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Budin Michov

ELECTROPHORESIS FUNDAMENTALS

ESSENTIAL THEORY AND PRACTICE

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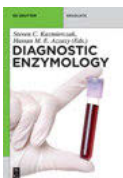
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Electrophoresis Fundamentals



Essential Theory and Practice

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To people who desire to study new techniques for exploring proteins and nucleic acids in our organisms.

Preface

Electrophoresis is referred to as moving of charged dissolved particles in an electric field, which causes their resolution as a result of velocities and interaction with the separation medium. The resolving is performed by charge, size, or binding affinity.

Numerous electrophoresis techniques are known. They are used for resolving proteins and nucleic acids, chromosomes, viruses, cell organelles (mitochondria, ribosomes), cells (red cells, tissue cells, and parasites), *etc.*

The electrophoresis methods are used in medicine, molecular biology, biochemistry, proteomic and genomic studies, microbiology, and forensics.

The proposed book *Electrophoresis Fundamentals* is based on my recent book *Electrophoresis. Theory and Practice*, published in 2020 by Walter de Gruyter in Berlin and Boston. While the published book is intended for specialists, the present one is a book for laboratory technicians, students, young scientists, biochemists, medical doctors, and more.

It was a challenge and a hard work for me to write the book *Electrophoresis Fundamentals* after the great progress in protein and nucleic acid electrophoresis in the last years. I will be happy, if it will help people who want to understand the basis of electrophoresis and desire to use its methods in the practice.

Nuremberg, Sofia, 2022
Budin Michov

About the Author



Prof. Budin M. Michov, MD, PhD, DSc, studied medicine and specialized in biochemistry. He worked as a chemist and biochemist 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 became the chairman of the Department of Biochemistry at the Medical Faculty of Sofia University, Bulgaria.

Prof. 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's 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 a buffer recirculator for electrophoresis.

Prof. Michov has published several books: *Electrophorese*, Walter de Gruyter, Berlin and New York (1996), in German; *Molecular Mechanisms of Inflammation and Allergy* (1977); *Electrophoresis. Theory and Practice* (2020), Walter de Gruyter, Berlin and Boston; and more.

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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 $\text{m}^2/(\text{s V})$
μ_i	Mobility of ion i , in $\text{m}^2/(\text{s V})$. [No difference exists between the terms ionic mobility and electrophoretic mobility.]
μ_i'	Effective mobility of ion i , in $\text{m}^2/(\text{s V})$
[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
[H ⁺]	Dimensionless proton concentration. $[\text{H}^+] = [\text{H}^+]/[\text{H}^+]_0$, where $[\text{H}^+]_0$ is the standard proton concentration of $1 \text{ mol}/\text{dm}^3$
[OH ⁻]	Equilibrium concentration of hydroxide ion, in mol/dm^3 (mol/L) or kmol/m^3
[OH ⁻]	Dimensionless concentration of hydroxide ion. $[\text{OH}^-] = [\text{OH}^-]/[\text{OH}^-]_0$, where $[\text{OH}^-]_0$ is the standard hydroxide concentration of $1 \text{ mol}/\text{dm}^3$
A	Adenine
ACES	N-(2-acetamido)-2-aminoethanesulfonic acid
$a_{i(p)}$	Electrokinetic radius of ion i (polyion p)
ALP	Alkaline phosphatase
Ammediol	2-Amino-2-methyl-1,3-propanediol
APS	Ammonium peroxydisulphate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BES	N,N-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid
BICINE	N,N-bis(2-hydroxyethyl)-glycine
BIS	N,N'-methylenebisacrylamide, N,N'-methylenediacrylamide
BISTRIS	Bis(2-hydroxyethyl)-amino-tris-(hydroxymethyl)-methane
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
C	Crosslinking
CA	Cellulose acetate
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-propanesulfonate
CHES	2-(cyclohexylamino)ethanesulfonic acid
c_i	Volume concentration of ion i , in mol/dm^3 (mol/L) or kmol/m^3
DEAE	Diethylaminoethyl
DMS	Dimethyl sulfate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid(s)
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenedinitrilotetraacetate (ethylenediaminetetraacetate)
Eq.	Equation(s)
G	Guanine
GABA	γ -Aminobutyric acid
GlyGly	Glycylglycine
[H ⁺]	Dimensionless concentration of hydrogen ion. $[\text{H}^+] = [\text{H}^+]/[\text{H}^+]_0$, where $[\text{H}^+]_0$ is the standard hydrogen ion concentration of $1 \text{ mol}/\text{dm}^3$.
HDL	High density lipoproteins
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid

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XXX — Abbreviations

HRP	H orseradish peroxidase
I	Ionic strength, in mol/kg or mol/dm ³ (mol/L)
I	Dimensionless ionic strength. $I = I/I_0$, where I_0 is the standard ionic strength of 1 mol/dm ³
I_c	Volume ionic strength in mol/dm ³ (mol/L)
IEF	I soelectric focusing
Ig	I mmunoglobulin
I_m	Mass ionic strength in mol/kg
IPG	I mmobilized pH gradient
K	Thermodynamic equilibrium constant; different dimensions
K	Dimensionless thermodynamic equilibrium constant. $K = K/K_0$, where K_0 is the standard thermodynamic equilibrium constant, which has the dimensions of K .
K_c	Concentration equilibrium constant; different dimensions
K_c	Dimensionless concentration equilibrium constant. $K_c = K_c/K_0$, where K_0 is the concentration equilibrium constant with dimensions of K_c .
kb	K ilobases
kbp	K ilobases pairs
K_R	Retardation coefficient
K_w	Ionic product of the water, in (mol/dm ³) ² , (mol/L) ²
LDL	L ow density lipoproteins
Mbp	M egabases pairs
MES	2-(N -morpholino)ethanesulfonic acid
m_i	Mass concentration (molality) of ion i , in mol/kg
MOPS	3- M orpholino-propanesulfonic acid
M_r	Relative molecular (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.
NBT	N itro blue tetrazolium
Nonidet	N on-ionic detergent
OD	O ptical density
PAGE	P olyacrylamide gel electrophoresis
PAS	P eriodacid- S chiff's reagent
pH	Hydrogen exponent (p ondus, p otentia hydrogenii) – the negative logarithm of [H ⁺], dimensionless
pH(I), pI	Isoelectric point (p ondus i soelectricus), dimensionless
pK	Negative logarithm of K , dimensionless
pK'	Negative logarithm of K' , dimensionless
pK _c	Negative logarithm of K_c , dimensionless
PBS	P hosphate- b uffered saline
PCR	P olymerize chain reaction
PVC	P olyvinyl chloride
PVDF	P olyvinylidene difluoride
r	Molecular radius
R_μ	Relative mobility
R_f	R atio to front
$r_{i(pi)}$	Geometric radius of ion i (p olyion pi)
RNA	R ibonucleic acid(s)
s	Number of ionic species in a solution, dimensionless
SDS	S odium d odecyl sulfate

SDS-PAGE	SDS polyacrylamide gel electrophoresis
<i>T</i>	Total monomeric concentration (polyacrylamide concentration)
T	Thymine
<i>TAE</i>	TRIS-acetate-EDTA
TBE	TRIS-borate-EDTA
TBS	TRIS-buffered saline
TCA	Trichloroacetic acid
TEA	Triethanolamine
TES	N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid
TMEDA (TEMED)	N,N,N',N'-tetramethylethylenediamine, 1,2-bis(dimethylamino)ethane
TRICINE	N-[tris(hydroxymethyl)methyl]-glycine
TRIS	Tris(hydroxymethyl)-aminomethane
UV	Ultraviolet light
VLDL	Very low density lipoproteins
z_i	Charge number (electrovalence) of ion <i>i</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/dm³ (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/dm³ (1 mol/L). The same is valid for the ionic strengths.

1 Fundamentals of electrophoresis

- Overview on electrophoresis — 1
- Separation media — 3
- Electrophoresis resolution and sharpness — 3
- Detection of resolved bands — 3
- References — 4

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 [1–3]. In its current form, the electrophoresis is connected with the studies of Tiselius [4] in the 1930s.

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

The electrophoresis is used for resolving of proteins and nucleic acids, chromosomes, viruses, cell membranes (plasma, lysosomal, nuclear, and other), cell organelles (mitochondria, ribosomes), cells (red cells, tissue cells, and parasites), *etc.*, it takes place in biochemistry, proteomic and genomic studies, forensics, molecular biology, and microbiology. By electrophoresis, more than the half of all separations and almost all separations of blood proteins and DNA are carried out.

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 (Figure 1.1).

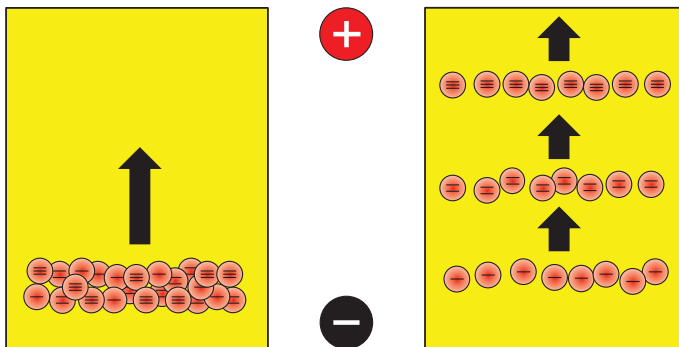


Figure 1.1: Electrophoresis of unipolar particles carrying different electric charges. *Left* – start of electrophoresis; *Right* – end of electrophoresis.

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Commonly, polyions that are to be resolved are applied onto a separation medium, which in its turn is placed into an electrophoresis cell that is connected to a power supply. When electric current is turned on, the larger and less charged polyions move slower through the medium, while the smaller and more charged polyions move faster.

Mostly, electrophoresis is carried out in agarose or polyacrylamide gels, soaked with buffers. Other separation media are: starch gel, cellulose acetate, and paper. Today they have lost their actuality. Electrophoresis can also be carried out in buffers only. The buffers are electrolyte solutions, which maintain constant pH values, e.g. TRIS-borate buffer, TRIS-histidinate buffer, Goods buffers, and more.

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. At electrophoresis of negatively charged proteins, Bromophenol blue, Xylene cyanol, which runs slower than Bromophenol blue, or Orange G are used; at electrophoresis of positively charged proteins Bromocresol green or Methylene blue are used.

Proteins can be separated also in pH gradients, where they stop at their isoelectric (pI) points. This electrophoresis, called *isoelectric focusing*, can be carried out in two types of pH gradients: produced by carrier ampholytes, or by immobilines.

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

Beside electrophoresis cells and power supplies, thermostats for reserving the resolving medium temperature, and densitometers or scanners for analyzing the *pherograms* (the resolved polyions in the medium) are used. Semi-automated 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; and the viscosity of the resolving medium depends mainly on the medium structure and temperature.

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

Electrophoresis should be carried out at voltage and electric current, when the heating could be drawn out from the electrophoresis cell.

After electrophoresis, *blotting* of proteins and nucleic acids can be made. Using this technique, the resolved bands can be immobilized onto blot membranes and treated afterward. The blotting methods are carried out in four steps: electrophoretic separation of proteins or nucleic acid fragments; their transfer and immobilization onto blot membranes; binding of analytical probes to the blotted substances; and visualization of the blotted bands.

The blotting of DNA bands is called Southern blotting, the blotting of RNA bands is called Northern blotting, and the blotting of protein bands is called Western blotting. The blotting membrane consists usually of nitrocellulose, nylon or polyvinylidene difluoride (PVDF).

Separation media

The electrophoresis can be carried out in a solution, but the diffusion there is too strong. In order to limit the diffusion, solid media are used. The earliest solid medium was cellulose contained in the filter paper. The paper electrophoresis was invented by Kunkel and Tiselius [5] in 1951. Cellulose acetate membranes were the next step in the electrophoresis progress. Today most common are agarose and polyacrylamide gel electrophoresis.

Electrophoresis resolution and sharpness

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

The *sharpness* of electrophoresis is referred to as the reciprocal value of the bandwidth; narrow the bands, the higher the sharpness.

Detection of resolved bands

The majority of polyion bands are not visible to the naked eye, with a few exceptions. Direct optical detection of resolved bands can be applied, for example, to hemoglobins (which are red colored). Therefore, a couple of methods are created for detection, localization, and quantitation of separated bands.

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

The proteins can be stained in gels. Common dye is Coomassie brilliant blue, which can detect 0.3 μg of protein in a spot. DNA is detected usually by fluorescent intercalating of ethidium bromide. Both proteins and DNA can react with silver ions to form black bands. The silver methods are 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 of the gel background to remove unbound dye. In some cases, placing the gel on an **ultraviolet (UV)** lightbox or under a UV lamp can reveal UV absorbing bands, or UV fluorescence bands.

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

The most modern methods of both detection and characterization of resolved proteins involve mass spectrometry.

The electrophoresis results can be recorded with a computer operated camera, and the intensity of a band or spot of interest can be compared against markers in the same gel, using specialized software.

After electrophoresis, the gels with bands can be saved in dry forms.

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1.1 Electric double layer of a charged particle

- 1.1.1 Electric charges of polyions — 5
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1.1.1 Electric charges of polyions

Polyions carry electric charges. In a solution, two types of electric charges exist: total and net charge. The *total charge* of a polyion is the sum of all electric charges, which the polyion carries: the charges of the amino acid residues, metal ions, cofactors, *etc.* These charges can be observed in an infinitely dilute solution.

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

$$Q_i = z_i e \quad (1.1.1)$$

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 $1.602\,1892 \times 10^{-19}$ C. Since z_i has a positive or negative value, the value of Q_i can have different signs.

The *net charge* is less (in absolute units) than the total charge. The oppositely charged ions (counter-ion) in the solution, which form ionic atmosphere are responsible for this. The surface of a charged and solvated (hydrated) ion or polyion, and its ionic atmosphere build an electric double layer [1, 2]. The electric field strength inside the electric double layer can be from 0 to over 10^9 V/m.

Several models describe the electric double layer. Most common are the models of Helmholtz, Gouy–Chapman, Debye–Hückel, and Stern.

1.1.2 Model of Helmholtz

The first model of the electric double layer was developed by Helmholtz [3] and Perrin [4]. According to it, the counter-ions are located at a certain distance from the charged surface. As a result, the charged particle can be considered as a ball capacitor. It consists of two concentric spheres similarly to a capacitor electric double layer, and the electric potential is decreased linearly by the distance from the charged surface (Figure 1.1.1).

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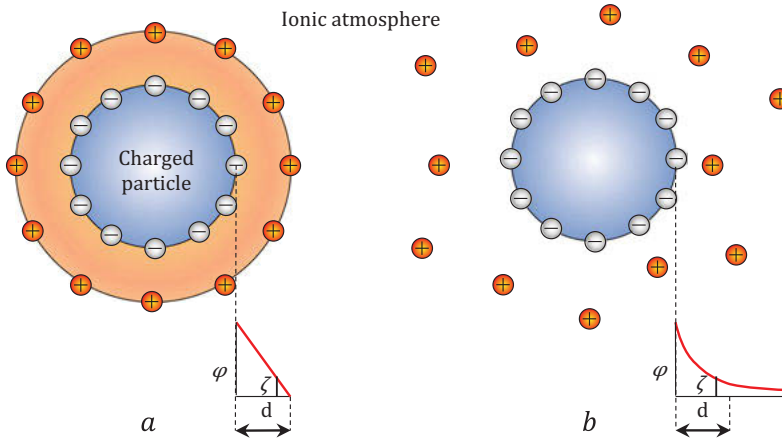


Figure 1.1.1: Models of the electric double layer according to Helmholtz (a) and Gouy–Chapman (b).

The electric potential of the surface of a charged particle (the inner electric potential of Helmholtz)

$$\varphi_{\text{in}} = \frac{Q}{4\pi\epsilon a} \quad (1.1.2)$$

where Q is the electric charge of the particle, in C; ϵ is the (di)electric permeability of the solvent (water), which is equal to the product of the relative (di)electric permeability ϵ_r and the (di)electric-constant ϵ_0 ($8.854\,187\,817 \times 10^{-12}$ 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)

$$\varphi_{\text{ex}} = \frac{Q}{4\pi\epsilon(a+d)} \quad (1.1.3)$$

where d is the thickness of the electric double layer. It follows from eqs. (1.1.2) and (1.1.3) that the electrokinetic potential ζ of a charged particle and its ionic atmosphere may be described by the expression

$$\zeta = \frac{Q}{4\pi\epsilon a} - \frac{Q}{4\pi\epsilon(a+d)} = \frac{Qd}{4\pi\epsilon a(a+d)} = \frac{Q}{4\pi\epsilon(1 + \frac{a}{d})} \quad (1.1.4)$$

1.1.3 Model of Gouy–Chapman

Gouy [5] and Chapman [6] have shown that the fixed arrangement of the counterions in the model of Helmholtz does not exist, because they are moving to and from the charged particle. According to the model of Gouy–Chapman, the number

of counter-ions, and thereby the electric potential of the double layer, decreases exponentially with the distance from the charged surface (Figure 1.1.1).

The relationship between the counter-ion concentration at the charged surface, c , and the counter-ion concentration in the solution, c_0 , is

$$c = c_0 e^{-\left(\frac{ze\varphi}{kT}\right)} \quad (1.1.5)$$

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

1.1.4 Theory of Debye–Hückel

Later Debye and Hückel [7] developed a similar theory of the electric double layer. According to it, the thickness of the ionic atmosphere

$$\frac{1}{\kappa} = \frac{1}{F} \left(\frac{\varepsilon RT}{2I} \right)^{1/2} \quad (1.1.6)$$

where κ is the parameter of Debye–Hückel in m^{-1} , F is the Faraday constant (96,484,554.61 C/mol), R is the molar gas constant [8,318.41 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. (1.1.6) that the thickness of ionic atmosphere depends exclusively on the ionic strength, i.e. on the concentration and 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 eq. (1.1.4) is replaced by the magnitude $1/\kappa$. Then, the following equation is obtained:

$$\zeta = \frac{Q}{4\pi\varepsilon a(1 + \kappa a)} \quad (1.1.7)$$

1.1.5 Model of Stern

In 1924, Stern [8] 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 counter-ions, which are adsorbed on the hydrated charged surface. The diffuse layer is composed of the remaining counter-ions, 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).

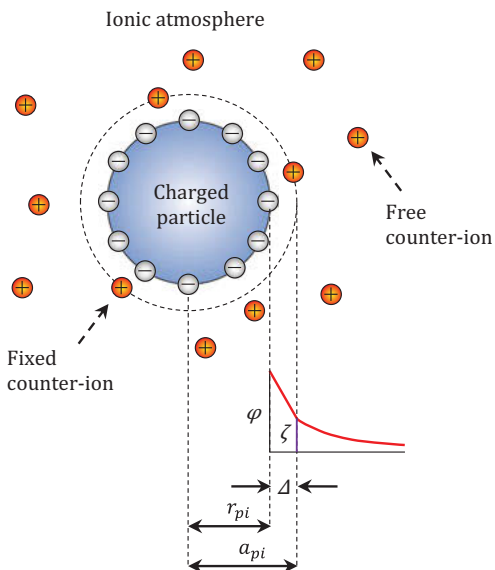


Figure 1.1.2: Spherical protein polyion of radius r_{pi} possessing a negative total charge of $-12e$ is covered by the fixed part of its ionic atmosphere of Δ thickness. The fixed part consists of 3 counter-ions with a positive net charge of $3e$. The diffuse part, which has a thickness of $(1/\kappa - \Delta)$, contains the remaining counter-ions, 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 counter-ions 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 diffuse part of the ionic atmosphere, and then decreases exponentially [9–11].

The model of Stern explains best the polyion properties. It is known that the pK_c values of some chemical groups of proteins differ by up to 1.5 pH units from the pK_c values of the same groups in corresponding amino acids. This can be explained so: If a protein polyion has a positive total (sum) 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. If 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 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 [12, 13].

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 [14]. Smoluchowski 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

$$\kappa a \gg 1 \quad (1.1.8)$$

When the Debye length is much larger (for example, in some nanocolloids), the following expression is obtained:

$$\kappa a < 1 \quad (1.1.9)$$

1.1.6 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 [15].

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

In accordance 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 different ions 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 counter-ion, and $A - R = \Delta$ is 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 has another electric potential, which we will call *electrokinetic potential* (Figure 1.1.3). The electrokinetic potential φ is a function not only of the distance from it, but also of the ionic strength of the solution.

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

$$\varphi_A = \varphi_R \frac{1 - \kappa(A - R)}{1 + \kappa R} \quad (1.1.10)$$

where φ_R is the electric potential of the geometric ion and its ionic atmosphere. The electric field of the ionic atmosphere (the counter-ions) 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

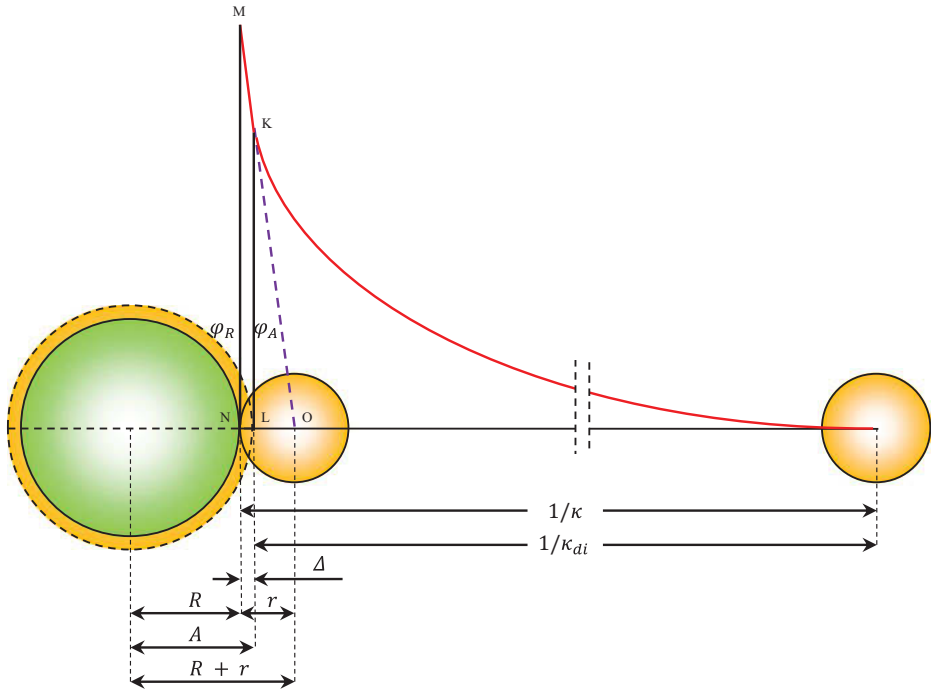


Figure 1.1.3: Electric potentials and radii of the central ion and its ionic atmosphere. φ_R – electric potential of the geometric ion and its ionic atmosphere; φ_A – electric potential of the electrokinetic ion and its ionic atmosphere; R and r – geometric radii of the central ion and its counter-ion, 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.

$$\varphi_R = \varphi_{R_0} + \varphi_{1/\kappa} \quad (1.1.11)$$

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

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

$$\varphi_A = \varphi_R(1 - \kappa r) \quad (1.1.12)$$

Using the above equations, we proved that

$$A = R + \kappa r^2 \quad (1.1.13)$$

where $r \leq \kappa^{-1} \leq \infty$, i.e. $0 \leq \kappa r \leq 1$.

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

$$\Delta = A - R = \kappa r^2 \quad (1.1.14)$$

Assuming that $R = nr$, it follows that

$$A = nr + \kappa r^2 = r(n + \kappa r) \quad (1.1.15)$$

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

$$a = r(1 + \kappa r) \quad (1.1.16)$$

where a is the electrokinetic radius of the central ion, and r is the geometric radius of the central ion and its counter-ion.

Having in mind the existence of two radii for each charged particle, we [16] integrated the differential equations of Henry's function [17, 18] in the interval $r, 2r$, and obtained the new function

$$f(\kappa r) = \frac{1 + \kappa a}{1 + \kappa r} = \frac{1 + \kappa r + (\kappa r)^2}{1 + \kappa r} \quad (1.1.17)$$

which helps us to define the electrokinetic potential as

$$\zeta = \frac{1.5\mu\eta}{\varepsilon} \frac{1 + \kappa r}{1 + \kappa a} \quad (1.1.18)$$

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 I of the solution, 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 , i.e. to the square of the geometric radius r of the counter-ion. When the thickness of the ionic atmosphere $1/\kappa \rightarrow \infty$, i.e. when $\kappa \rightarrow 0$, then $A \rightarrow R$; and when $1/\kappa \rightarrow 0$, i.e. when $\kappa \rightarrow 1/r$, then $A \rightarrow R + r$.

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1.2 Proteins and nucleic acids form polyions in solution

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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

Proteins are made up of amino acid residues and can in addition contain non-amino acid compounds. To the first group belong the globular proteins (soluble proteins with oval-shaped molecules, such as albumins and globulins), and some fibrous proteins (scleroproteins), such as collagens and keratins, which are water-insoluble. The second group includes protein complexes that are composed of a protein part and external compounds, such as hemoglobins.

1.2.1.1 Masses and electric 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 110, the relative molecular mass of the proteins is approximately 10,000 to 100,000 and more.

The proteins are amphoteric polyelectrolytes that form in an aqueous solution 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*.

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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 solution pH value: the first case is predominating at a high pH value; the second case is prevailing at a low pH value.

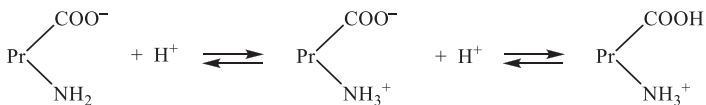
The total charge (the number of elementary charges) of a protein polyion is calculated as a sum of the negative and positive charges. For example, if a proteate has 200 negative and 180 positive charges, its total number of elementary charges is

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

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 the groups ϵ -NH₂ and $-C(NH)NH_2$ are protonated. The groups $-C_3N_2H_3$, α -NH₂, $-SH$, 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

The proteins have amphoteric molecules. That is why they can be considered as zwitterions and are denoted by the formula $H_3^+N-Pr-COO^-$. Their charges vary when the proton concentration in the solution is changed. The carboxyl groups at low pH values and the amino, imidazole and amidino groups at higher pH values are neutral:



The pH value, at which the negative charges of an amphoteric polyion neutralize its positive charges and the 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 [1].

The absence of a total 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, the protein precipitates. 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 [2]. 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 structures of proteins determine their biological activity (enzyme and hormone reactions, antigen-antibody reactions, oxygen transport, *etc.*). This is their native state. Changes in the quaternary, tertiary, and secondary structure of proteins is referred to as *denaturation*.

The denaturation of proteins may be caused by organic solvents (alcohols), decreasing pH (acid denaturation), high temperature (heat denaturation), ionic or non-ionic detergents, and high-energy radiation (UV, X-rays, 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.

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 a 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 (A) and guanine (G), pyrimidine bases are cytosine (C), uracil (U), and thymine (T). Uracil is not presented in the deoxyribonucleic acids (DNA) and thymine is not presented in the ribonucleic acids (RNA).

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 conformation of the DNA molecule exerts influence on DNA movement. For example, supercoiled DNA migrates faster than relaxed DNA because supercoiled DNA is more compact. The velocity of DNA movement changes under various electrophoresis conditions [3].

1.2.2.1 Masses and electric 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. Each mononucleotide residue has a negative charge in neutral or alkaline

solutions. Therefore, the number of elementary charges can be calculated when the relative nucleate mass is divided by -330 .

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 DNA and RNA polyions is constant; therefore, their mobilities in a solution are equal [4].

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 $-15.1 \times 10^{-9} \text{ m}^2/(\text{s V})$ at an ionic strength of 0.1 mol/L and $-21.7 \times 10^{-9} \text{ m}^2/(\text{s V})$ at an ionic strength of 0.01 mol/L (for denatured DNA from calf thymus, these data are -13.3×10^{-9} and $-19.0 \times 10^{-9} \text{ m}^2/(\text{s V})$, respectively). The increased temperature also diminishes the nucleate mobilities because the denaturation increases the nucleate volume.

1.2.2.2 Native and denatured nucleic acids

The nucleic acids, as the proteins, can be in native or denatured state (Figure 1.2.1). 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 native conditions, whereas single-stranded nucleic acids can occur under native or denaturing conditions.

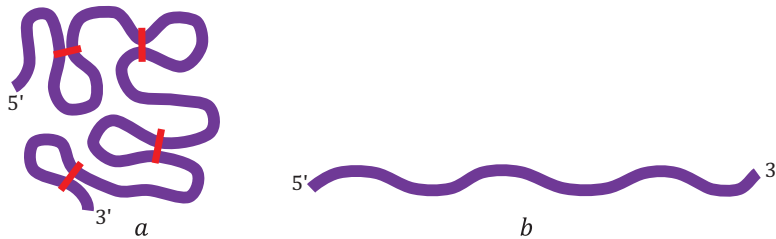


Figure 1.2.1: Native (a) and denatured (b) nucleic acid (rRNA). The base pairs are shown in red.

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 (for example, by increasing the ionic strength), the double helix is stabilized.

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, high 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; therefore, is called *DNA melting*. The DNA melting occurs at 70–90 °C in a saline solution and 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.

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.

Methylmercury hydroxide, glyoxal, or formaldehyde. These chemicals are volatile and toxic, especially methylmercury hydroxide; therefore, they should 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.

Urea and formamide. Highly concentrated urea (H₂C–CO–NH₂), alone or in combination with formamide (OHC–NH₂), 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.

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1.3 Electrophoresis is running in buffers

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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 Brønsted [1], buffers are systems of weak bases and their conjugated 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.

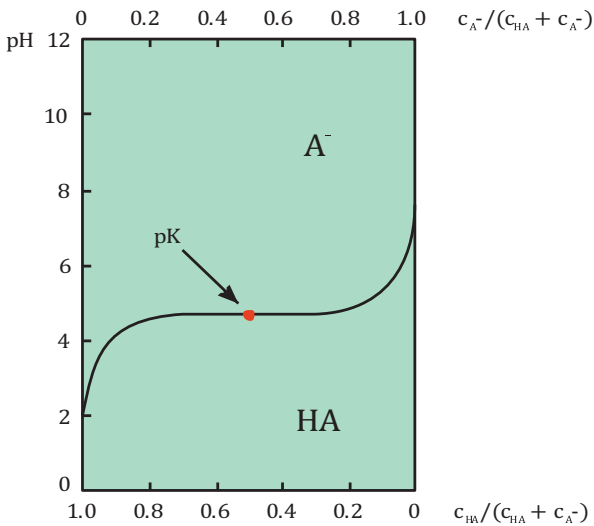


Figure 1.3.1: Titration of acetic acid (HA). The buffer zone of acetate buffer is between pH = 4 and pH = 5.4.

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The buffers obey the equation of Henderson–Hasselbalch

$$\text{pH} = \text{p}K_c + \log \frac{[\text{Base}]}{[\text{Acid}]} \quad (1.3.1)$$

With the aid of this equation, the buffer pH value can be calculated, if $\text{p}K_c$ of the buffering acid and the equilibrium concentrations of the base and its conjugated acid are known. When $\text{pH} = \text{p}K_c$, the concentrations of the base and acid are equal, i.e. half of the protolyte is ionized.

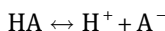
1.3.1.1 Buffer capacity

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

$$\beta = \frac{dc_A}{d\text{pH}} \quad (1.3.2)$$

where c_A is the base concentration.

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



the total base concentration in the buffer solution is

$$c_A = [\text{A}^-] + [\text{OH}^-] - [\text{H}^+] \quad (1.3.3)$$

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

$$K_c = \frac{[\text{H}^+][\text{A}^-]}{[\text{HA}]} \quad (1.3.4)$$

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

$$c = [\text{HA}] + [\text{A}^-] \quad (1.3.5)$$

it follows from eqs. (1.3.4) and (1.3.5) that

$$[\text{A}^-] = \frac{cK_c}{K_c + [\text{H}^+]} \quad (1.3.6)$$

It is known that

$$[\text{OH}^-] = \frac{K_w}{[\text{H}^+]} \quad (1.3.7)$$

where K_w is the ionic product of water, equal to about 10^{-14} (mol/L)² at 25 °C. Hence, eqs. (1.3.2), (1.3.6), and (1.3.7) can be united into

$$\beta = \frac{d\left(c\frac{K_c}{K_c + [H^+]} + \frac{K_w}{[H^+]} - [H^+]\right)}{d(-\log[H^+])} = \ln 10 \left(c \frac{K_c [H^+]}{(K_c + [H^+])^2} + \frac{K_w}{[H^+]} + [H^+] \right) \quad (1.3.8)$$

In neutral, weakly acidic, or weakly basic buffers, the concentrations of the proton and hydroxide ion are relatively low. Therefore, they can be neglected in eq. (1.3.8) that gives

$$\beta = \ln 10 c \frac{K_c [H^+]}{(K_c + [H^+])^2} = \ln 10 c \frac{10^{-pK_c - \text{pH}}}{(10^{-pK_c} + 10^{-\text{pH}})^2} \quad (1.3.9)$$

According to eqs. (1.3.8) and (1.3.9), the buffer capacity depends on the concentration and pH value of the buffer. When $\text{pH} = pK_c$, it follows from eq. (1.3.8) that

$$\beta = \ln 10 \left(\frac{c}{4} + \frac{K_w}{[H^+]} + [H^+] \right) \quad (1.3.10)$$

and from eq. (1.3.9) that

$$\beta = \ln 10 \frac{c}{4} \quad (1.3.11)$$

How a buffer counteracts a pH change? 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

$$\text{pH} = pK_c + \log \frac{x}{c - x} \quad (1.3.12)$$

To calculate the pH sensitivity of a buffer, the first derivative of eq. (1.3.12) should be derived. Using the relationship between the natural and decimal logarithms

$$\log n = \frac{\ln n}{\ln 10} \quad (1.3.13)$$

eq. (1.3.12) gives

$$\frac{d\text{pH}}{dx} = \frac{1}{\ln 10} \left[\frac{1}{x} - \frac{1}{c - x} (-1) \right] = \frac{c}{\ln 10 x (c - x)} \quad (1.3.14)$$

It can be determined from this expression that a buffer is more effective, if its first derivative is in its minimum, i.e. when its reciprocal value, named buffer capacity (see above), has a maximum value. To find when the quotient $d\text{pH}/dx$ has its minimum, the second derivative of eq. (1.3.12) should be derived:

$$\frac{d^2\text{pH}}{dx^2} = \frac{c(2x-c)}{\ln 10 x^2 (c-x)^2} \quad (1.3.15)$$

According to eq. (1.3.15), the second derivative is equal to 0 when $x = \frac{c}{2}$, hence, when the added base neutralizes half of the weak acid. If the x value is substituted in the Henderson–Hasselbalch equation, then

$$\text{pH} = \text{p}K_c + \frac{1}{\ln 10} \ln \frac{2c}{2c} = \text{p}K_c \quad (1.3.16)$$

1.3.2 Buffers used in electrophoresis

In electrophoresis, acidic, neutral, or alkaline buffers are used (Table 1.3.1). The alkaline buffers are most widespread because the most proteins and all nucleic acids are acidic.

Table 1.3.1: Buffers used in electrophoresis.

Buffer and pH	Concentrations	Preparation
Acidic buffers		
Acetate buffer pH = 5.3, 10x	0.60 mol/L sodium acetate CH ₃ COOH	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.
Neutral buffers		
TRIS-citrate-EDTA buffer pH = 7.2, 10x	0.25 mol/L TRIS Citric acid 0.01 mol/L Na ₂ EDTA	Adjust 30.29 g TRIS with 2 mol/L citric acid to pH = 7.2 and add 3.72 g Na ₂ EDTA · H ₂ O. Add deionized water to 1,000.0 mL.
Hydrogen phosphate buffer pH = 7.3, 10x	0.0034 mol/L KH ₂ PO ₄ 0.0071 mol/L Na ₂ HPO ₄	Mix 1.36 g/L KH ₂ PO ₄ and 1.78 g/L Na ₂ HPO 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 ₂ PO ₄ 0.01 mol/L Na ₂ EDTA	42.40 g TRIS 49.69 g Na ₂ HPO ₄ 3.72 g Na ₂ EDTA · H ₂ O 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 1.3.1 (continued)

Buffer and pH	Concentrations	Preparation
TRIS-borate-EDTA buffer pH = 8.3, 10x	0.89 mol/L TRIS 0.89 mol/L boric acid 0.02 mol/L Na ₂ EDTA	107.81 g TRIS 55.03 g boric acid 7.44 g Na ₂ EDTA · H ₂ O 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 ₂ EDTA	68.32 g TRIS 97.86 g taurine 0.74 g Na ₂ EDTA · H ₂ O 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	Solve 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-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.

We proved that the TRIS-borate buffer contains a complex compound formed by a condensation reaction between boric acid and TRIS [2]. The complex compound, which we called *TRIS-boric acid*, has a zwitterionic structure. It dissociates a hydrogen ion and a *TRIS-borate* ion whose mobility was calculated [3]. The existence of TRIS-boric acid in the TRIS-borate buffer was reproved 30 years later by a large scientific group in Paris [4].

The formation of TRIS-boric acid and the dissociation of its protonated amino group take place according to the following scheme (Figure 1.3.2)

1.3.3 Biological buffers

The major intracellular buffer is the hydrogen phosphate buffer ($\text{HPO}_4^{2-}/\text{H}_2\text{PO}_4^-$), often incorrectly referred to as phosphate buffer. Its maximum buffer capacity is at pH = 6.7. The fluids around the cells of living organisms have also constant pH. To

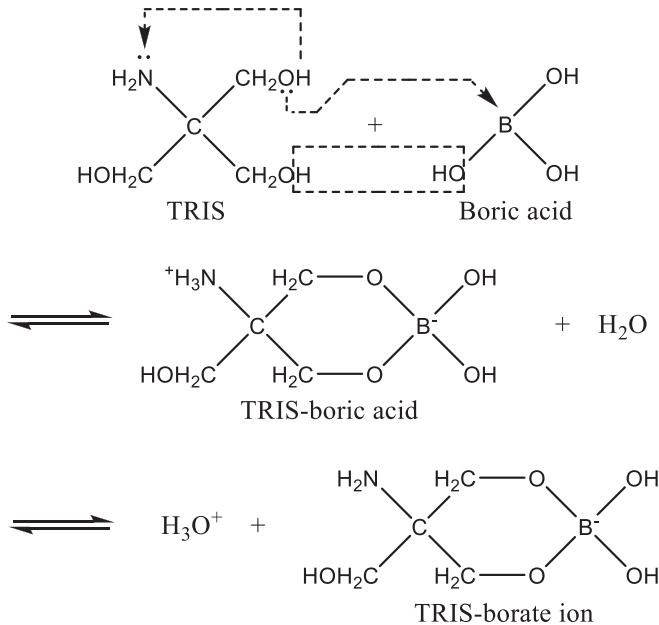


Figure 1.3.2: Formation and dissociation of TRIS-boric acid.

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 [5–7]. Most of Good buffers are solutions of zwitterionic compounds.

The specifications of biological buffers used in the electrophoresis techniques are given in Table 1.3.2. Their pK_a values are temperature and concentration dependent.

Table 1.3.2: Biological buffers used in electrophoresis.

Buffer	Full compound name	Chemical formulas	M_r	pK_a at 25 °C	Buffering range
MES	2-(<i>N</i> -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

Table 1.3.2 (continued)

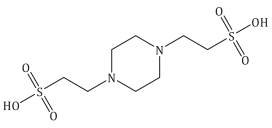
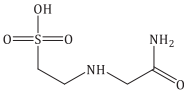
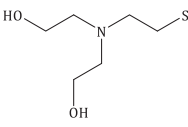
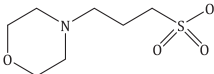
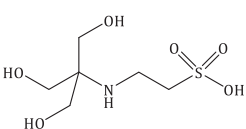
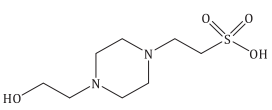
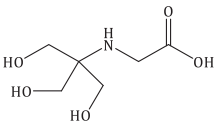
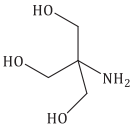
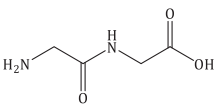
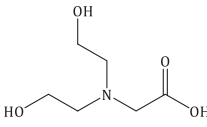
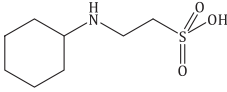
Buffer	Full compound name	Chemical formulas	M_r	pK_a at 25 °C	Buffering range
PIPES	1,4-Piperazinediethanesulfonic acid		302.37	6.76	6.1–7.5
ACES	2-(Carbamoylmethylamino)ethanesulfonic acid		182.20	6.78	6.1–7.5
BES	<i>N,N</i> -Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid		213.25	7.09	6.4–7.8
MOPS	3-Morpholinopropane-1-sulfonic 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
TRICINE	<i>N</i> -(2-Hydroxy-1,1-bis-(hydroxymethyl)ethyl)glycine		179.17	8.05	7.4–8.8
TRIS	2-Amino-2-hydroxymethylpropane-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

Table 1.3.2 (continued)

Buffer	Full compound name	Chemical formulas	M_r	pK_a at 25 °C	Buffering range
BICINE	2-(Bis(2-hydroxyethyl)-amino) acetic acid		163.17	8.26	7.6–9.0
CHES	2-(Cyclohexylamino)-ethanesulfonic acid		207.29	9.49	8.8–10.2

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1.4 Polyions are moving in electric field

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The mobilities play important role in electrophoresis of proteins and nucleic acids.

1.4.1 Ionic and polyionic mobility

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

$$F_e = F_f \quad (1.4.1)$$

It is known that

$$F_e = QE = z_\infty eE \quad (1.4.2)$$

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

$$F_f = f v_\infty \quad (1.4.3)$$

where f is the friction coefficient (in Js/m²), and v_∞ is the velocity of a charged particle (in m/s) in an infinitely dilute solution. For spherical particles, according to Stokes' law,

$$f = 6\pi\eta r \quad (1.4.4)$$

where η is the dynamic viscosity of the solvent (in Pa s), and r is the radius of the charged particle (in m). From eqs. (1.4.1)–(1.4.4), the equation

<https://doi.org/10.1515/9783110761641-005>

$$z_{\infty}e = 6\pi\eta r \frac{v_{\infty}}{E} \quad (1.4.5)$$

is obtained.

It is known also that

$$\frac{v_{\infty}}{E} = \mu_{\infty} \quad (1.4.6)$$

where μ_{∞} is the absolute mobility of the particle, in $\text{m}^2/(\text{s V})$, i.e. in an infinitely dilute solution. Then, it follows in from eqs. (1.4.5) and (1.4.6) that

$$\mu_{\infty} = \frac{z_{\infty}e}{6\pi\eta r} \quad (1.4.7)$$

In the reality, a charged particle moves together with a part of its ionic atmosphere. Therefore, the electrokinetic potential ζ , measured on the charged particle, 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 counter-ions), and the diffuse part of its ionic atmosphere. When the ionic strength is increased, the mobility of the charged particle decreases:

$$\mu = \frac{v}{E} = \frac{ze}{6\pi\eta r} \quad (1.4.8)$$

At the isoelectric point, the electric charge of a protein and its mobility are equal to zero.

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

1.4.1.1 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 is the total electrolyte concentration, in mol/dm^3 [1, 2]:

$$\mu' = \alpha\mu \quad (1.4.9)$$

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

1.4.2 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.

1.4.2.1 Equation of Smoluchowski

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

$$\mu = \frac{\zeta \varepsilon}{\eta} \quad (1.4.10)$$

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 ($8.854\,187\,817 \times 10^{-12}$ F/m); and η is the dynamic viscosity of the solution (in Pa s).

1.4.2.2 Equation of Hückel

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

$$\mu = \frac{\mu_\infty}{1 + \kappa a} = \frac{\zeta \varepsilon}{1.5 \eta} \quad (1.4.11)$$

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

1.4.2.3 Henry's function

Comparing the equations of Smoluchowski (1.4.10) and Hückel (1.4.11), we could 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 obtained that

$$\mu = \frac{\zeta \varepsilon}{1.5 \eta} f(\kappa a) \quad (1.4.12)$$

where

$$f(\kappa a) = 1 + \frac{(\kappa r)^2}{16} - \frac{5(\kappa r)^3}{48} - \frac{(\kappa r)^4}{96} + \frac{(\kappa r)^5}{96} - \frac{11}{96} e^{\kappa r} \int_{\infty}^{\kappa r} \frac{e^{-t}}{t} dt \quad (1.4.13)$$

According to Henry's function, the mobility of an ion or polyion depends not only on the ionic strength of the solution, which takes place in the parameter κ , but also on the radius of the charged particle a . When $\kappa a \rightarrow \infty$, then $f(\kappa a) \rightarrow 1.5$ and eq. (1.4.12) is transformed into the equation of Smoluchowski (1.4.10); when $\kappa a \rightarrow 0$, then $f(\kappa a) \rightarrow 1.0$ and eq. (1.4.12) gives the Hückel eq. (1.4.11).

1.4.2.4 New expression of Henry's function

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

$$a = r(1 + \kappa r) \quad (1.4.14)$$

where $0 \leq \kappa r \leq 1$. In accordance with this, we established that every ion and its ionic atmosphere are characterized by four electric potentials: φ_0 , φ_r , ζ , and φ_a . Using the theories of Gouy [9], Chapman [10], and Debye-Hückel [11], we proved that the geometric potential

$$\varphi_0 = \frac{ze}{4\eta \varepsilon r} \quad (1.4.15)$$

the potential of the geometric ion and its ionic atmosphere

$$\varphi_r = \frac{ze}{4\eta \varepsilon a} = \frac{ze}{4\eta \varepsilon r(1 + \kappa r)} = \frac{\varphi_0}{1 + \kappa r} \quad (1.4.16)$$

the electrokinetic potential

$$\zeta = \frac{ze}{4\eta \varepsilon r(1 + \kappa a)} = \frac{\varphi_0}{1 + \kappa a} = \varphi_r \frac{1 + \kappa r}{1 + \kappa a} \quad (1.4.17)$$

and the potential of the electrokinetic ion and its ionic atmosphere

$$\varphi_a = \frac{ze \exp[-\kappa(a - r)]}{4\pi \varepsilon a(1 + \kappa r)} = \frac{\varphi_r}{1 + \kappa a} = \frac{\zeta}{1 + \kappa r} = \zeta \frac{r}{a} \quad (1.4.18)$$

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 $f(\kappa r)$ [12]:

$$f(\kappa r) = \frac{1 + \kappa a}{1 + \kappa r} = \frac{1 + \kappa r + (\kappa r)^2}{1 + \kappa r} \quad (1.4.19)$$

Now eq. (1.4.12) could be converted into

$$\mu = \frac{\zeta \varepsilon}{1.5 \eta} f(\kappa r) = \frac{\zeta \varepsilon}{1.5 \eta} \frac{1 + \kappa a}{1 + \kappa r} \quad (1.4.20)$$

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

$$\mu = \frac{\zeta \varepsilon}{1.5 \eta} \quad (1.4.21)$$

i.e., the equation of Hückel occurs; and when $\kappa r \rightarrow 1$, then $f(\kappa r) \rightarrow 1.5$, and

$$\mu = \frac{\zeta \varepsilon}{\eta} \quad (1.4.22)$$

i.e., the equation of Smoluchowski occurs.

The graphics of $f(\kappa r)$ as a function of κr and of its logarithmic value are given on Figure 1.4.1.

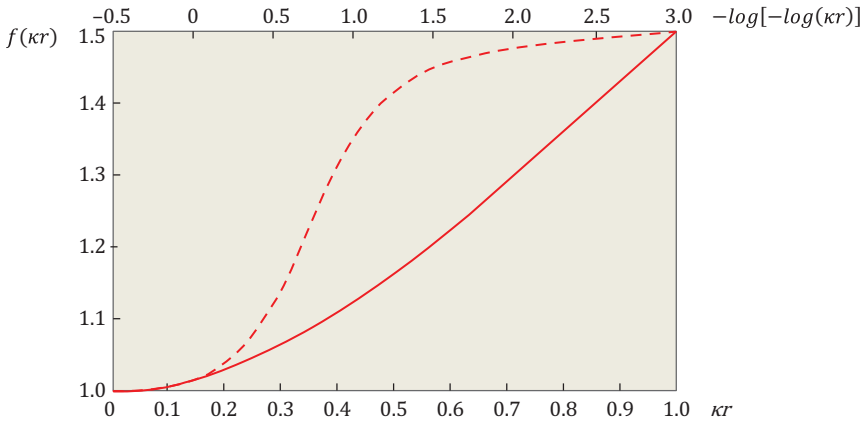


Figure 1.4.1: The dependence of function $f(\kappa r)$ on κr (solid line) and on its logarithmic value $-\log[-\log(\kappa r)]$ (dashed line).

1.4.2.5 Equation of Onsager

Onsager [13, 14] proposed that the polyionic 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 toward 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 1.4.2).

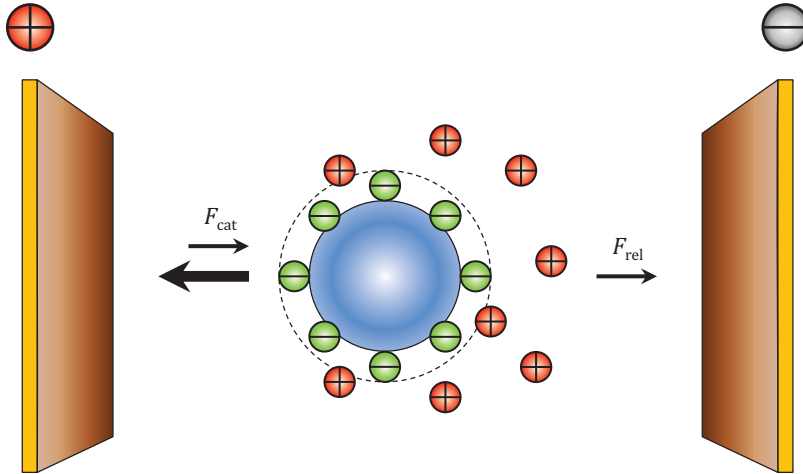


Figure 1.4.2: Influence of the cataphoretic strength (F_{cat}) and relaxation strength (F_{rel}) on the polyionic mobility.

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

$$\mu = \mu_{\infty} - (\mu_{cat} + \mu_{rel}) = \mu_{\infty} - z s I^{1/2} \quad (1.4.23)$$

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).

1.4.2.6 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 [15] can be applied in a good agreement with the experiment:

$$\mu = \mu_{\infty} - \frac{s I^{1/2}}{1 + \kappa a} \quad (1.4.24)$$

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 could have different values, depending on the experimental conditions.

1.4.2.7 Parametric equation

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

$$\mu = \mu_{\infty} - p \quad (1.4.25)$$

where p is the parameter of the ionic (polyionic) mobility [16] expressed as

$$p = \frac{ze\kappa}{6\pi\eta} \quad (1.4.26)$$

For weak electrolytes, the above equation should be transformed into

$$\mu' = \alpha(\mu_{\infty} - p) \quad (1.4.27)$$

1.4.2.8 Quadratic equation

If eq. (1.4.14), which represents our concept of existing of two radii of a charged particle (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:

$$\mu = \mu_{\infty} - \frac{sI^{1/2}}{1 + \kappa r + \kappa^2 r^2} \quad (1.4.28)$$

The experimental values of the ionic mobilities can be well expressed by the parametric and 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.

1.4.3 Mobilities of ions used in electrophoretic methods

In Table 1.4.1, the absolute mobilities of ions, which are used in the electrophoretic methods, are listed [17–19].

Table 1.4.1: Absolute ionic mobilities, in $\text{m}^2/(\text{s V})$, of ions, at two temperatures.


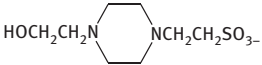
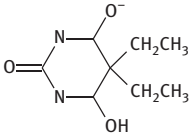
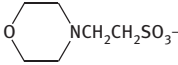
Ions	Chemical formulas	$\mu_{\infty} \times 10^9 (0 \text{ }^{\circ}\text{C})$	$\mu_{\infty} \times 10^9 (25 \text{ }^{\circ}\text{C})$
Hydronium ion	H_3O^+	248.74	362.39
Potassium ion	K^+	39.57	76.18
Sodium ion	Na^+	27.40	51.95
Imidazolium ion		26.65	43.74
Alaninium ion	$^+\text{H}_3\text{NCH}(\text{CH}_3)\text{COOH}$	17.46	32.67
1/2 Calcium ion	Ca^{2+}	15.55	30.84
β -Alaninium ion	$^+\text{H}_3\text{NCH}_2\text{CH}_2\text{COOH}$	17.22	29.30
Ammediolium ion	$\text{HOCH}_2(^+\text{H}_3\text{N})\text{C}(\text{CH}_3)\text{CH}_2\text{OH}$	13.37	27.86
TRIS ion	$(\text{HOCH}_2)_3\text{CNH}_3^+$	12.75	27.86
BISTRIS ion	$(\text{HOCH}_2\text{CH}_2)_2\text{NH}^+\text{C}(\text{CH}_2\text{OH})_3$	12.00	24.01
HEPESate ion		-9.52	-22.85
TRICINEate ion	$(\text{HOCH}_2)_3\text{CNHCH}_2\text{COO}^-$	-10.51	-24.01
Barbitalate (veronalate) ion		-13.99	-24.97
TESate ion	$(\text{HOCH}_2)_3\text{NHCH}_2\text{CH}_2\text{SO}_3^-$	-12.75	-26.90
ACESate ion	$\text{H}_2\text{NCOCH}_2\text{NHCH}_2\text{CH}_2\text{SO}_3^-$	-14.24	-27.86
MESate ion		-13.49	-28.19
1/2 Hydrogen phosphate ion	HPO_4^{2-}	-14.15	-29.71
Glycylglycinate ion	$\text{H}_2\text{NCH}_2\text{CONHCH}_2\text{COO}^-$	-16.97	-30.26
4-Aminobutirate ion (GABAate ion)	$\text{H}_2\text{NCH}_2\text{CH}_2\text{CH}_2\text{COO}^-$	-16.22	-32.19
Borate ion	$\text{B}(\text{OH})_4^-$	-18.21	-33.63
Taurinate ion	$\text{H}_2\text{NCH}_2\text{CH}_2\text{SO}_3^-$	-17.71	-34.11
Dihydrogen phosphate ion	H_2PO_4^-	-17.46	-34.28

Table 1.4.1 (continued)

ions	Chemical formulas	$\mu_{\infty} \times 10^9 (0 \text{ }^{\circ}\text{C})$	$\mu_{\infty} \times 10^9 (25 \text{ }^{\circ}\text{C})$
BICINEate ion	$(\text{HOCH}_2\text{CH}_2)_2\text{NCH}_2\text{COO}^-$	-19.70	-36.04
Glycinate ion	$\text{H}_2\text{NCH}_2\text{COO}^-$	-20.94	-38.45
Lactate ion	$\text{CH}_3\text{CHOHCOO}^-$	-18.95	-40.22
1/2 Sulfate ion	SO_4^{2-}	-21.25	-41.47
Acetate ion	CH_3COO^-	-22.18	-42.36
Formate ion	HCOO^-	-30.88	-56.60
Chloride ion	Cl^-	-42.95	-79.09
Hydroxide ion	OH^-	-108.83	-204.92

1.4.3.1 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 [20]. Different ions have different shapes but in order to simplify the equations we suppose that all ions are spherical.

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 , *etc.* 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 ionic spheres with the formula $V = \frac{4}{3}\pi r^3$ and simplifying the equation, the following expression is obtained:

$$r_x^3 = r_a^3 + r_b^3 \quad (1.4.29)$$

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

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 [21]. Transforming this equilibrium, we obtain the equation

$$r = \frac{ze}{6\pi\eta\mu_{\infty}} \text{ [m]} \quad (1.4.30)$$

where z is the ionic electrovalency, e is the electron charge ($1.6021892 \times 10^{-19}$ C), η is the dynamic viscosity of the water, and μ_{∞} is the absolute ionic mobility, *i.e.* the mobility in an extremely diluted solution. Hence, eq. (1.4.29) could be represented as

$$\left(\frac{z_x}{\mu_{\infty x}}\right)^3 = \left(\frac{z_a}{\mu_{\infty a}}\right)^3 + \left(\frac{z_b}{\mu_{\infty b}}\right)^3 \quad (1.4.31)$$

which gives that

$$\mu_{\infty x} = z_x \left[\left(\frac{z_a}{\mu_{\infty a}}\right)^3 + \left(\frac{z_b}{\mu_{\infty b}}\right)^3 \right]^{-1/3} [\text{m}^2/(\text{s V})] \quad (1.4.32)$$

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. (1.4.32) could be transformed into

$$\mu_{\infty x} = 2^{-1/3} \mu_{\infty a} [\text{m}^2/(\text{s V})] \quad (1.4.33)$$

Eqs. (1.4.32) and (1.4.33) could be applied for extremely diluted solutions. However, every ion is surrounded by counter-ionic atmosphere whose radius

$$\kappa^{-1} = 1.9885 \times 10^{-12} (\epsilon_r T / I)^{1/2} [\text{m}] \quad (1.4.34)$$

where ϵ_r is the relative electric permittivity, T is the thermodynamic temperature, and I is the ionic strength of the solution [22]. This atmosphere diminishes the absolute mobility of an ion $(1 + \kappa r)$ times, *i.e.*

$$\mu = \frac{\mu_{\infty}}{1 + \kappa r} [\text{m}^2/(\text{s V})] \quad (1.4.35)$$

From eqs. (1.4.30) and (1.4.35) follows that

$$\mu_{\infty} = \frac{\mu}{2} \pm \left[\frac{\mu}{2} \left(\frac{\mu}{2} + \frac{ze\kappa}{3\pi\eta} \right) \right]^{1/2} [\text{m}^2/(\text{s V})] \quad (1.4.36)$$

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. (1.4.36) could be transformed into

$$\mu_{\infty} = \mu + \frac{ze\kappa}{6\pi\eta} [\text{m}/(\text{s V})] \quad (1.4.37)$$

We gave evidences for the existence of a complex ion in TRIS-borate buffers, which we called TRIS-borate ion [23]. TRIS-borate ion $[(\text{HO})_2\text{B}^-(\text{OCH}_2)_2\text{C}(\text{CH}_2\text{OH})\text{NH}_2, \text{TB}^-]$ could be considered as composed of a TRIS ion $[(\text{HOCH}_2)_3\text{CN}^+\text{H}_3, \text{HT}^+]$ and a borate ion $[\text{B}(\text{OH})_4^-, \text{B}^-]$ when a proton and two water molecules are split off.

Let us calculate the mobility of TRIS-borate ion at 25 °C and $I = 0.01$ mol/L [24]. It is known that at 25 °C (298.15 K) and $I = 0.01$ mol/L $\mu_{\text{HT}^+} = 24.06 \times 10^{-9}$ and $\mu_{\text{B}^-} = -29.84 \times 10^{-9}$ m²/(s V) [4]. Taking into account that at this temperature $\epsilon_r = 78.54$ and

$\eta = 0.8904 \times 10^{-3}$ Pa s, we could calculate that $z\epsilon\kappa/(6\pi\eta) = (\pm)3.14 \times 10^{-9}$ m²/(s V). Hence, it follows from eqs. (1.4.37) and (1.4.32) that at 25 °C $\mu_{\infty\text{HT}^+} = 27.20 \times 10^{-9}$ m²/(s V), $\mu_{\infty\text{B}^-} = -32.98 \times 10^{-9}$ m²/(s V), and $\mu_{\infty\text{HB}^-} = -23.45 \times 10^{-9}$ m²/(s V), and when $I=0.01$ mol/L

$$\mu_{\text{TB}^-} = -23.45 \times 10^{-9} + 3.14 \times 10^{-9} = -20.31 \times 10^{-9} \text{ m}^2/(\text{s V})$$

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1.5 Electrophoresis is carried out in different solid media

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

Different solid media were used for electrophoresis. Nowadays, cellulose acetate and first of all polyacrylamide (PAA) and agarose gels are used.

1.5.1 Cellulose acetate

Cellulose acetate (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 fibrous molecules are formed, which are linked each with other by hydrogen bonds. CA 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 were used first by Joachim Kohn [1]. They do not counteract the diffusion, as a result of which the resolution of CA electrophoresis is of low grade.

1.5.2 Agarose gel

Agarose is one of the two main components of agar, which is extracted from red seaweed [2]. It consists of linear polysaccharide chains, which are built of approximately 400 residues of agarobiose. Agarobiose contains an α -(1 \rightarrow 3)-linked β -D-galactopyranose and a β -(1 \rightarrow 4)-linked 3,6-anhydro-L- α -galactopyranose [3]. The 3,6-anhydro-L- α -galactopyranose represents L-galactose that possess an anhydrobridge

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between the third and sixth positions. Some D- and L-galactose units can be methylated; ionizable groups are also found in small amounts [4] (Figure 1.5.1).

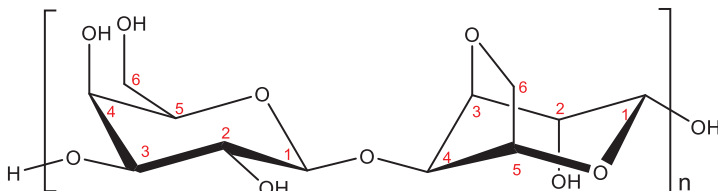


Figure 1.5.1: Structure of the residue of an agarobiose monomer.

Each polysaccharide chain contains about 800 galactose residues, and has M_r between 100,000 and 200,000 [5]. Many chains form helical fibers, which build supercoiled structures of a radius of 20–30 nm [6]. The fibers are quasi-rigid and have different length that depends on the agarose concentration [7]. They form pores of different diameter. This structure is held together by hydrogen bonds and can be disrupted by heating that melts the gel to give a liquid.

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 easily 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 [8]. 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 were introduced in the electrophoresis by Hjerten [9]. They are optimal for electrophoresis of proteins with M_r larger than 200,000 [10]. 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 are suitable for electrophoresis of 0.2–1 kb DNA fragments.

The agarose gels are usually cast on support films. The support films are made of 0.18–0.20 mm polyester film that binds to the melt agarose. The supported gels can be cut in different sizes, 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 [11].

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. The low-melting agarose is chemically modified agarose that possess hydroxyethyl

groups in its polysaccharide chains and fewer sulfate groups [12]. It has a lower resolution and lower mechanical strength than the standard agarose [13]. The low-melting agarose allows the 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 low-concentrated agarose gel are needed. However, when the agarose concentration falls, the gels degrade easily. The mechanical strength of an agarose gel is expressed in g/cm^2 , i.e. 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, an agarose type 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.

Charge free agarose. As mentioned, the agarose gels contain charged groups, which cause electroosmosis. To prevent this phenomenon, chemically modified agarose was invented that has no acidic groups [14, 15].

Electroosmosis. Electroosmosis is referred to as movement of the solvent (water) when located in an electric field [16]. It is caused in the following way: Residual chemical groups of sulfuric, pyruvic, or carbonic acid on an agarose molecule split hydrogen ions in neutral and alkaline buffers, and transform themselves into the negatively charged sulfate, pyruvate, or carboxylate groups. The negatively charged agarose gel does not migrate to the anode (stationary phase); however, its positively charged counter-ions migrate, together with their own hydration envelopes, to the cathode (mobile phase). As a result, the cathodic gel pole binds water (hydrates) while the anodic gel pole loses water (dehydrates).

1.5.3 Polyacrylamide gel

The **polyacrylamide** (PAA, poly(2-propenamide)) gel, introduced in 1959 by Raymond and Weintraub [17], is a copolymer of the monomer acrylamide and the comonomer bisacrylamide. It is the best medium for electrophoretic separations [18, 19]. The PAA gel is relatively nontoxic, because it contains minute residual amounts of acrylamide after its production [20].

The PAA gel has some important advantages in comparison with other gels: it is hydrophilic and electrical neutral; it does not show electroosmosis, since it carries almost no electric charges; it is transparent to light of wavelengths above 250 nm; it is chemically inert and thermo-stable; it can be prepared with desired pore size, if the concentration of acrylamide and bisacrylamide is changed; and it does not interact with polyions to be resolved, or with dyes.

1.5.3.1 Acrylamide

Acrylamide (prop-2-enamide, $M_r = 71.08$) (Figure 1.5.5) crystallizes into white crystals, which are soluble in water, methanol, glycerol, acetone, trichloromethane, and other solvents, and has a boiling point of 84.5 ± 0.3 °C. It is highly neurotoxic and accumulates in the body. Acrylamide polymerizes spontaneously during prolonged storage.

1.5.3.2 Bisacrylamide

Bisacrylamide (BIS), (*N,N'*-methylene-bis-acrylamide, *N,N'*-methylene-diacrylamide, $M_r = 154.17$) (Figure 1.5.5) is a white less toxic cross-linker, also soluble in water. It melts at 185 °C and also polymerizes spontaneously during prolonged storage.

1.5.3.3 The magnitudes T and C

Acrylamide (the monomer) and BIS (the comonomer) are often referred to as *monomers* and their solution as *monomeric solution*. Hjerten [21] improved formulas for the *total monomeric concentration* T and the *cross-linking degree* C of the PAA gel, which we have transformed into:

$$T = a + b \text{ (g/dL)} \quad (1.5.1)$$

and

$$C = \frac{b}{a + b} \text{ (dimensionless)} \quad (1.5.2)$$

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

The total monomeric concentration T and the cross-linking C determine the pore size.

1.5.3.4 Alternative cross-linkers

The BIS cross-linked PAA gels have high optical transparency, good mechanical properties at low concentrations, are electric neutral, and may possess variable porosities. However, their relatively low stability in alkaline solutions, and their reduced optical transparency at high concentrations limit their application. Therefore, besides BIS, alternative cross-linkers are developed: DATD [22], BAP (PDA) [23], DHEBA [24], BAC [25], AcrylAide (*FMC*, Rockland, Me.), *etc.* (Table 1.5.1).

Some of the cross-linkers are used for liquefaction of PAA gel to free the separated polyions after electrophoresis. If a gel contains DHEBA [24], the liquefaction is

carried out by oxidation with periodic acid; if it contains BAC [25], the BAC disulfide bonds should be split with thiols.

Table 1.5.1: Some alternative cross-linkers.

Cross-linkers	Chemical formulas	M_r
DATD (<i>N,N'</i> -diallyltartardiamide)		228.25
BAP (1,4-bis-acryloyl-piperazine), or PDA (piperazine-diacrylamide)		194.23
DHEBA (<i>N,N'</i> -(1,2-dihydroxyethylen)- bis-acrylamide)		200.20
BAC (<i>N,N'</i> -bis-acryloylcystamine)		260.38

Irrespective of this, the alternative cross-linkers have many disadvantages. Therefore, BIS is still the most used comonomer.

1.5.3.5 Initiator-catalyst systems

The copolymerization of acrylamide and BIS requires free radicals, which are generated by initiator-catalyst systems [26]. Among them, the APS-TMEDA system is optimal (Figure 1.5.2).

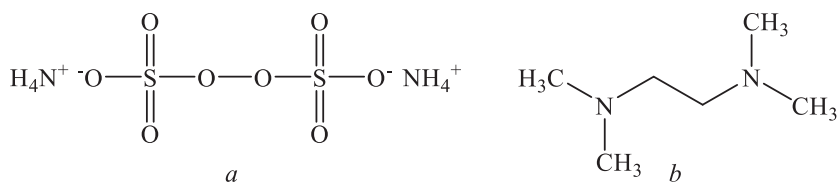


Figure 1.5.2: Chemical formulas of APS (*a*) and TMEDA (*b*).

The *initiator* APS (ammonium peroxydisulfate, ammonium persulfate, $M_r = 228.18$) dissociates in solution giving two ammonium cations and a peroxydisulfate anion. The peroxydisulfate anion forms two sulfate free radicals [27] (Figure 1.5.3):

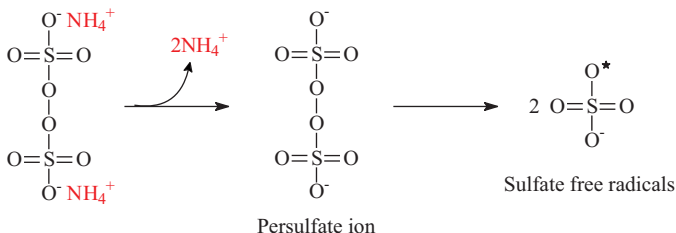


Figure 1.5.3: Building of sulfate free radicals from APS.

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. We propose that TMEDA concentration should remain constant, while the APS concentration should be changed, according to the formula:

$$c_{\text{APS}} = 0.32 (\text{g/dL})^2 / T [\text{g/dL}] \quad (1.5.3)$$

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' -tetramethylethylenediamine, TEMED, 1,2-bis(dimethylamino)-ethane, $M_r = 116.21$] is the most common 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.

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

Riboflavin (riboflavin-5'-phosphate) also generates free radicals in a photochemical reaction, often in combination with TMEDA. When irradiated with blue to ultraviolet light in the presence of oxygen, it converts into a leuco (colorless) form, which initiates the copolymerization (Figure 1.5.4). This is referred to as *photochemical copolymerization*.

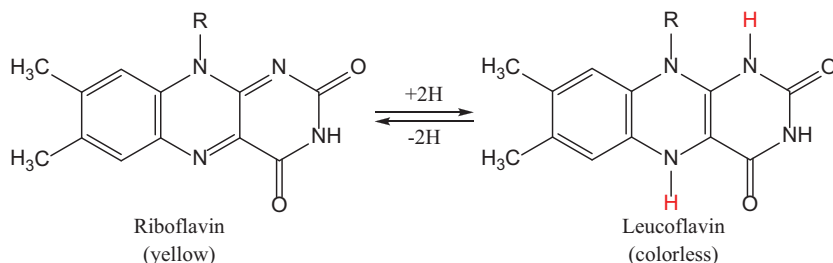


Figure 1.5.4: Riboflavin and its leuco form.

1.5.3.6 Copolymerization of acrylamide and BIS

The copolymerization of acrylamide and BIS proceeds in two steps. At first, the sulfate radicals bind to the alkene (the vinyl groups of acrylamide) molecules forming sulfate ester radicals, which take part in the polymerization producing long polymeric chains [28]. Then, the polymeric chains are cross-linked by BIS molecules. So, the spatial structure of the PAA gel is formed, which can be compared to a sponge [29, 30] (Figure 1.5.5).

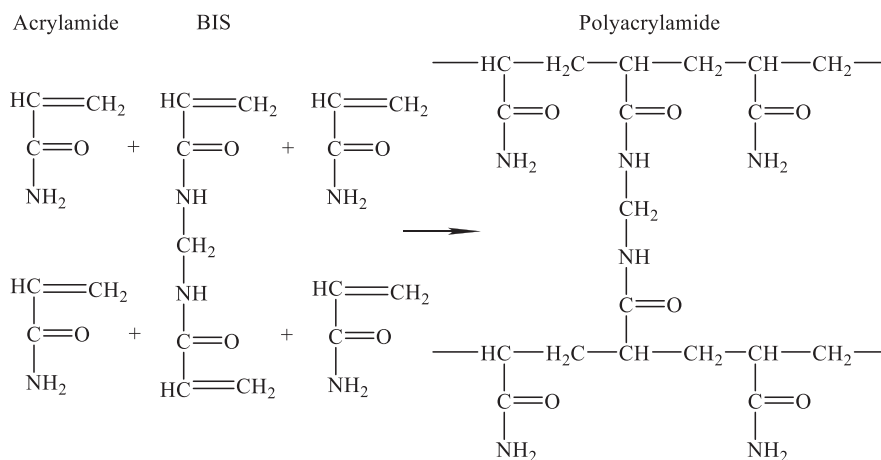


Figure 1.5.5: Copolymerization of acrylamide and BIS. A little part of the neutral carboxamide groups in the polyacrylamide gel can hydrolyze giving negatively charged carboxylate groups.

The viscosity, elasticity, and strength of a PAA gel depend on the ratio between the length of PAA chains and the frequency of the cross-linkages, i.e. 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 PAA 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 PAA chains.

When T is increased at fixed C , the number of PAA chains increases and the pore size decreases in a nearly linear relationship. 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 $C = 0.05$. 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.

For most proteins and SDS-proteins the value of C should be about 0.0026 (37.5:1); for most native DNA and RNA value of C should be about 0.033 (29:1); for most denaturing DNA and RNA the value of C should be about 0.05 (19:1 acrylamide/BIS). Gel with $T = 4$ g/dL and $C = 0.03$ have no sieving properties. It is important that total acrylamide is polymerized. If this is not the case, the nonpolymerized acrylamide binds covalently to the polyions during electrophoresis [31].

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.

Inhibitors of the copolymerization are the oxygen and peroxides, the last 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.

Similar to the PAA gel is the gel, formed by the monomer (*N*-acryloyl-tris(hydroxymethyl)aminomethane [32, 33].

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

1.5.3.7 Homogeneous polyacrylamide gels

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

1.5.3.8 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 [34]: 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, could be placed on the gel; the staining and destaining procedures are quickly performed.

The thin and ultrathin horizontal gels [35] on support films can be cut into strips and can be stored dry. However, they are not suitable for electroblotting. Alternatives for this purpose are the fabric-supported gels [36]. The fabric is commonly made from polyester fibers, which form pores with a diameter of 10–60 μm [37].

1.5.3.9 Gradient polyacrylamide gels

The gradient PAA gels (pore gradient PAA gels) were introduced by Margolis and Kenrick [38]. Their concentration varies continuously from one gel end to the other gel end, resulting in a continuous changing of the pore diameters [39, 40]. The T -value of the gradient gels is usually of 4–28 g/dL.

There are two types of gradient gels: linear and exponential. In the gradient gels with linearly increased total concentration T , at constant cross-linking degree C , the average pore radius decreases exponentially. The maximum pore radius $r_{\text{max}} = a - T^{-b}$, where a and b are empirical constants [41].

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1.6 General theory of electrophoresis

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

$$\mu = \frac{\zeta \varepsilon}{\eta} = \frac{\zeta \varepsilon_r \varepsilon_0}{\eta} \quad (1.6.1)$$

where μ (in $\text{m}^2/(\text{s V})$) 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 the (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 ($8.854\ 187\ 818 \times 10^{-12}$ 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

$$v = \mu E \quad (1.6.2)$$

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].

If direct voltage is applied between two electrodes, the electric field

$$E = \frac{U}{l} = \frac{J}{\gamma} \quad (1.6.3)$$

where U is the voltage (in V), l is the distance between the electrodes (in m), J is the electric current density (in A/m^2), and γ is the specific conductivity of the buffer (in S/m). The distance remains unchanged during electrophoresis; therefore, the strength of the electric field depends only on the voltage.

1.6.1 What is the polyionic mobility depending on?

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

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

$$A = R + \kappa r^2 \quad (1.6.4)$$

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 (in m) is the geometric radius of the counter-ion.

Besides, we [7] have proved that the polyionic mobility

$$\mu = \frac{\zeta \varepsilon}{1.5 \eta} f(\kappa r) \quad (1.6.5)$$

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

$$f(\kappa r) = \frac{1 + \kappa a}{1 + \kappa r} = \frac{1 + \kappa r + (\kappa r)^2}{1 + \kappa r} \quad (1.6.6)$$

where a and r are the electrokinetic and geometric radii of the counter-ion, respectively. The function $f(\kappa r)$ is similar to the Henry's function $f(\kappa a)$ [8, 9], however, in contrast to the Henry's function $f(\kappa a)$, it has a real and definite value.

If we introduce eq. (1.6.6) in (1.6.5), the following expression is obtained:

$$\mu = \frac{\zeta \varepsilon}{1.5 \eta} \frac{1 + \kappa a}{1 + \kappa r} \quad (1.6.7)$$

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 temperature.

Buffer pH. A buffer keeps the pH value, i.e. the proton concentration of a solution, unchanged. For strong electrolytes, which are completely ionized, the pH value 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

$$\mu_{i(\text{pi})}' = \alpha \mu_{i(\text{pi})} \quad (1.6.8)$$

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

Ionic strength of buffer. The polyionic mobility decreases when the ionic strength of a buffer increases [10]. This can be explained with the concept of the existing of geometric and electrokinetic radius (see 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 1.6.1).

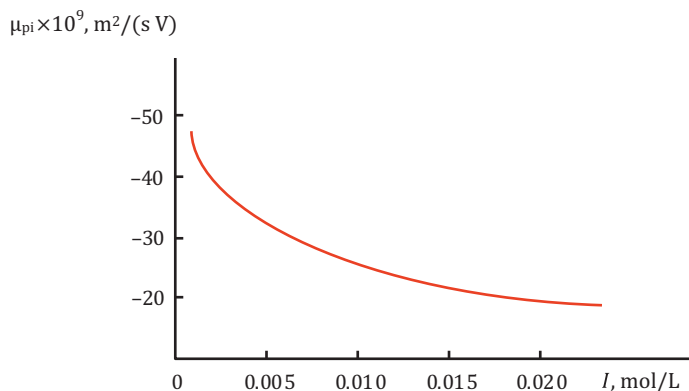


Figure 1.6.1: Dependence of polyionic mobility on buffer ionic strength.

(Di)electric permittivity of 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.

Temperature of 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

$$\eta = Ae^{b/T} \quad (1.6.9)$$

where A and b are empirical constants. Studies indicate that a temperature increase of 1 K increases the ionic mobility of about 3%.

1.6.1.3 Influence of medium

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

1.6.1.4 Electroosmosis

Electroosmosis (electroendosmosis, **electroosmotic flow**, EOF) is an essential component of the electrophoresis techniques. It 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. Electroosmosis was first described in 1809 by the Russian scientist Reuss [11].

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

1.6.2 Ionic boundaries

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

1.6.2.1 Moving boundary

The moving ionic boundary [12–14] across a buffer system is characterized by the magnitude W , which expresses its volume V swept by the passed electric charge Q [15]:

$$W = \frac{V}{Q} \text{ (m}^3/\text{C)} \quad (1.6.10)$$

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

$$v' = W \frac{I}{S} \quad (1.6.11)$$

where I (in A) is the total electric current, and S (in m²) is the cross section.

1.6.2.2 Stationary boundary

The stationary ionic boundary [16] does not move in a buffer system. It can also be obtained in a same buffer if the buffer parts have different pH values [17].

1.6.3 Regulating function

The regulating function of Kohlrausch [18], valid for strong and weak mono- and multivalent electrolytes, can be expressed by the relationship [19]

$$\omega(x) = \sum_i \frac{c_i'(x)z_i}{\mu_i} = \text{const}(x) \quad (1.6.12)$$

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 of the time of the electric current passage. Hence, the composition of the moving boundary is regulated automatically.

1.6.4 Diffusion

With the time, the resolved bands are broadened by diffusion. After a certain time t , a Gaussian concentration profile is observed as the result of diffusion. It may be characterized [20] by the standard deviation σ given by the relationship

$$\sigma = \sqrt{2Dt} \quad [\text{m}] \quad (1.6.13)$$

where D is the *diffusivity* or diffusion coefficient (in m^2/s).

1.6.5 Joule heating

The Joule heating (the electric power) that is contained in the equation

$$P = IU = I^2R \quad [\text{W, watts}] \quad (1.6.14)$$

is also important for the electrophoresis. The heating causes convection currents, diffusional broadening, evaporation, viscosity and pH changes, thermal denaturing of polyions, especially proteins, gel drying, and even fire. The temperature difference between the center and the outer parts of the resolving medium can reach more than 10 °C. This can be diminished by cooling systems, or using dilute buffers and low voltages.

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1.7 Electrophoresis instrumentation

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A modern electrophoresis labor has at its disposal: electrophoretic cell, DC power supply, thermostat, scanner or densitometer, and computer, casting cassettes, gradient maker, blotter, and other devices (Figure 1.7.1).

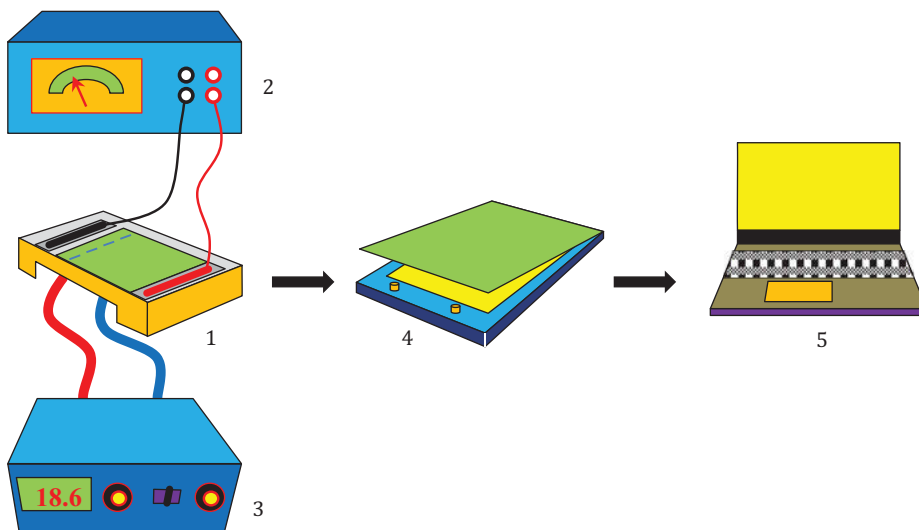


Figure 1.7.1: Apparatuses used for electrophoresis.

1. Electrophoresis cell; 2. power supply; 3. thermostat; 4. scanner; 5. computer.

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, pump, heating plate, magnetic stirrer, magnetic rods, height-adjustable platforms (laboratory boy), shaker, centrifuge, scissors, spatula, microwave oven, dryer or incubator, UV lamp, fan, water pump, paper cutting machine, and more.

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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, and glass. Platinum wires or graphite rods are used as electrodes. For protection, the electric current is automatically stopped in most cases 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 two glass plates separated by spacer strips. The electrophoretic cells for slab gels are preferable if compared with the electrophoretic cells for cylindrical gels, because the slab gels are easier to be analyzed by a densitometer or scanner and are suitable for immunoelectrophoresis and autoradiography. In addition, in slab gels, the separated polyions are faster stained and the slab gels can be dried as evidence.

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

1.7.2 Power supplies

There are different types of power supplies. A power supply suitable for all types of electrophoresis should produce a voltage of 50–5,000 V, and a direct electric current of 5–100 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

The cooling of an electrophoretic cell is carried out by 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.

1.7.4 Scanners and 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 to determine with the naked eye the exact differences between the intensities (concentrations) of the bands or spots. This can be carried out with the aid of densitometers.

In the analytical chemistry, the term **optical density** (OD, dimensionless) is used. It is different from the 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 transmitted radiation, depending on whether the measurement is carried out before or behind the medium.

Today, scanners are mostly used for analyzing the polyionic fractions obtained by electrophoresis. The pherograms are scanned and the scans are “read” using appropriate computer programs.

1.7.5 Gel casting cassettes

A casting cassette is constructed by two glass plates and a U-shaped spacer (Figure 1.7.2), which are held together by brackets.

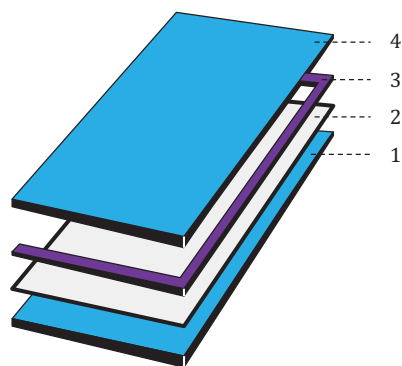


Figure 1.7.2: Assembly of a cassette for casting thin and ultrathin gels.

1. Lower glass plate; 2. support film; 3. U-shaped spacer; 4. upper glass plate.

Gel thickness can be varied by spacers inserted between the glass plates prior to the gel formation. The spacers are made of rubber or silicone. They can be glued to one of the glass plates. The distance between the glass 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 (for example, of Desaga, Heidelberg).

1.7.6 Gradient makers

A simple and widespread gradient maker consists of two communicating tubes: a mixing chamber and a reservoir, located at the same level (Figure 1.7.3).

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 its solution volume created during the twisting of the magnetic bar in the mixing chamber. 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.

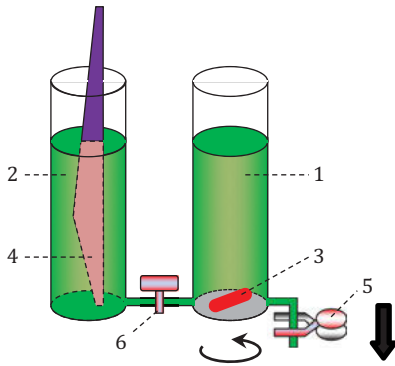


Figure 1.7.3: Gradient maker.

1. Mixing chamber; 2. reservoir; 3. magnetic bar;
4. compensation bar; 5. outlet clamp; 6. pinchcock.

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

1.7.7 Buffer mixers

During electrophoresis, changes in the ionic composition and pH value of the electrode buffers occur. To avoid this, numerous companies have produced different electrode buffer mixers (recirculators). Most recirculators operate using a same principle: a pump sucks buffer from one electrode tank and pumps it out into 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 have proposed a water-driven buffer recirculator [5]. Its construction is presented on Figure 1.7.4.

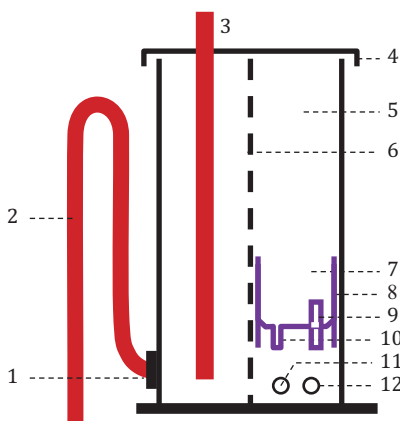


Figure 1.7.4: Scheme of recirculator.

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 water fills up this recirculator through the hose and flows out through its syphon. 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. When it is in the upper part of the recirculator, it feeds the buffer through the outlet tubing into the electrophoretic device.

If the vessels 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 vessel, spills over into the lower vessel and passes into the recirculator float. If the two electrode vessels are on the same level, as in the horizontal electrophoretic devices, the circulating buffer is poured out into one of the vessels, then it passes through tubing into the second vessel and afterwards in the recirculator float.

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

1.7.8 Blotters

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

1.7.9 Equipment for semi-automatic electrophoresis

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

The first semi-automated electrophoresis system was 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. It contains also a blotting unit with graphite electrodes for electrophoretic transfer.

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

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1.8 Classification of electrophoretic methods

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

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 electrode strips soaked with buffer instead of tank buffers; selection of application points, which is important for native electrophoresis and isoelectric focusing (IEF); free access to the gel surface during electrophoresis, *etc.*

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

According to the buffer, all electrophoresis methods can be classified as: zone electrophoresis, isotachophoresis (ITP), and IEF. There are also combinations between these methods.

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1.8.1 Zone electrophoresis

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

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 from each other forming zones (layers) in the two legs of the tube.

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 in turn are focused onto a photographic plate. Today Tiselius electrophoresis has little practical usage.

1.8.1.2 Capillary electrophoresis

The capillary electrophoresis (CE), named also high performance capillary electrophoresis, is carried out in capillaries or micro- and nanofluidic channels [2, 3]. Commonly, CE refers to capillary zone electrophoresis, but capillary gel electrophoresis, capillary IEF, capillary isotachophoresis, and micellar electrokinetic chromatography 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 free-flow electrophoresis (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. Free-flow isotachophoresis and free-flow isoelectric focusing are also known.

1.8.1.4 Solid media electrophoresis

The electrophoresis can be run also in solid 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.

1.8.1.5 Cellulose acetate electrophoresis

The cellulose acetate electrophoresis [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 agarose gel electrophoresis (AGE) [8, 9] is widespread. Depending on the polyion nativity, two types of AGE are known: electrophoresis of native polyions, i.e., of polyions with intact structure and properties; and electrophoresis of denatured polyions, i.e., of polyions with altered structure and properties.

1.8.1.7 Pulsed-field electrophoresis

The AGE is the preferred method for resolving DNA fragments of about 1,000–23,000 bp. For larger fragments, for example, chromosomes, the **pulsed-field gel electrophoresis (PFGE)** is used [10]. In the pulsed-field gel electrophoresis, DNA fragments greater than 23 kbp are forced by a pulsing electric field so that they relax and expand. The separation takes place many hours or even days.

1.8.1.8 Immuno electrophoresis and immunofixation

The **immuno electrophoresis (IE)** is a combination between a zone electrophoresis in an agarose gel and immune reactions. The immunoglobulins are located in the agarose gel. The **immunofixation (IF)** is an immuno electrophoretic method, during which immunoglobulins are applied on the gel surface after the electrophoresis.

1.8.1.9 Affinity electrophoresis

The **affinity electrophoresis** (AE) is a zone electrophoresis carried out in a ligands containing agarose gel [11, 12]. It is based on the interactions between chelating compounds and their ligands.

1.8.1.10 Polyacrylamide gel electrophoresis

The polyacrylamide gel, in contrast to the agarose gel, has no electric charges and, as a result, shows no electroosmosis. The **polyacrylamide gel electrophoresis** (PAGE) [13, 14] is a zone electrophoresis that is carried out for separation of native proteins and nucleic acids.

1.8.1.11 Iontophoresis

The **iontophoresis** (IP) is a type of zone electrophoresis, during which ions are introduced into the human body. This chemical flux is measured commonly in $\mu\text{mol}/\text{cm}^2\text{h}$. The reverse IP is a technique, which helps to remove ions from the human body.

1.8.2 Isotachopheresis

The term **isotachopheresis** (ITP) originates from the Greek words *isos* (same), *tachos* (speed), and *phorein* (to carry). It means moving of ions at the same velocity.

ITP takes place in a buffer system consisting of leading and trailing buffers, which form the moving ionic boundary (function) of Kohlrausch [15, 16]. The polyions arrange themselves in the moving boundary according to their effective mobilities [17, 18]. In contrast to the continuous electrophoresis, the electric field strength is distributed discontinuously and the pH value changes during the electrophoresis.

1.8.2.1 Disc-electrophoresis

There are native and denatured disc-electrophoresis methods.

1.8.2.2 Native disc-electrophoresis

The native disc-electrophoresis is a combination between ITP and zone electrophoresis. In the beginning of electrophoresis, the polyions are concentrated into a very thin stack, which is then resolved in fractions. It is based on the Ornstein theory [19].

1.8.2.3 SDS disc-electrophoresis

Typical denatured disc-electrophoresis is the **sodium dodecyl sulfate (SDS)** electrophoresis. It resembles a native disc-electrophoresis carried out in a SDS containing gel buffer system. 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.

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

1.8.3 Isoelectric focusing

The **isoelectric focusing (IEF)** is based on the migration of proteins 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).

1.8.3.1 Isoelectric focusing with carrier ampholytes

The carrier ampholytes are moving in an electric field until they lose their electric charges at their isoelectric points and stop. As a result, stationary pH gradients are formed, in which analyzed substances can be separated and focused into narrow bands. Usually IEF with carrier ampholytes is carried out in polyacrylamide gels. Agarose gels are also used for IEF, because they have large pores [20, 21].

1.8.3.2 Isoelectric focusing in immobilized pH gradients

The immobilines are acrylamide derivatives, which contain buffering groups. They can be copolymerized into polyacrylamide gels, so that **immobilized pH gradients (IPG)** are produced. In these gels IEF can also be run.

1.8.3.3 Two-dimensional electrophoresis

The two-dimensional electrophoresis (2D-electrophoresis) represents a combination between IEF and SDS disc-electrophoresis. They are carried out sequentially in two mutually perpendicular directions [22, 23]. Depending on the pH gradient, there are two types of 2D-electrophoresis: ampholyte 2D-electrophoresis, and immobiline 2D-electrophoresis.

1.8.4 Dielectrophoresis

The term *dielectrophoresis* (DEP) was first adopted by Pohl [24]. It contains the Greek words *di* (two), *electron* (amber, hence electricity), and *phorein* (to carry). Nowadays DEP is referred to as a method for separating dielectric particles in a non-uniform electric field [25–27]. The particles are not required to be charged. Many articles are dedicated on the theory, technology, and applications of DEP. Prominent among them is the review article by Ronald Pethig [28].

DEP is based on the fact that an inhomogeneous electric field polarizes all particles into *dipoles*, according to Maxwell [29, 30] and Hatfield [31], creating dipole moments [32]. The magnitude of the force exerted on the dielectric particles depends on their electric properties, shape, and volume; on the medium; and on the frequency of the electric field.

If the particles are moving in the direction of an increasing electric field, the DEP is referred to as positive DEP; if the particles are moving in the direction of a decreasing electric field, it is referred to as negative DEP.

1.8.4.1 Dielectrophoretic force

According to most scientists [33, 34], the time-average DEP force F_{DEP} , which acts on a spherical particle, is

$$F_{\text{DEP}} = 2\pi\epsilon r^3 F_{\text{CM}} \nabla E^2 \quad (1.8.1)$$

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 [35, 36] related to the polarizability of the particle; ∇ represents the gradient operator; and E is the electric field strength. As seen, F_{DEP} depends on the square of the applied electric field strength, which indicates that this process can be observed using either direct-current or alternative-current field.

Most particles, especially biological ones, are not homogeneous. Bacteria and cells have so-called multishell structure [37]. 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).

In accordance with this theory, the permittivity of a cell (e.g., human erythrocyte) is given by the expression

$$\epsilon_{\text{cell}} = \epsilon_{\text{mem}} \frac{\left(\frac{r_{\text{mem}}}{r_{\text{cyt}}}\right)^3 + 2 \frac{\epsilon_{\text{cyt}} - \epsilon_{\text{mem}}}{\epsilon_{\text{cyt}} + 2\epsilon_{\text{mem}}}}{\left(\frac{r_{\text{mem}}}{r_{\text{cyt}}}\right)^3 - \frac{\epsilon_{\text{cyt}} - \epsilon_{\text{mem}}}{\epsilon_{\text{cyt}} + 2\epsilon_{\text{mem}}}} \quad (1.8.2)$$

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.

1.8.4.2 DEP technology

The developing of the DEP technology includes the fabrication of microelectrodes, introduction of silicone polymers, fabrication of microfluidics devices for DEP, and developing “funnel” electrodes.

The *microelectrodes* are fabricated by photolithography and metallic vapor. They have interdigitated and castellated geometry, and use modest voltage [38]. In older techniques (Figure 1.8.1a), the particles are directed into flow paths [39]. Later Yasukawa *et al.* [40] modified this method to separate particles according to their size (Figure 1.8.1b). By coupling acoustic waves into an interdigitated microelectrode system, particles can first be concentrated and then focused into flow channels. These electrodes can be used for both positive and negative DEP of cells [41].

The *introduction of silicone polymers* was a new DEP trend. Cheng *et al.* [42] 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 made by film techniques, photo and electron beam lithography, laser ablation, complementary metal-oxide-semiconductor technology [43, 44], scanning force DEP [45], and assembling the nanoparticles in two or more differing surfaces [46].

“Funnel” electrodes were developed by Fiedler *et al.* [47] (Figure 1.8.2). With their help, the particles were guided by angled electrodes to a small exit gap, at which the particles were concentrated prior to be introduced into the device. Angled electrodes are employed in a DEP microchip design for filtering and sorting of bioparticles [48–50].

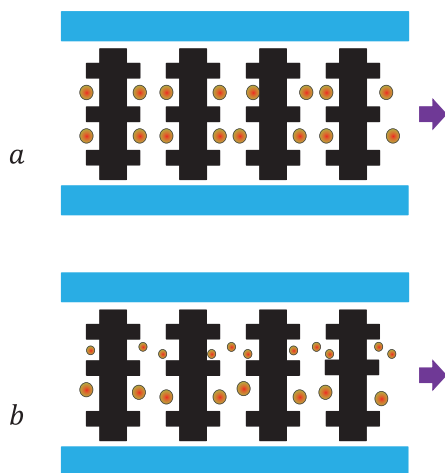


Figure 1.8.1: Microelectrodes.

(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.

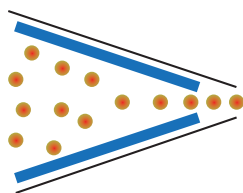


Figure 1.8.2: DEP funnel electrode design for concentrating particles in a flowing aqueous suspension.

1.8.4.3 Applications of dielectrophoresis

DEP is applied in medical diagnostics, particle filtration, biosensors, nanoassembly, microfluidics, cell therapeutics, and more. It has made possible the separation of cancer cells [51], proteins [52, 53], DNA [54–56], chromosomes [57], red and white blood cells [58–60], stem cells [61–63], neurons [64, 65], pancreatic β -cells [66], bacteria and yeast [67–69], and viruses [70, 71]. DEP can also be used for drug discovery and delivery [72], and for detecting apoptosis by measuring the changes in the electrophysiological properties of the cells [73].

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2 Electrophoresis of proteins

The 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 applied for electrophoresis, however, it is alkaline and, as a result, causes a strong electroosmotic flow.

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.

Starch gel electrophoresis is completely detached by the polyacrylamide gel electrophoresis because of the widely varying starch properties, lack of reproducibility, and inconvenience of handling. Nevertheless, it can be used for separations 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

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

Cellulose acetate electrophoresis is used for separation of proteins in clinical laboratories.

2.1.1 Theory of cellulose acetate electrophoresis of proteins

The cellulose acetate membranes have very large pores and, as a result, no screening effect on proteins to be resolved. The electrophoretic separation depends only on the electric charges of proteins. However, the resultant zones are broader than the start zone due to convection.

The theory of cellulose acetate electrophoresis is the theory of the zone electrophoresis. The electrophoretic separation also depends on the nature of the buffer, the voltage applied, and the distance between the electrodes.

2.1.2 Practice of cellulose acetate electrophoresis of proteins

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

Generally, 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).

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Therefore, it should be replaced by a buffer that contains no barbiturate. Such a buffer is the TRIS-taurinate buffer [2].

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

A similar kind of electrophoresis is Cellogel electrophoresis. It is carried out in Cellogel strips. The Cellogel strips contain cellulose, too, but are thicker than the CA membranes. Like the cellulose acetate membranes, the Cellogel strips have very large pores and, therefore, exert no screening effect on the proteins to be resolved.

2.1.3 Protocols

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 acetate (CA) membranes

Ponceau S

Trichloroacetic 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 1,000.00 mL

Ponceau S staining solution

Ponceau S 0.3 g

Trichloroacetic acid 3.0 g

Deionized water to 100.0 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 onto the cathode side of the membrane.
- Run the electrophoresis at 200–250 V (about 3 mA per membrane) for 25–30 min.
- After the electrophoresis, place the CA membrane into the Ponceau S staining solution for 5 min.
- Destain the membrane three times in 5 mL/dL acetic acid.
- Place the membrane in a Petri with clearing solution for 5 min.
- Place the membrane on 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.

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2.2 Agarose gel electrophoresis of proteins

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The agarose gels, compared to the polyacrylamide gels, have larger pores. Therefore, they are suitable for separation of native proteins with very high masses [1]. The agarose gels are simpler in production than the polyacrylamide gels; are nontoxic; and the electrophoresis run is only 20–30 min. The agarose gel electrophoresis is used in research and especially in diagnostics.

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2.2.1 Theory of agarose gel electrophoresis of proteins

The theory of agarose gel electrophoresis of proteins [2–3] does not differ from the general theory of electrophoresis. However, on 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 start line), while γ -globulins are moving back to the cathode (behind the start line). Nevertheless, electroosmosis helps to separate proteins with small electric charge.

Agarose gels are used generally in concentrations of 0.7–1.2 g/dL. In 1 g/dL agarose gel, particles with M_r up to 50×10^6 and with radii of up to 30 nm can be separated. This means that they are suitable for resolving globular and membrane proteins with high molecular masses [4], and viruses [5].

The most common electrophoresis on agarose gels is the zone electrophoresis, which is run in various buffers [6–7]. Among them, barbitalate buffers (veronalate buffers) are the most used. However, the usage of barbituric acid derivatives has been restricted by the Medicines Act [8]. Therefore, buffers containing no barbituric acid compounds are preferred. Such a buffer is the TRIS-taurinate buffer [9].

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

Horizontal agarose gel electrophoresis is a standard method for separation of proteins in serum, cerebrospinal fluid (CSF), urine, and other fluids.

2.2.2 Agarose gel electrophoresis of serum proteins

Serum protein electrophoresis (SPE) is a method used 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 γ -globulins (Figure 2.2.1). The ratio between the mass concentrations of albumin and globulins is 1.5–3:1.

SPE can be used for diagnosing different diseases, for example, multiple myeloma, macroglobulinemia, amyloidosis, hypogammaglobulinemia, and more.



Figure 2.2.1: Pherogram of serum proteins obtained by agarose gel electrophoresis.

2.2.2.1 Albumin

Albumin, together with the other serum proteins, is synthesized in the liver. Its relative molecular mass is approximately 69,000 and its isoelectric point is 4.9.

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 observed in the nephrotic syndrome (Figure 2.2.2) when albumin passes easily through the damaged glomerular membrane into the urine due to its low molecular mass. The albumin concentration is also low in liver diseases, malnutrition, nutrient absorption disorders, and loss of protein in enteropathy. Increased albumin concentration can be observed in acute alcoholism, during pregnancy, and in puberty.

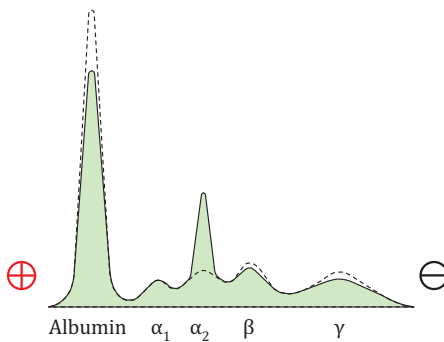


Figure 2.2.2: Nephrotic syndrome. The concentration of α_2 -globulins is increased; the concentration of albumin is decreased.

Before albumin, *transthyretin* (prealbumin) is moving. It transfers the hormone thyroxine. The concentration of transthyretin is too low; therefore, it is usually hidden when using common staining methods. Mutations of transthyretin cause familial amyloidosis.

Intermediate zone of albumin- α_1 -globulins

The intermediate zone of albumin- α_1 -globulins contains α_1 -lipoproteins (high-density lipoprotein (HDL)) and α -fetoprotein. The concentration of α_1 -lipoproteins decreases at severe inflammation, acute hepatitis, and liver cirrhosis; the concentration of α -fetoprotein increases 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. α_1 -Antitrypsin is a protein of the acute phase of inflammation.

α_1 -Lipoproteins (α -lipoproteins, HDL, $M_r = 200,000$) transport phospholipids, cholesterol, triacylglycerols, fat soluble vitamins such as A and E, and some hormones.

Acidic α_1 -glycoprotein ($M_r = 44,100$), called also orosomuroid, is an acute phase protein, too. Orosomuroid and α_1 -antitrypsin move together, but orosomuroid is stained worse. 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 during the clotting process.

The concentration of α_1 - and α_2 -globulins increases in malignant tumors while the concentration of albumin decreases (Figure 2.2.3).

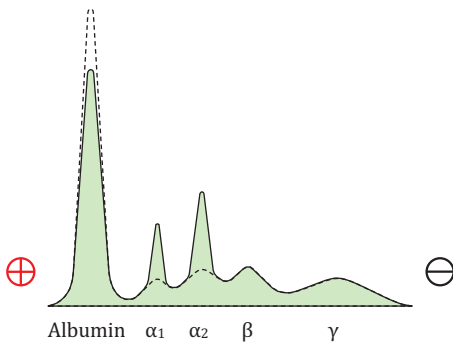


Figure 2.2.3: Increased concentrations of α_1 - and α_2 -globulins in malignant tumors. The albumin concentration decreases.

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; 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 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 through into the urine. Therefore, its concentration increases significantly in nephrotic syndrome.

Haptoglobins ($M_r = 100,000$) bind hemoglobins after red blood cells are destroyed and thus prevent the loss of iron. They represent the largest part of the acute phase proteins, causing an increase in the α_2 -globulin concentration in acute inflammation (Figure 2.2.4). On the contrary, their concentration decreases in hemolytic anemia.

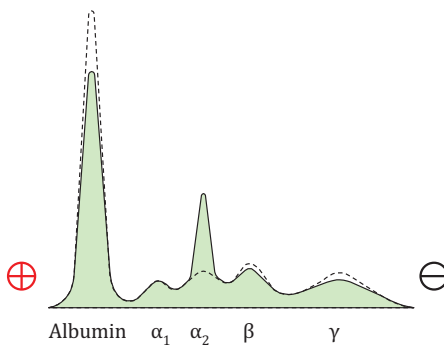


Figure 2.2.4: Increased concentration of α_2 -globulins in acute inflammation.

α_2 -Lipoproteins (prebeta-lipoproteins, VLDL, $M_r = 5\text{--}20 \times 10^6$) transport lipids, mainly triacylglycerols.

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 -zone, combined with that of α_1 -zone, increases in patients with malignant tumors and cirrhosis, while the albumin concentration decreases.

Intermediate zone of α_2 - β -globulins

The intermediate zone of α_2 - β -globulins contains prebeta-lipoproteins (HDL, VLDL). Their concentration is increased in type II hypercholesterolemia, hypertriglyceridemia,

and nephrotic syndrome. Here, 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 the heme of hemoglobins.

Transferrin ($M_r = 80,000$) transfers iron. It is an acute phase protein.

β -Lipoproteins (LDL, $M_r = 2.4 \times 10^6$) transport lipids, fat-soluble vitamins, and hormones.

Plasminogen ($M_r = 143,000$) destroys fibrin in the blood clot, when activated.

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 at liver cirrhosis, together with that of γ -globulins (β - γ -bridge) (Figure 2.2.5).

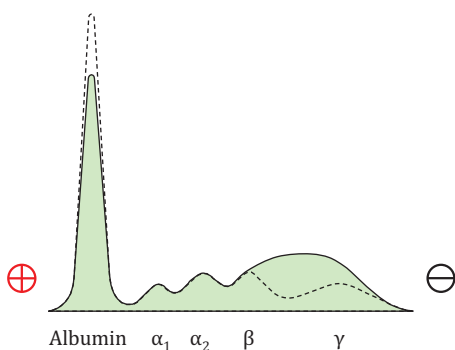


Figure 2.2.5: Beta-gamma bridge in liver cirrhosis.

In more precise agarose gel electrophoresis, β -globulins are separated in two fractions: β_1 and β_2 .

Beta-1-globulins 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 are presented predominantly by the complement 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 five classes of immunoglobulins (Ig): IgG, IgA, IgM, IgD, and IgE.

IgG ($M_r = 160,000$) bind specifically to antigens of viral, bacterial, or parasitic origin, also to bacterial toxins, Rh antibodies, insulin, *etc.*

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, and more. They are the fastest moving of antibodies; therefore, are always in the beginning of gamma zone. When their concentration is increased, IgA cause a β - γ -bridge, too. Their concentration is increased in patients with cirrhosis, respiratory infections, skin disease, or rheumatoid arthritis.

IgM ($M_r = 900,000$) include antibodies, as ABO isoagglutinines, antibacterial antibodies, Wassermann antibody, anti-thyroglobulin antibodies, *etc.*

The function of IgD ($M_r = 150,000$) is still unknown.

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 increases in viral hepatitis (Figure 2.2.6) as well as in multiple sclerosis.

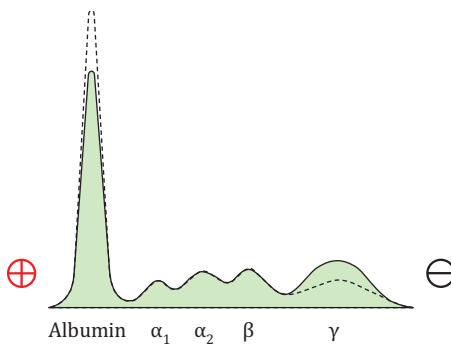


Figure 2.2.6: Increased concentration of γ -globulins and decreased concentration of albumin in virus hepatitis.

A spike-like enlargement of the gamma zone is typical for monoclonal gammopathy; a large basis increase indicates polyclonal gammopathy.

The narrow spike in monoclonal gammopathy (Figure 2.2.7) 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 show an increase of IgM-paraprotein concentration. However, 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*.

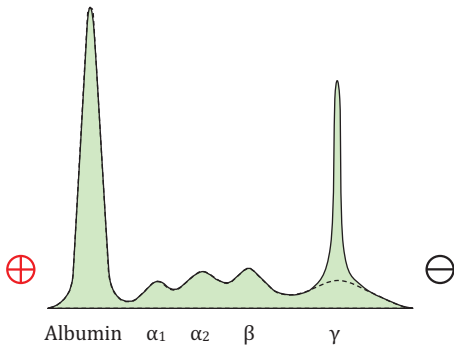


Figure 2.2.7: Increased concentration of immunoglobulins in monoclonal gammopathy.

Polyclonal gammopathy is usually a benign condition. It is typical for severe infections, chronic liver disease, rheumatoid arthritis, systemic lupus, and other connective tissue diseases.

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 γ -globulins is increased, while the concentration of albumin is decreased.

2.2.3 Agarose gel electrophoresis of lipoproteins

The 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, i.e. they originate from food, or they have endogenous origin, i.e. they are synthesized in the body.

2.2.3.1 High-density lipoproteins

High-density lipoproteins (HDL, α_1 -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 in the liver and small intestine.

2.2.3.2 Low-density lipoproteins

Low-density lipoproteins (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. By cholesterol are synthesized bile acids, steroid hormones, and steroid vitamins.

2.2.3.3 Intermediate-density lipoproteins

Intermediate-density lipoproteins (IDL) are formed during the degradation of very-low-density lipoproteins (VLDL). Their density is between that of low-density and that of VLDL. IDL are similar to the LDL and like them consist of protein that encircles triacylglycerols and cholesterol esters. The size of IDL is 25–35 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 particles.

2.2.3.4 Very-low-density lipoproteins

Very-low-density lipoproteins (VLDL, α_2 -lipoproteins, 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 constructed of a lipid droplet, which is 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 are composed usually by 18 straight-chain carbon atoms, containing one or more double bonds and many single bonds. The free fatty acids in blood, which are bound to albumin, serve as a raw material for the synthesis of endogenous triacylglycerols in the liver [10].

2.2.3.6 Hyperlipoproteinemias

Hyperlipoproteinemias are diseases that are characterized with increased concentration of lipoproteins. They are subdivided into five types [11].

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, HDL remains usually unchanged (Figure 2.2.8).

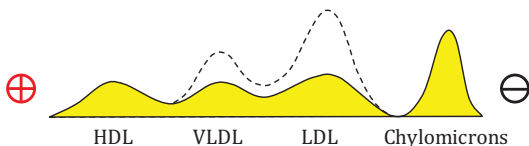


Figure 2.2.8: Hyperlipoproteinemia type I.

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 into 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 VLDL are increased (Figure 2.2.9).

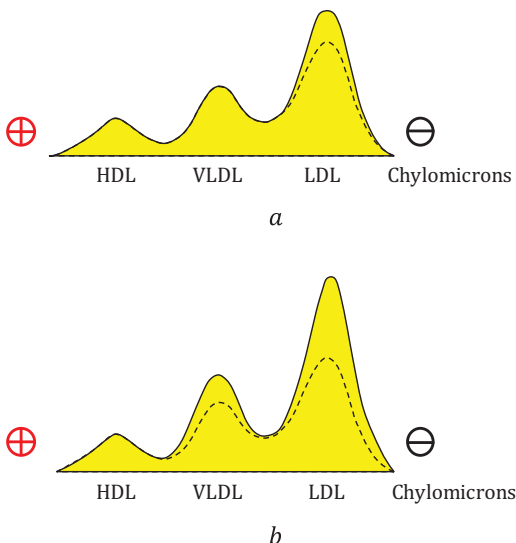


Figure 2.2.9: Hyperlipoproteinemia type IIa (a) and type IIb (b).

Hyperlipoproteinemia type III (hyper-LDL-hyper-VLDL-emia) is characterized with a broadband over the LDL and VLDL fractions (increased cholesterol and triacylglycerols), and a normal α -band fraction (Figure 2.2.10).

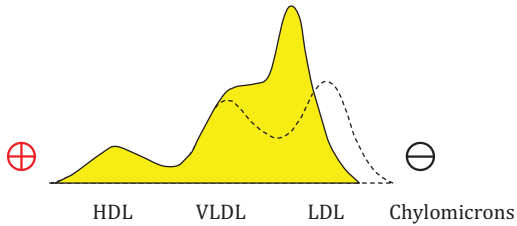


Figure 2.2.10: Hyperlipoproteinemia type III.

The hyperlipoproteinemia type IV (hyper-VLDL-emia) is accompanied by an increased VLDL fraction. The pheroogram shows normal LDL and HDL bands (Figure 2.2.11).

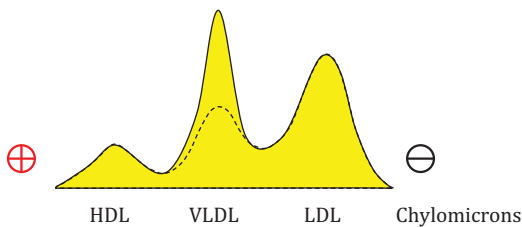


Figure 2.2.11: Hyperlipoproteinemia type IV.

In hyperlipoproteinemia type V (hyper-VLDL-emia and hyperchylomicronemia), a simultaneous increase of the concentration of exogenous triacylglycerols (chylomicrons) as well as of endogenous triacylglycerols (VLDL fraction) are seen (Figure 2.2.12).

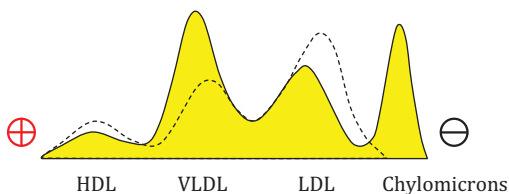


Figure 2.2.12: Hyperlipoproteinemia type V.

2.2.4 Agarose gel electrophoresis of hemoglobins

Electrophoresis, nearby chromatography, gives best information for **hemoglobins** (Hb). The hemoglobin electrophoresis is carried out most often in cellulose acetate membranes and agarose gels. Especially, good results are obtained by isoelectric focusing on polyacrylamide gel [12–13].

2.2.4.1 Normal hemoglobins

In the embryo, the following hemoglobins can be established: Gower 1 ($\zeta_2\varepsilon_2$), Gower 2 ($\alpha_2\varepsilon_2$), Portland I ($\zeta_2\gamma_2$), and Portland II ($\zeta_2\beta_2$). In the fetus, hemoglobin F ($\alpha_2\gamma_2$) can be found [14]. In adults, three types of hemoglobins exist: hemoglobin A (HbA), hemoglobin (HbA₂) and fetal hemoglobin F (HbF).

Hemoglobin A (HbA) is a tetramer with $M_r = 67,000$ that is composed of four polypeptide chains (globin chains), each of them with $M_r = 17,000$, and four 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 resembles that of myoglobin because they are related genetically.

HbA makes over 95% of the total hemoglobin in a healthy adult; the rest consists of HbA₂ and HbF (Figure 2.2.13). HbF dominates during the fetal development, however is replaced later by HbA. The formulas of HbA₂ and HbF are $\alpha_2\delta_2$ and $\alpha_2\gamma_2$, respectively. The synthesis of δ chain of HbA₂ begins late in the third trimester. In

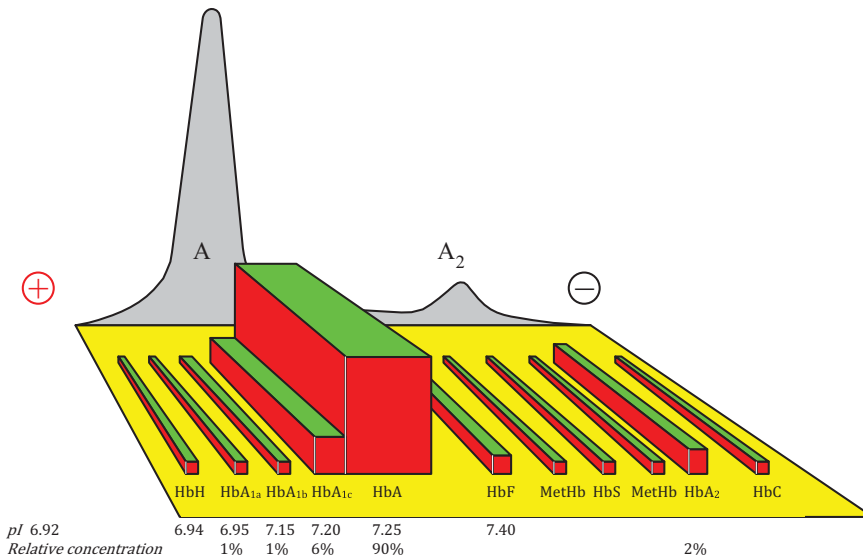


Figure 2.2.13: Hemoglobins.

adults, HbF is restricted in a limited population of red cells called F-cells. The concentration of HbF is increased in persons with sickle cell disease and β -thalassemia.

The hemoglobin concentration is 12.8–17.6 g/dL (on average 15 g/dL) in men, and 11.2–16.0 g/dL (on average 14 g/dL) in women.

HbA is partially glycosylated. The glycosylated hemoglobin, called HbA_{1c}, 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 three types of HbA_{1c}: 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 [15–16]. The concentration of HbA_{1c} reflects the average glucose concentration in the weeks before testing [17–18], whereas the 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.

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) occur. If they do not cause disorders, they are classified as abnormal hemoglobins; if they cause illnesses (hemoglobinopathias), they are called pathological hemoglobins [19].

The most widespread pathological hemoglobins are:

- Hemoglobin D-Punjab ($\alpha_2\beta_2^D$).
- Hemoglobin H (β_4), formed by a tetramer of β -chains; presents in variants of α -thalassemia.
- Hemoglobin Barts (γ_4), formed by a tetramer of γ -chains; presents in variants of α -thalassemia.
- Hemoglobin S ($\alpha_2\beta_2^S$), 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 ($\alpha_2\beta_2^C$), a product of a variation in the β -chain gene; causes a mild chronic hemolytic anemia.
- Hemoglobin E ($\alpha_2\beta_2^E$), a product of a variation in the β -chain gene; causes a mild chronic hemolytic anemia. It is characterized by replacing the glutamic acid residue at position 26 of the β -chain by lysine residue.
- Hemoglobin SC, a complex heterozygous form with one sickle gene and another encoding HbC gene.
- Hemoglobin Hopkins-2 [20], a variant form of hemoglobin that is sometimes established in combination with HbS in the sickle cell disease.

Sickle cell disease

Sickle cell disease (sickle cell anemia) was first described by Ernest Irons and James Herrick in 1910 [21]. In 1949, Linus Pauling [22] 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 [23]. It contains, 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 disease. It is located on the short (*p*) arm of chromosome 11 at position 15.5. People who receive the defective gene from both father and mother (homozygote persons) develop the disease. People who receive one defective and one healthy gene (heterozygote persons) remain healthy.

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 2.2.14).

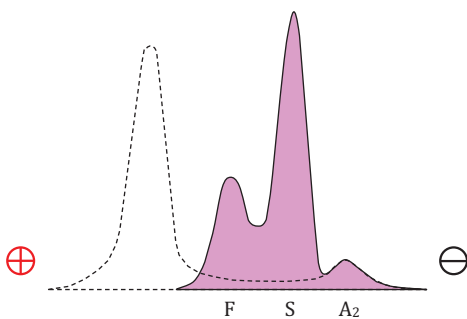


Figure 2.2.14: Sick cell disease.

In 1950, another pathological hemoglobin was identified – hemoglobin C [24]. 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. Later, hemoglobin E was identified and afterward hundreds of other hemoglobin variants.

Thalassemias

Thalassemias are diseases in which the synthesis of globin chains of HbA is disturbed. Instead of this, the body produces the globin chains of HbA₂ or HbF. Thalassemias occur more frequently than the hemoglobinopathies. The heterozygous thalassemia is called *thalassemia minor*. It is characterized with a mild anemia and a light increase of

the concentrations of HbA₂ or HbF. The homozygous thalassemia is called *thalassemia major*. It is a serious disorder that is widespread in the Mediterranean.

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 γ -chains in newborns, which lead to formation of the tetramers β_4 (HbH) or γ_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 2.2.15).

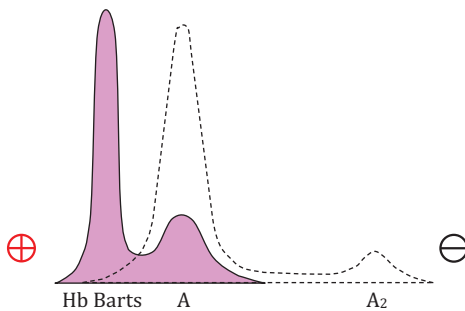


Figure 2.2.15: α -Thalassemia.

Beta-thalassemia. In β -thalassemias, the β -chains of HbA are not synthesized, which leads to increased production of α -, γ -, or δ -chains.

β -Thalassemia major, called Cooley anemia, leads to death in a few years, if not treated. It is accompanied by increased concentrations of HbA₂ and HbF (Figure 2.2.16).

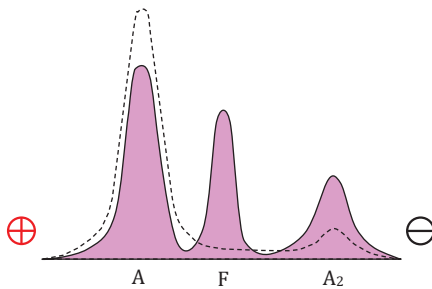


Figure 2.2.16: β -Thalassemia.

In addition to β -thalassemia major, β -thalassemia, β -fusion hemoglobinopathy (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. It has the mobility of hemoglobin S. In β -thalassemia, neither β -chains nor δ -chains are synthesized.

Hemoglobin E disease appears when a child inherits the HbE gene from both parents. In the first months of life, the fetal hemoglobin disappears and the amount of HbE increases, so that the patients start to have a mild β -thalassemia. People who are heterozygote for HbE (one normal allele and one abnormal allele) do not show any symptoms (there is no anemia or hemolysis) [25]. Patients who are homozygous for the HbE allele (have two abnormal alleles) suffer from a mild hemolytic anemia and mild enlargement of the spleen.

2.2.4.3 Running hemoglobin electrophoresis

Since the pI points of hemoglobins are at pH = 7.0, the hemoglobin electrophoresis should 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 2.2.17).

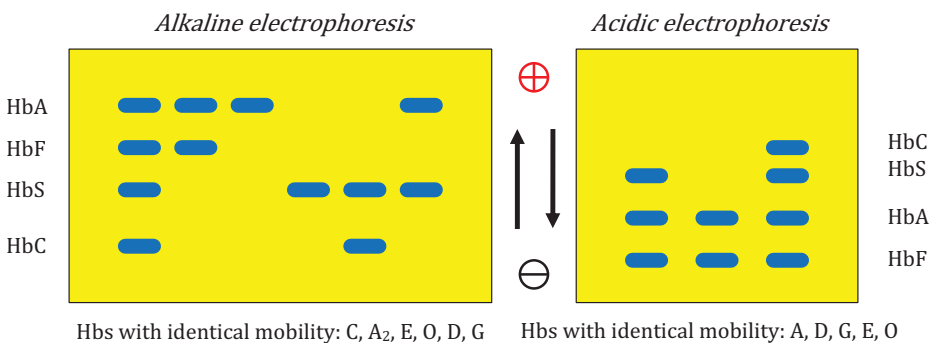


Figure 2.2.17: Hemoglobins obtained by alkaline and acidic electrophoresis.

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 the other buffer – TRIS-taurinate-EDTA buffer (also with pH = 8.5) [26] – are even better. In the alkaline hemoglobin electrophoresis, hemoglobins A, S, C, *etc.* can be separated.

The *acidic agarose gel electrophoresis* is used to separate hemoglobins that have equal migration velocities in alkaline electrophoresis. The hemolysates are applied at the anode, and the electrophoresis is carried out in a citrate buffer at pH = 6.1. HbC is separated from HbE, HbS from HbD or HbG, and HbS from HbC. Also, HbF is separated from HbA, which is impossible in the alkaline electrophoresis.

2.2.5 Agarose gel electrophoresis of cerebrospinal fluid proteins

The protein concentration in the CSF is about 250 times lower than that in the blood serum. Therefore, CSF should 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 be stained with silver, too.

The CSF represents an ultrafiltrate of the serum plasma. Therefore, the CSF pherogram is similar to the serum pherogram (Figure 2.2.18).

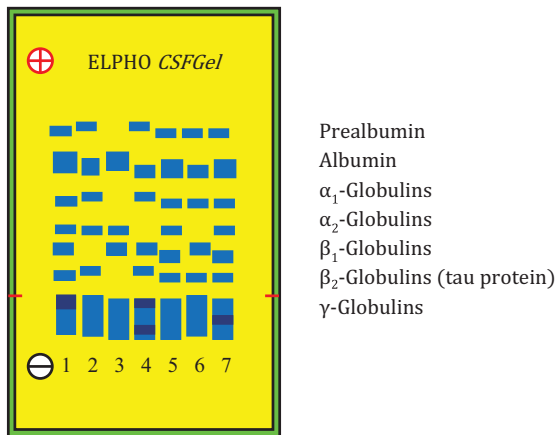


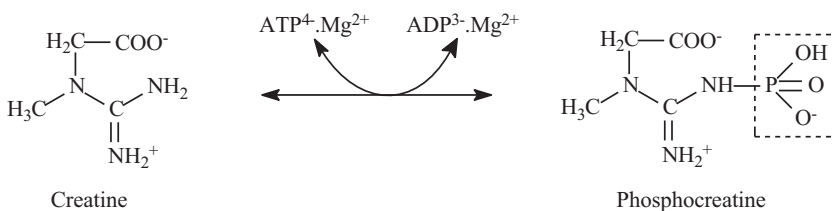
Figure 2.2.18: Electrophoretic bands of CSF proteins.

However, there are differences between both pherograms: The concentration of prealbumin in CSF is too high – it represents 4–5% of the total 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 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 γ -globulins are represented in significantly lower concentration in comparison to the serum γ -globulins. Often, a post-gamma fraction of low concentration, called gamma C, exists; and the lipoprotein concentration in the CSF is extremely low.

In multiple sclerosis, the agarose gel zone electrophoresis of CSF shows an oligoclonal IgG banding in the gamma area – up to seven IgG oligoclonal bands [27–28].

2.2.6 Electrophoresis of creatine kinase isoenzymes

Creatine kinase (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:



CK is composed of two polypeptide chains, B and M, making up of three forms: CK-BB, CK-MB, and CK-MM. They form high-molecular-mass complexes with IgG or IgA.

The isoenzyme CK-BB is contained in the cytosol of brain and nerve cells. It dominates over the other CK isoenzymes in bladder, kidney, prostate, uterus, gastrointestinal tract, lungs, placenta, spleen, liver, pancreas, and thyroid gland [29]. CK-BB is not detectable in normal adult serum. 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-MB and CK-MM [30]. The CK-BB activity in serum is greatly increased in different cancers (of prostate, breast, stomach, lungs, colon, testes, gall bladder, and leukemia) [31].

The isoenzyme CK-MB has its highest activity in the cardiac muscle. It is a specific indicator of the acute myocardial infarction. The elevated serum activity of CK-MB, in addition to the increased LDH₁/LDH₂ ratio (see below), the clinical history (previous chest pain), and the abnormal ECG (Q-wave) help to diagnose this severe disease (Figure 2.2.19).

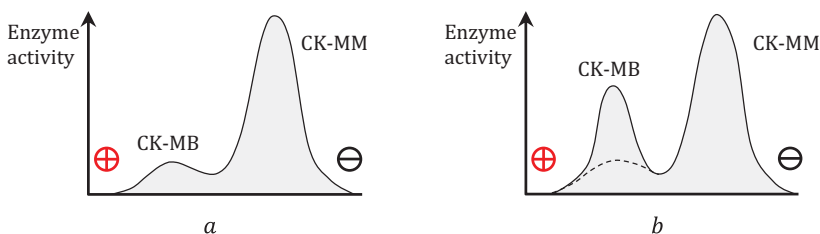


Figure 2.2.19: Activity of CK-MB and CK-MM.

(a) Activity in a healthy individual; (b) activity in a patient with acute myocardial infarction.

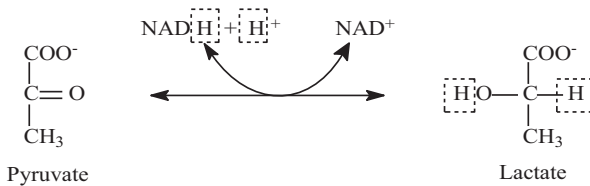
In acute myocardial infarction, an interruption or reduction of blood supply in the coronary arteries causes heart muscle necrosis. This damages the plasma membrane of the cardiac cells, which results in a release of CK-MB into the lymphatic

system and then into the peripheral blood. The serum concentration of CK-MB increases in 4–8 h after the beginning of infarction, and the high concentration remains up to the 12–18th h [32].

The isoenzyme 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 isoenzymes

Lactate dehydrogenase (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 **heart**) and M (from **muscle**). Five LDH isoenzymes exist that can be separated from each other by electrophoresis: LDH₁ (H₄), LDH₂ (H₃M), LDH₃ (H₂M₂), LDH₄ (HM₃), and LDH₅ (M₄). LDH₁, the isoenzyme with the highest negative charge, migrates fastest (its mobility is almost equal to that of serum albumin), while LDH₅ is the slowest isoenzyme (Figure 2.2.20). LDH₁ predominates in heart, kidney cortex, brain, and erythrocytes, while LDH₅ predominates in skeletal muscle and liver. LDH₂, LDH₃, and LDH₄ are present in the gall bladder, prostate, and uterus.

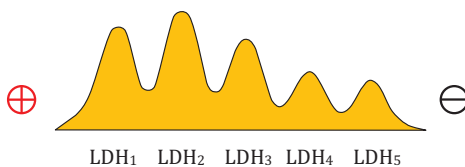


Figure 2.2.20: Normal pherogram of LDH isoenzymes.

In serum of a normal adult, the activity of LDH₂ is higher than that of the LDH₁, and the LDH₁/LDH₂ quotient is less than 0.76. In an acute myocardial infarction, LDH₁ is released from the myocardium cells into the blood. As a result, the LDH₁/LDH₂ quotient becomes in the first 24–48 h higher than 1, which may continue for days or weeks (Figure 2.2.21).

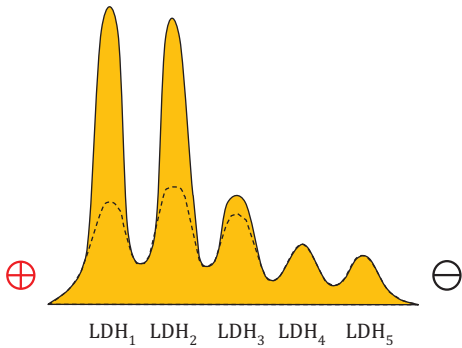


Figure 2.2.21: LDH isoenzymes in myocardial infarction.

The activities of LDH₂ and LDH₃ increase in pulmonary edema; of LDH₄ and LDH₅ in liver diseases; and of LDH₅ in renal diseases. In malignant neoplasms, all LDH isoenzymes show higher activity.

2.2.8 Agarose gel electrophoresis of urinary proteins

Normally, the urine contains no proteins, but certain diseases (for example, 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 2.2.22).

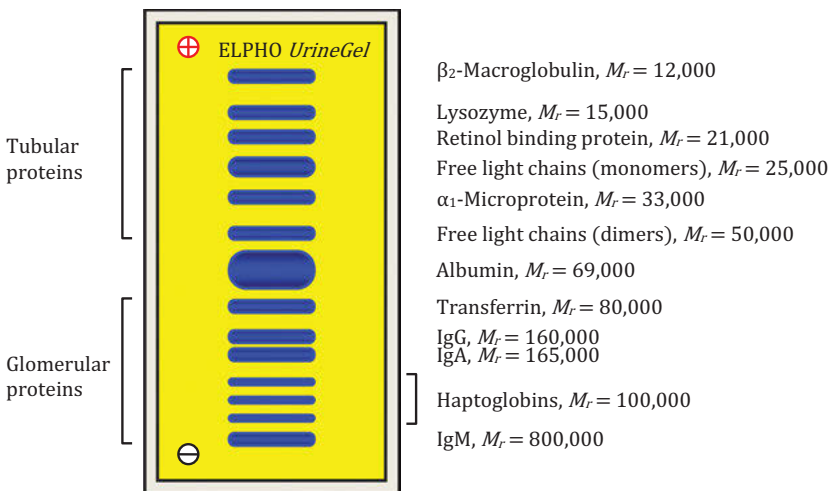


Figure 2.2.22: Pherogram of urinary proteins in a patient with mixed proteinuria.

In *glomerular proteinuria*, serum proteins are detected in urine: albumin, α_1 -acidic glycoprotein, α_1 -antitrypsin, α -lipoproteins, transferrin, haptoglobins, IgA, IgG, and more. They have high-molecular mass, which is greater than the albumin mass. β -Lipoproteins and IgM are not found [33–34] in the urine.

The *tubular proteinuria* occurs rare, when the tubular absorption fails. In this case, the proteins in urine have molecular masses that are less than the albumin mass, and, therefore, they run before albumin [35–36]: protein hormones [37] (gonadotropins, corticotropin, and erythropoietin), vitamin B₁₂-binding protein, blood group substances [38], retinol-binding protein, and some enzymes: lysozyme, amylase, plasmin, trypsin, pepsin, and LDH [39]. Compared to the glomerular proteins, they contain more carbohydrates.

2.2.9 Protocols

Agarose Gel Electrophoresis of Serum Proteins

The agarose gels for horizontal electrophoresis 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.

Materials and equipment

TRIS
 NaOH
 Formic acid
 Taurine
 Methylparaben
 NaN₃
 Amido black 10B (Naphthol blue black)
 Ethanol
 Acetic acid

TRIS-formate buffer (Gel buffer), pH = 8.8, I = 10 x 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, 10×	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, 10×	10.0 mL
Deionized water to	100.0 mL

Staining solution

Amido black 10B	0.2 g
Methanol	10.0 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

- Unpack an agarose gel and remove its cover film.
- Blot the gel surface with a filter paper strip where the application template should be laid on.
- 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.

- Place the bridge, together with the gel, into the electrode tanks of the electrophoresis cell, so that the gel contact directly with the electrode buffers in the cell (Figure 2.2.23).

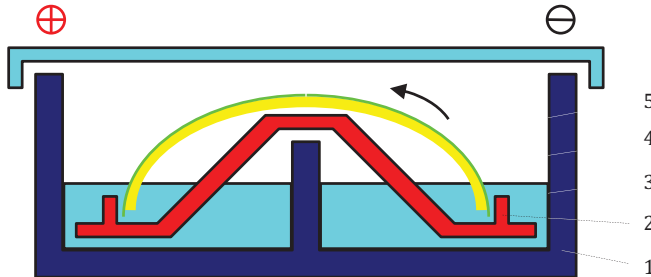


Figure 2.2.23: Direct contact between agarose gel and both electrode buffers.

1. Electrophoresis cell; 2. bridge; 3. electrode buffer; 4. agarose gel; 5. support film.

- Cover the electrophoresis cell with its lid, and switch on the power supply.
- 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 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.
- Evaluate the protein bands with a scanner or a densitometer at 600 nm.

Agarose Gel Electrophoresis of Lipoproteins

The agarose gel electrophoresis of lipoprotein is carried out as the agarose gel electrophoresis of serum proteins. However 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 serum albumin (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, 10×	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, 10×	10.0 mL
Deionized water to	100.0 mL

Staining solution

Sudan black B	0.2 g
Deionized water to <i>Filter.</i>	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 should 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, and switch on the power supply.
- 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 three times in 60 mL/dL ethanol for 5 min.
- Rinse the gel in deionized water.
- Dry the gel with a hair dryer or in a drying oven.

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)

2× Sample buffer

1.5 mol/L TRIS, 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, 10×	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, 10×	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 three 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 (hemolysed).
- Centrifuge after 30 min at 3,000 rev/min.
- Dilute the supernatant in a buffer and KCN, until the Hb concentration reaches 7.75×10^5 mol/L (10 g/L), and KCN concentration reaches 0.005 mol/L (0.326 g/L).
- Apply 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 (0.23 mol/L)

NaN_3 0.07 g (0.01 mol/L)

Deionized water to 100.00 mL

Solution B (0.2 mol/L citric acid)

Citric acid 4.20 g (mol/L)

NaN_3 0.07 g (0.01 mol/L)

Deionized water to 100.00 mL

Sodium citrate buffer, 10 \times , pH = 6.0, I = 10 \times 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

Sodium 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.

Agarose Gel Electrophoresis of Cerebrospinal Fluid Proteins

The agarose gel electrophoresis of CSF 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), pH = 8.8, I = 0.035 mol/L

TRIS-formate buffer, 10×	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, 10×	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 0.20–0.40 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 reaches 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 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.

Agarose Gel Electrophoresis of Urinary Proteins

Materials and equipment

TRIS

Formic acid

Agarose ($-m_r = 0.16\text{--}0.19$)

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, 10×	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, 10×	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 should 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, and switch on the power supply.
- 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.

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2.3 Immuno-electrophoresis

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Immuno-electrophoresis as well as affinity electrophoresis are carried out on agarose gels. They pass with binding of proteins to specific ligands. Therefore, both techniques are named *ligand electrophoresis*.

Immuno-electrophoresis presents a combination between agarose gel electrophoresis and immune reactions [1, 2]. It requires immunoglobulins (Ig, antibodies) [3] as ligands. The Ig recognize exogenous antigens (macromolecules in viruses, bacteria, tissues, etc.). They are produced by plasma cell lines (clones) of the immune system. The healthy organism possesses over 10,000 different clones. Agarose has been chosen as a gel of choice because it has large pores allowing the proteins to pass. For this technique, 1 g/dL agarose slabs are usually used, which contain buffers with pH = 8.6.

At certain antigen-antibody ratios, net-shaped insoluble immune complexes (immune precipitates) are formed in the gel (Figure 2.3.1). The non-precipitated proteins can be washed out. Immune precipitates are stained with dyes as Amido black 10B or

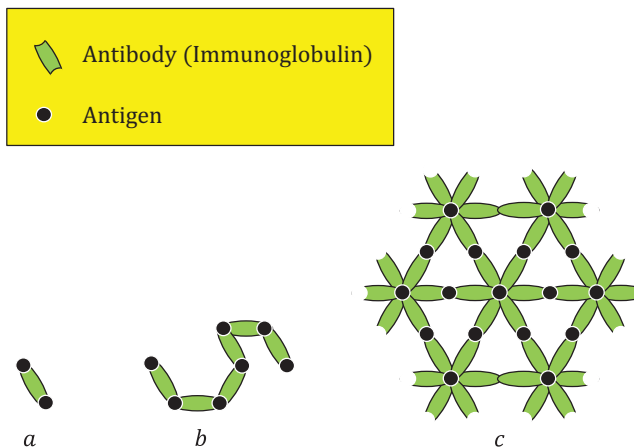


Figure 2.3.1: Hypothetical structure of soluble immune complexes (a and b) and insoluble immune precipitates (c). The ratio between the molar concentrations of antibodies and antigens is as follows: (a) 1:2; (b) 1:1; and (c) 3:1.

<https://doi.org/10.1515/9783110761641-013>

Coomassie brilliant blue R-250. Nowadays, the immunoelectrophoresis is replaced by electroblotting, because it requires fewer antibodies, and has higher sensitivity.

The most significant immunoelectrophoretic methods are immunoelectrophoresis according to Grabar and Williams, rocket-immunoelectrophoresis according to Laurell, immunofixation, and immunoprinting.

2.3.1 Immunodiffusion electrophoresis according to Grabar and Williams

Immunodiffusion electrophoresis according to Grabar and Williams [4] is a combination between agarose gel electrophoresis of proteins (antigens) and diffusion of their antibodies: after electrophoresis, antibodies are applied in channels next to the protein bands. The antibodies diffuse against the bands forming with them precipitate arcs (Figure 2.3.2).

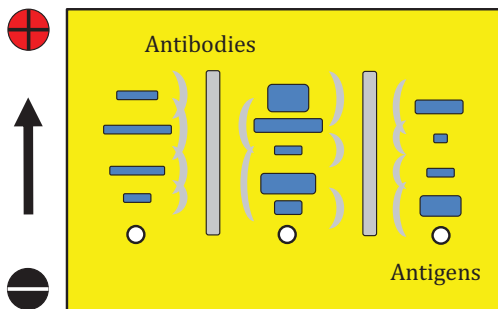


Figure 2.3.2: Immunodiffusion electrophoresis according to Grabar–Williams.

2.3.2 Rocket immunoelectrophoresis according to Laurell

The rocket immunoelectrophoresis was invented by Laurell [5] and modified by Eriksson *et al.* [6], and Yman *et al.* [7]. It is an electrophoresis of proteins (antigens) on agarose gel containing antibodies. The gel buffer is adjusted to pH = 8.6, so that only the antigens migrate, whereas at this pH value most antibodies cannot move because they are in their isoelectric points.

In the beginning of rocket immunoelectrophoresis, the antigens excess, which results in formation of soluble antigen–antibody complexes. Afterward, immune precipitates are formed, which are rocket-shaped figures whose surface (height) is proportional to the antigen concentrations (Figure 2.3.3). Therefore, the rocket immunoelectrophoresis is a quantitative immunoelectrophoresis, used for quantitation of human serum proteins.

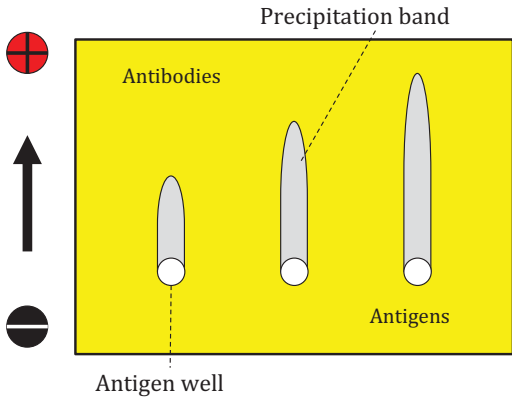


Figure 2.3.3: Rocket immunoelectrophoresis according to Laurell.

2.3.3 Immunofixation and immunoprinting

The immunofixation was described in 1964 [8, 9]. Later, in modified forms, it was used for identification of different proteins [10–12]. In immunofixation, the proteins first should be separated electrophoretically on agarose gel, and then they should be coated with corresponding antisera. So, immune complexes are built in the agarose gel [13]. The soluble proteins are washed out with physiological solution (Figure 2.3.4).

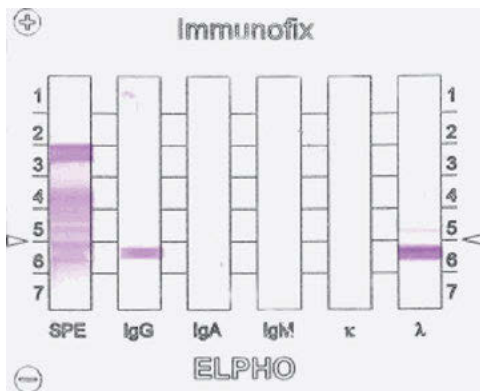


Figure 2.3.4: Immunofixation electrophoresis proving IgG paraproteins of λ -type in the blood serum.
SPE – Serum protein electrophoresis

In immunoprinting [14], first an agarose gel electrophoresis of antigens (proteins) is carried out. Then, the gel is overlaid with an antibody-containing agarose gel or cellulose acetate membrane. The resolved antigens diffuse into the antibody containing sheet and form immune precipitates.

2.3.4 Protocols

Immunofixation of Serum Proteins

Materials and equipment

TRIS-glycinate or TRIS-taurinate buffer

Agarose gel for immunofixation of serum proteins. It is situated between two films. The lower film is bound to the gel; the upper film can be easily removed from it.

Antisera: Anti-IgG, anti-IgA, anti-IgM, anti-kappa, and anti-lambda

Coomassie violet blue R-200 or Amido black 10B

Pipette

Staining solution

Coomassie violet R-200 0.2 g

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.
- 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 side markers (Figure 2.3.5) 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 **electrophoresis** (ELP) and next slots, and let the sample diffuse in the gel.
- After 5 min, blot the remaining sample volumes with filter paper.
- Remove the application template together with the paper strip from the gel.

Electrophoresis

- Fill the tanks of an ELP cell with electrode buffer.
- Hang the gel, with its support film up, on the bridge of an ELP cell.

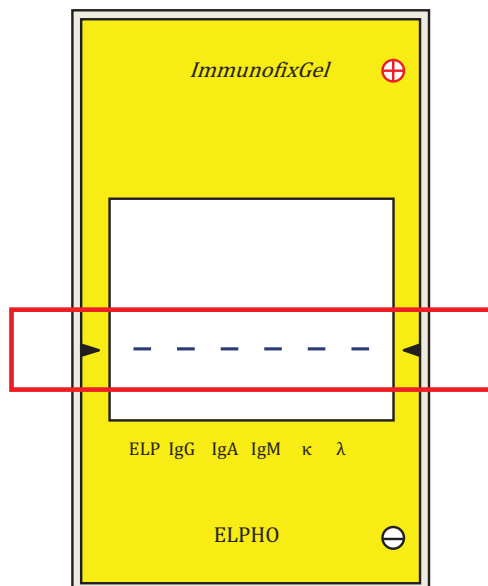


Figure 2.3.5: Application of a template for immunofixation onto an agarose gel.

- Insert the bridge with the gel into the tanks of the ELP cell. So, direct contact occurs between the gel and electrode buffers (see *Agarose gel electrophoresis of serum proteins*).
- Cover the ELP cell with its lid, and turn on the power supply.
- Run ELP at constant voltage or electric current (80 V, or 20–25 mA) at room temperature for 20–25 min.

Immunoprecipitation

- After the ELP, dry the agarose gel surface with a filter paper.
- Adjust an antiserum template on the gel, according to arrows on the support film, and press it gently against the gel (Figure 2.3.6).
- Apply with a pipette 50 μL of fixing solution (methanol-acetic acid-water, 3:1:6, V:V:V) in the ELP bed and 50 μL each of the antisera (G, A, M, κ , and λ) in their beds.
- Incubate the gel with the antisera on a wet filter paper at room temperature for 30 min.
- Remove the antisera template and press the gel dry. The gel dry pressing is carried out in the following way: Place 4–5 filter papers on the gel, a glass plate, and a mass of 1–2 kg for 10 min (Figure 2.3.7).
- Soak the gel in saline (0.9 g/dL NaCl) for 10 min, and press it again.
- Press the gel two more times and afterward dry it with a dryer or in a drying oven.

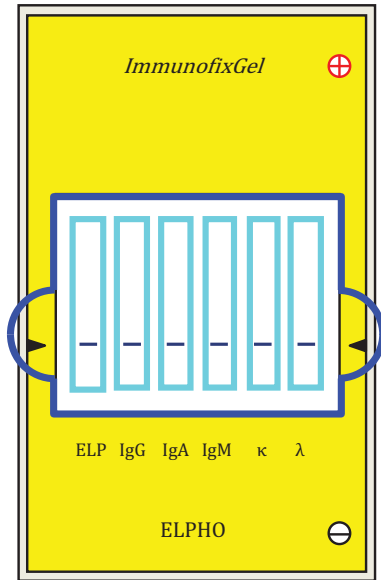


Figure 2.3.6: Adjustment of an antiserum template on an agarose gel after electrophoresis.

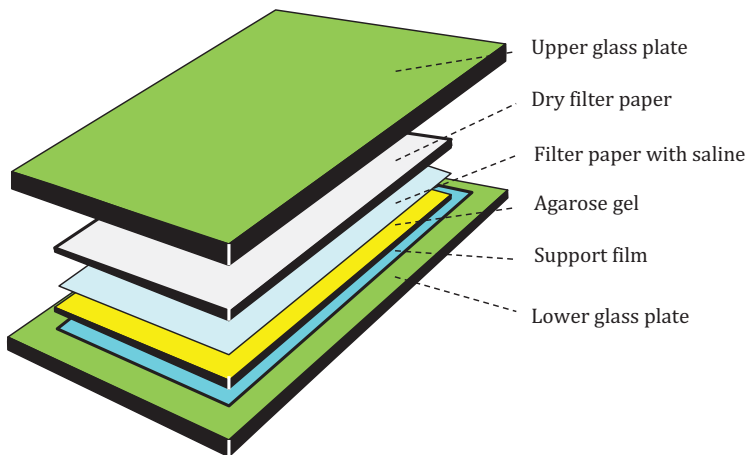


Figure 2.3.7: Dry pressing of an agarose gel.

Staining the protein bands

- Stain the gel in 0.2 g/dL Coomassie violet blue 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 two to three times for 2–3 min in 0.5 g/dL citric acid until the background is clear.
- Dry the gel with a dryer or in a drying oven.

Evaluation

Compare 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 0.5 to 1.5 mg/mL, and for λ chains is 1 to 2 mg/mL.

Immunofixation of Bence Jones Proteins**Materials and equipment**

TRIS-glycinate or TRIS-taurinate buffer

Agarose gel for immunofixation of Bence Jones proteins (light chains of paraproteins). It is located between two films. The lower film is bound to the gel, while the upper film can be easily removed from it.

Coomassie violet blue R-200 or Amido black 10B

Procedure**Sample preparing and applying**

- Use fresh urine samples. Most often, due to the low concentration of Bence Jones proteins, urine should be concentrated. A quick method for this purpose is a centrifugation in microcentrifuge concentrators. The final concentration of proteins in 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.
- Lay down a polyester template onto the blotted gel surface, according to both arrows on the support film (Figure 2.3.5), and press gently the template to remove the air bubbles underneath.
- Apply 2–3 μ L of the sample onto the ELP slot as well as onto the slots for GAM, κ , λ , κ free and λ free.
- Wait 5 min until the samples diffuse into the gel.
- Blot the exceeded sample with a filter paper and remove it, together with the template, from the gel.

Electrophoresis

- Hang the support film, with the gel 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 (see above).
- Close the electrophoretic cell with its lid and turn on the power supply.
- Run ELP at constant voltage (80 V) or constant electric current (20–25 mA) for 20–25 min at room temperature.

Immunoprecipitation

- After ELP, blot the gel surface with a thin filter paper.
- Place an antiserum template onto the gel and press it gently (Figure 2.3.6).
- Apply 50 μL of a polyvalent antiserum into the ELP reference bed and 50 μL each of the appropriate antisera (GAM, κ , κ -free, λ , and λ -free) into the test beds.
- Incubate the gel with antisera for 30 min on wet filter paper at room temperature.
- After incubation, remove the antiserum template and press the gel 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 (see above).
- Soak the gel in saline for 10 min.
- Press the gel and soak it in saline another two times.
- Dry the gel with a hairdryer or at room temperature.

Staining the serum immune complexes

- Stain the immune complexes in the gel with Coomassie violet blue R-200 or Amido black blue 10B (see above) for 10 min.
- Destain the gel background in a destaining solution.
- Dry the gel with a hairdryer or at room temperature.

Evaluation

The results can be evaluated by comparing the precipitation strips in the test beds with the precipitation strips in the reference ELP bed. The lower concentration limit for establishing monoclonal κ -chains is 0.5–1.5 mg/mL, and for α -chains is 1–2 mg/mL.

Immunoprobings with Avidin-biotin Coupling to Secondary Antibody**Materials and equipment**

TBS (TRIS-buffered saline)

TBST – mixture of TBS and Tween 20

Avidin

Biotinylated horseradish peroxidase (HRP) or alkaline phosphatase (ALP)

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 blotted proteins into a plastic bag containing the primary antibody solution.
- Incubate at room temperature for 30 min.
- Transfer the membrane from the plastic bag into 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 in 50 to 100 mL TTBS (for nitrocellulose or PVDF membranes) or TBS (for nylon membranes).
- Incubate at room temperature for 30 min, then wash.
- Prepare avidin-biotin-HRP (or avidin-biotin-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.
- Add TBST or TBS to 50 mL.
- 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.

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2.4 Affinity electrophoresis

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The affinity electrophoresis [1, 2] can be used for characterization of polyions, for example, for estimating binding constants in the lectin electrophoresis. It is suitable for studying isoenzymes (isozymes), e.g., of lactate dehydrogenase [3], alcohol dehydrogenase [4], or plasminogen [5], and above all of alkaline phosphatase [6].

2.4.1 Theory of affinity electrophoresis

The affinity electrophoresis represents zone electrophoresis in an agarose or polyacrylamide gel, which contains specific ligands, as lectins, enzyme substrates, or other substances. These ligands are not bound to the gel, opposite to the ligands in affinity chromatography. They form complexes with corresponding polyions, which complexes have other electric charges and 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 [7–9]. In lectin electrophoresis, wheat germ lectin (**w**heat **g**erm **a**gglutinin, WGA) is used for separating **a**lkaline **p**hosphatase (ALP) isoenzymes. ALP (orthophosphate monoester hydrolase, EC 3.1.3.1) is a

glycoprotein, which is built of two monomers connected by a Zn^{2+} . It catalyzes the hydrolytic splitting of terminal phosphate groups of organic phosphoric esters in alkaline solutions (Figure 2.4.1).

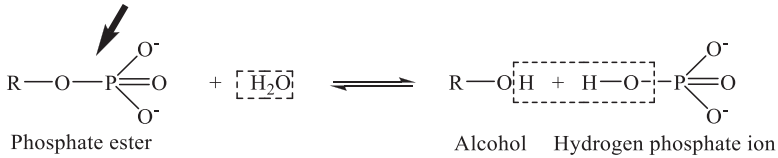


Figure 2.4.1: Hydrolysis of a phosphate ester by alkaline phosphatase.

The alkaline phosphatase consists of two polypeptide chains, which are chelated by a Zn^{2+} . The polypeptide chains are bound two by two forming a few alkaline phosphatase isoenzymes. They are encoded by three genes: a tissue unspecific gene, a colon gene, and a placental gene.

2.4.2.1 Electrophoresis of alkaline phosphatase isoenzymes on lectin agarose gels

Using affinity electrophoresis, following serum ALP isoenzymes are established: liver, bone, small intestine, placental, and macro-ALP isoenzyme (Figure 2.4.2).

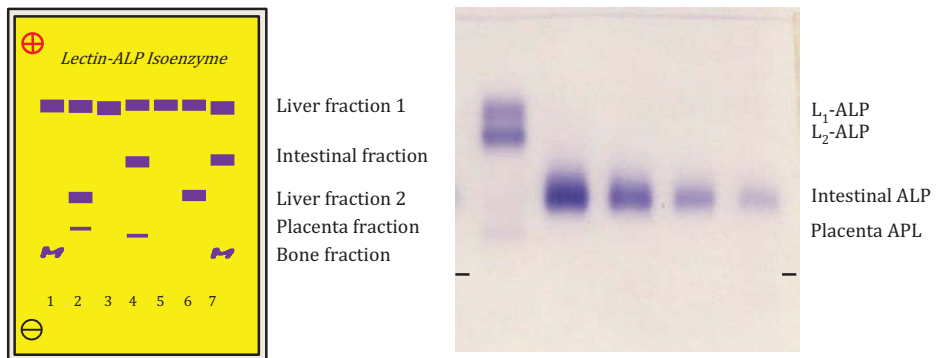


Figure 2.4.2: Alkaline phosphatase isoenzymes separated by affinity electrophoresis.

Liver ALP isoenzyme. The liver ALP isoenzyme contains *N*-acetylneuraminic (sialic) acid in very low concentration. It is heat stable and is present in two forms: L₁ or fast liver ALP; and L₂ or slow liver ALP. L₁-isoenzyme, together with the small intestine ALP isoenzyme, is found in healthy adult people. L₂-isoenzyme is rarely detected. It is located at the cathode side of L₁ in a lectin agarose gel. L₂ 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. Therefore, it binds immediately to the wheat germ lectin in agarose gels and comes to a standstill in front of the sample application place. The bone ALP isoenzyme is heat labile. Its mass is also twice greater than the mass of the liver ALP isoenzyme in both genders.

Small intestine ALP isoenzyme. The small intestine ALP isoenzyme is found in 10% of human population. In the blood serum, up to three bands of it can be detected.

Placental ALP isoenzyme. Placental ALP isoenzyme exists in two forms: P₁ (90%) and P₂ (10%). They appear in blood serum during pregnancy or at cancer of the ovary, pancreas, stomach, and colon, as well as at sarcomas [10, 11].

Macro-ALP isoenzyme. The macro ALP isoenzyme is rarely found. It is a complex between alkaline phosphatase and an immunoglobulin. Since its molecular mass is too large, the macro ALP isoenzyme remains at the application place.

As pointed out, the liver and bone ALP isoenzymes differ from each other only by the concentration of their *N*-acetylneuraminic acid. They can be separated electrophoretically from each other in the presence of Triton X-100 and wheat germ lectin. Triton X-100, a nonionic detergent, 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 stops in front of the sample application place and forms a tooth-shaped precipitate [12, 13].

After electrophoresis, colorimetric detection of the resolved isoenzyme bands can be carried out. For this purpose, disodium salt of 5-bromo-4-chloro-3-indolyl phosphate (Na₂BCIP) is hydrolyzed giving the dimer 5-bromo-4-chloro-3-indolyl (BCI)₂ and disodium hydrogen phosphate [14] (Figure 2.4.3).

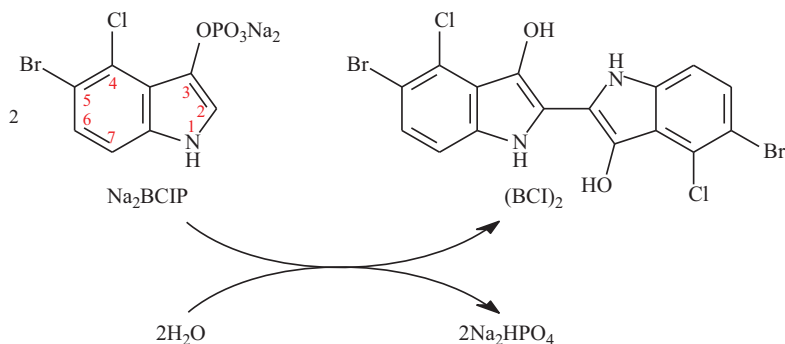


Figure 2.4.3: Hydrolysis of Na₂BCIP giving a dimer (BCI)₂ and disodium hydrogen phosphate.

Na₂BCIP is a colorless compound whereas (BCI)₂ is a blue dimer. The inorganic phosphate esterifies a serine residue in the neighborhood of the active site of alkaline phosphatase, which transfers afterward the inorganic phosphate onto the

aminoalcohol 2-**amino-2-methyl-1-propanol** (AMPro). So, the enzyme inhibition is avoided. The colored dimer shows which protein bands contain ALP activity.

The alkaline phosphatase is widely distributed in the human body. Its concentration is age-dependent and is elevated during the active bone growth. The activity of serum ALP is high in all bone disorders accompanied by increased osteoblastic synthesis, for example, in Paget's disease (osteitis deformans), hyperparathyroidism, rickets, osteomalacia, and osteoblastic tumors with metastases. Low serum ALP activity is found in hypothyroidism, hypophosphatasemia, pernicious anemia, and dwarfism.

2.4.3 Saccharide affinity electrophoresis

Glycosylation plays an important role in protein modification. To separate glycoproteins, Jackson *et al.* [15] used the boron compound [3-(methacryloylamino)-phenyl]boronic acid (MPBA) as ligands (probes) (Figure 2.4.4).

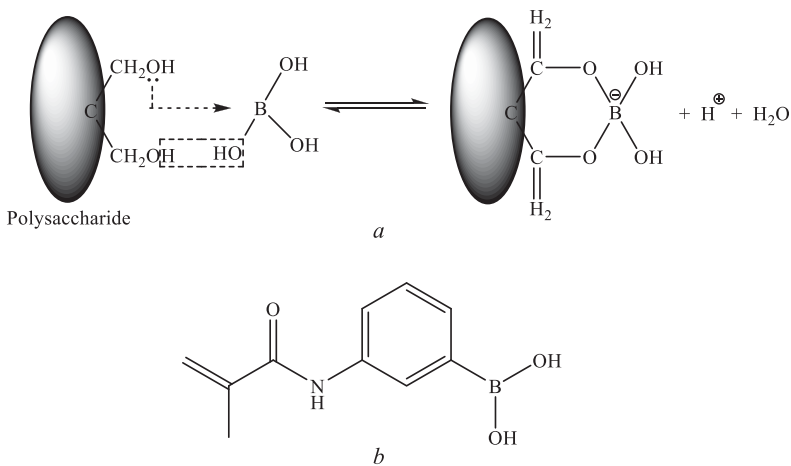


Figure 2.4.4: Saccharide affinity electrophoresis.

(a) Reversible bonding between a boron compound and a polysaccharide; (b) MPBA.

In **boronate affinity saccharide electrophoresis** (BASE), MPBA (in concentration of 0.5–1 g/dL) is immobilized by copolymerization in a polyacrylamide gel, where it operates as an affinity probe for fructose and linear polyalcohols of saccharide derivatives containing sialic acids. Phenyl boronic acids also function as saccharide receptors in aqueous solution [16–18] and in polyacrylamide gels when incorporated there [19–21]. They form cyclic boronic esters with various carbohydrates by reversible covalent interactions. As a result, the saccharides

migrate slower during gel electrophoresis. Morais *et al.* applied the same technique for serum glycoproteins of diabetic patients [22].

2.4.4 Affinity supported molecular matrix electrophoresis

To resolve mucins, Matsuno *et al.* established supported molecular matrix electrophoresis (SMME) [23–25]. In this method, the fibers of a porous hydrophobic polyvinylidene difluoride (PVDF) membrane, used as a support matrix, are coated with a hydrophilic polymer to resolve proteins or lipids. Later, Matsuno and Kameyama [26] combined SMME with affinity electrophoresis, binding to the hydrophobic fibers probes as lectins, glycolipids or antibodies, and hydrophilizing the fibers. Thus, they established the affinity SMME (ASMME). Migrating through this system, polyions interact with the probes and resolve (Figure 2.4.5).

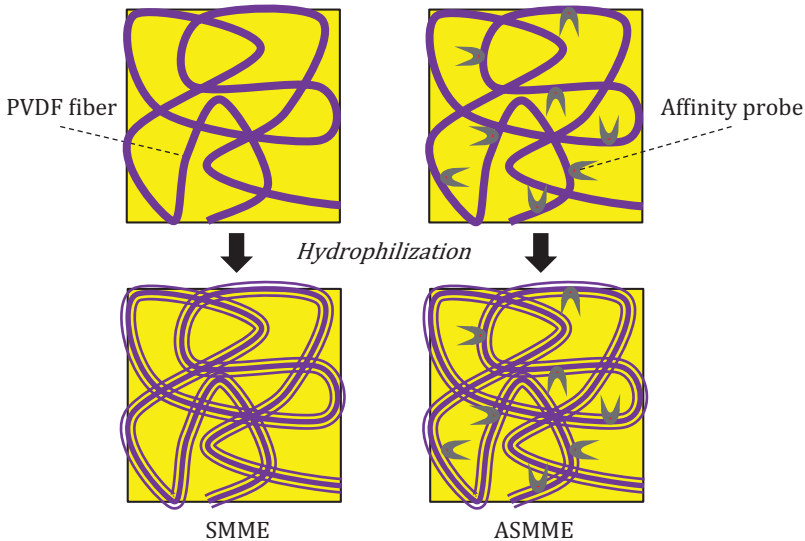


Figure 2.4.5: SMME and affinity SMME.

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 hydrophilization of the PVDF membrane.

2.4.5 Phosphate affinity electrophoresis

In phosphate affinity electrophoresis (Phos-tag PAGE), Phos-tag [27] binds specifically to the divalent phosphate ions in neutral solutions (Figure 2.4.6). It ($M_r = 595$)

is developed by Hiroshima University for simultaneous analysis of a phosphoprotein isoform and its non-phosphorylated counterpart.

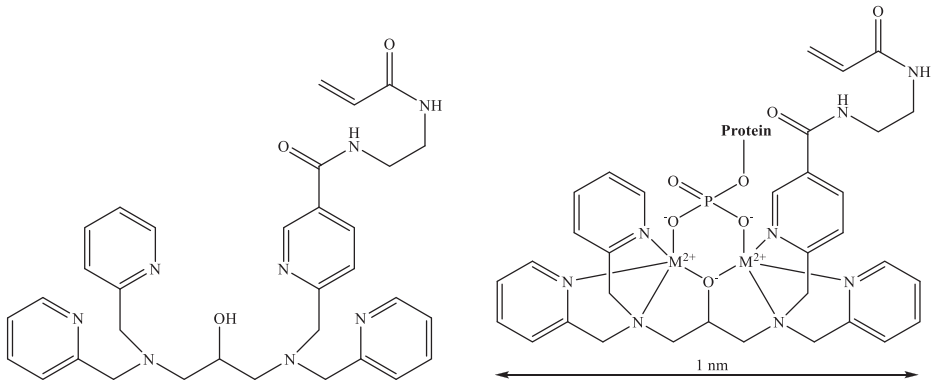


Figure 2.4.6: Phos-tag (*left*) and Phos-tag bound to phosphoprotein (*right*).

By applying the Laemmli SDS system [28] in Phos-tag gels, the Phos-tag SDS PAGE is developed [29, 30]. During it, a M^{2+} -Phos-tag complex (Figure 2.4.7) is formed, which binds to phosphate groups in gels with $\text{pH} > 9$.

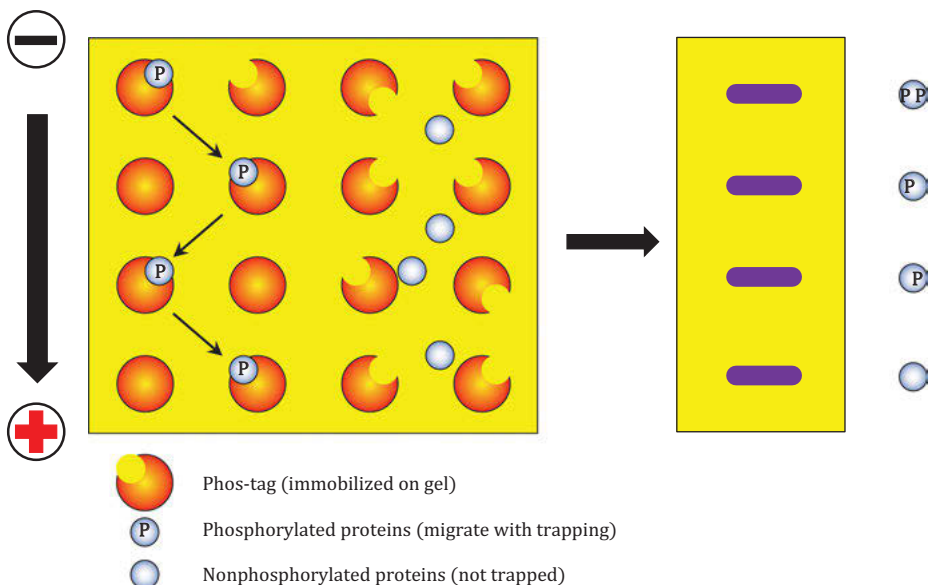


Figure 2.4.7: Phosphate affinity SDS electrophoresis (Phos-tag SDS PAGE).

The copolymerization of Phos-tag with acrylamide creates a gel that binds phosphorylated proteins and resolves them. $M^{2+} = \text{Zn}^{2+}$ or Mn^{2+} .

Using Mn^{2+} -Phos-tag SDS PAGE, the myosin light chain can be analyzed quantitatively. 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.

2.4.6 Capillary affinity electrophoresis

In capillary affinity electrophoresis (CAE) samples and probes are mixed, and the resulting complexes are separated by capillary electrophoresis (Figure 2.4.8). The 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 resolved bands.

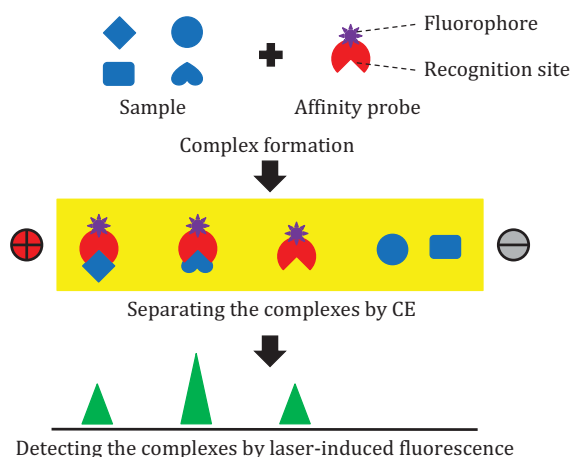


Figure 2.4.8: Principle of capillary affinity electrophoresis.

Shimura and Karger [31] used the fluorophore-labeled fragment **antigen-binding** (Fab) region of an immunoglobulin as an affinity probe for CAE of human growth hormone. Later, Shimura and Kasai [32] prepared an affinity probe from a recombinant immunoglobulin against human insulin.

2.4.7 Affinity-trap electrophoresis

The affinity-trap PAGE (AT-PAGE) was developed by Awada *et al.* [33]. In this method protein samples are separated by normal PAGE and then 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 2.4.9).

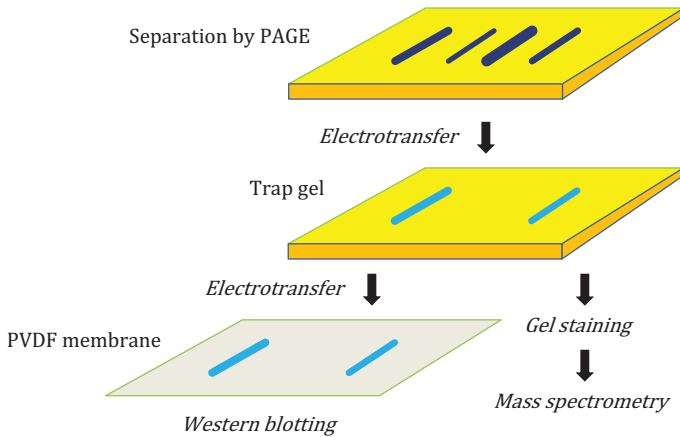


Figure 2.4.9: Principle of affinity-trap electrophoresis.

AT-PAGE is used for analyses of expressional proteins and their modifications.

2.4.8 Charge shift electrophoresis

Charge shift electrophoresis (CSE) is a simple and rapid method for distinguishing hydrophilic and amphiphilic proteins. It was introduced by Helenius and Simons [34]. They proved that the electrophoretic mobility of hydrophilic proteins stayed unaffected in detergent solutions; however, the mobility of amphiphilic proteins shifted anodally in Triton X-100-deoxycholate system and cathodally in Triton X-100-cetyltrimethylammonium bromide system.

2.4.9 Mobility shift electrophoresis

Mobility shift electrophoresis (MSE, band shift assay) is an affinity electrophoresis technique used to study if a protein or a protein mixture is capable of binding to a given DNA or RNA sequence. With its help, 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 was described by Garner and Revzin [35], and Fried and Crothers [36].

In the MSE, a protein-nucleate complex is built that is larger than the single protein polyion. Therefore, it moves slower forming another band that is shifted up in the gel (Figure 2.4.10).

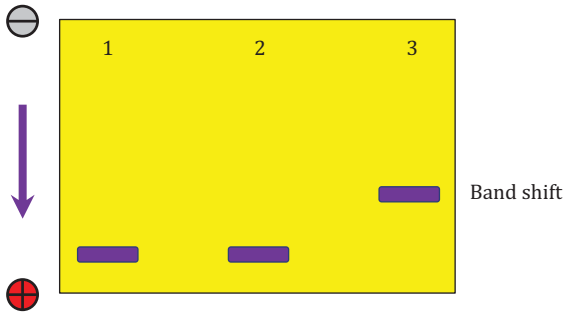


Figure 2.4.10: Mobility shift electrophoresis.

Lane 1 contains genetic material. Lane 2 contains a protein as well as a DNA fragment that does not interact with the protein. Lane 3 contains a protein and a DNA fragment that react with each other forming a larger, heavier and slower-moving complex. 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 the MSE are TRIS-acetate-EDTA and TRIS-borate-EDTA buffers. Inclusion of **bovine serum albumin (BSA)** or other carrier proteins in the gel improves the stability of some complexes during electrophoresis.

The MSE 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 have $T = 4\text{--}5$ g/dL and $C = 0.025$.
- Binding of a protein mixture to a DNA probe.
- Electrophoresis of the protein-DNA complexes in a gel, which afterward is dried and autoradiographed.

To create larger complexes with greater shift, an antibodies against the protein can be added to the protein-nucleate mixture. This method is referred to as a *mobility super-shift electrophoresis*, and is used to identify proteins in protein-nucleic acid complexes.

2.4.10 Protocols

Electrophoresis of ALP Isoenzymes on Lectin Agarose Gels

Materials and equipment

Ready-to-use agarose gels

Electrophoresis cell

Power supply

Procedure

- Remove the cover film from an ALP isoenzyme agarose gel.
- Blot the gel briefly with a filter paper strip where samples will be applied.
- Lay down an application template on the blotted place adjusting it according to the arrows on the support film.
- Press gently the template with fingers to push out air bubbles between the template and gel.
- Dilute the sera up to an ALP activity of 200–1 000 U/L and mix them with a sample buffer at a volume ratio of 1:1. If cerebrospinal fluid or urine is analyzed, concentrate it up to the same activity and mix it with the sample buffer.
- Apply 5 μ L of serum, cerebrospinal fluid or urine in the template slots and let the samples diffuse in the gel for 5 min.
- Blot the rest of the samples with a filter paper strip and remove the template and strip.
- Fill the tanks of an electrophoresis cell with electrode buffer.
- Hang the film-supported gel on the bridge of the electrophoresis cell, the gel side down.
- Place the bridge with the gel into the electrophoresis cell so that 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.

Staining the ALP Isoenzymes

Materials and equipment

To stain ALP isoenzymes, two substrate solutions are needed: solution 1 and solution 2. Substrate solution 1 is an AMPro-Cl buffer (95% 2-**a**mino-2-**m**ethyl-1-**p**ropanol corrected with HCl to pH = 10.2). Substrate 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: Contents of solutions for ALP isoenzymes.

Substrate solution 1, 6x		Substrate solution 2, 24x	
AMPro	14.23 mL	98% NBT	0.83 g
37% HCl	4.14 mL	Methanol to	100.00 mL
0.1 g/dL ZnCl ₂	6.82 mL		

Table 2.4.1 (continued)

Substrate solution 1, 6x	Substrate solution 2, 24x
10 g/dL MgCl ₂	1.02 mL
10 g/dL Na ₂ BCIP*	2.57 mL
NaN ₃	0.10 g
Deionized water to	100.00 mL

*Na₂BCIP = 5-bromo-4-chloro-3-indolyl phosphate disodium salt.

Procedure

- Make 30 mL (4:1:5, V:V:V) substrate solution (5.0 mL Substrate solution 1 + 1.25 mL Substrate solution 2 + 23.75 mL deionized water).
- Immerse the gel (gel surface upward) in the substrate solution.
- Incubate at 37 °C for 30 min.
- Inactivate the ALP isoenzymes in methanol-acetic acid-water (4:1:5, V:V:V) for 30 min. To make 30 mL solution, mix 12 mL methanol with 3.0 mL acetic acid and 15.0 mL deionized water).
- Wash the gel in tap water for 5 min and rinse it three times in deionized water.
- Dry the gel using a hair dryer, a drying oven, or at room temperature.
- Document the ALP isoenzyme bands using a scanner, photo camera, or densitometer at 580 nm.

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2.5 Polyacrylamide gel zone electrophoresis of proteins

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The polyacrylamide gel zone electrophoresis of proteins can be carried out in homogeneous or gradient gels.

2.5.1 Homogeneous gel zone electrophoresis of proteins

The **polyacrylamide gel electrophoresis (PAGE)** is used for separating proteins with relative molecular masses M_r from 5×10^3 to 2×10^6 . The separation depends on the protein mobilities and polyacrylamide gel properties. The *native PAGE* is used for native proteins, for example, enzymes. The *denaturing PAGE* is used to separate proteins, denatured by SDS or other detergents.

The gels for *vertical electrophoresis* usually have 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 should contain 10–20 g/dL glycerol or sucrose to delay their diffusion in the electrode buffer.

The gels for *horizontal electrophoresis* are usually cast on a support film. The samples are applied onto the gel using a silicone template. The horizontal electrophoresis in thin homogeneous or gradient gels, cast on support films, has many advantages over the vertical electrophoresis [1]: they are easily 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.

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2.5.1.1 Theory of polyacrylamide gel zone electrophoresis of proteins

The polyacrylamide gel zone electrophoresis is running in one buffer. It is contained in the gel, sample, and electrode tanks. The resolution of the electrophoresis depends on the volume and electric charge of the proteins. The electric charge of a protein is a function of the dissociation degree of its chemical groups and depends on the pH value and ionic strength of the buffer.

The ionic strength of a buffer should be relative low to keep heat generation at a minimum. On the other hand, the very low ionic strength lowers electrophoretic resolution. Therefore, the ionic strength of electrophoresis buffers should have middle values 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, because they are negatively charged; in acidic buffers, they migrate to the cathode, because they are positively charged. The sample is applied on the cathode or anode side of the gel, respectively.

In electrophoresis in alkaline buffers, Bromophenol blue Na salt could be added to the sample; and in electrophoresis in acidic buffers, Fuchsin red could be added to the sample. These dyes overrun the proteins and show their movement in the gel.

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).

Solutions	Concentrations	<i>T</i> = 5 g/dL	<i>T</i> = 12.5 g/dL	<i>T</i> = 20 g/dL
Buffer–gel system for acidic proteins				
TRIS-glycinate buffer, 4x (0.8 mol/L, pH = 8.3) or TRIS-chloride buffer, 4x (1.5 mol/L, pH = 8.8)	0.2 mol/L	4.0 mL	4.0 mL	4.0 mL
Monomeric solution (<i>T</i> = 50 g/dL, <i>C</i> = 0.03)	<i>T</i> = 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

Table 2.5.1 (continued)

Solutions	Concentrations	$T = 5 \text{ g/dL}$	$T = 12.5 \text{ g/dL}$	$T = 20 \text{ g/dL}$
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 ($T = 50 \text{ g/dL}$, $C = 0.03$)	$T = 5\text{--}20 \text{ g/dL}$	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

c_{APS} Was calculated according to the formula $c_{\text{APS}} = 0.32/T$, which we propose.

2.5.1.2 McLellan buffers

McLellan proposed a set of buffers, which are applicable for electrophoresis of native proteins [2]. These buffers cover a 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

Other buffers that have been used for native zone electrophoresis are: TRIS-glycinate (pH range 8.3–9.5) [3], TRIS-acetate (pH range 7.2–8.5) [4], and TRIS-borate (pH range 8.3–9.3) [5] buffers. Borate ions $[B(OH)_4^-]$ form complexes with some sugars; therefore, they influence the resolution of glycoproteins.

2.5.1.3 Running electrophoresis

The contact between the electrodes and polyacrylamide gel can be realized *via* filter paper bridges impregnated with electrode buffer. They should be parallel to the gel ends, and overlap 1 cm of the gel ends.

An alternative to the paper bridges are the electrode strips. They can be composed of gel (usually polyacrylamide gel) or thick filter paper. The strips contain electrode buffer(s). The electrodes are placed onto the electrode strips (Figure 2.5.1).

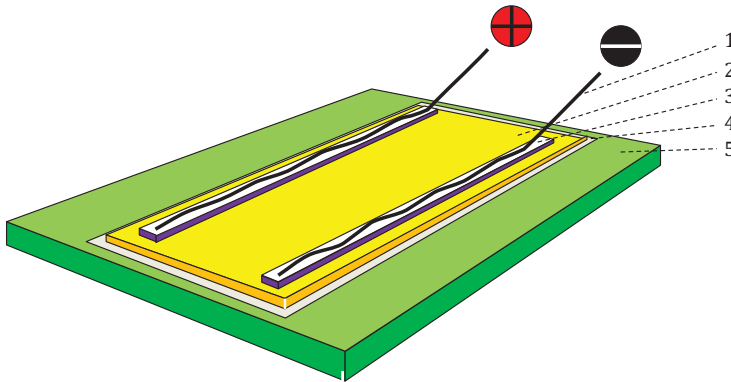


Figure 2.5.1: Electrode strips as contacts between gel and electrodes. 1. Electrode; 2. gel; 3. electrode strip; 4. support film; 5. cooling plate.

The electrophoretic conditions depend on buffer composition and gel thickness. The voltage can be in the range 200–500 V and should not be changed during electrophoresis. The cooling temperature is recommended to be 10 °C. If required, the gels can be cooled to 2–4 °C.

2.5.2 Gradient gel zone electrophoresis of proteins

The gradient polyacrylamide gels were introduced by Kolin [6] and improved by Margolis and Kenrick [7]. The electrophoresis is usually carried out in same buffers. The *T*-concentration of the polyacrylamide gels increases continuously from one gel

end to the other gel end, which results in a continuous decrease of the pore diameters. Therefore, the samples, which are applied on the large porous gel end, first are moving fast, later, however, their velocities decrease gradually because they begin to migrate through the small porous gel.

The gradient gels can be used to identify the molecular masses and radii of native proteins [8], comparing the velocities of tested proteins with the velocities of proteins with known masses and radii.

2.5.2.1 Theory of gradient gel zone electrophoresis

Let us consider the movement of a charged particle in a linear concentration gradient. The velocity v of the 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

$$v = \frac{dl}{dt} = al \quad (2.5.1)$$

hence

$$\frac{dl}{l} = a dt \quad (2.5.2)$$

The integration of eq. (2.5.1) in the intervals l_1, l_2 and t_1, t_2 gives the expressions

$$\int_{l_1}^{l_2} \frac{dl}{l} = \int_{t_1}^{t_2} a dt \quad (2.5.3)$$

and

$$\ln \frac{l_2}{l_1} = at \quad (2.5.4)$$

which can be transformed in

$$e^{at} = \frac{l_2}{l_1} \quad (2.5.5)$$

It follows from eq. (2.5.1) and (2.5.5) that

$$l_1 = \frac{v_1}{a} \quad (2.5.6)$$

and

$$l_2 = \frac{v_1}{a} e^{at} \quad (2.5.7)$$

So, the distance

$$l_2 - l_1 = \frac{v_1}{a} (e^{at} - 1) \quad (2.5.8)$$

and the modified linear velocity

$$v_2 - v_1 = v_1 (e^{at} - 1) \quad (2.5.9)$$

Let us now analyze the movement of the zone l_1, l_2 through a linear concentration gradient, if the initial zone width $l'_1 - l_1 = d_1$, and the final zone width $l'_2 - l_2 = d_2$. Then, it follows from eq. (2.5.5) and eq. (2.5.9) that

$$d_2 = e^{at} (l'_1 - l_1) = d_1 e^{at} = d_1 \frac{v_2}{v_1} \quad (2.5.10)$$

The polyionic velocity decreases during electrophoresis, i.e. $v_2 < v_1$. Therefore, according to eq. (2.5.10), the final width of the band is narrower than its initial width, i.e. $d_2 < d_1$.

The gradient gel typically has $T = 4\text{--}30$ g/dL (0.5 to 4.22 mol/L) (Figure 2.5.2). Margolis and Kenrick used the TRIS-borate-EDTA buffer of pH = 8.3 in the resolving gel as well as in the electrode tank. Other buffers for gradient zone electrophoresis are the TRIS-glycinate, TRIS-barbitalate, and TRIS-taurinate buffers.

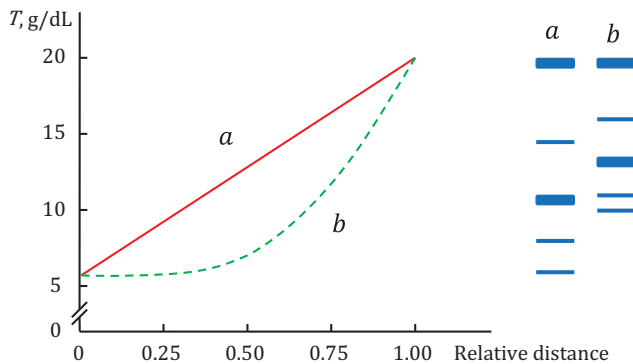


Figure 2.5.2: Proteins separated in a linear (a) and an exponential (b) gradient gels. The polyacrylamide concentration T is 5–20 g/dL.

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.

2.5.2.2 Ferguson plots

The dependence of the polyionic mobilities on the gel concentration was studied by Kenneth Ferguson who analyzed the movement of the pituitary hormone in starch gels [9]. He described the linear dependence of the mobilities on polyion 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, i.e., they have 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), according to the equation

$$\mu_r = \frac{d}{d_0} = \frac{\mu}{\mu_0} \quad (2.5.11)$$

The line slope is referred to as the retardation coefficient K_R , i.e., 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 depend on the peptide chain length (its mass).

The relationship between the relative protein mobility and the gel concentration may be expressed by the equation

$$\log \mu_r = \log \mu_{0(r)} - K_R T_r \quad (2.5.12)$$

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 magnitudes.

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 straight 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 2.5.3).

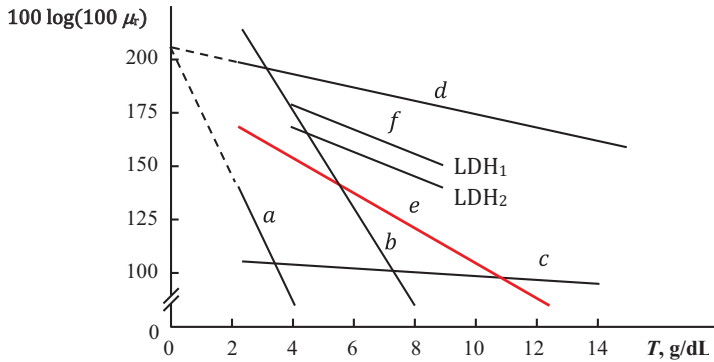


Figure 2.5.3: Ferguson plots: relative polyionic mobilities as a function of the polyacrylamide gel concentration T . The slopes of the lines are equal to K_R . (a) Polyion with a large mass and a low charge; (b) polyion with a large mass and a large charge; (c) polyion with a small mass and a small charge; (d) polyion with a small mass and a large charge; (e) polyion with an intermediate mass and an intermediate charge (typical case); (f) LDH₁ and LDH₂ – isoenzymes of lactate dehydrogenase, which have same masses but different charges.

2.5.2.3 Determination of Stokes radii and masses of native proteins

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

$$\{[\ln(\ln d_{r(\max)})]\}^{-1} = \alpha r_s + \beta \quad (2.5.13)$$

where α and β are constants. In order to determine the relative maximum migration distance, linear gradient gels with $T = 3\text{--}30$ g/dL and constant concentration of the cross-linker (BIS) is necessary. Simultaneously, the Stokes radius of a native protein is linked to its relative molecular mass M_r over the relationship

$$r_s = \epsilon M_r^{1/3} \quad (2.5.14)$$

where ϵ is a constant. Substituting eq. 2.5.13 in eq. 2.5.14, the equation

$$\ln\{[\ln(\ln d_{r(\max)})]\}^{-1} = \gamma (M_r)^{1/3} + \delta \quad (2.5.15)$$

is obtained where γ and δ are constants. The last equation shows γ 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 M_r of a protein from the relative maximum migration distance $d_{r(\max)}$ (Figure 2.5.4).

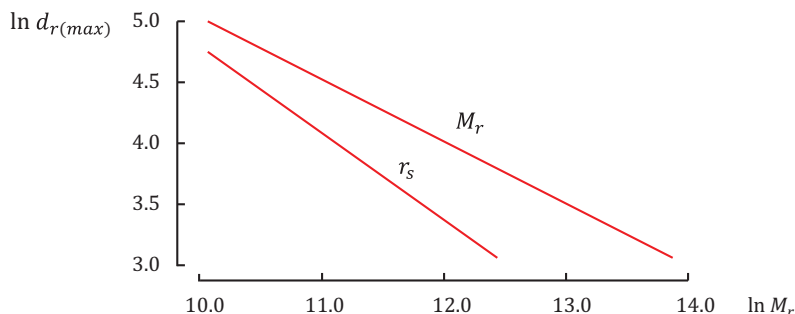


Figure 2.5.4: Calibration straight lines used for determination of Stokes radii r_s or relative molecular masses M_r of carbonic anhydrase from mammalian erythrocytes [1].

[1] Wagner H, Blasius E. Praxis der elektrophoretischen Trennmethode, 1989, 79.

Gradient gels give a better separation and sharpening of protein bands [10] under non-denaturing conditions. Best results are obtained in 3–30 g/dL gels [11] where the relative molecular masses of proteins, in the range of 13,000–950,000, can be determined.

When the mobility and radius of a protein are determined, its electric charge can also be calculated [12].

2.5.3 Blue native polyacrylamide gel electrophoresis

Blue native polyacrylamide gel electrophoresis (BN-PAGE) is a technique for precise resolving of proteins in native gels. It uses the anionic blue dye Coomassie brilliant blue G-250. Because of its hydrophobic properties, Coomassie brilliant blue G-250 binds unspecifically to all proteins, inclusively membrane proteins, and covers them with negative charges. The hydrophobic proteins lose their hydrophobicity 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 [13, 14]. After BN-PAGE, the visualization of proteins can be achieved by additional Coomassie brilliant blue staining, silver staining, or immunoblotting. Then, the protein bands can be analyzed by mass spectrometry [15].

BN-PAGE allows also detection, quantitation, and purification of 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) [16]. These enzymes, together with 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 [17, 18, 19].

2.5.4 Protocols

Horizontal Electrophoresis of Proteins on Gradient Gels

Materials and equipment

TRIS

HCl

Light and heavy monomeric (acrylamide/BIS) solutions

Sucrose

TMEDA

APS

Casting cassette with glass plates, spacers, and clamps

Gradient maker

4x TRIS-HCl buffer, pH = 8.8

TRIS 182.0 g (1.5 mol/L)

Deionized water 600.0 mL

Adjust with HCl to pH = 8.8.

Deionized water to 1,000.0 mL

Table 2.5.3: Light monomeric solutions (cited in the text).

Stock solution	Light monomeric solution (g/dL)									
	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

Procedure

- Assemble the casting cassette.
- Set up the gradient maker, close all its valves, and place a stir bar in the mixing chamber.
- Place the gradient maker on a magnetic stirrer.

Table 2.5.4: Heavy monomeric solutions (cited in the text).

Stock solution	Heavy monomeric solution (g/dL)											
	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	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	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	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	0.03	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	15.00

- Connect the outlet tubing of the gradient maker to a micropipette tip and place it onto the casting cassette.
- Pipette the high-concentration (heavy) solution into the mixing chamber and the low-concentration (light) solution into the reservoir chamber, and add APS.
- 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.
- Disassemble the casting cassette and take out the gel with the support film.
- Place the gel on a horizontal separation cell.
- Put polyacrylamide or paper strips, soaked with buffers, onto the gel poles.
- Place a silicone template on the gel in front of the cathode strip.
- Add to samples 10–20 g/dL glycerol and apply them in the slots of the template.
- Run the electrophoresis.
- Stain the protein bands with Coomassie brilliant blue or silver.

Blue Native Polyacrylamide Gel Electrophoresis

Preparation of Dialyzed Cell Lysate

Materials and equipment

BISTRIS
 6-Aminohexanoic acid (ϵ -aminocaproic acid)
 Sodium chloride
 Na_2EDTA
 Brij 96
 Digitonin
 Triton X-100
 Dialysis membrane for $M_r = 10,000\text{--}50,000$
 Centrifuge
 Microcentrifuge tubes
 Magnet stirrer
 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

The buffer should have pH = 7.4, if prepared properly.

BN-lysis (BN-dialysis) buffer, pH = 7.0

BISTRIS	0.42 (20 mmol/L)
ϵ -Aminocaproic acid	6.56 g (500 mmol/L)
Na_2EDTA	0.07 g (2 mmol/L)
NaCl	0.12 g (20 mmol/L)
Glycerol	10.00 mL
Deionized water to	100.00 mL

Adjust to pH = 7.0 with HCl. Store at 4 °C.

Detergent

0.5–2.0 g/dL Digitonin, 0.1–0.5 g/dL Brij 96, 0.1–0.5 mL/dL Triton X-100, 0.1–0.5 g/dL Dodecylmaltoside

The detergent should be determined empirically. Store in 5 mL aliquots at –20 °C.

Protease and phosphatase inhibitors

PMSF (phenylmethylsulfonyl fluoride)	0.02 g (1.0 mmol/L)
Sodium orthovanadate	0.01 g (0.5 mmol/L)
Deionized water to	100.00 mL

Procedure

- Obtain a cell pellet (10×10^6 cells) at 350 g and 4 °C for 5 min.
- Wash the pellet three times with ice-cold phosphate-buffered saline (PBS) buffer and centrifuge.
- Resuspend the pellet in 250 μ L 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 a heated Pasteur pipette, then place the tube on ice to cool down to 4 °C.
- Transfer the supernatant into the tube through the cap hole.
- Place a dialysis membrane for $M_r = 10,000$ on top of the opened tube, close the cap, and cut off the excess dialysis membrane.
- Seal the cap with Parafilm.
- Centrifuge the tubes at the lowest speed possible at 4 °C for 10 s.
- Prepare a 100 mL beaker with 10 mL of cold BN-dialysis buffer per 100 μ L sample.
- Affix the tube with tape upside-down inside the beaker, and remove air bubbles from the hole beneath the cap using a Pasteur pipette.
- Place the beaker onto the magnet stirrer, switch it on, and leave it run in a cold room overnight.
- Collect the dialyzed cell lysate in a new chilled microcentrifuge tube.

Casting Gradient BN Gels**Materials and equipment**

BISTRIS

HCl

Acrylamide/BIS solution ($T = 40$ g/dL, $C = 0.03$)

TMEDA

APS

Gradient maker

Silicone template

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

Procedure

- Close the clamp on the channel between the cylinders of the gradient maker, and the tubing clamp.
- Place a magnetic rod into the mixing cylinder.
- Input a syringe needle between the two glass plates of the casting cassette.
- Add APS to the light and heavy solutions.
- Pour the heavy solution into the mixing cylinder, and the light solution into the reservoir of the gradient maker.
- Switch on the magnetic stirrer.
- Open the clamps and force out the air bubble inside the channel between the cylinders.
- Let the mixed solution flow slowly between the glass plates to fill two-third of the cassette.
- Overlay the gradient solution in the cassette with deionized water.
- Let the solution polymerize at room temperature for 60 min.
- Add APS to the stacking solution.
- Pour the stacking solution with a pipette onto the gradient separating 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 brilliant blue G-250 (CBB G250)

Silicone template

Gel electrophoresis system

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.

Procedure

- Place paper strips, soaked with electrode buffer, onto the anode and cathode ends of the BN gel.
- Place the silicone template onto the stacking gel in front of the cathode strip.
- Load 1–40 μ L of the dialyzed lysate and 10–20 μ L of a marker mix into 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 (3–4 h for a minigel, and 18–24 h for a large gel).

Stain, if necessary.

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2.6 Isotachophoresis of proteins

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Isotachophoresis (ITP) is electrophoresis, where all co-ions (ions of same sign charges) migrate with same velocities.

2.6.1 Theory of isotachophoresis of proteins

The ITP is based on the theory of Kohlrausch [1]. In 1897, he studied a system of two strong electrolyte solutions, where the solution containing the faster (leading) ion was located in front of the solution containing the slower (trailing) ion. Kohlrausch noted that, if electric current was passed through this system, a moving ionic boundary was formed between the two solutions over the time. He called this phenomenon *persistent function* (now bearing his name – *Kohlrausch regulating function*) and derived electrochemical equations for the ion concentrations at the boundary. The proteins or other polyions arrange themselves between the leading and trailing ions.

Later, Longworth [2], Svensson [3], Alberty [4], and Dismukes and Alberty [5] obtained equations for moving boundaries 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. The leading ion is usually smaller, for example, chloride ion; and the trailing ion is usually larger, for example glycinate ion.

2.6.1.1 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; the mobility of ion A^- being higher than the mobility of ion B^- , i.e. $|\mu_{A^-}| > |\mu_{B^-}|$. Both buffers contain the same base B that associates a proton to build the counter-ion HC^+ (Figure 2.6.1).

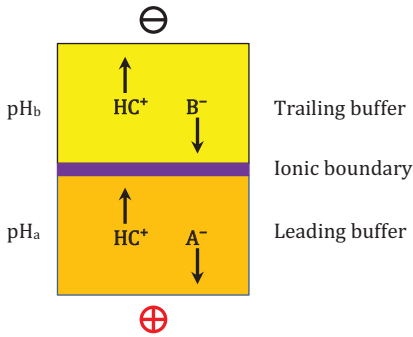


Figure 2.6.1: Scheme of buffer system for isotachopheresis.

A⁻ – leading ion; B⁻ – trailing ion; HC⁺ – counter-ion; pH_a and pH_b – pH values of the leading and trailing buffers, respectively

Let us determine when the ions A⁻ and B⁻ will move with same effective velocity v' , i.e., when

$$v'_{A^-} = v'_{B^-} \quad (2.6.1)$$

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 , i.e.

$$v'_{A^-(B^-)} = \mu'_{A^-(B^-)} E_{a(b)} = \alpha_{HA(HB)} \mu_{A^-(B^-)} E_{a(b)} \quad (2.6.2)$$

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 m²/(s V); and $E_{a(b)}$ is the electric field strength in the leading (trailing) buffer. The strength of the electric field is given by the following equation

$$E_{a(b)} = \frac{J}{\gamma_{a(b)}} \quad (2.6.3)$$

where J is the density of the total ionic current, in A/m²; and $\gamma_{a(b)}$ is the specific electric conductivity of the leading (trailing) buffer, in S/m.

It follows from eqs. (2.6.1)–(2.6.3) that

$$\frac{\alpha_{HB} \mu_{B^-}}{\gamma_b} = \frac{\alpha_{HA} \mu_{A^-}}{\gamma_a} \quad (2.6.4)$$

The specific conductivity

$$\gamma_{a(b)} = F \sum c_{A^-(B^-)} z_{A^-(B^-)} \mu_{A^-(B^-)} \quad (2.6.5)$$

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⁻).

From eqs. (2.6.4) and (2.6.5), the equation

$$\frac{\alpha_{HB}\mu_{B^-}}{\alpha_{HB}C_{HB}z_{B^-} - \mu_{B^-} + \alpha_{Cb}C_{Cb}z_{HC^+} + \mu_{HC^+}} = \frac{\alpha_{HA}\mu_{A^-}}{\alpha_{HA}C_{HA}z_{A^-} - \mu_{A^-} + \alpha_{Ca}C_{Ca}z_{HC^+} + \mu_{HC^+}} \quad (2.6.6)$$

results, where $c_{Ca(b)}$ is the concentration of base C, in mol/L, in the leading (trailing) buffer.

According to the law of electrical neutrality of a chemical solution,

$$\alpha_{HA(HB)}C_{HA(HB)}z_{A^-(B^-)} + \alpha_{Ca(b)}C_{Ca(b)}z_{HC^+} = 0 \quad (2.6.7)$$

Hence, eq. (2.6.6) can be transformed into the equation of Ornstein

$$\frac{C_{HB}}{C_{HA}} = \frac{z_{A^-} - \mu_{B^-} (\mu_{A^-} - \mu_{HC^+})}{z_{B^-} - \mu_{A^-} (\mu_{B^-} - \mu_{HC^+})} = F_k \quad (2.6.8)$$

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, the trailing base B, and the common acid HC, the Ornstein equation obtains the following form:

$$\frac{C_B}{C_A} = \frac{z_{HA^+} + \mu_{HA^+} (\mu_{HA^+} - \mu_{C^-})}{z_{HB^+} + \mu_{HA^+} (\mu_{HB^+} - \mu_{C^-})} = F_k \quad (2.6.9)$$

At first glance, the Kohlrausch regulating function, i.e. the dependence of the ionic concentration on the ionic mobility, looks incomprehensible. However, it can be explained with the discontinuously distributed electric field strength: its strength in the zone of the trailing buffer is higher than in the zone of the leading buffer. So, a potential gradient is formed in the ionic boundary between the leading and trailing ions.

If a leading ion enters the zone of trailing buffer, it begins to move quicker at the higher voltage and overtakes the ionic boundary. If a trailing ion penetrates into the zone of leading buffer, its velocity decreases due to the lower voltage and the ionic boundary overtakes it. Polyions, which have intermediate effective mobilities, staple in a decreasing turn between the leading and trailing ions and build with them a migration sandwich-like structure (Figure 2.6.2).

To concentrate a polyion, the effective mobility of leading ion A^- has to be greater, and the effective mobility of trailing ion B^- has to be smaller than the effective mobility of the polyion, hence, the following inequation

$$|\alpha_{HA}\mu_{A^-}| > |\alpha_{Hn}P\mu_{P^{n-}}| > |\alpha_{HB}\mu_{B^-}| \quad (2.6.10)$$

should be respected, where P^{n-} is the polyion to be analyzed. For example, if at 0 °C protein polyion P^{20-} [$\mu_{P^{20-}} = -5 \times 10^{-9} \text{ m}^2/(\text{s V})$] has to be concentrated at a front of chloride ion [$\mu_{Cl^-} = -37 \times 10^{-9} \text{ m}^2/(\text{s V})$] against TRIS-ion [$\mu_{HT^+} = 9 \times 10^{-9} \text{ m}^2/(\text{s V})$], the Kohlrausch regulating function will be

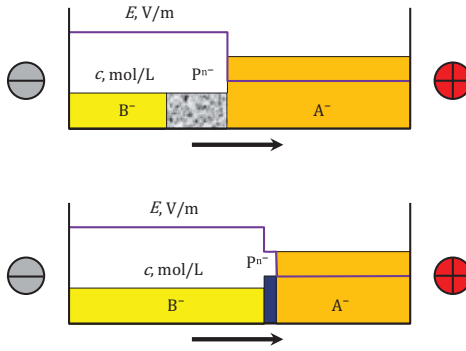


Figure 2.6.2: Start of electrophoresis (top) and concentrating of polyions p^{n-} in the moving ionic boundary (bottom). A^- – leading ion; B^- – trailing ion

$$F_k = \frac{c_{H_2O P}}{c_{HCl}} = \frac{(-1)(-5 \times 10^{-9})(-37 \times 10^{-9} + 9 \times 10^{-9})}{(-20)(-37 \times 10^{-9})(-5 \times 10^{-9} + 9 \times 10^{-9})} = 0.022$$

This means that, if the concentration of HCl is 0.06 mol/L, the protein concentration will reach a concentration of

$$0.022 \times 0.06 = 0.0013 \text{ mol/L}$$

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2.7 Disc-electrophoresis of proteins

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Discontinuous electrophoresis (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 is discontinuously distributed.

The term *discontinuous* buffer system was introduced in 1957 by Poulik [1] after he found that the electrophoretic resolution increases, if the starch gel contains a citrate buffer and the electrode tanks contain a borate buffer. We claim that one buffer can also form a discontinuous buffer system, if the buffer is divided in two or more parts of different ionic strengths and different pH values [2].

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During disc-electrophoresis, the sample polyions are stacked at first by isotachopheresis, and then are resolved by zone electrophoresis. The isotachopheresis 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); the zone electrophoresis takes place in a small-pore polyacrylamide gel (resolving gel) with a T -concentration of 0.7–4.2 mol/L (5–30 g/dL). A gradient resolving gel can also be used, where the protein bands become sharper [3].

After disc-electrophoresis, the protein bands can be stained or blotted. The bands can be quantified also by a gel imaging device or by visualizing with UV light.

The disc-electrophoresis is used in medicine, molecular biology, genetics, microbiology, biochemistry, and forensics, for separating proteins in blood sera, cerebrospinal fluids, urines, or tissue extracts.

2.7.1 Theory of disc-electrophoresis

When the concentration of the leading electrolyte is given, the concentration of the trailing electrolyte can be calculated using the Ornstein equation. To do this, it is necessary to know the mobilities of the leading, trailing, and counter-ions. Thereafter, the pH values and the concentrations of the base and 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

$$\alpha_{\text{HB}} = \frac{\mu'_{\text{B}^-}}{\mu_{\text{B}^-}} \quad (2.7.1)$$

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

$$\text{pH}_b = \text{p}K_{\text{c}_{\text{HB}}} + \log \frac{\alpha_{\text{HB}}}{1 - \alpha_{\text{HB}}} \quad (2.7.2)$$

The dissociation degree of the base C in the trailing buffer, α_{C_b} , can also be determined from the Henderson–Hasselbalch equation

$$\alpha_{\text{C}_b} = \left(1 + 10^{\text{pH}_b - \text{p}K_{\text{c}_{\text{HB}^+}}}\right)^{-1} \quad (2.7.3)$$

The concentration of the base in the trailing buffer can be calculated from the equation of electrical neutrality of a solution, according to which

$$c_{\text{C}_b} = -c_{\text{HB}} \frac{\alpha_{\text{HB}} z_{\text{B}^-}}{\alpha_{\text{C}_b} z_{\text{HC}^+}} \quad (2.7.4)$$

It follows from the theory of moving ionic boundary and eq. (2.7.4) that

$$\frac{\alpha_{\text{HB}} c_{\text{HB}}}{c_{\text{HA}}} = \alpha_{\text{HB}} F_k = \frac{[\text{H}_3\text{O}^+_{\text{b}}]}{[\text{H}_3\text{O}^+_{\text{a}}]} \quad (2.7.5)$$

hence

$$\text{pH}_a = \text{pH}_b + \log(\alpha_{\text{HB}} F_k) \quad (2.7.6)$$

The dissociation degree of the base in the leading buffer, α_{Ca} , can also be calculated from the Henderson–Hasselbalch equation

$$\alpha_{\text{Ca}} = (1 + 10^{\text{pH}_a - \text{pK}_{\text{HC}^+a}})^{-1} \quad (2.7.7)$$

The concentration of the base in the leading buffer can be obtained from the equation of electrical neutrality of a solution

$$c_{\text{Ca}} = -c_{\text{HA}} \frac{\alpha_{\text{HA}} z_{\text{A}^-}}{\alpha_{\text{Ca}} z_{\text{HC}^+}} \quad (2.7.8)$$

The cationic buffer systems, which consist of bases A and B, and a common acid HC, obey similar equations.

The disc-electrophoresis can be carried out in native or denatured (SDS) polyacrylamide gels.

2.7.2 Native disc-electrophoresis

Two native 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 called disc-electrophoresis in one buffer at two pH values.

2.7.2.1 Disc-electrophoresis according to Ornstein and Davis

The theoretical basis of disc-electrophoresis was developed by Leonard Ornstein [4], and its practical implementation was made by Baruch Davis [5]. It requires three different buffers: stacking, resolving, and electrode buffers.

To explain the disc-electrophoresis according to Ornstein–Davis, we assume that the stacking and resolving buffers are assembled by the strong acid HA and weak base C; and the electrode buffer is composed of the weak acid HB and the same base. Hence, the stacking and resolving buffers contain the leading ion A^- and counter-ion HC^+ , and the electrode buffer contains the trailing ion B^- and the same counter-ion HC^+ (Figure 2.7.1).

Let us calculate the concentrations and pH values of a buffer system, which is suitable for disc-electrophoresis of serum proteins at 0 °C. Most serum proteins have

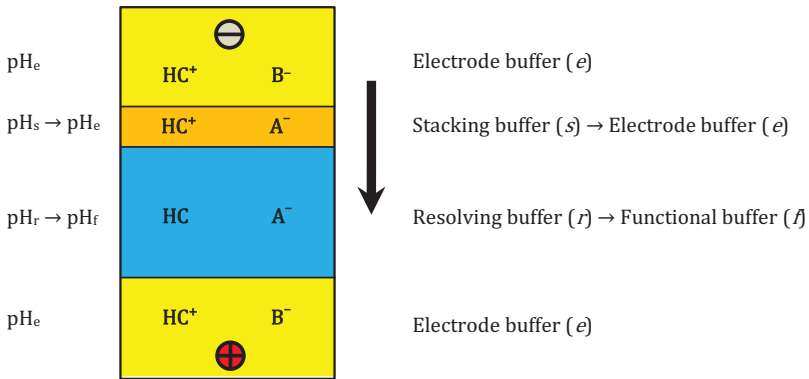


Figure 2.7.1: Scheme of the buffer system for disc-electrophoresis according to Ornstein–Davis. A^- – leading ion; B^- – trailing ion; HC^+ – counter-ion; pH_e , pH_s , pH_r , and pH_f – pH values of the electrode, stacking, resolving, and functional buffer, respectively. The two electrode buffers can be different or same (as on the figure).

negative total charges at the physiological pH value of the blood, i.e., 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 $0\text{ }^\circ\text{C}$, the effective mobilities of serum proteins are between -0.6×10^{-9} and $-7.5 \times 10^{-9} \text{ m}^2/(\text{s V})$. This means that the effective mobility of glycinate ion, which serves as a trailing ion, in the electrode buffer should be $-0.5 \times 10^{-9} \text{ m}^2/(\text{s V})$, and in the functional buffer should be $-10 \times 10^{-9} \text{ m}^2/(\text{s V})$. However, the effective mobility of the proteins in the resolving gel falls by half; therefore, it is sufficient if the effective mobility of glycinate ion in the resolving gel is $-5 \times 10^{-9} \text{ m}^2/(\text{s V})$.

The mobility of the glycinate ion μ_{G^-} at $0\text{ }^\circ\text{C}$ is equal to $-15 \times 10^{-9} \text{ m}^2/(\text{s V})$. So, the dissociation degree of glycine in the electrode buffer should be

$$\alpha_{HG_e} = \frac{-0.5 \times 10^{-9}}{-15 \times 10^{-9}} = \frac{1}{30}$$

and the dissociation degree of glycine in the functional buffer should be

$$\alpha_{HG_f} = \frac{-5 \times 10^{-9}}{-15 \times 10^{-9}} = \frac{1}{3}$$

From the above-derived equations, it can be calculated that pH_e should be 8.3 and pH_f should be 9.5 (Figure 2.7.2).

TRIS (T) is a suitable buffering base because the pK value of its protonated form (8.1), namely pK_{HT^+} , is approximately equal to the calculated pH_e value. Considering

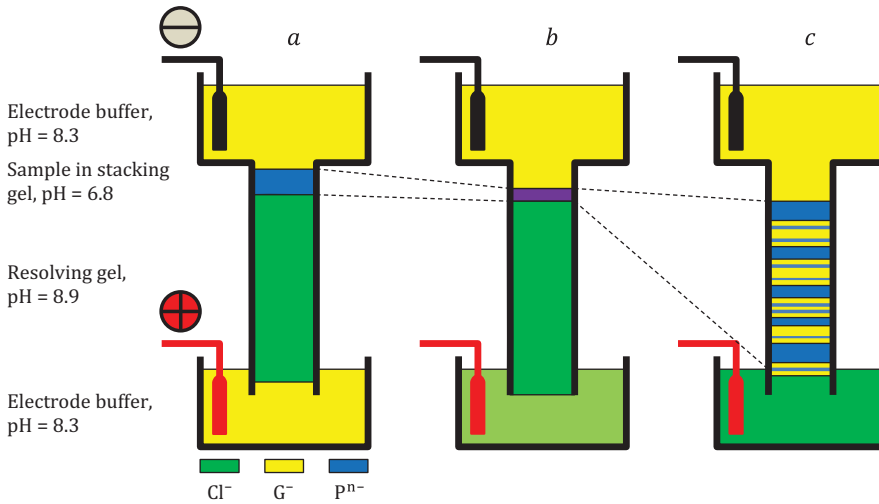


Figure 2.7.2: Scheme illustrating disc-electrophoresis of serum proteins. (a) Starting the disc-electrophoresis; (b) concentrating the proteins in the stacking gel; (c) separating the proteins in the resolving gel. Cl⁻ – chloride ion; G⁻ – glycinate ion; Pⁿ⁻ – protein polyions (polyanions).

that at 0 °C $\mu_{HT^+} = 9 \times 10^{-9} \text{ m}^2/(\text{s V})$, and if leading acid is HCl [$\mu_{Cl^-} = -37 \times 10^{-9} \text{ m}^2/(\text{s V})$ 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 $c_{HCl} = 0.06 \text{ mol/L}$, it follows from the above equations that $c_{HG} = 0.047 \text{ mol/L}$. It can also be calculated that $\text{pH}_s = 6.8$, and $\text{pH}_r = 8.9$, $\alpha_{Ce} = 0.37$, $c_{Te} = 0.004 \text{ mol/L}$, $\alpha_{Cs} = 0.96$, $c_{Ts} = 0.062 \text{ mol/L}$, $\alpha_{Cr} = 0.14$, and $c_{Tr} = 0.439 \text{ mol/L}$.

2.7.2.2 Buffer-gel systems for disc-electrophoresis according to Ornstein and Davis

Numerous 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 [7]. It is used for separating acidic proteins with relative molecular masses M_r of 10^4 – 6 , stable at $\text{pH} = 8.9$, in 1 mol/L (7 g/dL) polyacrylamide gel. Such proteins are the serum proteins. They are resolved at $\text{pH} = 9.5$. A disc-pherogram obtained with the buffer-gel system of Davis is shown in Figure 2.7.3.

The buffer-gel system of Reisfeld *et al.* [8] is used for separating alkaline proteins with relative molecular masses M_r of approximately 2×10^4 , stable at $\text{pH} = 4.3$, in 2.1 mol/L (15 g/dL) polyacrylamide gel. Such proteins are histones.

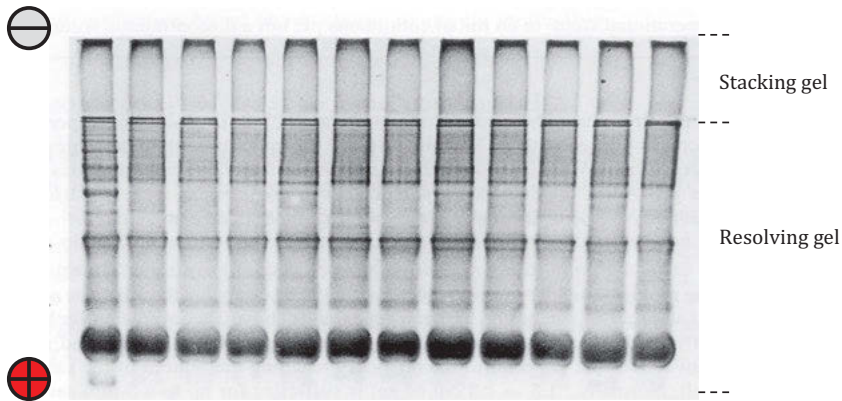


Figure 2.7.3: Disc-pherogram of serum proteins according to Davis.

2.7.2.3 Disc-electrophoresis according to Allen *et al.*

During the disc-electrophoresis according to Allen *et al.* [9, 10], the samples are concentrated by the effect of Hjerten and then separated in a step-gradient gel.

2.7.2.4 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 (stacking buffer) and diminish their velocity (focus) on the boundary with the buffer of high ionic strength (resolving buffer).

2.7.2.5 Buffer-gel systems for disc-electrophoresis according to Allen *et al.*

The buffer system of disc-electrophoresis according to Allen *et al.* also contains leading and trailing ions, but the pH value of the buffer system is everywhere constant. The stacking power of this electrophoresis is lower than this of the disc-electrophoresis according to Ornstein–Davis. However, the gradient resolving gel of a concentration of 0.4–1.7 mol/L (3–12 g/dL) sharpens the bands. In addition, a cover gel is recommended, which prevents the sample to diffuse and reduces the velocity of the trailing ion (Figure 2.7.4).

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,

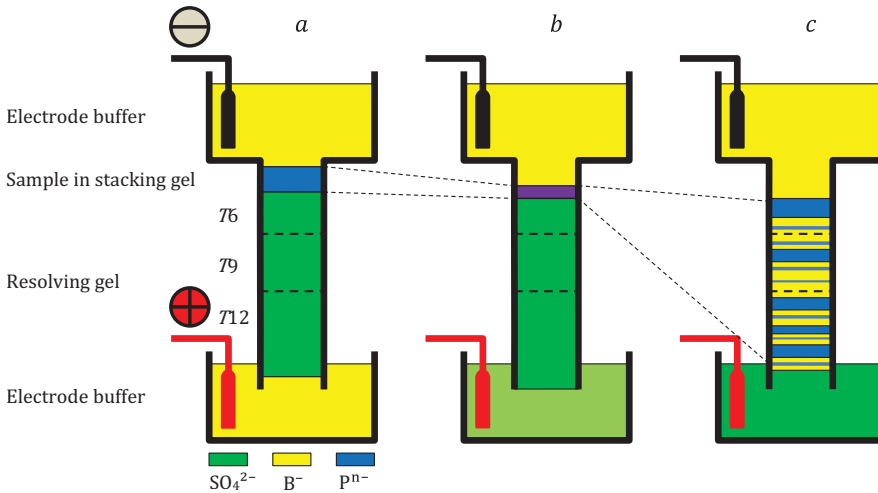


Figure 2.7.4: Effects during disc-electrophoresis according to Allen *et al.* (a) Starting the electrophoresis; (b) protein polyions are stacked by a moving ionic boundary; (c) protein polyions are resolved in a step-gradient gel. SO₄²⁻ – sulfate ion; B⁻ – borate ion; Pⁿ⁻ – proteinate polyions.

which show a tendency to aggregate; a chloride-glycinate buffer system (pH = 8.5) for resolving enzymes; and a potassium-β-alanine buffer system (pH = 4.0) for resolving basic proteins.

2.7.2.6 Disc-electrophoresis in one buffer at two pH values according to Michov

In the disc-electrophoresis in one buffer at two pH values according to Michov [13], the polyions stack between the same leading and trailing ions and are resolving in a zone electrophoresis.

2.7.2.7 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 stacking and resolving buffers, containing a same ion of the polyion’s polarity (Figure 2.7.5).

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. We have proved that the leading and trailing ions can

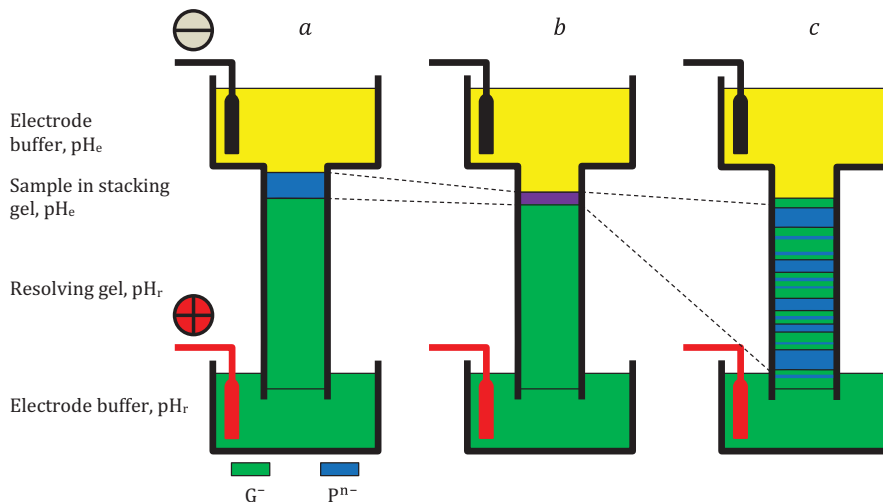


Figure 2.7.5: Disc-electrophoresis in one buffer at two pH values. (a) Starting the disc-electrophoresis; (b) stacking the polyions in a stationary ionic boundary between stacking and resolving gels; (c) resolving the polyions in a resolving gel. G⁻, glycinate ion; Pⁿ⁻, proteinate polyions.

be the same, e.g., 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

$$v_{A^-_a} = v_{A^-_b} \quad (2.7.9)$$

The velocity of ion i can be expressed by the equation

$$v_i = \mu'_i E = \alpha \mu_i E \quad (2.7.10)$$

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 from both equations that

$$\frac{\alpha_{HAa}}{\gamma_a} = \frac{\alpha_{HAb}}{\gamma_b} \quad (2.7.11)$$

The specific conductivity corresponds to the sum $F \sum c_i z_i \mu_i$, where F is the Faraday constant (96,484.55461 C/mol), c_i is the concentration of ion i , and z_i is the number

of its electric charges (electrovalency). Using this sum and the equation of electrical neutrality of a solution, eq. (2.7.11) can be simplified into

$$c_{\text{HAa}} = c_{\text{HAb}} = c_{\text{HA}} \quad (2.7.12)$$

The last equation shows that ion A^- moves with the same velocity in all parts of a buffer system, if the concentration of the electrolyte, which builds it, is the same everywhere.

From eqs. (2.7.11) and (2.7.12), the following equation is obtained:

$$\frac{c_{\text{Ba}}}{c_{\text{Bb}}} = \frac{\alpha_{\text{HAa}}\alpha_{\text{Bb}}}{\alpha_{\text{HAb}}\alpha_{\text{Ba}}} \quad (2.7.13)$$

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 should 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. This means that if the protein polyion P^{n-} should be concentrated, then

$$|\mu'_{A-a}| > |\mu'_{P^{n-}}| > |\mu'_{A-b}| \quad (2.7.14)$$

When these conditions are available, the polyions arrange themselves in the stationary boundary until the equation

$$c_{\text{HnP}} = c_{\text{HA}} \frac{\alpha_{\text{HA}}z_{A^-}}{\alpha_{\text{HnP}}z_{P^{n-}}} \quad (2.7.15)$$

is fulfilled. Here, c_{HnP} is the concentration of the stacked polyion, α_{HnP} is its ionization degree, and $z_{P^{n-}}$ is its electrovalency.

The values of α_{HAa} and α_{HAb} can be calculated from the effective mobilities of ion A^- in the resolving and electrode buffers, i.e., from μ'_{A-a} and μ'_{A-b} , respectively. Then the concentration of the base in the resolving and electrode gels can be determined. The value of $\alpha_{\text{Ba(b)}}$ can be computed from the Henderson–Hasselbalch equation.

2.7.2.8 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 $\text{pH} \geq 8.0$ the effective mobilities of serum protein

polyions μ'_{pn-} are -15×10^{-9} to -1.2×10^{-9} m²/(s V). Since the value of $\mu'_{pn- (max)}$ 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 -10×10^{-9} m²/(s V), and in the stacking gel μ'_{A-b} should be -10^{-9} m²/(s V).

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 $\mu_{G-} = -27.87 \times 10^{-9}$ m²/(s V) [17]. Then, it follows from the above equations that $\alpha_{HGa} = 0.3588$, $\alpha_{HGb} = 0.0359$, $c_{HG} = 0.2787$ mol/L, and $I_b = 0.01$ mol/L. Also at 25 °C, $pK_{HG} = 9.78$ and $pK_{HT+} = 8.07$. Hence, $pK_{HGa} = 9.54$, $pK_{HGb} = 9.69$, $pK_{HT+a} = 8.31$, 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. Next we can calculate, also from the above equations, 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 should be freshly prepared and last added. We propose that its concentration (in g/dL) should be calculated according to the formula

$$c_{APS} = \frac{0.32 \text{ (g/dL)}^2}{T} \text{ [g/dL]} \quad (2.7.16)$$

where T is the total monomeric concentration in g/dL.

After 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 (5.08 g/dL) 5-sulfosalicylic acid dehydrate for 30 min. Then 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 (Figure 2.7.6).

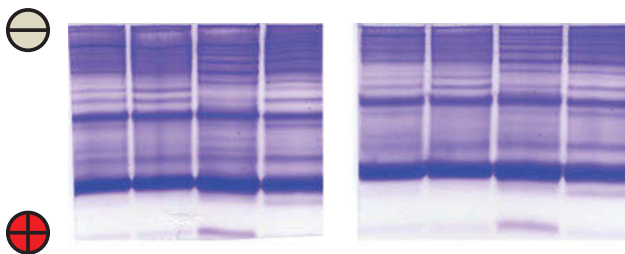


Figure 2.7.6: Serum proteins separated in a TRIS-glycinate buffer expanded stationary boundary of pH = 7.89–9.13, obtained in a 7 g/dL polyacrylamide slab gel. Staining with Coomassie brilliant blue R-250.

2.7.3 Denatured SDS disc-electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was introduced by Shapiro *et al.* [18]. It is widely used in biochemistry, forensics, genetics, molecular biology, and biotechnology, for separating denatured biological macromolecules, usually proteins or nucleic acids. Whereas the mobility of native polyions depends on their conformation and mass-to-charge ratio, the mobility of denatured polyions depends only on their mass-to-charge ratio.

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 (continuous) electrophoresis, which can be carried out in homogeneous as well as in gradient gels [19]. The second method is referred to as SDS disc-electrophoresis [20, 21]. SDS disc-electrophoresis does not distinguish from the disc-electrophoresis developed by Ornstein [22] and Davis [23] (see there). The only difference is that it contains SDS in addition. The SDS-protein complexes are concentrated also by a moving ionic boundary in the stacking gel where they arrange themselves according to their decreasing mobilities. Afterward, the SDS-protein complexes migrate through the resolving gel, where they are fractionated depending on their electric charges and masses.

2.7.3.1 Detergents

The detergents [24, 25], 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 (CMC)** and aggregation number (Figure 2.7.7).

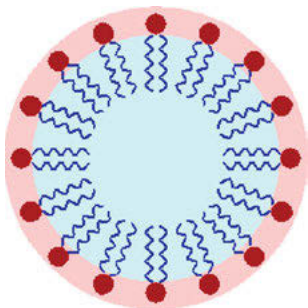


Figure 2.7.7: Schematic structure of an oil micelle in water. The detergent hydrophobic tails project into the oil, while the hydrophilic heads remain in contact with water.

CMC is the concentration of a detergent, in which it forms micelles. The lower the CMC value, the greater is the detergent strength. The **aggregation number (N_a)** is the number of detergent molecules, which form a micelle. The smaller the N_a , the less

the lipoprotein mass is increased. The CMC and the aggregation number are influenced by the pH value, temperature, and ionic strength.

Depending on their electric charges, the detergents can be divided into three groups: anionic, cationic, and zwitterionic detergents. Additionally, there are detergents that do not have electric charges – nonionic detergents (Table 2.7.1).

Table 2.7.1: Detergents used in electrophoresis.


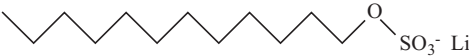
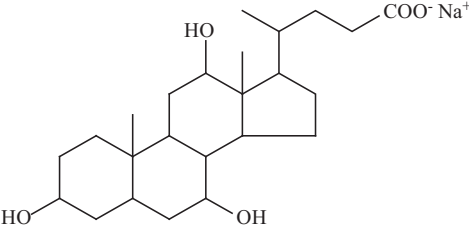

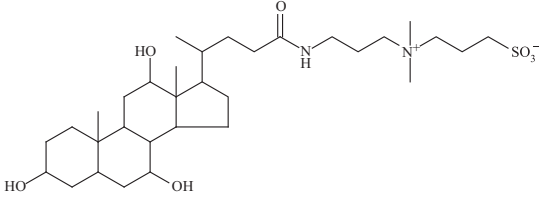
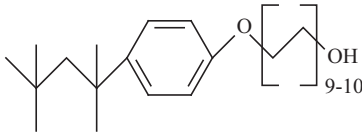
Detergents	Chemical formulas	Properties at 25 °C
Anionic detergents		
SDS (sodium dodecyl sulfate)		$M_r = 288.37$ CMC = 8.2×10^{-3} mol/L $N_a = 62$
LDS (lithium dodecyl sulfate)		$M_r = 272.33$ CMC = 8.8×10^{-3} mol/L $N_a = 90$
Sodium cholate		$M_r = 430.57$ CMC = 1.4×10^{-2} mol/L $N_a = 3$
Cationic detergents		
CTAB (<i>N</i> -Cetyl- <i>N,N,N</i> -trimethylammonium bromide)		$M_r = 364.46$ CMC = 0.2×10^{-4} mol/L $N_a = 169$

Table 2.7.1 (continued)

Detergents	Chemical formulas	Properties at 25 °C
Zwitterionic detergents		
CHAPS (3-[[3- cholamidopropyl]- dimethylammonio]-1- propanesulfonate)		$M_r = 614.89$ 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 $N_a = 143$

M_r – relative molecular mass; CMC – critical micelle concentration; N_a – aggregation number

Sodium dodecyl sulfate

The most commonly used detergent is **sodium dodecyl sulfate** (SDS, sodium lauryl sulfate, sodium dodecane sulfate) (Figure 2.7.8).

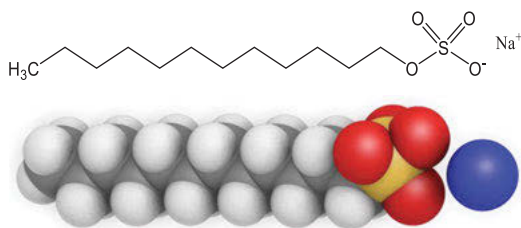


Figure 2.7.8: Chemical formula ($\text{NaC}_{12}\text{H}_{25}\text{SO}_4$) and structure of SDS.

SDS is the sodium salt of *dodecyl hydrogen sulfate* – the ester of dodecyl alcohol and sulfuric acid. It consists of a 12-carbon tail bound to a sulfate group that is electrostatically connected to a sodium ion. This structure gives the compound's amphiphilic properties that allow it to form micelles. CMC of SDS in pure water is 8.2 mmol/L at 25 °C [26], its aggregation number is about 62 [27], and its degree of micelle ionization α is around 0.3 (30 g/dL) [28, 29].

SDS ions cover the protein molecules and form with them SDS-protein complexes. Approximately 1.4 g of SDS is connected with 1 g of protein (one SDS ion is bound to two amino acid residues). So the proteins constitute about 42% of the total mass of the SDS-protein complexes [30]. The hydrophobic membrane proteins bind up to 4.5 g of SDS per gram of protein [31]. The glycoproteins depart from this rule since their hydrophilic glycan moiety reduces the hydrophobic interactions between proteins and SDS.

When a protein mixture is heated to 100 °C in the presence of SDS, SDS wraps around the proteins giving them negative electric charges. The hydrophobic tails of SDS ions remain inside the SDS-protein complex, but their heads face outward to water dipoles. The protein polypeptide chains unfold and linearize to become rod-like structures [32, 33] (Figure 2.7.9). With another words, SDS denatures the secondary and tertiary protein structure.

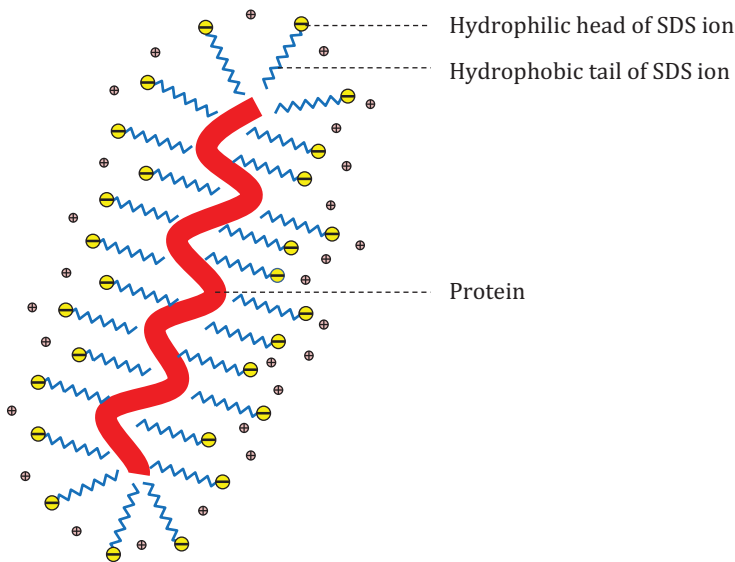


Figure 2.7.9: Structure of SDS-protein complex.

The protein polyions obtain certain numbers of negative electric charges per mass (in C/g) [34, 35], and, therefore, all SDS-protein complexes run to the anode. Since the conformation of all denatured proteins, except histones [36], become similar, the protein velocity through polyacrylamide gel depends only on their masses.

The cationic detergent CTAB has been used as an alternative to SDS for gel electrophoresis of proteins.

Sample preparation for SDS polyacrylamide gel electrophoresis

If proteins are a part of a solid tissue, the solid tissue should 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 [37, 38]. Besides SDS, the samples should contain gel buffer, glycerol, and Bromophenol blue as a trailing dye.

The sample preparation can be nonreducing, reducing, or reducing with alkylation.

Nonreducing sample preparation

During the nonreducing sample preparation, the disulfide bonds in the proteins remain intact (Figure 2.7.10). This preparation is carried out in a nonreducing buffer, where the proteins, in a concentration of 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 should be centrifuged to remove debris.

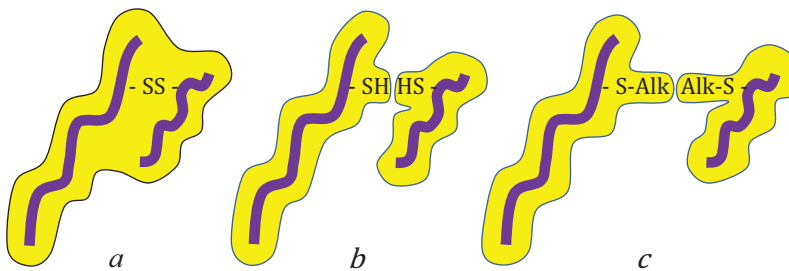
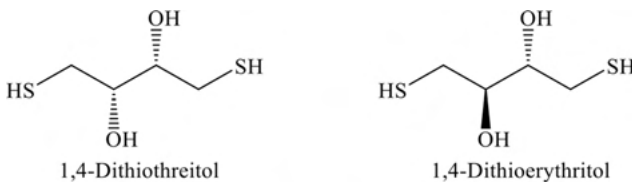


Figure 2.7.10: SDS-treated proteins. The polypeptide chains are presented as thick lines surrounded by SDS ions: (a) without reduction; (b) with reduction; and (c) with reduction and alkylation. –SS–, disulfide bridge; –SH, thiol group; Alk, alkyl group.

Reducing sample preparation

The reducing sample preparation takes place, if a reducing agent, most often 1,4-**di**thiothreitol (DTT), or 2-mercaptoethanol (**beta**-mercaptoethanol, BME), or 1,4-**dithi**oerythritol (DTE), in concentrations of 0.1–1.0 g/dL is added to the sample buffer.



The samples should be boiled in a closed vessel containing 1 g/dL SDS and 5 mmol/L DTT for 3–5 min. So the disulfide bridges between the cysteine residues of proteins are cleaved (Figure 2.7.11). As a result, the proteins unfold, stretch their polypeptide chains, and form rod-like structures with SDS ions. The quaternary protein structure (oligomeric structure) breaks too. If proteins should not be heated at 100 °C, the samples are left at room temperature for a few hours or overnight.

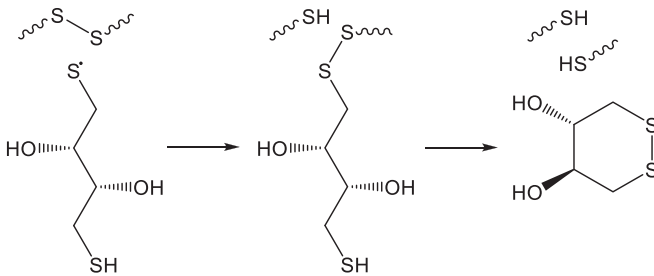


Figure 2.7.11: Reducing a disulfide bond by DTT *via* two sequential thiol-disulfide exchange reactions.

Reducing sample preparation with alkylation

The SH groups that occur after reducing treatment may be oxidized again by atmospheric oxygen or other oxidizing agents to form disulfide bridges. Therefore, they should be alkylated (to be bound to difficult reacting alkyl groups). The alkylation can be carried out by iodoacetamide, iodoacetic acid, or vinylpyridine [39] (Figure 2.7.12). After alkylation, the proteins increase their molecular masses slightly, because the relative molecular mass M_r of iodoacetamide is 184.96.

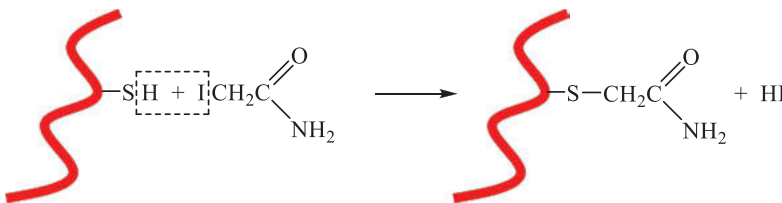


Figure 2.7.12: Alkylation of a thiol group by iodoacetamide. The polypeptide chain is shown as a thick line.

The alkylation is carried out at pH = 8.0 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.

2.7.3.2 Practice of SDS disc-electrophoresis

The original SDS electrophoresis was carried out in vertical round gels [40], and later in vertical slab gels [41, 42]. The wells in the vertical slab gels are formed by a comb and are filled with glycerol containing samples under the top (cathode) buffer. The introduction of thin (0.5–1.0 mm) and ultra-thin (0.1–0.5 mm) horizontal polyacrylamide gels [43] on support films [44, 45], e.g., on pretreated polyester films [46], was an important step forward.

The concentration of SDS in the gel should be 0.1 g/dL. The electrode buffer for horizontal electrophoresis can be contained in the tanks of electrophoresis cell or in porous electrode strips. The porous electrode strips can be constructed of gel (polyacrylamide or agarose gel) or paper. 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 should be concentrated.

The most SDS-PAGE separations are carried out under constant electric current. Now three buffer systems are used for SDS disc-electrophoresis in homogeneous gels: TRIS-chloride-glycinate system according to Laemmli, TRIS-acetate-TRICINEate system according 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 and in gradient gels.

SDS disc-electrophoresis in TRIS-chloride-glycinate buffer system according to Laemmli

The SDS disc-electrophoresis was described in 1970 by Laemmli [47] who added SDS to the discontinuous buffer system of Ornstein and Davis. It is characterized by a moving ionic boundary between chloride ions, as leading ions, and glycinate ions, as trailing ions, against TRIS ions as counter-ions, and is running through two gels: stacking and resolving (Figure 2.7.13).

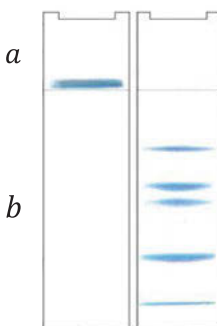


Figure 2.7.13: Laemmli electrophoresis: (a) stacking gel and (b) resolving gel.

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.

Protein samples, in concentrations of 0.2–0.6 mg/mL, are dissolved by heating in a sample solution. It contains 50 mmol/L TRIS-HCl, pH = 6.8, 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 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.

Proteins whose M_r is smaller than 12,000 resolve badly in the Laemmli system because they do not leave the band of SDS micelles that are formed behind the leading ions front.

SDS disc-electrophoresis in TRIS-acetate-TRICINEate buffer system according to Schägger–Jagow

The SDS disc-electrophoresis in TRIS-acetate-TRICINEate buffer system was empirically developed by Schägger and Jagow [48, 49]. This system uses TRICINEate ion as a trailing ion in place of the glycinate ion in the Laemmli system. It gives better results than the classical TRIS-chloride-glycinate buffer system of Laemmli when low molecular mass proteins ($M_r < 14,000$) are separated. 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 stacking gel is homogeneous ($T = 5$ g/dL, $C = 0.03$), whereas the resolving gel is linearly gradient ($T = 8-18$ 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).

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 gradient gels are irregular and gels roll up during drying.

SDS disc-electrophoresis in TRIS-formate-taurinate buffer system according to Michov

To eliminate the disadvantages of gradient gels, a new SDS disc-electrophoresis was developed in homogeneous slab gels [50]. This electrophoresis is carried out in a TRIS-formate-taurinate buffer system that has a higher buffer capacity than the prior buffer systems and is suitable for resolving of proteins with relative molecular masses of 6,000–450,000.

The horizontal SDS disc-electrophoresis in TRIS-formate-taurinate buffer system can be carried out using paper electrode strips. The handling of paper electrode strips is easier than the handling of gel electrode strips. Besides, they are nontoxic, while the gel electrode strips contain traces of acrylamide.

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 ionic strength of 0.1 mol/L, the mobilities of the formate, taurinate, and TRIS-ion are as follows: $\mu_{A^-} = -44.22 \times 10^{-9}$, $\mu_{B^-} = -23.53 \times 10^{-9}$, and $\mu_{HC^+} = 17.27 \times 10^{-9} \text{ m}^2/(\text{s V})$ [51].

To assemble a TRIS-formate-taurinate buffer system, we should calculate the function of Kohlrausch [52], the degree of taurine dissociation, 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 gels 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 $c_{HA} = 0.10 \text{ mol/L}$, then $c_{HB} = 0.0802 \text{ mol/L}$.

The SDS-protein complexes move in a T13-resolving gel with effective mobilities lower than $-4 \times 10^{-9} \text{ m}^2/(\text{s V})$. Therefore, the effective mobility of taurinate ion μ'_{B^-} in the stacking gel as well as in the resolving gel should be $-4 \times 10^{-9} \text{ m}^2/(\text{s V})$; the dissociation degree of taurine in the trailing (cathode) buffer should be 0.17; and its ionic strength I_b should be $0.17 \times 0.0802 = 0.0136 \text{ mol/L}$.

According to the theory of Debye–Hückel [53], at $I_b = 0.0136 \text{ mol/L}$ $\text{p}K_{\text{CHC}^+b}$ should be equal to 8.17 and $\text{p}K_{\text{CHB}}$ should be equal to 8.96. Therefore, it follows from the Henderson–Hasselbalch equation that $\text{pH}_b = 8.3$ and $\text{pH}_a = 7.4$. The stacking gel forms approximately 1/3, and the resolving gel forms approximately 2/3 of the gel volume (Figure 2.7.14).

On the two gel ends, corresponding paper electrode strips are placed, which contain the anode and cathode buffers. On the stacking gel, 10 mm in front of the cathode strip, an application template, best of silicone, should be placed and lightly pressed to create a good contact with the gel. In each template 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. Results obtained by this method are shown in Figure 2.7.15.

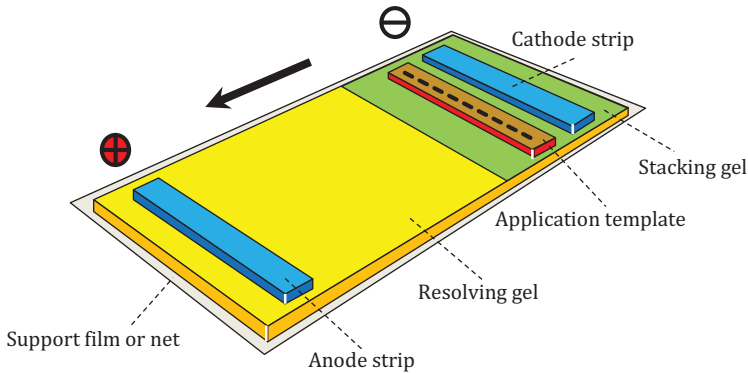


Figure 2.7.14: Scheme of a gel for SDS disc-electrophoresis in a TRIS-formate-taurinate buffer system.

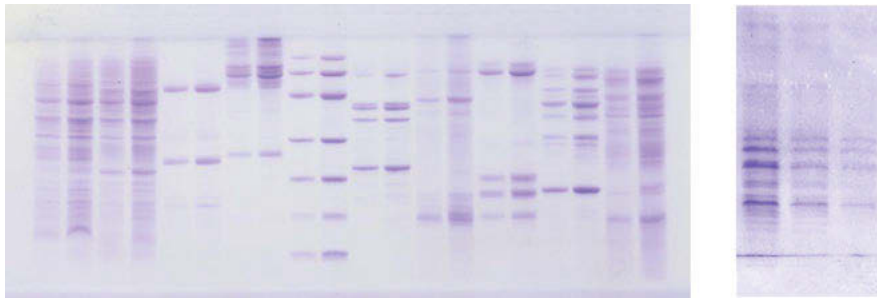


Figure 2.7.15: Electrophoretic results in a 13 g/dL homogeneous SDS slab gel according to Michov. *Left:* on a film-supported polyacrylamide gel; *right:* on a net-supported polyacrylamide gel. Applied volumes of 5 μ L each. Electrophoresis at constant electric current of 80 mA (100–500 V) and temperature of 10 $^{\circ}$ C. Running time 90 min. Staining with Coomassie brilliant blue R-250.

SDS disc-electrophoresis in one buffer at two pH values according to Michov

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 after Michov [54], the leading and trailing ions are same, e.g., 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. So a stationary ionic boundary is formed between a resolving buffer and a stacking buffer, not a moving ionic boundary. The polyions are concentrated at this boundary and then are separated from each other in the resolving gel (see *Native disc-electrophoresis in one buffer at two pH values according to Michov*).

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*).

SDS disc-electrophoresis in gradient gels

The pore-gradient gels for SDS disc-PAGE are characterized with continuous decrease of gel pore diameters. As a result, the proteins move in the beginning through the gel with larger pores but stop later in the small pores because they cannot pass through. In this electrophoresis, the discontinuous buffer system of Laemmli is used, too. Gradient gels with $T = 4\text{--}15$ g/dL are suitable for SDS proteins with $M_r = 40,000\text{--}200,000$; and gradient gels with $T = 4\text{--}20$ g/dL are suitable for SDS proteins with $M_r = 10,000\text{--}200,000$.

2.7.3.3 Staining of SDS protein bands

After Bromophenol blue has reached the anodic end of the gel, the electrophoresis should be stopped and the protein bands could be stained with Coomassie brilliant blue or silver. A protein band should 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.

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 (see 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 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 the method proceeds overnight [55].

Determination of molecular masses of SDS-denatured proteins

SDS–PAGE can be used to estimate the relative molecular masses M_r of denatured proteins [56–58]. This is based on the dependence of protein mobility μ on the total polyacrylamide gel concentration T_0 (undimensional), expressed by the equation

$$\log M_r = a \log T_0 + b \quad (2.7.17)$$

where a and b are undimensional constants. The larger the protein, the slower it migrates in a gel. In practice, the mobilities of investigated proteins are compared to the mobilities of standard proteins (Table 2.7.2) [59, 60].

Table 2.7.2: Relative molecular masses (M_r) of standard proteins used for 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 (<i>E. coli</i>)	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 <i>a</i> (muscle)	100,000	Myoglobin (sperm whale)	16,800
Phosphorylase <i>b</i> (rabbit muscle)	97,400	Hemoglobin	15,500
Lactoperoxidase	93,000	Ribonuclease <i>b</i>	14,700
Plasminogen	81,000	Lysozyme (hen egg white)	14,300
Transferrin	76,000	α -Lactalbumin	14,200
Bovine serum albumin	66,200	Cytochrome <i>c</i> (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 standard proteins are plotted 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 semilogarithm 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 passed by a protein in the resolving gel divided by the distance of the front. When R_f values of unknown proteins are plotted on the calibration straight line, the logarithm of their relative molecular masses can be obtained (Figure 2.7.16).

Useful SDS homogeneous gels for estimating the relative molecular masses of proteins in SDS-PAGE are as follows: $T = 7.5$ g/dL for $M_r = 40\text{--}200 \times 10^3$; $T = 10$ g/dL for $M_r = 30\text{--}100 \times 10^3$; $T = 12$ g/dL for $M_r = 15\text{--}90 \times 10^3$; and $T = 15$ g/dL for $M_r = 10\text{--}70 \times 10^3$.

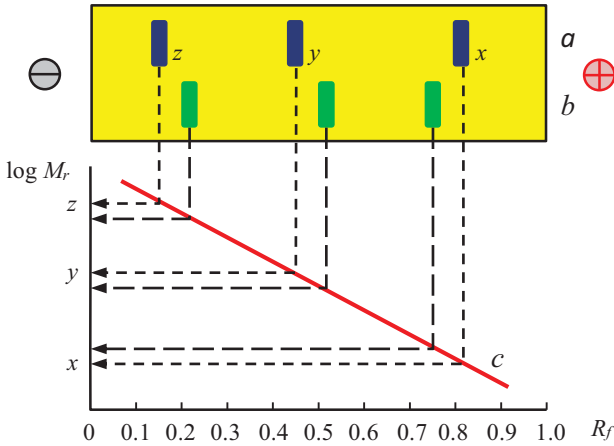


Figure 2.7.16: Determination of relative molecular masses of proteins by SDS electrophoresis using protein standards. (a) Protein bands *x*, *y*, and *z* in the pherogram; (b) bands of protein standards; (c) calibration straight line. M_r , relative molecular mass; R_f , relative migration distance.

2.7.4 Protocols

Disc-electrophoresis in Alkaline Buffer-gel System According to Ornstein–Davis

Materials and equipment

Buffers

Acrylamide and BIS

TMEDA

APS

Trichloroacetic acid (TCA)

5-Sulfosalicylic acid

Coomassie brilliant blue R-250

Methanol

Acetic acid

0.5 mm U-form silicone gasket

Support film for polyacrylamide gel

250 mL side-arm flask for degassing gel solutions

Glass plates

Clamps

Water-jet pump

Table 2.7.3: Stacking gel, $T = 4$ g/dL, $C = 0.03$.

Solutions	Preparation	Ingredients
TRIS-chloride buffer, 4× 0.25 mol/L TRIS HCl to pH = 6.8	Solve 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
Monomeric solution $T = 50$ g/dL, $C = 0.03$	Solve 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

Table 2.7.4: Resolving gel, $T = 12.5$ g/dL, $C = 0.03$.

Solutions	Preparation	Ingredients
TRIS-chloride buffer, 4× 1.5 mol/L TRIS HCl to pH = 8.8	Solve 18.17 g TRIS in 40 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
Monomeric solution $T = 50$ g/dL, $C = 0.03$	Solve 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

Table 2.7.5: Electrode buffer.

Solutions	Preparation	Ingredients
TRIS-glycinate buffer, 10× 0.025 mol/L TRIS 0.192 mol/L glycine pH = 8.3 (The resolving buffer can be used as anode buffer.)	Solve 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

Procedure

- Assemble a casting cassette: roll a support film for polyacrylamide gel on a glass plate, place on the film 0.5 mm U-form silicone gasket, put over a second glass plate, and fix the cassette by clamps.
- Make the resolving solution in the side-arm flask and degas it with the water-jet pump.
- Pipette resolving solution with the catalysts into the casting cassette until the solution fills 2/3 of the cassette (Figure 2.7.17).

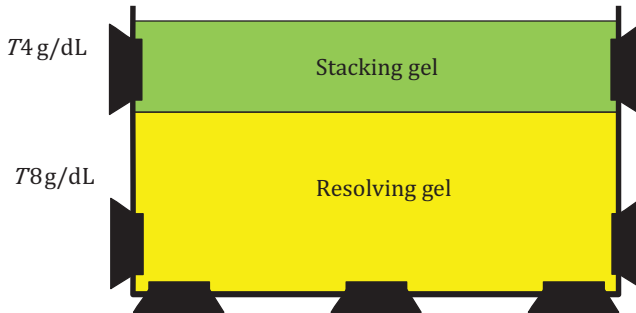


Figure 2.7.17: Casting a homogeneous gel for disc-electrophoresis.

- Pipette 2.0 mL deionized water or resolving buffer onto the resolving solution.
- Let the resolving solution polymerize at a room temperature for 45 min.
- Soak the liquid on the resolving gel using the water-jet pump.
- Pipette the stacking solution onto the resolving gel.
- Let the stacking solution polymerize at room temperature for another 45 min.
- Disassemble the casting cassette and take the polyacrylamide gel.
- Run disc-electrophoresis for 1–2 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.

Disc-electrophoresis in One Buffer at Two pH Values According to Michov**Materials and equipment**

Buffers

Acrylamide and BIS

TMEDA

APS

Glycine

Trichloroacetic acid

5-Sulfosalicylic acid

Coomassie brilliant blue R-250

Methanol

Acetic acid

250 mL side-arm flask for degassing gel solutions

Support film for polyacrylamide gel

Glass plates

0.5 mm U-form silicone gasket

Clamps

Water-jet pump

Table 2.7.6: Solutions for electrophoresis.

	Solution 1, 2×, pH = 9.3	Solution 2, 2×, pH = 8.3	Solution 3
TRIS	12.72 g	2.79 g	–
Glycine	2.70 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

Solutions 1 and 2 are double concentrated resolving and electrode buffers, respectively. For casting resolving and stacking gels, solution 3 is also needed, where the total monomeric concentration $T = 50$ g/dL, and the cross-linking degree $C = 0.03$.

Table 2.7.7: 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

Table 2.7.7 (continued)

	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 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

Procedure

- Assemble a casting cassette: roll a support film for polyacrylamide gel on a glass plate, place on the film 0.5 mm U-form silicone gasket, put over a second glass plate, and fix the cassette by clamps.
- Make the resolving solution in the side-arm flask and degas it with the water-jet pump.
- Pipette resolving solution with the catalysts into the casting cassette until the solution fills 2/3 of the cassette (see above).
- Pipette 2.0 mL deionized water or resolving buffer onto the resolving solution.
- Let the resolving solution polymerize at a room temperature for 45 min.
- Soak the liquid on the resolving gel using the water-jet pump.
- Pipette the stacking solution onto the resolving gel.
- Let the stacking solution polymerize at room temperature for another 45 min.
- Disassemble the casting cassette and take the polyacrylamide gel.
- Run disc-electrophoresis for 1–2 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.

SDS Disc-electrophoresis According to Laemmli**Materials and equipment**

TRIS
 HCl
 Acrylamide and BIS
 SDS
 Glycerol
 TMEDA
 APS
 Glass plates
 Support film
 0.5 mm U-form spacer
 Clamps
 Kerosene
 Paper strip electrodes
 Horizontal electrophoresis unit
 Constant current power supply
 Coomassie brilliant blue or silver staining

4× Stacking gel buffer, pH = 6.8

TRIS 6.05 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.

4× 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, 0.03 C)

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 in a refrigerator up to 2 weeks.

Table 2.7.8: 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
4× Stacking gel buffer, pH = 6.8, mL	3.75	–	–	–	–	–	–	–	–
4× Resolving gel buffer, pH = 8.8, mL	–	3.75	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	5.17
10 g/dL TMEDA, mL	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
Deionized water to, mL	15.00	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 $M_r = 60,000$ – $200,000$, 10 g/dL gel for proteins with $M_r = 16,000$ – $70,000$, and 15 g/dL gel for proteins with $M_r = 12,000$ – $45,000$.

10× 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. Prior to use, mix 100 mL of the electrode buffer with 900 mL of deionized water.

Procedure

- Assemble a sandwich of a lower glass plate, a support film for polyacrylamide gel, a 0.5 mm spacer, and an upper glass plate (see above).
- Using a syringe or a pipette pour 10 mL of the resolving gel solution in the casting cassette and overlay with deionized water.
- Allow the solution to polymerize for 30 min.
- Drain off the overlaid water.
- Using a syringe or a pipette pour 5 mL of the stacking gel solution into 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 2× SDS stacking gel buffer (1:1, V:V) and heat in a sealed screw-cap microcentrifuge tubes at 100 °C for 3–5 min. Dissolve molecular mass standards in 1× SDS stacking gel buffer.
- Pipette a few mL 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 4× electrode buffer onto the gel ends.
- 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 1 h.
- Stain with either Coomassie brilliant blue or silver nitrate or electroblot the proteins onto a membrane for immunoblotting. If the proteins are radiolabeled, they could be detected by autoradiography.

SDS Electrophoresis in TRIS-formate-taurinate Buffer System According to Michov

Materials and equipment

TRIS

Formic acid

Taurine

SDS

87% glycerol

TMEDA

APS

Glass plates

0.5 mm U-shaped gasket

Clamps

Horizontal electrophoresis unit

Power supply

BPB (**B**romophenol **b**lue Na salt)

Table 2.7.9: Electrode solutions for SDS disc-gel electrophoresis in a TRIS-formate-taurinate buffer system.

	4× Anode buffer pH = 7.8, I = 4 × 0.30 mol/L	4× 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

Nonreducing sample buffer, pH = 7.8

4× Anode buffer, pH = 7.8	2.50 mL
SDS	0.10 g
87% Glycerol	3.00 mL
Coomassie brilliant blue G-250	0.02 g
Deionized water to	10.00 mL

Monomeric solution (T = 50 g/dL, C = 0.03)

Acrylamide	48.5 g
BIS	1.5 g
Deionized water to	100.0 mL

Table 2.7.10: Stacking and resolving gels.

	Stacking gel		Resolving gel			
	T = 5 g/dL	T = 9 g/dL	T = 11 g/dL	T = 13 g/dL	T = 15 g/dL	T = 17 g/dL
4× Anode buffer pH = 7.8, mL	25.00	25.00	25.00	25.00	25.00	25.00
Monomeric 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 2.7.11: Gradient gels.

	Light solution $T = 6 \text{ g/dL}, C = 0.03$	Heavy solution $T = 20 \text{ g/dL}, C = 0.03$
4× Gel buffer, pH = 7.8 $I = 0.30 \text{ mol/L}$, 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 ($T = 8\text{--}18 \text{ g/dL}$) are cast using a gradient maker. The mixing chamber of the gradient maker is filled with the heavy solution; its reservoir is filled with the light solution. The cassette is filled from below.

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.
- Pour 10 mL of the resolving gel solution into the casting cassette using a syringe or a pipette and overlay with deionized water.
- Allow the solution to polymerize for 30 min.
- Drain off the overlaid water.
- Pour 5 mL of the stacking gel solution into the cassette using a syringe or a pipette.
- 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 3–5 min.
- Apply prior to electrophoresis 0.5–1.0 mL kerosene or silicone oil DC 200 onto the cooling plate of the electrophoretic cell.
- Place the gel with its support film down onto the cooling plate so that the air bubbles between the support film and cooling plate are pushed away.

- Place paper strips with the 4× anode and 4× cathode buffer onto the two opposite ends of the gel.
- Place onto the stacking gel, 10 mm in front to 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 into each slot 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/mm², 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 using autoradiography.

Coomassie Brilliant Blue R-250 Staining of Proteins Resolved in SDS Gels

Materials and equipment

Fixing solution (5 g/L glutardialdehyde 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; and 20 min for 0.5 mm polyacrylamide gels with $T = 16$ g/dL.
- 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 (agarose gels without cellophane membrane) at room temperature, under a hair dryer or in a drying oven.

If polyacrylamide gels are cast on net, the times should be halved.

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2.8 Isoelectric focusing of proteins

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Isoelectric focusing (IEF), known also as *electrofocusing*, is an electrophoresis technique for separating proteins in pH gradients. Moving through pH gradients, the proteins reach pH values (their isoelectric points, pI, pH(I)) where they lose their net electric charge and stop. Usually pH gradients are obtained in polyacrylamide gels with low concentration, where any sieving effect is eliminated.

The history of IEF began with Kolin [1]. He obtained unstable pH gradients *via* diffusion of two buffers with different pH values where separated proteins in sharp bands. Later, Svensson (Rilbe) published his theoretical works regarding the carrier (free) ampholytes [2,3]: hundreds of ampholytes with different isoelectric points should form in an electric field a pH gradient. To prove his theory he checked amino acids, synthetic peptides, and hydrolyzed proteins [4] but the problem remained open.

The first successful synthesis of carrier ampholytes (CA) was produced by Vesterberg [5–7], a student of Svensson. He synthesized a mixture of CA from residues of carboxylic acids and polyethylene amines. Later, Grubhofer proposed a method for synthesizing CA from oligoamino and oligosulfonic acid [8] and marketed them as *Servalys*. The next attempt came from Williams and Söderberg [9, 10] who established the *pharmalyte* CA. Later, CA were synthesized that contained in addition residues of phosphoric and sulfuric acids [11]. LKB offered them as *ampholines*.

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The next step was the binding of ampholytes to polyacrylamide gel, as a result of which immobilized pH gradients (IPG) were created. Nowadays, the IPG gels on plastic strips are preferred, because they can be used also for the second SDS dimension of the two-dimensional electrophoresis or in mass spectrometry.

2.8.1 Theory of isoelectric focusing

During IEF, the amphoteric polyions lose their net charges gradually until they stop at their isoelectric points (Figure 2.8.1).

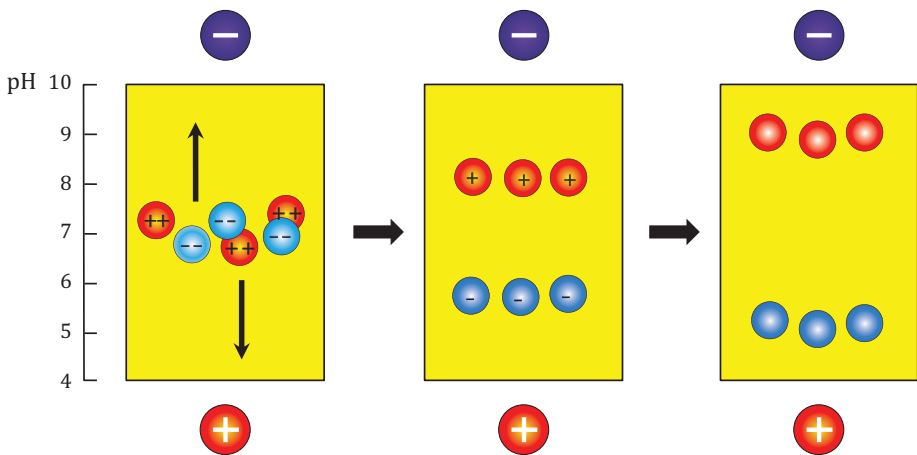
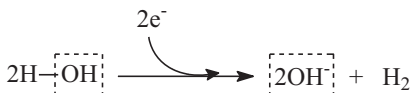


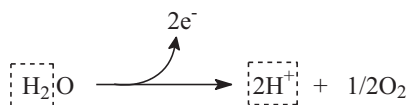
Figure 2.8.1: Isoelectric focusing of two proteins with pI values of 9.0 and 5.0.

At pH values above pI, the carboxyl groups are negatively charged ($-\text{COO}^-$), but the N-groups ($-\text{NH}_2$) carry no charges. As a result, the protein polyions have negative net charges. At pH values below pI, the carboxyl groups ($-\text{COOH}$) do not carry any charges; however, the N-groups are positively charged ($-\text{NH}_3^+$). 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 IEF on the cathode, where electron excess exists, hydroxide ions are formed:



and on the anode, where electron deficiency exists, protons are formed:



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 acidic solution at the anode end and an alkaline solution at the cathode end. When an acidic CA 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 through deprotonation. This continues until the numbers of its negative and positive charges become equal and, as a result, its net charge becomes zero. If the polyion moves away from its pI point by diffusion, it obtains again positive or negative charges and moves again to its pI point.

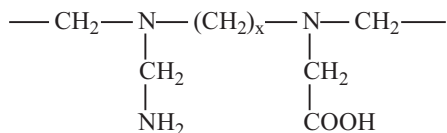
The isoelectric points of most proteins and peptides are located in the pH range of 3–12, but most of them are between pH = 4.0 and 7.0 [12, 13]. Narrow pH gradients can also be obtained. For example, if proteins have pI values from 5.2 to 5.8, they can be focused in a pH gradient of the range 5.0–6.0.

2.8.2 Isoelectric focusing with carrier ampholytes

IEF with CA can be carried out in different separation media: polyacrylamide gel [14], agarose gel [15, 16], acetyl cellulose films [17], and granulated gels [18, 19]. They should not have sieving effect.

2.8.2.1 Properties of carrier ampholytes

The CA represent a heterogeneous mixture of different low-molecular aliphatic oligoamino-oligocarboxylic acids (zwitterions) with close pI points:



They arrange themselves in an electric field according to their pI values and build a pH gradient. Since the pK values of CA and proteins to be analyzed are temperature

dependent, the IEF should be carried out at a constant temperature, preferably at 10 °C.

2.8.2.2 Formation of a pH gradient by carrier ampholytes

At first, the CA are in homogeneous distribution in the gel. After an electric field is applied, a pH gradient occurs in three steps (Figure 2.8.2):

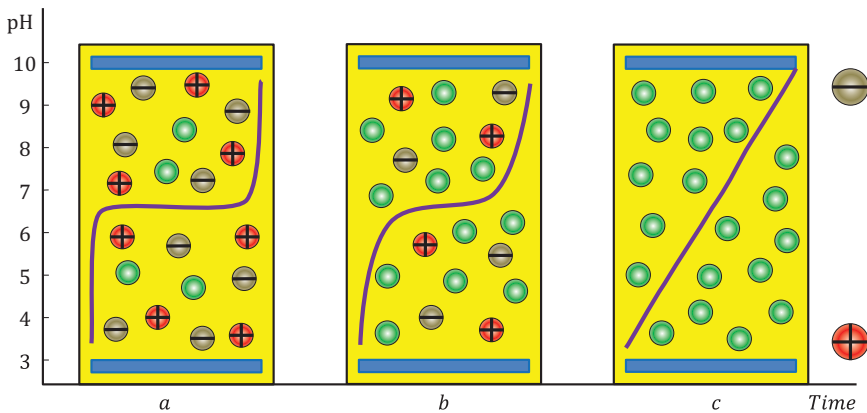


Figure 2.8.2: Steps (*a*, *b*, and *c*) of pH gradient building by carrier ampholytes.

- The CA begin to migrate according to their net charges to opposite electrodes. CA with pI lower than the pH value of separation medium carry negative charges; therefore, they migrate to the anode. CA with pI higher than the pH of separation medium have positive charges; therefore, they migrate to the cathode. As a result, two-step pH gradient is created with a pH plateau in the middle.
- The pH plateau disappears and
- Both pH gradients unite to give a continuous linear pH gradient.

Optimal pH gradients are formed by CA with M_r between 300 and 1,000. If M_r is less than 300, the pH gradient is unstable; if they are larger, they cannot be distinguished from proteins to be resolved. The pH gradient depends also on the number of CA molecules. The larger it is, the “smoother” the pH gradient. Ampholyte concentrations of about 2 g/dL give stable pH gradients. Glycerol, sorbitol, or sucrose can be added to the mixture, but they change the pH gradients if their concentrations are higher than 10–20 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), alkaline gradients (pH = 8.0–10.0), and very alkaline gradients (pH = 9.0–11.0).

Separator electrofocusing

The resolution of a pH gradient can be increased by adding appropriate separators, known as pH gradient modifiers [20,21]. The separators are amino acids or amphoteric electrolytes, which flatten the pH gradient near their isoelectric points. Separators are, e.g., tetraglycine (pI = 5.2), proline (pI = 6.3), threonine (pI = 6.5), β -alanine (pI = 6.9), 5-aminovaleric acid (pI = 7.5), histidine (pI = 7.6), and 6-aminocaproic acid (pI = 8.0). For example, with the help of 0.33 mol/L β -alanine in a pH gradient of 6–8, the glycated HbA_{1c} was separated from the closely adjacent HbA, the major hemoglobin band [22].

IEF is carried out on polyacrylamide gels or rarely on agarose gels.

2.8.2.3 IEF with carrier ampholytes on polyacrylamide gels

CA-IEF is usually carried out on polyacrylamide gels: at $T = 4\text{--}5$ g/dL and $C = 0.03$ for electrofocusing of proteins, and at $T = 10$ g/dL and $C = 0.025$ for electrofocusing of oligopeptides. The polyacrylamide gels show no electroosmotic effects. However, they 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 produced during electrophoresis. This problem was solved after the gel thickness was reduced, and thin (thickness of 0.5 mm) [23,24] and ultrathin (thickness less than 0.5 mm) [25,26] slab gels were produced on pretreated plastic sheets (support films). Because of their higher surface-to-volume ratio, the thin polyacrylamide or agarose gels dissipate the Joule heating better than the thick ones and, as a result, can be subjected to higher voltage. In addition, the sample volume is reduced, and the times for staining, destaining, and drying of the gel are shortened.

Rehydratable polyacrylamide gels for IEF with carrier ampholytes

The polymerization of a monomeric solution in the presence of CA requires higher concentrations of APS. Besides, when a gel was polymerized in the presence of CA, it dissolves partially or completely from the support film in an acidic solution. Nonionic detergents, which are often added to the monomeric solution to increase the solubility of hydrophobic proteins, inhibit the adhesion between the gel and film, too. To improve the adhesion of the gel to the support film, rehydratable gels are used [27, 28].

The rehydratable gels polymerize without addition of CA. Then they are washed with deionized water, are dried, and can be stored indefinitely at $-20\text{ }^{\circ}\text{C}$. Prior to IEF, the rehydratable gels should be swollen (rehydrated) in 2 g/dL CA with 20–30 mL/dL glycerol to their original thickness.

However, the rehydratable gels have some disadvantages: Their production (washing and drying) and their rehydrating take time. Besides, they free water on their surface during electrofocusing.

2.8.2.4 Sample preparation and application

The sample proteins should be completely dissolved, and in concentrations at which the pherogram bands will be visible. For Coomassie brilliant blue R-250 staining, the total protein concentration in the sample must be about 0.1 g/dL (1 mg/mL); for silver staining, it must be 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, 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 [29,30]. 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 (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 salt concentration in a protein sample, 20 mmol/L was specified [31], but it is better to be decreased to 5 mmol/L. If necessary, the samples can be desalted by dialysis, gel filtration, or ultrafiltration.

Among a lot of methods, the sample application into template slots is the most convenient technique. The template should be removed 10 min after the application. If residual sample is still on the gel, it should be blotted with blotting paper.

2.8.2.5 Electrode solutions

The contact between the gel and electrodes is realized *via* electrode solutions whose pH values are higher and lower than the maximum and minimum pI points of the CA. 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 should be blotted and then the strips are put onto the gel.

The anode strips should contain acidic solution, and the cathode strips should contain basic solution (Table 2.8.1). In addition, the electrode solutions should contain 30–40 mL/dL glycerol, which prevents the drying of electrode strips.

Table 2.8.1: 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 ₃ PO ₄	0.5 mol/L NaOH
		0.25 mol/L acetic acid	0.25 mol/L NaOH
		0.025 mol/L aspartic acid,	0.025 mol/L lysine
		0.025 mol/L glutamic acid	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

2.8.2.6 Running isoelectric focusing with carrier ampholytes

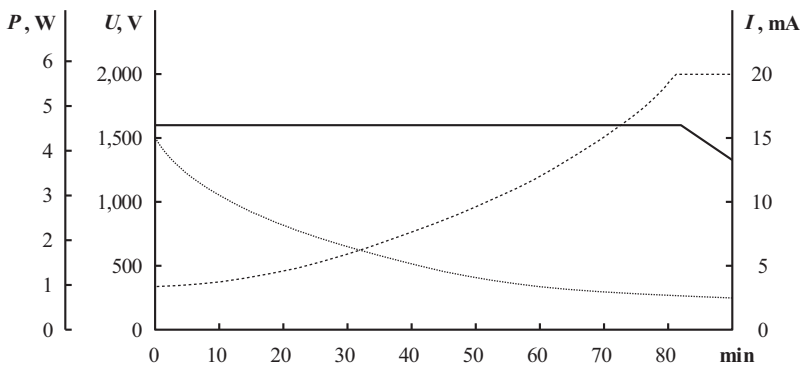
There is almost no difference between the IEF on polyacrylamide and agarose gels (Table 2.8.2). However, prior to IEF, the surface of agarose gel should be dried with a filter paper to remove the superficial liquid film. In addition, prefocusing of agarose gels should be avoided because the cathode drift here is much stronger than in polyacrylamide gels.

The IEF is run at constant power. In the beginning, the CA wear high net charges and the gel has a high conductivity. When the CA approach their isoelectric points and form a pH gradient, they lose their charges and mobilities. Therefore, the conductivity of the CA lowers, which causes the voltage to increase. Thus, a typical current–voltage relationship is obtained: the electric current falls, the voltage increases, and the electric power remains constant (Figure 2.8.3). In the presence of 8–9 mol/L urea, the temperature should not be below 15 °C to avoid the crystallization of urea.

After IEF, the gels are stained usually with Coomassie brilliant blue R-250, Serva violet 17, or silver (see there). In agarose gels, the silver staining is not enough successful because of the electroosmosis.

Table 2.8.2: Electrophoretic conditions for isoelectric focusing on polyacrylamide and agarose gels containing 2 g/dL carrier ampholytes at 10 °C; separation distance of 10 cm.

pH interval	Time	Power	Voltage	Electric current
Polyacrylamide gels				
<i>Prefocusing</i>				
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
<i>Focusing</i>				
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				
<i>Focusing</i>				
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

**Figure 2.8.3:** 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 $120 \times 250 \times 0.25$ mm, during a run at 10 °C for 90 min. — P , ----, U , I .

2.8.3 Isoelectric focusing in immobilized pH gradients

Gasparic, Bjellqvist, and Rosengren introduced the IPG [32]. They are acrylamido buffers that are copolymerized by their vinyl groups into polyacrylamide matrix to form IPG gels [33,34] (Figure 2.8.4). First, LKB Produkter AB produced and offered such buffers under the name of *Immobiline*.

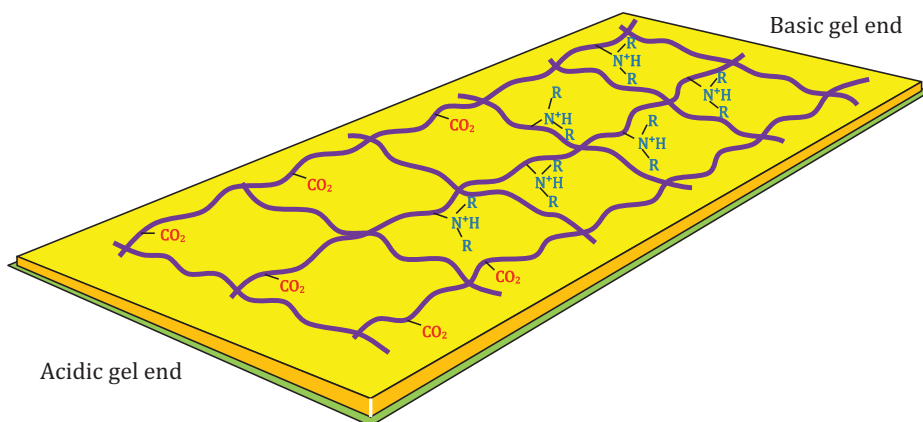


Figure 2.8.4: Polyacrylamide gel copolymerized with immobilines.

The distribution of immobilines in polyacrylamide gels takes place during the gel casting. Linear pH gradients are prepared using a gradient maker and two mixtures of immobilines: one relatively basic and one relatively acidic. Ultra-narrow pH gradients (up to 0.01 pH/cm) can also be produced.

The IPGs have several advantages over the pH gradients obtained with CA:

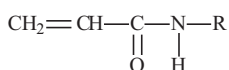
- IPG do not deform by any external influences (electroosmosis, high ionic strength of the samples, or protein overloads).
- The IPGs have a uniform buffering capacity and conductivity along the gel. This cannot be guaranteed with the CA pH gradients because not all of their components are present in same concentration.
- The IPG gels have a low conductivity, as the immobilines cannot move freely. As a result, a 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 gels with CA.
- The resolution of IEF in IPG exceeds the resolution of IEF with CA to 10–20 times and allows to resolve proteins that differ from each other by pI values of only 0.001–0.002 pH units [35]. While the resolution of IEF with CA is more than 0.02 pH units/cm, the resolution of IEF in IPG gels may reach 0.001 pH units/cm.

However, the IPG gels also have some disadvantages:

- The preparation of IPG is a complex process.
- The IPG gels can adsorb proteins.
- IEF in IPG gels requires very high voltage.
- The electrophoretic separation takes too long time.

2.8.3.1 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). They 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 [36]; therefore, it is recommended [37] that 0.005 mg/mL hydroquinone monomethyl should be added to the acidic immobilines ($pK = 1.0, 3.6, 4.4,$ and 4.6), and the neutral and basic immobilines ($pK = 6.2, 7.0, 8.5,$ and 9.3) to be dissolved in *n*-propanol. 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 IPGs are shown in Table 2.8.3.

Table 2.8.3: Structures of the acrylamido buffers used for preparing immobilized pH gradients.

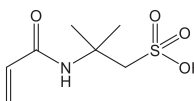
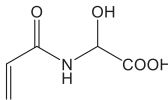
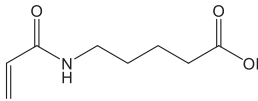
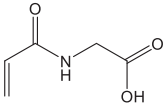
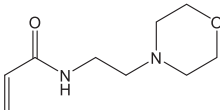
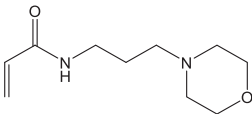
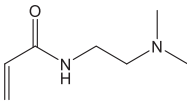
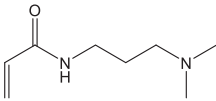
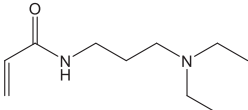
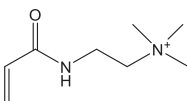
pH	Chemical formula	Name	M_r
Acidic acrylamido buffers			
1.2		2-Acrylamido-2-methylpropane sulfonic acid	207
3.1		2-Acrylamido-glycolic acid	145
3.6		<i>N</i> -Acryloyl-glycine	129

Table 2.8.3 (continued)

pH	Chemical formula	Name	M_r
4.6		4-Acrylamido-butyric acid	157
Neutral and basic acrylamido buffers			
6.2		2-Morpholino propylacrylamide	184
7.0		3-Morpholino propylacrylamide	199
8.5		<i>N,N</i> -Dimethyl aminoethyl acrylamide	142
9.3		<i>N,N</i> -Dimethyl aminopropyl acrylamide	156
10.3		<i>N,N</i> -Diethyl aminopropyl acrylamide	184
>12		<i>N,N,N</i> -Triethyl aminoethyl acrylamide	198

2.8.3.2 Casting IPG gels

The IPG gels are cast using a gradient maker and immobilines, wherein the heavy (with 25 mL/dL glycerol) solution is acidic and the light (with 5 mL/dL glycerol) solution is basic. The mixing chamber is filled with the heavy solution that builds the lower part of the IPG; the reservoir is filled with the light solution that builds the upper part of the IPG. For IEF of proteins, gels with $T = 10$ g/dL and $C = 0.025$ are recommended. In practice, thin (0.5 mm) as well ultrathin (<0.5 mm) IPG gels are used [38].

Rehydratable IPG gels

After casting, an IPG gel can be used immediately or can be dried. Prior to drying, the gel should be washed 3 times for 20 min each in deionized water and once in 2–3 mL/dL glycerol for 20 min. Then it is dried under a hair dryer and is sealed in a plastic wrap to be stored in the refrigerator at 4–8 °C for several days, or in the freezer at –20 °C for indefinite storing. The dried gel, which is referred to as a *rehydratable gel*, can be later rehydrated (reconstituted) in suitable solutions.

2.8.3.3 Running isoelectric focusing on IPG gels

The IPG IEF is carried out in the following way: 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 not be below 15 °C, since urea crystallizes at lower temperatures. As electrode solutions for IEF in IPG gels, as well as for hybrid IEF, 0.1 g/dL NaOH or 0.01 mol/L lysine (as a cathode solution), and 0.1 g/dL H₃PO₄ or 0.01 mol/L glutamic acid (as an anode solution) are used.

The IEF proceeds in two phases: During the *first phase*, the proteins migrate at a constant voltage into the gel. The electric power and electric current are set at maximum values, but the voltage is set on 120–300 V, so that the field strength should be 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 IPGs 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 2.8.5). This phase continues for 60 min.

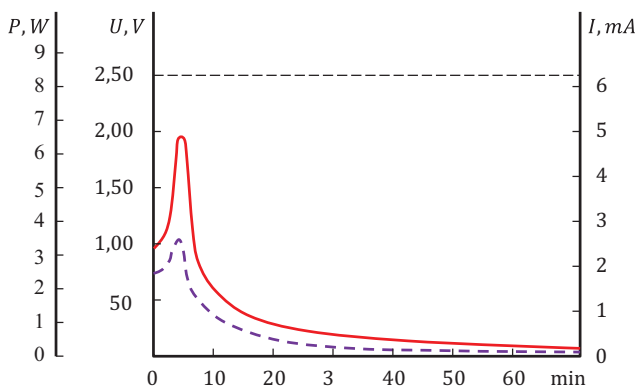


Figure 2.8.5: Electric power (P), voltage (U), and electric current (I) during the first 60 min of IEF in an immobilized pH gradient (pH = 4.5–4.8). — P , -- U , ... I .

During the *second phase* of IEF, the voltage should be set on maximum value of 5,000 V (2,000 V in hybrid IEF), the power should be set on maximum value of 10 W; then 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 CA. Therefore, the voltage in the IPG IEF should be higher and IEF should continue longer.

In the last years, an IEF microprocessor apparatus was described that is operating at 12,000 V maximum voltage, enabling to run high-speed IEF of proteins within 3 h [39]. In its standard configuration, up to six IPG strips can be run.

The electrofocusing time is reciprocally proportional to the pH units: 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 (1 pH unit or less).

After electrofocusing, the proteins in an IPG gel can be stained with conventional or colloidal Coomassie brilliant blue in phosphoric or sulfuric acid. If higher sensitivity is desired, the IPG gels can be stained with silver.

2.8.4 Isoelectric focusing in the clinical laboratory

With the help of IEF on polyacrylamide gels, more than 300 hemoglobin variants [40, 41, 42], lipoproteins [43, 44, 45], phenotypes of α_1 -antithrypsin [46, 47], urinary proteins [48, 49], globulins [50, 51], salivary proteins [52, 53], catalase [54], and many other clinically important proteins were analyzed. Besides, genetic investigations in forensic medicine [55] were performed. Meat proteins [56, 57], potato varieties [58, 59], and wheat proteins [60] were studied, too.

2.8.5 Protocols

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 Flavin mononucleotide (FMN, riboflavin-5'-phosphate)

Gel casting 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

Monomeric 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

- Pour monomeric solution into the casting cassette.
- Illuminate the monomeric solution in the cassette with the fluorescent lamp for 1 h.
- Open the 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.

Running Isoelectric Focusing with Carrier Ampholytes**Materials and equipment**

Flat-bed electrophoresis cell

Film-supported gels with carrier ampholytes

Power supply capable of delivering 2,000–3,000 V and 6W

Refrigerated water circulator, if required

1 N NaOH catholyte

1 N H₃PO₄ anolyte

Electrode strips

Sample application template

Procedure

- Pipette 0.5–1.0 mL of kerosene or silicone oil DC 200 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.

- Place an application template onto the gel centrum, and press it gently to provide a good contact.
- Prefocus the gel at 400–500–1,000 V per 10 cm gel for 30–60 min. So a pH gradient will be formed, and the unwanted ions, e.g., APS and TMEDA ions, will migrate toward the electrode strips.

Gels with higher glycerol concentration and the rehydrated gels do not require 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 2.8.6).

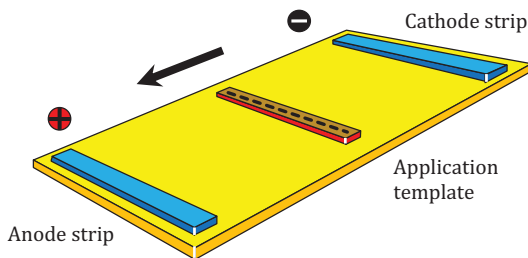


Figure 2.8.6: Gel with carrier ampholytes, paper electrode strips, and an application template.

- Increase gradually the field strength from 50–100 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, 5,000–20,000 Vh per 10 cm gel is used.
- Interrupt the IEF 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.

Isoelectric focusing of net-supported gels containing carrier ampholytes

- Place the net-supported gel onto the cooled to 15 °C cooling plate of the electrophoretic unit.
- Place the electrode strips containing the anode and cathode solutions on both ends of the gel.
- Place an application template onto the middle of the gel 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 IEF at 200 V (for gels with separation distance of 10 cm).
- Increase the electric voltage by 400–1,000 V every 30 min until a maximum voltage of 3,000 V is reached.

In gels with high glycerol concentration, the voltage can reach 6,000 V.

- Continue the electric focusing until the desired Vh product.

Casting Immobiline Gels

Materials and equipment

Acrylamide
BIS
TMEDA
APS
Immobilines
87% Glycerol
Gel cassette

Procedure

IPG gels with linear pH gradients are cast according to the recipes of Gianazza et al. [61, 62] (Table 2.8.4).

- 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 $-20\text{ }^{\circ}\text{C}$.

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 pharmalytes 3–10	5.00 mL
DTT	0.23 g (15.0 mmol/L)
Deionized water to	100.00 mL

Table 2.8.4: Volumes of the acidic (heavy) and basic (light) immobiline solution, which are used for casting of 15 mL slab gels (120 × 250 × 0.5 mm) with wide pH gradients (2–6 pH units). Both solutions contain monomers in same concentration but the heavy solution contains more glycerol.

		pH ranges												
		Acidic (heavy) solution 0.2 mol/L immobilines with diverse pK, in μL					Basic (light) solution 0.2 mol/L immobilines with diverse pK, 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

(continued)

Table 2.8.4 (continued)

		pH ranges										
		Acidic (heavy) solution 0.2 mol/L immobilines with diverse pK, in μL					Basic (light) solution 0.2 mol/L immobilines with diverse pK, 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
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

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 plus two layers of parafilm, 0.1 mm each).
- Clamp the cassette together and pour the rehydration solution.
- Cut dried IPG gels 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 to their original thickness (0.5 mm) (Figure 2.8.7) overnight at room temperature.

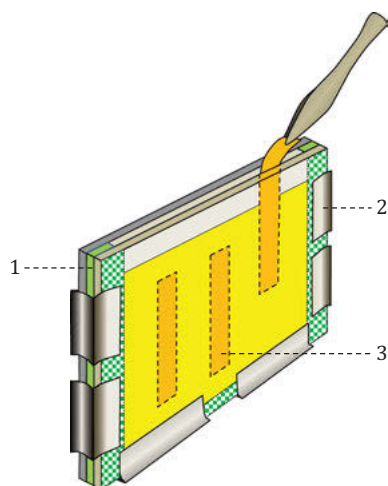


Figure 2.8.7: Rehydration of IPG gel strips.
1. U-formed gasket; 2. clamp; 3. gel strip.

- Take the rehydrated gel strips out of the cassette and place them gel up on a sheet of 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 of IEF chamber and place the IPG gel strips on it, 2 mm apart from each other. The acidic end of the IPG gel strips should face toward the anode.
- Cut two 3 mm thick filter paper to a 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 top of the IPG gel strips at the cathode and anode.
- 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 chamber 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 3,500 V overnight.

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2.9 Free-flow electrophoresis of proteins

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Free-flow electrophoresis (FFE) has been developed by Grassmann and Hannig [1–3]. It is a matrix-free electrophoretic technique, which resembles the capillary electrophoresis. Until the 1980s, it was a technology for separation of cells and organelles, but now is used for separation of proteins and other charged particles. Besides the (macro) FFE, miniaturized versions of FFE, called micro-FFE, are also known [4].

The advantage of FFE is the fast separation of proteins, which do not adhere to any matrix structure as agarose or polyacrylamide gel. The separations are highly reproducible and can be carried out under native or denaturing conditions [5]. The electrophoresis continues only several minutes; therefore, most proteins, for example, enzymes, preserve their activity. To succeed this, the pH value during electrophoresis should be kept within physiological limits, and sucrose, glucose, or other compounds should be added to maintain appropriate isosmotic pressure.

2.9.1 Theory of free-flow electrophoresis

The sample in FFE is injected in a thin buffer layer (<1 mm) that flows through a separation channel formed between two parallel plates [6, 7]. Perpendicularly to this flow, an electric field is applied in order to deflect charged compounds in different angles according to their electrophoretic mobilities. Therefore, the theory of FFE 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 (deflection angle) is the angle between the resultant line and y -axis. The deflection angle increases with the increase in the electric field strength and decreases with the increase in the buffer flow velocity (Figure 2.9.1). The separated compounds are collected continuously as fractions.

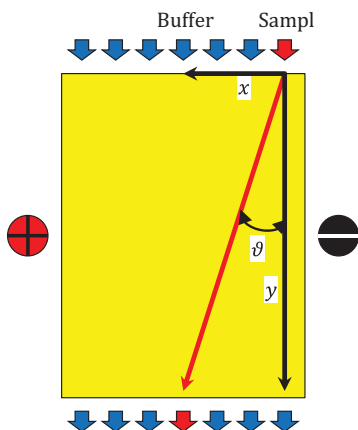


Figure 2.9.1: Scheme of free-flow electrophoresis.

The magnitude of $tg\theta$ is equal to the ratio between the distances x and y , hence, between the sample velocity v_s and the buffer velocity v_b :

$$tg\theta = \frac{x}{y} = \frac{v_s}{v_b} \quad (2.9.1)$$

The electrophoretic velocity of the sample in an electric field could be given by the equation

$$v_s = \mu_s E = \mu_s \frac{J}{\gamma} = \mu_s \frac{I}{q\gamma} \quad (2.9.2)$$

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. (2.9.1) and (2.9.2), it follows that

$$tg\theta = \frac{\mu_s I}{q\gamma v_b} \quad (2.9.3)$$

2.9.2 Types of free-flow electrophoresis

According to the electrophoretic principles, three types of FFE can be distinguished: **free-flow zone electrophoresis (FFZE)**, **free-flow isotachopheresis (FFITP)**, and **free-flow isoelectric focusing (FFIEF)**.

2.9.2.1 Free-flow zone electrophoresis

FFZE is carried out in one (continuous) buffer. The buffer should 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). The borate buffer should be carefully used because boric acid forms complexes with polyols [8].

The migration distance d of an analyte moving through the electric field is given by the equation

$$d = \mu_p Et \quad (2.9.4)$$

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 the band broadening (σ_T): 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]:

$$\sigma_T^2 = \sigma_{INJ}^2 + \sigma_D^2 + \sigma_{HD}^2 + \sigma_{ED}^2 + \sigma_{JH}^2 + \sigma_{EMD}^2 \quad (2.9.5)$$

The diffusional broadening is related to the residence time t of the analyte in the separation chamber [12]:

$$\sigma_D^2 = 2Dt \quad (2.9.6)$$

where D is the analyte diffusion coefficient [13].

2.9.2.2 Free-flow isotachopheresis

In FFITP, discontinuous buffer system is used (Figure 2.9.2). It consists of a leading and trailing buffer. The mobility of the leading ion should be greater, and the mobility of the trailing ion should be smaller than the mobility of the particles to be resolved. During the electrophoresis run, the sample components arrange themselves according to their descending mobilities and form sharp bands whose concentrations depend on the concentration of the leading ion. The counter-ion is usually an ion of a weak base or acid, which has high buffer capacity in the necessary pH range.

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.

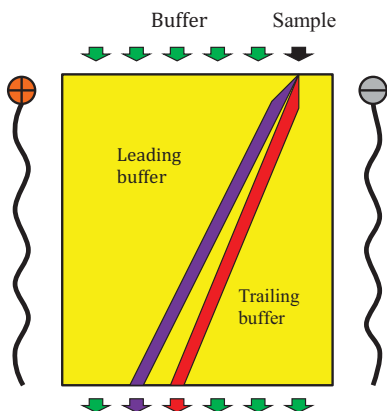


Figure 2.9.2: Scheme of free-flow isotachopheresis of two negatively charged particles.

2.9.2.3 Free-flow isoelectric focusing

In FFIEF, the sample is fractionated in a linear pH gradient [14] created by carrier ampholytes (Figure 2.9.3). The proteins to be resolved 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).

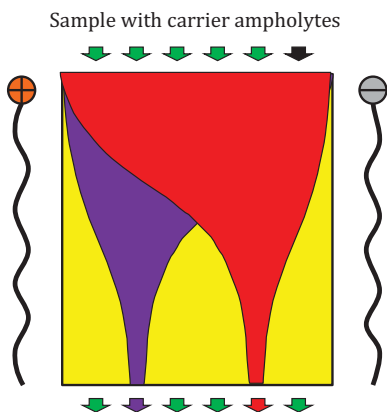


Figure 2.9.3: Free-flow isoelectric focusing in carrier ampholyte pH gradient.

The linear pH gradient is perpendicular to the flow direction. The sample components migrate through the pH gradient, due to the electric field, until they reach their pI. There they lose their electric charges and focus [15].

The equilibrium between the electrophoretic and diffusional transport during IEF is described by the following differential equation, valid under steady-state conditions [16]:

$$\frac{d(c\mu E)}{dx} = \frac{d}{dx} D \frac{dc}{dx} \quad (2.9.7)$$

where c [in mol/L] is the analyte concentration at position x in the separation chamber, μ [in $\text{m}^2/(\text{s V})$] is the analyte mobility at that point, E [in V/m] is the electric field strength, and D [in m^2/s] is the diffusion coefficient.

2.9.3 Device technology of free-flow electrophoresis

A FFE device has a front plate and a back plate. The front plate is made of poly(methylmethacrylate) (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 plate and the back plate is usually 0.1–0.5 mm and can be adjusted by membrane spacers, e.g., of cellulose nitrate.

The separation chamber is divided into three regions: separation region, anode bed, and cathode bed. The electrodes are placed outside the separation region avoiding the disturbance of generated oxygen and hydrogen bubbles. Usually, the device is located on a cooled plate in order to remove the heat generated by the electric current. This principle could be applied for smaller FFE systems that use injection molding and milling [17].

The buffer is fed with the help of a tubing pump and flows vertical in the narrow gap between the two plates [18–20]. The sample is applied in the upper part of the device with the aid of a dossier pump. The electric current is maintained by filter paper strips, which are immersed in lateral electrode tanks. The separated proteins are collected at the lower end of the device. The device is cooled with water.

2.9.4 Detection system of free-flow electrophoresis

The detection system of FFE consists of a scanner and computer with printer. The scanner measures the UV absorption of proteins flowing with the buffer. Prior to the electrophoretic separation, the absorbance of the pure buffer is measured to determine a baseline, which should be subtracted from the values obtained. The measurement signals are sent to a computer working with appropriate software.

2.9.5 Applications of free-flow electrophoresis

FFE is an excellent method for analyzing human plasma proteins [21]. It can also be used for separation of enzymes [22–23], cell organelles (lysosomes, mitochondria, ribosomes, and more) [24], membranes, chloroplasts, viruses, bacteria, and cells as platelets, lymphocytes, red cells, thymus cells, tumor cells, thyroid cells, and more [25–27]. The FFE is also a good technique for preparative separations of peptides [28–30], proteins [31–33], cellular components [34], and cells [35–37].

Using FFITP, human transferrin was isolated [38], and the purification of ovalbumin, lysozyme [39], and monoclonal antibodies [40] was reported. Using narrow pH gradients in FFI, antibody isoforms were separated that vary by less than 0.02 pH units [41].

2.9.6 Protocols

Free-flow Zone Electrophoresis of Human T and B Lymphocytes

Materials and equipment

TRIS

Phosphoric acid

NaCl

KCl

T and B lymphocytes

Apparatus for FFE

Resolving buffer, pH = 7.4

NaH₂PO₄·H₂O 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

NaH₂PO₄·H₂O 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

NaH₂PO₄·H₂O 2.76 g (0.02 mol/L)

Adjust with TRIS to pH = 7.0.

Deionized water to 1,000.00 mL

Procedure

- Suspend 2×10^7 cells of a sample in 1 mL resolving buffer.
- Fill the cathode and anode tanks with cathode and anode buffer, respectively.
- Give 240 V/cm field strength at 20 °C.
- Run FFE for 6 min.

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2.10 Capillary electrophoresis of proteins

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Capillary electrophoresis (CE) [1, 2] can be carried out in buffers or on gels containing native or denaturing buffers [3]. The interest in CE is based on its high resolution and speed. It is possible to separate reproducibly with the help of CE attomole quantities (10^{-18} to 10^{-21} mol) of proteins, nucleic acids, carbohydrates, and other compounds in minutes up to an hour [4, 5].

2.10.1 Theory of capillary electrophoresis of proteins

The theory of CE does not differ from the general electrophoretic theory [6, 7]. The analytes migrate in an electric field E with the velocity

$$v_{ep} = \mu_{ep}E = \frac{QE}{6\pi r\eta} \quad (2.10.1)$$

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. Other variables such as shape and hydrophobicity have also been shown to affect the velocity [8].

Simultaneously in a fused silica capillary, silanol ($-\text{Si}-\text{OH}$) groups ($\text{p}K \approx 3$) attached to the internal wall of the capillary are ionized giving negatively charged silanolate groups ($-\text{Si}-\text{O}^-$). The positive ions of the buffer are attracted to the negative silanolate groups. Therefore, an electric double layer with fixed and diffuse positive counter-ions is built (Figure 2.10.1). When an electric field is applied in a capillary,

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the diffuse positive counter-ions (cations) migrate to the negatively charged cathode through the electric field. Since they are hydrated, the water of the buffer migrates too, which causes **electroosmotic flow** (EOF). Therefore, in CE two opposite processes are acting: the electrophoretic velocity of polyions and EOF of the buffer [9].

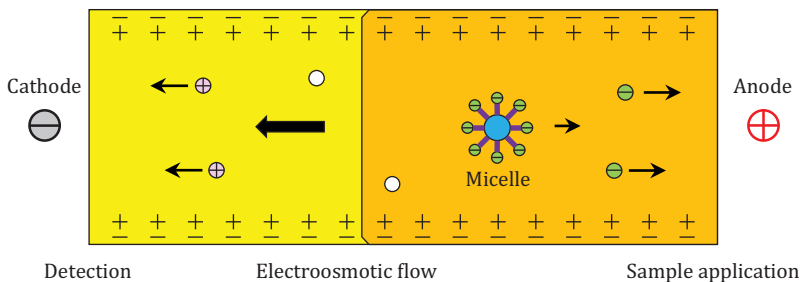


Figure 2.10.1: Depiction of the interior of a fused silica capillary filled with a buffer during electrophoresis.

The velocity of the EOF v_0 can be expressed as

$$v_{eo} = \mu_{eo} E \quad (2.10.2)$$

where μ_0 is the electroosmotic mobility. It is defined as

$$\mu_{eo} = \frac{\varepsilon \zeta}{\eta} \quad (2.10.3)$$

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 CE is

$$v = v_{ep} + v_{eo} = \left(\mu_{ep} + \mu_{eo} \right) E = \left(\frac{Q}{6\pi r} + \varepsilon \zeta \right) \frac{E}{\eta} \quad (2.10.4)$$

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 cations, then by neutral molecules, and finally by anions.

2.10.2 Instrumentation

A 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 from 7 to 100 cm long (usually 50 cm), and has about 375 μm outer diameter and usually 50 μm inner diameter. Teflon capillaries are also available.

The outside of the capillaries is coated with a thin layer of polyimide to get flexible. The inside of the capillaries is coated with polyacrylamide, methylcellulose, or other compounds. Thus, the adsorption of polyions on the capillary surface is avoided (Figure 2.10.2).

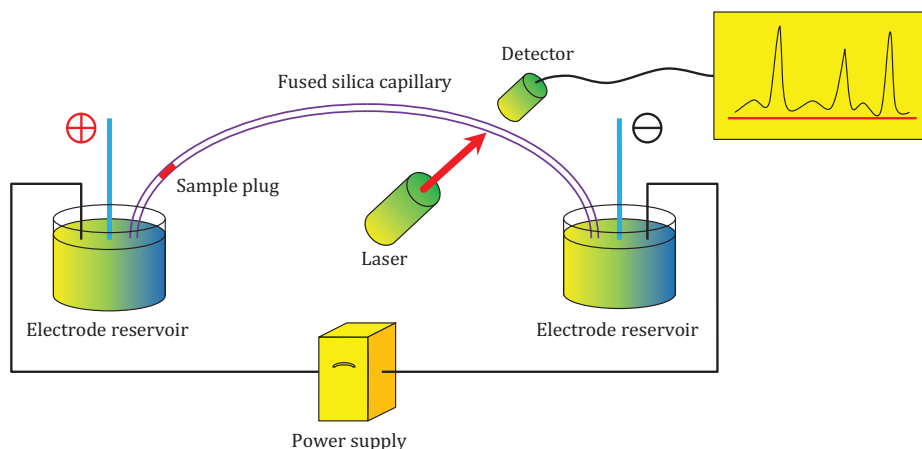
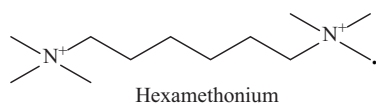


Figure 2.10.2: Capillary electrophoresis instrumentation.

The sample is introduced into the capillary by pressure, siphoning, or electrokinetically. Then the capillary is returned to the source vial. After electric voltage is applied, the sample components separate from each other and are detected at the outlet end of the capillary. The results are presented as peaks in a pherogram.

2.10.2.1 Coating the capillaries

During CE, noxious interactions appear between the proteins to be resolved and the capillary. For example, basic proteins adsorb onto the negatively charged internal wall of the fused silica capillary, which leads to impaired efficiency and poor resolution [10, 11]. In addition to the direct interaction with the capillary wall, the proteins are moved by EOF, which also hinders the resolution of CE. The electroosmotic flow can be slowed using ionic additives, such as Mg^{2+} and hexamethonium, which bind to the negatively charged silanulates and, as a result, lower the electric charge of the capillary



Another strategy to slow EOF is to coat the capillary wall with polymers. There are two types of coating: permanent and dynamic [12].

Permanent capillary coating is a complex process. Prior to it, the capillary surface should be cleaned and activated by etching, leaching, dehydration, and silylation [13]. The etching can be done with sodium hydroxide; the leaching can be carried out with hydrochloric acid; and 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 diminish EOF. During this coating, polymers are adsorbed on capillaries *via* physical bonds. The dynamic capillary coating can be performed using neutral or cationic polymers. Surfactants can also be used.

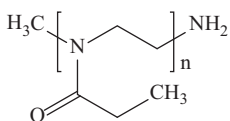
Neutral polymers [14] quench the protein binding to the silica wall in 50–60%; only the neutral hydrophobic polymer poly(*N,N*-**dimethylacrylamide**) [poly(DMA)] shows higher inhibition (ca. 85%). To the group of cationic polymers belong **polyethylenimine** (PEI) [15–17], **poly(*N*-hydroxyethylacrylamide)** (PHEA) [18, 19], chitosan [20, 21], **poly(dimethylsiloxane)** (PDMS) [22, 23], ***N*-methylpolyvinylpyridinium** quaternary ion (PVPy-Me) [24], and more.

Surfactants that can be used as coating agents could be divided into two groups: nonionic and zwitterionic surfactants (Table 2.10.1).

Besides the above-shown coating polymers, fluorinated polymers are also used as coating substances, for example, **poly(tetrafluoroethene)** (PTFE, or Teflon) and **fluorocarbon** (FC). They 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 [25, 26].

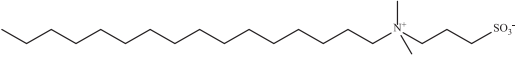
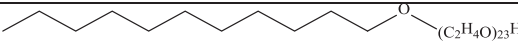
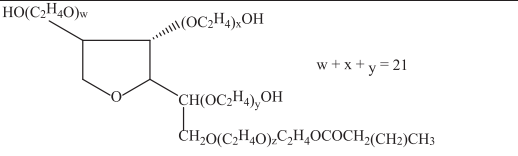
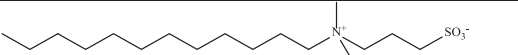
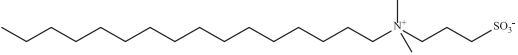
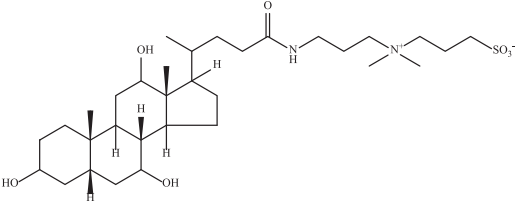
2.10.2.2 Sieving matrix in capillary electrophoresis

A sieving matrix that was proposed for CE is the polymer **poly(2-ethyl-2-oxazoline)** (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 CE [27]. PEOX has good hydrolytic stability because the amide is highly substituted.

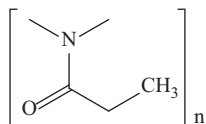


Poly(2-ethyl-2-oxazoline)

Table 2.10.1: Chemical formulas of nonionic and zwitterionic surfactants.

Detergent	Formula	CMC (mmol/L)	M_r
<i>Nonionic surfactants</i>			
Triton X-100 (polyethylene glycol tert-octylphenyl ether)		0.02-0.09	625
Brij 35 (polyoxyethylene lauryl ether)		0.05-0.1	1,225
Tween 20 (polyoxyethelene sorbitanmonolaurate)		0.06	1,228
<i>Zwitterionic surfactants</i>			
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
CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)		6-10	614.9

A homolog of PEOX is **poly(*N,N*-dimethylpropionamide)** (PDMPA).



Poly(*N,N*-dimethylpropionamide)

Hydroxypropyl cellulose was also used as a sieving matrix [28]. Another polymer that was used for separating single cells is **poly(ethylene oxide)** (PEO) [29]. It has $M_r = 100,000$ and low viscosity, which allows a hydrodynamic injection of proteins or cells into a coated capillary.

2.10.3 Practice of capillary electrophoresis

CE is carried out in three steps: injection of a sample into the capillary, separation, and detection of protein bands.

2.10.3.1 Injection

The injection of a sample is carried out by electromigration, gravity, or pressure [30]. To inject a sample into a capillary by electromigration, a sample applicator is needed. When the sample is applied at the anode, no positive ions are allowed to be present in the sample. If this is undesirable, the injection can be carried out by gravity. Then the sample vial and the corresponding capillary end should be raised to a higher level than the other capillary end, whereby the sample is sucked by the siphon effect.

2.10.3.2 Separation

The migration of proteins is due to a high-voltage power supply which creates electric field between the source and destination vials. All ions, positive or negative, are pulled through the capillary in the same direction by EOF and are detected near its outlet end. The output of the detector is connected with a computer or an integrator. The Joule heating is removed by a blower.

2.10.3.3 Detection

There are diverse techniques for detecting the separated bands: **ultraviolet (UV) detection**, **laser-induced fluorescence (LIF) detection**, **mass spectrometry (MS) detection**, **chemiluminescence (CL) detection**, and **whole-column imaging detection (WCID)**.

The UV detection is the most common detection. It is based on the UV absorbance and is used for analyzing the hemoglobin variants. Frequently employed wavelengths are 210, 280, and 415 nm. Zhu *et al.* [31–33] have found that the maximal absorbance of the heme group is at 415 nm wavelength.

The LIF is a method, in which an atom or a molecule is excited to a higher energy level by absorption of laser light followed by spontaneous emission of light [34–36]. Hb exhibits native fluorescence that relies on the fluorescence of aromatic amino acid residues [37].

The MS provides extremely high sensitivity of CE for very small sample concentrations. It includes ionization of chemical species and sorting the ions based on their mass-to-charge ratio. As a result, the compound masses within a sample are measured. MS analysis is used to measure Hb variants and amino acid sequences [38].

The CL detection is another method widely used in combination with chromatography, spectrometry, and immunoassay [39]. It is characterized by excellent sensitivity and selectivity, allowing high resolution and precise quantification [40].

The WCID has been successfully employed for the electrofocusing analysis of Hb. Here a short capillary (a few cm long) is used as a separation channel, which is connected with two pieces of a 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 molecules (such as proteins) inside the capillary. When high voltage is applied, the large molecule in the separation channel will be focused at their pI values. Then the focused bands are imaged with a charge coupled device (CCD) camera (Figure 2.10.3).

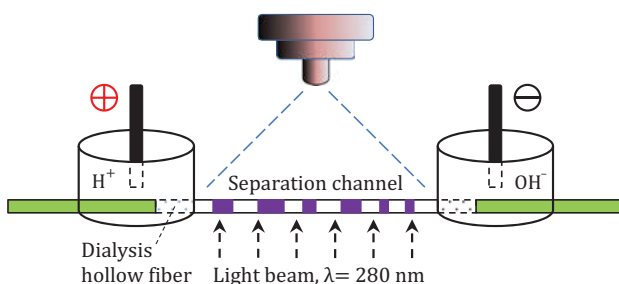


Figure 2.10.3: Principle of whole-column imaging detection.

2.10.4 Types of capillary electrophoresis

According to the main electrophoretic methods, three CE methods are distinguished: capillary zone electrophoresis (CZE), capillary isotachopheresis (CITP), and capillary isoelectric focusing (CIEF).

CZE was carried out in capillaries with an inner diameter of 40 μm [41]. It was run in phosphate buffer [42]. The phosphate buffer can cover a broad range of pH value (2.55–11.43) due to the three dissociation constants of phosphoric acid and its ions ($\text{p}K_{\text{H}_3\text{PO}_4} = 2.0$, $\text{p}K_{\text{H}_2\text{PO}_4^-} = 7.2$, and $\text{p}K_{\text{HPO}_4^{2-}} = 11.0$).

CITP [43–44] needs two buffers, which contain a leading ion (e.g., formate ion) and a trailing ion (e.g., taurinate ion). It is carried out in capillaries with inner diameters of 0.8–0.2 mm.

In CIEF, proteins with different isoelectric points are separated in a pH gradient [45]. It is important that EOF is equal to zero. This can be achieved by a modification of the capillary surface or at a suitable pH value of the buffer [46]. If the capillaries are not coated, the separation medium viscosity has to be increased by adding glycerol [47] or methylcellulose [48].

2.10.5 Applications of capillary electrophoresis

CE is widely used in the clinical and forensic medicine [49, 50]. An instrument for clinical CE (cCE) should have the capability for rinsing, washing, thermostating, and easy replacement of the capillaries as well as of accurate applying of nanoliter volumes of a sample. In addition, the cCE design should include dilution of the sample and automated buffer replenishment.

2.10.5.1 Serum protein analysis

Chen *et al.* [51] showed first that the CE is an alternative to agarose electrophoresis. Figure 2.10.4 shows the comparative profiles obtained with scanning densitometry of an agarose gel and direct analysis of a pooled serum sample by CE.

Prealbumin (transthyretin). Prealbumin transports thyroxine and triiodothyronine. Its concentration is an important indicator of nutritional status, inflammation, malignancy, liver cirrhosis, or Hodgkin's disease. It occurs in a relatively low concentration in "normal" serum (200–360 mg/L) but can be detected by CE at 214 or 200 nm wavelength.

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 into five density classes: **high-density lipoproteins** (HDL), **low-density lipoproteins** (LDL), **very-low-density lipoproteins** (VLDL), **intermediate-**

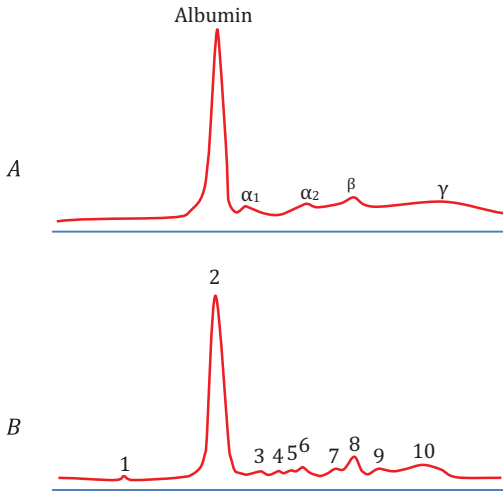


Figure 2.10.4: Serum protein analysis by agarose gel electrophoresis and capillary electrophoresis. (A) Agarose zone electrophoresis of normal serum proteins giving five fractions. (B) Capillary zone electrophoresis of the same proteins giving more fractions in TRIS-borate buffer, pH = 8.3. Detection 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.

density lipoproteins (IDL), and chylomicrons (see there). LDL are the major cholesterol depot in the blood. They carry cholesterol to the tissues [52]. HDL carry cholesterol in the reverse direction – from the tissues to the liver for excretion [53].

The concentrations of LDL and HDL are highly important in the clinical diagnostics. However, the adsorption of lipoproteins onto the fused silica capillary wall makes their separation difficult [54, 55]. Attempts have been made to diminish the adsorption by dynamic or permanent coating of capillaries [56, 57]. The dynamic coating is made with methylglucamine or SDS or polymers such as poly(ethyleneoxide) (PEO) or hydroxypropyl methylcellulose [58, 59].

Immunoglobulin subtypes. Klein and Jolliff [60] have shown that immunoglobulin subtypes (IgG, IgA, IgM, heavy chains, and light chains κ and λ) can be identified by CE. The serum samples should be incubated with a solid phase to which specific antibodies are bound. During the incubation, the immunoglobulin subtypes bind specifically to their antibodies.

2.10.5.2 Hemoglobins

Hemoglobins (Hb) ($M_r = 64,500$) are the major proteins in **red blood cells (RBC)**. CE has important significance in their analysis [61–63] (Figure 2.10.5) because of its high speed, low sample consumption, and high resolution.

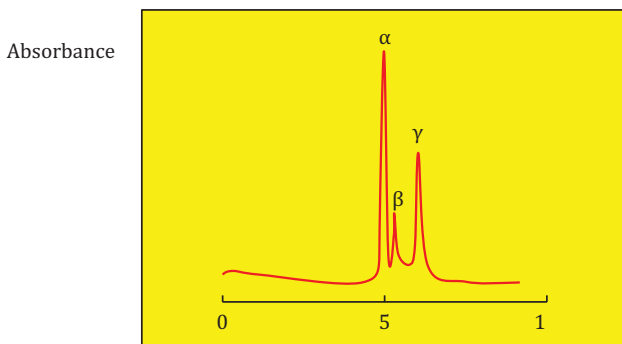


Figure 2.10.5: Separation of fetal α -, β -, and γ -globin chains.

The CZE methods for Hb separation use strongly acidic buffers (pH = 2.0–2.5) [64–66] or strongly alkaline buffers (pH = 11.8) [67]. Under these circumstances, the globin chains dissociate from the heme during electrophoresis [68, 69]. So the Hb variants A₂, F, and A_{1c} were studied [70]. Zhu *et al.* [71] demonstrated that using capillary isoelectric focusing α -thalassemias can be identified. The computer-assisted CIEF is also an excellent method for the clinical assessment of hemoglobinopathies because of its automatization, high resolution, and possibility of simultaneous quantification of Hb variants [72–74].

2.10.5.3 Isoenzymes

CE can also be used for isoenzyme assays [75], for example, of alkaline phosphatase, creatine kinase, A and B forms of *O*-*N*-acetylglucosaminidase, P and S types of amylase, proteolytic isoenzymes, γ -glutamyl transpeptidase, kallikrein, renin, cathepsin B, and 5'-nucleotidase.

2.10.5.4 Immune complexes

CE can be used for separation of immune complexes from unbound antibody and antigen [76]. Ultratrace detection is also possible when using fluorescence-tagged monoclonal antibodies and laser-induced fluorescence (LIF) detection [77]. As a result, the detection of cancer biomarkers in nanomolar or even femtomolar concentration was done. In addition, it was demonstrated that linear polyacrylamide (LPA)-coated capillaries are suitable for CE-MS analysis of IgG subunits [78]: the heavy and light chains of IgG molecules were separated on LPA-coated capillaries by more than 3 min, with peak width of about 45 s.

2.10.5.5 Single cell analysis

Jorgenson *et al.* [79] first demonstrated the potential of CE for analyzing cells differing in age, differentiation, subtype, *etc.* Olefirowicz and Ewing [80, 81] furthered these studies by using special capillaries for studying the cytoplasm of dopaminergic neurons.

Other applications of CE were 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 has been reported using polyvinyl alcohol-coated capillary [82, 83]. For interferon- β_1 proteoforms, instead of regular PEI-coated capillary, a cross-linked polyethylenimine coating has been used on the quantitative CE-MS analysis [84].

2.10.6 Protocols

Capillary IEF of Proteins

Materials and equipment

IEF markers

Ampholyte mixture, pH = 3.0–10.0

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 2,000–3,000 V and power of 6 W

Sodium borate buffer, 5 mmol/L, pH = 8.0

Sodium borate anhydrate 1.01 g (5 mmol/L)

Adjust 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)

Adjust 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)

Adjust 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

- Dilute a sample with 50 mmol/L sodium borate buffer to give 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 give 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.
- Place 10 mmol/L phosphoric acid in the anode reservoir, and 20 mmol/L NaOH in the cathode reservoir.
- Focus the sample at constant 10 kV 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.

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2.11 Two-dimensional electrophoresis

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The **two-dimensional** (2D) gel electrophoresis (2D PAGE) is a combination of two high-resolution electrophoretic methods: IEF and SDS-PAGE. It was introduced in 1975 by O'Farrell [1] and Klose [2].

2.11.1 Theory of 2D electrophoresis

In the first direction (dimension) of the 2D electrophoresis, the positively charged proteins will run toward the negative end of the gel, and the negatively charged proteins will run to the positive end of the gel. Afterward, the proteins are resolved in the second direction (dimension) by denaturing SDS discontinuous electrophoresis (disc-electrophoresis) according to their masses: Prior to the SDS disc-electrophoresis, the gel strip with the resolved proteins should be treated with SDS. This procedure unfolds (denatures) the proteins into long straight polyions and binds a number of SDS ions to them proportional to their masses. Since the SDS ions are negatively charged, all proteins will become approximately the same mass-to-charge ratio (Figure 2.11.1).

The IEF of the 2D electrophoresis can be done in pH gradient gels with carrier ampholytes or in IPG gels [3]. The 2D electrophoresis with carrier ampholytes is characterized by a shorter focusing run than the 2D electrophoresis in IPG gels; however, the 2D electrophoresis in IPG gels has higher reproducibility. The IEF of the 2D electrophoresis can also be carried out in hybrid IPG gels, which contain additional carrier ampholytes [4].

As a result of 2D electrophoresis, the protein polyions form a 2D pherogram of spots that looks like a geographical map. The position of each protein spot can be coordinated in the rectangular (Cartesian) system (Figure 2.11.2).

<https://doi.org/10.1515/9783110761641-021>

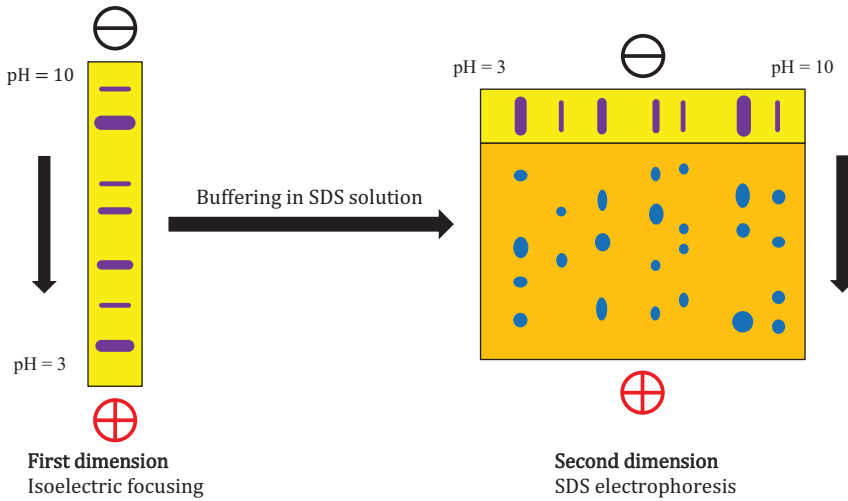


Figure 2.11.1: Principle of two-dimensional electrophoresis.

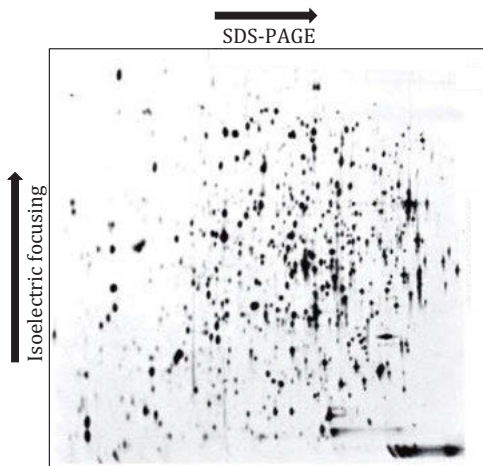


Figure 2.11.2: Protein spots obtained by 2D electrophoresis.

2.11.2 Isoelectric focusing in the first dimension

Prior to IEF in the first dimension, the protein sample should be appropriately prepared.

2.11.2.1 Sample preparation

Tissue or blood samples for a 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). The protein concentration in the sample should be about 10 mg/mL. In addition, 8.0–9.0 mol/L urea, 1.0–2.0 mL/dL Nonidet NP-40, 0.5 g/dL dithiothreitol, and 0.8–1.0 g/dL carrier ampholytes (if the first-dimension gel contains carrier ampholytes) should be added to the sample. The sample buffer should have low ionic strength.

2.11.2.2 ISO-DALT and IPG-DALT

If the IEF is performed by carrier ampholytes, the 2D electrophoresis is referred to as ISO-DALT system [5]. **Iso** is for the pI separation by IEF using carrier ampholytes, and **Dalt** is for the mass resolving by SDS disc-electrophoresis (Dalton is the old unit for mass, which has found no place in SI system). If the IEF is performed in an IPG gel, the 2D electrophoresis is referred to as IPG-DALT system.

The gels for IEF with carrier ampholytes have T concentration of 4–5 g/dL and a degree of cross-linking $C = 0.03$, and contain 8–9 mol/L urea and 1–2 g/dL carrier ampholytes. Their ends should be covered with paper or gel strip electrodes; the anode strip could contain 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); and the cathode strip could contain 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. Then a silicone template could be laid down onto the gel, and 10 μ L of samples each could be applied into its slots.

For IPG-Dalt system, immobilized pH gradient of pH = 4.0–10.0, 4.0–7.0, or 7.0–10.0 is used. They can be prepared as follows (Table 2.11.1)

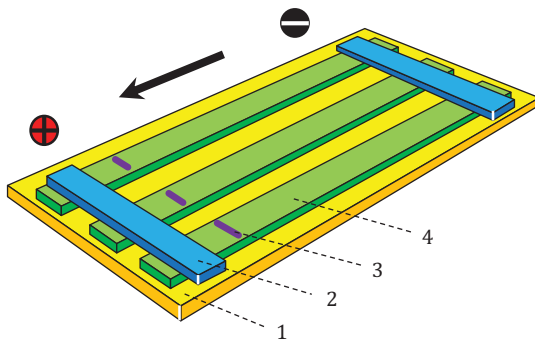
The IPG gel is prepared using a gradient maker at room temperature. Thereafter, the gel is removed from the casting cassette, and washed 3 times for 20 min each in deionized water, 1 time in 2 mL/dL glycerol, and finally dried at room temperature using a fan. At the end, the gel is put in a plastic wrap and stored at $-20\text{ }^{\circ}\text{C}$.

After rehydration at room temperature for 3–24 h, the swollen IPG gel strips are put between two filter papers to remove the excess rehydration solution so that urea should not crystallize on their surface. Then the strips are placed, the acidic ends facing the anode, onto the coated with kerosene cooling block of a horizontal chamber. Next, the IPG gel strips are covered with paper or gel electrode strips (Figure 2.11.3). At the end, the samples are applied, using a silicone template, onto the gel strips.

The IEF is carried out at $10\text{--}15\text{ }^{\circ}\text{C}$, wherein a prefocusing is needed only in IEF with carrier ampholytes; prefocusing for IEF on IPG gels is unnecessary. After IEF, the gel with carrier ampholytes or the IPG gel strips can be used immediately for the second dimension of the 2D electrophoresis, or can be stored in a freezer at $-80\text{ }^{\circ}\text{C}$ or in liquid nitrogen.

Table 2.11.1: Preparation of solutions for 250 × 120 × 0.5 mm IPG gels ($T = 4$ g/dL, $C = 0.03$) with different pH gradients.

	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	–
Monomeric 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 2.11.3:** IEF on IPG gel strips.

1. Separation chamber; 2. electrode strip; 3. sample; 4. IPG gel strip.

2.11.3 SDS disc-electrophoresis in the second dimension

After IEF in the first dimension, SDS electrophoresis should be run in the second dimension. So the focused proteins are separated according to their molecular masses.

Prior to the SDS electrophoresis, the gel strip with the focused proteins should be equilibrated in test tubes containing SDS solution. IEF gel strips, which contain carrier ampholytes, should be equilibrated for 2 min, and IPG gel strips should be equilibrated

twice for 15 min each. After the second equilibration of IPG gel strips, 0.26 mol/L 2-iodoacetamide is added to the equilibration solution to alkylate the protein SH groups and avoid 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 and is placed on the stacking SDS gel.

The SDS electrophoresis can be run in the TRIS-chloride-glycinate system of Ornstein [6], Davis [7], and Laemmli [8]; in the TRIS-chloride-TRICINEate system of Schägger [9]; or in the TRIS-formate-taurinate system of Michov [10]. The electrophoresis is fulfilled 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 SDS electrophoresis is continued 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 strip (Figure 2.11.4).

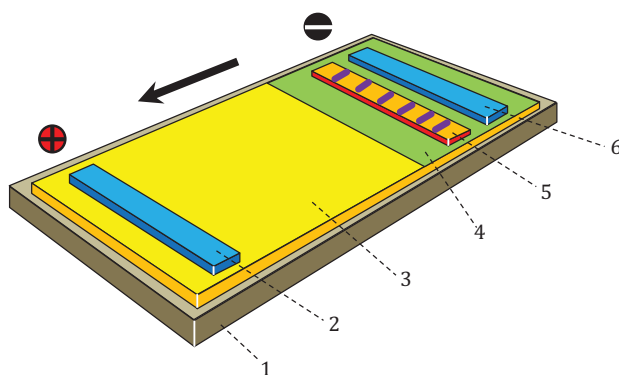


Figure 2.11.4: SDS electrophoresis after placing an equilibrated gel strip on an SDS stacking gel. 1. Separation chamber; 2. anode strip; 3. separating gel; 4. stacking gel; 5. gel strip with protein bands separated by IEF; 6. cathode strip.

For the second dimension of 2D electrophoresis, an SDS gradient or homogeneous gel [11] can be used. In both cases, the gel consists of a stacking part and a resolving part. The SDS stacking gel has usually $T = 5$ g/dL and $C = 0.02$ – 0.04 , and contains 0.125 mol/L TRIS-chloride buffer with pH = 6.8. The SDS resolving gel contains usually $T = 10$ – 15 g/dL and $C = 0.02$ – 0.04 , and contains 0.375 mol/L TRIS-chloride buffer of pH = 8.8.

After the electrophoresis, SDS is removed from the gel by incubating in a solution of Triton X-100.

2.11.4 Detection and evaluation of proteins in 2D pherograms

The 2D electrophoresis has the highest resolution available today. It is capable of resolving over 3,000–4,000 protein spots in a single gel and allows a separation of two protein polyions that differ one from another by only one charged amino acid residue. Therefore, the 2D electrophoresis is used for studying proteins of microorganisms [12, 13], plants [14, 15], or milk [16, 17], and is of great importance for the clinical diagnostics [18, 19].

2.11.4.1 Autoradiography and fluorography

The autoradiography and fluorography are the most sensitive detection methods for 2D pherograms. The *autoradiography* is referred to as emission of radioactively labeled proteins onto an X-ray film. For this purpose, proteins should be labeled with different isotopes: with ^3H -amino acids and with ^{14}C -amino acids or ^{35}S -methionine.

In *fluorography*, a visible light, produced by interaction between a radioactive radiation and a scintillation substance, blackens an X-ray film. For this purpose, 15 g/dL 2,5-diphenyloxazole (PPO) in **dimethyl sulfoxide** (DMSO) is used. To do this, the gel after the 2D electrophoresis 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 h, and in 5 g/dL glycine for 1 h. Then it is dried, as 10 wet sheets of filter paper, a wet cellophane membrane, the gel, a second wet cellophane membrane, and porous polyethylene sheets are placed on a perforated metal plate and covered with a rubber flap. All layers should be compressed under vacuum produced by a water-jet pump. The drying is carried out overnight at 50 °C. Then an X-ray film is placed on the dried gel and the film exposure takes place at –70 °C for 2–3 days. Finally, the spots on the film are obtained as follows: 6 min developing, 10 min fixing, and 10–20 min water cleaning.

2.11.4.2 Two-dimensional gel image analysis

Two-dimensional electrophoresis is used to obtain practical proteomics information. Modern 2D gel analysis software can rapidly analyze spots in the gel. With appropriate computer program, the entire analysis process from background correction to spot matching results takes minutes. Robots are also used for isolation of protein spots from 2D gels.

2.11.5 Protocols

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 1.5–2.0 mL buffer per 100 mg tissue and homogenize using strokes with a pestle.
- Transfer an aliquot to a 200 μ L centrifuge tube and centrifuge at 200,000 g for 1 h.
- Load the supernatant onto the first-dimensional gel.

First-dimensional gels (isoelectric focusing)

Materials and equipment

Acrylamide

Bisacrylamide

TMEDA

APS

Urea

Ampholytes, pH = 4.0 to 8.0

Nonidet P-40

NaOH

H₃PO₄

Casting cassette

Electrofocusing unit

Power supply

Monomeric 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 monomeric solution, 0.75 mL ampholytes (pH = 4.0–8.0), and 6.0 mL 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 vacuum for 2–3 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 monomeric solution to polymerize for 1 h.
- Fill 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 10–30 μ L protein samples onto the gel.
- Switch on the power supply.
- Focus at constant voltage for 16 h.

Second-dimensional gels (SDS electrophoresis)**Materials and equipment**

First-dimensional gel

Acrylamide

Bisacrylamide

SDS

TMEDA

APS

Equilibration buffer

Electrode buffer

Casting cassette

Horizontal electrophoresis apparatus

Power supply

Procedure

- Prepare gel mixing buffer, monomeric solution, SDS, TMEDA, APS, and deionized water (Table 2.11.2). Deaerate using vacuum for 5 min.
- Assemble glass plates with 1.0 mm spacers to produce a casting cassette.
- Pour the monomeric solution in the casting cassette to 5 mm below the top and overlay with deionized water.
- Allow the solution to polymerize for 1.5 h.
- Using a spatula, slide off a strip from the first-dimensional gel and equilibrate it in the equilibration buffer.
- Place the equilibrated gel strip on the SDS gel.

Table 2.11.2: Solutions for second-dimensional gels.

Stock solutions	Final monomeric 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

- Mount the gel in an electrophoresis unit.
- Fill the tanks with electrode solutions.
- Start cooling at 10–20 °C.
- Switch on the power supply.
- Electrophorese at 15–20 mA/gel until the tracking dye reaches the opposite end of the gel.
- Stain the gel and process it for immunoblotting or autoradiography.

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2.12 Preparative electrophoresis of proteins

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Preparative electrophoresis is a valuable method for isolation of diverse proteins, for example, ATP-dependent enzymes and respiratory complexes. Three types of preparative electrophoresis are used: disc-electrophoresis, isoelectric focusing (IEF), and **quantitative preparative native continuous polyacrylamide gel electrophoresis** (QPNC-PAGE).

2.12.1 Preparative disc-electrophoresis

The separated proteins can be eluted from the gel either during or after the electrophoresis.

2.12.1.1 Elution of proteins during electrophoresis

Elution of proteins from a gel during electrophoresis can be carried out, when the proteins are removed by a buffer flowing to a collector. For this purpose, a cassette with a narrow channel in its lower part can be used [1]. The channel is connected with a peristaltic pump. The pump pushes the buffer through the narrow channel and transports with it the proteins to the collector (Figure 2.12.1).

Another construction for protein elution during electrophoresis uses also a gel-casting cassette [2]: The cassette is divided by three vertical spacers (two outer spacers and one inner spacer), which form two compartments: a large compartment for the preparative gel and a small compartment for the detection gel (Figure 2.12.2). On both sides of the preparative polyacrylamide gel, polyethylene tubings are attached reaching its bottom. The detection gel is used to determine the protein

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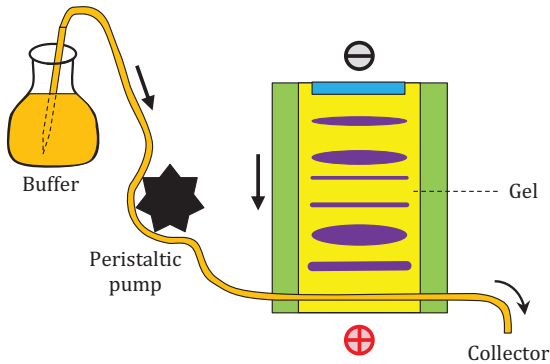


Figure 2.12.1: Scheme of a simple device for protein elution during electrophoresis.

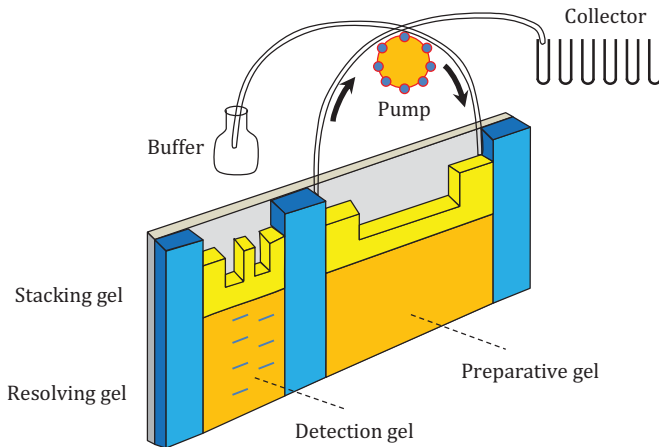


Figure 2.12.2: Cassette for elution of proteins during electrophoresis.

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 two-channel peristaltic pump, the protein fractions are transported from the dialysis membrane to a collector.

positions. When disc-electrophoresis is carried out, both gels consist of a stacking gel ($T = 4 \text{ g/dL}$, $C = 0.03$) and a resolving gel ($T = 10 \text{ g/dL}$, $C = 0.03$). The protein yield can reach 89–99%.

2.12.1.2 Elution of proteins after electrophoresis

Prior to elution, the protein bands after electrophoresis should be located in the gel. Then the bands could be excised and the proteins eluted. If the proteins are

colored (e.g., hemoglobins), it is easy to find them. If they are not colored, they should be stained or their location could be defined using UV absorbance [3, 4], fluorescence imaging [5–7], or phosphorescence at low temperatures [8]. An elegant way to identify enzymes in a gel is to use in-gel assays for enzyme activities [9–11].

The elution of proteins after electrophoresis can be done by diffusion, gel dissolving, or electroelution.

Elution by diffusion

The elution by diffusion is the simplest method to free proteins from a gel [12]. For this purpose, buffers without detergent should be used [13]. The monomers, TMEDA, and APS, which diffuse together with the proteins, can be removed using ion exchange chromatography [14, 15]. However, the elution by diffusion is time-consuming and incomplete because proteins still remain in the gel – the yield is low [16].

Elution by gel dissolving

Elution by gel dissolving (depolymerizing) is a complicated method leading to chemical modification of proteins. To obtain proteins from gels with the standard cross-linker 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 [17], irreversible damages of all proteins take place. Another alternative is to use a special cross-linker, for example, *N,N'*-diallyltartardiamide. This gel should be solubilized in 2 g/dL periodic acid (H_5IO_6), which is suited for elution of high-molecular-mass proteins [18]. Unfortunately, the usage of periodic acid is not adequate for most proteins [19–21].

Electroelution

The electroelution of proteins from a preparative gel is carried out in electric field driving the proteins to migrate in direction opposite to the electrophoresis direction into a dialysis bag [22–24]. This technique is characterized by a high yield. So insulin, myoglobin, and bovine serum albumin were eluted from the gel up to 95%.

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 [25] (Figure 2.12.3). Finally, the protein eluate can be concentrated and lyophilized.

An apparatus for *vertical-type elution* (Figure 2.12.3a) consists of a tube with buffer, sieve, a dialysis bag, and a tank with buffer [26]. The elution is carried out in the following steps: the dialysis bag is attached on the sieved tube, which is filled with buffer,

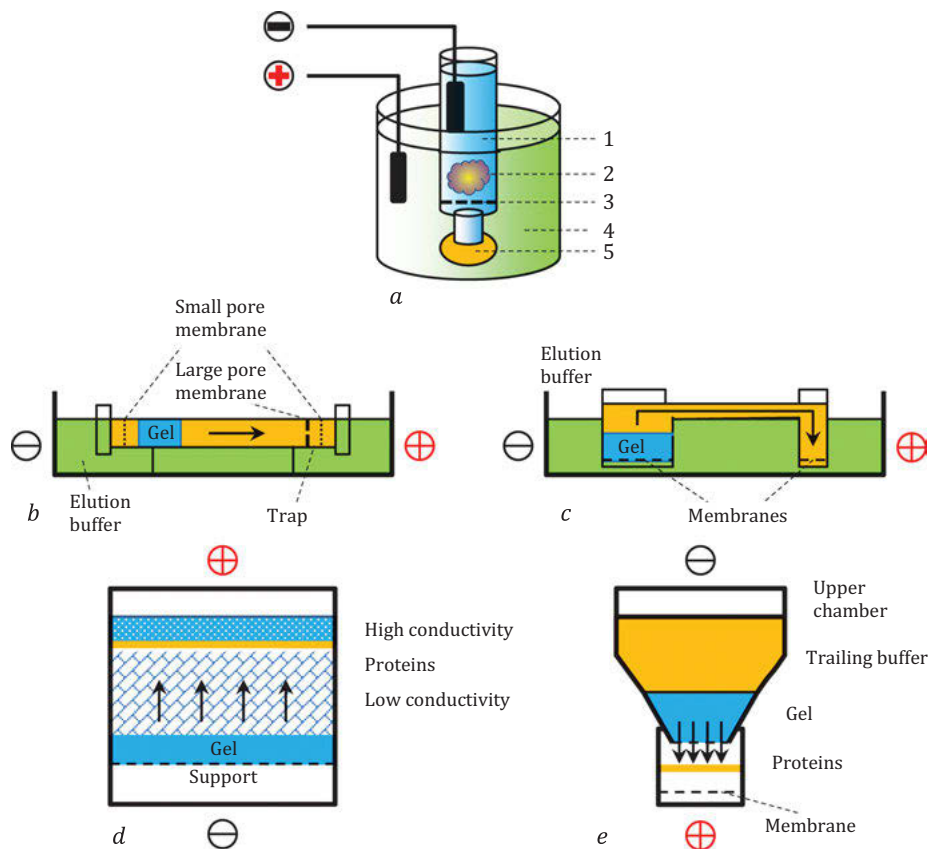


Figure 2.12.3: Different types of electroelution of proteins. The proteins are collected on a small-pore membrane that supports the gel.

(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 stacking elution: isotachopheresis is expanded to elute proteins in a funnel-shaped device.

and placed in a tank with the same buffer. The polyacrylamide gel is cut into small pieces and put into the tube. Thereafter, electric current is applied, which drives the proteins to penetrate through the sieve into the dialysis bag. The dialysis membrane can be omitted [27] using a layer of hydroxylapatite, which captures the protein. The bound proteins can then be eluted by phosphate buffer with other pH values.

For *horizontal-type elution* (Figure 2.12.3b), a flat-bed electrophoresis tank and a special column are used [28, 29]. Both ends of the column are sealed with semi-permeable membranes to form an elution chamber that is divided by a large-pore

membrane into two compartments. The gel pieces are placed in the large compartment. After applying an electric field, proteins migrate into the small compartment and are captured there by a small-pore membrane.

The construction for *bridge-type elution* (Figure 2.12.3c) consists of two separate horizontal chambers, which are connected by a bridge [30, 31]. Both chambers are sealed by dialysis membranes. The bigger chamber contains the gel material with the 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.

The *discontinuous conductivity gradient elution* (Figure 2.12.3d) does not use a dialysis membrane but a combination of two solutions. A low-conductivity glycerol layer is surrounding the gel. A second high-conductivity glycerol layer is set upon the first one [32–34]. 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. However, the high concentrated layer can cause a salt-ing-out effect, which diminishes the yield.

The *steady-state stacking elution* (Figure 2.12.3e) is based on the isotachopheresis, which can be expanded [35] to separate proteins in a funnel-shaped device [36, 37]. 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 [38]. As a result, proteins leave the gel pieces to be concentrated into the glycerol solution.

2.12.2 Preparative isoelectric focusing

Preparative IEF can be carried out with carrier ampholytes or in immobilized pH gradients.

2.12.2.1 Preparative IEF with carrier ampholytes in granulated gels

The preparative IEF using carrier ampholytes is accomplished in horizontal granular gels [39, 40] made of the dextran products Sephadex G-75, G-200, or best Ultradex [41]. It proceeds in the following steps: producing a granulated gel, introducing a sample into the granulated gel, IEF, and elution of proteins.

Prior to preparing the gel, Sephadex G-75 and G-200 should be washed with deionized water. When the difference between the conductivities of water before and after washing has reached a minimum, the washing process can be stopped. If Ultradex is used, washing is omitted.

The preparation of a horizontal granulated layer involves three phases: 1. mixing the washed gel with carrier ampholytes; 2. inserting electrode strips into the

mold and casting the gel with carrier ampholytes; and 3. drying partially the gel and carrier ampholytes (Figure 2.12.4).

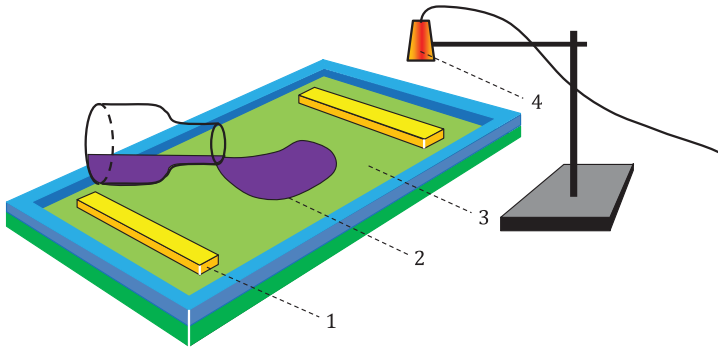


Figure 2.12.4: Producing a horizontal layer of granulated gel for preparative IEF with carrier ampholytes.

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 using a hair dryer (4).

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 ampholytes, is cast into a rectangular frame, and a part of it is taken off using a spatula forming a well. The gel part is transferred into a Becher glass where the mixture is mixed with the sample. Then the mixture is poured back into the well (Figure 2.12.5).

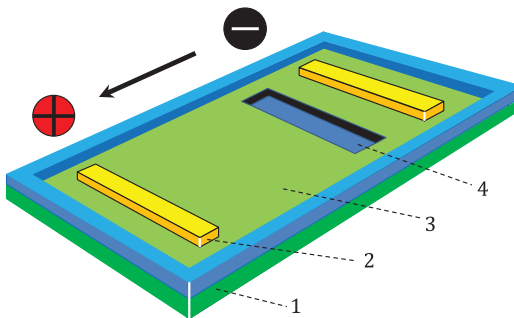


Figure 2.12.5: Application of sample as a zone in a granulated gel layer.

1. Glass plate with a silicone frame; 2. electrode strip; 3. granulated gel; 4. well in the gel layer.

Before starting the preparative IEF, moist filter paper strips should be placed on both electrodes. The anode filter paper strip is impregnated with 0.5 mol/L H_3PO_4 , and the cathode filter paper strip is impregnated with 0.5 mol/L NaOH. Thereafter,

the mold with the granulated gel layer is placed on the cooling plate of an electrophoresis unit. Afterward, the separation unit is covered with the safety lid and the electrophoresis unit is connected to the power supply.

Based on the newly established electrophoretic method called **divergent flow IEF** (DF IEF), a DF IEF instrument was proposed, which operates without carrier ampholytes [42]. In DF IEF, the proteins are separated, desalted, and concentrated in one step, and the proteins have been concentrated up to 16.8-fold.

After preparative electrofocusing, the visualization of protein bands in the mold could be done placing a filter paper onto the granulated gel, staining the protein blot, then placing it under the mold, and finally taking out the gel segment containing the desired protein. Then the protein can be extracted from the gel segment and analyzed.

Finally, the proteins should 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 the high mass proteins: dialysis, gel filtration, and ultrafiltration. At the end, the proteins could be lyophilized and frozen.

2.12.2.2 Preparative IEF in immobilized pH gradients

The preparative IEF can be carried out also in immobilized pH gradients [43–45]. An interesting technique of it is the channel focusing (Figure 2.12.6). It proceeds in the following steps: casting a preparative IPG gel, analytical IEF in the margins of the IPG gel, digging channels, running the preparative IEF, and elution of proteins.

Casting a preparative IPG gel. An IPG gel for preparative IEF is cast as an IPG gel for analytical IEF. Then two short slots for analytical electrophoresis of proteins of interest are made in the gel margins, and a long slot is made in the medium of the gel for the 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 underwent an analytical electrofocusing, and the obtained protein bands are stained to determine the protein position.

Digging channels. The remaining gel is rehydrated as the margin strips and is placed on 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 granular gel, which should collect the protein at its migration. If necessary, more channels can be cut for different proteins.

Running preparative IEF. The preparative IEF in an IPG gel is running 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. The gel is cleaned with a

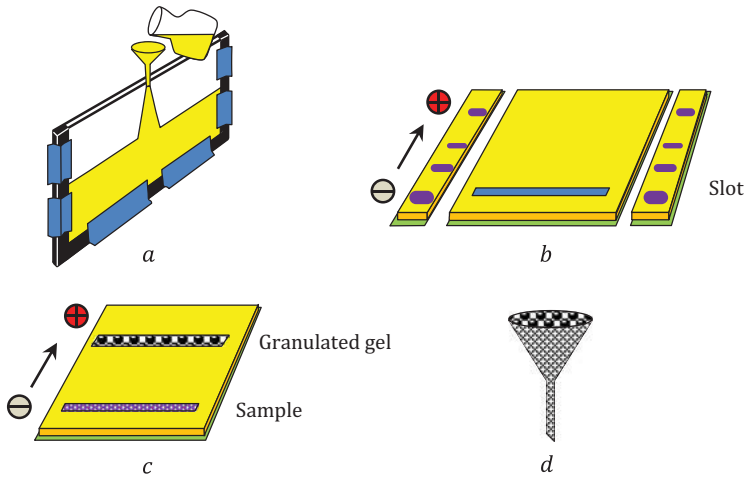


Figure 2.12.6: Channel focusing.

(a) Casting an IPG gel; (b) cutting margins of the IPG gel and running an analytical IEF to determine the position of proteins of interest; (c) making a well (channel), filling it with the granulated gel, and carrying out a preparative focusing; (d) extracting and eluting proteins from the granulated gel.

buffer from the rest of proteins, washed, and dried. It can be used for next channel IPG electrofocusing.

2.12.2.3 Recycling isoelectric focusing

The recycling **IEF** (RIEF) according to Bier *et al.* [46, 47] is based on the principle of continuous recirculation of a separation medium in a special apparatus. It consists of an IEF unit, a multichannel 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 the aid of a computer.

The IEF unit is filled with carrier ampholytes and the sample, which are then recycled through the pump, the pH monitor, and the 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 RIEF, is removed by the thermostat.

2.12.3 QPNC-PAGE

QPNC-PAGE (**q**uantitative **p**reparative **n**ative **c**ontinuous **p**olyacrylamide **g**el **e**lectrophoresis) is a variant of polyacrylamide gel zone electrophoresis. It is used to

isolate metalloproteins in biological samples and to resolve properly and improperly folded proteins or protein isoforms [48]. During the electrophoresis, the metalloproteins are not dissociated into apoproteins and metal cofactors.

QPNC-PAGE is based on the time of **polymerization** of **acrylamide** (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 ensure 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 [49].

QPNC-PAGE is used for isolating copper chaperone for superoxide dismutase (CCS), superoxide dismutase (SOD), prions, transport proteins, amyloids, and metalloenzymes, which are present in brain blood or other samples in Alzheimer's disease or amyotrophic lateral sclerosis [50]. 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+} , and Cd^{2+}) in organisms and thus balance oxidative and reductive processes in the cytoplasm [51].

2.12.4 Protocols

QPNC-PAGE

Materials and equipment

TRIS

HCl

Acrylamide

BIS

TMEDA

Ammonium peroxydisulfate (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 APS. Prepare freshly.

Electrophoresis buffer

20 mmol/L TRIS-HCl, 1 mmol/L NaN_3 , pH = 10.0. Keep at 4 °C.

Monomeric solution, $T = 4 \text{ g/dL}$, $C = 0.027$

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 monomeric solution to a level of 40 mm in a graduated glass column with an inner diameter of 28 mm. Finally, add APS. The time of polymerization is 69 h at room temperature.
- Mix 3.0 mL of a sample with 0.3 mL glycerol.
- Apply the mixture under the upper electrophoresis buffer and run the electrophoresis at 4 °C.
- Elute the separated proteins continuously in a special elution chamber and transport them to a fraction collector.

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The **microfluidic chip (microchip) electrophoresis (ME)** resembles a miniaturized capillary electrophoresis in a planar minidevice. Since the pioneering works of Harrison *et al.* [1, 2], the microchips are of considerable interest, owing to their portability, small sample consumption, and high speed of electrophoresis [3–5]. They are used in clinical and forensic diagnostics [6], proteomics [7], pharmaceutical analysis, environmental monitoring, *etc.*

A microchip contains narrow channels with diameters of about 50 μm , volumes of 10–50 μL , and size of 1–10 cm. They are filled with buffers, are arranged in the form of a cross, and are connected with four reservoirs (Figure 2.13.1). The short channel is dedicated to sample injection, whereas the long one represents the separating channel [8].

The injection of the sample into the microchip is achieved by the electrokinetic mode: First, the sample is pipetted into the sample load reservoir; then voltage is applied between the sample load reservoir and sample waste reservoir. When the

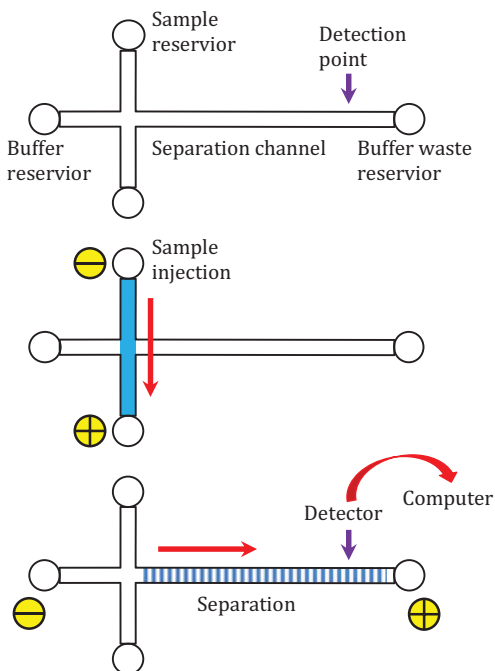


Figure 2.13.1: Events in microchip zone electrophoresis.

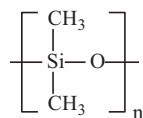
electrophoresis is run, the analytes resolve as a result of their mobilities and the electroosmotic flow, driven by the surface charges of the channel.

2.13.1 Microchip materials

The first microchips were fabricated from glass [9–11] or quartz [12–14] using photolithographic techniques [15]. However, their application was limited because of high cost and complicated procedures [16]. Nowadays, the microchips are made of rigid polymers [17–19]. They can be fabricated using *in situ* polymerization [20], laser ablation [21], imprinting [22], injection molding [23, 24], hot embossing [25–27], *etc.* The most popular polymers for preparing microchips are PDMS (**polydimethylsiloxane**), PMMA (**poly(methyl methacrylate)**), and PC (**polycarbonates**).

2.13.1.1 PDMS

PDMS belongs to the group of polymeric organosilicon compounds that are referred to as silicones [28, 29]. PDMS has the chemical formula $\text{CH}_3[\text{Si}(\text{CH}_3)_2\text{O}]_n\text{Si}(\text{CH}_3)_3$, where n is the number of repeating monomer units [30].

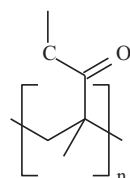


PDMS monomer

PDMS has many advantages over other polymer materials: it is nontoxic, inert, optically transparent, flexible, and nonflammable [31–33]. However, PDMS has also some disadvantages. The most prominent among them is its hydrophobicity [34, 35]. This hinders the introduction of aqueous solutions into the microchannels. To decrease the hydrophobicity of PDMS, oxygen plasma [36], or UV/ozone [37] 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 [38].

2.13.1.2 PMMA

PMMA, also known as acrylic glass as well as Plexiglas, Crylux, Acrylite, Lucite, and Perspex, was developed in 1928 in different laboratories by William Chalmers, Otto Röhm, Walter Bauer, and more. It is a transparent thermoplastic often used as an alternative to glass [39–41]. Chemically, PMMA is a synthetic polymer of methyl methacrylate.



PMMA monomer

PMMA is an ideal polymer for the fabrication of microfluidic chips because of low price, optical transparency, and good mechanical properties [42, 43]. Its surface can be modified by aminolysis, reduction, and photoactivation (Figure 2.13.2) [44].

2.13.1.3 PC

PC are a group of thermoplastic polymers containing carbonate groups [45–47]. They are strong, tough, robust, molded, and thermoformed materials. PC are pliable and resistant to chemicals. The precursor monomer of PC is **bisphenol A** (BPA).

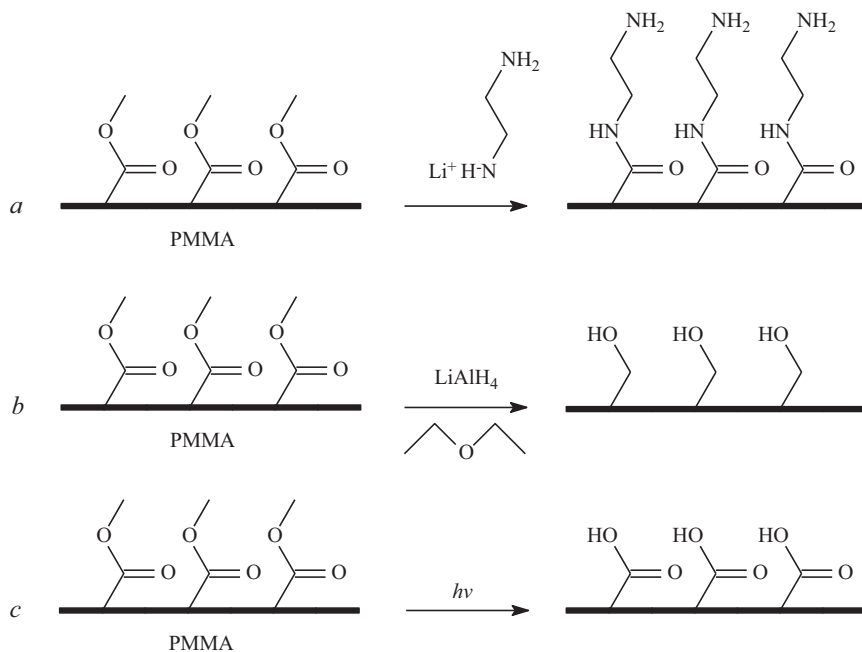
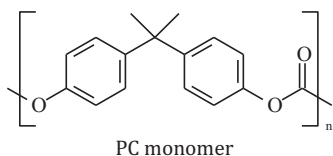
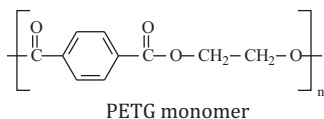


Figure 2.13.2: Modification of PMMA surface using aminolysis (a), reduction (b), and photoactivation (c).



Other polymers used for preparation of microchips are **polyethylene terephthalate glycol** (PETG), **polyethylene terephthalate** (PET) [48, 49], **polystyrene** (PS) [50–52], Mylar [53], **polyimide** (PI) [54, 55], **cyclic olefin copolymer** (COC), and more.



2.13.2 Microchip fabrication

The microchip fabrication includes fabrication of channel and cover plates, wall coating, and bonding the plates.

2.13.2.1 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, *etc.*

The technology of soft lithography is used for fabrication of PDMS microchips [56]: A computer-aided design (CAD) program is employed for the design of microchips. The CAD-generated patterns are printed on a transparency that is used as a photomask in UV photolithography to generate a master. The resulting relief structure serves as a master for fabricating PDMS molds into which a liquid PDMS prepolymer is poured.

The *in situ* polymerization of methyl methacrylate takes place in molds with the aids of UV light [57] and heat [58]. 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 (Figure 2.13.3).

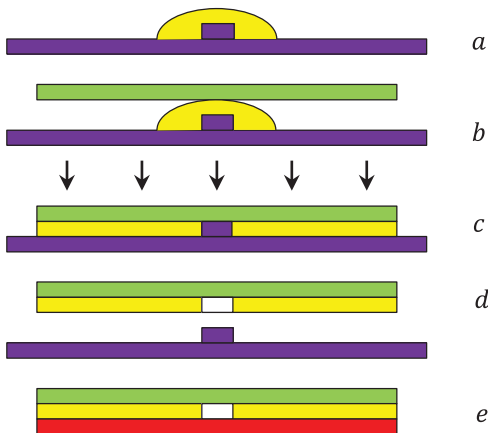


Figure 2.13.3: Scheme of microfabrication process.

(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 and thermal sealing of PMMA channel plate to form a complete microchip.

Chen *et al.* [59] 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. 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 expensive lithography-based techniques.

2.13.2.2 Fabrication of cover plate

The cover plate of PMMA microdevices can be fabricated by injection molding [60, 61]: The PMMA pellets are melted and injected under pressure into a mold cavity containing an inserted master. The masters 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 was also employed to fabricate PMMA microdevices using software [62, 63]. In this process, a beam of high-energy laser breaks bonds in polymer molecules. The PMMA plate is mounted on a table that moves in the *X* and *Y* directions while the focused laser beam scans.

Rapid fabrication of PMMA microfluidic chips has been made based on the *in situ* surface polymerization of methyl methacrylate [64]. 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.

2.13.2.3 Wall coating

For separation of proteins, the channel surface of microfluidic chips should be hydrophilic to limit the protein adsorption during electrophoresis. However, the polymers used for fabrication of microchips are hydrophobic. Therefore, they ought to be coated with hydrophilic substances. The wall coating can be dynamic or permanent.

Dynamic wall coating

In dynamic wall coating, hydrophilic substances in the buffer coat the wall of the microchip channels during electrophoresis. Diverse methods for dynamic wall coating are known: for glass microchips, PMMA microchips, and more.

For glass microchips, PDMA is used simultaneously as a wall coating material and a sieving matrix. Its presence reduces the electroosmosis to $0.5 \times 10^{-9} \text{ m}^2/(\text{s V})$ [65]. Dynamic coatings of the channel surface of PMMA microchips were made by dissolving positively or negatively charged surfactants, or hydrophilic neutral polymers in the electrophoresis buffers [66, 67]. A dynamic coating process using 2 g/dL hydroxyethyl

cellulose (HEC) for the surface modification of PMMA microfluidic chips was developed [68]. Mohamadi *et al.* [69] have described a dynamic coating method using methylcellulose and the nonionic detergent Polysorbate 20, which suppresses protein adsorption onto PMMA channel wall.

Permanent wall coating

Permanent wall coating happens when hydrophilic substances coat the wall of the microchip channels. Diverse methods for permanent wall coating are used for glass microchips, PMMA microchips, and other microchips.

The classical wall coating of glass microchips proposed by Hjerten was applied with some modifications [70–72]. After flushing channels with NaOH, they are filled with γ -methacryloxypropyltrimethoxysilane in diluted acetic acid and acetonitrile for 1 h. Then an aqueous solution of acrylamide with ammonium persulfate and TMEDA is pumped into the channel and polymerized at room temperature. Finally, the channels are flushed with water and dried by vacuum.

One of the earliest methods for modification of the PMMA surface was proposed by Wichterle [73]. His principle consists in reesterification of PMMA with polyfunctional hydroxyl compounds, such as ethylene glycol, glycerol, mannitol, and saccharose. 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 [74]: The polymer was hydrolyzed first with hot sulfuric acid containing sodium or potassium hydrogen sulfate and then the hydrolyzed PMMA was esterified with glycerol.

A covalent hydrophilization of channels was introduced by Soper *et al.* [75, 76] who imparted amine or octadecyl groups to the surface of PMMA microchannel *via* aminolysis and reactions with *n*-octadecylisocyanate. Using atom-transfer radical polymerization, polyethylene glycol was grafted onto the surface of PMMA channels, which also reduced the electroosmotic flow and the adsorption of proteins on the PMMA surface [77]. Kitagawa *et al.* [78] have developed a one-step covalent immobilization of poly(ethyleneimine) (PEI) onto PMMA substrates to achieve an efficient separation of basic proteins in microchip electrophoresis.

2.13.2.4 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.

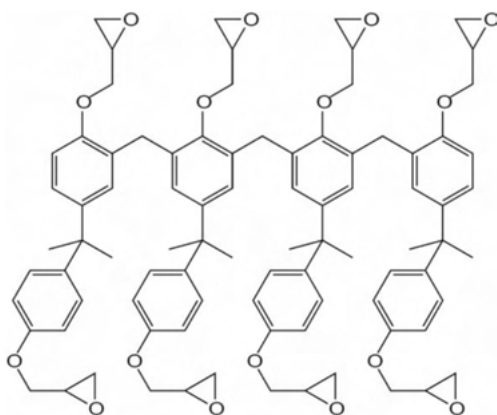
In the *thermal bonding* the channel and cover plates are assembled and heated to 105 °C in a convection oven and pressed together using a bonding device [79]. However, the pressure and temperature may cause microchannel deformation. In an attempt to overcome these limitations, hot-press bonding conducted in a vacuum [80] or in a boiling water bath [81] was developed.

Solvent bonding has also been reported for PMMA microchips [82–84]. Klank *et al.* [85] employed a plasma-enhanced ethanol bonding for such purpose. Lin *et al.* [86] sealed PMMA microfluidic chips utilizing a solvent composed of ethanol and 1,2-dichloroethane at room temperature.

The *polymerization bonding* is another method for bonding PMMA microchips [87]: 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. Finally, the bonding device was put in a 95 °C convection oven when the monomer methyl methacrylate was polymerized to realize the bonding.

Microwave bonding of PMMA microchips at low temperature was proposed by Lei *et al.* [88]. Later, Yussuf *et al.* [89] have reported bonding of PMMA microfluidic chips using microwave energy and conductive polyaniline.

Xu *et al.* [90] have fabricated PMMA microchips by *room-temperature imprinting*: The PMMA plate was placed on a silicon template, and the assembly was inserted between two polished aluminum plates and was hydraulic pressed at room temperature. After the pressure was released, the open channels on the plastic plate were sealed with a PDMS film. Later, Woolley *et al.* [91] developed a method for rapid prototyping of PMMA microfluidic chips using SU-8 photoresist: SU-8 is an epoxy-based negative photoresist. It was employed as a template for solvent imprinting on a glass slide while pressed into a solvent-wetted surface.



SU-8

2.13.3 Zone electrophoresis on microchip

The principle of the microchip zone electrophoresis is same as the principle of conventional electrophoresis: the separations of proteins are based on differences in their electrophoretic mobility, which generally correlate to their charge-to-mass ratio.

2.13.3.1 Free-flow electrophoresis on microchip

The first device for free-flow zone electrophoresis on microchips was developed by Raymond *et al.* [92, 93]. Using this device, the authors separated three rhodamine-B isothiocyanate-labeled amino acids: lysine, glutamine, and glutamic acid, and a mixture of human serum albumin, bradykinin, and ribonuclease A.

Later, Fonslow and Bowser [94] presented a free-flow electrophoresis (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.

Next, a device for FFE on glass microchip using laser-printing toner as a structural material was proposed [95]: The separation channel is 8 μm deep and has an internal volume of 1.42 μL . The Joule heating dissipation 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 $\text{mW}/\mu\text{L}$. 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 2.13.4).

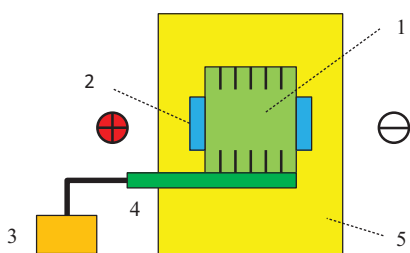


Figure 2.13.4: Diagram of free-flow electrophoresis on microchip.

1. Separation channel; 2. side reservoirs; 3. air compressor; 4. outlet reservoir; 5. glass plate.

2.13.3.2 Affinity- and immunoelectrophoresis on microchip

Affinity- and immunoelectrophoresis on microchips take place in affinity, most often in immune interaction [96]. Immunoelectrophoresis on microchips has allowed the fast quantitation of inflammatory cytokines in the cerebrospinal fluid of patients presenting a head trauma [97]. In this procedure, the reservoir was silanized to exhibit the amino terminal group and so to obtain an affinity area between the Fab fragments and fluorescently tagged analytes. Then a low-pH buffer was added to dissociate the antibody–antigen complexes and the electrophoresis was run.

2.13.4 Isotachophoresis on microchip

During isotachophoresis (ITP) the samples are concentrated between a leading ion and a trailing ion. The leading ion is chosen to have a higher mobility than any ions (of the same polarity) in the sample. The trailing ion is chosen to have a lower mobility than any ions (of the same polarity) in the sample. After applying an electric field, equilibrium is reached, in which all ions have the same velocity. The concentration of ions in the sample arises and is a consequence of the concentration of leading ion.

2.13.4.1 Disc-electrophoresis on microchip

Lin *et al.* [98] have carried out disc-electrophoresis on microchips integrating ITP pre-concentration with zone gel electrophoresis. Each microchip was designed with a sample injection channel to allow stacking the sample into a narrow zone. Thus, they separated carbonic anhydrase, ovalbumin, BSA, and conalbumin.

2.13.5 Isoelectric focusing on microchip

In isoelectric focusing on microchip, the charged proteins are separated according to their pI in a pH gradient generated by ampholytes. 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 [99, 100] is necessary to perform an in-channel detection of proteins. To avoid mobilization step, whole-column imaging (WCI) method may be employed. WCI detection is carried out using an epifluorescence microscope with a xenon lamp [101], a mercury lamp [102, 103], or LED [104–106].

2.13.6 Two-dimensional electrophoresis on microchip

Li *et al.* [107] combined IEF with SDS electrophoresis on a plastic planar microchip with dimensions of 2×3 cm. Thus, they carried out two-dimensional (2D) protein separation in less than 10 min. Later, a miniaturized instrument capable of performing 2D electrophoresis was described [108]. It consists of a compartment for the first-dimensional IEF polyacrylamide gel, which is connected to a second-dimensional SDS polyacrylamide gel. The focused samples are automatically transferred from the IEF gel to the SDS polyacrylamide gel by electromigration. Separated protein spots can be excised from stained gels, digested with trypsin, and identified by mass spectrometry.

Shadpour and Soper [109] carried out 2D electrophoretic separation of proteins using an embossed PMMA microchip. The separation in the first dimension was based on SDS electrophoresis, and the second dimension was carried out using a fast **micellar electrokinetic chromatography** (MEKC), which sorted the proteins depending on the differences in their interaction with SDS. Also, microfluidic 2D separation of proteins was described, which combined temperature gradient focusing with SDS polyacrylamide gel electrophoresis [110].

2.13.7 Protein separation technique on microchip

The protein electrophoresis on microchips, similar to the common gel electrophoresis, was carried out in three steps: concentrating the samples, running electrophoresis, and detecting separated proteins.

2.13.7.1 Concentrating the protein samples prior to microchip electrophoresis

The samples for microchip electrophoresis can be concentrated by filtering [111]: 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 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 are retained.

Wang *et al.* [112] have developed a microfluidic sample concentration device based on the electrokinetic trapping mechanism enabled by nanofluidic filters. 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.

2.13.7.2 Running protein electrophoresis

Various injection procedures are proposed, such as pinched or gating modes [113]. They 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 improves the control over the injected sample volume.

2.13.7.3 Detecting the proteins

The detection of resolved proteins can be done by staining, fluorescence, chemiluminescence (CL), and mass spectrometry.

Staining the proteins

The binding between dyes and proteins can be covalent or non-covalent.

Covalent binding of proteins can be realized between protein molecules and amine or other groups (carboxylic or thiol groups) of the dyes. Gottschlich *et al.* [114] have developed a microchip where naphthalene-2,3-dicarboxaldehyde was used for labeling of the insulin chains A and B, produced after disulfide reducing.

Non-covalent binding of proteins is performed with fluorogenic reagents. The labeling reagent should have low fluorescence in the unbound state and a high fluorescence enhancement when bound to protein. Bousse *et al.* [115] have developed a glass microchip for non-covalent fluorescent labeling method. Denatured SDS-protein complexes bound the fluorescent dye when the separation begins. The SYPRO Orange and Agilent dye exhibited a fluorescent enhancement upon binding to SDS micelles or SDS-protein complexes. At the end of the separation channel, in front of the detection point, an intersection was used to dilute SDS below its critical micelle concentration.

Fluorescence detection

The fluorescence detection is the most widely used method for protein detection in microchip electrophoresis due to its high sensitivity. The laser-induced fluorescence (LIF) detection is the important method for protein detection after electrophoresis on microchips [116, 117]. According to this, the laser is focused on very small protein volumes to obtain high irradiation. Lamp-based fluorescence detection

methods, following LIF detection, are used for optical detection of separated proteins. They use microscope-based detector setups using xenon or mercury lamps. For this purpose, epifluorescence microscopes were combined with **photomultiplier tube (PMT) detection** [118].

Vieillard *et al.* [119] 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. Using this technique, the separation and detection of β -lactoglobulin A and carbonic anhydrase II needed less than 1 min.

Chemiluminescence detection

Another detection method used in microfluidic electrophoresis is the **chemiluminescence (CL) detection**. Ren *et al.* [120] have described a new strategy to afford online CL detection of heme proteins on microchips. The detection principle is based on the catalytic effect of heme proteins on the CL reaction of luminol–H₂O₂ enhanced by 4-iodophenol. Cytochrome *c*, myoglobin, and horse radish peroxidase were separated within 10 min on a glass microchip.

Mass spectrometry detection

The possibility to integrate a microfluidic chip separation with **mass spectroscopy (MS) detection** has been studied by many researchers [121, 122]. A PMMA microfluidic-based gel protein recovery system, in which fluid transports protein from a PAGE gel piece to a collection reservoir *via* a microfluidic channel, has been developed [123]. The sample proteins were mobilized out of the gel into a microfluidic channel by electric field. Afterward, the proteins were detected using **matrix-assisted laser desorption/ionization MS (MALDI MS)** over gel loads of 0.1–10 pg.

2.13.8 Microchips in clinical diagnostics

A 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) were resolved on microchip, in less than 60 s [124].

Giordano *et al.* [125] have developed a method for dynamic labeling of proteins with NanoOrange in microchip SDS zone electrophoresis of serum albumin. Later, the group of Chan [126] has proposed a method for quantifying urinary albumin, a marker of microalbuminuria, which is a risk factor of cardiovascular diseases.

MALDI-based techniques were successfully used with the goal of identifying proteins in connection with HER-2/neu-positive, aggressive-type breast cancer [127]. Tumor cells were collected by laser-captured microdissection (LCM) from HER-2/neu-positive and -negative tumors.

The protein microarray system could simultaneously determine the concentration of two viral antigens (HBsAg and HBeAg) and seven antiviral protein antibodies (HBsAb, HBcAb, HBeAb, HCVAb, HDVAb, HEVAb, and HGVAb) of human hepatitis viruses in human sera within 20 min. The results were confirmed by ELISA [128].

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2.14 Blotting of proteins

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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 are accessible to reagents or high-molecular-mass ligands such as lectins, antibodies, antigens, DNA, or RNA.

2.14.1 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.

Blotting is carried out in three steps: transfer of proteins onto a blot membrane, blocking the free binding sites on the blot membrane, and detection of the blotted proteins (Figure 2.14.1). The unoccupied binding sites on the blot membrane have to 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.

<https://doi.org/10.1515/9783110761641-024>

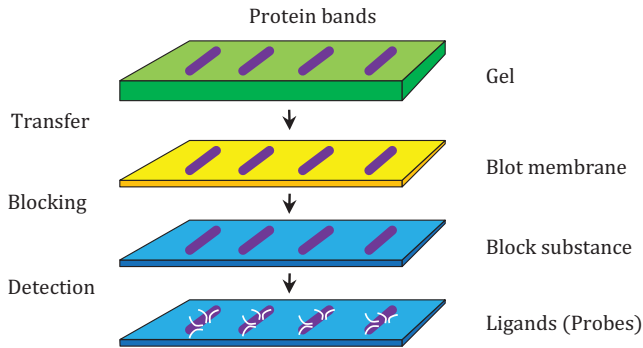


Figure 2.14.1: Steps of blotting.

2.14.2 Blot membranes

For blotting, special blot membranes are used: **nitrocellulose (NC)**, **polyvinylidene difluoride (PVDF)** membrane, nylon, **diazobenzoyloxymethyl (DBM)** cellulose, **diazophenylthioether (DPT)** cellulose, or activated glass fiber paper:

- The NC membrane is the most commonly used blot membrane [1, 2]. Typically, its pores are 0.2 or 0.45 μm wide. The smaller the pores, the greater the binding capacity of the blot membrane.
- PVDF membrane possesses high binding capacity and is mechanically stable [3, 4]. It allows easier stripping than the NC membrane.
- Nylon has good mechanical stability [5]. It binds the proteins electrostatically by hydrophobic interactions. This causes a strong background when staining the proteins.
- Diazobenzoyloxymethyl paper [6] and diazophenylthioether paper [7] bind proteins electrostatically and covalently. Today, they are replaced by nylon.

2.14.3 Transfer of proteins

The most widespread transfer of proteins is the electrotransfer. Another transfer process is the capillary transfer. It is used also for transferring of nucleic acids (see there).

2.14.3.1 Electrotransfer of proteins

The electrotransfer (electroblotting) was patented in 1987 by William Littlehales [8]. It uses electric current to pull proteins from the gel onto a PVDF or an NC membrane.

The electrotransfer is the fastest way of blotting and can be carried out with polyacrylamide or agarose gels.

The film-supported gels are problematic for electrotransfer, since the film should be removed after electrophoresis [9]. Meanwhile, there are net-supported gels [10, 11], which are suitable for electroblotting.

The electroblotting has two variants: tank blotting and semidry blotting.

Tank blotting of proteins

To carry out tank blotting [12], the gel with the electrophoretically separated proteins and the blot membrane are placed vertically between filter papers and set down in a tank with buffer. Thereafter, the gel is blotted between two electrodes (Figure 2.14.2). The transfer continues usually overnight.

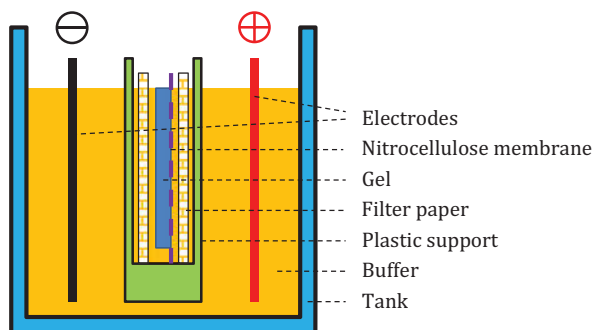


Figure 2.14.2: Tank blotting.

The blot sandwich is constructed of a buffer-saturated sheet of filter papers, gel, membrane, and another buffer-saturated sheet of filter papers. It is placed in a tank containing transfer buffer. The transfer is carried out at a voltage of 100 V for 1–2 h or at 15 V overnight.

Semidry blotting of proteins

The semidry blotting is very popular [13, 14]. The gel and blot membrane are placed between filter papers soaked with transfer buffer and the resulting blot sandwich is inserted between the horizontal graphite plates of a blotter. Blotters with glassy carbon plates are available too, which are more solid than the graphite plates.

The semidry blotting can be carried out, similarly to electrophoresis, in a continuous buffer or in a disc-buffer system.

In the *continuous semidry buffer*, a same buffer is used on both sides of the blot membrane. Under these conditions, the blotted bands on the membrane are not enough sharp.

In the *disc-semidry blotting*, between both plates of the blotter, following matters are placed: a lower thick filter (anode) paper soaked with anode buffer, a blot membrane, a net gel (with the gel side down), a cellophane membrane, and an upper thick filter paper soaked with the cathode buffer. Then the upper (cathode) plate of the blotter is weighted with 1 kg mass. Finally, the power supply is turned on to start the blotting (Figure 2.14.3).

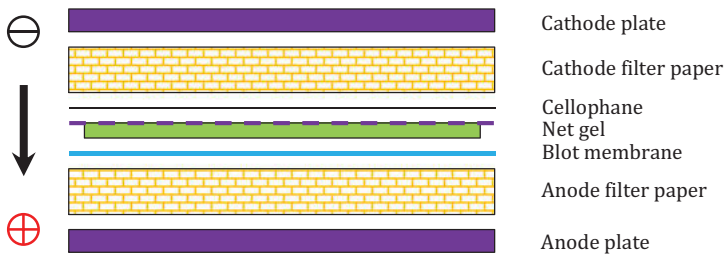


Figure 2.14.3: Disc-semidry blotting.

During the disc-semidry blotting, the electrophoretically separated proteins are overtaken by a moving ionic boundary. Therefore, the protein bands are seen very sharp on the blot membrane. We recommend the discontinuous TRIS-formate-taurinate buffer system for semidry blotting [15] (Table 2.14.1). It can be used for native proteins (after disc-electrophoresis or isoelectric focusing) or denatured proteins (after SDS electrophoresis).

2.14.3.2 Capillary transfer of proteins

The capillary transfer was introduced by Edwin Southern (Southern blotting) in 1975 [16], when he transferred DNA fragments onto an NC membrane. In this procedure [17], 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 onto the gel and sucks its buffer together with the resolved bands (Figure 2.14.4). The transfer needs a long time, usually overnight.

After the protein transfer is complete, the blot membrane should be blocked and the proteins detected.

Table 2.14.1: TRIS-formate-taurinate buffer system for disc-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	–

**Figure 2.14.4:** Capillary transfer of proteins.

2.14.4 Blocking

To cover 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 **bovine serum albumin** (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, and 10 g/dL fetal calf serum. For PVDF membranes, liquid fish gelatin is preferred [18].

2.14.5 Detection

After transfer and blocking, the blotted proteins can be detected by dyes or high-molecular-mass ligands (probes).

2.14.5.1 Detection by dyes

Nonspecific staining of proteins on blot membranes can be carried out with the anionic dyes such as Ponceau S, Amido black 10B, Coomassie brilliant blue R-250, India ink, Fast green FCF, with chelates, and metals (see there).

The staining with Ponceau S [19–21] is easy to use. The staining with Amido black 10B (Naphthol blue black) is most prevalent [22–24]. The staining with Coomassie brilliant blue R-250 [25–27] has a limited use for the blot membranes, since it binds to most membranes and cannot be completely freed from them.

Simple to use is the India ink staining of proteins blotted on NC membrane [28, 29]. It is more sensitive than the staining with Coomassie brilliant blue R-250, Amido black 10B, and Fast green, and its sensibility is almost as high as the silver staining method. The proteins on membrane appear as black bands on a gray background.

Fast green FCF is used in a concentration of 0.1 g/dL dissolved in 1 mL/dL acetic acid [30, 31].

Chelates can also serve as dyes for blotted proteins. Such an ability has, e.g., the purple-colored chelate ferrozine/ferro ion [32].

The metal (colloidal gold and silver) staining of membranes has the highest sensitivity but long incubation and troublesome preparation. Copper iodide staining is also sensitive but needs complicated steps to prepare the necessary reagents [33].

2.14.5.2 Detection by probes

Probes are antibodies, antigens, lectins, nucleic acids, and more. A few methods of detection by probes are used: colorimetric, chemiluminescent, radioactive, and fluorescent detection.

During the *colorimetric detection*, the blot membrane is probed for proteins of interest with antibodies, linked to enzymes (e.g., 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. In enzyme blotting, native enzymes are blotted and detected using specific coupled color reactions [34, 35].

The *chemiluminescence detection* needs the incubation of Western blot with a substrate that will luminesce when exposed to a reporter on a secondary antibody. The light is detected by charge-coupled device (CCD) cameras, which capture a

digital image of the Western blot or photographic film. The image is analyzed by densitometry, which evaluates the stained protein. Nowadays, software allows even molecular mass analysis (Figure 2.14.5).

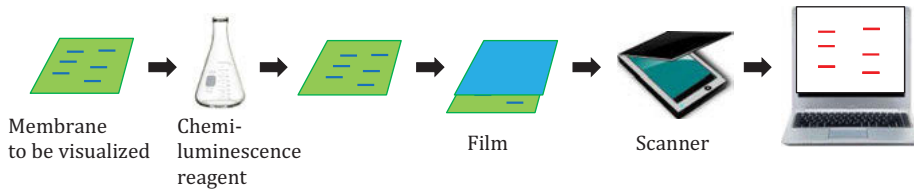


Figure 2.14.5: Chemiluminescence detection.

The *radioactive detection* does not require enzyme substrates but labeled proteins. After placing of an X-ray film onto the Western blot, dark regions are created, which correspond to the proteins of interest. The importance of this method is declining due to its hazardous radiation; therefore, it is rarely used now.

For *fluorescent detection*, a fluorescently labeled probe is excited by light and the appeared emission is detected by a photosensor such as a CCD camera equipped with appropriate filters. This technique is considered to be one of the best methods for quantification, but is less sensitive than chemiluminescence method [36].

2.14.5.3 Immunoblotting

In the immunoblotting [37, 38], the antigens (proteins) are transferred to the blot membrane. There they form precipitation bands with specific immunoglobulins. The precipitation bands can be visualized, if the proteins are labeled, for example, with ^{125}I [39].

2.14.6 Making the blot membranes transparent

After staining, a NC blot membrane can be made transparent without changing the color intensity of the stained bands [40]. For this purpose, the blot membrane should be impregnated with a monomeric solution, which has the same refractive index as NC. Thereafter, the monomer is polymerized using a photoinitiator and UV irradiation.

The transparency is achieved by the following procedure:

- A few drops of the monomeric solution (usually of 2 g/dL benzoin methyl ether in **trimethylolpropane trimethacrylate**, TMPTMA) with a photoinitiator are pipetted on the blot membrane and a second PVC film is placed on.

- The air bubbles between the blot membrane and the PVC films are removed with a photo roller;
- Both sides of the resulting sandwich are irradiated for 15 s each with UV light.

2.14.7 Blotting techniques

Different blotting techniques for proteins are known: **Western blotting** (WB), **Far-western blotting** (FWB), **Southwestern blotting** (SWB), **Northwestern blotting** (NWB), and **Eastern blotting** (EB).

2.14.7.1 Western blotting

WB (protein immunoblot) is a technique for transferring native or denatured proteins onto a NC or PVDF membrane separated by gel electrophoresis, where they bind to specific antibodies (Figure 2.14.6). It originates from the laboratory of George Stark at Stanford University [41] and the laboratory of Harry Towbin at the Friedrich Miescher Institute [42]. The name of Western blotting was given by Neal Burnette [43] as a nod to Southern blotting, developed earlier by Edwin Southern [44].



Figure 2.14.6: Scheme of Western blotting.

The HIV test contains a WB 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; the free antibodies are washed away. Finally, a secondary anti-human antibody linked to an enzyme signal is added.

The WB is also used as a definitive test for **bovine spongiform encephalopathy** (BSE, commonly referred to as *mad cow disease*). It can be used also for diagnosing some forms of Lyme disease (Lyme borreliosis), hepatitis B infection, and HSV-2 infection caused by herpes type 2.

2.14.7.2 Far-Western blotting

FWB was derived from the standard WB to detect protein–protein interactions *in vitro*. After proteins are transferred on the membrane, it is blocked and probed, usually with purified bait proteins. The bait proteins form complexes with prey proteins [45]. Then, an antibody probe is used to detect the presence of these complexes.

Unlike most methods using cell lysates (e.g., co-immunoprecipitation, co-IP) or living cells (e.g., fluorescent resonance energy transfer FRET), FWB determines whether two proteins bind to each other directly.

2.14.7.3 Southwestern blotting

SWB is based on Southern blotting, which is used for DNA detection, and on WB, which is used for protein detection. It was first described by B. Bowen and colleagues in 1980 [46, 47]. The SWB is carried out on NC membranes. With its aid, DNA-binding proteins are identified and characterized by their ability to bind to specific oligonucleotide probes.

Proteins are separated on a polyacrylamide gel containing SDS, renatured by removing SDS in the presence of urea, and blotted onto an NC membrane by diffusion. The genomic DNA region of interest is digested by restriction enzymes. In their activity, 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.

2.14.7.4 Northwestern blotting

NWB is a hybrid of Western and Northern blotting. It is used to detect interactions between proteins that are immobilized on an NC membrane and RNA for studying the gene expression.

After separating by electrophoresis on agarose or polyacrylamide gel, RNA-binding proteins are transferred onto an NC membrane [48]. The blot is soaked in a blocking solution, such as TRIS-HCl (pH = 7.5), containing Mg acetate, DDT, Triton X-100, nonfat milk, or BSA [49]. Then a specific competitor RNA is applied, and the blot is incubated at room temperature [50]. During this time, the competitor RNA binds to the RNA-binding proteins on the membrane. After the incubation, the blot is washed in order to remove the unbound RNA. Common wash solutions are phosphate-buffered saline (PBS) buffer or a Tween 20 solution [51]. Finally, the blot is developed by X-ray or similar autoradiography methods [52].

2.14.7.5 Eastern blotting

EB is a biochemical technique used to analyze protein posttranslational modifications leading to synthesis of lipoproteins, phosphoproteins, and glycoproteins: phosphorylation, acetylation, acylation, alkylation, nitroalkylation, arginylation, biotinylation, formylation, geranylgeranylation, glutamylation, glycosylation, hydroxylation, isoprenylation, lipoylation, methylation, selenation, succinylation, sulfation, transglutamination, ubiquitination, and more [53, 54]. It is similar to lectin blotting (i.e., detection of carbohydrate epitopes on proteins) [55].

2.14.8 Protocols

Western Blotting

Materials and equipment

NC or PVDF membrane

Filter paper

Semidry transfer unit

Power supply

10× TBST (TRIS-buffered saline containing Tween 20), pH = 7.4

NaCl	80.0 g (1.37 mol/L)
KCl	2.0 g (0.03 mol/L)
TRIS	30.0 g (0.25 mol/L)
Tween	10.0 mL (0.02 mol/L)

Dissolve in 800 mL deionized water. Adjust pH to 7.4 with HCl. Add deionized water to 1 L.

TBST buffer, pH = 8.0

TRIS	0.30 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 (0.1 mmol/L)
Deionized water to	100.00 mL

Cell lysis buffer

TRIS-HCl (pH = 7.5)	0.24 g (20 mmol/L)
NaCl	8.77 g (150 mmol/L)
NP-40	1.00 mL

EDTA	0.58 g (2.0 mmol/L)
Leupeptin	100.00 µg
Aprotinin	100.00 µg
Na ₃ PO ₄	0.02 g (1 mmol/L)
PMSF	0.02 g (1 mmol/L)
NaF	0.02 g (5 mmol/L)
Na ₄ P ₂ O ₇	0.08 g (3 mmol/L)
Deionized water to	100.00 mL

Transfer buffer

TRIS	3.0 g (0.025 mol/L)
Glycine	14.4 g (1.19 mol/L)
Methanol	200.0 mL
Deionized water to	1,000.0 mL

Blocking buffer, and primary and secondary antibody dilution buffer

TBST with 5% nonfat dry milk

Procedure

- Place cells in a microcentrifuge tube and centrifuge to collect cell pellet.
- Lyse the cell pellet with 100 µL of cell lysis buffer on ice for 30 min (for 10⁶ cells use 100 µL of cell lysis buffer).
- Centrifuge at 14,000 rpm (16,000 g) for 10 min at 4 °C.
- Transfer the supernatant to a new tube and discard the pellet. Remove 20 µL of supernatant and mix with 20 µL of 2× sample buffer.
- Boil for 5 min. Cool at room temperature for 5 min. Microcentrifuge for 5 min.
- Load up to 40 µL of sample to each well of a 1.5 mm thick gel.
- After electrophoresis transfer proteins onto an NC or PVDF membrane according to the manufacturer instructions.
- Place the blot membrane in blocking buffer consisting of 5% nonfat dry milk/TBST.
- Incubate the blot membrane with agitation at room temperature for 1 h, or at 4 °C overnight.
- Dilute the primary antibody to the recommended concentration in 5% nonfat dry milk/TBST. Place the membrane in the primary antibody solution and incubate at room temperature for 2 h, or at 4 °C overnight with agitation.
- Wash three times for 5 min each with wash buffer (TBST, pH = 7.4).
- Incubate the membrane at room temperature for 30 min with horseradish peroxidase (HRP)-conjugated secondary antibody, diluted to 1:1,000–1:5,000 in 5% nonfat dry milk/TBST.

- Wash 4 times each with TBST for 10 min and once with PBS for 2 min.
- Incubate membrane (protein side up) with 10 mL of enhanced chemiluminescence substrate for 1–2 min. The final volume required is 0.125 mL/cm².
- Drain off the excess detection reagent and wrap up the blots.
- Place the wrapped blots (protein side up) in an X-ray film cassette and expose to X-ray film from 5 s to 60 min.

Semidry Blotting

Materials and equipment

Transfer buffer

Transfer membrane: 0.45 µm NC, PVDF, and 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 NCs are used, do not use methanol because it dissolves NC.
- 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 paper stack soaked with electrode buffer.
- Place the anode.
- Place on the anode a 5 kg weight.
- Set the voltage to 15 V, limit the electric current to 0.4 mA per cm² 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.

Capillary Blotting

Materials and equipment

NC paper (pore size 0.20–0.45 μm)

Transfer buffer

Transfer membrane: 0.45 μm NC, PVDF, and neutral or positively charged nylon

Methanol

Filter paper

Glass plates

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

Destaining solution

Methanol 40.0 mL

Acetic acid 10.0 mL

Deionized water to 100.0 mL

Procedure

- Place two trays with blotting buffer on the table.
- Place onto the trays a 25 × 20 cm glass plate and six layers of chromatographic 3 mm filter paper on it.
- Dip the two ends of the paper in the blotting buffer and allow the papers to wet completely.
- Place the gel with proteins on the wetted filter paper.
- Wet an NC membrane of the same size as the gel that is to be blotted in the blotting buffer.
- Place the gel onto the wetted NC membrane, avoiding air bubbles trapped in between.
- Lay six layers of chromatographic 3 mm filter paper on the gel and place over another glass plate.

- Place a weight of approximately 500 g over the setup and leave the set for 1–2 days.
- Free the NC membrane and immerse it in the Amido black solution for 10 min with shaking.
- Destain in the destaining solution.
- Dry the blot between sheets of filter paper.

India Ink Staining of Proteins on Membrane

Materials and equipment

TBST

India ink

Acetic acid

Tween 20

Rocking platform

TBST buffer, pH = 8.0

TRIS 0.30 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 200.05 mL

Deionized water to 100.00 mL

Staining solution

India ink 0.10 mL

Acetic acid 1.00 mL

TBST solution to 100.00 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.

Detecting Proteins by Immunoblotting

Materials and equipment

TBST

Methanol

SDS

Recombinant protein or unlabeled translated protein for probe

5 g/dL nonfat instant dry milk in TBST

Primary antibody specific for protein probe

Alkaline phosphatase (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 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 L with deionized water. Store at room temperature.

Procedure

- Transfer the proteins onto a blot membrane and block the free places.
- Incubate the blot in 5 mL/dL nonfat 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 ALP buffer.
- Incubate the blot in developing solution for 1–15 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.

Immunoprobng with Avidin–biotin Coupling to Secondary Antibody

Materials and equipment

NC or PVDF membrane
Blocking buffer
TBS (TRIS-buffered saline)
TBST
Avidin
Biotinylated HRP or ALP
Primary antibody
Biotinylated secondary antibody
Heat 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 NC or PVDF membranes, incubate at room temperature for 30–60 min. For nylon membrane, incubate at 37 °C for 2 h.
- Prepare primary antibody in 5 mL TBST (for NC or PVDF membrane) or TBS (for nylon membrane). Dilutions generally range from 1/100 to 1/100,000.
- 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 NC or PVDF membrane) or TBS (for nylon membrane) for 15 min.
- Dilute two drops of the biotinylated secondary antibody in 50–100 mL of TBST (for NC or PVDF membrane) or TBS (for nylon membrane).
- Transfer the membrane into a plastic bag containing secondary antibody solution.
- Incubate at room temperature with slow rocking for 30 min and 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 NC 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 the appropriate visualization protocol.

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2.15.1 Qualitative evaluation of protein pherograms

Most proteins are colorless in nature. Therefore, they are usually stained after electrophoresis. The nonspecific staining involves fixing, staining, destaining of the background, and drying of the gel.

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2.15.1.1 Fixing of proteins

The fixing causes denaturation and precipitation of proteins. For this purpose, methanol, ethanol, acetic acid, trichloroacetic acid (TCA), 5-sulfosalicylic acid, or glutardialdehyde are used.

Mixture of alcohol, acetic acid, and water. The alcohol, methanol or ethanol, dehydrates the proteins, while acetic acid diminishes the pH value of the solution. Thus, the hydration shell of proteins is damaged, and most of them are put at or near their pI points. As a result, the protein solubility decreases and the proteins precipitate. Usually, two mixtures are used: 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. TCA in a concentration of 10–20 g/dL settles all proteins having relative molecular mass M_r above 5,000. 5-Sulfosalicylic acid in a concentration of 2–5 g/dL precipitates also most proteins, except serum α_1 -acidic glycoprotein. Better results are obtained when using a mixture of 10 g/dL TCA and 5 g/dL 5-sulfosalicylic acid.

Glutardialdehyde. Glutardialdehyde, in a concentration of 0.5–1.0 mL/dL, cross-links 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 (Figure 2.15.1). This reaction takes place at pH = 6.0–7.0.

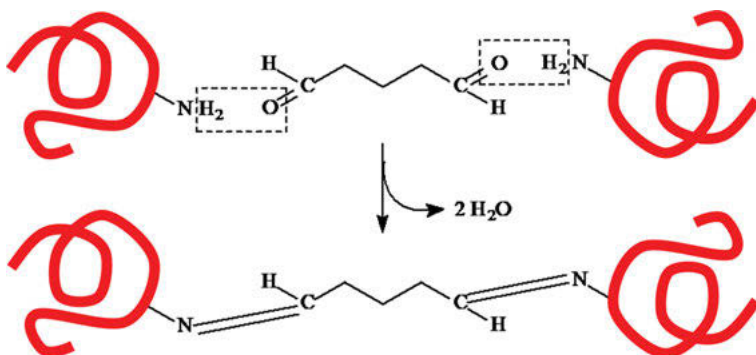


Figure 2.15.1: Glutardialdehyde as a cross-linker of proteins.

2.15.1.2 Staining of proteins

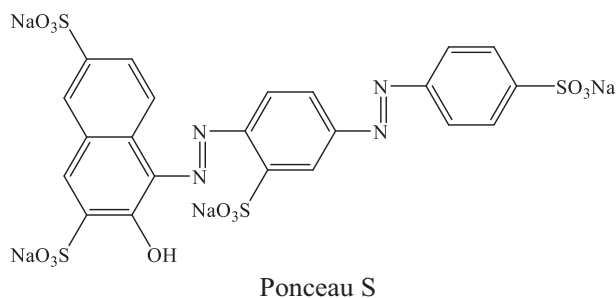
The proteins in a gel can be found (visualized) using anionic dye staining, counterionic dye staining, metal staining, autoradiography, fluorography, immunoprobng, or other methods.

Anionic dye staining of proteins

To the anionic dye staining methods belong the staining methods with Ponceau S, Amido black 10B, Coomassie brilliant blue (CBB) R-250, Fast green FCF, India ink, and more.

Ponceau S staining of proteins

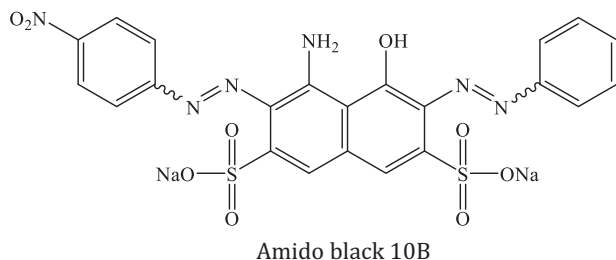
The staining with Ponceau S, $M_r = 760.58$, is used usually after electrophoresis on cellulose acetate membranes [1–3].



The fixing and staining are carried out in 0.3 g/dL Ponceau S in 3 g/dL TCA; the destaining in 0.5 g/dL TCA; and drying at room temperature.

Amido black staining of proteins

The staining with Amido black 10B (Naphthol blue black), $M_r = 616.50$, is prevalent for agarose and polyacrylamide gels [4, 5], rarely for nitrocellulose blot membranes [6].



Prior to staining, the agarose gel should be dried after fixation. Thus, the proteins will be stained faster, and the gel absorbs less dye.

Coomassie brilliant blue staining of proteins

Originally, the Coomassie dyes were used for staining silk and wool. They were introduced to color electrophoretically separated proteins in the 1960s – first CBB R-250 [7–9], then CBB G-250 [10], and finally Coomassie violet R-200 [11–13] (Table 2.15.1).

Table 2.15.1: Coomassie dyes used in electrophoresis.

Dyes	Chemical formulas	Properties
Coomassie brilliant blue R-250 (Acid blue 83), sodium salt		$M_r = 825.97$ $\lambda = 560 \text{ nm}$ Soluble in methanol and ethanol Sensitivity: 10–50 ng/mm ²
Coomassie brilliant blue G-250 (Acid blue 90), sodium salt		$M_r = 854.03$ $\lambda = 580 \text{ nm}$ 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

CBB R-250 is the most popular protein stain. In acidic solutions, it binds to the amino groups of proteins, which are contained in lysine, arginine, and histidine residues. The high intensity of CBB R-250 can be explained with the secondary binding between the dye molecules. The proteins are detected as blue bands on a clear background.

There are two staining methods with Coomassie dyes: conventional and colloidal staining.

Conventional Coomassie brilliant blue staining of proteins

The conventional Coomassie staining methods are relatively fast and less complicated. Their sensitivity reaches 100 ng protein/band.

First, Fazekas de St. Groth *et al.* described a procedure for staining proteins on a cellulose acetate sheet using CBB R-250 [14]. They suggested that the negatively charged sulfonate groups of CBB R-250 bind electrostatically to the positively charged protonated amino groups of proteins and that the positively charged quaternary ammonium groups of CBB R-250 bind to the negatively charged protein carboxyl groups. In these processes, hydrophobic interactions and hydrogen bonding take part, too. Later, Meyer and Lambert [15] and Chrambach *et al.* [16] 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, or a solution of 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 [17].

Colloidal Coomassie brilliant blue staining of proteins

Chrambach *et al.* [18] and Diezel *et al.* [19] replaced CBB R-250 by colloidal CBB G-250 dissolved in 12.5 g/dL TCA, to reduce the gel background. Neuhoff *et al.* [20, 21] employed the same staining principle including ammonium sulfate and phosphoric acid in the protocol. Their modification alleviated the problem of background staining and enabled the detection of very weak spots. Neuhoff dye (“blue silver”) recipe contains 2 g/dL H_3PO_4 , 10 g/dL $(\text{NH}_4)_2\text{SO}_4$, 20 mL/dL methanol, and 0.1 g/dL CBB 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 long time [22].

India ink staining of proteins

The staining with carbon (India ink) [23, 24] is more sensitive than the Coomassie staining and comes closely to the sensitivity of silver staining. It is used mostly for staining of blot membranes (see there).

Counter-ionic dye staining of proteins

The counter-ionic dye staining method employs two oppositely charged dyes (a negatively charged acidic dye and a positively charged basic dye), which form an ion-pair complex [25]. The ion-pair complex forms a colloidal staining solution. During the counter-ionic dye staining, the equilibrium exists between the ion-pair complexes and the free forms of dyes.

A counter-ionic dye staining method is the EV-ZC staining [26]. It uses the basic dye ethyl violet (EV), and the acidic dye zincon (ZC). The detection limit of this method is 4–8 ng of protein in about 60 min.

Metal staining of proteins

To the metal staining of proteins belong the silver staining and chelate dye staining.

Silver staining of proteins

The vast application of silver staining to detect proteins after electrophoresis was established by Merrill and colleagues who used formaldehyde to reduce silver ions into silver atoms under alkaline conditions [27, 28]. It is some 100 times more sensitive than Coomassie staining at detection limit of 1–0.1 ng protein per mm² of band area (50 ng protein in a band). The main disadvantage of silver staining is the poor correlation between staining intensity and protein concentration, because this method is not an end-point staining method as the less sensitive Coomassie or SYPRO Ruby staining [29]. Besides, there are proteins that react little or do not react with the silver ions [30] because staining depends on the complexation of Ag⁺ with glutamate, aspartate, and cysteine residues [31].

The mechanisms of silver staining are similar to those of a photographic process [32]: The gel matrix with the separated proteins is saturated with Ag⁺, which bind preferentially to basic amino acid residues of proteins. When Ag⁺ are reduced to elementary Ag, the proteins obtain a typical brownish-gray-black color [33,34].

The silver staining comprises the following steps: fixing, sensitizing, developing, and stopping. Finally, the gel is dried.

The *fixing* step does not differ from that at Coomassie staining. It denatures the proteins to precipitate. For this purpose, weak acids, e.g., acetic acid and alcohol, are usually used.

During the *sensitizing* step (impregnating), the silver ions are bound to the precipitated proteins to form silver nuclei.

The *developing* step (visualizing) requires a reducing agent, such as formaldehyde, which reduces the silver ions to metallic silver (Figure 2.15.2). The reaction takes place near the silver nuclei much faster than in the gel, since the silver nuclei catalyze the reduction and transform the protein bands into dark brown to black bands on a slightly yellowish to colorless background. The developing step takes place at higher pH values, for example, in a sodium carbonate solution.

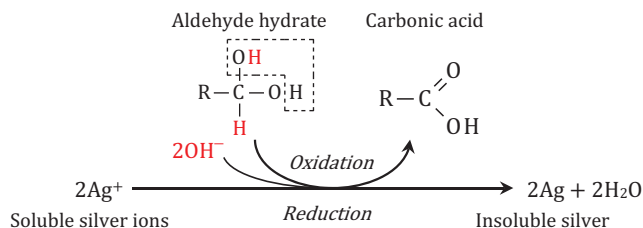


Figure 2.15.2: Mechanism of silver staining.

After the developing step, the reduction should be *stopped*. Otherwise, all silver ions will be reduced to metallic silver in the gel, which will obtain a black color. In principle, the stopping step is caused by a strong decrease in the pH value.

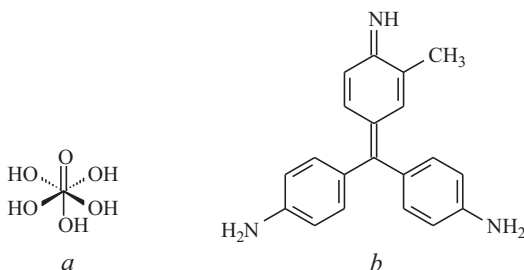
The *drying* step of the colored pherogram is similar to that after the Coomassie staining. It is carried out usually under a hair dryer. If the polyacrylamide concentration is high, the wet gel should be covered, free of air bubbles, with water-swollen cellophane.

Chelate dye staining of proteins

Metal chelates are organometallic complexes that bind to proteins [35]. They appear at acidic pH and can be used to detect proteins on nitrocellulose, polyvinylidene difluoride, and nylon membranes, as well as in polyacrylamide gels. An example for the chelate dye staining of proteins is the zinc-imidazole staining [36]. Similarly, a zinc-imidazole staining procedure was proposed for **poly(ethylene glycol) (PEG)**-linked proteins separated in SDS polyacrylamide gels [37–39]. After electrophoresis, the gels were rinsed with deionized water and incubated in an imidazole-SDS solution. Then, they were soaked in zinc sulfate solution. The PEGylated protein bands remained transparent.

Staining of glycoproteins

The detection of polysaccharides in glycoproteins, glycolipids, mucins, and glycogen is carried out with the **periodic acid–Schiff (PAS)** test [40]. The Schiff reagent is a reaction product of fuchsine and sodium bisulfite. Periodic acid oxidizes the vicinal diols in the sugars, usually breaking up the bond between two adjacent carbon atoms not involved in the glycosidic linkage, and creating a pair of aldehydes at the two free tips of each broken monosaccharide ring. The aldehydes react with the Schiff reagent to give a purple-magenta color.



Periodic acid (a) and fuchsine (b).

A new variant of PAS test is the staining test of Hart *et al.* [41]. 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 degree of protein glycosylation, making it 8–16-fold more sensitive than the standard colorimetric PAS test. 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.

Staining of lipoproteins

The staining of lipoproteins in a gel is carried out with Sudan Black B. First, the gel is dried at 60 °C, and then it is stained in freshly prepared staining solution (Sudan black and sodium chloride in ethanol). If a precipitate is formed, ethanol should be added until the precipitate dissolves. Finally, the gel is destained in a solution of NaCl in ethanol. After washing in deionized water, the gel is dried again at 60 °C.

Staining of enzymes

Enzymes may be detected directly in the gel or indirectly by color reactions. In the direct detection, the gel with the electrophoretically separated enzymes is placed in a solution with specific substrates coupled to a diazo dye. In the indirect detection, 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 are used for the detection of different enzymes: alkaline phosphatase [42, 43], alcohol dehydrogenase [44, 45], amylase [46, 47], cellulase [48], deoxyribonuclease [49], glucosidase [50], glycosyl transferase [51, 52], catalase [53, 54], creatine kinase [55], lactate dehydrogenase [56], malate dehydrogenase [57], peroxidase [58, 59], phosphoglucomutase [60], peptide hydrolases [61, 62], acid phosphatase [63, 64], trypsin [65], and more.

Autoradiography of proteins

Autoradiography is the most sensitive detection method for proteins. In this method, X-ray film is used to visualize radioactive molecules that have been electrophoresed on agarose or polyacrylamide gels, or hybridized (e.g., on immunoblots). A photon or a β -particle from a radioactive molecule reduces silver bromide crystals on a film emulsion forming grains of silver (Table 2.15.2).

Table 2.15.2: Isotopes and their sensitivities.

Isotope	Sensitivity
^{32}P	50
^{125}I	100
^{14}C	400
^{35}S	400
^3H	8,000

The autoradiography can be used directly or indirectly.

For *direct autoradiography*, the proteins are marked with the isotopes ^{14}C , ^{35}S , ^{32}P , or ^{125}I [66]. 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.

In *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” consisting of the separation gel, an X-ray film, and an intensifying screen is incubated at $-70\text{ }^\circ\text{C}$. The radiation that penetrates the film hits the screen and excites emission of light quanta. They enhance the blackening on the film.

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 of a certain wavelength and releases this energy by emitting photons. Most commonly utilized fluorogenic reagents are **o**-phthaldialdehyde (OPA) and naphthalene dicarboxaldehyde (NDA) [67]. OPA is used in the presence of a thiol, e.g., **β** -mercaptoethanol (BME) [68], 3-mercapto-1-propanol [69], dithiothreitol (DTT) [70], ethanethiol [71], or *N*-acetyl-L-cysteine (NAC) [72].

In *fluorography*, a gel with the electrophoretically separated proteins, which have been previously labeled with ^3H , ^{14}C , or ^{35}S , is impregnated with scintillating substances such as 2,5-diphenyloxazole [73] or sodium salicylate [74]. The gel is dried and is let to act on X-ray film at $-70\text{ }^\circ\text{C}$. The interaction between the radioactive radiation and the scintillating substance produces visible light that blackens the film. It is also possible to treat the sample, prior to electrophoresis, with a fluorescent marker [75, 76], which makes the bands visible under UV light.

SYPRO Ruby staining of proteins

SYPRO Ruby staining was first introduced in 2000 by Berggren *et al.* [77]. Its sensitivity is better than other protein stainings and as sensitive as the best silver staining procedures [78]. SYPRO Ruby stain is a fluorescent ruthenium-based metal chelate dye, which interacts with basic amino acid residues (of lysine, arginine, or histidine). It is less sensitive if the proteins are native. Therefore, it is better if the native gels are soaked in 0.05 g/dL SDS for 30 min and then stained with SYPRO Ruby stain.

SYPRO Ruby stain is compatible with **matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy**. MALDI mass spectrometry requires little sample preparation and can be automated using robotic liquid-handling systems. MALDI mass spectroscopy is utilized for biomarkers, for example, prostate cancer biomarker [79].

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.

Agarose gels, stained for glycoproteins with PAS reagent, are destained in 7 mL/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 CBB R-250, are destained in a mixture of methanol–acetic acid–water (3:1:6, V:V:V) or of ethanol–acetic acid–water (4:1:5, V:V:V).

Polyacrylamide gels, stained with colloidal CBB G-250, are destained in 0.1 mol/L TRIS-hydrogen phosphate buffer with pH = 6.5.

Polyacrylamide gels, stained with silver, are destained in deionized water.

Polyacrylamide gels, stained with SYPRO Ruby stain, are destained by incubating the gel in 0.1 mL/dL Tween 20 or in 7.5 mL/dL acetic acid overnight.

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 gel should be covered with a cellophane membrane. The gradient polyacrylamide gels are placed on 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 cellophane membrane should be folded onto the underside of the glass plate.

2.15.2 Quantitative evaluation of a protein pherogram

The presence of a stained band could 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 has to be done by densitometry of pherograms or scanning them.

2.15.2.1 Densitometry

Densitometry means quantitative measurement of the absorbance (optical density, OD) of a material when light passes through it [80]. The maximum and minimum densities that can be measured are called D_{\max} and D_{\min} , respectively.

Densitometry is carried out on dry gels or gel photos. As a result, a densitogram is obtained. The densitogram is a curve that reflects the concentrations of bands obtained by cellulose acetate, agarose gel, or polyacrylamide gel electrophoresis (Figure 2.15.3).

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 in a reduction of its intensity into I , in cd. According to the Beer–Lambert law [81, 82], the natural logarithm of the ratio between the irradiated and transmitted light is referred to as absorbance A (OD):

$$A = \ln \frac{I_0}{I} \quad (2.15.1)$$

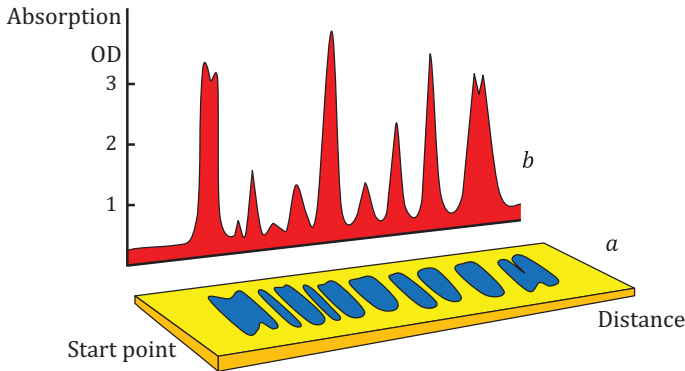


Figure 2.15.3: Densitometry: (a) pherogram and (b) densitogram.

In densitometry, white light or laser light is used. The white light should be adjusted, using a suitable filter, because the dyes have different absorption maxima (Figure 2.15.4). 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.

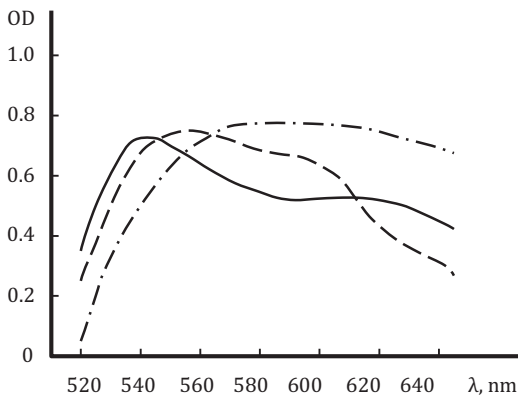


Figure 2.15.4: Absorption spectra of different dyes. — Coomassie brilliant blue R-250; — — Coomassie brilliant blue G-250; — · — Amido black 10B (Naphthol blue black B).

The baseline of a densitogram defines its background. Two methods are used to define the baseline (Figure 2.15.5): 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 for unevenly colored background. The first method is simpler, but the manual baseline brings precise results, as it excludes the influence of the background.

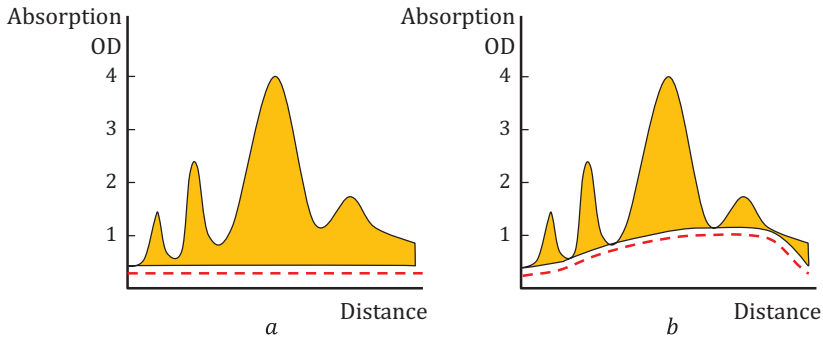


Figure 2.15.5: Different baselines. (a) Horizontal baseline; (b) manual baseline. -- Baseline.

Evaluating a densitogram, absolute or relative protein concentrations can be determined. In the first case, a calibration curve is required; in the second case, a relationship between the total band surface (total concentration) and a band surface is needed. The relative concentration is often more important than the absolute concentration. In the regions of low protein concentrations, a linear relationship exists between the concentration and the corresponding band area.

In the one-dimensional (1D) densitometry, the bands of a pherogram are scanned only in one direction. In the two-dimensional (2D) densitometry, the spots of a pherogram are scanned in two directions; then the information is translated into digital signals and processed in a computer [83, 84]. In the 1D densitometry, a mountain-shaped curve is obtained on one baseline; in the 2D densitometry, a mountain-shaped curve is obtained on a base surface. Therefore, the 2D densitogram looks like a geographic map.

In the 1D densitometry, the evaluation of protein concentrations uses the proportionality between the protein concentration and the band surface; in the 2D 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 2D densitometry, which takes into account the base surface and the spot volumes [85, 86].

Densitometers

The densitometers work usually in a transmission mode, since gels, blot membranes, or autoradiograms are usually transparent. In this case, it determines the OD of a sample placed between a light source and a photoelectric cell [87]. A high-resolution densitometer can scan and analyze bands using a computer and an appropriate software. The molecular masses and isoelectric points of proteins can also be determined.

The resolution of a densitometer depends above all on the width of light beam.

The *width of light beam* has a crucial importance for the resolution of a densitometer when bands lie close together. The wider the light beam, the more the measured absorption differs from the band reality on the gel. 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 , and a laser beam should not be thinner than 50 μm .

On the market, *microdensitometers* are also available. They measure OD in microscopic domains [88, 89]. An alternative version to microdensitometers is the beam expanded laser microdensitometer [90, 91]. It has increased data collection speed, increased depth of focus, and superior signal-to-noise ratios [92].

2.15.2.2 Scanning

Using a standard scanner and appropriate software, the resolved bands on a gel can be quantified. The image produced by a scanner should be an accurate representation of both positions and intensities of the bands.

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 allows automatically analyzing gel electrophoresis images turning a scanner into a densitometer. It can also quantify western blots, agarose gels, PCR gels, *etc.* 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.

2.15.3 Protocols

Coomassie Brilliant Blue Staining of Proteins in Polyacrylamide Gels

Materials and equipment

TCA

CBB 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

CBB 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 nonionic detergents (Nonidet NP-40, Triton X-100) are to be fixed in 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–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 cellophane membrane using a roller. Place the edges of the cellophane membrane around the glass plate.
- Dry at room temperature.

Colloidal Coomassie Staining**Materials and equipment**

Glutardialdehyde

CBB G-250

Ammonium sulfate [(NH₄)₂SO₄]

Phosphoric acid (H₃PO₄)

Methanol or ethanol

Purification of CBB G-250

Solve 4 g of CBB 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 with 2 mL of 5 g/dL CBB G-250. 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.

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 ($\text{Na}_2\text{S}_2\text{O}_3$)

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 in 0.2 g/L $\text{Na}_2\text{S}_2\text{O}_3$ 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 the gel 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.

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 10–60 min.
- Stain 1D and 2D gels for a minimum of 3 h. Stain IEF gels overnight.
- Destain gel background by incubating overnight in the destaining solution to minimize fluorescence.
- Incubate the gel in a 2 mL/dL glycerol for 30 min.
- Dry the gel using a gel dryer.

Staining of Lipoproteins with Sudan Black B

Materials and equipment

Sudan black 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

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 mixture for 15 min.
- Destain in the destaining solution for 15 min.
- Wash in deionized water for several seconds.
- Dry the gel at 60 °C.

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2.16 Precast gels for protein electrophoresis. Rehydratable gels

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Agarose and polyacrylamide (PAA) gels are most widespread precast gels.

2.16.1 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 thickness is usually 0.5 mm. The precast agarose gels are most commonly used in the clinical diagnosis: they are suitable for zone electrophoresis of proteins or for immunofixation and affinity electrophoresis.

The *precast agarose gels for zone electrophoresis* of proteins are used for separation of serum, cerebrospinal fluid, and urine proteins. Besides, they can be used for separation of hemoglobins and isoenzymes of creatine kinase, lactate dehydrogenase, and alkaline phosphatase (ALP). 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 *precast gels for affinity electrophoresis* of proteins contain lectin. They are used for separation of the isoenzymes of alkaline phosphatase.

2.16.2 Precast polyacrylamide gels

The precast PAA 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 PAA gels can be stored at 4 °C for months, regardless of whether they are intended for disc-electrophoresis (discontinuous electrophoresis), SDS disc-electrophoresis, isoelectric focusing with carrier ampholytes, isoelectric focusing with immobilines, or for rehydratable PAA gels.

The *precast PAA gels for disc-electrophoresis* of native electrophoresis consist of a stacking gel and a resolving gel, or contain no stacking gel. The resolving gel has a PAA concentration (T) from 8 to 18 g/dL or even more. They are supplied with gel

<https://doi.org/10.1515/9783110761641-026>

or paper strip electrodes. The precast PAA gels for disc-electrophoresis are cast on a film or a net of different sizes.

The *precast gels for SDS disc-electrophoresis of proteins* can contain the Laemmli or other buffer system. The gels from ELPHO, Nuremberg, are 0.5 mm thick. They are cast on a film or net [1], and are covered with a 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), also 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 gels 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 [2], and consist of a stacking part and a resolving part. The electrode contact with the gel is transmitted through gel electrode strips, which contain buffer.

The *precast PAA gels for isoelectric focusing with carrier ampholytes* are 0.15–0.50 mm thick. Their PAA concentration *T* is 5 g/dL and the cross-linking degree *C* is 0.03. The precast gels for isoelectric focusing with carrier ampholytes form different pH gradients (Figure 2.16.1). They are cast on a support film or a net. The net gels are suitable for blotting.

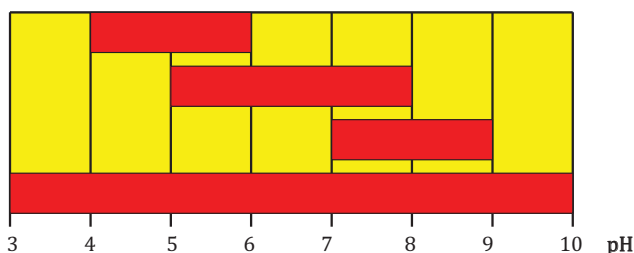


Figure 2.16.1: pH gradients, which can be obtained with precast gels.

The *precast PAA gels for isoelectric focusing with immobilines* are usually 0.5 mm thick. They are cast on a film or a net, and are supplied with gel or 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 dimensions of 51 × 82, 100 × 0, 125 × 125, and 250 × 125 mm.

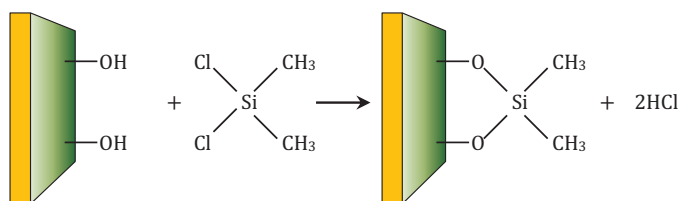
2.16.3 Rehydratable polyacrylamide gels

The rehydratable PAA gels (CleanGels) represent rehydratable homogeneous PAA 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. They can be stored at -20 °C almost indefinitely. Immobiline DryStrips for pH gradients 3.0–10.0, 3.0–10.5, and 4.0–7.0 are also offered.

2.16.4 Protocols

Silanization of Glass Plates

The upper glass plate of a casting cassette comes in contact with the gel; therefore, it should be hydrophobic. To do this, it should be coated with Repel-silane (Pharmacia Biotech, Uppsala) or dichlorodimethyl silane (Merck, Darmstadt) (Figure 2.16.2).



Glass plate

Figure 2.16.2: Binding of dichlorodimethyl silane (Repel-silane) to a glass surface.

Materials and equipment

Glass plates

Repel-silane or dichlorodimethyl silane

Procedure

- 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 silanization, is removed.

Casting Agarose Gels on Support Film by Capillary Technique

The capillary technique is used for casting thin (0.5 mm) agarose gels.

Materials and equipment

Agarose
Support film
Glass plates
Repel-silane
Silicone spacers
Clamps
Syringe or pipette

Procedure

- Place some drops of water onto a glass plate.
- Lay a support film down on the glass plate and roll over so that any air bubbles are removed.
- Hydrophobize a second glass plate with a repellent solution and mount 0.5 mm silicone spacer on its long sides.
- Fix the glass plates together with clamps to make a cassette.
- Heat the cassette at 60 °C in a drying oven.
- Place the sandwich horizontal onto a flat surface.
- Let the agarose solution penetrate into the cassette using a preheated syringe or pipette.
- Let the cast solution harden at room temperature for 30 min and then 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.

Casting Thin PAA Gels by Cassette Technique

The cassette technique [3] is used for casting 0.5–1.0 mm (Figure 2.16.3).

Materials and equipment

Glass plates
Repel-silane
Support film for PAG
Photo roller
U-form silicone gasket
Clamps
Monomeric solution

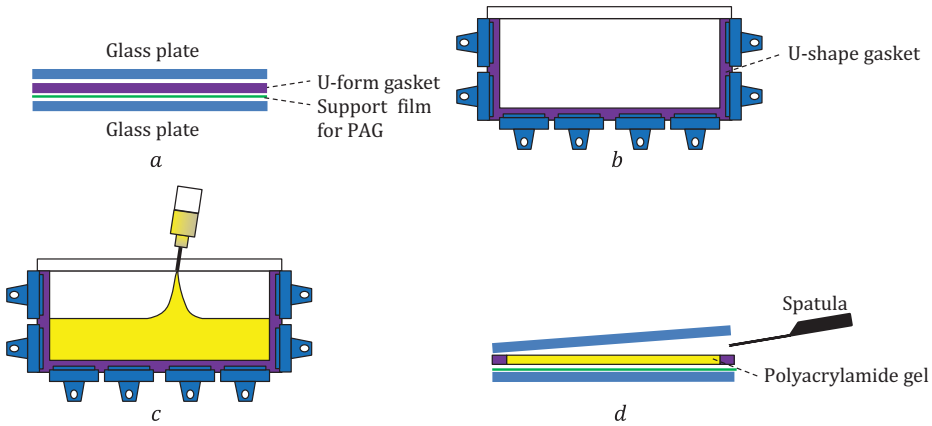


Figure 2.16.3: Steps (a, b, c, and d) of casting polyacrylamide gel by a cassette technique.

Procedure

- Put a few drops of water onto a glass plate, place a support film over it, with its hydrophilic side up, 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 2.16.4).

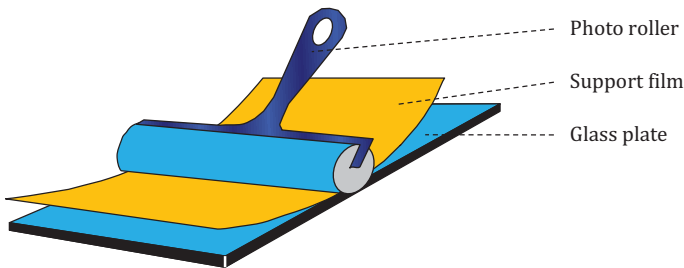


Figure 2.16.4: Rolling a support film.

- 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 cassette, fix it with clamps, and set it vertically.
- Cast the monomeric solution into the cassette using a syringe or a pipette. After 1 h, remove the clamps from the cassette, disassemble it, and take the gel off. The gel can be used immediately for electrophoresis or can be stored in a plastic envelope for weeks in the refrigerator at 4 °C.

Casting Ultrathin PAA Gels by Flap Technique

The flap technique [4] is suitable for production of ultrathin (0.05–0.30 mm) PAA gels, even with dimensions of 40 × 20 cm (Figure 2.16.5).

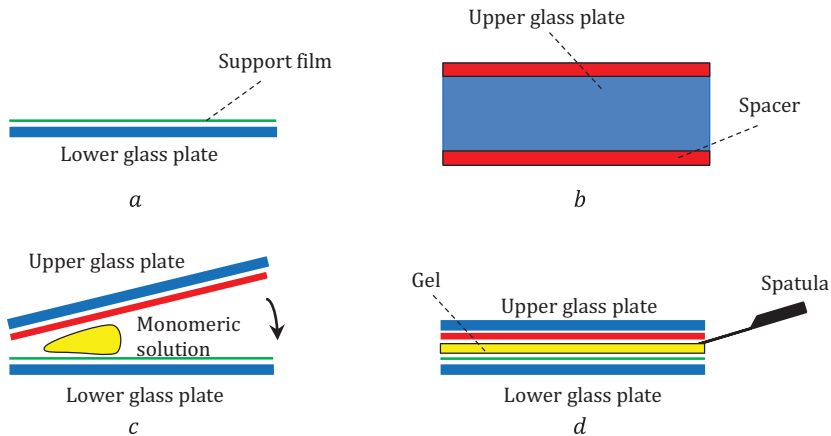


Figure 2.16.5: Steps (a, b, c, and d) of polyacrylamide gel casting by flap technique.

Materials and equipment

Glass plates
 Repel-silane
 Support film for PAG
 Silicone spacers
 Monomeric solution
 Spatula

Procedure

- Put a few drops of water onto a horizontal glass plate (lower glass plate) and place over a support film with its hydrophobic side down. Roll the support film with a photo roller to remove the air bubbles.
- 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.
- 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 with the help of a spatula and use it, or envelope it into a plastic bag and store it in the refrigerator at 4 °C for up to several weeks.

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3 Electrophoresis of nucleic acids

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The nucleic acid 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, whereas the electrophoresis in discontinuous buffer systems is referred to as disc-electrophoresis.

Double-stranded DNA fragments naturally look like rods, whose migration through a gel is a function of their size and gyration [1, 2]. Single-stranded DNA or RNA tends to fold up into structures with complex shapes, which migrate through a gel in a complicated manner because of their structure. Agents that disrupt hydrogen bonds, such as NaOH or formamide, are used to denature the nucleic acids and cause them to resemble long rods [3].

The electrophoretic separation of nucleic acids is carried out in weak acidic, neutral, or alkaline solutions with pH range of 6–10. Under these conditions the nucleic acids behave themselves as polyanions (nucleates) and move in an electrical field to the anode. If the pH value is too low, the bases cytosine and adenine will protonate and obtain positive electric charges; if the pH value is too high, the bases thymine and guanine will deprotonate and gain negative electric charges. In both cases, additional electric charges will cause denaturation of nucleic acids and will change their mobility.

Buffers for electrophoresis of nucleic acids

For DNA electrophoresis, buffers with pH values in the range of 7–9 are best suited. They should contain electrolytes whose pK_c values are close to the desired pH value. In Table 3.1, the most commonly used buffers for nucleate electrophoresis are given.

Borate ion in the **TRIS-borate-EDTA** (TBE) buffer is a strong inhibitor for many enzymes. As a result, a DNA sample in a TBE buffer can better keep its integrity and, therefore, the size of DNA nucleates can be better analyzed.

TRIS-acetate-EDTA (TAE) buffer is used as a running buffer in denaturing gel electrophoresis methods for mutation analysis. High concentration of salts in the sample, for instance, NaCl, retards the DNA mobility.

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Table 3.1: Most commonly used buffers for nucleate electrophoresis.

Buffer's name	Description	Composition
TBE (TRIS-borate-EDTA) buffer pH = 8.3	Low ionic strength buffer which can be used for both preparative and analytical agarose gel electrophoresis. Most commonly used for polyacrylamide gel electrophoresis. Rarely used for sequencing electrophoresis.	For 1 L 5× buffer TRIS 54.0 g Boric acid 27.5 g 0.5 mol/L EDTA 20.0 mL <i>Adjust pH to 8.3 with HCl</i> Deionized water to 1,000.0 mL
TAE (TRIS-acetate-EDTA) buffer pH = 8.0	Used for analytical and preparative agarose gel electrophoresis when DNA is purified by glass beads. Sometimes it is used in pulse-field electrophoresis.	For 1 L 50× 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

Polymerase chain reaction

The **polymerase chain reaction** (PCR) was developed in 1983 by Kary Mullis [4, 5], who was awarded the Nobel Prize in Chemistry in 1993. It is a technique to amplify DNA segments *in vitro* [6, 7]. PCR mimics the DNA replication, but confines it to DNA sequences of interest. It requires about 500-fold less DNA than Southern blotting and is less time-consuming.

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. The **quantitative PCR** (qPCR) techniques are applied to determine quantitatively the levels of gene expression. An interesting method is the combination of reverse transcription and 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 in PCR [8] increasing the detection of alterations of gene expression in microbes, tumors, or other diseases [9].

Procedure

A PCR setup requires several components [10]:

- A *buffer* providing a suitable pH value and ions necessary for the DNA polymerase optimum activity. The following buffer is often used: 10–50 mmol/L TRIS-HCl with pH = 8.3–9.0 containing up to 50 mmol/L KCl and 0.5–5 mmol/L MgCl₂. Instead of Mg²⁺, Mn²⁺ can also be used.

- 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 short synthetic oligonucleotides, which contain from 14 to 40 mononucleotides. The optimal concentration of the primers is 0.1–1 $\mu\text{mol/L}$.
- *Deoxynucleoside (deoxynucleotide) triphosphates* dATP, dGTP, dTTP, and dCTP. They take place in the synthesis of new DNA strands with the help of DNA polymerase according to the principle of complementarity. The four dTTPs should be present at equimolar concentrations in the reaction mixture. Their optimal concentration is 20–200 $\mu\text{mol/L}$.
- *DNA polymerase* – an enzyme that catalyzes the synthesis of new DNA strands. It binds to a double-stranded region of DNA and uses the primers. The heat-resistant Taq polymerase is commonly used, as it retains its activity at 94 °C for 45–60 min; however, its optimal temperature is 72 °C. The Taq polymerase was first isolated by Chien *et al.* from the microorganism *Thermophilus aquaticus* (Taq) [11].

The PCR 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. Finally, bacterial thermostable Taq polymerase catalyzes the elongation of each primer using four **deoxynucleoside triphosphates** (dNTP).

Every PCR cycle consists of several temperature steps. The temperature in each cycle conforms to the enzyme used for DNA synthesis, the concentration of bivalent ions, dNTPs in the reaction, and the melting temperature of the primers [12]. The cycle steps are as follows:

- *Initialization*: This step is used for thermostable DNA polymerases that require heat activation [13]. During this step, the reaction chamber is heated up to 94–98 °C for 1–10 min.
- *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 hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules (Figure 3.1).
- *Annealing*: 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 on the 3'-end of each strand.
- *Elongation*: The temperature for this step depends on the DNA polymerase used. The optimum temperature for the thermostable Taq polymerase is approximately 75–80 °C [14, 15]. 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. 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

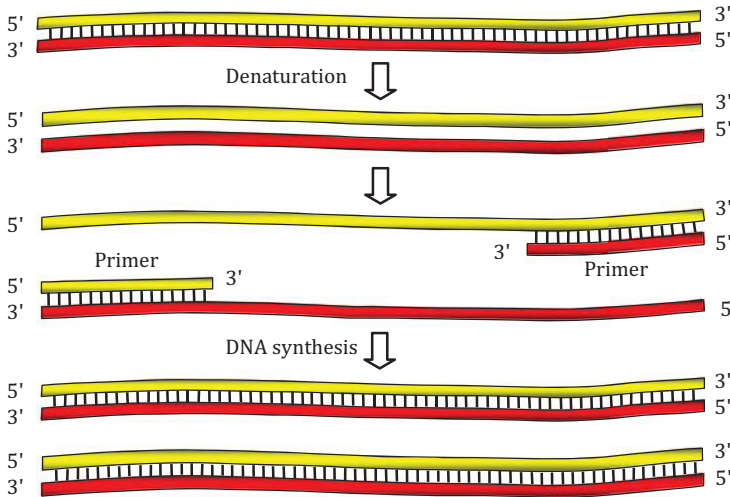


Figure 3.1: Steps of polymerase chain reaction.

The strands of double DNA are separated from each other by heating (denaturation), and primers are joined complementarily to their ends. 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.

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*: The reaction chamber is cooled to 4–15 °C.

In automating the process, the time for one cycle is a few minutes. For 20 cycles, the amount of starting DNA is increased 10^6 times and for 30 cycles it increased 10^5 times. Most PCR methods amplify DNA fragments between 0.1 and 10 kilobase pairs (kbp), although some techniques allow for amplification of up to 40 kbp [16]. These DNA copies are sufficient in quantity to be used in hybridization techniques.

Applications

PCR has been applied in many areas of molecular genetics:

- Known segments of DNA can easily be produced with the help of PCR.
- PCR is used for studying the gene expression.
- Using PCR, deletions, insertions, translocations, or inversions of DNA can be analyzed.
- PCR generates probes for Southern or northern blot hybridization.

- Although DNA breaks down over time, PCR has been successfully applied to clone ancient genes. For example, 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.

PCR has also 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 can also be used as a sensitive test for tissue typing, which is vital for organ transplantation. There is a proposal to replace the antibody-based tests for blood type with PCR-based tests [17].
- The PCR methods are widely performed to detect the presence of a viral infection soon after infection and before the onset of disease [18]. This is very important because the presence of antibodies to the virus circulating in the bloodstream do not appear until weeks after infection. For this purpose, the primers used should be specific to the targeted sequences in the viral DNA.
- Forensic DNA typing is an effective way of identifying or exonerating criminal suspects due to the analysis of criminal evidence discovered. 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 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 [19].

Surface electrophoresis

A new DNA electrophoresis, called surface electrophoresis (SE), was emerged by Han and Craighead [20]. It involves electrostatic interactions between nucleates and surfaces [21, 22].

Compared with the existing methods, SE 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 [23]. In addition, the resolution of this technique is independent from the bp number. However, the resolution of SE is poor because of the strong diffusion.

SE 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. The friction can be controlled by coating the silicon surface with silane monolayer film, which permits the separation of different DNA sizes [24].

A single DNA chain on a surface is composed of loops, trains, and tails. Its length correlates with the ionic strength: higher ionic strength leads to a smaller flexible DNA chain. According to Li *et al.* [25], when migrating on a surface, a DNA chain experiences three different forces: driving force, buffer friction force, and surface friction force (Figure 3.2).

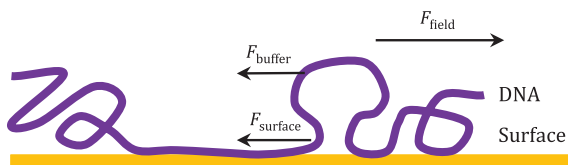


Figure 3.2: Forces experienced by a single DNA chain migrating in buffer on surface under electric field.

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 1 bp and the surface, and α as the train ratio. Then, the driving force

$$F_{\text{field}} = EQN_{\text{bp}} \quad (3.1)$$

the buffer friction force

$$F_{\text{buffer}} = f_{\text{b}}vN_{\text{bp}} \quad (3.2)$$

and the surface friction force

$$F_{\text{surface}} = f_{\text{s}}\varepsilon\alpha N_{\text{bp}} \quad (3.3)$$

If these three forces are in balance, then

$$EQN_{\text{bp}} = f_{\text{b}}vN_{\text{bp}} + f_{\text{s}}\varepsilon\alpha N_{\text{bp}} \quad (3.4)$$

Taking into account that the mobility

$$\mu = \frac{v}{E} \quad (3.5)$$

it follows from eqs. (3.4) and (3.5) that

$$\mu = \mu_0 \left(1 - \frac{f_s \varepsilon \alpha}{QE} \right) \quad (3.6)$$

where

$$\mu_0 = \frac{Q}{f_b} \quad (3.7)$$

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

$$E_{\min} = \frac{f_s \varepsilon \alpha}{Q} \quad (3.8)$$

According to eq. (3.6), when $\alpha = 0$, i.e., when there is no adsorption of DNA on the surface, μ is the free-buffer mobility. When $\alpha = 1$, i.e., when there is full 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. Also according to eq. (3.6), μ increases with the electric field strength and would approach the free-buffer mobility, when the electric field strength overwhelms the surface friction force.

A device for SE contains separation cell, Pt electrode, silicon substrate, TBE buffer, laser source, dichroic mirror, bandpass filter, and computer. Lee and Kuo [26] fabricated a gel-free microchannel electrophoresis device for surface separation of DNA fragments ranging from 3.5 to 21.2 kbp. Another gel-free microchannel device for SE of DNA was fabricated with polydimethylsiloxane [27].

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3.1 Agarose gel electrophoresis of nucleic acids

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Native agarose gel electrophoresis of nucleic acids is an analytical technique for separating 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 nucleic acids to be separated can be prepared in several ways. When the DNA nucleates are very large, they should be cut into smaller fragments using DNA restriction endonucleases. Xylene cyanol or Bromophenol blue is added often to DNA samples to build a moving front. These dyes run with DNA fragments that are 5,000 bp or 300 bp in length, respectively. Other 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 a pH range from 6 to 10. If the pH value is very low, the bases cytosine and adenine bind protons and are positively charged; if the pH value is too high, the bases thymine and guanine split protons and are negatively charged. In both cases, the additional electric charges cause denaturing of nucleic acids and change their mobilities.

DNA is commonly visualized after reacting with ethidium bromide and viewed under UV light. Dyes such as SYBR green, GelRed, methylene blue, and crystal

<https://doi.org/10.1515/9783110761641-028>

violet are also available. The measurement and analysis of the resolved nucleates are mostly carried out using specialized software.

3.1.1 Zone polyacrylamide gel electrophoresis of native nucleic acids

The zone polyacrylamide gel electrophoresis of native nucleic acids is carried out in one buffer.

3.1.1.1 Theory of sieving migration

The widely accepted Ogston model treats the gel matrix as a sieve (Ogston sieving) consisting of randomly distributed network of interconnected pores [1, 2]: A random coil DNA moves almost free through the connected pores, but the movement of larger molecules is impeded and slowed down, which leads to separation of nucleates of different sizes. The more the radius of nucleate gyration approximates the size of a gel pore, the more likely the nucleate will pass through the pore.

The Ogston model, however, breaks down for very large molecules moving through pores significantly smaller than the nucleate size. For DNA molecules greater than 1 kb, a *reptation model* is most commonly used [3]. This model assumes that DNA polyions crawl in a snake-like fashion (reptation) through the pores. At higher electric field strength, the leading end of the molecule becomes strongly biased in the forward direction, and pulls the rest of the molecule along.

Double-stranded DNA fragments behave as long rods. Therefore, their migration through a gel is relative to their size or, for cyclic DNA fragments, their radius of gyration. Single-stranded DNA or RNA tends to fold up into different tertiary structure molecules and migrate through the gel in a complicated manner. Therefore, agents 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.

Factors affecting migration of nucleic acids

A few factors that affect the migration of nucleic acids are: ionic strength of buffer, gel concentration, electric field strength, and concentration of ethidium bromide, if used during electrophoresis [4].

Ionic strength of buffer. The mobility of nucleic acids decreases when the buffer ionic strength increases [5]. This can be explained with the concept of the geometric and electrokinetic radius [6]. When the ionic strength increases, the electrokinetic radius of a nucleate, a_{pi} , grows, and its 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.

Electric field strength. The mobility of high-molecular-mass DNA fragments increases when the electric field strength increases whereas the effective range of separation decreases. However, the voltage is limited because it heats the gel, which may cause DNA to melt.

Concentration of ethidium bromide. Ethidium bromide in the gel more strongly affects circular DNA than linear DNA. It intercalates into circular DNA, which has different charge, length, and superhelicity. Increasing ethidium bromide concentration can change DNA from a negatively supercoiled through a relaxed form to positively coiled superhelix [7].

3.1.1.2 Practice of zone agarose gel electrophoresis of native nucleic acids

Zone agarose gel 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.

Submarine electrophoresis of nucleic acids

The agarose gel electrophoresis is commonly carried out horizontally in a submarine mode whereby the slab gel is completely submerged in a buffer [8]. The submarine electrophoresis is a standard method for nucleic acid analyzing. Using it, DNA and RNA fragments can be separated and identified, and restriction fragments length polymorphism (RFLP) analysis [9] can be performed (see below).

The unit for submarine electrophoresis contains two buffer tanks, connected with each other by a flat bridge. The agarose gel, cast on a support film, is placed on the bridge [10] (Figure 3.1.1).

In practice, 0.8 g/dL agarose gels are used. Highly concentrated agarose gels (2–4 g/dL) are applied for analysis of PCR products. Higher ionic strength increases the resolution [11], but generates a lot of heat. This may destroy the helix structure of DNA.

The most common buffer for submarine zone electrophoresis is the **TRIS-borate-EDTA** buffer (TBE), which was introduced by Peacock and Dingman [12]. It has a pH value of 8.3 and TRIS concentration of 50–100 mmol/L. Michov [13] has established that the TRIS-borate buffers contain a complex compound formed by a condensation

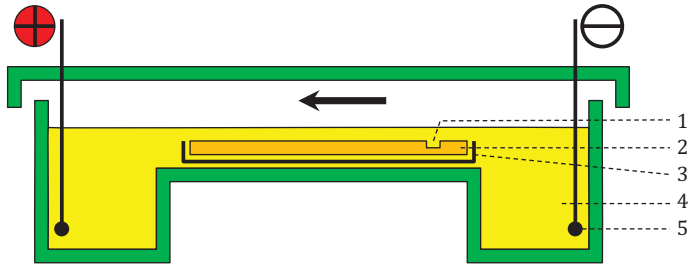


Figure 3.1.1: Unit for submarine electrophoresis.

1. Wells for samples; 2. agarose gel; 3. gel dish; 4. electrode buffer; 5. electrode wire.

reaction between boric acid and TRIS. The complex compound, which was referred to as *TRIS-boric acid*, has a zwitterionic structure. Its existence was reproved 30 years later [14].

According to Michov, another buffer, which can be used for nucleic acid separation, is the **TRIS-taurinate-EDTA** buffer (TTE) [15]. 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 ₂ EDTA · 2H ₂ O	0.37 g
Deionized water to	1,000.00 mL

Restriction fragment length polymorphism

RFLP is a laboratory technique, with which differences between samples of homologous DNA molecules can be illustrated. It begins with fragmenting a sample double-helical DNA by restriction enzymes (restrictases) in sequences of lengths of 4–6 base pairs, in a process known as restriction digest. The restriction fragments are separated by length using agarose gel electrophoresis, and are transferred onto a membrane (Southern blotting). Hybridization between them and labeled DNA probe determines their length. *Hybridization* is a process of recombination of complementary chains of different origin (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 *probe*. The probes are labeled either with an isotope (³²P, ³⁵S, or ³H) or with fluorescent ligands. A probe will hybridize with a matrix, if their 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.

Variable number tandem repeat

A **variable number tandem repeat** (VNTR) is the location in a genome where a short nucleotide sequence is organized as a tandem repeat. It exists on many chromosomes, and often shows variations in length (number of repeats) among individuals. VNTR is useful in genetics and biology research, forensics, and DNA fingerprinting.

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 3.1.2). Allele *a* has four VNTR repeats, and the probe detects a longer fragment between the two restriction sites. In allele *b*, there are only two VNTR repeats, 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.

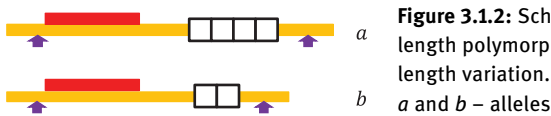


Figure 3.1.2: Schematic diagram of restriction fragment length polymorphism by variable number tandem repeat length variation.
a and *b* – alleles

VNTR analysis is used to study genetic diversity.

DNA profiling

DNA profiling (DNA fingerprinting, DNA testing, or DNA typing) is a process of determining individual's DNA characteristics, which are 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 [16–18]. 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 [19]. DNA profiling is also used to help clarify paternity [20] and in genealogical or medical research.

DNA profiling uses repetitive sequences that are highly variable (VNTR). VNTR are similar between closely related individuals, but unrelated individuals are extremely unlikely to have the same VNTR. The DNA testing is carried out after collecting buccal cells using a swab. The swab has wooden or plastic stick handle with

cotton on synthetic tip. The swab should rub 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 or razor) can be used. Next, DNA in the sample should be amplified, which is achieved by the polymerase chain reaction (see there).

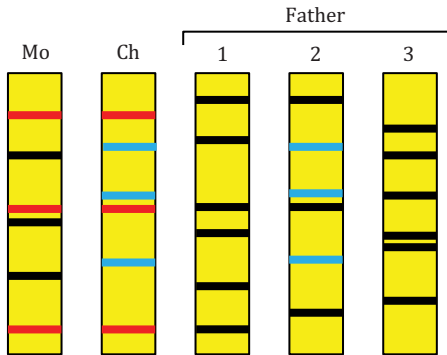


Figure 3.1.3: Example of DNA profiling in order to determine the father of a child. Child's DNA sample (Ch) should contain DNA bands of both parents: mother (Mo) and suspected father (Fa). In this case, man #2 is the father.

For paternity DNA profiling, samples obtained from mother, child, and possible fathers are analyzed. The child's DNA is a composite of its parent DNA. Electrophoretic bands in the child's DNA fingerprint that are not present in the mother's DNA should be contributed by the father (Figure 3.1.3).

DNA profiling may also be used to determine evolutionary relationships among organisms [21].

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 individual genome or nuclear DNA, because it is contained in the nucleus. Beside the nuclear DNA, the mitochondria in the cells also have their own genetic material termed mitochondrial DNA. The mitochondrial DNA comes only from the mother, without any shuffling. Comparison of the mitochondrial genome is easier than that based on the nuclear genome. However, testing the mitochondrial genome can prove only, if two individuals are related 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 passes directly from father to son.

Interpretation of DNA test results

A paternity or maternity test report lists the genetic profiles of each tested party. It also lists the **relationship index (RI)** for each marker – a statistical measure of how powerful a match is at a particular locus. Then RI are multiplied with each other to produce the **combined paternity index (CPI)**, which is the genetic odds that the tested man is more likely to be the biological father. It is generally accepted by government agencies that a CPI of 100 and a probability of 99% or higher is strong proof that the tested man is the biological father.

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 DNA from the second suspect and sperm DNA had the same profile (Figure 3.1.4).

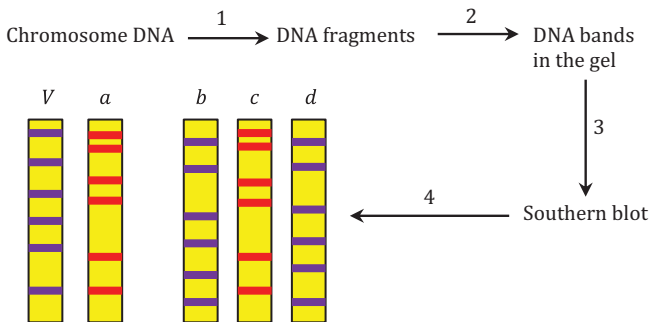


Figure 3.1.4: Comparative analysis of victim's DNA and DNA from three suspected men. The test proves that the murder is the suspected man *c*.

1 – DNA restriction with restriction enzymes; 2 – Gel-electrophoresis of DNA fragments;
3 – Southern blotting; 4 – Hybridization with radioactive sample, and autoradiography
V – Victim's DNA; *a* – Foreign DNA isolated from victim's body; *b*, *c*, *d* – DNA from suspicious men.

Many criminal cases were solved using the DNA profiling. For example, in 1994, Anna Anderson, who claimed to be Grand Duchess Anastasia Nikolaevna of Russia, was tested after her death using samples of her tissue that had been stored at Virginia hospital. The tissue was tested using DNA fingerprinting, which showed that she bore no relation to the Romanovs [22].

3.1.2 Zone agarose gel electrophoresis of denatured nucleic acids

The separation of DNA strands from each 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 higher temperature than double-stranded DNA that contains predominantly A–T base pairs (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.

Denaturing agarose gel electrophoresis is used for single-stranded DNA or RNA, which consist of 500 to 20,000 mononucleotides, e.g. for messenger **RNA** (mRNA). RNA samples are most commonly separated on agarose gels containing formaldehyde as a denaturing agent to save the RNA secondary structure [23]. Another method for separation of high-molecular-mass RNA such as ribosomal RNA and their precursors by agarose-formaldehyde gel electrophoresis was described [24]. It can be used for subsequent analysis of RNA by Northern blotting.

3.1.3 DNA sequencing

Two DNA sequencing methods are known: Maxam–Gilbert method and Sanger method. In addition, other methods as single-strand conformation polymorphism (SSCP) analysis, deoxyribonuclease (DNase) footprinting assay, and nuclease protection assay are used for detailed DNA analysis.

3.1.3.1 Maxam–Gilbert sequencing method

The Maxam–Gilbert sequencing method, called chemical mode, is based on nucleobase-specific partial chemical modification of DNA and subsequent cleavage of the DNA backbone at sites adjacent to the modified nucleotides [25].

A DNA nucleate should be labeled at its 3'- or 5'-end. In the rule, a λ -³²P-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 a terminal *transferase* and an α -³²P-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. Then the labeled fragments are separated by gel electrophoresis and eluted from the gel.

The chemical treatment generates breaks at a small proportion of one or two of the four nucleotide bases in each of the four reactions (G, A + G, C, and C + T).

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 3.1.5).

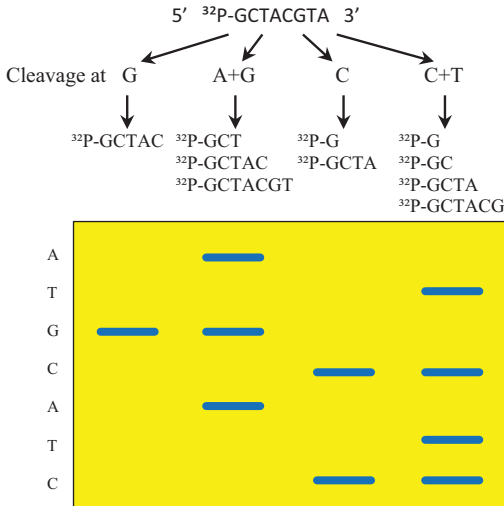


Figure 3.1.5: Maxam–Gilbert sequencing reaction.

Cleaving a tagged segment of DNA at different points yields tagged fragments of different size. The fragments are separated by gel electrophoresis.

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 radio-labeled 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.* [26].

3.1.3.2 Sanger sequencing method

Sanger sequencing (chain termination) method is based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during DNA replication [27, 28]. 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 **dideoxynucleoside triphosphates** (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 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 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 [29]. 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 [30]. Thus, the migrating bands of each separating track are registered in their order, i.e., the sequence of the separated DNA, in a computer (Figure 3.1.6).

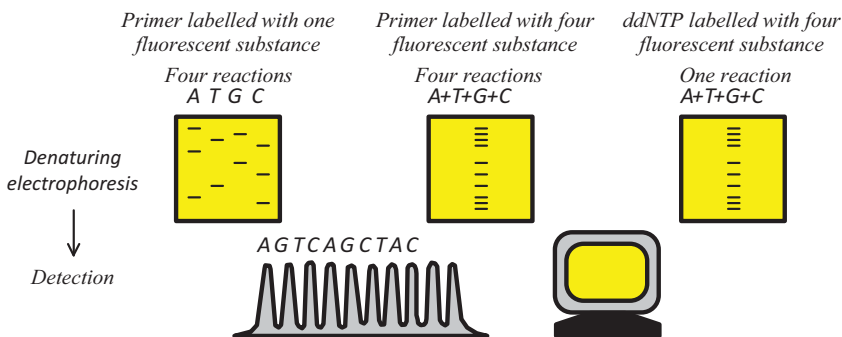


Figure 3.1.6: Principle of DNA sequencing according to Sanger.

3.1.3.3 Single-strand conformation polymorphism method

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 [31].

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 two single-stranded DNA strands with different sequences can cause them to 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 [32] (Figure 3.1.7).

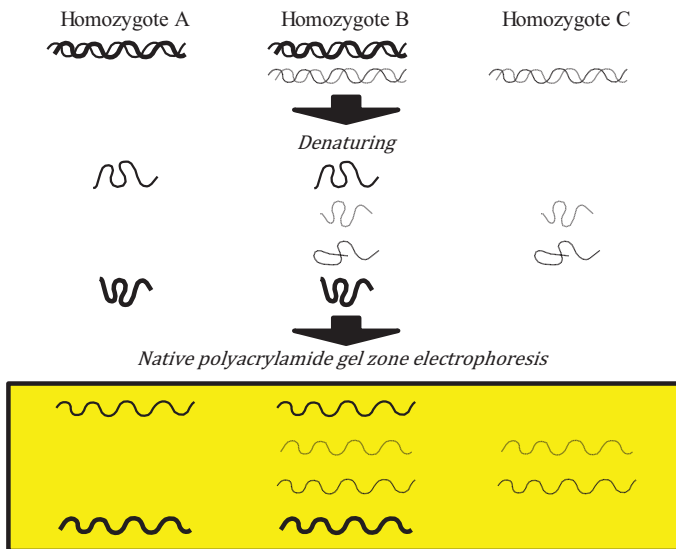


Figure 3.1.7: Scheme of SSCP analysis.

SSCP analysis is used in molecular biology, for example in genotyping to detect homozygous individuals of different allelic states, as well as heterozygous individuals [33]. Sequence variations such as small deletions or insertions and even single base substitutions can change the DNA conformation; therefore, they 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 [34].

Single-stranded DNA weakly binds ethidium bromide. Therefore, PCR is needed to amplify 100–500 bp genomic DNA or complementary **DNA** (cDNA) before SSCP analysis. This can be made with the help of reverse transcription on an mRNA.

3.1.3.4 DNase footprinting assay

DNase footprinting assay [35, 36] 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 DNase cuts a radioactively end-labeled DNA fragments. Then, the DNA fragments are resolved by gel electrophoresis. 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”. If DNA is marked at its ends, a marked and an unmarked single strand will be obtained (Figure 3.1.8). The unlabeled strand is not visible on the autoradiogram.

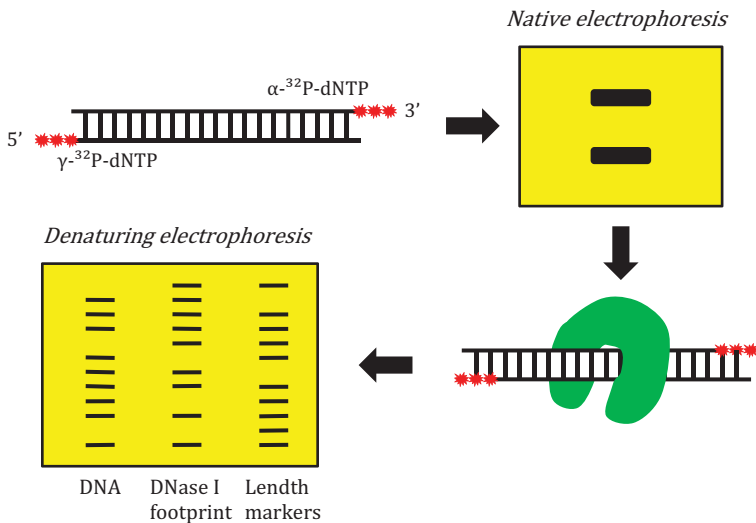


Figure 3.1.8: Principle of DNase footprinting assay.

Using DNase footprinting assay, the transcriptional control of MUC1 gene was investigated [37]. It encodes DF3 glycoprotein that is highly expressed in human breast cancer cells.

For analysis of RNA–protein interactions, similar “toeprinting” assay is used [38–40]. In this method, the primer extension reaction is used to examine the binding of protein to RNA, from its 3'- (toe) to the other 5'- (heel) side.

3.1.3.5 Nuclease protection assay

The nuclease protection assay is a technique used to identify RNA molecules of known sequence in a heterogeneous RNA sample [41, 42]. RNA is first mixed with DNA or RNA probes that are complementary to the sequence of interest. They form hybridized double-stranded DNA-RNA or RNA-RNA hybrids. Then, the mixture is exposed to ribonucleases that cleave the rest of probe and single-stranded RNA to individual mononucleotides but have no activity against the hybrids. They are analyzed by electrophoresis in a denaturing polyacrylamide gel, and are detected autoradiographically (Figure 3.1.9).

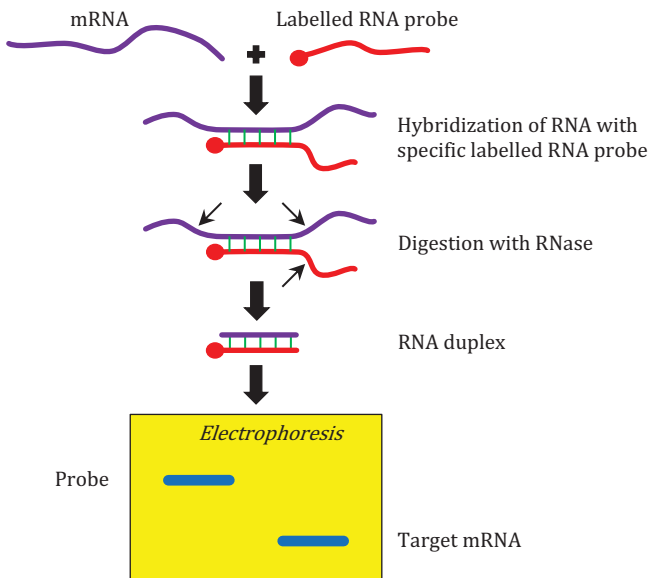


Figure 3.1.9: Principle of the nuclease protection assay: mRNA and probes, which are not complementary to each other, are degraded by nucleases. Only hybrids between mRNA and their probes remain to be separated by denaturing electrophoresis.

If the probe is labeled radioactively, the gel should be dried after electrophoresis to be prepared for autoradiography.

S₁-nuclease protection assay

In order to find the 5'-end of an mRNA, S₁-nuclease protection assay is used. During the process, labeled DNA probes hybridize with mRNA sample. Then, the single-stranded DNA probe is destroyed by the S₁-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 3.1.10).

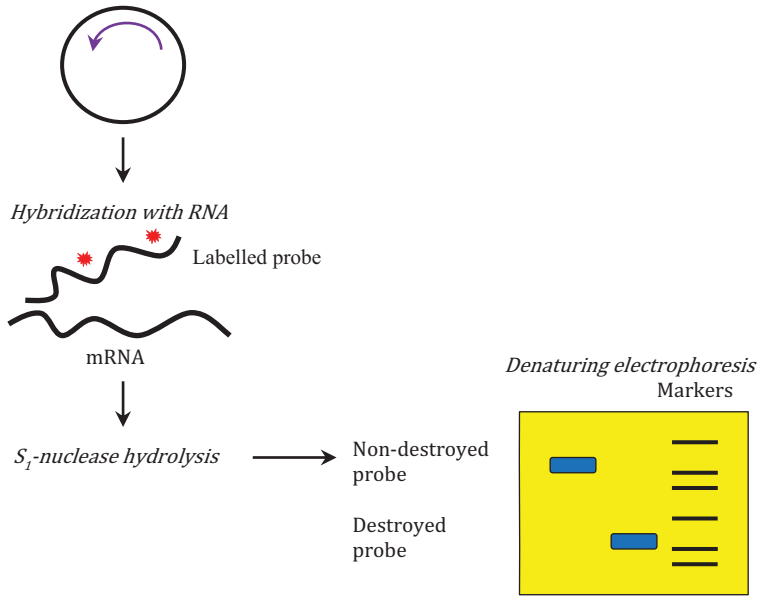


Figure 3.1.10: Principle of S₁-nuclease protection assay.

Using the S₁-nuclease protection assay, the promoter region of mouse gene δ /YY1 has been described [43], which is responsible for a transcription factor.

3.1.4 RNA separation

The separation of fragmented RNA finds place on polyacrylamide gels containing urea [44]. An RNA ladder is often run alongside the samples to show the size of fragments obtained. The ribosomal subunits can also act as size markers. 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 [45].

A method for resolving RNA fragments [46] is based on the migration of RNA fragments on a polycationic polyacrylamide gel, produced by incorporating positively charged immobilines into a neutral polyacrylamide backbone. The separation is carried out in a 0–10 mmol/L immobiline (p*K* = 10.3) gradient under denaturing conditions (6 mol/L urea). Using it, good separations of single-stranded RNA of 21–23 mononucleotides in length, which appear to regulate the gene expression, are obtained.

3.1.4.1 Primer extension assay

In the **primer extension assay** (PEA), the transcription start site for a gene is determined experimentally by identifying the 5'-end of the encoded (mRNA). The process begins with a primer, usually a synthetic oligonucleotide of about 20 residues, which is complementary to an mRNA sequence of 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 a RNA sample.

Reverse transcriptase (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 cDNA products are analyzed by denaturing polyacrylamide gel electrophoresis, followed by autoradiography. 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 PEA 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.

3.1.5 Protocols

Native DNA Electrophoresis on Agarose Gels

Materials and equipment

TRIS-borate-EDTA (TBE) buffer, pH = 8.0

DNA samples and markers

10 $\mu\text{g}/\text{mL}$ ethidium bromide

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.
- Place an application template onto the gel in front of 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 electrophoresis at a constant current and room temperature according to the following electrophoresis program until the tracking dye reaches the anode.

Gel size (mm)	Current (mA)	Voltage (V)
51 × 82	10	80
260 × 125	78	120

- 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.

Sanger Sequencing Reactions Using *Taq* DNA Polymerase

Materials and equipment

10x *Taq* polymerase buffer (500 mmol/L TRIS-HCl, pH = 9.0)

Taq Sanger mixtures A, C, G, and T

Taq DNA polymerase

dNTP chase

50–75 °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.
- Prior to use, dilute *Taq* DNA polymerase to 2.5 U/µL in the *Taq* polymerase buffer, and keep on ice.
- Add
2 µL 10 mCi/mL α-³⁵S-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 mixture.

- 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.

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3.2 Pulsed-field gel electrophoresis of nucleic acids

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Agarose gel electrophoresis is the method of choice regarding DNA fragments in size 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) [1]. Now this method is known as **pulsed-field gel electrophoresis (PFGE)**. The PFGE allows separation of linear double-stranded DNA nucleates of 20 kbp up to 10 Mbp, for example, of whole chromosomes [2]. This is important for the mapping of human genome, which comprises about 3,000 Mbp.

3.2.1 Theory of pulsed-field gel electrophoresis of nucleic acids

PFGE 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° on both sides. So the long DNA nucleates migrate at first as reptiles, and then unfold their molecules and enlarge their volume (Figure 3.2.1). Whereas smaller nucleates have enough time to fold and unfold, and as a result migrate quickly through the gel, greater nucleates need more time to fold and unfold and as a result move slowly.

The pulse times are equal for each direction. They can be increased for extremely large DNA nucleates (up to 2 Mbp). PFGE takes between 10 h and several days depending on the size of DNA fragments. For folding and unfolding, larger nucleates need longer time compared to smaller nucleates [3].

<https://doi.org/10.1515/9783110761641-029>

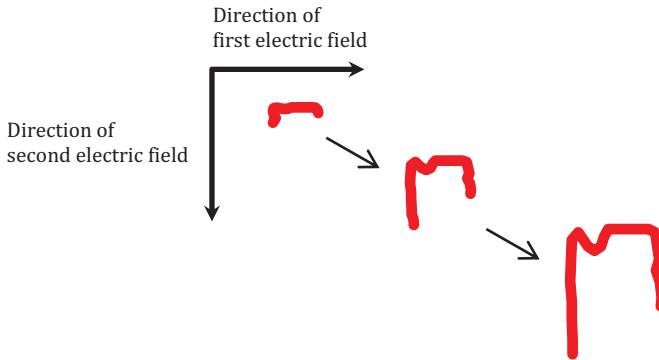


Figure 3.2.1: Enlargement of a nucleic acid molecule.

3.2.2 Types of pulsed-field gel electrophoresis

PFGE 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 PFGE are known: gel electrophoresis in contour-clamped homogeneous electric fields (CHEF), field inversion gel electrophoresis (FIGE), transverse alternating field gel electrophoresis (TAFGE), and rotating gel electrophoresis (RGE) in a constant electric field [4] (Figure 3.2.2).

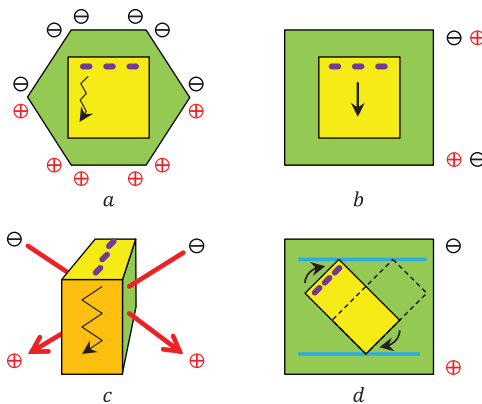


Figure 3.2.2: Types of pulsed-field gel electrophoresis.

(a) Gel electrophoresis in a contour-clamped homogeneous electric fields, 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.

CHEF gel electrophoresis [5, 6] is a type of PFGE that enables the resolution of DNA fragments with lengths from 10 kb up to 9 Mb.

In FIGE, the electric field is reversed in certain pulse frequencies [7, 8]. Besides 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 PFGE 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.

In TAFGE [9, 10], two electrodes are located on each side of a vertical agarose gel. The gel is located in a large Plexiglas tank, where the electrode wires stretch.

RGE is a technique for separating large DNA fragments [11–13]. 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.

3.2.3 Mapping the human genome

Due to PFGE, genome mapping is carried out [14–16]. 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. PFGE is also used for karyotyping and analysis of chromosomes.

3.2.3.1 STR analysis

The people have different numbers of short DNA sequences. The **short tandem repeats** (STR) 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 resulting DNA fragments are separated by electrophoresis.

3.2.3.2 AmpFLP

Amplified fragment length polymorphism (AmpFLP) [17] relies on **variable number tandem repeat** (VNTR) polymorphisms to distinguish various alleles, which are separated on a gel in the presence of an allelic ladder. The bands obtained can be visualized by silver staining. AmpFLP allows for easy creation of phylogenetic trees based on comparing individual samples of DNA. It can be automated.

3.2.3.3 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 STR. They are short DNA pieces that characterize the individuals.

3.2.3.4 Y-chromosome analysis

The Y chromosome is found only in males. For its analysis in forensics, paternity, and genealogical DNA testing, STR on the Y-chromosome (Y-STR) analysis is used. 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 [18].

3.2.3.5 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 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 (see above).

3.2.4 Protocols

Pulsed-field Gel Electrophoresis of Chromosomal DNA

Materials and equipment

Cells

TAE or TBE buffer

Low melting agarose

Molecular mass markers: chromosomes (1.05–3.13 Mbp), *Schizosaccharomyces* (3.5–5.7 Mbp), and yeast chromosome PFG (225–1,900 kbp)

Procedure

- Centrifuge cells at 4,000 g for 5 min.
- Resuspend the pellet to concentration of 8.6×10^7 cells/mL.
- Add 3.1 mL of 37% formaldehyde into 40 mL centrifuge tubes and place them on ice.
- Centrifuge at 4,000 g for 5 min.
- Wash samples by resuspending them in 10 mL of buffer with 50 mmol EDTA and following centrifugation at 4,000 g for 5 min. Repeat the wash step 3 times.
- Resuspend infected cells in 0.5 mL of buffer.
- Add to the cells 0.5 mL of 2% low melting agarose kept at 45 °C, mix well, and pour the mixture into plug molds.
- Place plug molds in refrigerator for 15 min to solidify.
- Remove agarose blocks from the mold and place them into buffer with 1 mg/mL proteinase K.
- Incubate the agarose blocks for 24 h at 50 °C.
- Wash the agarose blocks for 30 min 4 times. Cut the blocks in small pieces to fit gel wells.
- Prepare 1% agarose gel in TAE buffer using a casting stand.
- Load the agarose blocks into the gel wells and seal them with melted 1% low melting agarose in running buffer.
- Separate the chromosomal DNA in an electrophoresis unit with TAE running buffer.
- Run electrophoresis at 3 V/cm with pulse time ramping from 250 to 900 s for 60 h. Change the buffer every 24 h.
- Stain the nucleates with 0.5 mg/L ethidium bromide for 20 min.

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3.3 Capillary electrophoresis of nucleic acids

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The capillary electrophoresis (CE) is effective for DNA sequencing, fragment analysis, and DNA typing [1]. In CE methods, nucleates are separated according to their electrophoretic mobility and non-covalent interactions with a phase in capillaries. CE has many advantages over slab gel electrophoresis in terms of speed, resolution, sensitivity, and data handling.

3.3.1 Theory of capillary electrophoresis of nucleic acids

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 the DNA chain length, which results in decrease of the DNA electrophoretic mobilities (Figure 3.3.1).

A strong denaturant to cleave intramolecular hydrogen bonds in RNA is required for RNA size separation. Sumitomo *et al.* [2] have found that carboxylic acids (for example acetic acid) were strong denaturants for RNA and that RNA separation performance was dramatically improved by CE 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 that of either 2.5 mol/L formaldehyde or 7.0 mol/L urea by estimating DNA melting temperature.

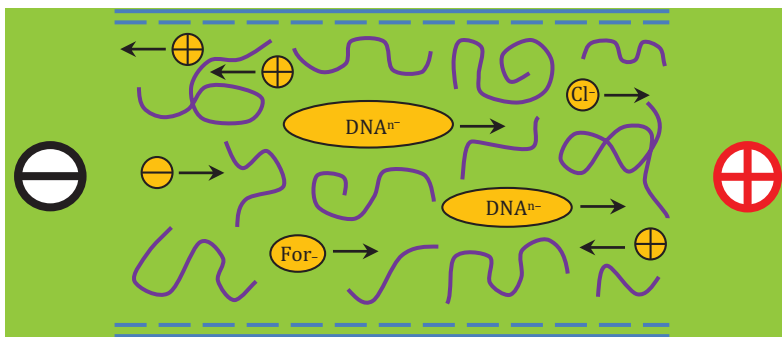


Figure 3.3.1: DNA migrates through entangled strands of a polymer twisting and tumbling through the gel. Small ions such as chloride (Cl^-) and formate (For^-) have higher electrophoretic mobility.

3.3.2 Instrumentation for capillary electrophoresis

The CE instrument contains capillaries, coating polymers, and gels. It also requires a suitable sample injection module, a detector, adequate temperature control, and protection from high voltages is used.

3.3.2.1 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 its 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.

3.3.2.2 Coating polymers

A capillary should be coated to eliminate charge effects that are contributed by the native silica surface. This can be achieved by cellulose-derived polymers or modified acrylamide polymers, which interact strongly with the inner surface of the bare fused-silica capillary. The synthesis and characterization of a hydrophilic, replaceable, self-coating polymer matrix, poly-*N*-hydroxyethylacrylamide (PHEA), for application in DNA CE was reported [3].

3.3.2.3 Gels

An ideal separation medium for DNA separations using CE should possess the following properties: high sieving ability, dynamic coating ability, and relatively low viscosity. A separation medium with these specifications can be used for automation of CE and for further enhancing of its performance [4]. For this purpose, homopolymers, such as linear polyacrylamide (LPA) and its derivatives [5–7], polyvinylpyrrolidone (PVP) [8], polyethylene oxide (PEO) [9], and cellulose and its derivatives [10–12] are used.

LPA is hydrophilic. It forms strongly entangled networks that are excellent for high-quality DNA separations of long sequencing read lengths (up to 1,300 bases) [13]. Doherty *et al.* [14, 15] showed that by adding very small amounts of cross-linking monomers into the polymer solution to form LPA “nanogels,” sequencing performance could be improved compared with LPA synthesized in the absence of any cross-linker. 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.

In addition to linear polyacrylamide, the acrylamide derivative poly(*N,N*-dimethylacrylamide) (pDMA) has also been demonstrated as a high-quality polymer for sequencing DNA [16–18]. 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 on the capillary surface prior to 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 [19].

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 and are not removed from the capillary between runs. These gels are preferred when larger DNA molecules should be resolved – e.g., 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. Flowable polymers can be used for oligonucleotides and double-stranded DNA (dsDNA) fragment analysis.

3.3.3 Running capillary electrophoresis

The CE can be run when a fused-silica capillary, filled with gel or liquid, joins two buffer tanks and is placed at a high voltage (10–30 kV). The resulting electric field drives the nucleic acids along the capillary. Generally, the capillaries are 20–50 cm long and have 50–100 μm internal diameter.

The CE of nucleic acids is carried out in three steps: injection, separation, and detection of the separated nucleic acids.

3.3.3.1 Injection

DNA samples are injected electrokinetically or by pressure into the sample tube. Then, they are introduced into the capillary using a voltage of 5–15 kV for a few seconds. The samples should be diluted with formamide or water [20] to reduce the ionic strength.

3.3.3.2 Separation

The separation of DNA is carried out in a small-diameter (50–100 μm) quartz capillary under a high voltage in a few minutes up to an hour [21–23]. The separation is performed in TBE buffer with alkaline pH value. Urea is often included as a denaturant when analyzing single-stranded DNA (ssDNA). ssDNA interacts efficiently with the sieving polymer, and its size is proportional to its length. Denaturants prevent also the complementary sequences within ssDNA molecules to bind to each other. This could create loops and hairpins. The temperature should be controlled, as it strongly influences DNA mobility and the calculated allele size [24]. The separation times range from 10 to 45 min at 1–10 kV. In the presence of appropriate standards with known electrophoretic mobility, the physical properties of DNA can be examined [25].

3.3.3.3 Detection

CE 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 is captured by a photoarray detector. Each dye has a characteristic emission spectrum.

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 [26] or rhodamine derivatives should be covalently attached to the DNA prior to electrophoresis or added to the electrophoresis buffer.

3.3.4 Pulsed-field capillary electrophoresis

Another type of CE is **pulsed-field capillary electrophoresis** (PFCE) [27]. It can be used not only for resolution of DNA chains containing above 1 kbp [28, 29], but also for resolution of DNA chains into the Mbp range. PFCE can be carried out in a gel or in an ultradilute polymer solution. 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 the electric field strength. At constant field, a DNA fragment collides with **hydroxyethyl cellulose** (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. The 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.

3.3.5 Applications of capillary electrophoresis

Schwartz *et al.* [30] 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.* [31] showed that CE is an effective method for genomic analysis. They indicated that it is suitable for prenatal diagnosis of X-linked recessive disorders.

Optimized CE and fluorescence-labeled PCR appear to be the methods of choice in **Huntington disease** (HD) and **sickle cell disease** (SCD) diagnostics, often combined with Southern blotting.

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 deficiency turns into dementia [32]. In 1993, a research group, using genetic linkage analysis [33], isolated the causal gene at 4p16.3 [34].

SCD, is a group of genetically blood disorders. It is characterized by a hemoglobin abnormality. This leads to a sickle-like erythrocytes under certain circumstances

[35]. Temperature changes, dehydration, high altitude and more may develop pain attacks (sickle cell crises), anemia, bacterial infections, and stroke [36]. SCD 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 [37].

The analysis of dsDNA fragments, such as those found in polymerase chain reaction products and DNA restriction digests [38, 39], has led to the development of a complete map of the human genome.

3.3.6 Protocols

Quantitative PCR Analysis

The quantitative PCR analysis can be used in conjunction with CE separation to amplify and quantitate DNA target sequence by conjugating an intercalating dye with the samples or by using a covalently modified and fluorescently labeled oligonucleotide primer.

Materials and equipment

TBE buffer

Resolving gel

65 cm 100 μm i.d. coated capillary

Standard 174 DNA ladder (in a concentration of 10 $\mu\text{g}/\text{mL}$ and stored at $-20\text{ }^\circ\text{C}$)

CE instrument with fluorescent detection

Procedure

- Prepare gel buffer mixture and add intercalating dye. Store at $4\text{ }^\circ\text{C}$ 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.
- Load standard DNA ladder into the capillary at low pressure for 10 s.
- Electrophorese at 9.4 kV and $25\text{ }^\circ\text{C}$ for 30 min.
Replace inlet gel reservoir after 30 injections.

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3.4 Polyacrylamide gel electrophoresis of nucleic acids

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If small double-stranded DNA fragments are to be resolved on agarose gels, 2 g/dL agarose gels should be used. However, these gels are opalescent and, as a result, the sensitivity of the ethidium bromide proving is reduced. In addition, the zone electrophoresis on agarose gels runs for several hours. Therefore, low-molecular-mass DNA fragments are separated in native polyacrylamide gels.

The native polyacrylamide gel electrophoresis is used for the separation of double-stranded DNA with a 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, additional electric charges cause denaturation of nucleic acids and change their mobility.

3.4.1 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 continuous strength of the electric field during electrophoresis. With the help of the native zone electrophoresis on polyacrylamide gel, DNA nucleates, oligonucleotides, RNA nucleates, and viroids are resolved

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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).

3.4.1.1 Practice of zone polyacrylamide gel electrophoresis of native nucleic acids

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 is rolled by a roller. Then, it is framed with a U-shaped spacer and covered with another glass plate. The resulting cassette is clamped together and placed upright. A buffer, for example TRIS-borate-EDTA buffer, is mixed with a monomeric solution, TMEDA, and APS. The mixture is cast into the cassette and covered with deionized water to prevent air-oxygen diffusion into the mixture.

If the monomeric concentration T is 50 g/dL, the polyacrylamide gels for zone electrophoresis can be prepared as follows (Table 3.4.1):

Table 3.4.1: Producing polyacrylamide gels.

Reagents	Total monomeric 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
Monomeric 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

After 1 h of polymerization at room temperature, the polyacrylamide gel should be removed from the cassette: the cassette is opened and the cover glass plate is raised up using a spatula. Thereafter, the spacer is removed and the gel is covered with a hydrophobic polyester film, or packed in a wrap.

Prior to electrophoresis, a thermostat is set to 10 °C and the cooling plate of a separation unit is covered with 0.5–1 mL of kerosene or silicone oil DC-200 as a contact fluid. The gel, with its support film down, is placed onto the cooling plate so that the bubbles between the plate and support film are removed. Then, the cover film is removed from the gel and paper electrodes strips, impregnated with electrode buffer, are placed onto the cathode and anode ends of the gel. Before the cathode strip, at a distance of 1 cm, a silicone template is placed and lightly pressed

against the gel. Afterward, 0.1 mg/mL DNA containing 0.025 g/dL of Na salt of Xylene cyanol or Bromophenol blue is applied in the template slots.

Electrophoresis conditions. The electrophoresis is carried out 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 the opposite end of the gel.

On principle, native double-stranded DNA nucleates move faster than denatured (single-stranded) DNA with the same masses. Moreover, smaller DNA fragments move together with Bromophenol blue, while larger DNA fragments follow Xylene cyanol [3]. This can be explained by the structures of Xylene cyanol and Bromophenol blue (Figure 3.4.1): The molecular mass of Xylene cyanol Na salt ($M_r = 538.61$) represents approximately 3/4 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.

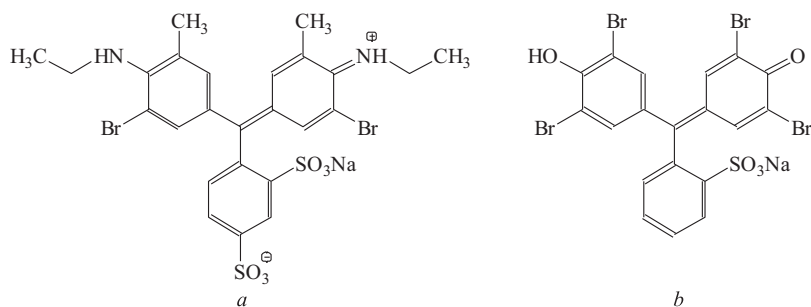


Figure 3.4.1: Chemical structures of the sodium salts of (a) Xylene cyanol and (b) Bromophenol blue.

The relationship between the polyacrylamide concentration and the masses of nucleic acids to be resolved can be seen in Table 3.4.2. It shows that there are differences between the behavior of native and denatured DNA nucleates during electrophoresis.

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 or radioactive labeling.

With the help of native zone electrophoresis on polyacrylamide gel, DNA nucleates, oligonucleotides, RNA nucleates, and viroids are resolved in accordance with 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).

Table 3.4.2: 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			

3.4.2 Disc-electrophoresis of native nucleic acids

The disc-electrophoresis is used for separation of native 20 to 60 bp double-stranded DNA [4–6], and RNA [7]. It gives sharper bands than the zone (continuous) electrophoresis [8] in TRIS-borate-EDTA buffer [9, 10].

3.4.2.1 Theory of disc-electrophoresis of native nucleic acids

The negative charges of phosphate backbones cause DNA nucleates to move toward the positively charged anode. The migration in a solution, in the absence of gel, is independent of molecular masses, i.e., no separation by size happens [11, 12]. During a native disc-electrophoresis in a gel, however, the nucleic acids are concentrated in an ionic boundary between the leading and trailing ion, and in the resolving gel, they are overtaken by the ionic boundary. Thereafter, the electrophoresis runs as a zone electrophoresis. If the concentration of the leading ion is enough high, nucleates run slower but the resolution of electrophoresis is higher [13].

3.4.2.2 Running disc-electrophoresis of native nucleic acids

Prior to electrophoresis, the nucleic acid to be separated can be prepared in several ways: In the case of large DNA molecules, DNA is cut by DNA restriction endonuclease into smaller fragments. In PCR-amplified samples, the enzymes which are present in the sample are to be removed prior to analysis. Once the nucleic acid is

properly prepared, the samples are placed into wells of the gel or into slots and a voltage is applied through the gel.

The DNA fragments of different lengths are visualized using ethidium bromide, a specific DNA fluorescent dye. Under UV light, these gels show bands corresponding to nucleic acid bands.

After electrophoresis, DNA nucleates can be eluted. For this purpose, their bands can be cut out from the gel with a scalpel or razor blade and transferred into a centrifuge tube containing 200 μL of sterile deionized water. After several hours or overnight extraction at 80 $^{\circ}\text{C}$, 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 in 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.

3.4.2.3 Disc-electrophoresis of double-stranded PCR products

Nucleates of greater length cannot be separated by native polyacrylamide gel electrophoresis. Therefore, they should be broken by endonucleases, and the nucleate fragments should be multiply in the **polymerase chain reaction (PCR)** [14]. The products of the PCR can be later separated in horizontal polyacrylamide gels using, for example, disc-electrophoresis in a TRIS-formate-borate buffer system [15], or TRIS-formate-taurinate buffer system [16].

The TRIS-formate-taurinate buffer system consists of a TRIS-formate buffer as leading buffer (the anode buffer), and a TRIS-taurinate buffer as trailing buffer (the cathode buffer). The TRIS-formate buffer is in the polyacrylamide gel, and the TRIS-taurinate buffer is 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 3.4.2).

The recipes for the TRIS-formate-taurinate buffer system and the preparation of polyacrylamide gels for disc-electrophoresis are presented in Tables 3.4.3 and 3.4.4, and some results are shown on Figure 3.4.3.

A simple gel electrophoresis apparatus for size-selective separations of DNA fragments in both polyacrylamide and agarose gels was described [17]. It employs a microslab gel format with a novel gel casting technique that eliminates the need for combs to define sample loading wells. 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 **light-emitting diode (LED)** and using an appropriate emission filter.

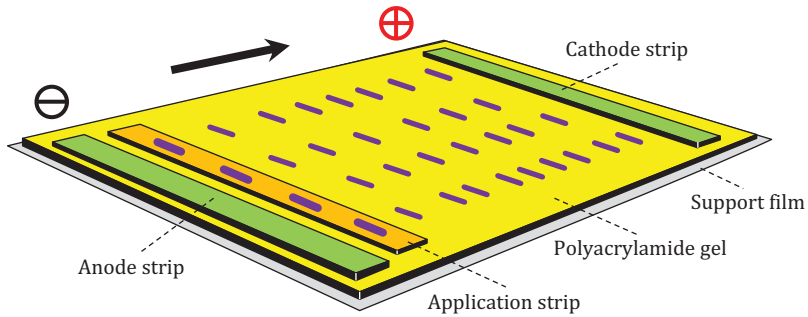


Figure 3.4.2: Disc-electrophoresis of PCR products.

Table 3.4.3: Recipes for the TRIS-formate-taurinate buffer system for disc-electrophoresis of nucleic acids.

	Anode buffer, pH = 7.4	Cathode buffer pH = 8.3	Monomeric solution $T = 50 \text{ g/dL}$, $C = 0.03$
TRIS, g	16.32	2.23	–
99 mL/dL Formic acid, mL	4.57	–	–
Taurine, g	–	6.02	–
Acrylamide, g	–	–	48.50
BIS, g	–	–	1.50
87% Glycerol, mL	10.00	10.00	–
$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, g	0.45	0.22	–
NaN_3 , g	0.06	0.03	–
Deionized water to, mL	100.00	100.00	100.00

Table 3.4.4: Recipes for polyacrylamide gels containing TRIS-formate-taurinate buffer system for disc-electrophoresis of nucleic acids.

	$T = 5 \text{ g/dL}$ pH = 7.4	$T = 7 \text{ g/dL}$ pH = 7.4	$T = 9 \text{ g/dL}$ pH = 7.4	$T = 11 \text{ g/dL}$ pH = 7.4	$T = 13 \text{ g/dL}$ pH = 7.4
Anode buffer, mL	16.67	16.67	16.67	16.67	16.67
Monomeric 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

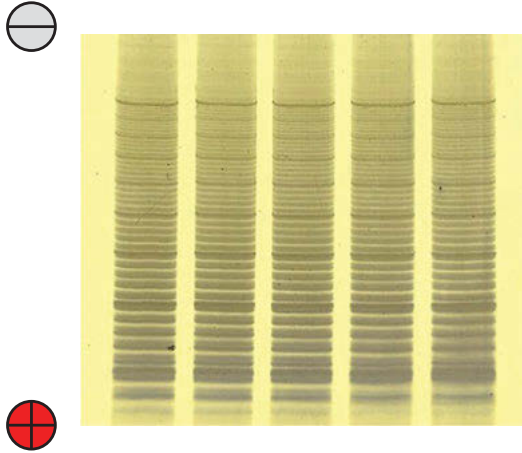


Figure 3.4.3: Disc-electrophoresis of 20 to 100 bp DNA fragments in a 7% polyacrylamide gel. Silver staining.

After electrophoresis, the PCR products can be detected by various methods. The most common among them is the silver staining [18], which is more sensitive than the ethidium bromide method [19]. The PCR products can also be labeled with fluorescent substances [20, 21], and the labeled PCR products can be visualized by the chemoluminescence digoxigenin system [22].

3.4.3 Electrophoretic mobility shift assay

The **electrophoretic mobility shift assay** (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 [23], and Fried and Crothers [24, 25]. It is used to deduce the affinities of a protein for one or more DNA or RNA sites or to compare the affinities of different proteins for same nucleate site.

The proteins should be in solutions with physiological ionic strength and physiological pH value to recognize specific sequences of DNA/RNA. On the other hand, DNA should be native and double-stranded to interact with them. Therefore, EMSA is carried out in buffers.

If a double-stranded DNA fragment contains a detection sequence for some protein, the protein is bound to this DNA fragment during electrophoresis. The resulting DNA–protein complexes migrate slower through the gel and, therefore, displace from the DNA band – *band shift* (Figure 3.4.4). When the DNA fragment binds more than one protein poliyon, the mobility of the complex reduces further.

To visualize EMSA, the nucleic acid fragments in the gel are usually labeled radioactively or fluorescently, or are bound to biotin. While the isotopically ^{32}P -labeled

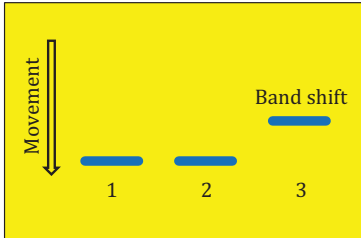


Figure 3.4.4: Scheme of the electrophoretic mobility shift assay.

Lane 1 contains only genetic material. *Lane 2* contains protein and DNA fragments, which do not interact each with other. *Lane 3* contains protein and DNA fragments that have reacted with each other to build a slower moving complex.

DNA has less or no effect on the protein binding affinity, the non-isotopic labels including fluorophores or biotin can alter the affinity and the stoichiometry of the protein–DNA interaction. The biotin label uses streptavidin, conjugated to an enzyme, such as horseradish peroxidase.

If the starting concentrations of a 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 [26]. 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.

3.4.4 Clinical applications

EMSA seems 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 [27]. It was applied also to study the MUC1 promoter in breast cancer cells [28]. The MUC1 gene encodes the DF3 protein, which represents a high-molecular-mass glycoprotein. Membrane proteins can also be identified by the EMSA using charged detergents.

3.4.5 Protocols

DNA Disc-electrophoresis in TRIS-taurinate Buffer at Two pH Values According to Michov

Materials and equipment

TRIS
Taurine
Acrylamide
BIS

87% Glycerin

Bromophenol blue (BPB) Na salt

TMEDA

Ammonium peroxydisulfate (APS)

Casting cassette

Buffer 1 (Anode buffer), pH = 8.6, I = 2×0.20 mol/L

TRIS 14.52 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 6.93 g (0.06 mol/L)

Taurine 13.01 g (0.10 mol/L)

Deionized water to 1,000.00 mL

Monomeric solution, T = 50 g/dL, C = 0.03

Acrylamide 48.50 g

BIS 1.50 g

Deionized water to 100.00 mL

The gel solutions should be prepared according to Table 3.4.5.

Table 3.4.5: 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, pH = 7.6, I = 0.02 mol/L	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 = 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	–	–	–	–	–
Monomeric 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
Deionized water, mL, to	100.00	100.00	100.00	100.00	100.00	100.00

Procedure

- Place some drops of water onto a glass plate.
- Lay a support film down on the glass plate and roll over so that any air bubbles are removed.
- Place on the film 0.5 mm U-form silicone gasket, put over a second hydrophobized glass plate, and fix the cassette by clamps.
- Pour stacking solution into the vertical casting cassette until one-third of it is filled.
- Overlay the stacking solution with some deionized water.
- After the stacking solution has gelled, pour out the deionized water and overlay the stacking gel with resolving solution.
- Overlay the resolving solution with some deionized water.
- Allow the resolving solution to gel.
- Run the electrophoresis at 20 mA for 120 min.
Use paper electrode strips with undiluted anode buffer and cathode buffer, respectively.

Separation of PCR Products by Polyacrylamide Gel Disc-Electrophoresis According to Michov**Materials and equipment**

TRIS-formate buffer, pH = 8.3

TRIS-taurinate buffer, pH = 7.4

Monomeric solution ($T = 50$ g/dL, $C = 0.03$)

10 g/dL TMEDA

10 g/dL APS

DNA samples

DNA ladder

Gel electrophoresis cell

Glass plates, spacers and combs

DC power supply

Ethidium bromide or SYBR Green EMSA nucleic acid gel stain

Procedure

- Cast a gel on support film as above.
- Put 0.5–1.0 mL of contact fluid, for example, kerosene or silicone oil DC 200, onto the cooling plate of the electrophoretic cell.
- Place the film-supported gel, together with its cover film, onto the cooling plate.
- 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 on an application template (preferably of silicone) in front of the cathode strip.
- Apply 5–8 μL of nucleic acid samples with a concentration of 10 mg/mL into the slots of the application template.
- Place the electrodes on the corresponding paper electrode strips.
- Run disc-electrophoresis at constant electric current and a temperature of 10 °C until the dye front reaches the opposite strip.
- Stain the DNA bands with ethidium bromide or SYBR Green EMSA nucleic acid gel stain.

Electrophoretic Mobility Shift Assay

Materials and equipment

TRIS-borate-EDTA buffer (TBE)

10,000x Concentrate in dimethylsulfoxide

Trichloroacetic 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)

Na₂EDTA 0.04 g (1 mmol/L)

Deionized water to 100.0 mL

Procedure

- Peel the tape from the bottom of casting cassette.
- Pull the comb from the gel.
- Fill the upper buffer chamber with TBE running buffer. The buffer level should exceed the level of the wells.
- Load DNA samples and DNA ladder.
- Fill the lower buffer chamber 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 μ L of 10,000x SYBR green EMSA gel stain concentrate into 50 mL TBE buffer and pour into gel staining tray.
- Transfer the gel into the staining tray.
- Incubate for 20 min on orbital shaker, protected from light.
- 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 with 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 with deionized water for 10 s.

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.

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3.5 Microchip electrophoresis of nucleic acids

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(Micro)chip electrophoresis (ME, microfluidic electrophoresis, lab-on-a-chip) is one of the most powerful analytical tools in recent years [1]. Since its introduction in the early 1990s by Manz *et al.* [2] and Harrison *et al.* [3], it has become an important method for analyzing DNA fragments because of speed, miniaturization, small reagent volumes, high resolution, and automation [4–6]. Using this method, DNA can be sized, sequenced, and genotyped [7].

3.5.1 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 [8, 9]:

$$v = \mu E \quad (3.5.1)$$

Since E is equal to the ratio between the voltage U and the length l between the electrodes, eq. (3.5.1) can be transformed to give

$$v = \mu \frac{U}{l} \quad (3.5.2)$$

As the length between the electrodes is a constant, the last equation shows that only the voltage controls the velocity of nucleates.

3.5.2 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. It begins with a buffer inlet reservoir and ends with buffer outlet reservoir (buffer waste). The injection channel can be 8.0 mm long. It

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 3.5.1) [10].

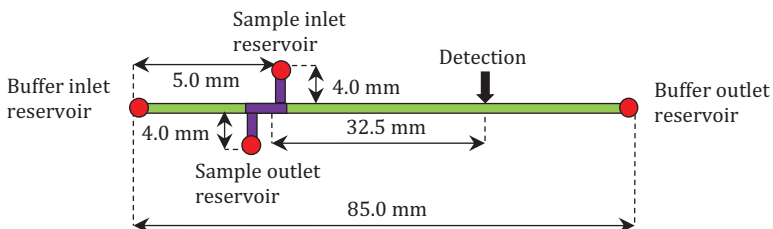


Figure 3.5.1: Schematic diagram of a microfluidic chip.

All microchips are fabricated by hot embossing method with a master fabricated on soda-lime glass using photolithography techniques. The reservoirs are created by drilling. The sequencing chip has a four-layer construction, consisting of three 100 mm diameter glass wafers and a **polydimethylsiloxane (PDMS)** membrane. Prior to experiments, the microchip should be rinsed with 1 mol/L NaOH and then with deionized water.

3.5.2.1 Polymers

The polymer solutions used as DNA sieving matrices define the quality of the separation [11, 12]. Among them, the *N*-alkoxyalkylacrylamide polymers – **poly(*N*-methoxyethylacrylamide)** (pNMEA) and **poly(*N*-ethoxyethylacrylamide)** (pNEEA) – were investigated [13]. The polymers pNMEA and pNEEA were synthesized *via* free-radical polymerization from the monomers ***N*-methoxyethylacrylamide** (NMEA) and ***N*-ethoxyethylacrylamide** (NEEA), respectively (Figure 3.5.2). The concentrations of the monomers are presented by their mass parts in the total monomeric mixture; e.g., NMEA90-NEEA10 means 90% NMEA monomer and 10% NEEA monomer in the total mixture.

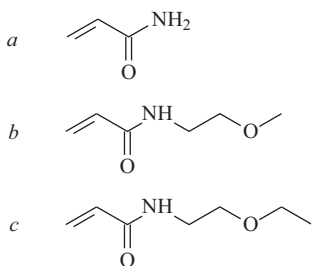


Figure 3.5.2: Monomer structures. (a) Acrylamide; (b) NMEA; (c) NEEA.

3.5.3 Running microchip electrophoresis of nucleic acids

Various preconcentration techniques were developed and applied for ME to increase the sensitivity, including high salt stacking [14], field-amplified sample stacking [15–17], sweeping [18], base stacking [19], porous membrane stacking [20], and transient isotachopheresis TITP [21–23].

Kang *et al.* [24] proposed a TRIS-borate-EDTA (TBE) buffer (pH = 8.3) with 0.5 µg/mL ethidium bromide (EtBr) for the ME run. The dynamic coating matrix was made by dissolving 0.5 g/dL of $M_r = 1,000,000$ polyvinylpyrrolidone (PVP), and the sieving matrix was made by dissolving 0.5 g/dL of $M_r = 8,000,000$ polyethylene oxide (PEO). The DNA sample was injected into the injection region by applying a voltage of 480 V.

3.5.4 Applications of DNA microchip electrophoresis

Lin *et al.* [25] separated dsDNA by a PMMA electrophoresis microchip using polymer solutions containing gold nanoparticles. Later, Xu *et al.* [26, 27] 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.

The analysis of single-base mutations in genomic DNA was carried out using a PMMA chip for detection of the allele-specific ligation products using a universal array platform. Sung *et al.* [28] have used PMMA ME for analyzing PCR products. Lee *et al.* [29] have developed a hot-embossed PMMA-PCR chip for amplifying a human cancer tumor-suppressing DNA sequence. Liu *et al.* [30] have reported a method based on PMMA microchips for separation of multiplex PCR products.

A fast diagnosis by ME was evaluated, using programmed field strength gradients (PFSG) in a conventional glass double-T microfluidic chip [31]. Compared to ME with a constantly applied electric field, ME-PFSG achieved 15-fold faster analysis.

Highly sensitive and rapid analysis of the methylated p16 gene in plasma and tissue DNA in cancer patients was realized by Zhou *et al.* [32] in a PMMA microchip. Hashimoto *et al.* [33] have performed PCR, ligase detection reaction, and hybridization assays using flow-through PMMA microchip for the detection of low-abundant DNA point mutations.

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3.6 Blotting of nucleic acids

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Blotting of nucleic acids is used to understand the organization, sequences, and hybridization of nucleic acids, as well as the gene expression, inherited diseases and infectious agents.

3.6.1 Blotting principles

As with proteins, nucleic acids blotting runs in three steps: transfer of nucleate bands onto a blot membrane, blocking the free (unoccupied) binding sites of the blot membrane by blocking agents, and detection of the blotted nucleates by specific high molecular ligands (probes).

3.6.1.1 Transfer

The transfer of electrophoretically separated nucleates onto a membrane is necessary because nucleates in a gel have difficulty reacting with probes. Polyacrylamide gels should be blotted in a transfer buffer of low ionic strength. There are a few transfer methods: diffusion transfer, capillary transfer, vacuum transfer, and electrotransfer. Among them, capillary and vacuum transfer are most commonly used, whereas the diffusion transfer and electrotransfer are limited used.

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Diffusion transfer

In the diffusion transfer (blotting) [1], the blot membrane is placed onto the gel surface and the nucleate transfer is carried out by diffusion (Figure 3.6.1). The blotting time takes a long time – up to 36–48 h. The gel could be also blotted between two blot 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 more used as it takes too much time.

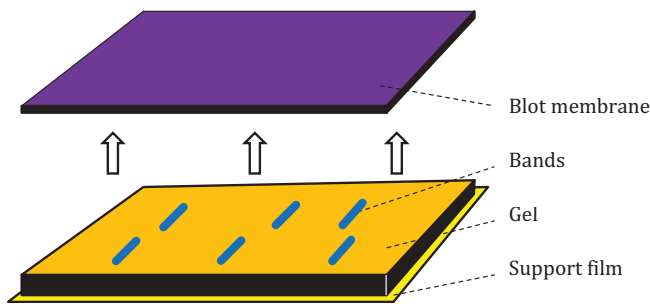


Figure 3.6.1: Diffusion blotting.

Capillary transfer

In the capillary transfer (blotting) [2], a buffer is sucked up into a stack of dry papers, which are placed onto a blot membrane covering a gel with the resolved nucleates. The paper stack is usually covered with a glass plate and the extra ballast. The paper stack acts with its capillary force on the gel, sucks the gel buffer upstairs and, as a result, the resolved nucleates stop on the blot membrane. The transfer is carried out for a long time, usually overnight (Figure 3.6.2).

In addition to the upward capillary transfer, a downward capillary transfer is known (see *Protocols*).

Vacuum transfer

The vacuum transfer (blotting) [3, 4] requires a vacuum blotter that creates negative pressure (incorrect named vacuum) of 200–400 Pa (20–40 cm of water) (Figure 3.6.3). For this purpose, a water-jet pump can be used. The vacuum transfer should be made carefully, since the gel could be broken. It takes place in 30–40 min.

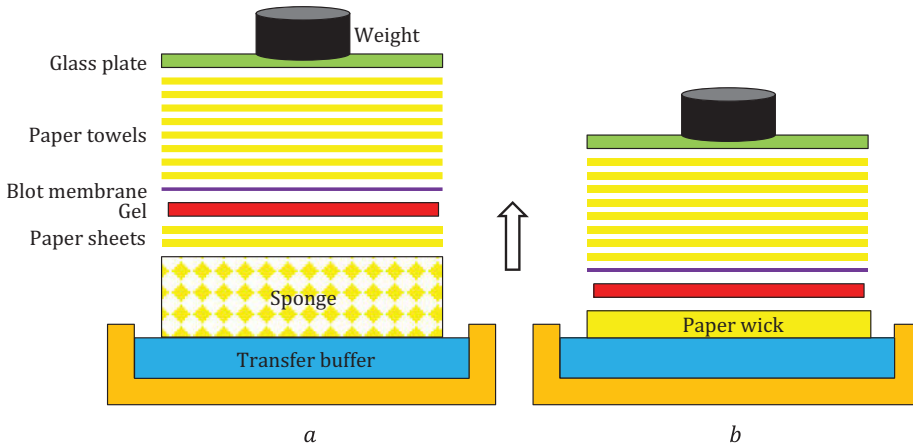


Figure 3.6.2: Upward capillary transfer.
(a) Sponge method; (b) Wick method.



Figure 3.6.3: Vacuum blotting.

Electrotransfer

The electrophoretic transfer (electrotransfer, electroblotting) of nucleic acids is carried out when the gel, containing electrophoretically separated DNA or RNA, is clamped to a blotting membrane in a cassette, and is put between two plate electrodes creating an electric field. It is faster than the capillary transfer and takes place in a few minutes to 24 h. If the gel is connected to a support film, it should be freed from it [5, 6]. Meanwhile, net-supported gels [7, 8] are available, which are suitable for electroblotting.

The electrotransfer has two versions: tank transfer (blotting) and semidry transfer (blotting).

The *tank transfer* [9] of nucleates is preferred in comparison with the semidry transfer. Prior to it, the gel with the resolved nucleates and the blot membrane are placed between filter papers soaked with buffer. Then, the blot sandwich is introduced into a grid cassette and put vertically between two platinum electrode wires that are in a tank containing a 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.

In the *semidry transfer* [10, 11], the electrodes have a direct contact to the gel – nitrocellulose membrane sandwich to make a fast transfer. The polyacrylamide gels should be equilibrated in the transfer buffer to remove electrophoresis 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 out in horizontal location without a buffer tank or gel cassettes.

3.6.1.2 Blocking

The unoccupied binding sites on 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, for example, is the Denhardt’s buffer [12]. It contains 10 to 50 g/mL of heterogeneous DNA with 0.02 g/dL of bovine serum albumin, 0.02 g/dL of ficoll, 0.02 g/dL of polyvinyl pyrrolidone, 1 mmol/L Na₂EDTA, and 50 mmol/L NaCl in a 10 mmol/L TRIS-chloride buffer with pH = 7.0.

3.6.1.3 Detection by probes, dyes, and autoradiography

After transfer and blocking, the nucleate bands can be made visible using specific high molecular ligands (probes) or dyes. Besides, radiolabeled DNA or RNA probes bind (hybridize) with complementary DNA or RNA. To prevent radioactivity, the same probes can be labeled 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 left to expose (hours to weeks) in the dark and cold. If required, the gel can be overlaid on the negative and the radioactive spots obtained can be cut up, eluted, and scintillated.

There are different blotting methods for nucleic acids: Southern blotting, Northern blotting, reverse Northern blotting, Middle-eastern blotting, Eastern-western blotting, Eastern blotting, Far-eastern blotting, and other (Table 3.6.1).

Table 3.6.1: The famous blotting methods for nucleates.

Blotting methods	Goals
Southern blotting	DNA
Northern blotting	RNA
Reverse Northern blotting	RNA
Middle-eastern blotting	polyA RNA

3.6.2 Southern blotting

Southern blotting is created by the British biologist Edwin Southern [13]. 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.
- The gel is placed into an alkaline solution to denature the double-stranded DNA.
- A nitrocellulose membrane is placed onto the gel. Then, a stack of filter papers and a weight are put above to ensure good contact between gel and membrane. As a result of originated capillary forces, the buffer moves from the gel into the filter papers and transfer 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 DNA hybridization probe. In some cases, RNA can also be used as a 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 obtained 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 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.

Southern blotting allows also detection of larger gene alterations such as deletions, insertions or rearrangements. Hereditary motor and sensory neuropathy type I, for example, are 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 **peripheral myelin protein 22** [14].

3.6.3 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 [15]. The name of this technique comes from the similarity between it and the Southern blotting. The difference between two methods is that in Northern blotting RNA, rather than DNA, is analyzed (Figure 3.6.4).

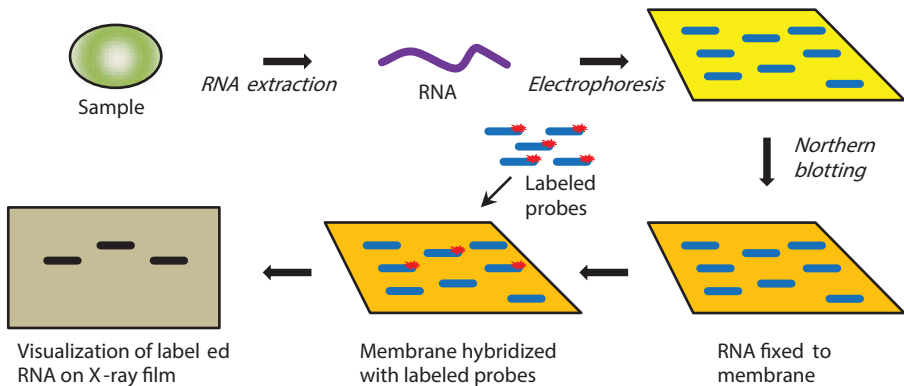


Figure 3.6.4: RNA detection by Northern blotting.

The procedure of Northern blotting starts with the extraction of total RNA from a homogenized tissue or cells. mRNA can be isolated from the total RNA using oligo-(dT)-cellulose chromatography to maintain only RNAs with poly(A) tails [16, 17].

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. The nylon membrane is most effective since it is positive charged while RNA carries negative charges. The transfer buffer contains formamide that lowers the annealing temperature of the probe-RNA interaction preventing the RNA degradation at high temperatures [18].

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 the end, the membrane is washed to remain only the complexes between RNA and the probe. The hybrid signals are detected by an X-ray film and are quantified by densitometry.

The probes are labeled either with radioactive isotopes (^{32}P) or with chemiluminescent substances. The chemiluminescent labelling can occur in two ways: either the probe is attached to **alkaline phosphatase** (ALP) or **horseradish peroxidase** (HRP), or is labeled with biotin, whose ligand (avidin or streptavidin) is attached to the enzyme (e.g. HRP). An X-ray film can detect both the radioactive and chemiluminescent signals. Many researchers prefer chemiluminescent signals because they are faster, more sensitive, and reduce the health hazards that go along with radioactive labels [19].

Northern blotting is used to determine gene expression rates during differentiation and morphogenesis, as well as in abnormal or diseased conditions [20]. It also allows observing the gene expression in tissues, organs, developmental stages, pathogen infection, and over the course of treatment [21, 22]. This technique has also been used to explore the overexpression of oncogenes and downregulation of tumor-suppressor genes in cancerous cells, as well as the gene expression in the rejection of transplanted organs. Besides, the Northern blotting can show which region of RNA is missing [23].

A variant of the Northern blotting is the reverse Northern blotting. In it, DNA rather than RNA is first immobilized on a blot membrane and then is detected with labeled RNA probes. The 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 [24]. However, the reverse Northern blotting is limited by its ability to probe only with one mRNA at a time, while q-PCR requires transcripts to be long enough to generate primers for the sequence and probes can be costly.

3.6.4 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 [25].

3.6.5 Protocols

Southern Blotting by Downward Capillary Transfer

Materials and equipment

TRIS-borate-EDTA (TBE) buffer

Saline-sodium citrate (SSC) 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, followed by a few pieces of the same paper, and then a chromatographically clean filter paper prewetted with transfer buffer.
- Wet the membrane and place it onto the filter paper, followed by the gel (Figure 3.6.5).

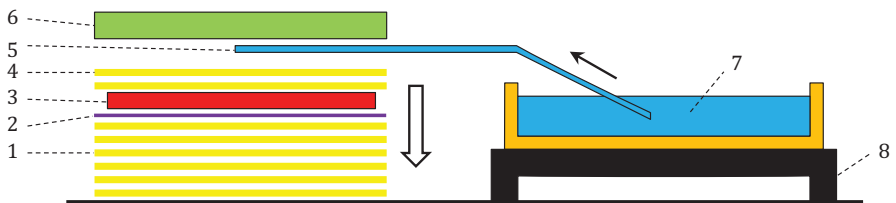


Figure 3.6.5: Downward capillary transfer.

1. Paper towels; 2. blot membrane; 3. gel; 4. paper sheets; 5. tubing; 6. glass plate; 7. transfer buffer; 8. support

- Place two pieces of soaked paper onto the top so that one end is submerged in a glass dish containing transfer buffer. Cover the sandwich with a glass plate to reduce evaporation, and transfer for 1 h.
- Remove paper towels and filter papers. 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.
- Rinse in SSC buffer, 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.

Electroblotting of Polyacrylamide Gel onto Nylon Membrane

Materials and equipment

TRIS-borate-EDTA (TBE) buffer

Saline-sodium citrate (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 buffer for 20 min.
- Cut a few pieces of chromatographically clean filter paper to the same size as the gel.
- Wet a piece of a nylon membrane with dimensions of the gel in water for 5 min.
- Cover the gel with the 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 three soaked filter papers, carefully removing trapped bubbles by rolling a glass pipette over the surface.
- Flood the filter paper carrying the gel with TBE-buffer and place on top of the stack, gel side up. Flood the gel with TBE-buffer and place the prewetted membrane on top. Flood the surface with $0.5 \times$ TBE buffer and place a few 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.

- 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.

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3.7 Evaluation of nucleic acid pherograms

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The nucleic acids, which are colorless, should be made visible after electrophoresis. This can be realized by counter-ion dye staining, silver staining, fluorescent substances, autoradiography, or protein probes. The results can be evaluated quantitatively by densitometry or photography.

3.7.1 Counter-ion dye staining of nucleic acids

The counter-ion dye staining of nucleic acids in agarose and polyacrylamide gels [1, 2] is a result of the interaction between negatively charged phosphate groups on DNA or RNA and cationic dyes, which intercalate between the base pairs of nucleic acids.

The main counter-ion dyes for staining of DNA in gels are **Methylene blue** (MB) [3, 4], **Brilliant cresyl blue** (BB) [5], **Crystal violet** (CV) [6], **Nile blue** (NB) [7, 8], and **Iodine blue** (IB) [9]. It is simple, but its sensitivity is inferior to ethidium bromide (EB) staining (see below).

More sensitive is the staining with a mixture of IB and methyl orange. IB has a positively charged group and six aromatic rings while methyl orange has a negatively charged group. The staining mixture has pH = 4.7, consists of 0.008 g/dL Indoin blue and 0.002 g/dL Methyl orange, and contains 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.

3.7.2 Silver staining of nucleic acids

Silver staining of nucleic acids in agarose and polyacrylamide gels [10–12] enables the detection of 0.03 ng DNA or RNA per mm² [13, 14], which corresponds to less than 50 pg nucleic acids per band. It provides higher sensitivity than EB fluorescence

<https://doi.org/10.1515/9783110761641-034>

method, however, requires multiple steps, preparation of prior to use solutions, and the toxic formaldehyde [15, 16].

The silver staining of nucleic acids, as the silver staining of proteins, passes in the same steps: fixing, sensitizing, developing, stopping, and drying the gel.

The *fixing* causes precipitation of the nucleic acids by denaturation. For this purpose, weak acids, such as acetic acid, are used, with or without alcohol. The low pH value of these solutions avoids the protons dissociation from the nucleic acids. As a result, the nucleic acids lose their negative charges and precipitate.

During the *sensitizing* (impregnation), the precipitated nucleic acids bind silver ions. This process is usually carried out in a neutral solution of silver nitrate and allows the building of silver nuclei.

The *developing* (visualizing) requires a strong reducing agent, such as formaldehyde, which reduces the silver ions to metallic silver. The reaction takes place faster in the vicinity of silver nuclei than in the gel, since the silver nuclei catalyze it. During the developing, 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 [17, 18].

The developing should be quickly *stopped*. Otherwise, all silver ions will be reduced to metallic silver, resulting in a black background. The stopping is caused by changing the pH value.

The *drying* is carried out under a hair dryer or in a dryer. If the polyacrylamide concentration of the gel is high, the wet gel should be placed with the support film down onto 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 the underside of the glass plate and the gel should be dried. The ultrathin polyacrylamide gels and agarose gels do not need to be covered by cellophane.

According to the fast method of Han *et al.* [19], nitric acid and ethanol in the impregnation step eliminate the need for prior treatment of polyacrylamide gels with a fixing solution, followed by water rinsing. The procedure can be completed within 10 min. The sensitivity of this method is significantly improved by the silver-ion sensitizer **eriochrome black T** (EBT), which reduces the gel background staining and increases the sensitivity of the method [20]. Compared to the conventional silver staining methods, the method of Han *et al.* saves time and displays high sensitivity (Table 3.7.1).

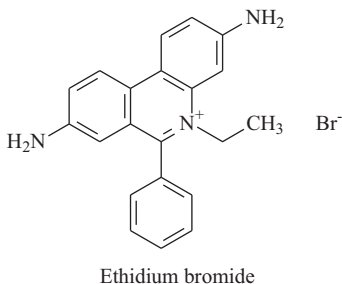
3.7.3 Fluorescence methods for detecting nucleic acids

The localization of nucleic acids in gels is most often carried out by fluorescence detection methods. For this purpose usually EB is used.

Table 3.7.1: Procedures and comparison of different methods for DNA silver staining.

	Method of Bassam <i>et al.</i> [21]	Method of Carlos <i>et al.</i> [22]	Method of Qu <i>et al.</i> [23]	Method of Han <i>et al.</i> [24]
<i>Fixing</i>	10 mL/dL ethanol and 0.5 mL/dL acetic acid for 3 min	10 mL/dL acetic acid for 20 min	–	–
<i>Rinsing</i>	H ₂ O for 2 min, twice	H ₂ O for 2 min, thrice	–	–
<i>Impregnating</i>	10 mL/dL ethanol, 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 for 30 min	25 mL/dL ethanol, 1 g/dL HNO ₃ and 0.2 g/dL AgNO ₃ for 2–5 min	1 g/dL HNO ₃ and 0.1 g/dL AgNO ₃ for 5 min
<i>Rinsing</i>	H ₂ O for 2 min, twice	H ₂ O for 20 s, optional	H ₂ O for 2 min	H ₂ O for 5 s, twice
<i>Developing</i>	3 g/dL NaOH and 0.1 g/dL HCHO for 5 min	3 g/dL Na ₂ CO ₃ , 1.5 mL 37 g/dL HCHO and 2 mg/L Na ₂ S ₂ O ₃ for 2–5 min	3 g/dL Na ₂ CO ₃ and 0.2 g/dL HCHO for 2–5 min	2 g/dL NaOH, 0.04 g/dL Na ₂ CO ₃ , 2 mL 0.0025 g/dL EBT and 1.5 mL 37 g/dL HCHO for 5 min
<i>Stopping</i>	10 mL/dL ethanol and 0.5 mL/dL acetic acid for 5 min, then in H ₂ O for 10 min	10 mL/dL acetic acid for 5 min	10 mL/dL acetic acid for 2–5 min	2.5 g/dL ampicillin for 5 s, then H ₂ O
<i>Sensitivity denat. DNA</i>	3.50 ng	0.44 ng	0.44 ng	0.11 ng
<i>Sensitivity native DNA</i>	14 ng	14 ng	3.5 ng	1.75 ng
<i>Background</i>	lightest	dark	light	golden yellow
<i>Used time</i>	~40 min	~60 min	~20 min	~10 min

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.



In intercalated state, nucleates absorb **ultraviolet (UV)** light and emit it again in the visible range. As a result, the nucleic acid-EB complexes are seen in agarose gels as orange-red fluorescent bands on a dark background [25] (Figure 3.7.1).

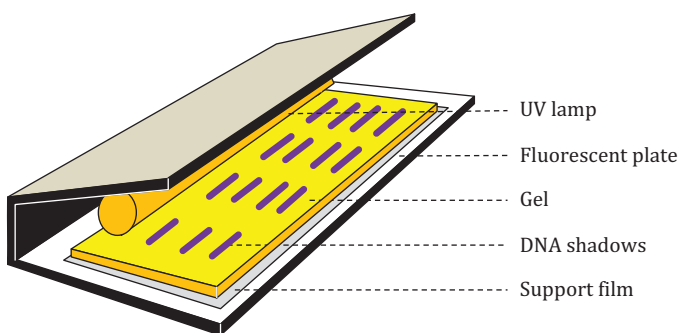


Figure 3.7.1: Visualizing the separated nucleic acids by ethidium bromide under UV light.

EB can be added to a sample prior to or after electrophoresis. If EB is added 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-EB complexes move by about 15% slower than the “uncolored” DNA. If EB is added after the electrophoresis, the agarose gel should be kept in a TRIS-borate-EDTA buffer containing 0.5 $\mu\text{g}/\text{mL}$ EB at room temperature for 45 min. The nucleates, “colored” with EB, are usually photographed under UV light.

EB is a strong mutagen. The UV irradiation may also damage both the DNA in the samples and the experimenters [26–28]. To avoid the mutagenic effect of EB, a fluorescent method using **berberine/Mordant Yellow 3 R** (BB/M3R) has been introduced [29].

In addition to EB, three other high sensitivity fluorescent substances (SYTO 13, TOTO-1, and YOYO-1) are used for staining of DNA and RNA [30, 31]. TOTO-1 and YOYO-1 represent dimers of the compounds TO-PRO-1 (benzothiazolium-4-quinolinolium) and YO-PRO-1 (benzoxazolium-4-quinolinolium). DNA can be “colored”

with these substances prior to electrophoresis because they dissociate difficultly from nucleic acids.

In recent years, it was found that the nucleic acid dye GelRed™ is the most sensitive and safest dye under UV light and blue light excitation [32].

3.7.4 Autoradiography of nucleic acids

Autoradiography is the most sensitive detection method for nucleic acids. It requires isotope labeled nucleic acids. After electrophoresis, the resolving gel should be dried before contacting with an X-ray film. 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 reduces the silver ions to atomic (metal) silver in form of grains, which blacken on the X-ray film (Figure 3.7.2). Then, the film is scanned by a special detector, which transmits the information into a computer.

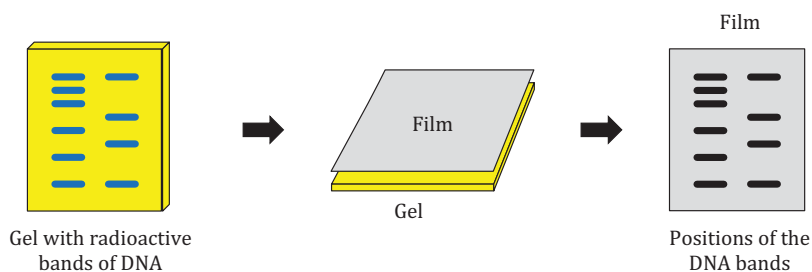


Figure 3.7.2: Autoradiogram of electrophoretically separated deoxynucleates.

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 the oxygen atom. Therefore, oxygen atoms in the α -phosphate residues of nucleoside triphosphates can be replaced with ^{35}S atoms and later be incorporated by DNA or RNA polymerases in nucleotide chains.

The isotopes ^{32}P , ^{33}P , and ^{35}S release high-energy electrons named β -particles (Table 3.7.2). The isotope ^{32}P radiates highest energy whereas the isotope ^{33}P radiates much weaker energy. The energy of the isotope ^{35}S is less than that of the isotope ^{33}P .

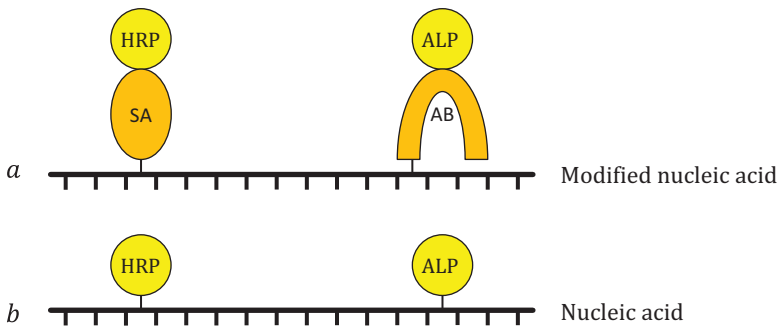
Table 3.7.2: Characteristics of isotopes used in autoradiography.

Isotope	Strength of β -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

3.7.5 Labeling of nucleic acids with proteins

The nucleic acids can be labeled (coupled) with enzymes. Typically **horseradish peroxidase (HRP)** and **alkaline phosphatase (ALP)** are used, which are often referred to as reporter enzymes. These enzymes can be detected by colorimetric or chemiluminescent substances. In the chemical luminescence, a chemical reaction produces visible light, which can last for several hours. It can be recorded on an X-ray film, similarly as in the autoradiography.

The labeling of nucleic acids with proteins (enzymes) may be indirect or direct (Figure 3.7.3).

**Figure 3.7.3:** Labeling of nucleic acids with enzymes.

Horseradish peroxidase (HRP) or alkaline phosphatase (ALP) can be coupled to nucleic acids indirectly (a) or directly (b). SA – streptavidin; AB – antibody.

The *indirect coupling* of proteins with nucleic acids is based on modified nucleotides of DNA or RNA. Under these circumstances special proteins, for example, **streptavidin (SA)** or **antibodies (AB)** can be bound to the nucleotide chain, which in turn can fix HRP or ALP. For chemical modifications of nucleic acids, three substances are used: biotin, fluorescein, and digoxigenin. If nucleic acids are labeled with biotin, they can bind the bacterial protein SA. Biotin should be at the end of the nucleic acid; only then, biotin can bind to the large SA molecule. So, the steric hindrance is

avoided and the SA molecule can come undisturbed closely to the biotin ligand. SA conjugates with the modified nucleic acid alone or bound to a reporter enzyme.

The *direct coupling* of proteins on nucleic acids is used also for the reporter enzymes HRP and ALP [33]. The direct coupling changes the DNA mobility. The advantage of the direct DNA–protein coupling is that it is made quickly, because the nucleotides should not be labeled with SA or AB. 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.

3.7.6 Absorption spectroscopy of nucleic acids

The absorption of nucleic acids at 260 or 280 nm wavelengths can be used to calculate the nucleic acid concentrations. Absorption at 325 nm indicates particles in the solution or dirty cuvettes. Contaminants containing peptide bonds or phenol absorb at 230 nm.

3.7.7 Protocols

Detection of DNA and RNA in Gel Using Ethidium Bromide

Materials and equipment

Agarose gel

DNA or RNA

Ethidium bromide

Ethidium bromide is a mutagen. Handle 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)

Na₂EDTA 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 1–2 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.
- Pour agarose solution into a cassette to gel.

Procedure

- After electrophoresis run, place the agarose 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.

Fast and Sensitive Silver Staining of DNA Bands According to Han *et al.* [34]

Materials and equipment

Polyacrylamide slab gel

HNO₃

AgNO₃

NaOH

Na₂CO₃

Eriochrome black T (EBT)

HCHO

Ampicillin

Procedure

- Impregnate the gel in a mixture of 0.1 g/dL AgNO₃ and 1 g/dL HNO₃ 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 Na₂CO₃, 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 turns 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.

Autoradiography of Radiolabeled DNA in Gels and Blots

Materials and equipment

Dried gel or membrane (e.g., after immunoblotting)

Developer

Fixer

Film or paper cassette with particle-board supports and metal binder clips

Plastic wrap

X-ray 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 3.7.4).



Figure 3.7.4: Autoradiography setup.

- Place an X-ray film on top of the sample, and 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 intensifying screens (Table 3.7.3). Time of exposure depends on the strength of radioactivity in the sample. A Geiger counter can be used to detect the relative amount of radioactivity in the sample.

Table 3.7.3: Film choice and exposure temperature for autoradiography.

Isotope	Enhancement method	Film	Exposure temperature
^3H	Fluorography	Double-coated	$-70\text{ }^\circ\text{C}$
^{35}S , ^{14}C , ^{32}P	None	Single-coated	Room temperature
^{35}S , ^{14}C , ^{32}P	Fluorography	Double-coated	$-70\text{ }^\circ\text{C}$
^{32}P , ^{125}I	Intensifying screens	Double-coated	$-70\text{ }^\circ\text{C}$

- After exposure, return the cassette into the darkroom and remove the film for developing.
- Immerse the film in $20\text{ }^\circ\text{C}$ developer for 5 min, and wash in running water for 1 min.
- Immerse the film in $20\text{ }^\circ\text{C}$ fixer for 5 min, and wash in running water for 15 min.
- Hang the film to dry.

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3.8 Precast gels for nucleic acid electrophoresis

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- 3.8.3 Protocols — 396
 - Casting Mini- and Midi-agarose Gels — 396

The precast gels for nucleic acid electrophoresis are as important, as the precast gels for protein electrophoresis. They can contain agarose or polyacrylamide.

3.8.1 Precast agarose gels

The precast agarose gels on film or net for native or denaturing zone electrophoresis of nucleic acids contain buffers. In principle, they are 0.5 mm thick. The precast gels are available in different dimensions: 51 × 82, 80 × 100, 125 × 125, 125 × 200, 125 × 250 mm or more. The concentration of the agarose can be 0.60, 0.80, 1.00 g/dL or other.

The precast agarose gels are located between two polyester films. The cover film is loosely bound to the gel. It should be removed prior to the application of a template on the gel. The bottom film is chemically bound to the gel.

3.8.2 Precast polyacrylamide gels

The precast polyacrylamide gels on film or net for native or denaturing disc-electrophoresis of nucleic acids are usually 0.5 mm thick. They are supplied together with paper strips. The gels and paper strips contain buffers: the gel and anode strip contain, for example, TRIS-formate-EDTA buffer, and the cathode strip contains, for example, TRIS-taurinate-EDTA buffer.

The polyacrylamide concentration T of the precast gels may be 5, 7, 9, 11, or 13 g/dL, at dimensions corresponding to the dimensions of the precast agarose gels. Film-supported gels are used for electrophoresis and diffusion blotting, whereas net-supported gels are suitable for electroblotting.

Similarly to the precast agarose gels, the precast polyacrylamide gels are located between two polyester films. The cover film should be removed prior to the application of a template on the gel. The lower film of a net-supported gel, which is loosely bound to the gel, should be removed after electrophoresis for electroblotting.

Precast polyacrylamide gels should be stored at 4–8 °C in the refrigerator.

3.8.3 Protocols

Casting Mini- and Midi-agarose Gels

Materials and equipment

Appropriate buffer

Agarose

Casting cassette for vertical mini- or midi-agarose gels

Procedure

- Fulfill the casting cassette (for example, with inner dimensions of $80 \times 50 \times 2$ mm) with agarose solution (10 mL).
- Place a comb into the solution.
- After gelling, remove carefully and under buffer the comb from the gel.

4 Iontophoresis

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The term *iontophoresis* stems from Greek words “ion” and “phorein” (to carry) and is referred to as inserting ions into the body driven by electric field.

The advantages of iontophoresis are: painless drug delivering in the body, without injections; reduced risk of infection because the skin and outer mucosae remain unhurt; direct drug delivery into the ill place; and no risks of fibrosis in contrast to the injection drug delivering. Iontophoresis has, however, some disadvantages. The most common is that the iontophoretic procedures make the skin dry. Skin irritation, blistering, and peeling might also occur.

Reverse iontophoresis is also known. It is a technique for removing ions or neutral molecules from the body. Reverse iontophoresis can be caused when the skin is negatively charged, which makes it permeable for cations (sodium and potassium ions). They, in turn, cause electroosmosis that pushes solvent toward the anode. The reverse iontophoresis can be applied to extract glucose across the skin in diabetic patients to monitor the blood glucose concentration [1, 2]. This is used in the GlucoWatch device [3].

4.1 Theory of iontophoresis

In iontophoresis, charged drugs are inserted into skin or mucosae for an appropriate length of time [4]. This is carried out with the help of electric field [5]. The electric current should be direct and below the level of patient’s pain threshold. Besides, the drugs should have relatively small molecules ($M_r < 8,000$).

The ions to be driven into a tissue should be placed under the electrode with the same polarity. If so, the anode repels cations, and the cathode repels anions (Figure 4.1). The ground electrode might be placed elsewhere on the body surface. In principal, the negative electrode (cathode) is larger than the positive electrode

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(anode) to avoid skin irritation. For example, the surface of the cathode can be twice larger than the surface of the anode.

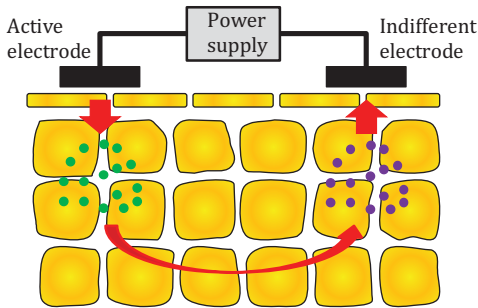


Figure 4.1: Ions of the polarity as the polarity of stimulating electrode are repelled into the skin, and on the contrary.

Active ion; Counter-ion

4.2 Factors affecting iontophoresis

Factors affecting iontophoresis have physicochemical and biological origin.

4.2.1 Physicochemical factors

Physicochemical factors that affect iontophoresis are: concentration, pH value and ionic strength of the buffer, solution viscosity, and electric current.

Concentration. The drug should be water soluble and ionizable. Smaller molecules are more mobile.

Buffer pH. The pH value of the buffer containing the drug should have such value, at which the drug molecules are ionized [6]. This pH value is called pH optimum of the drug.

Buffer ionic strength. If a drug is dissolved in a buffer, the buffer ions compete with the drug ions in the electric field. This process has a prominent role in iontophoresis because the buffer ions are generally smaller than the drug ions; therefore, they are faster than the drug ions.

Solution viscosity. The migration of a drug is inversely related to the viscosity of the solution to be introduced.

Electric current. The electric current can be direct, alternate or pulsed. The alternating electric current (AC) iontophoresis shows better results than the conventional direct electric current (DC) iontophoresis [7].

4.2.2 Biological factors

The biological factors, which influence iontophoresis, are the thickness and permeability of skin or mucosa. Sweat glands in the skin are in principal the path for inserting charged ions. This fact was demonstrated by Papa and Kligman, who proved this using Methylene blue [8].

4.3 Calculating the iontophoretic current

The electric current density J 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 should be delivered through an electrode surface S of 6 cm^2 , then the electric current density at either the anode or cathode should be

$$J = \frac{I}{S} = \frac{2}{6} = 0.33 \text{ mA/cm}^2 \quad (4.1)$$

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 is 0.5 mA/cm^2 , then, according to eq. (4.1), the maximum electric current I should be

$$I = J \times S = 0.5 \times 6 = 3 \text{ mA} \quad (4.2)$$

4.4 Iontophoresis device

An iontophoresis device consists of a microprocessor-controlled battery, two electrodes, and a reservoir.

The iontophoresis *battery* creates low voltage – usually 9 V .

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).

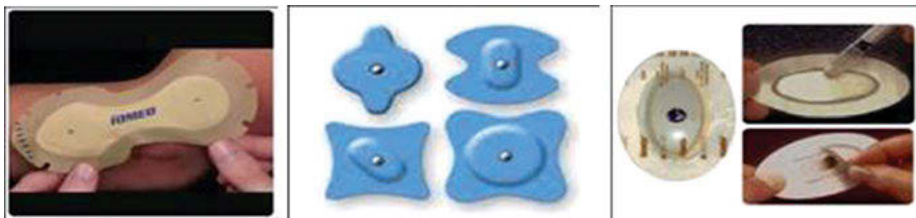


Figure 4.2: Electrodes for iontophoresis.

The *reservoir* contains a gauze or gel pad, to which a drug solution is applied or injected through.

Two types of iontophoresis are run: diagnostic and therapeutic iontophoresis.

4.5 Diagnostic iontophoresis

For diagnostic purposes, pilocarpine iontophoresis is often used to stimulate sweat secretion, as part of the diagnosis of *cystic fibrosis* [9–11].

Acetylcholine iontophoresis is used for testing the endothelium by stimulating endothelium-dependent synthesis of NO and subsequent *microvascular vasodilation* [12]. Acetylcholine ions are positively charged; therefore, they are placed in the anode chamber.

Iontophoresis can be used for testing *contact eczema* [13]: The test substances are administered through epidermis into dermis.

Iontophoresis of epinephrine for assessment of neuromuscular synapsis response improves the diagnosis of *myasthenia gravis*. For this purpose, an improved method called iontophoresis of epinephrine with repetitive nerve stimulation (RNS) was successfully used [14].

4.6 Therapeutic iontophoresis

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.

Most significant types of therapeutic iontophoresis are transdermal and ocular iontophoresis.

4.6.1 Transdermal iontophoresis

Iontophoresis for transdermal delivery of drugs has been used for more than a century [15–17]. It is applicable to small ions, and polyions of M_r up to a few thousand. The ions are driven into the skin *via* the sweat gland ducts, which have low resistance, and hair follicles.

In transdermal iontophoresis, low voltage is applied between an iontophoretic chamber (containing a same-polar drug) on the skin and another electrode chamber also on the skin (Figure 4.3).

The transdermal drug delivery can be controlled by a microprocessor [18]. The most treated disease is hyperhidrosis.

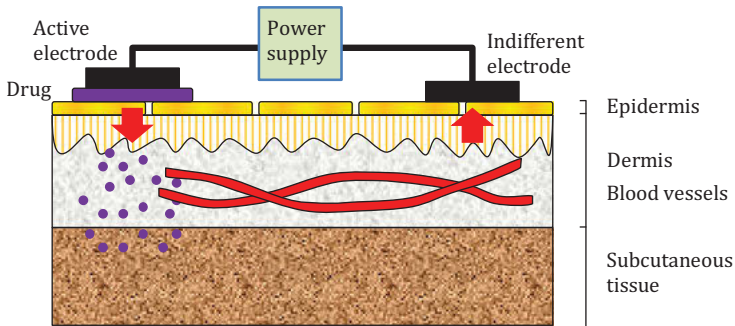


Figure 4.3: Transdermal drug delivery by iontophoresis.

Hyperhidrosis iontophoresis

Hyperhidrosis is characterized with excessive sweating under no reason. This disease affects about 4% of the population. Hyperhidrosis can occur anywhere on the body (hands, feet, underarms, and face) and can affect all genders and ages, but is most common in teenagers and young adults.

Iontophoresis blocks temporarily the sweat glands and stops the sweating. Patients with hyperhidrosis should undergo several iontophoresis applications per week, lasting from 20 to 40 min [19]. In serious cases of hyperhidrosis, water solutions with cholinergic inhibitors, such as methyl sulfate [20], atropine [21], or glycopyrronium bromide [22–24], can be used.

Other transdermal applications

Myasthenia gravis is another disease that can be treated iontophoretically. It is a result of a genetic defect in the neuromuscular synapsis [25]. In most cases, myasthenia gravis is caused by circulating antibodies that block acetylcholine receptors on the postsynaptic membrane, inhibiting so the neurotransmitter acetylcholine. Acetylcholine ion is positively charged; therefore, it can be forced out from the pre-synaptic terminal by a microcathode [26].

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* [27–29].

Iontophoresis is also used for treatment of *plantar fasciitis*, *bursitis*, lateral and medial *epicondylitis* (commonly referred to as tennis elbow and golfers elbow, respectively), and other diseases [30].

Some *viral infections* are also positively influenced by iontophoresis. Iontophoresis can be an alternative for delivering of acyclovir to treat *herpes labialis* [31].

Planar warts can be successfully treated with sodium salicylate iontophoresis [32]. Iontophoretic application of idoxuridine is effective in *herpes simplex* treatment [33].

Hyperkeratosis with fissuring of palms and soles is treated with 5–10 g/dL sodium salicylate and shows improvement within 3–4 weeks [34].

Iontophoresis with 1 g/dL meladine at patients with *vitiligo* shows marked repigmentation [35].

Iontophoresis with hyaluronidase leads to temporary increased skin softness and flexibility and decreases the cold sensitivity in patient with *scleroderma* [36].

Methyl prednisolone iontophoresis is used for healing of erosive *lichen planus* [37].

Iontophoresis can be used in the treatment of *lymphedema* of the limbs [38].

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.

Muscle spasm is treated with 2–5 g/dL CaCl_2 on the negative pole. Calcium ions stabilize membranes, and decrease excitability threshold of peripheral nerves and skeletal muscle. Also 2 g/dL MgSO_4 (ointment) on the same pole can be used, because magnesium ions push out calcium ions. As a result, muscles relax.

Iontophoretic patches enable patients to administer a bolus of fentanyl for *pain relief* [39]. Soft tissue pain can be treated with 4–5 g/dL lidocaine (ointment) on anode.

Dentists have used it for anesthesia of the oral mucosa [40, 41]. The anesthesia of the skin can be achieved by local administration of lidocaine and epinephrine [42, 43].

4.6.2 Ocular iontophoresis

Iontophoresis is also used for delivery of therapeutic drugs into the eye. It has the capacity to provide high drug concentration safely, while only 1% of the same drugs delivered *per os* reach the eye. Two types of ocular iontophoresis are known: transcorneal and transscleral.

Transcorneal iontophoresis can deliver drugs 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 [44].

The lens-iris diaphragm is the main barrier of local 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. This treatment is useful for posterior ocular disorders, such as *retinitis*, *optic nerve atrophy*, *uveitis*, *endophthalmitis*, *pediatric retinoblastoma*, and **age-related macular degeneration** (AMD).

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5 History of electrophoresis and iontophoresis

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The history of electrophoresis and iontophoresis continues a few centuries.

5.1 History of electrophoresis

5.1.1 Discovery of electrophoresis

The movement of charged particles in an electric field was first observed as long ago as 1807 by the Russian chemists Pyotr Ivanovich Strakhov (1757–1813) and Ferdinand Frederic Reuss (1778–1852) at the Moscow State University [1]. Using a microscope, they noticed that the application of a constant electric field caused dispersed in water clay particles to migrate. Reuss discovered also the opposite flow of water (electroosmosis).

G. 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 solution pH value.

In 1892, H. Picton and S. Linder [4] observed that hemoglobin, a colored protein, was moving in a U-tube, filled with an electrolyte solution and placed in an electric field. In 1899, Hardy reported the movement of serum globulins in 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, 6].

In 1909, L. 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* (to carry).

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In 1985, B. Michov drew up the parameter of ionic mobility, which can be used for calculating the ionic mobilities at different ionic strengths [7]. In 1988, he simplified the Henry's function [8].

5.1.2 Zone electrophoresis of Tiselius

The history of modern electrophoresis began with the works of Arne Tiselius in Sweden. In 1937, he published a new method for analyzing bio-colloids, and developed the "Tiselius apparatus" for moving boundary electrophoresis [9]. Tiselius detected separated but colorless protein bands using a Schlieren-scanning system.

Over time, the electrophoresis in solutions was replaced by electrophoresis in solid media. At first, filter paper was used, then cellulose acetate membrane, and finally gels.

5.1.3 Paper and cellulose acetate electrophoresis

Paper electrophoresis was introduced by P. König in 1937 [10]. He soaked paper in a buffer for zone electrophoresis and suggested UV detection. Using the paper electrophoresis, Linus Pauling and colleagues [11] proved in 1949 that patients with sickle cell disease contained an abnormal hemoglobin.

In 1957, J. Kohn introduced cellulose acetate membranes as a solid support for hemoglobin electrophoresis [12].

5.1.4 Gel electrophoresis

In 1955, O. Smithies introduced partially hydrolyzed starch gels for electrophoretic separation of serum proteins [13, 14]. Later, agarose, polyacrylamide, and other gels enabled efficient separation of proteins and nucleic acids.

Agarose gel electrophoresis

Agarose, a purified form of agar, was introduced in 1961 by Stellan Hjerten [15] as separation medium for electrophoresis. It continues to be applied for separation of proteins and nucleic acids. In 1967, S. Hjerten [16] used 3 mm i.d. tubes for capillary electrophoresis. Later, in 1981, J. Jorgenson and K. Lukacs [17] demonstrated high-performance capillary electrophoresis in 75 μm i.d. capillaries. During 1988 to 1990, Karger's group [18, 19, 20] separated single stranded DNA in gel-filled capillaries.

Polyacrylamide gel electrophoresis

Polyacrylamide gel has been introduced as electrophoresis medium in 1954 by G. Oster [21]; however, credit for this development is usually given to S. Raymond and L. Weintraub [22] who published their work in 1959. Later S. Hjerten gave detailed formulae and recipes for preparation of polyacrylamide gels with sieving characteristics [23].

In 1967, A. Shapiro *et al.* [24] showed that the detergent sodium dodecyl sulfate (SDS) improved the resolution of polyacrylamide gel electrophoresis. In 1969, K. Weber and M. Osborn [25] optimized this method. The explanation of the processes in SDS electrophoresis was presented by J. Reynolds and C. Tanford [26].

The discovery of disc-electrophoresis is a great progress in the electrophoresis history. Its theoretical basis was developed by L. Ornstein [27]; its practical implementation was improved by B. Davis [28]. Later, U. Laemmli [29] used the Ornstein buffer system for SDS disc-electrophoresis of proteins.

During 1982 to 1986, B. Michov established the complex compound TRIS-boric acid in the TRIS-borate buffers and calculated the mobility of TRIS-borate ion [30, 31, 32]. In 1989, he described a disc-electrophoresis in one buffer at two pH values [33].

5.1.5 Isoelectric focusing

The isoelectric focusing of proteins was applied by Alexander Kolin [34, 35]. He developed it in a column containing a sucrose gradient where two buffer solutions were allowed to diffuse into one another to generate a pH gradient.

A few years later, H. Svensson (H. Rilbe) created the theoretical basis of the isoelectric focusing and gave the name of carrier ampholytes [36]. He obtained the first carrier ampholyte solution (a polypeptide mixture obtained from partially hydrolyzed blood) and separated there hemoglobins [37].

Next, O. Vesterberg [38, 39] patented a method of creating synthetic carrier ampholytes. They were mixtures of aliphatic aminocarboxylic acids, which formed a continuous pH gradient when placed in an electric field.

5.1.6 Two-dimensional electrophoresis

In the two-dimensional electrophoresis, proteins are separated by their pI values in a pH gradient in a first dimension, and then are separated due to their molecular sizes using SDS-PAGE in a second dimension. The method was introduced in 1975 simultaneously by three independent groups of researchers, namely P. O'Farrell [40], J. Klose [41], and G. Scheele [42], although the credit usually goes to O'Farrell.

5.1.7 Blotting

The blotting of polyions, resolved by electrophoresis, was discovered by E. Southern [43]. Later, many scientists created similar techniques for different substances.

Blotting of proteins

Many forms of protein blotting are used: Western, Southwestern, Northwestern, Far-Western, Eastern, and Far-Eastern.

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 J. Renart and J. Reiser [44], published in July 1979 this method. They used passive transfer of proteins, then ¹²⁵I-labeled protein A for detection.

Harry Towbin’s group in Basel, Switzerland, including also T. Staehelin (at Hoffman–La Roche) and J. Gordon (at Friedrich-Miescher-Institut) [45] published in September 1979 a similar technique. They transferred proteins from a SDS-PAGE gel to blot membrane using electric current and secondary antibodies for detection.

W. Neal Burnette, working in Robert Nowinski’s laboratory 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 [46] after his friends repeated the process [47]. W. Neal Burnette named the technique “Western blotting” as a nod to Southern blotting and because his laboratory was on the west coast [48,49].

So, Stark’s group published first; Towbin’s group developed the most common method, including the electrophoretic transfer, as well as the use of secondary antibodies; and Burnette popularized the technique under the name Western blotting.

Southwestern blotting is a technique, which was first described by B. Bowen and J. Steinberg *et al.* in 1980 [50]. It is used to characterize proteins that bind to specific DNA probes.

Northwestern blotting, also known as *Northwestern assay*, is a hybrid technique of Western blotting and Northern blotting. It detects interactions between RNA and proteins [51].

Far-Western blotting is 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. So Far-Western blotting is employed to detect protein: protein interactions.

Eastern blotting was created by H. Tanaka *et al.* [52]. It is a technique used to analyze protein post-translational modifications and to detect carbohydrate epitopes.

Far-Eastern blotting was named in 2000 by D. Ishikawa and T. Taki [53]. It is based on antibody or lectin proving 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 of DNA was discovered in 1975 by the British biologist E. Southern at University of Edinburgh [54] and carries his name.

Northern blotting of RNA was developed in 1977 by George Stark's group at Stanford University [55].

Middle-Eastern blotting was described in 1984 [56]. It was developed for blotting and immobilizing of polyA-RNA using DNA probes.

Eastern-Western blotting was introduced in 1996 by M. Bogdanov *et al.* [57]. It is based on a method of Towbin, created in 1984 [58], and a work by T. Taki *et al.* in 1994 [59]. During the Eastern-Western blotting, phospholipids are blotted on PVDF or nitrocellulose membrane, and then proteins are transferred by Western blotting onto the same membrane where they are probed with specific antibodies.

5.1.8 Staining methods

The detection of resolved proteins relies on dyes such as Bromophenol blue [60], Azocarmine B [61], and Amido black 10B [62]. A better staining assay is that of M. Bradford [63] who bound the dye Coomassie brilliant blue (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.* [64]. CBB R-250 was employed for staining of protein bands in polyacrylamide gels by A. Chrambach *et al.* in 1967 [65].

In 1979, C. Merrill *et al.* [66] and R. Switzer *et al.* [67] applied silver staining, which is 10–100 times more sensitive than CBB R-250, for protein detection in pherograms.

5.1.9 Outline history of electrophoresis

Year	Researcher(s)	Development
1807–1809	Strakhov and Reuss [68]	Observed movement of dispersed clay particles in an electric field – the discovery of electrophoresis in Moscow State University
1859	Quincke [69, 70]	Studied the electroosmosis and discovered the electric double layer

(continued)

Year	Researcher(s)	Development
1879	Helmholtz [71]	Proposed the first model of the electric double layer
1887	Arrhenius [72]	Developed the theory of electrolyte dissociation
1892	Picton and Lindner [73]	Invented the boundary electrophoresis
1896	Hardy [74]	Observed the movement of globulin in a U-tube by electric current
1897	Kohlrausch [75]	Described the “persistent” function in a system of two or more electrolyte solutions
1903	Smoluchowski [76, 77]	Derived the first equation of ion mobility
1904	Perrin [78]	Used the Helmholtz model for dissolved particles
1910–1913	Gouy [79] and Chapman [80]	Developed, independently from each other, the theory of the diffuse counter-ionic atmosphere of the electric double layer
1923	Debye and Hückel [81]	Created the theory of electrolyte solutions and the theory of electric double layer
1923	Brönsted [82]	Developed the proteolytic theory of acids and bases
1924	Hückel [83]	Derived the second equation of ion mobility
1924	Stern [84]	Developed the current theory of the electric double layer
1926	Onsager [85, 86]	Derived the equation of conductivity of an ionic solution
1931	Henry [87, 88]	Combined the equations of Smoluchowski and Hückel in a mathematical function
1937	Tiselius [89, 90]	Carried out the free electrophoresis of serum proteins and improved an apparatus for their detection
1937–1948	König [91, 92], Wieland and Fischer [93]	Introduced the paper electrophoresis and an apparatus for it
1942	Martin [94]	Developed the isotachopheresis
1949	Pauling <i>et al.</i> [95]	Proved, using electrophoresis, that an abnormal hemoglobin caused sickle cell disease
1950	Grassmann and Hannig [96, 97]	Developed the free-flow electrophoresis

(continued)

Year	Researcher(s)	Development
1953	Grabar and Williams [98]	Developed the immunoelectrophoresis
1954	Kolin [99, 100]	Introduced artificial pH gradients for IEF
1959	Robinson and Stokes [101]	Completed the Onsager equation
1955	Smithies [102, 103]	Described electrophoresis in a starch gel
1957	Kohn [104, 105]	Introduced cellulose acetate as an electrophoretic medium
1957	Poulik [106]	Used the TRIS-citrate-borate buffer system as a discontinuous buffer system for electrophoresis
1959	Raymond and Weintraub [107]	Introduced the polyacrylamide gel as an electrophoresis medium
1961	Hjerten [108]	Used agarose gel for electrophoresis
1961	Svensson [109, 110, 111]	Developed the theory of isoelectric focusing
1962	Hjerten [112]	Gave detailed formulae for preparation of gels with specific sieving characteristics
1963	Fazekas <i>et al.</i> [113]	Introduced Coomassie brilliant blue as a highly sensitive dye for detection of electrophoretically separated proteins
1964	Ornstein [114] and Davis [115]	Invented the theory and practice of disc-electrophoresis in a TRIS-chloride-glycinate buffer system
1965	Hjerten [116]	Described the concentration of polyions on the boundary to a solution with higher ionic strength – Hjerten's effect
1965–1967	Laurell [117, 118] and Clarke and Freeman [119]	Described the rocket immunoelectrophoresis
1966	Thorne [120]	Used agar as separation medium
1966	Vesterberg [121, 122]	Synthesized carrier ampholytes and introduced the isoelectric focusing of proteins in the practice
1967–1969	Shapiro <i>et al.</i> [123] and Maizel [124]	Developed SDS-PAGE in continuous buffers for determination of molecular masses of proteins
1967–1971	Margolis and Kenrick [125, 126], Kopperschläger <i>et al.</i> [127], Slater [128, 129] and Rodbard <i>et al.</i> [130]	Introduced gradient gels in the electrophoretic practice and analyzed theoretically the gradient electrophoresis

(continued)

Year	Researcher(s)	Development
1969	Weber and Osborn [131]	Optimized the SDS electrophoresis for separation of protein subunits
1969	Allen <i>et al.</i> [132, 133]	Introduced the disc-electrophoresis in step-gradient gels
1970	Laemmli [134, 135]	Used SDS in the discontinuous buffer system of Ornstein and Davis, and created so the SDS disc-electrophoresis
1972	Aaij and Borst [136]	Introduced agarose gels with ethidium bromide for DNA electrophoresis
1973	Jovin and Chrambach [137, 138]	Calculated mobilities of ions used in electrophoresis
1975	O'Farrell [139], Klose [140] and Scheele [141]	Introduced independently the two-dimensional electrophoresis, which combines isoelectric focusing with SDS electrophoresis
1975	Southern [142]	Carried out blotting of DNA
1975–1982	Gasparic <i>et al.</i> [143] and Bjellquist <i>et al.</i> [144]	Used immobilized pH gradients for isoelectric focusing
1976	Lambin <i>et al.</i> [145, 146] and Lasky [147]	Used gradient gels for SDS electrophoresis
1978	Hannig [148, 149]	Invented the free-flow electrophoresis
1979–1987	Mikkers <i>et al.</i> [150], Jorgensen and DeArman [151], Tsuda <i>et al.</i> [152], Hjerten [153], Gebauer <i>et al.</i> [154], Terabe <i>et al.</i> [155], Green and Jorgenson [156], Hjerten <i>et al.</i> [157, 158]	Developed the capillary electrophoresis
1979	Rosen <i>et al.</i> [159]	Used agarose gels of low electroosmosis as a medium for isoelectric focusing
1979–1981	Towbin <i>et al.</i> [160], Renart <i>et al.</i> [161] and Burnette [162]	Invented Western blotting
1979–1981	Switzer <i>et al.</i> [163] and Merril <i>et al.</i> [164]	Introduced the silver method as a highly sensitive staining method for electrophoretically resolved bands
1981	Jorgenson and Lukacs [165]	Demonstrated high performance capillary electrophoresis separations in 75 μm i.d. capillaries

(continued)

Year	Researcher(s)	Development
1982–1986	Michov [166, 167, 168]	Established the complex compound TRIS-boric acid in the TRIS-borate buffers, and calculated the mobility of TRIS-borate ion.
1983	Mullis [169]	Invented the polymerase chain reaction (PCR)
1984	Schwartz and Cantor [170]	Introduced the pulsed-field gel electrophoresis for separation of large DNA nucleates
1985	Michov [171]	Drew up the parameter of ionic mobility, which can be used for calculating the ionic mobilities at different ionic strengths
1987	Schägger and Jagow [172]	Published the TRIS-acetate-TRICINEate buffer system for SDS-electrophoresis in gradient gels
1988	Michov [173]	Simplified the Henry's function
1988–1990	Karger's group [174, 175, 176]	Separated single stranded DNA oligonucleotides in gel-filled capillaries
1989	Booth [177]	Separated chromosomes in agarose gels by pulsed-field electrophoresis
1989	Michov [178]	Described a disc-electrophoresis in one buffer at two pH values
1992	Harrison <i>et al.</i> [179]	Introduced the microchip-based capillary electrophoresis (MCE)
2004	Kastenholz [180]	Improved electrophoresis of metaloproteins
2013	Michov [181, 182]	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

5.2 History of iontophoresis

Iontophoresis exists as a medical method after the early 1900s when French biologist Stephane Leduc (1853–1939), professor at the Ecole de Medecine de Nantes, published his works [183, 184, 185]. Fritz Frankenhäuser (born 1868) is said to have introduced the term “iontophoresis.”

R. Wirtz employed iontophoresis in ophthalmology in 1908. He passed electric current through electrolyte-saturated cotton sponges, placed over the eye globe, for treatment of corneal ulcers, keratitis, and episcleritis [186].

In 1942, S. Witzel *et al.* applied iontophoresis for delivery of a variety of antibiotics such as tetracyclines, chloramphenicol, penicillin, streptomycin, neomycin, and bacitracin in rabbits [187].

In 1959, L. Gibson and R. Cooke demonstrated that sweating could be induced by pilocarpine iontophoresis [188].

In 1973, M. Corneau *et al.* used iontophoresis for local anesthesia of the ear [189].

In 1986, L. Gangarosa and J. Hill applied iontophoresis of vidarabine monophosphate for treatment of herpes orolabialis [190].

In 1992, W. Rigano *et al.* demonstrated treating of burned ears by gentamicin iontophoresis [191].

In 2013, M. 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 [192].

5.2.1 Outline history of iontophoresis

Year	Researcher(s)	Development
1900	Leduc [193, 194, 195]	Published works on the physiological effects of electric current on the body
1908	Wirtz [196]	Employed iontophoresis in ophthalmology
1942	Witzel <i>et al.</i> [197]	Applied iontophoresis for delivery of antibiotics in rabbits
1959	Gibson and Cooke [198]	Demonstrated that sweating could be induced by pilocarpine iontophoresis
1973	Corneau and Brummett [199]	Used iontophoresis for local anesthesia of the ear
1986	Gangarosa and Hill [200]	Applied iontophoresis of vidarabine monophosphate for treatment of herpes orolabialis
1992	Rigano <i>et al.</i> [201]	Demonstrated treating of burned ears by gentamicin iontophoresis
2003	Monti <i>et al.</i> [202]	Studied the iontophoresis on the permeation of β -blocking agents across rabbit corneas <i>in vitro</i>
2013	Patane <i>et al.</i> [203]	Treated inflammatory disorders of the orbit with repeated transscleral iontophoresis of dexamethasone phosphate in the rabbit eye

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6 Troubleshooting

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6.1 Protein electrophoresis troubleshooting

Problem	Cause	Solution
CA electrophoresis		
The surface of CA membrane is spotty.	The drying of cellulose acetate membrane has taken too long.	Shorten the drying.
No protein bands on CA membrane.	The proteins have left CA membrane.	Monitor the running of dye front.
	The proteins have precipitated on application site.	Let electrophoresis run in another buffer with a different pH value.
Agarose electrophoresis		
The agarose gel is too soft.	The agarose concentration is too low.	Check the recipe. Increase the 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.
Air bubbles in the agarose gel.	The support film or glass plates of casting cassette were unclean.	Do not touch the hydrophilic side of support film with fingers. Wash the glass plates before use.
	The glass plates or agarose solution were too cold.	Heat the glass plates to 60–70 °C before casting.
The agarose gel separates from support film.	An incorrect support film was used.	Do not exchange the support films for agarose and polyacrylamide gels.
	The gel was cast on the wrong side of the support film.	Cast the gel on the hydrophilic side of the support film. Check the sides with water drops.

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(continued)

Agarose electrophoresis

The agarose 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.
The gel “sweats” (is covered with water drops) during electrophoresis run.	Strong electroosmosis.	Add 20 g/dL glycerol or 10 g/dL sorbitol to the agarose gel.

Polyacrylamide electrophoresis**Prior to electrophoresis**

The monomeric solution polymerizes too slowly or does not polymerize.	The concentration of TMEDA or APS in the monomeric solution is too low.	Increase the concentration of TMEDA or APS in the monomeric solution.
	The APS solution is too old or is stored improperly. The gelling temperature is too low. Too much oxygen in the monomeric solution. The pH value for the gel polymerization is not optimal.	Use a new APS solution. Store the APS solution in the refrigerator. Cast gels at 20–25 °C. Vent the monomeric solution with a water-jet pump. Increase the concentration of TMEDA, if the pH value of the monomeric solution is too low.
The monomeric solution polymerizes too quickly.	The concentration of TMEDA or APS in the monomeric solution is too high. The polymerization temperature is too high.	Check the recipe for gel casting. Reduce the concentrations of TMEDA or APS. Cast gels at 20–25 °C.
The polyacrylamide gel is too soft.	The concentration of monomeric solution was too low.	Increase the concentration of monomeric solution.
	The APS solution was too old or stored improperly.	Use a new APS solution. Store the APS solution in the refrigerator.
The polyacrylamide gel is sticky.	The concentration of acrylamide or BIS in the monomeric solution is too low. The APS solution is too old or stored improperly.	Check the composition of the monomeric solution. The usable life of an APS solution is a week. Store the APS solution in the refrigerator.
	The atmospheric oxygen inhibited the polymerization of gel surface.	Degas the monomeric solution with a water-jet pump and overlay the monomeric solution with deionized water after casting.

(continued)

Prior to electrophoresis		
Air 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 the air bubbles in the casting cassette by vigorous knocking on it.
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 monomeric solution on the hydrophilic side of the support film; check first the side with water drops.
	The support film was stored incorrectly or too long.	Store the support film dry and in the dark at room temperature.
The polyacrylamide 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.
During electrophoresis		
The electric current does not flow or flows too little during the electrophoresis run.	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. The cooling is insufficient.	Reduce the electric power. Check the coolant temperature (10–15 °C are recommended). The cooling plate should be made of glass, metal or best of ceramic.
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.
	Poor contact between the gel and the electrode bridges.	Weight the electrode bridges on the gel with a glass plate.

(continued)

After electrophoresis

No protein bands in the gel.	Proteins have left the gel. The protein concentration in the sample was very low. The cathode and anode were exchanged. The detection sensitivity of the staining method is too low.	Monitor the movement of dye front. Apply more sample volume or concentrate the sample. Check the connections to the power supply. Use another staining method, e.g., silver staining.
Precipitates on the place of sample application.	The proteins were not dissolved 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. Dilute the sample and desalt.
The protein bands are pale.	The protein concentration or sample volume was low. The sample was not completely dissolved. The sensitivity of the staining method is low.	Concentrate the sample or apply more volume of the sample. Treat the sample with ultrasound; centrifuge, if there is opacity. Stain more time. Use another staining method, for example, silver staining.
The protein bands have formed tails.	Precipitates or solid components in the sample. The sample was not dissolved. The sample was old.	Centrifuge the sample before applying. Treat the sample with ultrasound. Use fresh sample.
The samples from adjacent tracks have run into each other.	The sample volumes were too large. The application template did not laid tight on the gel.	Concentrate the samples and apply fewer volumes. Press lightly the application template toward the gel to remove any air between the template and gel.
The protein bands are blurred.	Too much protein in the sample. The separated proteins diffused in the gel.	Apply smaller volume or dilute the sample. Fix the proteins immediately after electrophoresis.
The gel rolls on during drying.	The gel contracts.	Add 5 g/dL glycerol in the final wash solution.
The gel dissolves from the support film during staining.	The bonds between the support film and the gel were partially hydrolyzed by strong acids (TCA) in some staining solutions.	The concentrated gels ($T > 10$ g/dL) should have cross-linking degree $C = 0.02$.

(continued)

After electrophoresis

The Coomassie color has insufficient intensity.	SDS was not completely removed from the proteins. The alcohol concentration in the destaining solution was too high.	Clean the gels more time to remove SDS, and color longer time. Reduce the concentration of ethanol or methanol in the destaining solution, or use a colloidal staining method.
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6.2 IEF troubleshooting

Problem	Cause	Solution
Prior to isoelectric focusing		
Polyacrylamide gel has not polymerized or is sticky.	The concentration of monomers was too low.	Increase monomeric concentration.
	The concentration of TMEDA or APS in the monomeric solution was too low.	Use 5 μ L of 10 g/dL TMEDA and 5 μ L of 10 g/dL APS per 1 mL of monomeric solution.
	The APS solution was too old or improperly stored.	Use new APS solution. Store the APS solution in the refrigerator at 4–8 °C for a week.
	The concentration of air oxygen in the monomeric solution was too high.	Degas the monomeric solution with a water-jet pump.
Monomeric solution has polymerized too fast.	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 add them.
	The concentration of TMEDA or APS in the monomeric solution was too high.	Check the recipe for gel casting.
Polyacrylamide gel separates from the support film.	The environment temperature was too high.	Cast gels at 20–25 °C.
	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 sides with drops of water.
	The monomeric 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 later.

(continued)

Problem	Cause	Solution
Prior to isoelectric focusing		
The gel does not adhere to the support film, but to the glass plate.	The glass plate is hydrophilic.	Clean the glass plate and treat it with Repel-silane.
Air bubbles in the gel.	The glass plate that comes in contact with the monomeric solution was dirty. The support film was unclean.	Wash the glass plate before use. Do not touch the hydrophilic side of the support film with fingers.
During isoelectric focusing		
It does not flow or flow too little electric current.	One of the connectors has no or poor contact. There is a bad contact with the electrode strips.	Check all connections. The default setting for IPG gel is 5,000 V, 1.0 mA, and 5.0 W. Put a glass plate onto the gel.
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 cell.	The electric power is too high. The cooling is insufficient.	The electric power should not exceed 2.5 W/mL gel. 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 sorbitol to the monomeric 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. Check the cooling temperature. The cooling block should be made of glass, metal, or best of ceramic.
Urea crystallizes in IPG gels.	The IEF temperature is too low. The surface dries out.	Focus at 10–15 °C. Add 0.5 mL/dL Nonidet NP-40 to the rehydration solution.

(continued)

During isoelectric focusing		
Iso-pH-lines in the gel.	The salt concentration in the sample is too high. The concentration of APS was too high.	Desalinate the samples. Reduce the APS concentration or use rehydratable gels.
After isoelectric focusing		
The protein bands are not sharp.	The concentration of the carrier ampholytes was too low. The electrofocusing time was too short.	The concentration of a carrier ampholytes in the gel should be at least 2 g/dL. Prolong the electrofocusing time.
The bands pull tails.	There were precipitates or particles in the samples.	Centrifuge the sample.
The bands are crooked.	The salt concentration in the samples was too high.	Desalinate the samples.
The samples from adjacent tracks run into each other.	The sample volumes were too large. The application template was not placed tight on the gel.	Concentrate the samples and apply less volumes. Press gently the application template toward the gel to remove any air bubbles between the template and gel.
The background remains blue after Coomassie staining.	Basic immobiline groups bind to Coomassie dyes.	Use colloidal or silver staining method.

6.3 Nucleic acid electrophoresis troubleshooting

Problem	Cause	Solution
Prior to electrophoresis		
The consistency of agarose gel is insufficient.	The agarose concentration is too low. The gelling time of agarose was too short.	Check the recipe. Keep agarose dry because it is hygroscopic. The gelling of agarose solution should continue at least 60 min at room temperature or better overnight.

(continued)

Problem	Cause	Solution
Prior to electrophoresis		
Air bubbles in agarose gels.	The support film or glass plates that came into contact with the agarose solution were dirty. The agarose solution or glass plates were cold – the agarose solution gelled during casting.	Do not touch the hydrophilic side of the support film or glass plate with fingers. Prior to use wash the glass plates with dishwashing detergent. Preheat the agarose solution and glass plates at 70 °C.
The agarose 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 gel only on the hydrophilic side of support film. Prior to casting, check the film side with water drops.
The monomeric solution does not polymerize or polymerizes to slow.	The concentration of TMEDA or APS in the monomeric solution was too low. The APS solution was too old or stored improperly. The room temperature was too low.	Check the composition of the monomeric solution. Increase the concentration of TMEDA or APS. Use new APS solution. Store the APS solution in the refrigerator. Cast gels at 20–25 °C.
The polyacrylamide gel is too soft and sticky.	The concentration of acrylamide or BIS was too low. The concentration of TMEDA or APS in the monomeric solution was too low. The APS solution was old. There was too much oxygen in the monomeric solution.	Increase the concentration of acrylamide or BIS. Use 5 µL of 10 g/dL TMEDA and 5 µL of 10 g/dL APS per 1 mL of monomeric solution. The maximum storage for an APS solution in the refrigerator is a week. Degas the monomeric solution using a water-jet pump.
Air bubbles in the polyacrylamide gel.	The support film was unclean. The glass plate that came in contact with the monomeric solution was dirty.	Do not touch the hydrophilic side of support film with fingers. Wash the glass plate with dishwashing detergent or ethanol before use.

(continued)

Problem	Cause	Solution
Prior to electrophoresis		
The polyacrylamide gel separates from support film.	An improper support film was used. The gel was cast onto the wrong side of the support film. The support film was stored incorrectly or too long. The glass plate that came into contact with the monomeric solution was unclean.	Do not exchange the support film for agarose and polyacrylamide gels. Cast gel only on the hydrophilic side of the support film, first checking the side with water drops. Store the support film at room temperature in the dark. Prior to use, wash the glass plate with dishwashing detergent or ethanol.
The gel does not adhere to the support film, but to the glass plate.	The glass plate is too hydrophobic.	Treat the glass plate with Repel-silane.
During electrophoresis		
It does not flow or flows too little electric current.	One of the connectors has no or poor contact. Poor contact between the electrode strips and gel.	Check all connections. Put a glass plate onto the electrodes on the gel.
The gel “sweats” – is covered with water drops.	No water binding additives in the gel.	Add 20 g/dL glycerol or 10 g/dL sucrose to the monomeric 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.	The polyacrylamide gel contains thin spots because the support film was not fixed on the glass plate of casting cassette.	Prior to casting, roll the support film on a wet glass plate to get an even contact between the film and plate.

After electrophoresis

No nucleic acid bands on the gel.	<p>The cathode and anode were changed.</p> <p>The nucleic acids have left the gel.</p> <p>The mass of the nucleic acid was too large – the nucleic acid could not enter the gel.</p> <p>Insufficient quantity of DNA on the gel.</p> <p>DNA was degraded.</p> <p>Improper light source was used for visualization of ethidium bromide-stained DNA.</p>	<p>Check the connections to the power supply.</p> <p>Monitor the passage of the dye front.</p> <p>Use agarose gel or reduce the total concentration <i>T</i> of the polyacrylamide gel.</p> <p>Increase the amount of DNA; don't exceed 50 ng/band.</p> <p>Avoid nuclease contamination.</p> <p>Use a short wavelength (254 nm) light.</p>
The nucleic acid bands are very weak.	The concentration of nucleic acids or the sample volume was too small.	Concentrate the sample, apply more volume, and use more sensitive detection methods.
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.	Excess salt in the samples.	Check the salt concentrations in the gel and electrode buffers.
DNA bands are smeared.	<p>DNA was degraded.</p> <p>Too much DNA was loaded on the gel.</p> <p>Too much salt in the DNA sample.</p>	<p>Avoid nuclease contamination.</p> <p>Decrease the DNA concentration.</p> <p>Remove excess salts using ethanol precipitation.</p>
The nucleic acid bands form tails.	<p>The concentration of nucleic acids in the sample was too high.</p> <p>Diffusion after the separation.</p>	<p>Apply small sample volume or dilute the sample.</p> <p>Shorten the time between the end of electrophoresis and detection procedure.</p>
The samples from adjacent tracks ran into each other.	<p>The sample volumes were too much.</p> <p>The application template was not tight on the gel.</p>	<p>Concentrate the samples and apply smaller volumes.</p> <p>Press lightly the application template toward the gel to push away air bubbles between the template and gel.</p>

(continued)

After electrophoresis

The DNA fragments are arranged in a curved manner – “smiling” effect. Autoradiogram is too light.	The temperature in the central part of the gel was higher than in the gel margins. Old label Not enough primer Not enough template DNA Low enzyme activity	Reduce the voltage. Apply the samples at some distance from the gel margins. Use new label. Check the primer concentration. Check the template concentration. Use ~0.5 pmol template DNA per reaction. Use fresh enzyme.
Amplified DNA concentration is lower than expected.	Amplification cycle setting is low. Low MgCl ₂ concentration. Low sample concentration. Inhibitors in the template. PCR reagents are contaminated or expired. Degraded primers.	Add three to five cycles. Increase the MgCl ₂ concentration. Increase the sample concentration. Purify the template. Use fresh PCR reagents. Store unused primers at –20 °C.
PCR inhibition.	Sample contains PCR inhibitors: hemoglobin, heparin, and polysaccharides.	Dilute the sample before amplification to reduce the concentration of PCR inhibitors.
Intensive background.	Impure reagents were used. The water quality was poor.	Use reagents p.a. Use only deionized water.

6.4 Blotting troubleshooting**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.
Nitrocellulose membrane is prewet unevenly.	The nitrocellulose membrane is old or contaminated.	Use new nitrocellulose membrane.
Nitrocellulose membrane is getting fragile after baking.	The nitrocellulose membrane is overbaked.	Check oven temperature.

(continued)

During blotting

No electric current or too small electric current.	One of the connectors had no or poor contact.	Check all connections.
Electric power is too high.	Electric current flows around the blot sandwich.	Cut filter papers and blot membrane according to gel size.
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.
Blotter becomes hot during blotting.	Electric current intensity is very high.	Blot at 0.8–1.0 mA/cm ² .

After blotting

No transfer on the blot membrane.	The direction of blotting was wrong.	Blot toward the anode in an alkaline buffer.
Transfer is incomplete.	The transfer time was too short. The electric current passed around the blot sandwich.	Extend the transfer time. Cut the blot membrane and filter papers according to the gel size.
Transfer is unregularly.	The electric current flow was irregular because the electrode plates were dry.	Prior to blotting wet the electrode plates with deionized water.
Loss of protein bands.	Low molecular mass proteins ($M_r < 20,000$) may be lost during the post transfer washes.	Use glutardialdehyde fixation and smaller pore size nitrocellulose membranes (0.2 μm).
Proteins have left the gel, but are not found on the blot membrane.	Low-molecular peptides have been washed during the detection procedure.	When using a nylon membrane, the fixative should contain glutardialdehyde.
Strong background on the blot membrane.	Blocking was ineffective. Cross-reactions with the blocking agent.	Block longer or use higher temperature (37 °C). Use a different blocking agent, i.e. fish gelatin or skim milk.

(continued)

After blotting

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 \approx 300–350 mg of total RNA (3–5%). Use optimal hybridization temperature. Use freshly synthesized probes. Increase exposure time.
More bands after RNA hybridization instead of one expected.	Cross-hybridization. The hybridization temperature was very low.	The probe concentration was too high. Increase the hybridization temperature up to 52 °C.
Specks and splotches.	Contaminated membrane. Bubbles in hybridization solution.	Use new membrane. Avoid bubbles, mix during hybridization.

6.5 Iontophoresis troubleshooting

Problem	Cause	Solution
Prior to isoelectric focusing		
Device does not power up.	Battery Battery contact	Check the battery. If necessary, change it. Check both contacts between the battery and biological tissue.
Iontophoresis device is shutting down.	Treatment site has high resistance.	Leave the electric current to 0.5 mA.
	Treatment site is callused.	Wet the callused site.
Battery indicator lights when device is turned on.	Battery voltage is too low.	Replace the battery.
Battery indicator lights during treatment.	Battery voltage is decreasing during treatment.	Replace the battery.
Electric current stops.	Treatment has paused by decreasing electric current to zero mA.	Restart the treatment.
Resistance limits.	Skin resistance at the electrode site is too high.	Set the electric current level as high as possible and prolong the time to deliver desired dose.

Problems

- 1 Fundamentals of electrophoresis
- 2 Electrophoresis of proteins
- 3 Electrophoresis of nucleic acids

1 Fundamentals of electrophoresis

- 1.1 pK value of dihydrogen phosphate ($\text{H}_2\text{PO}_4^- \leftrightarrow \text{H}^+ + \text{HPO}_4^{2-}$) is equal to 7.20 at 25 °C. Calculate its pK_c value at $I = 0.1$ mol/L.
- 1.2 Calculate the ionic strength of a solution containing 0.1 mol/L CH_3COONa and 0.2 mol/L NaCl .
- 1.3 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.
- 1.4 Calculate the buffer capacity of a buffer with $\text{pH} = 5.21$ 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.
- 1.5 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 $12.75 \times 10^{-9} \text{ m}^2/(\text{s V})$ at 0 °C and $27.86 \times 10^{-9} \text{ m}^2/(\text{s V})$ at 25 °C. Use the ionic mobility parameter of the linear equation for calculation.
- 1.6 Using the equation $\mu = \frac{dl}{tU}$, calculate the mobility of albumin polyion $\mu_{\text{albumin}^{n-}}$, 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).
- 1.7 Which is the most important buffer in the cell?
 - A) Hydrogen carbonate buffer
 - B) Hemoglobinate buffer
 - C) Proteinate buffer
 - D) Hydrogen phosphate buffer
- 1.8 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

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- 1.9 Which buffer is most significant for the blood plasma?
- A) Proteinate buffer
 - B) Hydrogen carbonate buffer
 - C) Hemoglobinate buffer
 - D) Hydrogen phosphate buffer
- 1.10 How high 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
- 1.11 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
- 1.12 How high 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

2 Electrophoresis of proteins

- 2.1 How can be prepared a monomeric solution of acrylamide and BIS with a total monomeric 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, if the concentration of the leading electrolyte c_{HA} is equal to 0.10 mol/L?
- 2.3 Which concentration will reach the protein H_nP, if the concentration of the strong leading acid $c_{HA} = 0.10$ mol/L and the Kohlrausch function $F_k = 0.02$?

- 2.4 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, which are located from each other at a distance l of 10 cm.
- 2.5 At what pH value borate, taurinate, and glycinate ions will follow certain proteins, if the slowest of them has a velocity of $-13 \times 10^{-9} \text{ m}^2/(\text{s V})$ and is running in a solution with I of 0.1 mol/L at 25 °C? At 25 °C the absolute mobilities of these ions are equal to -33.63×10^{-9} , -34.11×10^{-9} , and $38.45 \times 10^{-9} \text{ m}^2/(\text{s V})$, respectively. The pK values of the corresponding acids at the same temperature and $I = 0.1 \text{ mol/L}$ are 9.02, 8.84, and 9.56, respectively.
- 2.6 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 $\text{pK}_{\text{HTRIS}^+}$ and glycine pK_{HG} at 25 °C are 8.07 and 9.78, respectively.
- 2.7 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 an alpha-helix
 - D) Hydrogen bonds, which are perpendicular to the axis of the polypeptide chain
- 2.8 Which amino acid forms disulfide bridges in proteins?
- A) Methionine
 - B) Serine
 - C) Cysteine
 - D) Threonine
- 2.9 The isoelectric point pI (pH(I)) of pepsin (an enzyme in gastric juice) is approximately equal to 1. Which amino acids are present in it?
- A) Tryptophan and tyrosine
 - B) Serine and alanine
 - C) Aspartic and glutamic acid
 - D) Lysine and arginine
- 2.10 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 speed in buffers of different pH values
 - D) Proteins are separated according to their molecular masses

- 2.11 Blotting techniques are used to identify
- A) Unique proteins
 - B) Nucleic acid sequences
 - C) Both A and B
 - D) None of the above
- 2.12 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

- 3.1 How many base pairs have a double-helical DNA with M_r of 2×10^6 and 2×10^7 ?
- 3.2 How many electric charges have single-stranded RNAs with M_r of 4×10^4 and 4×10^5 , respectively?
- 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 Calculate the electrophoretic mobility μ of DNA, which has run a distance d of 127 mm in an electric field with a distance between the electrodes of 20 cm at voltage U of 250 V in one hour electrophoresis.
- 3.7 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
- 3.8 Which are the bonds between neighboring bases in a DNA strand?
- A) Ester bonds
 - B) Covalent bonds
 - C) Hydrogen bonds
 - D) Hydrophobic interactions

- 3.8 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
- 3.10 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
- 3.11 Which bonds between both DNA strands are broken at denaturation?
- A) 3',5'-Phosphodiester bonds
 - B) Covalent bonds
 - C) Hydrogen bonds
 - D) Ionic bonds
- 3.12 Southern blotting is a method for checking the presence of
- A) DNA
 - B) RNA
 - C) Protein
 - D) None of the above

Solution of problems

- 1 Fundamentals of electrophoresis
- 2 Electrophoresis of proteins
- 3 Electrophoresis of nucleic acids

1 Fundamentals of electrophoresis

- 1.1 It follows from the relationship between pK and the ionic strength that

$$pK_c = 7.20 - \frac{2 \times 0.1^{1/2}}{1 + 0.1^{1/2}} = 7.20 - 0.48 = 6.72$$

- 1.2 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

$$I = \frac{1}{2} [0.3 \times 1^2 + 0.1(-1)^2 + 0.2(-1)^2] = 0.3 \text{ mol/L}$$

- 1.3 If 0.01 mol/L HCl is added to a buffer,

$$pH = 4.64 + \log \frac{0.1 - 0.01}{0.1 + 0.01} = 4.55$$

If 0.01 mol/L NaOH is added to a buffer,

$$pH = 4.64 + \log \frac{0.1 + 0.01}{0.1 - 0.01} = 4.73$$

- 1.4 The pH value of the buffer is equal to the value of the pK_c of pyridinium ion. Therefore, $\alpha = 0.5$, i.e., the equilibrium concentrations of pyridine and pyridinium ion are equal. Then it follows that

$$\beta = \ln 10 \times 0.5(1 - 0.5) \times 0.1 = 0.0576 \text{ mol/L}$$

- 1.5 At 0 °C (273.15 K), the values of η and ϵ_r of water are equal to 1.787×10^{-3} Pa s, and 87.74, respectively, i.e., the parameters of the ionic mobility $p_i = 15.45 \times 10^{-9} z_i I^{1/2} \text{ m}^2/(\text{s V})$. At 25 °C (298.15 K), the values of η and ϵ_r of water are equal to 0.8904×10^{-3} Pa s, and 78.30, respectively, i.e., the parameter of the ionic mobility $p_i = 31.42 \times 10^{-9} z_i I^{1/2} \text{ m}^2/(\text{s V})$. Then, at 0 °C and $I = 0.01$ mol/L

$$\mu_{\text{HTRIS}^+} = (12.75 - 15.45 \times 0.01^{1/2}) \times 10^{-9} = 11.20 \times 10^{-9} \text{ m}^2/(\text{s V})$$

and at the same temperature but at $I = 0.07 \text{ mol/L}$

$$\mu_{\text{HTRIS}^+} = (12.75 - 15.45 \times 0.07^{1/2}) \times 10^{-9} = 8.66 \times 10^{-9} \text{ m}^2/(\text{s V})$$

At 25°C and $I = 0.01 \text{ mol/L}$

$$\mu_{\text{HTRIS}^+} = (27.86 - 31.42 \times 0.01^{1/2}) \times 10^{-9} = 24.72 \times 10^{-9} \text{ m}^2/(\text{s V})$$

and at the same temperature but at $I = 0.07 \text{ mol/L}$

$$\mu_{\text{HTRIS}^+} = (27.86 - 31.42 \times 0.07^{1/2}) \times 10^{-9} = 19.55 \times 10^{-9} \text{ m}^2/(\text{s V})$$

- 1.6 Taking into account that $25 \text{ mm} = 25 \times 10^{-3}$, $100 \text{ mm} = 100 \times 10^{-3}$, and $1 \text{ h} = 3,600 \text{ s}$, according to the ionic mobility equation,

$$\mu_{\text{albumin}^{n-}} = \frac{25 \times 10^{-3} \times 100 \times 10^{-3}}{3,600 \times 250} = 2.8 \times 10^{-9} \text{ m}^2/(\text{s V})$$

1.7 D

1.8 D

1.9 B

1.10 C

1.11 C

1.12 A

2 Electrophoresis of proteins

- 2.1 The total monomeric 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, in g/dL. According to the problem, these equations can be transformed into

$$a + b = 50$$

and

$$0.03(a + b) = b$$

where from 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

$$C_{HB} = F_k \times C_{HA} = 0.8 \times 0.10 = 0.08 \text{ mol/L}$$

- 2.3 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

$$C_{H_nP} = F_k \times C_{HA} = 0.02 \times 0.10 = 0.002 \text{ mol/L}$$

- 2.4 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

$$\mu_{alb} = -\frac{0.038 \times 0.1}{3,600 \times 250} = -4.2 \times 10^{-9} \text{ m}^2/(\text{s V})$$

2.5 From the equation of the ionic mobility parameter follows that at $I = 0.1$ mol/L and $25\text{ }^\circ\text{C}$, the mobility of borate, taurinate, and glycinate ion is -28.51×10^{-9} , -24.17×10^{-9} , and -23.69×10^{-9} $\text{m}^2/(\text{s V})$, respectively. Therefore, the dissociation degrees of the corresponding acids

$$\alpha_{\text{boric acid}} = \frac{-13 \times 10^{-9}}{-23.69 \times 10^{-9}} = 0.55$$

$$\alpha_{\text{taurine}} = \frac{-13 \times 10^{-9}}{-24.17 \times 10^{-9}} = 0.54$$

and

$$\alpha_{\text{glycine}} = \frac{-13 \times 10^{-9}}{-28.51 \times 10^{-9}} = 0.46$$

Hence, according to the Henderson–Hasselbalch equation, glycine, taurine, and boric acid should be dissolve at $\text{pH} = 9.49, 8.91,$ and 9.11 , respectively.

2.6 From the Debye–Hückel equation follows that

$$\text{p}K_{\text{cHTRIS}^+} = 8.07 + \frac{0.01^{1/2}}{1 + 0.01^{1/2}} = 8.16$$

and

$$\text{p}K_{\text{cHG}} = 9.78 + \frac{0.01^{1/2}}{1 + 0.01^{1/2}} = 9.69$$

Then, according to Henderson–Hasselbalch equation,

$$\alpha_{\text{TRIS}} = (1 + 10^{\text{pH} - \text{p}K_{\text{c}}})^{-1} = (1 + 10^{8.30 - 8.16})^{-1} = 0.420$$

and

$$\alpha_{\text{HG}} = (1 + 10^{\text{p}K_{\text{c}} - \text{pH}})^{-1} = (1 + 10^{9.69 - 8.30})^{-1} = 0.039$$

Hence,

$$c_{\text{TRIS}} = \frac{0.01}{0.420} = 0.024 \text{ mol/L}$$

and

$$c_{\text{glycine}} = \frac{0.01}{0.039} = 0.256 \text{ mol/L}$$

2.7 A

2.8 C

2.9 C

2.10 A

2.11 C

2.12 C

3 Electrophoresis of nucleic acids

- 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. 2×330 . Therefore, a double-helical DNA with M_r of 2×10^6 contains

$$\frac{2 \times 10^6}{2 \times 330} \approx 3,000$$

base pairs, and a double-helical DNA with M_r of 2×10^7 contains

$$\frac{2 \times 10^7}{2 \times 330} \approx 30,000$$

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 4×10^4 has

$$\frac{4 \times 10^4}{330} \approx 120$$

negative charges, and a single-stranded RNA with M_r of 4×10^5 has

$$\frac{4 \times 10^5}{330} \approx 1,200$$

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:

$$\frac{\mu}{\mu_0} = 10^{-K_R c}$$

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 mobility of DNA

$$\mu_{\text{DNA}} = \frac{v_{\text{DNA}}}{E} = \frac{d/t}{U/l} = \frac{dl}{tU}$$

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, $t = 3,600$ s, and $U = 250$ V. Therefore,

$$\mu_{\text{DNA}} = \frac{0.127 \times 0.2}{3,600 \times 250} = 28.2 \times 10^{-9} \text{ m}^2/(\text{s V})$$

3.7 D

3.8 D

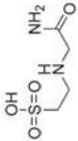


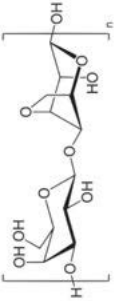
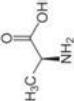
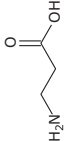
3.9 A

3.10 B

3.11 C

3.12 A

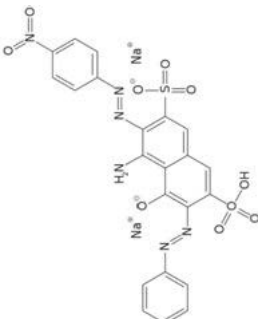


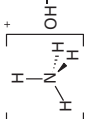

Reagents for electrophoresis

Reagent	Molecular formula	M_r	Density, d	pK_{25C}	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		630.55			
L-Alanine		89.09		9.69	9.0–10.4
β -Alanine		89.09		10.19	9.5–10.9

(continued)

<https://doi.org/10.1515/9783110761641-041>

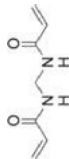
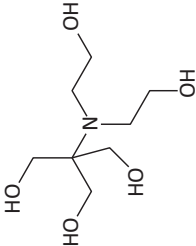
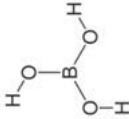
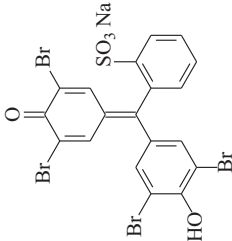
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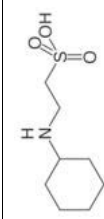
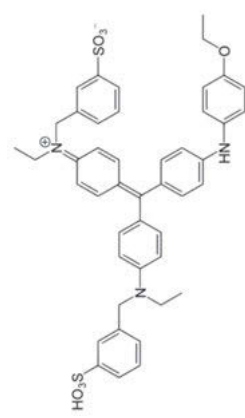
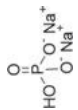
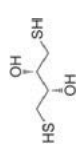
Reagent	Molecular formula	M_r	Density, d	pK_{25C}	Useful pH range
Amido black 10B		616.49			
4-Aminobutyric acid (GABA)		103.12	10.43		9.7–11.1
Ammediol (2-amino-2-methyl-1,3-propanediol)		105.14			
Ammonium hydroxide		35.05			
Ammonium sulfate		132.13			

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

(continued)

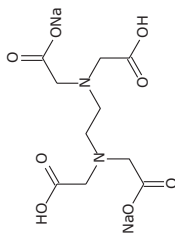
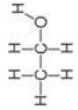

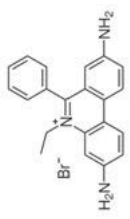


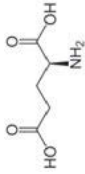
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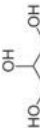
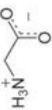
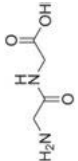
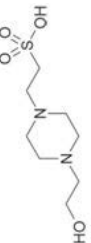
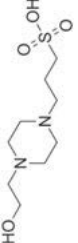
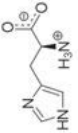


Reagent	Molecular formula	M_r	Density, d	pK_{25C}	Useful pH range
BIS (Bisacrylamide, Diacrylamide)		127.13			
BISTRIS		209.24	6.46	5.8-7.2	
Boric acid		61.83	9.24	8.5-9.9	
Bromophenol blue Na salt		691.94			

Calcium lactate		218.22	
Carrier ampholytes, pH = 3-10			8.6-10.0
CHES		207.29	9.30
Coomassie brilliant blue R-250		825.97	
Disodium hydrogen phosphate		141.96	
1,4-Dithiothreitol (DTT)		154.24	

(continued)




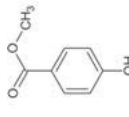


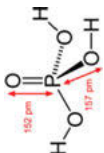
(continued)

Reagent	Molecular formula	M_r	Density, d	pK_{25C}	Useful pH range
EDTA, Na ₂		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			

Glutarialdehyde 25%		100.12	1.06
Glycerol 87%		92.09	1.23
Glycine		75.07	9.78
Glycylglycine		132.12	8.25
HEPES		238.30	7.48
HEPPS		252.33	8.00
L-Histidine		155.16	8.97
Hydrochloric acid 37%	H—Cl	36.46	1.18
Imidazole		68.08	6.95
Immobilines, pH = 4-10			
2-Iodoacetamide		184.96	

(continued)



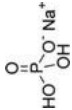
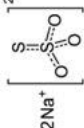
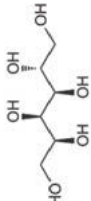
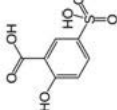
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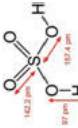
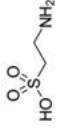
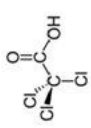
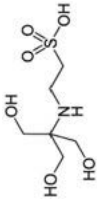
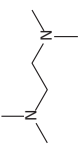
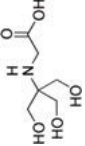
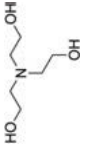
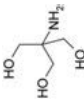
Reagent	Molecular formula	M_r	Density, d	pK_{25C}	Useful pH range
Lactic acid		90.08		3.84	
MES		195.23		6.16	5.5-6.9
Methanol		32.04	0.79		
Methylparaben (Nipagin, methyl 4-hydroxybenzoate)		152.15			
MOPS		209.26		7.20	6.5-7.9
Naphthol blue black – see <i>Amido black 10B</i>					
Nitric acid		63.01	1.40		
Phosphoric acid		98.00	1.69	2.15	

PIPES		302.36	6.76	6.1-7.5
Potassium dihydrogen phosphate		136.08	7.20	6.5-7.9
Potassium hydroxide	K^+OH^-	56.11		
L-Proline		115.13		
Pyridine		79.10	5.21	4.5-5.9
Pyronin Y (G)		302.80		
SDS		288.38		
Silver nitrate		169.87		

(continued)

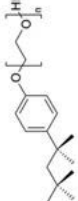
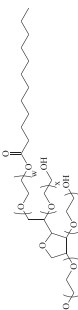
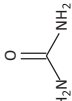

(continued)

Reagent	Molecular formula	M_r	Density, d	pK_{25C}	Useful pH range
Sodium acetate		82.03			
Sodium carbonate		105.99			
Sodium dihydrogen phosphate		119.98			
Sodium hydroxide	$\text{Na}^+\text{O}^- \text{H}$	40.00			
Sodium thiosulfate		158.10			
D-Sorbitol		182.17			
5-Sulfosalicylic acid		254.21			

Sulfuric acid		98.07		
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
Triethanolamine		149.19	7.74	
TRIS		121.14	8.07	7.4-8.8

(continued)

(continued)

Reagent	Molecular formula	M_r	Density, d	pK_{25C}	Useful pH range
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	Relative density, d
Acids			
HCOOH	46.03	90	1.21
		98	1.22
CH ₃ COOH	60.05	99.6	1.05
H ₃ PO ₄	98.00	85	1.70
HCl	36.46	36	1.18
H ₂ SO ₄	98.07	98	1.835
HNO ₃	63.01	70	1.42
Bases			
NH ₄ OH	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
<i>Check pH. Store at room temperature.</i>	

Electrophoresis buffer, pH = 4.7

Pyridine	2.50 mL
Acetic acid	2.50 mL
<i>n</i> -Butanol	5.00 mL
Deionized water to	100.00 mL
<i>Check pH. Store at room temperature.</i>	

Sodium-acetate buffer, 4x, pH = 5.0

Sodium acetate·3H ₂ O	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/10.1515/9783110761641-042>

Sodium acetate (SA) buffer, 3 mol/L, pH = 6.0

Na·acetate·H₂O 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 (Na₂HPO₄·7H₂O, M_r = 268.07).
 Add sodium phosphate monobasic monohydrate (NaH₂PO₄·H₂O, M_r = 137.99).
 Adjust solution to desired pH using HCl or NaOH.
 Add deionized water to 1,000 mL.

pH = 6.6

Na₂HPO₄·7H₂O 10.76 g (0.04 mol/L)
 NaH₂PO₄·H₂O 8.26 g (0.06 mol/L)
 Deionized water to 1,000.00 mL

pH = 7.0

Na₂HPO₄·7H₂O 15.48 g (0.06 mol/L)
 NaH₂PO₄·H₂O 5.83 g (0.04 mol/L)
 Deionized water to 1,000.00 mL

pH = 7.4

Na₂HPO₄·7H₂O 20.21 g (0.08 mol/L)
 NaH₂PO₄·H₂O 3.39 g (0.02 mol/L)
 Deionized water to 1,000.00 mL

Hydrogen phosphate buffer, 0.5 mol/L, pH = 6.8

Na₂HPO₄ 3.55 g
 NaH₂PO₄ 3.45 g
 Deionized water to 100.00 mL

Sodium chloride-sodium citrate (SSC) buffer, 20x, pH = 7.0

Na₂citrate·2H₂O 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

Phosphate-buffered saline (PBS), 10x, pH = 7.3

Na ₂ HPO ₄ ·7H ₂ O	1.15 g (4.3 mmol/L)
KH ₂ PO 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 (see there)	
Tween-20	0.50 mL
Deionized water to	100.00 mL

Store at room temperature.

TRIS-EDTA-Na (TEN) buffer, pH = 7.4

TRIS	0.61 g (0.05 mol/L)
<i>Add 1 mol/L HCl to pH = 7.4.</i>	
NaCl	0.88 g (0.15 mol/L)
Na ₂ EDTA	3.36 g (0.01 mol/L)
Deionized water to	100.00 mL

Store at room temperature up to 6 months.

TRIS-EDTA-SDS (TES) buffer, pH = 7.5

TRIS	0.12 g (0.01 mol/L)
<i>Add 1 mol/L HCl to pH = 7.5.</i>	
Na ₂ EDTA	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.

Low-salt buffer (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

TRIS-buffered saline (TBS), pH = 7.5

TRIS	1.21 g (0.10 mol/L)
<i>Add 1 mol/L HCl to pH = 7.5.</i>	
NaCl	0.88 g (0.15 mol/L)
Deionized water to	100.00 mL

Store at 4 °C up to several months.

Sodium chloride-TRIS-EDTA (STE) buffer, pH = 8.0

TRIS	0.12 g (10 mmol/L)
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Add 1 mol/L HCl to pH = 8.0.

Na ₂ EDTA	0.03 g (1 mmol/L)
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NaCl	0.58 g (0.1 mol/L)
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Deionized water to	100.00 mL
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TRIS-borate-EDTA (TBE) buffer, 10x, pH = 8.0

TRIS	108.00 g (0.89 mol/L)
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Boric acid	55.00 g (0.89 mol/L)
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Add 1 mol/L HCl to pH = 8.0.

Na ₂ EDTA	6.72 g (0.02 mol/L)
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Deionized water to	1,000.00 mL
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TRIS-buffered saline with Triton X-100 (TBST), pH = 8.0

TRIS	0.12 g (0.01 mol/L)
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Add 1 mol/L HCl to pH = 8.0.

NaCl	0.88 g (0.15 mol/L)
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Triton X-100	0.05 mL
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Deionized water to	100.00 mL
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Store at room temperature up to 6 months.

TRIS-EDTA (TE) buffer, pH = 8.0

TRIS	0.12 g (0.01 mol/L)
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Bring with HCl to pH = 8.0.

Na ₂ EDTA	0.03 g (1 mmol/L)
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Deionized water to	100.00 mL
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Triethanolamine (TEA) buffer, pH = 8.0

Triethanolamine	1.33 g (0.1 mol/L)
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Deionized water	80.00 mL
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Adjust pH to 8.0 with 1 mol/L

HCl

Deionized water to	100.00 mL
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Prepare fresh daily.

TRIS-borate-EDTA (TBE) buffer, 5x, pH = 8.3

TRIS	5.45 g (0.45 mol/L)
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Boric acid	2.78 g (0.45 mol/L)
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Na ₂ EDTA	0.17 g (0.005 mol/L)
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Deionized water to	100.00 mL
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Store at room temperature up to several weeks.

TRIS-acetate-EDTA (TAE) buffer, 50x, pH = 8.5

TRIS	24.20 g (0.2 mol/L)
Acetic acid	5.72 mL (0.1 mol/L)
Na ₂ EDTA	3.36 g (0.1 mol/L)
Deionized water to	100.00 mL

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,

$I = 10 \times 0.08 \text{ mol/L}$

Solution A (0.2 mol/L Na₃ citrate)

Na ₃ -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, $I = 10 \times 0.012 \text{ mol/L}$

TRIS	5.40 g (0.045 mol/L)
H ₃ BO ₃	1.55 g (0.025 mol/L)
Na ₂ EDTA·2H ₂ O	0.29 g (0.008 mol/L)
NaN ₃	0.10 g (0.015 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 (0.3 mol/L)
NaOH	1.48 g (0.4 mol/L)
Taurine	11.60 g (0.9 mol/L)
NaN ₃	0.65 g (0.10 mol/L)
Deionized water to	100.00 mL

TRIS-barbitalate buffer for agarose electrophoresis, 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 Na₂EDTA)

TRIS	7.20 g (0.60 mol/L)
Sodium barbitalate	10.30 g (0.25 mol/L)
[8.46 Barbital + 1.84 NaOH]	
Barbital	1.84 g (0.10 mol/L)
Na ₂ EDTA	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

Alkaline phosphatase (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)

MgCl₂ 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.

BES-buffered solution (BBS), 2x, pH = 6.95

BES 1.07 g (0.05 mol/L)

Na₂HPO₄ 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-Bromo-4-chloro-3-indolyl phosphate (BCIP) solution

BCIP 0.50 g

Dimethylformamide 10.00 mL

Store at -20 °C in aliquots.

4-Nitro blue tetrazolium (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)

MgCl₂ 0.56 g (0.059 mol/L)

Deionized water to 100.00 mL

NBT-BCIP substrate buffer, pH = 9.5

ALP buffer (see there) 50.00 mL

NBT buffer (see there) 0.22 mL

BCIP solution (see there) 0.17 µL

Prepare fresh.

Developing solution

NBT stock (see there) 66.00 µL

ALP buffer (see there) 10.00 mL

BCIP stock (see there) 33.00 µL

Solutions for native disc-electrophoresis

Acrylamide/bisacrylamide (T30, CO.03)

Acrylamide	29.00 g
Bisacrylamide	1.00 g
Deionized water to	100.00 mL

Wear gloves when working.

Acrylamide-BIS solution (T50, CO.03)

Acrylamide	48.50 g
BIS	1.50 g
Deionized water to	100.00 mL

Buffers for disc-electrophoresis according to Michov

Resolving buffer, 10x, pH = 6.5

[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, 10x, pH = 7.4

[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, 2x, pH = 6.5, 2 x 0.20 mol/L

Electrode buffer a	25.00 mL
Na ₂ EDTA	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

Solutions for SDS disc-electrophoresis

Nonreducing sample buffer for SDS electrophoresis, pH = 6.5, 0.20 mol/l

Electrode buffer <i>a</i>	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 x 0.2 mol/L

Electrode buffer <i>a</i>	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

SDS sample buffer, 2x, pH = 6.8

TRIS	4.00 mL (0.125 mol/L)
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Add 1 mol/L HCl to pH = 6.8.

Glycerol	2.00 mL
Na ₂ EDTA	0.02 g (0.006 mol/L)
SDS	0.40 g (0.14 mol/L)
2-Mercaptoethanol	0.40 mL (0.57 mol/L)

or

0.2 mol/L DTT	0.40 mL (0.008 mol/L)
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NaBPB	0.02 g
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Deionized water to	10.00 mL
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Filter and store at -20 °C up to 6 months.

Alkylating solution for SDS electrophoresis, 2x, for SDS-electrophoresis

2-Iodacetamide	3.70 g (0.20 mol/L)
Deionized water to	100.00 mL

Buffers for SDS electrophoresis according to Michov

Resolving buffer, 10x, pH = 6.5

[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, 10x, pH = 7.4

[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.

Solutions for IEF

Sample solution for native IEF

Carrier ampholytes 3-10	1.00 mL
Carrier ampholytes 4-9	0.25 mL
87% Glycerol	40.00 mL
Amberlite MB-3	1.00 g
<i>(Amberlite MB-3 deionizes the solution.)</i>	
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
<i>(Amberlite MB-3 deionizes the solution.)</i>	
Deionized water to	100.00 mL

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 ₂ O]	
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)
[x 2/3 = 32.03 mL]	
1,4-Dithiothreitol	0.62 g (0.04 mol/L)
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	5.00 mL (0.50 mol/L)
Methanol	20.00 mL
Deionized water to	100.00 mL

Degas before transfer.

Blocking buffer, pH = 7.4

TRIS	0.12 g (0.01 mol/L)
<i>Add 1 mol/L HCl to pH = 7.4.</i>	
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	1.21 g (0.1 mol/L)
<i>Add 1 mol/L HCl to pH = 7.8.</i>	
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.

Fixative solutions*Fixative TS (trichloroacetic acid with 5-sulfosalicylic acid), 10x*

TCA	50.00 g (3.06 mol/L)
SSA dihydrate	10.00 g (0.39 mol/L)
Deionized water to	100.00 mL

Fixative A (acetic acid)

Acetic acid	7.00 mL (1.11 mol/L)
Deionized water to	100.00 mL

Fixative GE (glutardialdehyde-ethanol), 10x

25 mL/dL Glutardialdehyde	10.00 mL (0.5 mol/L)
Ethanol	50.00 mL
Deionized water to	100.00 mL

Fixative CE (citric acid-ethanol), 2x

Citric acid	1.00 g (0.05 mol/L)
Ethanol	60.00 mL
Deionized water to	100.00 mL

Fixative AE (acetic acid-ethanol)

Acetic acid	10.00 mL (1.75 mol/L)
Ethanol	40.00 mL
Deionized water to	100.00 mL

Staining solutions*Ponceau S staining solution*

Ponceau S	0.30 g
Trichloroacetic acid	3.00 g
Deionized water to	100.00 mL

Ponceau S – TCA–sulfosalicylic acid (PTS) staining solution, 10x

Ponceau S	3.00 g
Trichloroacetic acid	30.00 g
Sulfosalicylic acid	30.00 g
Deionized water to	100.00 mL

Store at room temperature. Prior to use dilute tenfold with deionized water.

Coomassie brilliant blue R-250 (CBB) solution

CBB R250	0.20 g
Methanol	30.00 mL
Acetic acid	10.00 mL
Deionized water	60.00 mL

Coomassie brilliant violet R-200 (CBV) solution

CBV R-200	0.20 g
Methanol	30.00 mL
Acetic acid	10.00 mL
Deionized water	60.00 mL

Amido black 10B (AB) solution

Amido black 10B	0.20 g
Methanol	30.00 mL
Acetic acid	10.00 mL
Deionized water	60.00 mL

Sudan black B (SB) solution, 10x

Sudan black B	1.00 g
Ethanol to	100.00 mL

Bromophenol blue (BPB) solution

NaBPB·	0.10 g
[0.1 g/dL BPB + 0.01 g/dL NaOH]	
Citric acid	0.50 g
Deionized water to	100.00 mL

Filter.

Destaining solutions*Destaining solution A (acetic acid), 10x*

CH ₃ CO ₂ H	50.00 mL
Deionized water to	100.00 mL

Destaining solution AM (acetic acid–methanol)

Acetic acid	10.00 mL
Methanol	20.00 mL
Deionized water to	100.00 mL

Destaining solution AE (acetic acid–ethanol), 2x

Acetic acid	20.00 mL
Ethanol	60.00 mL
Deionized water to	100.00 mL

Destaining solution C (citric acid), 10x

Citric acid	5.00 g
Deionized water to	100.00 mL

Destaining solution P (phosphoric acid), 10x

Phosphoric acid	3.00 g
Deionized water to	100.00 mL

Silver staining solutions*Preparing solution for silver staining, 10x*

Na ₂ S ₂ O ₃ ·5H ₂ O	3.00 g (0.12 mol/L)
Deionized water to	100.00 mL
[2Na ₂ S ₂ O ₃ + AgBr → Na ₃ [Ag(S ₂ O 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 ₄ NO ₃	0.20 g (0.024 mol/L)
Silicotungstic acid·aq, H ₄ [Si(W ₃ O ₁₀) ₄]·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 ₂ CO ₃	8.00 g (0.755 mol/L)
[2[Ag(NH ₃) ₂]OH + HCHO → 2Ag + HCOONH ₄ + 3NH ₃ + H ₂ O]	
Deionized water to	100.00 mL

Silver stain kit for Paa gels, 10x

AgNO ₃	1.00 g (0.006 mol/L)
Deionized water to	100.00 mL

Store in a brown flask.

Developer 1 for silver staining of Paa gels, 10x

Na ₂ CO ₃	29.60 g (2.8 mol/L)
Deionized water to	100.00 mL

Developer 2 for silver staining of Paa gels

37 g/dL HCHO	10.00 mL
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Other solutions*Electrode buffer for CA (cellulose acetate) electrophoresis, pH = 8.6, I = 0.10 mol/L*

Sodium barbitalate	2.06 g (0.10 mol/L)
[16.94 Barbital + 3.68 NaOH]	
Barbital	0.40 g (0.02 mol/L)
NaN ₃	0.14 g (0.02 mol/L)
Deionized water to	100.00 mL

Denhardt's solution, 100x

Ficoll 400	10.00 g
Polyvinylpyrrolidone	10.00 g
BSA (Fraction V)	10.00 g
Deionized water to	500.00 mL

Filter sterilize and store at -20 °C in aliquots.

*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

Sucrose solution, 10 g/dL, pH = 7.5

TRIS	0.24 g (0.02 mol/L)
Deionized water to	80.00 mL
<i>Add 1 mol/L HCl to pH = 7.5.</i>	
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

Lysis buffer, pH = 8.0

TRIS	0.12 g (0.01 mol/L)
Deionized water to	80.00 mL
<i>Add 1 mol/L HCl to pH = 8.0.</i>	
NaCl	0.82 g (0.14 mol/L)
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.**Elution buffer, pH = 8.0*

TRIS	0.61 g (0.05 mol/L)
Deionized water to	80.00 mL
<i>Add 1 mol/L HCl to pH = 8.0.</i>	
Na ₂ EDTA	0.34 g (0.01 mol/L)
SDS	1.00 g (0.03 mol/L)
Deionized water to	100.00 mL

Filter sterilize and store at room temperature.

SI units and physical constants used in electrophoresis

The International system of units SI (Système International) was introduced in 1960 [1–3]. It contains seven *base units* (Table 1).

Table 1: SI base units.

Quantity	Abbreviation	Name of unit	SI unit
Mass	<i>m</i>	Kilogram	kg
Length	<i>l, b, h, δ, d, r, s, λ</i>	Meter	m
Time	<i>t</i>	Second	s
Thermodynamic temperature	<i>T</i>	Kelvin	K
Amount of substance	<i>n</i>	Mole	mol
Electric current	<i>I</i>	Ampere	A
Luminous intensity	<i>I_v</i>	Candela	cd

The *mol* is referred to as an amount *n* of a substance, which contains as much particles as 0.012 kg carbon isotope ¹²C. The particles can be of different nature: atoms, molecules, ions, or electrons. The following relationship exists between the mass *m*, in kg, and the amount, in mols, of a homogeneous substance *x*:

$$m = nM_x = nN_A m_x = nN_A M_r m_u \quad (1)$$

where $M_x = N_A m_x$ is the molar mass of the particle, in kg/mol; N_A ($6.022\,045 \times 10^{23} \text{ mol}^{-1}$) 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 ¹²C.

The derived units of the SI base units and the physical constants, which are used in the electrophoresis methods, are listed in Tables 2 and 3, respectively.

Table 2: Derived units of the 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, l (liter)
Volume flow rate	$q_v = dV/dt$	m^3/s
Velocity (rate) of migration	$v = ds/dt$	m/s
Velocity of an ion	$v_i = ds_i/dt$	m/s
Mobility of an ion	$\mu_i = v_i/E$	$m^2/(s V)$
Absolute mobility of an ion	$\mu_{\text{ooi}} = v_{\text{ooi}}/E$	$m^2/(s V)$
Effective mobility of an ion	$\mu'_i = \alpha\mu_i$	$m^2/(s V)$
Mechanics		
Density	$\rho = m/V$	kg/m^3
Force	$F = ma$	N (Newton) = $kg m/s^2 = CV/m$
Pressure	$p = F/A$	$Pa = N/m^2 = J/m^3$
Dynamic viscosity	$\eta = \tau dz/dv$	$Pa s = kg/(m s)$
Relative viscosity	$\eta_r = \eta/\eta_s$	Dimensionless
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	$^{\circ}C$ (degree Celsius)
Joule heating	$P = UI$	W
Electricity		
Electric charge	Q	$C = As$
Charge number (electrovalence)	z	Dimensionless

Table 2 (continued)

Derived quantity	Abbreviation and equation	SI unit
Electric current area density	$J = \frac{dl}{dS} = F \sum c_i z_i v_i$	A/m ²
Electric field strength (intensity)	$E = F/Q = dV/dr$	V/m = N/C
Electric potential	$\varphi, V = A/Q = Q/(4\pi\epsilon r)$	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	$F = QE = Q_1 Q_2 / (4\pi\epsilon r^2)$	N
Electric capacity	$C = dQ/dU = \epsilon S/d$	F (farad) = C/V
(Di)electric permittivity	$\epsilon = D/E$	F/m
Relative (di)electric permittivity	$\epsilon_r = \epsilon/\epsilon_0$	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 /(s V)
Specific conductivity	$\gamma, \sigma = \frac{1}{\rho} = F \sum c_i z_i \mu_i$	S/m
Molar conductivity	$\Lambda_m = \frac{\gamma}{c} = F(z^+ \mu^+ + z^- \mu^-)$	S m ² /(k)mol
Physical chemistry		
Amount of substance	n, ν	(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

Table 2 (continued)

Derived quantity	Abbreviation and equation	SI unit
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	$pH = -\log \frac{[H^+]}{[H^+]_o}$	Dimensionless
Isoelectric point	$pI, pH(I)$	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	$1.6605655 \times 10^{-27}$ kg
Elementary charge	e	$1.6021892 \times 10^{-19}$ C
(Di)electric constant	ϵ_0	$8.854187818 \times 10^{-12}$ F/m
Avogadro constant	N_A	6.022045×10^{26} kmol ⁻¹
Faraday constant	$F = N_A e$	9.648455461×10^7 C/kmol
Molar gas constant	$R = N_A k$	8,314.41 J/(kmol K)
Boltzmann constant	$k = R/N_A$	1.380662×10^{-23} J/K

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Electrophoresis terms

<i>Acid</i>	Proton donor. An acid gives a proton to a base.
<i>Affinity electrophoresis</i>	Zone electrophoresis in usually agarose gel containing specific ligands.
<i>Agarose</i>	Natural polymer composed of D-galactose and 3,6-L-anhydrogalactose. Agarose gel is used as separation medium in different electrophoresis methods, especially in the clinical routine.
<i>Agarose gel electrophoresis</i>	Zone electrophoresis on agarose gel.
<i>Allele</i>	Alternative form of a gene.
<i>Ampholyte</i>	Chemical compound having acidic and basic properties.
<i>Analytical electrophoresis</i>	Electrophoresis used for resolving of polyions as bands.
<i>Autoradiography</i>	Blackening on an X-ray film by irradiation of labeled with isotopes polyions, which are separated each from other by gel electrophoresis.
<i>Base</i>	Proton catcher. The base binds a proton from an acid.
<i>Blotter</i>	Device where transfer of proteins or nucleic acids onto a blot membrane is carried out.
<i>Blotting</i>	Method for transferring electrophoretically resolved bands onto a membrane where they can react with non-specific or specific reagents. Blotting is a proofing method, not an electrophoresis method.
<i>Buffer</i>	Solution of a base and its conjugate acid, which keeps constant pH value.
<i>Buffer capacity</i>	The capacity of a buffer to maintain constant pH value when the buffer is diluted or bases (acids) are added.
<i>Capillary electrophoresis</i>	Electrophoresis (zone electrophoresis, isotachopheresis, or isoelectric focusing) in capillaries.
<i>Carrier ampholytes</i>	Synthesized ampholytes that reach their isoelectric points in an electric field to form a stable pH gradient.
<i>Cellulose acetate</i>	Natural compound used for production of film-formed electrophoretic medium.
<i>Cellulose acetate electrophoresis</i>	Zone electrophoresis in cellulose acetate membranes.
<i>Densitometry</i>	Method for determining the number and concentration of electrophoretically separated bands by measuring their optical density using a light or laser beam.
<i>Deoxyribonucleates</i>	DNA polyions.
<i>Disc-electrophoresis</i>	Combination of isotachopheresis and zone electrophoresis, which takes place in appropriate buffer systems.
<i>DNA (deoxyribonucleic acids)</i>	Polynucleotides that consist of specifically arranged deoxyribonucleotide residues. They are negatively charged in a solution.
<i>DNase (deoxyribonuclease)</i>	Enzyme that catalyzes the hydrolysis of DNA.
<i>Effect of Hjerten</i>	Concentrating of polyions on the boundary between solutions with a lower and higher ionic strengths.

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(continued)

<i>Effect of molecular sieve</i>	Separation of polyions in a gel, depending on its pores.
<i>Effective mobility</i>	Ionic mobility multiplied by the dissociation degree of the electrolyte building the ion.
<i>Electric double layer</i>	Electric layer formed by a charged surface and its counter-ion(s).
<i>Electroblotting</i>	Blotting carried out in an electric field.
<i>Electrolyte</i>	Chemical compound that forms ions in a solution.
<i>Electrophoresis</i>	Separation of dissolved electrically charged particles in an electric field.
<i>Exons</i>	mRNA fragments containing genetic information for proteins. In most eukaryotes and in some prokaryotes, the exons are divided by introns.
<i>Fibrillar proteins</i>	Usually insoluble proteins whose polypeptide chains form filaments.
<i>Fluorography</i>	Blackening on a photosensitive film, caused by visible light irradiation, emitted by polyions labeled with isotopes.
<i>Free-flow electrophoresis</i>	Electrophoresis in a flowing buffer. It can be carried out as zone electrophoresis, isotachopheresis, or isoelectric focusing.
<i>Gel</i>	Porous soft material.
<i>Genome</i>	Total genetic information contained in the nucleic acids of an organism.
<i>Globular proteins</i>	Soluble proteins whose polypeptide chains are folded in the space.
<i>Gradient gel</i>	Gel with linearly or exponentially changeable concentration.
<i>Gradient gel electrophoresis</i>	Electrophoresis in a gradient gel.
<i>Gradient maker</i>	Device for casting gradient gels.
<i>Henderson–Hasselbalch equation</i>	Equation expressing the relationship between the pH value, on the one hand, and the pK_c value and ratio between the base and its conjugated acid, on the other.
<i>Homogeneous gel</i>	Gel of same concentration in all its parts.
<i>Homogeneous gel electrophoresis</i>	Electrophoresis in a homogeneous gel.
<i>Immuno-electrophoresis</i>	Zone electrophoresis on an agarose gel containing or receiving immunoglobulins. As a result, the electrophoretically resolved polyions take part in precipitation reactions.
<i>Introns</i>	mRNA fragments that are located between exons.
<i>Ionic strength</i>	Mathematical term that takes into account the concentration and electrovalence of all ions in a solution.
<i>Ionization constant</i>	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.
<i>Isoelectric focusing</i>	Electrophoresis in a pH gradient. The moving polyions stop at their isoelectric points.
<i>Isoelectric focusing with carrier ampholytes</i>	Isoelectric focusing in a pH gradient created by carrier ampholytes.
<i>Isoelectric focusing in immobilized pH gradients</i>	Isoelectric focusing in a pH gradient created by bound immobilines.

(continued)

<i>Isoelectric point</i>	pH value at a certain ionic strength and temperature, where the sum of all electric charges of a polyion is equal to zero.
<i>Isotachophoresis</i>	Electrophoresis that is carried out in a system of two or more buffers, which form a moving ionic boundary between them.
<i>mRNA (messenger RNA)</i>	Type of RNA. The information of mRNA is translated by ribosomes onto proteins.
<i>Net charge</i>	It is less (in absolute value) than the total charge due to the counter-ions, which are bound to the polyion surface, according to Stern model.
<i>Oligonucleotides</i>	Short nucleic acids, similar to oligopeptides, which serve as primers.
<i>Paper electrophoresis</i>	Zone electrophoresis on paper.
<i>Persistent function of Kohlrausch</i>	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.
<i>Polyacrylamide</i>	Polymer consisting of acrylamide and bis-acrylamide residues. The polyacrylamide gel has no electroosmosis and is the most widely used separation medium in electrophoresis.
<i>Polyacrylamide gel electrophoresis</i>	Zone electrophoresis in homogeneous polyacrylamide gels.
<i>Polyion</i>	Large particle, e.g. nucleate or proteinate, which has negative, positive, or both charges.
<i>Precast gels</i>	Polyacrylamide or agarose gels that are cast on a support film or support net prior to electrophoresis.
<i>Preparative electrophoresis</i>	Quantitative electrophoresis, during which the polyions are separated from each other into separate solutions.
<i>Protein</i>	Macromolecule that is composed of one or more polypeptide chains. It has many and different electric charges in a solution.
<i>Protolyte</i>	Electrolyte that gives or receives one or more protons in a solution.
<i>Pulsed-field electrophoresis</i>	Zone electrophoresis that is used for separation of chromosomes or other large particles in an electric pulsed-field.
<i>3'-Poly (A)</i>	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.
<i>Rehydratable gel</i>	Dried gel, usually of polyacrylamide, that may be converted into usable wet gel in an appropriate solution.
<i>Relaxation</i>	Dislocation of the electric charges of a particle in an electric field whereby its velocity slows down.
<i>Restriction endonucleases</i>	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.
<i>Ribonucleates</i>	RNA nucleates.

(continued)

<i>RNA (ribonucleic acid)</i>	Polynucleotides that consist of specifically arranged ribonucleotide residues. They carry negative charges in a solution.
<i>RNases (ribonucleases)</i>	Enzymes that degrade RNA.
<i>rRNA (ribosomal RNA)</i>	Two types of rRNA are known in eukaryotes: 28S and 18S rRNA. The bacteria contain also two types of rRNA: 23S and 16S rRNA.
<i>SDS disc-electrophoresis</i>	Disc-electrophoresis whose buffer system contains the denaturing agent SDS.
<i>SDS zone electrophoresis</i>	Zone electrophoresis whose buffer contains the denaturing agent SDS.
<i>Splicing process</i>	Process during which intron transcripts are cut off from the RNA copy of a gene, whereby the exon transcripts are joined together.
<i>Starch gel</i>	Natural polymer that consists of D-glucose residues.
<i>Starch gel electrophoresis</i>	Zone electrophoresis in a starch gel. Today it is replaced by the electrophoresis in polyacrylamide or agarose gels.
<i>Submarine electrophoresis</i>	Agarose zone electrophoresis for separation of nucleates that is carried out under a buffer.
<i>Support film</i>	Chemically pretreated film, usually of polyester, which is used for chemical fixation of a gel.
<i>Support net</i>	Chemically pretreated net, usually of polyester, which is used for chemical fixation of a gel.
<i>Total charge</i>	Sum of all the electric charges of a polyion in an infinitely dilute solution.
<i>tRNA (transfer RNA)</i>	RNA used for transporting amino acids in the process of protein synthesis.
<i>Two-dimensional electrophoresis</i>	Combination between isoelectric focusing with carrier ampholytes or in immobilized pH gradients, and SDS disc-electrophoresis.
<i>Zone electrophoresis</i>	Electrophoresis that is run in one buffer, hence at continuous electric field strength.
<i>Zwitterion</i>	Ion that carries a positive and a negative charge.

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