine
- C Cytosine and thymine
- D Adenine and thymine

3.12 Which bases contain purine?
- A Adenine and guanine
- B Cytosine and guanine

C Cytosine and thymine
D Adenine and thymine

3.13 Which bonds bind the mononucleotide residues in polynucleotide chains?
A Peptide bonds
B N-glycosidic bonds
C O-glycosidic bonds
D 3′,5′-Phosphodiester bonds

3.14 How are charged the mononucleotides at physiological pH value?
A Negative
B They have no charges
C Positive
D They have positive and negative charges

3.15 What are the bonds between neighboring bases in a DNA strand?
A Ester bonds
B Covalent bonds
C Hydrogen bonds
D Hydrophobic interactions

3.16 Which bonds stabilize the double helix structure of DNA?
A Hydrogen bonds and hydrophobic interactions between the bases
B Ionic bonds between the phosphate residues
C Hydrogen bonds between the deoxyriboses and bases
D Disulfide bridges

3.17 What are the names of enzymes that split mononucleotides from the end of a polynucleotide chain?
A Endonucleases B Exonuclease C Ligase D Polymerase

3.18 What is true?
A DNA contains uracil, and RNA contains thymine
B DNA has a greater mass and volume than RNA
C Nucleases attack DNA, but not RNA
D Phosphodiester bonds in DNA are stable against alkalis, on the contrary in RNA

3.19 Which bonds between both DNA strands are broken at denaturation?
A 3′,5′-Phosphodiester bonds
B Covalent bonds
C Hydrogen bonds
D Ionic bonds

3.20 A DNA molecule contains adenine whose quantity is 20% of all bases. How high is the percentage of cytosine?
A 10 B 20 C 30 D 40

3.21 When is DNA more resistant to denaturation?
A When it contains more AT pairs
B When it contains more GC pairs
C When it contains equal numbers of GC and AT pairs
D Denaturation does not depend on the type of base pairs

3.22 Eastern blotting is a method for checking the presence of
A DNA B RNA C Protein D Protein post translational modifications

3.23 Northern blotting is a method used for checking the presence of
A DNA B RNA C Protein D None of the above

3.24 Southern blotting is a method for checking the presence of
A DNA B RNA C Protein D None of the above

4 Iontophoresis

Iontophoresis is a method, which is closed to electrophoresis. It is characterized with ionic flows in tissues or organs driven by an electric field.

The term *iontophoresis* stems from Greek words “ion” and “phorein” (bear) and is referred to as inserting of ions into the body.

Iontophoresis has advantages and disadvantages against other electrophoresis methods.

The advantages of iontophoresis are: painless drug delivering in the body; patients do not receive injections; reduced risk of infection because the skin and outer mucosae remain unhurt; drug delivery is made directly into the ill place; treatment continues in minutes; and no risks of fibrosis in contrast to drug delivering with continuous injection.

However, iontophoresis has also some disadvantages. The most common among them is that the iontophoretic procedures make the skin dry. Blistering, peeling, and irritation might also occur on the skin. These side effects are usually treated by moistening the skin after each session. An over-the-counter hydrocortisone cream can also help. In addition, if the electric current is too high, it can cause the skin burn and can create extreme pH values, making the proteins to precipitate in sweat ducts. Histopathological changes, such as hemorrhagic necrosis and infiltration of polymorphonuclear cells, can also be observed.

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4.1 Theory of iontophoresis

In iontophoresis, charged agents (drugs) are inserted in organic tissues (skin) usually as ionic flow [1]. This is carried out with the help of an electric field [2]. The electric current should be below the level of the patient's pain threshold and should be allowed to flow for an appropriate length of time. Besides, the charged agents must have relatively small molecules (M_r < 8,000) and the power supply must produce direct electric current.

The ions to be driven into a tissue should be placed under the electrode with the same polarity, that is, positively charged cations should be placed under the anode and the negatively charged anions should be placed under the cathode. If so, the anode repels cations and the cathode repels anions (Figure 4.1). The ground electrode can be placed elsewhere on the body surface.

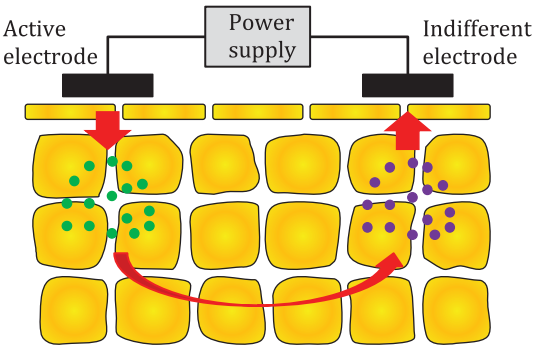

Figure 4.1: Ions with the same polarity as the polarity of stimulating electrode are repelled into the skin, and on the contrary.
● Active ion; ● Counter-ion

It is considered that the penetration of ions into tissues is likely to be less than 1 mm. There is no evidence that electric current is responsible for penetrations beyond this level.

4.2 Factors affecting iontophoresis

Factors affecting iontophoresis have physicochemical and biological origin.

4.2.1 Physicochemical factors

Physicochemical factors are: concentration, pH value and ionic strength of the buffer, solution viscosity, and electric current.

Concentration. The drug should be water soluble, of low dose, and ionizable with a high charge density. Smaller molecules are more mobile but large molecules are also iontophoresable. When drug concentration is increased, greater drug delivery results.

Buffer pH. If a drug is dissolved in a buffer, the pH value of the buffer must have such pH value at which the most drug molecules are ionized [3]. This pH value is called pH optimum for an appropriate drug.

Buffer ionic strength. If a drug is dissolved in a buffer, the buffer ions compete with the drug ions for the delivery. This process takes a significant place in iontophoresis because the buffer ions are generally smaller than the drug ions and, therefore, are more mobile than the drug ions.

Solution viscosity. The migration of a drug is inversely related to the viscosity of the solution be introduced.

Electric current. The electric current can be direct, alternate, or pulsed. It can have various waveforms, including square, sinusoidal, triangular, or trapezoidal. **A**lternating electric **c**urrent (AC) iontophoresis shows better results than conventional **d**irect electric **c**urrent (DC) iontophoresis. Constant conductance AC iontophoresis shows reduced flux drift and less variability compared to conventional constant DC iontophoresis [4].

4.2.2 Biological factors

The biological factors are the thickness and permeability of the skin or mucosa. Sweat glands in the skin are the most significant path for conducting charged ions. This fact was demonstrated by Papa and Kligman, who proved this using Methylene blue [5].

4.3 Calculating the iontophoretic current

The values of the applied electric current variables used in the iontophoretic treatment of diseases are given in Table 4.1.

Table 4.1: Electric current variables used in iontophoresis [6].

Electric current type	DC
Electric current amplitude	1–4 mA
Treatment duration	20–40 min
Total electric current delivered	40–80 mA/min

The concentration of a drug or other agent should be 2–5 g/dl, the electric current density at the cathode should not be above 0.5 mA/cm^2, and the electric current density at the anode should not be above 1.0 mA/cm^2.

If an electric current I of 2 mA must be delivered through an electrode surface S of 6 cm^2, then the electric current density at either the anode or the cathode should be

$$J = \frac{I}{S} = \frac{2}{6} = 0.33\,\mathrm{mA/cm^2} \tag{4.1}$$

On the contrary, it is possible to calculate the electric current that should be applied. If the surface S of the active electrode is 6 cm^2, and the maximum electric current density J is 0.5 mA/cm^2, then, according to eq. (4.1), the maximum electric current I should be

$$I = J \bullet S = 0.5 \bullet 6 = 3\,\mathrm{mA} \tag{4.2}$$

4.4 Reverse iontophoresis

Reverse iontophoresis is a technique in which ions or neutral molecules in the body are removed. The negative charge of the skin makes it permeable for cations, such as sodium and potassium ions, and allows electro(endo)osmosis, hence, solvent flows toward the anode.

4.4.1 Electroosmosis

Under definitive circumstances, when electric current is applied, water flows from the electrode reservoir into the skin, or in the opposite direction. As a result, any nonionized molecules (nonionized drugs) that are in the solution follow this flow. If only nonionized drugs are to be inserted, it may be necessary to add NaCl to the drug solution to enhance its conductivity and in turn to establish electroosmotic flow.

By electroosmosis, diverse neutral molecules, including glucose, can be extracted from the skin (s. below).

4.5 Iontophoresis device

An iontophoresis device consists of a microprocessor-controlled battery, two electrodes, and a reservoir.

4.5.1 Battery

The iontophoresis battery creates commonly low voltage – usually 9 V.

4.5.2 Electrodes

The electrodes of an iontophoresis device are active and passive. They are made of metal or gel and possess special wells or receptacle areas for drugs (Figure 4.2).

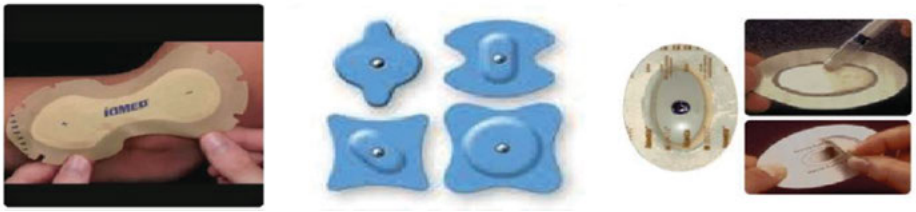

Figure 4.2: Electrodes for iontophoresis.

The electrode patch is preconfigured to deliver smaller electric current than is normally employed in the clinic. The patch is applied for 12–24 h (depending on the intended dose) after which is removed and discarded (it cannot be reused).

The electrodes are connected to the iontophoresis device by wires. Nowadays wireless devices are gaining popularity, especially for home use.

4.5.3 Reservoir

The reservoir contains a gauze or gel pad, to which a drug solution is applied or injected through.

The iontophoresis unit can be set for electric current and time.

Two types of iontophoresis are run: diagnostic and therapeutic iontophoresis.

4.6 Diagnostic iontophoresis

For diagnostic purposes, acetylcholine iontophoresis is used for testing the endothelium by stimulating endothelium-dependent synthesis of NO and subsequent *microvascular vasodilation* [7]. Acetylcholine ions are positively charged (s. below) and, therefore, are placed in the anode chamber.

Pilocarpine iontophoresis is often used to stimulate sweat secretion, as part of the diagnosis of *cystic fibrosis* [8,9,10]. Pilocarpine iontophoresis causes sweating for minutes.

Iontophoresis can be used for testing *contact eczema* [11]: The test substances are administered through epidermis into dermis. So, the disadvantages of the traditional patch test procedure, such as prolonged wearing of the test strips, are eliminated.

Iontophoresis of epinephrine for assessment of neuromuscular synapsis response is a new technique that improves the diagnosis of *myasthenia gravis*. Patients with myasthenia gravis are more sensitive to iontophoresis of epinephrine and demonstrate decrement findings in the low repetition stimulus tests in comparison to normal people. An improved method called iontophoresis of epinephrine with **r**epetitive **n**erve **s**timulation (RNS) was successfully used in assessments of the disease [12].

The reverse iontophoresis can also be applied for diagnostic purposes. As mentioned, the electroosmosis can be used for extracting neutral molecules, including glucose, across the skin for monitoring the subdermal concentration of blood glucose in diabetic patients [13,14]. This event is used in some devices, for example GlucoWatch device, which allow establishing the glucose concentration in the blood across the skin [15].

4.7 Therapeutic iontophoresis

The **E**lectro**m**otive **D**rug **A**dministration (EMDA) permits a medicine or other chemical to be delivered through the skin [16]. This mode of delivering resembles an injection without a needle; hence, it is a noninvasive injection. It differs from dermal patches, which do not rely on electric field.

Usually, iontophoresis for disease treatment is carried out with drugs in low concentrations – up to 5 g/dl, and under electric current up to 5 mA. Treatment times are in the 20–40-min range.

Principled, the negative electrode (cathode) is larger than the positive electrode (anode) to avoid skin irritation. For example, the surface of the cathode can be twice larger than the surface of anode.

Although the drug delivery rate cannot be well controlled, the total amount of drug administration can be controlled, because the total amount of drug is limited by the battery capacity.

Most significant types of therapeutic iontophoresis are transdermal and ocular iontophoresis; and most treated disease is hyperhidrosis.

4.7.1 Transdermal iontophoresis

Iontophoresis for transdermal (across skin) delivery of drugs has been used for more than a century [17,18,19]. It plays an important role in modern therapies.

Transdermal iontophoresis is applicable to small ions and polyions of relative molecular masses up to a few thousand. The ions are driven into the skin *via* the sweat gland ducts and hair follicles. The ducts have lower resistance than the stratum corneum; therefore, the greater part of electric current passes through them. The transdermal drug delivery can be microprocessor controlled [20].

The transdermal iontophoresis is run by three mechanisms: (*a*) the electric field drives ions through the skin; (*b*) the flow of electric current increases the skin permeability; and (*c*) electroosmosis [21,22]. Electroosmosis acts in the following way: Since the human skin is negatively charged, water flows from anode to cathode. Therefore, the electroosmotic flow helps transdermal delivery of negative ions and proteins from the anode compartment, but counteracts the ionic delivery from the cathode compartment [23,24].

In transdermal iontophoresis, a low voltage is applied between an iontophoretic chamber (containing a same-polar drug) on the skin and another electrode chamber also on the skin. As a result, the drug creates electric current through the tissue (Figure 4.3).

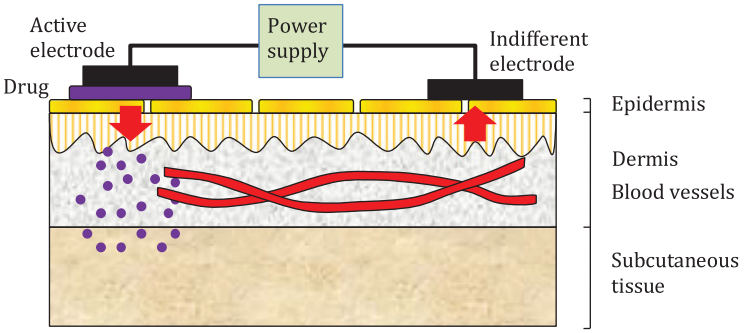

Figure 4.3: Transdermal drug delivery using iontophoresis.

The skin, being a semipermeable membrane, allows a small amount of drug ions to penetrate [25]. To accelerate this process, ultrasound is applied. The formation of undesirable vesicles and bullae in the skin can be avoided by periodically interrupting the electric current with a short electric current in the opposite direction. The rate of drug delivery can be controlled by a microprocessor or by the patient [26].

Prior to application, the skin area should be carefully washed. The electrode pads should be soaked in the appropriate solution. Then, required electric current should be slowly turned up, and after the treatment time it should be slowly turned down.

4.7.1.1 Hyperhidrosis iontophoresis

Everyone sweats, but some people sweat more than others. People who suffer from excessive sweating under no reason have the disorder *hyperhidrosis*. This disease affects about 4% of the population. Hyperhidrosis can occur anywhere on the body (hands, feet, underarms, and face) and affects all genders and ages, but is most common in teenagers and young adults.

Iontophoresis is an extremely effective, drug-free method for treating all types of hyperhidrosis since the 1930s. It blocks temporarily the sweat glands and stops the sweating. Patients with hyperhidrosis must undergo several iontophoresis sessions per week, lasting from 20 to 40 min. This treatment must be done regularly. Also, a portable machine can be used at home. The practical aspects of hyperhidrosis treatment are well described [27].

In most common cases of hyperhidrosis, the iontophoresis uses tap water under the positive and negative pole, as conducting medium with the skin [28,29,30]. The mechanism of action of tap water iontophoresis remains unknown. A hypothesis was published that tap water iontophoresis inhibits the sweating by mechanical blockage of sweat ducts with keratin plugs in stratum corneum of palms, soles of feet, and axilla [31]. However, later works by Hill *et al.* [32] have cast doubt on this explanation. They examined, by light and electron microscopy, sweat glands from the palm of a patient with hyperhidrosis before and after iontophoresis treatment and found no changes.

In serious cases of hyperhidrosis, water solutions with cholinergic inhibitors, such as methyl sulfate [33], atropine [34], and glycopyrronium bromide [35,36,37], can be used. They have a longer-lasting effect than the tap water.

4.7.1.2 Other transdermal applications

Iontophoresis is used for treatment of *plantar fasciitis, bursitis*, lateral and medial *epicondylitis* (commonly referred to as tennis elbow and golfers elbow, respectively), and other diseases [38].

Iontophoresis with histamine, 2 g/dl tolazoline hydrochloride, or 20 g/dl ZnO (ointment) on the positive pole of the iontophoretic device is carried out in treatment of *trophic ulcers* [39,40,41]. Tolazoline ion stimulates local blood flow, and zinc ion has antiseptic effect.

Iontophoresis with $CuSO_4$ on the negative electrode is used for treatment of *epidermatophytosis* [42], and KI iontophoresis is used for treatment of *sporotrichosis* [43].

Some *viral infections* are also positively influenced by iontophoresis. Iontophoresis can be an alternative for delivering of acyclovir to treat *herpes labialis* [44].

Planar warts can be successfully treated with sodium salicylate iontophoresis [45]. Iontophoretic application of idoxuridine is effective in *herpes simplex* treatment [46].

Hyperkeratosis with fissuring of palms and soles is treated with 5–10 g/dl sodium salicylate and shows improvement within three to four weeks (six to eight sittings of 10–15 min each) [47]. Salicylate ions act anti-inflammatory.

Iontophoresis with 1 g/dl meladine at patients with *vitiligo* shows marked repigmentation [48].

Iontophoresis with hyaluronidase leads to temporary increased skin softness and flexibility, and decreases the cold sensitivity in patient with *scleroderma* [49]. Hyaluronidase hydrolyzes connective tissue and increases its permeability.

Local *edema*, and *subacute and chronic inflammations*, are healed with 150 μg/ml hyaluronidase in 0.9 g/dl NaCl on anode. This enzyme increases the permeability in connective tissues.

Methyl prednisolone iontophoresis is used for healing of erosive *lichen planus* [50].

For some kinds of inflammation, 4 mg/ml dexamethasone (on cathode) or 0.5 g/dl hydrocortisone (on anode), as ointment, are used. Hydrocortisone acts anti-inflammatory.

Calcific tendinitis (myositis ossificans) is treated with 2–5 g/dl acetic acid at the negative electrode. Acetate ion increases solubility of calcium deposits in tendons.

Iontophoresis can be used in the treatment of *lymphedema* of the limbs [51].

Iodine iontophoresis reduces *scar tissue* [52], and administrates antibiotics (penicillin), which help *burn patients* with infected wounds [53]. For patients with microbial infections and *adhesive capsulitis*, 5–10 g/dl iodine (or ointment) on cathode are used. It acts as a broad spectrum antiseptic.

Myasthenia gravis is another disease that can be treated iontophoretically. It is caused by a genetic defect in the neuromuscular synapses [54] and is characterized with fluctuating muscle weakness and fatigue. In most cases, myasthenia gravis is caused by circulating antibodies that block acetylcholine receptors on the postsynaptic membrane, inhibiting the neurotransmitter acetylcholine. Since acetylcholine ion is positively charged, it can be forced out from the presynaptic terminal by a microelectrode to help patients with acetylcholine relief disorders [55].

The enzyme acetylcholinesterase catalyzes the hydrolysis of acetylcholine to acetic acid and choline. Therefore, inhibitors of acetylcholinesterase increase the acetylcholine concentration in the synapses and can be used for treatment of patients with myasthenia gravis. Such inhibitor is neostigmine, which blocks the active site of acetylcholinesterase, and the enzyme cannot break down the acetylcholine molecules (Figure 4.4).

Muscle spasm is treated with 2–5 g/dl $CaCl_2$ on the negative pole. Calcium ions stabilize membranes and decrease excitability threshold in peripheral nerves and skeletal muscle. Also 2 g/dl $MgSO_4$ (ointment) on the same pole can be used. Magnesium ions push out calcium ions. As a result, muscles relax and their neuromuscular activity is reduced.

For *muscle and joint pain*, 2–3 g/dl sodium salicylate or 10 g/dl trolamine salicylate ointment on the cathode is used. Salicylate ion inhibits the synthesis of prostaglandins, which take part in inflammation and pain.

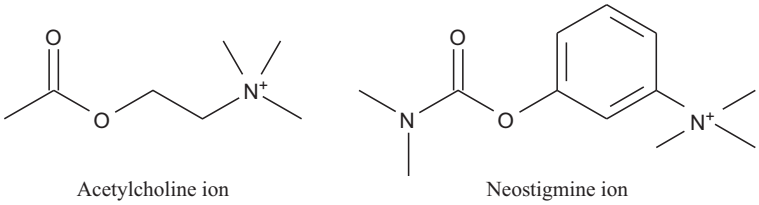

Figure 4.4: Structural similarity between acetylcholine and neostigmine ions.

Iontophoretic patches enable patients to administer a bolus of fentanyl for *pain relief* [56]. Soft tissue pain can be treated with 4–5 g/dl lidocaine (ointment) on anode.

Iontophoresis is applied also for sport injuries. For this purpose, a mild electric current is used. Most sessions last 10 to 15 min for a week or two until the injury is healed.

Otolaryngologists use iontophoresis of local anesthetics for *anesthesia* of the middle ear, and dentists use it for anesthesia of the oral mucosa [57,58].

The anesthesia of the skin can be achieved with local administration of lidocaine and epinephrine [59,60]. Skin anesthesia is best obtained with solutions containing 1 to 4 g/dl lidocaine and between 1/10,000 and 1/50,000 epinephrine. The anesthetic iontophoresis is valuable especially for children.

Aphthous stomatitis is healed with triamcinolone acetonide – a synthetic corticosteroid, which is about eight times as potent as prednisone.

Dermabraded tattoos are treated with iron and titanium oxide on anode.

4.7.2 Ocular iontophoresis

Iontophoresis is one of the noninvasive techniques for delivery of therapeutic drugs into the eye. It has the capacity to provide high drug tissue concentration safely, while minimizing the systemic drug exposure. So drugs in solutions, ointments, and suspensions become efficient, while only 1% of the same drugs delivered per os reach the eye. This minimizes the risk of infection, inflammation, and trauma.

The most commonly method is as follows: A 5–10 mm eye cup is filled with drug solution and a metal electrode is submerged. Then the cup is placed over the eye. During iontophoresis, the drug is continuously infused to the cup through a port for drug delivery, and air bubbles are aspirated through other port. The opening of the eye cup is 13 mm, which avoids contact with the cornea [61,62,63].

Two types of ocular iontophoresis are known: transcorneal and transscleral.

4.7.2.1 Transcorneal iontophoresis

Transcorneal iontophoresis delivers a drug in high concentration to the anterior segment of the eye (cornea, aqueous humor, ciliary body, iris, and lens), for treatment of anterior segment diseases. It is used for the treatment of *glaucoma*, *dry eyes*, *keratitis*, *corneal ulcers*, and *ocular inflammations*. However, the transcorneal iontophoresis cannot insert drugs into the posterior segment of the eye [64].

4.7.2.2 Transscleral iontophoresis

The lens-iris diaphragm is the main barrier of local applied drugs to pass through the choroid in the posterior eye tissues such as vitreous and retina. Transscleral iontophoresis overcomes this barrier and delivers drugs to the vitreous and retina. The iontophoretic electrode is placed on the conjunctiva to avoid electric current damage in the retina. This treatment is an effective alternative for multiple intravitreal injections or systemic therapy used for posterior ocular disorders, such as *retinitis*, *optic nerve atrophy*, *uveitis*, *endophthalmitis*, *pediatric retinoblastoma*, and ***a**ge-related **m**acular **d**egeneration* (AMD).

4.8 Troubleshooting

Problem	Cause	Solution
Device is shutting down.	Treatment site has high resistance.	Leave the electric current to 0.5 mA.
	Treatment site is callused.	Soak treatment site for 20–30 min prior to treatment. Wet the callused site.
Device does not power up.	Battery.	Check the battery. If necessary, change it.
	Battery contact.	Check both contacts between battery and biological tissue.
Electrode indicator lights.	Loose electric connection at one or both electrodes.	Turn electric current knob clockwise to pause treatment.
	One or both electrodes have pulled away from the skin.	Correct the problem.
	Insufficient medication in drug electrode.	Add medication and restart treatment.
	Drug electrode is not hydrated.	Hydrate the drug electrode.
Battery indicator lights when device is turned on.	Battery voltage is too low.	Replace battery.

(continued)

Problem	Cause	Solution
Battery indicator lights during treatment.	Battery voltage is decreasing during treatment.	Replace battery.
Electric current stops.	Treatment has paused by decreasing electric current to zero mA.	Turn electric current knob to restart treatment.
Dose limits.	Maximum electric current dose of 80.0 mA/min has been reached.	Use dosage recommendations.
Electric current limits.	Maximum electric current of 4.0 mA has been reached.	Use recommendations on electric current settings.
Resistance limits.	Skin resistance at the electrode site is too high.	Set electric current level as high as possible and prolong time to deliver desired dose.

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5 Danger during electrophoresis

Electrophoresis can be accompanied by body damages as irritation, erosion, inflammation, or intoxication. They can be obtained by objects to be examined and separation medium, or during electrophoretic treatment and electrophoresis run.

5.1 Danger by objects to be examined

Danger can come from objects to be analyzed, such as bacterial toxins, viruses, blood pathogens, for example HIV viruses, and pathological genes. These objects should be examined in appropriate laboratories, for example, viral and bacteriological laboratories.

5.2 Danger by separation medium

Polyacrylamide gels are produced by copolymerization of acrylamide and BIS. Acrylamide is neurotoxic, penetrates through the skin and by inhalation, and accumulates in the body. The disease symptoms appear soon or later. The central nervous system shows drowsiness, disturbance of balance, and mental changes characterized by confusion, hallucinations, memory loss. The peripheral nervous system shows symptoms of polyneuropathy, such as numbness of the lower limbs, tingling of the fingers, tenderness to the touch, weak or absent tendon reflexes, such as knee jerk, ataxic gait, foot drop, and muscular atrophy of the extremities.

Acrylamide is genotoxic, too. It causes chromosomal aberrations, sister chromatid exchanges and unscheduled DNA synthesis. Besides, it has reproductive toxicity: Testicular atrophy and decreased fertility have been reported in male mice given acrylamide by mouth. Additional laboratory studies have shown that acrylamide causes a variety of tumors in rats and mice. Therefore, acrylamide is classified as a possible human carcinogen.

Acrylamide should not be in contact with the skin, neither be inhaled. When working with it, rubber gloves and goggles must be used, and the preparations must be made under an outlet. *Never pipette an acrylamide solution by mouth*! Allow unused acrylamide solutions to polymerize and discard the resultant gels.

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The polyacrylamide gel is not toxic. Nevertheless it is not allowed to touch it with bare hands, because of a risk that it still contains some amount of acrylamide. This risk is diminished significantly, if polyacrylamide gels are previously washed and dried to obtain rehydratable gels.

Immobilines, lectins, and related chemicals are toxic, too.

The electrophoretic buffer can also be dangerous. A toxic buffer is, for example, the widespread barbitalate buffer, which contains a narcotic.

5.3 Danger during electrophoretic treatment

During sample preparing and the operations after the electrophoresis (fixation, staining, destaining), toxic chemicals can be used, for example SDS, 1,4-dithiothreitol, trichloroacetic acid, methanol, and more.

5.4 Danger during electrophoresis run

The electrophoresis unit and power supply must be held on a dry place. In some electrophoretic methods, such as isoelectric focusing on gels with carrier ampholytes, or on IPG gels, dangerous voltages are used. All electrophoresis methods should be carried out in separation chambers closed safely. The connectors of the separation chambers must be so installed that the electric current would immediately stop, if the chamber is accidentally opened.

The most important roles for carrying electrophoresis run are given in the following lines:

- Carefully inspect all cables and connections between the electrophoresis chamber and power supply. Cables and connectors must be insulated.
- Replace exposed or frayed wires.
- Never remove or insert leads unless the power supply is turned off. Do not insert or remove leads with both hands, as this can shunt lethal electricity through the heart.
- Do not touch the power supply with both hands during operation. The power supply ought to turn off automatically at short circuit.
- Always start the electrophoresis with the power supply turned off.
- Connect the gel apparatus to the power supply and then turn the power supply on.
- Turn up the electric voltage, current, or power to the desired level.
- Reverse the process after the electrophoresis has finished: turn the power supply down to zero, turn off the power supply, and then disconnect the gel apparatus.

6 Future of electrophoresis

The development of electrophoresis in the future is characterized by certain trends. They concern the methods, separation and precast gels, blotting methods, automation, and microchip electrophoresis.

The horizontal electrophoresis of proteins and nucleic acids is gaining on importance over the vertical electrophoresis. Combinations between electrophoretic methods and other analytical methods have future.

6.1 Protein electrophoresis

The shorter separation lengths [1,2] and the shorter separation times are of importance in the clinical routine as well as in the research. The free-flow electrophoresis [3,4] is used more and more due to its valuable analytical and preparative results. The isoelectric focusing in immobilized pH gradients replaces more and more the isoelectric focusing with carrier ampholytes.

6.1.1 Gel electrophoresis of proteins

The paper, starch, and agar gel electrophoresis were detached by the agarose and polyacrylamide gel electrophoresis.

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6.1.1.1 Agarose gel electrophoresis of proteins

Agarose gel was the most important medium for electrophoretic separations for a long time. The possibility to vary its pore diameter allows its usage for separation of protein polyions of different sizes. Agarose gel is and will remain the common medium for the serum protein electrophoresis.

6.1.1.2 Polyacrylamide gel electrophoresis of proteins

Other co-monomers, instead of BIS, will be used in the polymerization process to obtain polyacrylamide gels appropriate for certain proteins.

The electrophoresis in slab gels wins on importance against the electrophoresis in round gels.

The thick gels are replaced by thin and ultrathin gels and the significance of the precast gels on film or net used in the electrophoresis and isoelectric focusing will increase.

The 2D-electrophoresis is in a rapid development, too. In this connection, two factors are important: the precise immobilized pH gradients and the use of computer programs for evaluation of two-dimensional pherograms (protein maps). The databases of 2D-pherograms are already a good start of this trend [5].

6.1.1.3 Blotting of proteins

Several methods for blotting of proteins exist. They will be improved.

Western blotting

The Western blotting is most common used. It is an analytical technique for detection of specific proteins. At first, native or denatured proteins are resolved by gel electrophoresis; then they are transferred onto a membrane (typically nitrocellulose or PVDF), where the proteins bind to specific antibodies [6,7].

Western blotting has future in protein quantification. The combination of highly selective antibodies, faster blotting protocols, and quantitative imaging allow Western blotting to continue to be a method of interest.

6.1.2 Capillary electrophoresis of proteins

The use of capillary electrophoresis will rise [8,9] because of its high resolution, short analysis time, and quantitative computer-controlled evaluations. It is already translocated from the analytical laboratory to the clinical environment. CE has the potential for improving standard analyses (routine and specialized) in the clinical laboratory.

A simple approach was presented recently based on the in-line coupling between magnetic particles-based **s**olid-**p**hase **e**xtraction (SPE) and CE [10]. Silica-

coated iron oxide particles were synthesized and used as a reverse-phase sorbent for in-line SPE-CE. Magnets were used to immobilize the sorbents inside the capillary. The results obtained have showed that this strategy enhances the detection sensitivity in the range of 125–700-fold compared with CE. The method, which has been applied for urine samples from drug abusers, has future.

6.1.3 Protein microchip electrophoresis

The automation of the protein electrophoresis has begun with the PhastSystem [11,12]. An automated electrophoresis station houses all electrical, optical, and hardware components necessary for the separation, staining, destaining, and reserving the results. Connected to computer software, it accomplishes electrophoretic analysis in 30–40 min.

Today, LabChip microfluidic systems for automated protein electrophoresis are available. They permit separation, detection, and data analysis of much smaller samples using much smaller reagent quantities than the standard methods.

Within each microchip, microchannels connect the sample wells to a separation channel and buffer wells. A set of electrodes applies a voltage across the microchannels, causing charged molecules in the samples to migrate through the separation microchannel. The microchannels are filled with gel that sieves the samples. Samples are run sequentially, with a sufficient lag between them to prevent cross-contamination.

In the presence of **l**ithium **d**odecyl **s**ulfate (LDS), proteins migrate through the separation channel at a rate based on their size. During separation, a fluorescent dye associates with the LDS micelles coating the proteins with free micelles. Traditionally, in SDS-PAGE, samples are stained in the gel once separation is completed; the trend now is the proteins to be stained with a fluorescent dye during separation.

6.2 Nucleic acid electrophoresis

A variety of DNA electrophoresis methods have been developed. They serve for the diagnosis of more than 3,000 monogenic diseases [13]. This trend will gain in the future.

6.2.1 Gel electrophoresis of nucleic acids

Nucleic acid gel electrophoresis is used to separate DNA or RNA fragments by size and reactivity. Longer fragments migrate slower through the gel because they experience more resistance within the gel, and on the contrary.

6.2.1.1 Agarose gel electrophoresis of nucleic acids

Agarose gel will save its position for establishing nucleic acid fragment sizes and for PCR reactions because of its great pores and simplicity of manipulation. For diagnostic applications, however, favorable alternatives are used.

6.2.1.2 RFLP

In **r**estriction **f**ragment **l**ength **p**olymorphism (RFLP) analysis, the DNA sample is broken by restriction enzymes into pieces and the resulting fragments are separated according to their lengths by agarose gel electrophoresis [14]. This method allows the detection of different alleles in DNA and will be in use for genetic diseases caused by mutations. RFLP analysis is important for the genome mapping and the localization of genes in genetic disorders. However, it is less efficient as compared with the primer extension [15] and **s**ingle **n**ucleotide **p**olymorphism (SNP) genotyping based on DNA microchip technology [16].

6.2.1.3 Polyacrylamide gel electrophoresis of nucleic acids

The resolution of DNA electrophoresis on polyacrylamide gels is higher as compared with the electrophoresis on agarose gels. Radioactive labeling of PCR products and subsequent autoradiography is most commonly used. Detection systems for automated sequencing of DNA fragments, labeled by fluorochromes, and laser detection during PAGE will be applied in the future.

6.2.1.4 SSCP

The **s**ingle-**s**trand **c**onformation **p**olymorphism (SSCP) is a method for analyzing the conformational difference of single-stranded nucleotide sequences of identical length. The single-stranded nucleotides can be distinguished by gel electrophoresis according to their different conformations [17].

Using SSCP, the sequence of a target gene can be screened in order to identify specific mutations. In the future, the high-throughput microchip sequencing will render the role of the SSCP method in human genetic diagnostics.

6.2.1.5 DNA sequence analysis using slab-gel electrophoresis

The DNA sequencing allows detection of unknown base pair substitutions as well as small deletions or insertions. As handling and time-effectiveness of sequencing is much more convenient in capillary electrophoresis systems, sequencing on a PAGE basis will be replaced by this modern method. Furthermore, DNA microarrays are developed for high-throughput sequencing. Sequencing with the help of DNA microchips is largely restricted to analysis of known mutations [18,19]. Future options will comprise sequencing the whole human genome on a single microchip.

6.2.1.6 DGGE and TGGE

Denaturing **g**radient **g**el **e**lectrophoresis (DGGE) [20,21,22] and **t**emperature **g**radient **g**el **e**lectrophoresis (TGGE) [23] are forms of electrophoresis, which use either a chemical or temperature gradient to denature the sample as it moves through a polyacrylamide gel. DGGE and TGGE can be applied to nucleic acids and (less commonly) to proteins. DGGE was the original technique, and TGGE is a refinement of it.

DGGE and TGGE are methods employed for investigating DNA variations among tumor cells [24,25] and a variety of mutation screening schedules in DNA diagnostics [26].

The separation principle of DGGE and TGGE is based on the melting characteristics of DNA fragments. Fragments differing by only a single base pair migrate at different speed in a denaturing polyacrylamide gradient gel due to their differing melting temperatures [27,28]. In DGGE, PCR products dissociate with increasing concentrations of urea, while in TGGE, DNA fragments dissociate with increasing temperatures. As a result of the melting, DNA spreads through the gel and can be analyzed for single components, even those as small as 200–700 bp.

Attachment of a **g**uanine and **c**ytosine (GC) rich tail (the so-called GC clamp), which does not denaturize DNA, improves the sensitivity of these methods. Large PCR fragments up to 1,000 bp can be screened efficiently [29,30]. The significance of these methods will increase.

6.2.1.7 Blotting of nucleic acids

The nucleic acids blotting will increase becoming the method of choice when it comes to specific nucleates. The introduction of net-supported thin and ultrathin polyacrylamide gels will help this trend to increase [31,32].

Southern blotting

Southern blotting combines RFLP and hybridization with a probe [33]. DNA is denaturized, size-separated, and transferred on nylon membranes for hybridization with specific probes that can be radiolabeled or visualized by chemoluminescent treatment. Nowadays, the quantitative PCR techniques and **m**ultiplex **l**igation-dependent **p**robe **a**mplification (MLPA) [34] represent alternatives of Southern blotting for detection of genomic deletions or insertions.

MLPA will gain a success in the future. Its kits for a large variety of genes with known occurrence of small insertions/deletions are now available and their number will increase.

Northern blotting

Northern blotting allows investigation of RNA expression or corresponding splice variants. In comparison with Southern blotting, total RNA or poly(A)+mRNA is

proved instead of genomic DNA [35]. Nowadays, the field of RNA expression analysis is occupied by real-time PCR applications. Therefore, Northern blotting has lost its previous importance in genetic diagnostics.

6.2.2 Capillary electrophoresis of nucleic acids

The introduction of **c**apillary **e**lectrophoresis (CE) in the late 1980s [36] has provided the basis for the decipherment of the complete euchromatic sequence of the human genome [37]. CE offers superior resolution, sensitivity, and potential for automation as compared to classical slab-gel electrophoresis, resulting in a tenfold increase of throughput.

The genomic information accumulated by CE allows clarifying genetic causes for many diseases. It has paved the way for expanded genetic diagnostics in DNA sequencing.

CE replaces the DNA sequencing *via* slab-gel electrophoresis due to the next significant improvements:

- The hands-on time is reduced in CE compared to slab gels. Modern CE sequencers provide automated injection of polymer and capillary array installation.
- The run times are reduced by five- to tenfold due to the high voltage. The relatively small dimensions of the capillaries allow many parallel runs in hundreds of capillaries.
- The increased sensitivity of CE enables to analyze smaller amounts of template as compared to slab-gel applications [38].

Capillary electrophoresis will enter diagnostic areas that are occupied by traditional genetic methods. In the future, fluorescence-labeled PCR and optimized CE appear to be the methods of choice in **H**untington's **d**isease (HD) and **s**ickle-**c**ell **a**nemia (SCA) diagnostics.

6.2.3 DNA microchip electrophoresis

The separation time of electrophoresis in a slab gel is impractically long. Therefore, in the last years the DNA separation has been orientated toward the use of microchips (microfluidic chips). They have been under rapid technological progress.

The small chips with all electronic parts integrated enhance the impact of portable analytical instruments. Because of the narrow sample zone and highly controlled injections, the microchips make a five- to tenfold increase in DNA sequencing rate over even **c**apillary **a**rray **e**lectrophoresis (CAE) [39,40]. They can deliver reasonably long DNA sequencing reads, for example 550 bases in 27 min [41,42,43].

In microchips, the fragments of DNA or RNA are bound to fluorescent dyes and detected as they pass a laser and photodiode detector (laser-induced fluorescence). The data are automatically collected, saved, and presented by software. The high-speed and fidelity resolution of microchip electrophoresis of nucleic acids will have a large place in the future biotechnologies and drug industry.

The microchip electrophoresis will be the widespread form of electrophoresis in the next years. This trend demands additional steps to decrease their expensive price.

6.3 Digital documentation

Traditionally, the electrophoresis results are documented using silver halide-based photography. While this remains an excellent method for qualitative documentation of single-gel results, digital capture offers a number of significant advantages when documentation requires quantitation analysis. Digital images of gel pherograms can be obtained using an image-capture device, and the images can be manipulated using image analysis software [44].

The electrophoretic bands are recorded with a computer-operated camera; the intensity of the bands or spots is measured with specialized software and compared against markers image separated on the same gel. They can be quantitatively analyzed by a gel imaging device or by visualizing the gel with UV light.

The digital documentation will replace completely the photography as a method of documentation. In future, the clinical as well as the research electrophoresis will be supported by improved devices and computer programs for digital gel documentation.

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History of electrophoresis and iontophoresis

The history of electrophoresis is an interesting story that continues already a few centuries.

History of electrophoresis

Discovery of electrophoresis

The movement of charged particles in an electric field was first observed as long ago as 1807–1809 by the Russian chemist of German nationality Ferdinand Friedrich Reuss (1778–1852) at the Moscow State University [1]. Using a microscope he observed the migration of colloidal clay particles in an electric field. He also discovered the opposite flow of water (electroosmosis).

Quincke [2,3] showed that the migration speed of a charged particle in an electric field is linearly related to the potential gradient and is affected by the pH value of the solution.

In 1892, Picton and Linder [4] observed that hemoglobin, a colored protein, moved in a U-tube, filled with an electrolyte solution and placed in an electric field. In 1899, Hardy reported the movement of serum globulins under the influence of an electric field and showed that the electric charge of a protein could be changed from positive to negative when pH value was varied [5].

In 1909, Leonor Michaelis introduced the term *electrophoresis*. This term is composed of the Greek words *elektron* (amber), which was connected in the ancient times with the electricity, and *phorein* (bear).

In 1929, the movement of both red blood cells [6,7] and bacteria was reported.

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Zone electrophoresis of Tiselius

The history of modern electrophoresis began with the works of Arne Tiselius in Sweden. In 1937, he published his classic paper describing a new method for analyzing bio-colloids, and developed the "Tiselius apparatus" for moving boundary electrophoresis [8]. He detected separated but colorless proteins using a Schlieren-scanning system. With the help of it, he obtained refraction shadows of resolved proteins on a photographic plate.

Later, instead of solutions, new methods used solid or gel media for separating charged particles into stable bands (zones). In 1950, Tiselius dubbed these methods "zone electrophoresis". At first, as a supporting medium, a filter paper was used; later, gels were used. Gel electrophoresis and related techniques became the basis for a wide range of biochemical methods, such as protein fingerprinting, blotting procedures, DNA sequencing, and many more.

In 1948, Tiselius was awarded the Nobel Prize.

Paper electrophoresis

The first report on paper electrophoresis appeared by König in 1937 [9]. He introduced paper soaked in a buffer for zone electrophoresis and suggested UV detection. In 1939, von Klobusitzky and König separated the components of snake venom by paper electrophoresis [10]. In 1949, Pauling and colleagues [11] proved using electrophoresis that patients with sickle cell disease contain an abnormal hemoglobin.

Since the 1950s, new electrophoresis methods were developed: affinity electrophoresis, capillary electrophoresis, electroblotting, electrophoretic mobility shift assay, isotachophoresis, pulsed-field gel electrophoresis, preparative electrophoresis, and more.

In 1957, Kohn introduced cellulose acetate membranes as a solid support for hemoglobin electrophoresis [12]. Next years significant contributions to electrophoresis variants were made: in immunoelectrophoresis, counter immunoelectrophoresis, radial immunodiffusion, protein blotting, and immunofixation.

Gel electrophoresis

In 1955, significant progress in the electrophoresis was made by Oliver Smithies who introduced partially hydrolyzed starch gels for separation of serum proteins [13,14]. Starch gel (and later agarose, polyacrylamide, and other gels) enabled efficient separation of proteins, making it possible to analyze complex protein mixtures with relatively simple technology.

Agarose gel electrophoresis

Agarose, a purified form of agar, was introduced as separation medium for electrophoresis in 1961 by Hjerten [15]. It continues to be applied for separation of proteins and nucleic acids.

Polyacryalamide gel electrophoresis

Polyacrylamide gel has been introduced as electrophoresis medium in 1954 by Oster [16] who advanced his photopolymerization techniques over the subsequent years. However, credit for this development is usually given to Raymond and Weintraub [17] who published their work in 1959. Later, Hjerten gave detailed formulae and recipes for preparation of polyacrylamide gels with specific sieving characteristics [18].

In 1967, Shapiro *et al.* [19] showed that the anionic detergent **s**odium **d**odecyl **s**ulphate (SDS) improved the electrophoresis resolution of proteins on the basis of their molecular masses in polyacrylamide gels. In 1969, this method was optimized by Weber and Osborn who confirmed that it gave relatively true molecular masses data [20]. The explanation of this process was presented by Reynolds and Tanford [21].

Disc-electrophoresis

The discovery of disc-electrophoresis is a substantial progress in the electrophoresis history. Its theoretical basis was developed by Leonard Ornstein [22], and its practical implementation was improved by Baruch Davis [23].

Later, Laemmli [24] used the Ornstein buffer system for SDS disc-electrophoresis of proteins. SDS disc-electrophoresis is a successful, simple, and useful technique for determining the molecular masses of macromolecules.

Capillary electrophoresis

Hjerten used in 1967 [25] 3 mm i.d. tubes for capillary electrophoresis. Later, in 1981, Jorgenson and Lukacs [26] demonstrated first high-performance capillary electrophoresis separations with 75 μm i.d. capillaries.

In 1988–1990, Karger's group [27,28,29] separated single-stranded DNA oligonucleotides in gel-filled capillaries.

Isoelectric focusing

Isoelectric focusing of proteins was applied by Kolin [30,31]. He developed it in a column containing a sucrose gradient to limit the band mixing, where two buffer solutions were allowed to diffuse into one another to generate a pH gradient.

A few years later, Svensson created the theoretical basis of isoelectric focusing and gave the name of carrier ampholytes [32]. The first carrier ampholytes studied was a polypeptide mixture obtained by partially hydrolyzed blood, where he separated hemoglobins [33].

Next, Vesterberg [34,35] patented a method of creating synthetic carrier ampholytes. They were mixtures of aliphatic aminocarboxylic acids, which rapidly formed a continuous pH gradient when placed in an electric field. His ampholytes were commercialized by LKB under the name "Ampholine". The resultant pH gradients in polyacrylamide gels were relative stable.

Two-dimensional electrophoresis

In the best 2D-technique, the proteins are separated by their pI values in a pH gradient in the first dimension, and then are separated due to their molecular sizes/charges using SDS-PAGE in the second dimension. The method was introduced in 1975 simultaneously by three independent groups of researchers, namely O'Farrell [36], Klose [37], and Scheele [38], although the credit usually goes to O'Farrell. Isoelectric focusing was achieved in a porous tubular gel in the presence of ampholytes, and then the tube with the focused proteins was placed onto a SDS gel and the proteins were once more separated.

Blotting

The blotting of polyions resolved by electrophoresis was discovered by Edwin Southern. Later, many scientists created similar techniques for different substances.

Blotting of proteins

There are many forms of protein blotting: Western, Southwestern, Northwestern, Far-western, Eastern, and Far-Eastern.

Western blotting

Western blotting (called also "Protein blotting" or "Protein immunoblot") was developed in 1979 by three groups of researchers: in Stanford, Basel, and Seattle, which worked independently.

George Stark's group at Stanford University, including also Jaime Renart and Jakob Reiser, published first [39] in July 1979 this method. They used passive transfer of proteins, and then ^{125}I-labeled protein A for detection.

Harry Towbin's group in Basel, Switzerland, also including Julian Gordon (at Friedrich-Miescher-Institut) and Theophil Staehelin (at Hoffman–La Roche), published in September 1979 [40] a similar technique. They transferred proteins from SDS gel to blot membrane using electric current [41], and used secondary antibodies for detection. So they developed a technique, which is now widespread.

W. Neal Burnette, working in Robert Nowinski's lab at the Fred Hutchinson Cancer Research Center in Seattle, submitted in 1979 a similar paper, which was at first rejected. This paper was published in 1981 [42] after he had already become famous through distribution of preprints to friends who repeated the process [43]. Burnette gave the technique the name "Western blotting" as a nod to Southern blotting and because his lab was on the west coast. His version is simpler and universal [44,45].

So, Stark's group published first. Towbin's group developed what appears to be the most common method, including the electrophoretic transfer method and buffers, as well as the use of secondary antibodies. Burnette gave the technique the name and popularized the Western blotting.

Southwestern blotting

Southwestern blotting is a technique that was first described by Bowen, Steinberg, and colleagues in 1980 [46]. It is used to identify and characterize proteins that bind to DNA by their ability to bind to specific oligonucleotide probes.

Northwestern blotting

Northwestern blotting, known also as *Northwestern assay*, is a hybrid technique of Western blotting and Northern blotting. It detects interactions between RNA and proteins [47].

Far-Western blotting

Far-Western blotting was based on the technique of Western blotting. While Western blotting uses an antibody to detect a protein of interest, Far-Western blotting uses a non-antibody protein for the same purpose. Thus, Far-Western blotting is rather employed to detect protein: protein interactions.

Eastern blotting

Eastern blotting was created by Tanaka *et al.* [48]. It is mentioned in an immunology textbook, which compares the common blotting methods (Southern, Northern, and Western) and states that "the Eastern blot, however, exists only in test questions" [49].

Far-Eastern blotting

Far-Eastern blotting seems to have been named in 2000 by Ishikawa and Taki [50]. The method is based on antibody or lectin proving of lipids transferred to PVDF membranes.

Blotting of nucleic acids

Two main types of nucleic acid blotting exist: Southern and Northern blotting. Beside them Middle-Eastern and Eastern-Western blotting are known.

Southern blotting

Southern blotting of separated by electrophoresis DNA polyions was discovered in 1975 by the British biologist Edwin Southern at University of Edinburgh [51] and carries his name.

Northern blotting

Northern blotting of RNA was developed in 1977 by George Stark's group at Stanford University [52].

Middle-Eastern blotting

Middle-Eastern blotting was described in 1984 [53]. It was developed for blotting and immobilizing of polyA-RNA (resolved by agarose) using DNA probes.

Eastern-Western blotting

Eastern-Western blotting was introduced in 1996 by Bogdanov *et al.* [54]. Using it, phospholipids are blotted on PVDF or nitrocellulose membrane prior to transfer of proteins by Western blotting onto the same membrane and probing with specific antibodies. Eastern-Western blotting is based on the earlier work by Taki *et al.* in 1994 [55], and on the previous method of Towbin, created in 1984 [56].

Staining methods

The detection of resolved proteins relied on dyes such as Bromophenol blue [57], Azocarmine B [58], and Amido black 10B [59]. On this basis, visible wavelength scanning densitometers were introduced by USA companies as Brinkman and Beckman.

The best staining assay is that of Bradford [60] who bound the dye **C**oomassie **B**rilliant **b**lue (CBB) R-250 to proteins. This dye was developed as an acid wool dye. Its binding to proteins was first studied by Fazekas de St Groth *et al.* [61]. CBB has

been employed for staining of protein bands in polyacrylamide gels by Chrambach *et al*. in 1967 [62].

In 1979, Switzer and colleagues [63] applied silver staining for high-sensitivity protein detection in electrophoresis. In the following year, they applied it to the analysis of proteins in human cerebrospinal fluid [64] showing that it is 10–100 times more sensitive than Coomassie Brilliant blue R-250.

Outline history of electrophoresis

The history of electrophoresis is shown in Table 1.

Table 1: Outline history of electrophoresis.

Year	Researcher(s)	Development
1807–1809	Ferdinand Friedrich Reuss [65]	Observed movement of colloids in an electric field – the discovery of electrophoresis in Moscow State University.
1858	Hittorf [66,67]	Measured the transport numbers of ions and the conductivity of solutions.
1859–1861	Quincke [68,69]	Studied the electroosmosis and discovered the electric double layer.
1879	Helmholtz [70]	Proposed the first model of the electric double layer.
1887	Arrhenius [71]	Developed the theory of electrolyte dissociation.
1892	Picton and Lindner [72]	Invented the boundary electrophoresis.
1897	Kohlrausch [73]	Described the “persistent” function in a system of two or more electrolyte solutions and empirically discovered the square root dependence of the equivalent conductivity of the electrolyte concentration.
1899	Hardy [74]	Observed the movement of globulin in a U-tube by electric current.
1903	Smoluchowski [75,76]	Derived the first equation of ionic mobility.
1904	Perrin [77]	Used the Helmholtz model for dissolved particles.
1910–1913	Gouy [78] and Chapman [79]	Developed, independently from each other, the theory of the structure of the diffuse counterionic atmosphere of the electric double layer.

Table 1 (continued)

Year	Researcher(s)	Development
1923	Debye and Hückel [80]	Created the theory of electrolyte solutions and the theory of electric double layer in an ionic solution.
1923	Brønsted [81]	Developed the proteolytic theory of acids and bases.
1924	Hückel [82]	Derived the second equation of ion mobility.
1924	Stern [83]	Developed the current theory of the electric double layer.
1926	Onsager [84,85]	Derived the equation of conductivity of an ionic solution.
1931	Henry [86,87]	Combined the equations of Smoluchowski and Hückel in a mathematical function.
1933–1937	Tiselius [88,89]	Carried out the free electrophoresis of serum proteins. Improved apparatus for moving boundary studies including ultraviolet detection of proteins.
1937–1948	König [90,91], Wieland and Fischer [92]	Introduced the paper electrophoresis and an apparatus for it.
1942	Martin [93]	Developed the isotachophoresis.
1949	Pauling *et al*. [94]	Using electrophoresis proved that abnormal hemoglobin caused sickle cell disease.
1950–1961	Grassmann and Hannig [95,96]	Developed the free-flow electrophoresis.
1953	Grabar and Williams [97]	Combined the separation of proteins with antigen-antibody reactions in agar and thus developed immunoelectrophoresis.
1954	Kolin [98,99]	Introduced artificial pH gradients for IEF.
1955	Robinson and Stokes [100]	Completed the Onsager equation.
1955–1959	Smithies [101,102]	Described electrophoresis in a starch gel.
1957	Kohn [103,104]	Introduced the cellulose acetate as an electrophoretic medium.
1957	Poulik [105]	Used the TRIS-citrate-borate buffer system as a discontinuous buffer system for electrophoresis.
1959	Raymond and Weintraub [106]	Introduced the polyacrylamide gel as an electrophoresis medium.
1961	Hjerten [107]	Used the agarose gel for electrophoresis.
1961–1962	Svensson [108,109,110]	Developed the theory of isoelectric focusing.

Table 1 (continued)

Year	Researcher(s)	Development
1962	Hierten [111]	Gave detailed formulae for preparation of gels with specific sieving characteristics.
1963	Fazekas *et al.* [112]	Introduced Coomassie brilliant blue as a highly sensitive dye for detection of electrophoretically separated proteins.
1964	Ornstein [113] and Davis [114]	Invented the theory and practice of disc-electrophoresis in a TRIS-chloride-glycinate buffer system.
1965	Hjerten [115]	Described the concentration of polyions on the boundary to a solution with higher ionic strength – Hjerten's effect.
1965	Laurell [116,117] as well as Clarke and Freeman [118]	Described the rocket immunoelectrophoresis.
1966 1966–1969	Thorne [119] Vesterberg [120,121]	Used agar as separation medium. Synthesized carrier ampholytes and introduced the isoelectric focusing of proteins in the practice.
1967	Hjerten [122]	Invented capillary electrophoresis.
1967–1969	Shapiro *et al.* [123] and Maizel [124]	Developed the SDS-PAGE in continuous buffers for determination of molecular masses of proteins.
1967–1971	Margolis and Kenrick [125, 126], Kopperschläger *et al.* [127], Rodbard *et al.* [128], Slater [129,130]	Introduced gradient gels in the electrophoretic practice and analyzed theoretically the gradient electrophoresis.
1967	Hjerten [131]	Invented a free electrophoresis, which served as a basis for the capillary electrophoresis.
1967–1987	Mikkers *et al* [132], Jorgensen and DeArman [133], Tsuda *et al.* [134], Hjerten [135], Gebauer *et al.* [136], Terabe *et al.* [137], Green and Jorgenson [138], Hjerten [139,140,141]	Developed the capillary electrophoresis.
1969	Weber and Osborn [142]	Optimized the SDS electrophoresis for separation of protein subunits.
1969	Allen *et al.* [143,144]	Introduced the disc-electrophoresis in step-gradient gels.
1970–1973	Laemmli [145,146]	Used SDS in the discontinuous buffer system of Ornstein and Davis and created the SDS disc-electrophoresis.

Table 1 (continued)

Year	Researcher(s)	Development
1972	Aaij and Borst [147]	Introduced agarose gels with ethidium bromide for DNA electrophoresis.
1973–1979	Jovin and Chrambach [148,149]	Calculated mobilities of ions, which are used in electrophoresis, and p*K* values of protolytes, which form them.
1975	O'Farrell [150], Klose [151], and Scheele [152]	Introduced independently the two-dimensional electrophoresis, which combines isoelectric focusing with SDS electrophoresis.
1975	Southern [153]	Performed blotting of DNA.
1975–1982	Gasparic *et al.* [154], and Bjellquist *et al.* [155]	Used immobilized pH gradients for isoelectric focusing.
1976–1978	Lambin *et al.* [156,157], and Lasky [158]	Used gradient gels for SDS electrophoresis.
1978	Hannig [159,160]	Invented the free-flow electrophoresis.
1979	Rosen *et al.* [161]	Used agarose gels of low electroosmosis as a medium for isoelectric focusing.
1979	Towbin *et al.* [162], and Renart *et al.* [163]	Invented protein blotting.
1979–1981	Switzer *et al.* [164], and Merril *et al.* [165]	Established the silver as a highly sensitive staining method for electrophoretically resolved bands.
1981	Jorgenson and Lukacs [166]	Demonstrated high-performance capillary electrophoresis separations in 75 μm i.d. capillaries.
1982–1986	Michov [167,168,169]	Established that the complex compound TRIS-boric acid persists in TRIS-borate buffers, and calculated the mobility of TRIS-borate ion.
1983	Mullis [170]	Invented the **p**olymerase **c**hain **r**eaction (PCR).
1984	Schwartz and Cantor [171]	Introduced the pulsed-field gel electrophoresis for separation of large DNA nucleates.
1985	Michov [172]	Developed the parameter of ionic mobility, which can be used for calculating the ionic mobility at different ionic strengths.

Table 1 (continued)

Year	Researcher(s)	Development
1987	Schägger and Jagow [173]	Published the TRIS-acetate-TRICINEate buffer system for SDS-electrophoresis in gradient gels.
1988	Michov [174]	Simplified the Henry function.
1988–1990	Karger's group [175,176,177]	Separated single stranded DNA oligonucleotides in gel-filled capillaries.
1989	Booth [178]	Separated chromosomes in agarose gels by pulsed-field electrophoresis.
1989	Michov [179]	Described a disc-electrophoresis in one buffer at two pH values.
1992	Harrison *et al.* [180]	Introduced the **m**icro**c**hip-based capillary **e**lectrophoresis (MCE) – the earliest format of microfluidics.
1994	Wilkins [181]	Coined the term *proteomics*.
2004	Kastenholz [182]	Improved electrophoresis of metaloproteins.
2013	Michov [183,184]	Proved that every ion or polyion has two radii and two electric potentials: geometric and electrokinetic, and established the dependence of the electrokinetic radius on the ionic strength.

History of iontophoresis

Most authors accept that iontophoresis exists as a medical method after the works of the French biologist Stephane Leduc (1853–1939), professor at the Ecole de Medecine de Nantes in the early 1900s. Fritz Frankenhäuser is said to have introduced the term "iontophoresis."

In 1900 and later, Leduc [185,186,187] has published works on the physiological effects of electric current on the body. These papers led to establishment of ionic medication as a form of medical treatment.

Wirtz employed iontophoresis in ophthalmology in 1908. He passed electric current through electrolyte-saturated cotton sponges placed over the eye globe for treatment of conditions such as corneal ulcers, keratitis, and episcleritis [188].

In 1942, Witzel *et al.* applied iontophoresis for delivery of a variety of antibiotics such as tetracyclines, chloramphenicol, penicillin, streptomycin, neomycin, and bacitracin in rabbits [189].

In 1959, Gibson and Cooke demonstrated that sweating could be induced by pilocarpine iontophoresis [190].

In 1973, Corneau and Brummett used iontophoresis for local anesthesia of the ear [191].

In 1986, Gangarosa and Hill applied iontophoresis of vidarabine monophosphate for treatment of herpes orolabialis [192].

In 1992, Rigano *et al.* demonstrated treating of burned ears by gentamicin iontophoresis [193].

In 2003, Monti *et al.* studied the effect of iontophoresis on the permeation of the β-blocking agents **t**imolol **m**aleate (TM) and **b**eta**x**olol hydrochloride (BX) across rabbit corneas *in vitro*. They demonstrated that iontophoresis increased more the permeation of the hydrophilic TM than the lipophilic BX. The increased permeation after applying both drugs was accompanied by an increased corneal hydration [194].

In 2013, Patane *et al.* showed that repeated transscleral iontophoresis with dexamethasone phosphate in the rabbit eye could be used in the treatment of inflammatory disorders of the orbit, which require prolonged corticosteroid treatment [195].

Outline history of iontophoresis

The history of iontophoresis is shown in Table 2.

Table 2: Outline history of iontophoresis.

Year	Researcher(s)	Development
1900	Leduc [196,197,198]	Published works on the physiological effects of electric current on the body.
1908	Wirtz [199]	Employed iontophoresis in ophthalmology.
1942	Witzel *et al.* [200]	Applied iontophoresis for delivery of a variety of antibiotics in rabbits.
1959	Gibson and Cooke [201]	Demonstrated that sweating could be induced by pilocarpine iontophoresis.
1973	Corneau and Brummett [202]	Used iontophoresis for local anesthesia of the ear.
1986	Gangarosa and Hill [203]	Applied iontophoresis of vidarabine monophosphate for treatment of herpes orolabialis.
1992	Rigano *et al.* [204]	Demonstrated treating of burned ears by gentamicin iontophoresis.
2003	Monti *et al.* [205]	Studied the iontophoresis on the permeation of β-blocking agents across rabbit corneas *in vitro*.
2013	Patane *et al.* [206]	Treated inflammatory disorders of the orbit with repeated transscleral iontophoresis of dexamethasone phosphate in the rabbit eye.

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SI units and physical constants for electrophoresis

The International System of units SI (Système International) was introduced in 1960 [1,2,3]. It contains, in its current form, 7 *base units* (Table 1).

Table 1: SI base units.

Quantity	Abbreviation	Name of unit	SI unit
Mass	m	kilogram	kg
Length	$l, b, h, \delta, d, r, s, \lambda$	meter	m
Time	t	second	s
Thermodynamic temperature	T	kelvin	K
Amount of substance	n	mole	mol
Electric current	I	ampere	A
Luminous intensity	I	candela	cd

For electrophoresis, the unit *mol* has very important meaning. The mol is referred to as an amount of substance of a system, which contains as much particles as 0.012 kg carbon isotope ^{12}C. The particles can be of different nature: atoms, molecules, ions, electrons. Between the mass m, in kg, and the amount n, in mol, of a homogeneous substance x, the following relationship exists:

$$m = nM_x = nN_A m_x = nN_A M_r m_u \tag{1}$$

where $M_x = N_A m_x$ is the molar mass of the particle, in kg/mol; N_A (6.022 045•10^{23} mol^{-1}) is Avogadro constant (the number of particles in 1 mol); m_x is the mass of the particle x, in kg; M_r is the relative mass of the particle, dimensionless; and m_u is the atomic mass unit, in kg, equal to 1/12 of the mass of one atom of carbon isotope ^{12}C.

The derived units from SI base units and the physical constants, which are used in the electrophoretic analyses, are listed in Tables 2 and 3, respectively.

Table 2: Derived units from SI base units.

Derived quantity	Abbreviation and equation	SI unit
Space and time		
Area	$A, S = l^2$	m^2
Volume	$V = l^3$	m^3, l (liter)
Volume flow rate	$q_v = dV/dt$	m^3/s
Volumetric flux	$I_V = \frac{dV}{sS} = q_V/A$	m/s

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Table 2 (continued)

Derived quantity	Abbreviation and equation	SI unit
Velocity (rate) of migration	$v = ds/dt$	m/s
Velocity of an ion	$v_i = ds_i/dt$	m/s
Mobility of an ion	$\mu_i = v_i/E$	$m^2/(sV)$
Absolute mobility of an ion	$\mu_{\infty i} = v_{\infty i}/E$	$m^2/(sV)$
Effective mobility of an ion	$\mu'_i = \alpha\mu_i$	$m^2/(sV)$
Mechanics		
Density	$\rho = m/V$	kg/m^3
Force	$F = ma$	N (newton) = kg m/s^2 = CV/m
Pressure	$p = F/A$	Pa (pascal) = N/m^2 = J/m^3
Dynamic viscosity	$\eta = \tau dz/dv$	Pa s = kg/(m s)
Relative viscosity	$\eta_r = \eta/\eta_s$	dimensionless
Reduced viscosity	$\eta_{red} = \eta_{sp}/c_m$	l/g, m^3/kg
Intrinsic viscosity	$[\eta] = \lim_{c\to 0} \eta_{red}$	l/g, m^3/kg
Thermodynamics		
Energy, work, heat	$E, W = Fl$	J (joule) = Nm = Ws = CV
Quantity of heat	Q	J
Power	$P = dW/dt$	W (watt) = J/s
Celsius temperature	t	°C (degree Celsius)
Joule heating	$P = UI$	W
Heat flux density	$w = \frac{P}{S} = \frac{UI}{S} = \frac{RI^2}{S}$	W/m^2
Electricity		
Elementary charge	e	C
Electric charge	Q	C = As
Charge number (electrovalence)	z	dimensionless
Electric charge density	Q/S	C/m^2
Electric current area density	$J = \frac{dI}{dS} = F\sum^{c_i} z_i v_i$	A/m^2
Electric field strength (intensity)	$E = F/Q = dV/dr$	V/m = N/C
Electric potential	$\varphi, V = A/Q = Q/(4\pi\varepsilon r)$	V (volt) = W/A = J/C
Electrokinetic potential	ζ	V
Electric potential difference (voltage)	U	V = C/F
Electric energy	$W = QU$	J
Electric power	$P = UI$	W = VA
Force acting on an electric charge	$F = QE = Q_1Q_2/\left(4\pi\varepsilon r^2\right)$	N
Electric capacity	$C = dQ/dU = \varepsilon S/d$	F (farad) = C/V

Table 2 (continued)

Derived quantity	Abbreviation and equation	SI unit
(Di)electric permittivity	$\varepsilon = D/E$	F/m
Relative (di)electric permittivity	$\varepsilon_r = \varepsilon/\varepsilon_0$	dimensionless
Electric resistance	$R = U/I$	Ω (ohm) = V/A
Electric conductance	$G = 1/R$	S (siemens) = Ω^{-1}
Electrophoretic mobility	$\mu = v/E$	$m^2/(sV)$
Specific conductivity	$\gamma, \sigma = \frac{1}{\rho} = F\sum^{c_i} z_i\mu_i$	S/m
Molar conductivity	$\Lambda_m = \frac{\gamma}{c} = F(z^+\mu^+ + z^-\mu^-)$	$Sm^2/(k)mol$
Equivalent conductivity	$\Lambda_i = Fz_i\mu_i$	$Sm^2/(k)mol$
Absolute conductivity	$\Lambda_{\infty i} = Fz_i\mu_{\infty i}$	$Sm^2/(k)mol$
Effective conductivity	$\Lambda_i^{'} = Fz_i\mu_i^{'}$	$Sm^2/(k)mol$
Physical chemistry		
Amount of substance	n, v	(k)mol
Molar mass	$M = m/n$	kg/mol
Relative molecular mass	$M_r = M_x/m_u$	dimensionless
Molar volume concentration (molarity)	$c_B = n/V$	$kmol/m^3$, mol/l
Molar mass concentration (molality)	$m_B = n/m$	(k)mol/kg
Mass concentration	$c_m = m/V$	kg/m^3, g/l, g/dl
The equilibrium concentration of substance B	$[B]$	mol/l
Activity coefficient of substance B	γ_B	dimensionless
Relative activity of substance B	$a_B = \gamma_B[B]$	mol/l
Diffusion coefficient	D	m^2/s
Friction coefficient	$\mu = F/N$	dimensionless
Mass ionic strength (molal ionic strength)	$I_m = \frac{1}{2}\sum_{i=1}^{s} c_i {z_i}^2$	mol/kg
Volume ionic strength (concentration ionic strength)	$I_c = \frac{1}{2}\sum_{i=1}^{s} c_i {z_i}^2$	mol/l, $kmol/m^3$
Dissociation degree	α	dimensionless
Hydrogen exponent	$\mathrm{pH} = -log\frac{[H^+]}{[H^+{}_o]}$	dimensionless
pH gradient	$d\mathrm{pH}/dl$	1/m
Isoelectric point	$\mathrm{p}I, \mathrm{pH}(I)$	dimensionless
Persistent function of Kohlrausch	$\frac{v^+}{v^-} = \frac{z^+c^+}{z^-c^-}$	dimensionless

Table 3: Physical constants.

Physical constants	Symbol and equation	Value
Atomic mass unit	m_u	$1.660\ 5391 \bullet 10^{-27}$ kg
Elementary charge	e	$1.602\ 176\ 634 \bullet 10^{-19}$ C
(Di)electric constant	ε_0	$8.854\ 187\ 813 \bullet 10^{-12}$ F/m
Avogadro constant	N_A	$6.022\ 140\ 76 \bullet 10^{26}$ kmol^{-1}
Faraday constant	$F = N_A e$	$9.648\ 533\ 212 \bullet 10^{7}$ C/kmol
Molar gas constant	$R = N_A k$	8,314.46 J/(kmol K)
Boltzmann constant	$k = R/N_A$	$1.380\ 649 \bullet 10^{-23}$ J/K

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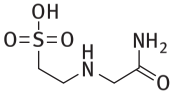
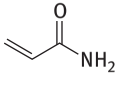
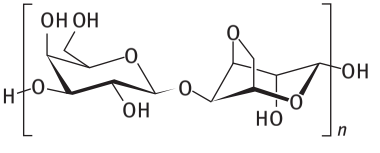
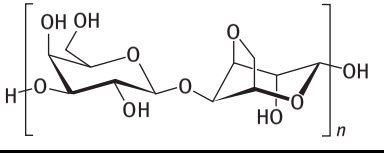
Reagents for electrophoresis

Reagent	Molecular formula	M_r	Density, d	p$K_{25}{}^{\circ}_{C}$	Useful pH range
ACES		182.19		6.78	6.1–7.5
Acetic acid		60.05	1.05	4.76	4.1–5.5
Acrylamide		71.08			
Agarose high electroosmosis		630.55			
Agarose low electroosmosis		630.55			

(continued)

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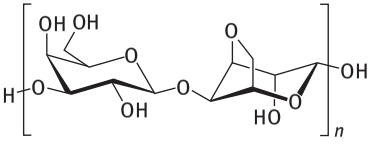
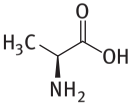
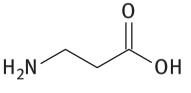
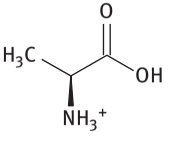
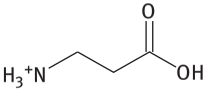
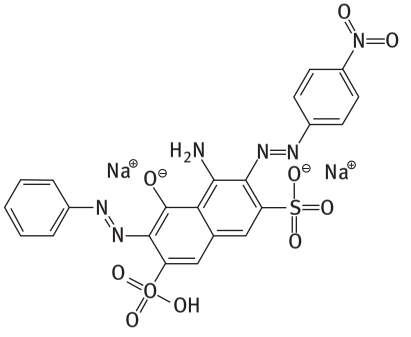
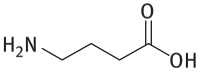
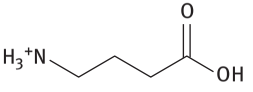
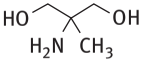
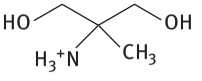
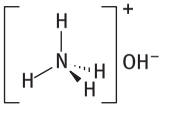
Reagent	Molecular formula	M_r	Density, d	$pK_{25}{}^{\circ}{}_{C}$	Useful pH range
Agarose without electroosmosis		630.55			
L-Alanine		89.09		9.69	9.0–10.4
β-Alanine		89.09		10.19	9.5–10.9
Alaninium ion		90.09		2.43	
β-Alaninium ion		90.09		3.63	

Amido black 10B		616.49		
4-Aminobutyric acid (GABA)		103.12	10.43	9.7–11.1
4-Aminobutyricium ion (GABA-ion)		104.12	4.23	3.5–4.9
Ammediol (2-amino-2-methyl-1,3-propanediol)		105.14		
Ammediolium ion		106.14	8.83	8.1–9.5
Ammonium hydroxide		35.05		

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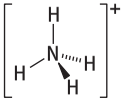
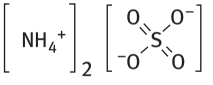
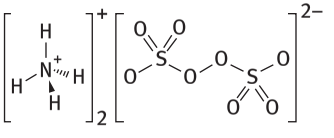
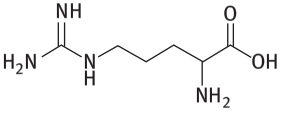
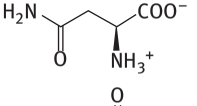
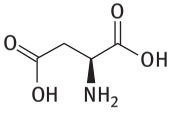
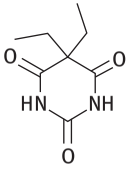
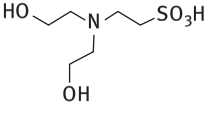
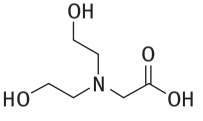
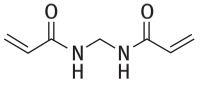
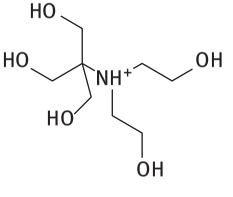
Reagent	Molecular formula	M_r	Density, d	p$K_{25}{}^{\circ}{}_C$	Useful pH range
Ammonium ion		18.04		9.25	8.6–10.0
Ammonium sulfate		132.13			
APS (Ammonium peroxydisulfate)		228.19			
L-Arginine		174.20			
Asparagine		132.12		8.86	8.2–9.6
L-Aspartic acid		133.10			

Barbital (Veronal)		184.20	7.98	7.3–8.7
BES [N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid]		213.25	7.09	6.4–7.8
BICINE		163.17	8.26	7.6–9.0
BIS (Bisacrylamide, Diacrylamide)		127.13		
BISTRIS ion		209.24	6.46	5.8–7.2

(continued)

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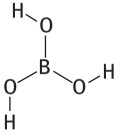
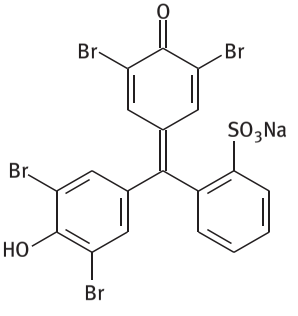
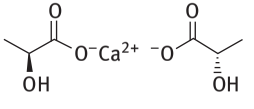
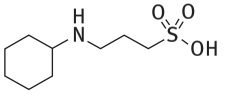
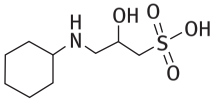
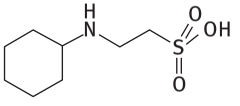
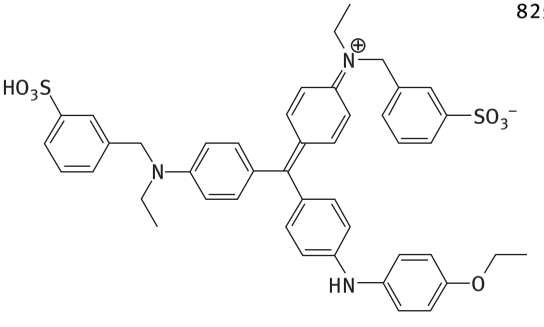
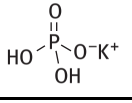
Reagent	Molecular formula	M_r	Density, d	p$K_{25}{}^{\circ}{}_{C}$	Useful pH range
Boric acid		61.83		9.24	8.5–9.9
Bromophenol blue Na salt		691.94			
Calcium lactate		218.22			
CAPS		221.32		10.40	9.7–11.1

CAPSO		237.31	9.60	8.9–10.3
Carrier ampholytes, pH = 3–10				
CHES		207.29	9.30	8.6–10.0
Coomassie brilliant blue R-250		825.97		
Potassium dihydrogen phosphate		136.08	7.20	6.5–7.9

(continued)

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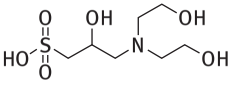
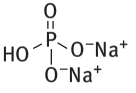
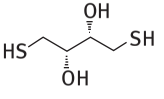
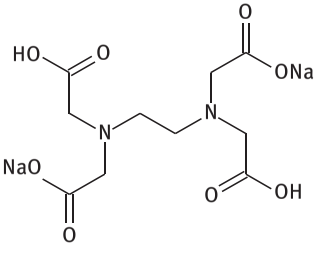
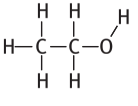
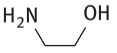
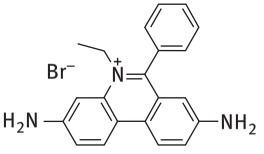
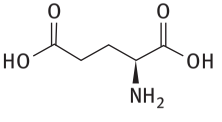
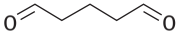
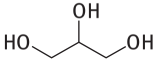
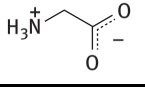
Reagent	Molecular formula	M_r	Density, d	$pK_{25}°_C$	Useful pH range
DIPSO		243.27		7.60	6.9–8.3
Disodium hydrogen phosphate		141.96			
1,4-**Dit**hiothreitol (DTT)		154.24			
Na_2EDTA		336.21			
Ethanol		46.07	0.79		

Ethanolamine		62.08	1.02	9.50	8.8–10.2
Ethidium bromide		394.32			
Formaldehyde 37%		30.03	1.09		
Formic acid		46.03	1.22	3.75	
L-Glutamic acid		147.13			
Glutardialdehyde 25%		100.12	1.06		
Glycerol 87%		92.09	1.23		
Glycine		75.07		9.78	9.1–10.5

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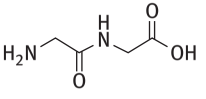
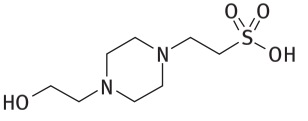
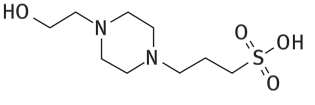
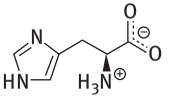
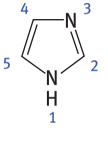
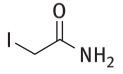
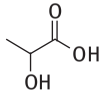
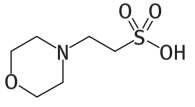
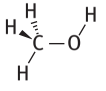
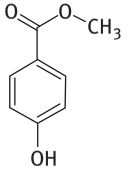
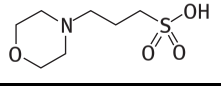
Reagent	Molecular formula	M_r	Density, d	p$K_{25}{}^{\circ}{}_C$	Useful pH range
Glycylglycine		132.12		8.25	7.6–9.0
HEPES		238.30		7.48	6.8–8.2
HEPPS		252.33		8.00	7.3–8.7
L-Histidine		155.16		8.97	8.3–9.7
Hydrochloric acid 37%		36.46	1.18		
Imidazole		68.08		6.95	6.2–7.8

Immobilines, pH = 4-10

2-Iodoacetamide		184.96			
Lactic acid		90.08		3.84	
MES		195.23		6.16	5.5–6.9
Methanol		32.04	0.79		
Methylparaben (Nipagin, methyl 4-hydroxybenzoate)		152.15			
MOPS		209.26		7.20	6.5–7.9

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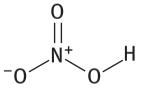
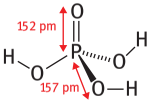
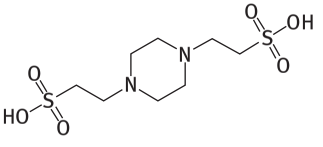
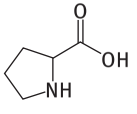
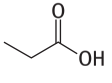
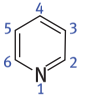
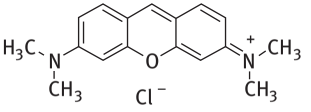
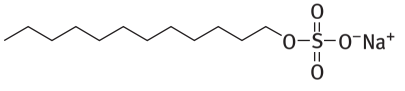
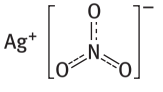
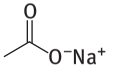
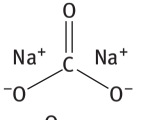
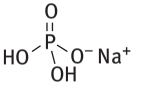
Reagent	Molecular formula	M_r	Density, d	pK_{25}°C	Useful pH range
Naphthol blue black – s. Amido black 10B					
Nitric acid		63.01	1.40		
Phosphoric acid		98.00	1.69	2.15	
PIPES		302.36		6.76	6.1–7.5
Potassium hydroxide		56.11			
L-Proline		115.13			
Propionic acid		74.08		4.87	4.2–5.6

Pyridine		79.10	5.21	4.5–5.9
Pyronin Y (G)		302.80		
SDS		288.38		
Silver nitrate		169.87		
Sodium acetate		82.03		
Sodium carbonate		105.99		
Sodium dihydrogen phosphate		119.98		
Sodium hydroxide		40.00		

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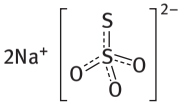
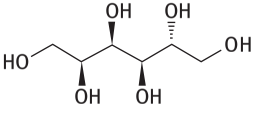
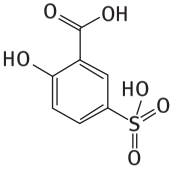
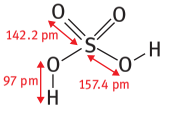
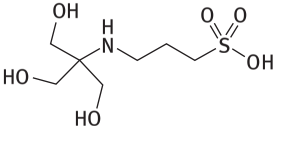
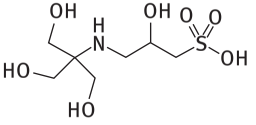
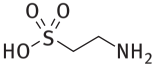
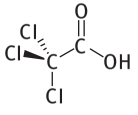
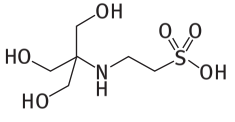
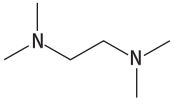
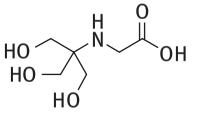
Reagent	Molecular formula	M_r	Density, d	$pK_{25°C}$	Useful pH range
Sodium thiosulfate		158.10			
D-Sorbitol		182.17			
5-Sulfosalicylic acid		254.21			
Sulfuric acid		98.07			
TAPS		243.27		8.40	7.7–9.1

TAPSO		259.27		7.64	6.9–8.3
Taurine		125.14		9.06	8.4–9.8
Trichloroacetic acid		163.38	1.62		
TES		229.25		7.55	6.9–8.3
TMEDA		116.21			
TRICINE		179.17		8.09	7.4–8.8

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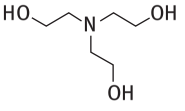
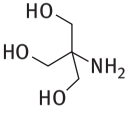
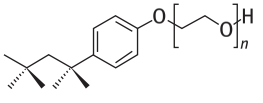
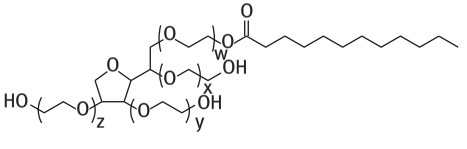
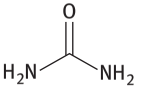
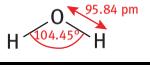
Reagent	Molecular formula	M_r	Density, d	p$K_{25^\circ C}$	Useful pH range
Triethanolamine		149.19		7.74	
TRIS		121.14		8.07	7.4–8.8
Triton X-100		250.38	1.07		
Tween 20 (Polysorbate 20)		1227.54			
Urea		60.06			
Water		18.02	1.00	15.74	

Recipes for electrophoresis solutions

Table R-1: Molarities and relative density of concentrated acids and bases.

Protolyte	M_r	% by mass	Rel. dens., *d*
Acids			
HCOOH	46.03	90	1.21
		98	1.22
CH_3COOH	60.05	99.6	1.05
H_3PO_4	98.00	85	1.70
HCl	36.46	36	1.18
H_2SO_4	98.07	98	1.835
HNO_3	63.01	70	1.42
Bases			
NH_4OH	35.0	28	0.90
NaOH	40.0	50	1.53
KOH	56.11	45	1.45
	56.11	50	1.51

Buffers

Electrophoresis buffer, pH = 3.5

Pyridine	0.50 ml
Acetic acid	5.00 ml
Deionized water to	100.00 ml

Check pH. Store at room temperature.

Electrophoresis buffer, pH = 4.7

Pyridine	2.50 ml
Acetic acid	2.50 ml
n-Butanol	5.00 ml
Deionized water to	100.00 ml

Check pH. Store at room temperature.

Sodium-acetate buffer, 4x, pH = 5.0

Sodium acetate · $3H_2O$	2.72 g (0.2 mol/l)
Acetic acid	1.16 ml (0.2 mol/l)
Deionized water to	100.00 ml

https://doi.org/10.1515/9783110330755-010

***S**odium **a**cetate (SA) buffer, 3 mol/l, pH = 6.0*

$Na \cdot acetate \cdot H_2O$	40.82 g (3 mol/l Na acetate)

Titrate with 3 mol/l acetic acid [17.24 ml acetic acid to 100 ml deionized water (3 mol/l)] to pH = 6.0

Deionized water to	100.00 ml

Store at room temperature up to 6 months.

Electrophoresis buffer, pH = 6.5

Pyridine	10.00 ml
Acetic acid	0.40 ml
Deionized water to	100.00 ml

Check pH. Store at room temperature.

Hydrogen phosphate buffer, 0.01 mol/l

Prepare 800 ml of deionized water in a suitable container.
Add sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$, $M_r =$ 268.07).
Add sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$, $M_r =$ 137.99).
Adjust solution to desired pH using HCl or NaOH.
Add deionized water to 1,000 ml.

pH = 6.6

$Na_2HPO_4 \cdot 7H_2O$	10.76 g (0.04 mol/l)
$NaH_2PO_4 \cdot H_2O$	8.26 g (0.06 mol/l)
Deionized water to	1,000.00 ml

pH = 7.0

$Na_2HPO_4 \cdot 7H_2O$	15.48 g (0.06 mol/l)
$NaH_2PO_4 \cdot H_2O$	5.83 g (0.04 mol/l)
Deionized water to	1,000.00 ml

pH = 7.4

$Na_2HPO_4 \cdot 7H_2O$	20.21 g (0.08 mol/l)
$NaH_2PO_4 \cdot H_2O$	3.39 g (0.02 mol/l)
Deionized water to	1,000.00 ml

Hydrogen phosphate buffer, 0.5 mol/l, pH = 6.8

Na_2HPO_4	3.55 g
NaH_2PO_4	3.45 g
Deionized water to	100.00 ml

***S**odium chloride-**s**odium **c**itrate (SSC) buffer, 20x, pH = 7.0*

$Na_2citrate \cdot 2H_2O$	8.80 g (0.3 mol/l)

Adjust pH to 7.0 with 1 mol/l HCl.

NaCl	17.50 g (3 mol/l)
Deionized water to	100.00 ml

***P**hosphate-**b**uffered **s**aline (PBS), 10x, pH = 7.3*

$Na_2HPO_4 \cdot 7H_2O$	1.15 g (4.3 mmol/l)
KH_2PO_4	0.20 g (1.4 mmol/l)
NaCl	8.00 g (137 mmol/l)
KCl	0.20 g (2.7 mmol/l)
Deionized water to	100.00 ml

PBS-Tween

PBS (s. *Recipe*)	
Tween-20	0.50 ml
Deionized water to	100.00 ml

Store at room temperature.

***T**RIS-**E**DTA-**N**a (TEN) buffer, pH = 7.4*

TRIS	0.61 g (0.05 mol/l)

Add 1 mol/l HCl to pH = 7.4.

NaCl	0.88 g (0.15 mol/l)
Na_2EDTA	3.36 g (0.01 mol/l)
Deionized water to	100.00 ml

Store at room temperature up to 6 months.

***T**RIS-**E**DTA-**S**DS (TES) buffer, pH = 7.5*

TRIS	0.12 g (0.01 mol/l)

Add 1 mol/l HCl to pH = 7.5.

Na_2EDTA	3.36 g (0.01 mol/l)
SDS	0.50 g (0.017 mol/l)
Deionized water to	100.00 ml

Store at room temperature.

***L**ow-**s**alt **b**uffer (LSB), pH = 7.5*

HEPES	0.48 g (0.02 mol/l)
NaCl	0.58 g (0.10 mol/l)

Store at 4 °C up to several weeks. Before use add:

2-Mercaptoethanol	0.01 ml (1.0 mmol/l)
PMSF	0.09 g (0.5 mmol/l)
Deionized water to	100.00 ml

***T**RIS-**b**uffered **s**aline (TBS), pH = 7.5*

TRIS	1.21 g (0.10 mol/l)

Add 1 mol/l HCl to pH = 7.5.

NaCl	0.88 g (0.15 mol/l)
Deionized water to	100.00 ml

Store at 4 °C up to several months.

***S**odium chloride-**T**RIS-**E**DTA (STE) buffer, pH = 8.0*

TRIS	0.12 g (10 mmol/l)
Add 1 mol/l HCl to pH = 8.0.	
Na_2EDTA	0.03 g (1 mmol/l)
NaCl	0.58 g (0.1 mol/l)
Deionized water to	100.00 ml

***T**RIS-**b**orate-**E**DTA (TBE) buffer, 10x, pH = 8.0*

TRIS	108.00 g (0.89 mol/l)
Boric acid	55.00 g (0.89 mol/l)
Add 1 mol/l HCl to pH = 8.0.	
Na_2EDTA	6.72 g (0.02 mol/l)
Deionized water to	1,000.00 ml

***T**RIS-**b**uffered **s**aline with **T**riton X-100 (TBST), pH = 8.0*

TRIS	0.12 g (0.01 mol/l)
Add 1 mol/l HCl to pH = 8.0.	
NaCl	0.88 g (0.15 mol/l)
Triton X-100	0.05 ml
Deionized water to	100.00 ml

Store at room temperature up to 6 months.

***T**RIS-**E**DTA (TE) buffer, pH = 8.0*

TRIS	0.12 g (0.01 mol/l)
Bring with HCl to pH = 8.0.	
Na_2EDTA	0.03 g (1 mmol/l)
Deionized water to	100.00 ml

***T**RIS-**b**orate-**E**DTA (TBE) buffer, 5x, pH = 8.3*

TRIS	5.45 g (0.45 mol/l)
Boric acid	2.78 g (0.45 mol/l)
Na_2EDTA	0.17 g (0.005 mol/l)
Deionized water to	100.00 ml

Store at room temperature up to several weeks.

***T**RIS-**a**cetate-**E**DTA (TAE) buffer, 50x, pH = 8.5*

TRIS	2.42 g (0.2 mol/l)
Acetic acid	5.72 ml (0.1 mol/l)
Na_2EDTA	3.38 g (0.1 mol/l)
Deionized water to	100.00 ml

Electrophoresis buffer, pH = 8.9

Ammonium carbonate	1.00 g
Deionized water to	100.00 ml

Check pH. Store at room temperature.

Solutions for agarose gel electrophoresis

Agarose gel, 1 g/dl

Agarose	1.00 g
Ethidium bromide	0.05 g
Gel buffer to	100.00 ml

Melt for several minutes.

Sodium-citrate buffer for acidic agarose electrophoresis of hemoglobins, 10x, pH = 6.0, I = 10 x 0.08 mol/l

Solution A (0.2 mol/l Na_3 citrate)

$Na_3 \cdot$ citrate	5.88 g (0.23 mol/l)
NaN_3	0.02 g (0.003 mol/l)
Deionized water to	100.00 ml

Solution B (0.2 mol/l citric acid)

Citric acid	4.20 g (0.22 mol/l)
NaN_3	0.02 g (0.003 mol/l)
Deionized water to	100.00 ml

Solution A	41.50 ml
Solution B	9.50 ml
NaN_3	0.02 g (0.003 mol/l)
Deionized water to	100.00 ml

TRIS-borate-EDTA buffer for agarose electrophoresis, 10x, pH = 8.6, I = 10 x 0.012 mol/l

TRIS	5.40 g (0.45 mol/l)
H_3BO_3	1.55 g (0.25 mol/l)
$Na_2EDTA \cdot 2H_2O$	0.29 g (0.08 mol/l)
NaN_3	0.10 g (0.15 mol/l)
Deionized water to	100.00 ml

Electrode buffer for alkaline agarose electrophoresis of hemoglobins and lipoproteins, 10x, pH = 8.8, I = 10 x 0.04 mol/l

TRIS	3.15 g (0.26 mol/l)
NaOH	1.48 g (0.37 mol/l)
Taurine	11.60 g (0.93 mol/l)
NaN_3	0.65 g (0.10 mol/l)
Deionized water to	100.00 ml

TRIS-barbitalate buffer for agarose electrophoiresis, 10x, pH = 8.9

TRIS	6.06 g (0.50 mol/l)
Barbital	2.58 g (0.14 mol/l)
Sodium barbitalate	10.31 g (0.50 mol/l)
Deionized water to	100.00 ml

Electrode buffer for agarose electrophoresis, 10x, pH = 9.0, I = 10 x 0.09 mol/l

Ammediol	18.34 g (1.74 mol/l)
BICINE	17.40 g (1.07 mol/l)
NaN_3	0.65 g (0.10 mol/l)
Deionized water to	100.00 ml

TRIS-taurinate-NaOH buffer for agarose electrophoresis, 10x, pH = 9.1, I = 10 x 0.06 mol/l

TRIS	1.82 g (0.15 mol/l)
NaOH	2.33 g (0.58 mol/l)
Taurine	12.17 g (0.49 mol/l)
NaN_3	0.65 g (0.10 mol/l)
Deionized water to	100.00 ml

TRIS-barbitalate-NaOH buffer for agarose electrophoresis, 10x, pH = 9.2, I = 10 x 0.06 mol/l (with Na_2EDTA)

TRIS	7.20 g (0.60 mol/l)
Barbital	1.84 g (0.10 mol/l)
Sodium barbitalate	10.30 g (0.25 mol/l)
[8.46 Barbital + 1.84 NaOH]	
Na_2EDTA	3.72 g (0.10 mol/l)
NaN_3	1.00 g (0.03 mol/l)
Deionized water to	100.00 ml

Solutions for affinity electrophoresis

***Al**kaline **p**hosphatase (ALP) buffer, pH = 9.5*

TRIS	1.21 g (0.1 mol/l)
Add 1 mol/l HCl to pH = 9.5.	
NaCl	0.58 g (0.1 mol/l)
$MgCl_2$	0.48 g (0.05 mol/l)
Tween 20	0.10 ml
Deionized water to	100.00 ml
Store at 4° C up to 3 months.	

***B**ES-**b**uffered **s**olution (BBS), 2x, pH = 6.95*

BES	1.07 g (0.05 mol/l)
Na_2HPO_4	0.02 g (0.0015 mol/l)
NaCl	1.64 g (0.28 mol/l)
Deionized water	80.00 ml
Adjust pH to 6.95 with 1 mol/l NaOH	
Deionized water to	100.00 ml
Store at −20 °C.	

*5-**B**romo-4-**c**hloro-3-**i**ndolyl **p**hosphate (BCIP) solution*

BCIP	0.5 g
Dimethylformamide	10.0 ml

Store at −20 °C in aliquots.

*4-**N**itro **b**lue **t**etrazolium (NBT) buffer, pH = 9.2*

TRIS	0.12 g (0.01 mol/l)

Add 80 ml deionized water and adjust pH to 9.2 with 1 mol/l HCl.

NBT chloride	0.41 g (0.005 mol/l)
BCIP	0.20 g (0.006 mol/l)
$MgCl_2$	0.56 g (0.059 mol/l)
Deionized water to	100.00 ml

NBT-BCIP substrate buffer, pH = 9.5

ALP buffer (s. *Recipe*)	50.00 ml
NBT buffer (s. *Recipe*)	0.22 ml
BCIP solution (s. *Recipe*)	0.17 µl

Prepare fresh.

Developing solution

NBT stock (s. *Recipe*)	66.0 µl
ALP buffer (s. *Recipe*)	10.0 ml
BCIP stock (s. *Recipe*)	33.0 µl

Solutions for native disc-electrophoresis

Acrylamide/bisacrylamide 29:1

Acrylamide	29.0 g
Bisacrylamide	1.0 g
Deionized water to	100.0 ml

Acrylamide is a neurotoxin. Always wear gloves when working.

Acrylamide-BIS solution (T50, C0.03)

Acrylamide	48.50 g
BIS	1.50 g
Deionized water to	100.00 ml

Sample solution for native IEF

Urea	24.02 g (4.0 mol/l)
Amberlite MB-3	1.00 g

(*Amberlite MB 3 deionizes the solution.*)

Deionized water to	100.00 ml

Sample solution for denaturing IEF

Carrier ampholytes 3-10	1.00 ml
Carrier ampholytes 4-9	0.25 ml
Urea	48.05 g (8.0 mol/l)
1,4-Dithiothreitol	0.31 g (0.02 mol/l)
87% Glycerol	40.00 ml
Amberlite MB-3	1.00 g
(*Amberlite MB-3 deionizes the solution.*)	
Deionized water to	100.00 ml

Solutions for SDS disc-electrophoresis

Resolving buffer for disc-electrophoresis, 10x, pH = 6.5, according to Michov

[Electrode buffer *a* (+)]

TRIS	19.64 g (1.62 mol/l)
Formic acid	6.04 ml (1.60 mol/l)
87% Glycerol	10.00 ml
NaN_3	0.10 g (0.015 mol/l)
Deionized water to	100.00 ml

Electrode buffer for disc-electrophoresis, 10x, pH = 7.4, according to Michov

[Electrode buffer *b* (–)]

TRIS	2.61 g (0.22 mol/l)
BICINE	21.98 g (1.35 mol/l)
87% Glycerol	10.00 ml
0.1 g/dl NaBPB	0.69 ml
NaN_3	0.10 g (0.015 mol/l)
Deionized water to	100.00 ml

Sample buffer for disc-electrophoresis of nucleic acids, 2x, pH = 6.5, 2 × 0.20 mol/l

Electrode buffer *a*	25.00 ml
Na_2EDTA	0.07 g (0.002 mol/l)
1 g/l NaBPB	2.77 ml (0.04 mmol/l)
87% Glycerol	20.00 ml
Deionized water to	100.00 ml

Nonreducing sample buffer for SDS electrophoresis, pH = 6.5, 0.20 mol/I

Electrode buffer *a*	12.50 ml (0.20 mol/l)
SDS	1.13 g (0.04 mol/l)
1 g/dl NaBPB	1.38 ml (0.02 mmol/l)
87% Glycerol	10.00 ml
Deionized water to	100.00 ml

Reducing sample buffer, 2x for SDS electrophoresis, pH = 6.5, 2 × 0.2 mol/l

Electrode buffer *a*	25.00 ml (0.40 mol/l)
SDS	2.27 g (0.08 mol/l)
87% Glycerol	20.00 ml
1,4-Dithiothreitol	0.31 g (0.02 mol/l)
1 g/l NaBPB	2.77 ml (0.04 mmol/l)
Deionized water to	100.00 ml

Alkylating solution for SDS electrophoresis, 2x, for SDS-electrophoresis

2-Iodacetamide	3.7 g (0.20 mol/l)
Deionized water to	100.0 ml

Resolving buffer for SDS electrophoresis, 10x, pH = 6.5, according to Michov

[SDS electrode buffer *a* (+)]

TRIS	17.68 g (1.46 mol/l)
Formic acid	5.44 ml (1.44 mol/l)
SDS	0.23 g (0.008 mol/l)
87% Glycerol	10.00 ml
Deionized water to	100.00 ml

Electrode buffer for SDS electrophoresis, 10x, pH = 7.4, according to Michov

[SDS electrode buffer *b* (–)]

TRIS	2.36 g (0.19 mol/l)
BICINE	19.78 g (1.21 mol/l)
SDS	0.23 g (0.008 mol/l)
87% Glycerol	10.00 ml
0.1 g/dl NaBPB	0.69 ml
Deionized water to	100.00 ml

SDS electrophoresis buffer, 5x, pH = 8.3

TRIS	1.51 g (0.12 mol/l)
Glycine	7.20 g (0.96 mol/l)
SDS	0.50 g (0.02 mol/l)
Deionized water to	100.00 ml

Store at 0 to 4 °C up to 1 month.

SDS lysis buffer, pH = 8.0

TRIS	0.61 g (0.05 mol/l)

Add 1 mol/l HCl to pH = 8.0.

SDS	0.58 g (0.02 mol/l)
Deionized water to	100.00 ml

Add fresh 1 mmol/l DTT.

SDS sample buffer, 2x, pH = 6.8

TRIS	4.00 ml (0.125 mol/l)
Add 1 mol/l HCl to pH = 6.8.	
Glycerol	2.00 ml
Na_2EDTA	0.02 g (0.006 mol/l)
SDS	0.40 g (0.14 mol/l)
2-Mercaptoethanol	0.40 ml (0.57 mol/l)
0.2 mol/l DTT	0.40 ml (0.008 mol/l)
NaBPB	0.02 g
Deionized water to	10.00 ml

Filter and store at −20 °C up to 6 months.

Solutions for IEF

Anode (+) fluid 3 for IEF

L-aspartic acid	0.17 g (0.0125 mol/l)
L-glutamic acid	0.18 g (0.0125 mol/l)
87% Glycerol	50.00 ml
NaN_3	0.02 g (0.003 mol/l)
Deionized water to	100.00 ml

Cathode (–) fluid 10 for IEF

L-arginine	0.22 g (0.0125 mol/l)
L-lysine	0.18 g (0.0125 mol/l)
	[0.21 g/dl L-lysine·H_2O]
Ethylene diamine	6.00 ml (0.9 mol/l)
87% Glycerol	50.00 ml
NaN_3	0.02 g (0.003 mol/l)
Deionized water to	100.00 ml

Rehydrating buffer for native PAGE, pH = 9.0

El. buffer, 10x, pH = 9.0	0.25 ml
Carrier ampholytes	0.40 ml
Carrier ampholytes 4-9	0.10 ml
Dextran 8	5.00 g
NaN_3	0.02 g (0.003 mol/l)
Deionized water to	100.00 ml

Rehydrating buffer for denatured PAGE, pH = 9.0

El. buffer, 10x, pH = 9.0	0.25 ml
Carrier ampholytes	0.40 ml
Carrier ampholytes 4-9	0.10 ml
Urea	48.05 g (8.0 mol/l)
1,4-Dithiothreitol	0.62 g (0.04 mol/l)

El. buffer, 10x, pH = 9.0	0.25 ml
Dextran 8	5.00 g
NaN_3	0.02 g (0.003 mol/l)
Deionized water to	100.00 ml

Prepare fresh.

Blotting solutions

Transfer buffer, pH = 10.0

CAPS	5.0 ml (0.50 mol/l)
Methanol	20.0 ml
Deionized water to	100.0 ml

Degas before transfer.

Blocking buffer, pH = 7.4

TRIS	0.12 g (0.01 mol/l

Add 1 mol/l HCl to pH = 7.4.

BSA	5.00 g
Hen ovalbumin	1.00 g
NaCl	0.88 g (0.15 mol/l)
NaN_3	0.02 g (0.003 mol/l)
Deionized water to	100.00 ml

Store at 4 °C for up to 6 months.

Blocking solution with BSA, pH = 7.8

TRIS	(0.1 mol/l)

Add 1 mol/l HCl to pH = 7.8.

BSA	5.00 g
NaCl	0.88 g (0.15 mol/l)
NaN_3	0.02 g (0.003 mol/l)
Deionized water to	100.00 ml

Prepare fresh.

***Tri*ethanol*a*mine (TEA) buffer, pH = 8.0**

Triethanolamine	1.33 g (0.1 mol/l)
Deionized water	80.00 ml

Adjust pH to 8.0 with 1 mol/l HCl.

Deionized water to	100.00 ml

Prepare fresh daily.

***TBS-T*ween 20 (TBST)**

TRIS-buffered saline	100.0 ml
Tween 20	0.1 ml

Store at 4 °C up to several months.

Urea solutions

9.5 mol/l urea

Urea	28.50 g
Deionized water to	50.00 ml

10 mol/l urea

Urea	30.03 g
Deionized water to	50.00 ml

*Electrode buffer for CA (**c**ellulose **a**cetate) electrophoresis, pH = 8.6, I = 0.10 mol/l*

Sodium barbitalate [16.94 Barbital + 3.68 NaOH]	2.06 g (0.10 mol/l)
Barbital	0.40 g (0.02 mol/l)
NaN_3	0.14 g (0.02 mol/l)
Deionized water to	100.00 ml

Fixative solutions

*Fixative TS (**t**richloroacetic acid with 5-**s**ulfosalicylic acid), 10x*

Tri**c**hloroacetic **a**cid (TCA)	50.0 g (3.06 mol/l)
5-**S**ulfo**s**alicylic **a**cid (SSA) dihydrate	10.0 g (0.39 mol/l
Deionized water to	100.0 ml

*Fixative A (**a**cetic acid)*

Acetic acid	7.0 ml (1.11 mol/l)
Deionized water to	100.0 ml

*Fixative GE (**g**lutardialdehyde-**e**thanol), 10x*

25 ml/dl Glutardialdehyde	10.0 ml (0.5 mol/l)
Ethanol	50.0 ml
Deionized water to	100.0 ml

*Fixative CE (**c**itric acid-**e**thanol), 2x*

Citric acid	1.0 g (0.05 mol/l)
Ethanol	60.0 ml
Deionized water to	100.0 ml

*Fixative AE (**a**cetic acid-**e**thanol)*

Acetic acid	10.0 ml (1.75 mol/l)
Ethanol	40.0 ml
Deionized water to	100.0 ml

Staining solutions

Ponceau S staining solution

Ponceau S	0.3 g
Trichloroacetic acid	3.0 g
Deionized water to	100.0 ml

***P**onceau S – **T**CA-**s**ulfosalicylic acid (PTS) staining solution, 10x*

Ponceau S	3.0 g
Trichloroacetic acid	30.0 g
Sulfosalicylic acid	30.0 g
Deionized water to	100.0 ml

Store at room temperature. Prior to use dilute tenfold with deionized water.

***C**oomassie **b**rilliant **b**lue R-250 (CBB) solution*

CBB R250	0.2 g
Methanol	30.0 ml
Acetic acid	10.0 ml
Deionized water	60.0 ml

***C**oomassie **b**rilliant **v**iolet R-200 (CBV) solution*

CBV R-200	0.2 g
Methanol	30.0 ml
Acetic acid	10.0 ml
Deionized water	60.0 ml

Amido black 10B solution

Amido black 10B	0.2 g
Methanol	30.0 ml
Acetic acid	10.0 ml
Deionized water	60.0 ml

Sudan black B solution, 10x

Sudan **b**lack B	1.0 g
Ethanol to	100.0 ml

***B**romo**p**henol **b**lue (BPB) solution*

NaBPB	0.1 g
[0.1 g/dl BPB + 0.01 g/dl NaOH]	
Citric acid	0.5 g
Deionized water to	100.0 ml

Filter.

Destaining solutions

*Destaining solution A (**a**cetic acid), 10x*

CH_3CO_2H	50.0 ml
Deionized water to	100.0 ml

*Destaining solution AM (**a**cetic acid-**m**ethanol)*

Acetic acid	10.0 ml
Methanol	20.0 ml
Deionized water to	100.0 ml

*Destaining solution AE (**a**cetic acid-**e**thanol), 2x*

Acetic acid	20.0 ml
Ethanol	60.0 ml
Deionized water to	100.0 ml

*Destaining solution C (**c**itric acid), 10x*

Citric acid	5.0 g
Deionized water to	100.0 ml

*Destaining solution P (**p**hosphoric acid), 10x*

Phosphoric acid	3.0 g
Deionized water to	100.0 ml

Silver staining solutions

Preparing solution for silver staining, 10x

$Na_2S_2O_3 \cdot 5H_2O$	3.0 g (0.12 mol/l)
Deionized water to	100.0 ml

$[2Na_2S_2O_3 + AgBr \rightarrow Na_3[Ag(S_2O_3)_2] + NaBr]$

Store in a brown flask.

Silver stain solution A (Sensitizer) for silver staining of agarose gels

$AgNO_3$	0.20 g (0.012 mol/l)
NH_4NO_3	0.20 g (0.024 mol/l)
Silicotungstic acid · aq $H_4[Si(W_3O_{10})_4] \cdot$ aq.	1.00 g (0.003 mol/l)
37 g/dl HCHO	0.89 ml (0.12 mol/l)
Deionized water to	100.00 ml

Silver stain solution B (Developer) for silver staining of agarose gels

Na_2CO_3	8.0 g (0.755 mol/l)

$[2[Ag(NH_3)_2]OH + HCHO \rightarrow 2Ag + HCOONH_4 + 3NH_3 + H_2O]$

Deionized water to	100.0 ml

Silver stain kit for Paa gels, 10x

$AgNO_3$	1.0 g (0.006 mol/l)
Deionized water to	100.0 ml

Store in a brown flask.

Developer 1 for silver staining of Paa gels, 10x

Na_2CO_3	29.6 g (2.8 mol/l)
Deionized water to	100.0 ml

Developer 2 for silver staining of Paa gels

37 g/dl HCHO	10.0 ml

Other solutions

Denhardt's solution, 100x

Ficoll 400	10.0 g
Polyvinylpyrrolidone	10.0 g
BSA (Fraction V)	10.0 g
Deionized water to	500.0 ml

Filter sterilize and store at −20 °C in aliquots.

*Detergent solution TX (**T**riton **X**-100), 10x*

Triton X-100	5.0 ml
Deionized water to	100.0 ml

*EDTA (**e**thylenediamine**t**etra**a**cetic acid) solution, pH = 8.0*

Na_2EDTA	16.81 g (0.5 mol/l)
Deionized water to	80.00 ml

Adjust pH to 8.0 with 0.5 mol/l NaOH.

Deionized water to	100.00 ml

Elution buffer, pH = 8.0

TRIS	0.61 g (0.05 mol/l)
Deionized water to	80.00 ml

Add 1 mol/l HCl to pH = 8.0.

Na_2EDTA	0.34 g (0.01 mol/l)
SDS	1.00 g (003 mol/l)
Deionized water to	100.00 ml

Filter sterilize and store at room temperature.

Lysis buffer, pH = 8.0

TRIS	0.12 g (0.01 mol/l)
Deionized water to	80.00 ml

Add 1 mol/l HCl to pH = 8.0.

NaCl	0.82 g (0.14 mol/l)

TRIS	
$MgCl_2$	0.14 g (0.015 mol/l)
Nonidet **P-40** (NP-40)	0.50 ml
Deionized water to	100.00 ml

Store at room temperature.

Sucrose solution, 10 g/dl, pH = 7.5

TRIS	0.24 g (0.02 mol/l)
Deionized water to	80.00 ml

Add 1 mol/l HCl to pH = 7.5.

Sucrose	10.00 g
NaCl	5.84 g (1 mol/l)
EDTA	0.17 g (0.005 mol/l)
Deionized water to	100.00 ml

Solution of problems

Solution of problems 1

1.1 Since $[Na^+] = 0.1 + 0.2 = 0.3$ mol/l, $[CH_3COO^-] = 0.1$ mol/l, and $[Cl^-] = 0.2$ mol/l, the ionic strength

$$I = \frac{1}{2}\left[0.3 \bullet 1^2 + 0.1(-1)^2 + 0.2(-1)^2\right] = 0.3 \text{ mol/l}$$

1.2 It follows from the relationship between pK and the ionic strength that

$$\mathrm{p}K_c = 7.20 - \frac{2 \bullet 0.1^{1/2}}{1 + 0.1^{1/2}} = 7.20 - 0.48 = 6.72$$

1.3 Carbonic acid H_2CO_3 is in equilibrium with carbon dioxide dissolved in deionized water ($CO_{2(dis)}$), according to the following scheme:

$$CO_{2(dis)} + H_2O \overset{\mathrm{p}K_0}{\leftrightarrow} H_2CO_3 \overset{\mathrm{p}K_{c1}}{\leftrightarrow} H^+ + HCO_3^- \overset{\mathrm{p}K_{c2}}{\leftrightarrow} 2H^+ + CO_3^{2-}$$

The balance between carbon dioxide and carbonic acid is shifted to carbon dioxide, because only 0.0026 of the dissolved carbon dioxide exists as carbonic acid, hence $\mathrm{p}K_0 = 2.6$. The value of $\mathrm{p}K_{c1}$ is equal to 3.8, as a result of which $\mathrm{p}K_c$ value of both reactions ($\mathrm{p}K_0 + \mathrm{p}K_{c1}$) is equal to 6.4. The value of $\mathrm{p}K_{c2}$ is almost 4 units higher than the value of $\mathrm{p}K_{c1}$, i.e., K_{c2} is approximately 10,000 times smaller than K_{c1}. Therefore, $\mathrm{p}K_{c2}$ can be ignored in the calculations. According to Henderson–Hasselbalch equation,

$$\mathrm{pH} = \mathrm{p}K_0 + \mathrm{p}K_{c1} + \log\frac{[HCO_3^-]}{[CO_{2(dis)}]}$$

The concentration of hydrogen ion is $10^{-\mathrm{pH}}$. Therefore, this equation can be transformed to give

$$\mathrm{pH} = \mathrm{p}K_0 + \mathrm{p}K_{c1} + \log\frac{10^{-\mathrm{pH}}}{[CO_{2(dis)}]} = 6.4 - \mathrm{pH} - \log 10^{-4}$$

from where follows that

$$\mathrm{pH} = \frac{6.4 + 4}{2} = 5.2$$

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1.4 If 0.01 mol/l HCl is added to a buffer,

$$\mathrm{pH} = 4.64 + \log\frac{0.1 - 0.01}{0.1 + 0.01} = 4.55$$

If 0.01 mol/l NaOH is added to a buffer,

$$\mathrm{pH} = 4.64 + \log\frac{0.1 + 0.01}{0.1 - 0.01} = 4.73$$

1.5 From Henderson–Hasselbalch equation follows that

$$5.50 = 4.64 + \log\frac{[\mathrm{CH_3COO^-}]}{[\mathrm{CH_3COOH}]}$$

hence

$$[\mathrm{CH_3COO^-}] = 10^{5.50 - 4.64}[\mathrm{CH_3COOH}] = 7.2444[\mathrm{CH_3COOH}]$$

It follows from the last equation and from the equation

$$[\mathrm{CH_3COO^-}] + [\mathrm{CH_3COOH}] = 0.2$$

that

$$[\mathrm{CH_3COO^-}] = c_{\mathrm{CH_3COONa}} = 0.1757\ \mathrm{mol/l}$$

and

$$[\mathrm{CH_3COOH}] = c_{\mathrm{CH_3COOH}} - \mathrm{CH_3COO^-} = 0.2 - 0.1757 = 0.0243\ \mathrm{mol/l}$$

1.6 It follows from Henderson–Hasselbalch equation that

$$10.00 = 10.33 + \log\frac{[\mathrm{CO_3^{2-}}]}{[\mathrm{HCO_3^-}]}$$

hence

$$[\mathrm{CO_3^{2-}}] = 10^{10.00 - 10.33}[\mathrm{HCO_3^-}] = 0.4677[\mathrm{HCO_3^-}]$$

The buffer capacity β is

$$0.3 = \ln 10\frac{[\mathrm{HCO_3^-}][\mathrm{CO_3^{2-}}]}{[\mathrm{HCO_3^-}] + [\mathrm{CO_3^{2-}}]}$$

Then, the following equation system can be written:

$$[\mathrm{CO_3^{2-}}] = 0.4677[\mathrm{HCO_3^-}]$$
$$\ln 10[\mathrm{HCO_3^-}][\mathrm{CO_3^{2-}}] = 0.3([\mathrm{HCO_3^-}] + [\mathrm{CO_3^{2-}}])$$

Hence, $[HCO_3^-] = 0.4089$ mol/l, $[CO_3^{2-}] = 0.1912$ mol/l, and the total buffer concentration is

$$c = 0.4089 + 0.1912 = 0.6001 \text{ mol/l}$$

1.7 The pH value of the buffer is equal to the value of pK_c of pyridinium ion. Therefore, α = 0.5, i.e., the equilibrium concentrations of pyridine and pyridinium ion are equal. Then it follows that

$$\beta = \ln 10 \bullet 0.5(1 - 0.5) \bullet 0.1 = 0.0576 \text{ mol/l}$$

1.8 At pH = 4.62, the buffer capacity

$$\beta_{\text{pH4.62}} = \ln 10 \bullet 0.5(1 - 0.5) \bullet 0.2 = 0.1151 \text{ mol/l}$$

To calculate the buffer capacity at pH = 5.50, the equilibrium concentrations of CH_3COO^- and CH_3COOH should be found. According to Henderson–Hasselbalch equation,

$$5.50 = 4.62 + \log \frac{[CH_3COO^-]}{[CH_3COOH]}$$

therefore,

$$[CH_3COO^-] = 10^{5.50 - 4.62}[CH_3COOH] = 7.5858[CH_3COOH]$$

From the equation system

$$[CH_3COO^-] + [CH_3COOH] = 0.2$$

$$[CH_3COO^-] = 7.5858[CH_3COOH]$$

follows that $[CH_3COO^-]$ = 0.1767 mol/l, and $[CH_3COOH]$ = 0.0233 mol/l. When pH = 5.50, the buffer capacity

$$\beta_{\text{pH5.50}} = \ln 10 \frac{0.1767 \bullet 0.0233}{0.2} = 0.0474 \text{ mol/l}$$

The ratio between the buffer capacities at pH = 4.62 and pH = 5.50 is

$$\frac{\beta_{\text{pH4.62}}}{\beta_{\text{pH5.50}}} = \frac{0.1151}{0.0474} = 2.4$$

1.9 At 0 °C (273.15 K), the values of η and ε_r of water are equal to $1.787 \bullet 10^{-3}$ Pa s, and 87.74, respectively, i.e., the parameter of the ionic mobility $p_i = 15.45 \bullet 10^{-9} z_i I^{1/2}$ m^2/(sV). At 25 °C (298.15 K), the values of η and ε_r of water are equal to $0.8904 \bullet 10^{-3}$ Pa s, and 78.30, respectively, i.e., the parameter of the ionic mobility $p_i = 31.42 \bullet 10^{-9} z_i I^{1/2}$ m^2/(sV). Then, at 0 °C and I = 0.01 mol/l

$$\mu_{\mathrm{HTRIS}^+} = \left(12.75 - 15.45 \bullet 0.01^{1/2}\right) \bullet 10^{-9} = 11.20 \bullet 10^{-9}\ \mathrm{m}^2/(\mathrm{sV})$$

and at the same temperature but at I = 0.07 mol/l

$$\mu_{\mathrm{HTRIS}^+} = \left(12.75 - 15.45 \bullet 0.07^{1/2}\right) \bullet 10^{-9} = 8.66 \bullet 10^{-9}\ \mathrm{m}^2/(\mathrm{sV})$$

At 25 °C and I= 0.01 mol/l

$$\mu_{\mathrm{HTRIS}^+} = \left(27.86 - 31.42 \bullet 0.01^{1/2}\right) \bullet 10^{-9} = 24.72 \bullet 10^{-9}\ \mathrm{m}^2/(\mathrm{sV})$$

and at the same temperature but at I = 0.07 mol/l

$$\mu_{\mathrm{HTRIS}^+} = \left(27.86 - 31.42 \bullet 0.07^{1/2}\right) \bullet 10^{-9} = 19.55 \bullet 10^{-9}\ \mathrm{m}^2/(\mathrm{sV})$$

1.10 Taking into account that 25 mm = $25 \bullet 10^{-3}$, 100 mm = $100 \bullet 10^{-3}$, and 1 h = 3,600 s, according to the ionic mobility equation,

$$\mu_{albumin^{n-}} = \frac{25 \bullet 10^{-3} \bullet 100 \bullet 10^{-3}}{3{,}600 \bullet 250} = 2.8 \bullet 10^{-9}\ \mathrm{m}^2/(\mathrm{sV})$$

1.11 A

1.12 C

1.13 B

1.14 D

1.15 A

1.16 B

1.17 D

1.18 B

1.19 C

1.20 C

1.21 A

1.22 C

1.23 B

1.24 C

Solution of problems 2

2.1 The total monomer concentration and the degree of crosslinking of a polyacrylamide gel are given by the equations

$$T = a + b$$

and

$$C = \frac{b}{a+b}$$

respectively, where a and b are the concentrations of acrylamide and BIS respectively, in g/dl. According to the problem, these equations can be transformed into

$$a + b = 50$$

and

$$0.03(a+b) = b$$

from where follows that 48.5 g of acrylamide and 1.5 g of BIS should be resolved in 100.0 ml water to obtain the desired T and C values.

2.2 The "persistent" function of Kohlrausch F_k is calculated according to the equation

$$F_k = \frac{c_{HB}}{c_{HA}}$$

Hence

$$c_{HB} = F_k \bullet c_{\mathrm{HA}} = 0.8 \bullet 0.10 = 0.08 \text{ mol/l}$$

2.3 Disc-electrophoresis is a combination between isotachophoresis and zone electrophoresis. The isotachophoresis takes place in a large-pore polyacrylamide gel (stacking gel); the zone electrophoresis is run in a small-pore polyacrylamide gel (resolving gel). In the stacking gel, the protein polyions focus in a moving ionic boundary (stacking effect) and migrate with constant velocity to the boundary between the stacking and resolving gel. Then, in the resolving gel, their velocity decreases, because the viscosity of the resolving gel exceeds the viscosity

the stacking gel. As a result, the ionic boundary overtakes the polyions, which separate in the resolving gel according to their volumes.

2.4 For the protein H_nP, the "persistent" function of Kohlrausch F_k can be calculated according to equation

$$F_k = \frac{c_{H_nP}}{c_{HA}}$$

Hence

$$c_{H_nP} = F_k \bullet c_{HA} = 0.02 \bullet 0.10 = 0.002 \text{ mol/l}$$

2.5 The mobility of albumin is

$$\mu_{alb} = \frac{v_{alb}}{E} = \frac{d/t}{U/l} = \frac{dl}{tU}$$

where v_{alb} (in m/s) is the velocity of albumin; E (in V/m) is the electric field strength; d (in m) is the distance between the electrodes; t (in s) is the time; U (in V) is the voltage, and l (in m) is the run distance. Since d = −0.038 m, l = 0.1 m, t = 3,600 s, and U = 250 V, it follows that

$$\mu_{alb} = -\frac{0.038 \bullet 0.1}{3{,}600 \bullet 250} = -4.2 \bullet 10^{-9} \text{ m}^2/(\text{sV})$$

2.6 The specific conductivity γ is equal to the sum $F \sum c_i z_i \mu_i$, where F is the Faraday constant (96,485.332 12 C/mol), and c_i, z_i and μ_i are the concentration, the number of electrons (electrovalence), and the mobility of ion i, respectively. Hence, the following equation should be taken into consideration:

$$F(c_{H^+} z_{H^+} \mu_{H^+} + c_{A^-} z_{A^-} \mu_{A^-}) = F(c_{H^+} z_{H^+} \mu_{H^+} + c_{B^-} z_{B^-} \mu_{B^-})$$

Because z_{H^+} is equal to 1, and z_{A^-} and z_{B^-} are equal to −1, the expression is simplified to give

$$\mu_{A^-} = \mu_{B^-}$$

This means that the conductivity of the strong monobasic acids HA and HB, if they are present in the same concentrations, and if their anions A^- and B^- have the same mobilities, is equal.

2.7 From the equation of the ionic mobility parameter follows that at I = 0.1 mol/l and 25 °C, the mobility of borate, taurinate, and glycinate ion is $-28.51 \bullet 10^{-9}$, $-24.17 \bullet 10^{-9}$, and $-23.69 \bullet 10^{-9}$ $\text{m}^2/(\text{sV})$, respectively. Therefore, the dissociation degrees of the corresponding acids

$$\alpha_{boric\ acid} = \frac{-13 \bullet 10^{-9}}{-23.69 \bullet 10^{-9}} = 0.55$$

$$\alpha_{taurine} = \frac{-13 \bullet 10^{-9}}{-24.17 \bullet 10^{-9}} = 0.54$$

and

$$\alpha_{glycine} = \frac{-13 \bullet 10^{-9}}{-28.51 \bullet 10^{-9}} = 0.46$$

Hence, according to the Henderson–Hasselbalch equation, glycine, taurine, and boric acid must be dissolve at pH = 9.49, 8.91, and 9.11, respectively.

2.8 The buffer capacity β depends on the concentrations and pK values of buffering substances: the higher their concentrations and the closer their pK value to the buffer pH value, the higher the buffer capacity. Since the concentrations of all buffers are equal, the buffer capacity depends only on the pK values. The base TRIS and the acid ACES have pK values, which are closest to pH = 7.0. Therefore, a TRIS-ACESate buffer will have the highest buffer capacity.

2.9 If the dissociation degree of TRIS α_{TRIS} and glycine $\alpha_{\mathrm{glycine}}$ are equal to α, it follows from the Henderson–Hasselbalch equation that

$$\mathrm{pH} = \mathrm{p}K_{\mathrm{HTRIS}^+} + \log\frac{1-\alpha}{\alpha} = \mathrm{p}K_{\mathrm{glycine}} + \log\frac{\alpha}{1-\alpha}$$

Then

$$10^{\mathrm{p}K_{\mathrm{HTRIS}^+} - \mathrm{p}K_{\mathrm{glycine}}} = \frac{(1-\alpha)(1-\alpha)}{\alpha^2}$$

hence, α = 0.8188 and pH = 8.93.

2.10 It follows from the Debye–Hückel equation that

$$\mathrm{p}K_{\mathrm{cHTRIS}^+} = 8.07 + \frac{0.01^{1/2}}{1 + 0.01^{1/2}} = 8.16$$

and

$$\mathrm{p}K_{\mathrm{cHG}} = 9.78 + \frac{0.01^{1/2}}{1 + 0.01^{1/2}} = 9.69$$

Then, according to Henderson–Hasselbalch equation,

$$\alpha_{\mathrm{TRIS}} = \left(1 + 10^{\mathrm{pH} - \mathrm{p}K_{\mathrm{c}}}\right)^{-1} = \left(1 + 10^{8.30 - 8.16}\right)^{-1} = 0.420$$

and

$$\alpha_{HG} = \left(1 + 10^{pK_c - pH}\right)^{-1} = \left(1 + 10^{9.69 - 8.30}\right)^{-1} = 0.039$$

Hence,

$$c_{TRIS} = \frac{0.01}{0.420} = 0.024 \text{ mol/l}$$

and

$$c_{glycine} = \frac{0.01}{0.039} = 0.256 \; mol/l$$

2.11 A

2.12 C

2.13 A

2.14 D

2.15 B

2.16 C

2.17 D

2.18 C

2.19 B

2.20 C

2.21 A

2.22 D

2.23 C

2.24 C

Solution of problems 3

3.1 The relative molecular mass M_r of the mononucleotide residues is 314, approximately 330. This means that two mononucleotide residues (a base pair) have a molecular mass of approx. $2 \bullet 330$. Therefore, a double-helical DNA with M_r of $2 \bullet 10^6$ contains

$$\frac{2 \bullet 10^6}{2 \bullet 330} \approx 3{,}000$$

base pairs, and a double-helical DNA with M_r of $2 \bullet 10^7$ contains

$$\frac{2 \bullet 10^7}{2 \bullet 330} \approx 30{,}000$$

base pairs.

3.2 A mononucleotide residue carries a negative electric charge. The relative molecular mass of a mononucleotide residue is approximately 330. Therefore a single-stranded RNA with M_r of $4 \bullet 10^4$ has

$$\frac{4 \bullet 10^4}{330} \approx 120$$

negative charges, and a single-stranded RNA with M_r of $4 \bullet 10^5$ has

$$\frac{4 \bullet 10^5}{330} \approx 1{,}200$$

negative charges.

3.3 If a DNA contains predominantly AT pairs, it melts at a lower temperature (70 °C) because two hydrogen bonds exist between A and T; when a DNA contains predominantly GC pairs, it melts at a higher temperature (90 °C) because three hydrogen bonds exist between G and C.

3.4 The equation, which expresses the linear relationship between the logarithm of DNA mobility μ and the agarose concentration c, is:

$$\frac{\mu}{\mu_o} = 10^{-K_R c}$$

or

$$\log \mu = \log \mu_0 - K_R c$$

where μ_0 is its electrophoretic DNA mobility in an agarose gel of concentration of 0 g/dl, and the slope K_R is the retardation coefficient. The value of K_R

depends on the ionic strength of the buffer and the properties of the gel. This equation becomes invalid when the agarose concentration is over 0.9 g/dl.

3.5 The migration of DNA fragments slows down in a gel in presence of ethidium bromide by about 15% because ethidium bromide intercalates between the bases and thus increases the mass of DNA.

3.6 The **p**olymerase **c**hain **r**eaction (PCR) is a highly efficient method for targeted increase of the concentration of nucleic acids (PCR products), which are later used for a subsequent sequencing. The PCR method requires very small amounts of biological material.

3.7 The mobility of DNA

$$\mu_{\mathrm{DNA}} = \frac{v_{\mathrm{DNA}}}{E} = \frac{d/t}{U/l} = \frac{dl}{tU}$$

where v_{DNA} (in m/s) is the DNA velocity; E (in V/m) is the strength of the electric field, d (in m) is the distance between the electrodes; t(in s) is the time; U (in V) is the voltage; and l (in m) is the run distance. In our example, d = 0.127 m, l = 0.2 m, t = 3600 s, and U = 250 V. Therefore,

$$\mu_{\mathrm{DNA}} = \frac{0.127 \bullet 0.2}{3{,}000 \bullet 250} = 28.2 \bullet 10^{-9} m^2/(sV)$$

3.8 It is known that

$$\alpha_{\mathrm{TRIS}} = \frac{\mu'_{\mathrm{HTRIS}^+}}{\mu_{\mathrm{HTRIS}^+}}$$

where α_{TRIS} is the dissociation degree of TRIS ion (HTRIS^+), and μ'_{HTRIS^+} and μ_{HTRIS^+} are its effective mobility and its mobility, respectively. Therefore,

$$\alpha_{\mathrm{TRIS}} = \frac{5 \bullet 10^{-9}}{24.06 \bullet 10^{-9}} = 0.2078$$

It follows from equation of Henderson–Hasselbalch that

$$\mathrm{pH} = \mathrm{p}K_c + \log\frac{1-\alpha_{\mathrm{TRIS}}}{\alpha_{\mathrm{TRIS}}} = 8.16 + \log\frac{1-0.2078}{0.2078} = 8.74$$

3.9 From Henderson–Hasselbalch equation follows that

$$\alpha_{\mathrm{TRIS}} = \left(1 + 10^{\mathrm{pH}-\mathrm{p}K_c}\right)^{-1} = \left(1 + 10^{8.0-8.24}\right)^{-1} = 0.635$$

Hence,

$$c_{\mathrm{TRIS}} = \frac{0.04}{0.635} = 0.063 \text{ mol/l}$$

The pH value of 8.0 is located above the pK_c value of acetic acid, therefore all its molecules are dissociated. This means that the concentration of acetic acid is equal to the ionic strength; hence it is 0.04 mol/l. Hence, the buffer composition should be:

TRIS	7.63 g
99% acetic acid	2.31 ml
$Na_2EDTA \cdot 2H_2O$	0.37 g
H_2O to	1,000.00 ml

3.10 It follows from Debye–Hückel equation that

$$\mathrm{p}K_c(\mathrm{HTRIS}^+) = 8.07 + \frac{0.1^{1/2}}{1 + 0.1^{1/2}} = 8.31$$

and

$$\mathrm{p}K_c\left(\mathrm{H_2PO_4^-}\right) = 7.20 - \frac{2 \bullet 0.1^{1/2}}{1 + 0.1^{1/2}} = 6.72$$

According to equation of Henderson–Hasselbalch, the dissociation degree of TRIS is

$$\alpha_{\mathrm{TRIS}} = \left(1 + 10^{\mathrm{pH} - \mathrm{p}K_c}\right)^{-1} = \left(1 + 10^{7.0 - 8.31}\right)^{-1} = 0.953$$

and the dissociation degree of dihydrogen phosphate ion is

$$\alpha_{\mathrm{H_2PO_4^-}} = \left(1 + 10^{\mathrm{p}K_c - \mathrm{pH}}\right)^{-1} = \left(1 + 10^{6.72 - 7.0}\right)^{-1} = 0.656$$

The ionic strength of TRIS-hydrogen phosphate-EDTA buffer is

$$I = \alpha_{\mathrm{TRIS}} c_{\mathrm{TRIS}} + c_{\mathrm{H_3PO_4}}\left(1 - \alpha_{\mathrm{H_2PO_4^-}}\right) + 4\left(\alpha_{\mathrm{H_2PO_4^-}}\, c_{\mathrm{H_3PO_4}}\right)^2 = 0.10$$

Simultaneously, according to the electroneutrality law of chemical solutions,

$$\alpha_{\mathrm{TRIS}} c_{\mathrm{TRIS}} = c_{\mathrm{H_3PO_4}}\left(1 + \alpha_{\mathrm{H_2PO_4^-}}\right)$$

Hence, it follows from the last two equations that the desired TRIS-hydrogen phosphate-EDTA buffer must contain 0.085 mol/l TRIS and 0.048 mol/l H_3PO_4.

3.11 C

3.12 A

3.13 D

3.14 A

3.15 D

3.16 A

3.17 B

3.18 B

3.19 C

3.20 B

3.21 B

3.22 D

3.23 B

3.24 A

Electrophoretic terms

Acid	A proton donor. An acid gives a proton to a base.
Affinity electrophoresis	Zone electrophoresis in an agarose gel containing usually specific ligands.
Agarose gel	A natural polymer composed of D-galactose and 3,6-L-anhydrogalactose. The agarose gel is used as separation medium in different electrophoresis methods, especially in the clinical routine.
Agarose gel electrophoresis	Zone electrophoresis in an agarose gel.
Allele	An alternative form of a gene.
Ampholyte	A chemical compound, which has acidic and basic properties.
Analytical electrophoresis	Electrophoresis used for resolving of polyions as bands.
Autoradiography	Blackening on an X-ray film by irradiation of labeled with isotopes polyions, which are separated each from other by gel electrophoresis.
Bacteriophage	A virus that infects bacteria.
Base	A proton catcher. The base binds a proton from an acid.
Blotter	A device where transfer of proteins or nucleic acids onto a blot membrane is carried out.
Blotting	A method for transferring electrophoretically resolved bands onto a membrane where they can react with nonspecific or specific reagents. Blotting is a proofing method, not an electrophoresis method.
Buffer (buffer solution)	A solution of a base and its conjugate acid, which keeps a pH value constant.
Buffer capacity	The capacity of a buffer to maintain a pH value constant, if the buffer is diluted or bases (acids) are added.
Capillary electrophoresis	Electrophoresis (zone electrophoresis, isotachophoresis, or isoelectric focusing) in capillaries.
Carrier ampholytes	Synthesized ampholytes that reach their isoelectric points in an electric field and form so a stable pH gradient.
cDNA	Cyclic DNA. It is formed by a reverse transcriptase as a copy of a mRNA.
Cellulose acetate	A compound used for producing film-formed electrophoretic medium.
Cellulose acetate electrophoresis	Zone electrophoresis in cellulose acetate membranes.
Densitometry	A method for determining the number and concentration of electrophoretically separated bands by measuring their optical density using a light or laser beam.
Deoxyribonucleates	DNA polyions.
Disc-electrophoresis	A combination of isotachophoresis and zone electrophoresis, which takes place in appropriate buffer systems.

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DNA (deoxyribonucleic acids)	Polynucleotides that consist of specifically arranged deoxyribonucleotide residues. They are negatively charged in a solution.
DNase (deoxyribonuclease)	Enzyme that catalyzes the hydrolysis of DNA.
Effect of Hjerten	Concentrating of polyions on the boundary between solutions with a lower and higher ionic strength.
Effect of molecular sieve	Separation of polyions in a gel, depending on its pores.
Effective mobility	Ionic mobility multiplied by the dissociation degree of electrolyte that builds the ion.
Electric double layer	Electric layer formed by a charged surface and its counter-ion(s).
Electroblotting	Blotting performed in an electric field.
Electrolyte	A chemical compound that forms ions in a solution.
Electrophoresis	Separation of dissolved and electrically charged particles in an electric field.
Exons	mRNA fragments containing genetic information for proteins. In most eukaryotes and in some prokaryotes, the exons are divided by introns.
Fibrillar proteins	Usually insoluble proteins whose polypeptide chains form filaments.
Fluorography	Blackening on a photosensitive film, caused by visible light irradiation, emitted by polyions labeled with isotopes.
Free-flow electrophoresis	Electrophoresis in a flowing buffer. It can be carried out as zone electrophoresis, isotachophoresis, or isoelectric focusing.
Gel	A porous soft material.
Genome	Total genetic information contained in the nucleic acids of an organism.
Globular proteins	Soluble proteins whose polypeptide chains are folded in the space.
Gradient gel	A gel with linearly or exponentially changeable concentration.
Gradient gel electrophoresis	Electrophoresis in a gradient gel.
Gradient mixer	A device for casting gradient gels.
Henderson–Hasselbalch equation	An equation expressing the relationship between the pH value, on the one hand, and the pK_c value and ratio between the base and conjugated acid, on the other.
Homogeneous gel	A gel of same concentration in all parts.
Homogeneous gel electrophoresis	Electrophoresis in a homogeneous gel.
Immunoelectrophoresis	Zone electrophoresis in an agarose gel containing or receiving immunoglobulins. As a result the electrophoretically resolved polyions take part in precipitation reactions.
Introns	mRNA fragments that are located between exons.
Ionic strength	Mathematical term that takes into account the concentration and electrovalence of all ions in a solution.
Ionization constant	Constant that characterizes an electrolytic reaction. It is a function of the equilibrium concentrations of the reactants and products at a certain temperature and pressure.

Isoelectric focusing	Electrophoresis in a pH gradient. The moving polyions stop at their isoelectric points.
Isoelectric focusing with carrier ampholytes	Isoelectric focusing in a pH gradient created by carrier ampholytes.
Isoelectric focusing in immobilized pH gradients	Isoelectric focusing in a pH gradient created by bound immobilines.
Isoelectric point	pH value at a certain ionic strength and temperature, when the sum of all electric charges of a polyion is equal to zero.
Isotachophoresis	Electrophoresis that is carried out in a system of two or more buffers forming moving ionic boundaries between them.
mRNA (messenger RNA)	Type of RNA. The information of mRNA is translated by ribosomes onto proteins.
Net charge	It is less (in absolute value) than the total charge due to the counterions which are bound to the polyion surface, according to Stern model.
Oligonucleotides	Short nucleic acids, similar to oligopeptides, which serve as primers.
Paper electrophoresis	Zone electrophoresis on paper.
Persistent function of Kohlrausch	An electrochemical effect according to which the concentration of an ion depends on its mobility. If certain conditions are met, leading and trailing ions are moving with same velocity forming an ionic boundary.
Polyacrylamide	A polymer consisting of acrylamide and bisacrylamide residues. The polyacrylamide gel has no electroosmosis and is the most widely used separation medium in electrophoresis.
Polyacrylamide gel electrophoresis	Zone electrophoresis in homogeneous polyacrylamide gels.
Polyion	A large particle, e.g., nucleate or proteinate, which has negative, positive, or both charges.
Precast gels	Polyacrylamide or agarose gels that are cast on a support film or support net prior to electrophoresis.
Preparative electrophoresis	Quantitative electrophoresis during which the polyions are separated from each other into separated solutions.
Protein	A macromolecule that is composed of one or more polypeptide chains. In a solution, it has many and different electric charges.
Protolyte	An electrolyte that gives or receives one or more protons in a solution.
Pulsed-field electrophoresis	Zone electrophoresis that is used for separation of chromosomes or other large particles in an electric pulsed field.
3′-Poly (A)	A tail of hundreds of adenosine mononucleotide residues hanging on the 3′ end of most eukaryotic mRNA. The poly (A) tails are involved in the translation control.
Ready-made kits	Kits that contain precast gels, solutions (buffer, fixing solution, staining solution, *etc.*) and other materials (application templates, blotting papers, *etc.*).

Rehydratable gel	A dried gel, usually of polyacrylamide, that may be converted into usable wet gels in a solution.
Relaxation	Dislocation of the electric charges of a particle in an electric field whereby its velocity slows down.
Restriction endonucleases	Enzymes, which are isolated from different bacterial species. They cut off specific sequences of DNA nucleates. The restriction endonucleases protect the bacteria from virus infections. DNA of the host bacterium is resistant, because it is methylated.
Ribonucleates	RNA polyions.
RNA (ribonucleic acids)	Polynucleotides that consist of specifically arranged ribonucleotide residues. They carry negative charges in a solution.
RNases (ribonucleases)	Enzymes that catalyze the hydrolysis of RNA.
rRNA (ribosomal RNA)	Two types of rRNA are known in eukaryotes: 28S and 18S. The bacteria contain also two types of rRNA: 23S and 16S.
Scanner	A device for determining the number and concentration of electrophoretically separated bands by measuring their optical density using a light or laser beam.
SDS disc-electrophoresis	Disc-electrophoresis whose buffer system contains the denaturing agent SDS.
SDS zone electrophoresis	Zone electrophoresis whose buffer contains the denaturing agent SDS.
Splicing process	Process during which intron transcripts are cut off from the RNA copy of a gene, whereby the exon transcripts are joined together.
Starch gel	A natural polymer that consists of D-glucose residues.
Starch gel electrophoresis	Zone electrophoresis in a starch gel. Today it is replaced by the electrophoresis in polyacrylamide or agarose gels.
Submarine electrophoresis	Agarose zone electrophoresis for separation of nucleates that is carried out under a buffer.
Support film	A chemically pretreated film, usually of polyester, which is used for chemical fixation of a gel.
Support net	A chemically pretreated net, usually of polyester, which is used for chemical fixation of a net.
Total charge	The sum of all the electric charges of a polyion in an infinitely dilute solution.
tRNA (transfer RNA)	RNA used for transporting amino acids in the process of protein synthesis.
Two-dimensional electrophoresis	A combination between isoelectric focusing with carrier ampholytes or in immobilized pH gradients, and SDS disc-electrophoresis.
Zone electrophoresis	Electrophoresis that is run in one buffer, hence at continuous electric field strength.
Zwitterion	An ion that carries a positive and a negative charge.

Subject Index

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