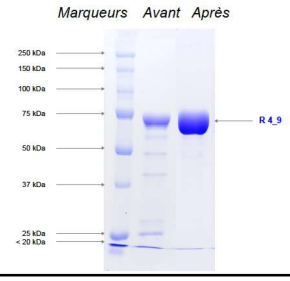
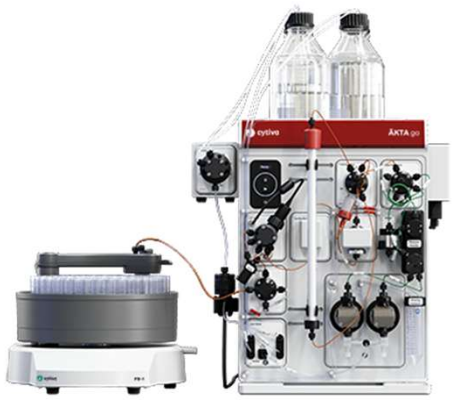
Separation/Purification of Biomacromolecules

Master I Applied Microbiology

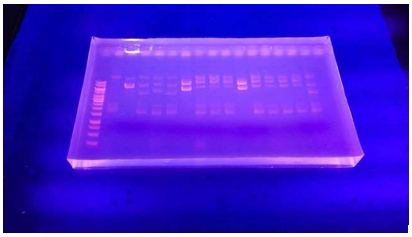
University of Jijel

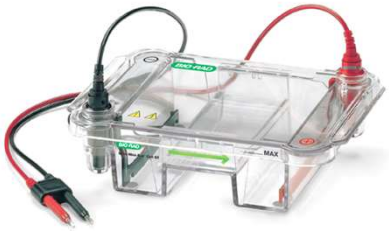
Pr H Ouled Haddar

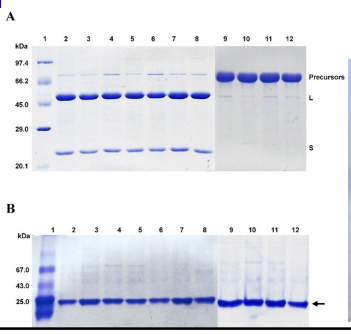


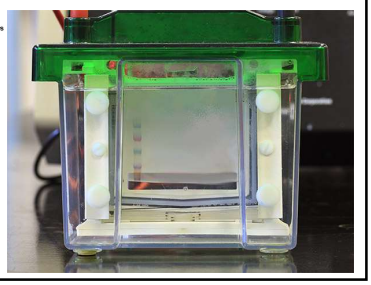


Chapter 6. Electrophoresis techniques



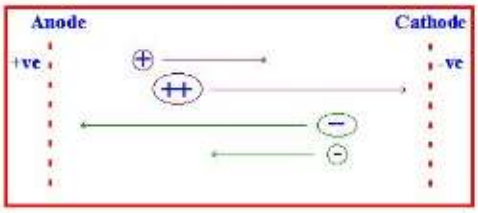






Theoretical basis of electrophoresis

The principle of electrophoresis is based on the movement of charged particles under the influence of an electric field. Electrophoresis is a widely used technique for separating and analyzing biomolecules, such as DNA, RNA, proteins, and other charged particles, based on their size, shape, charge, or mass-to-charge ratio.



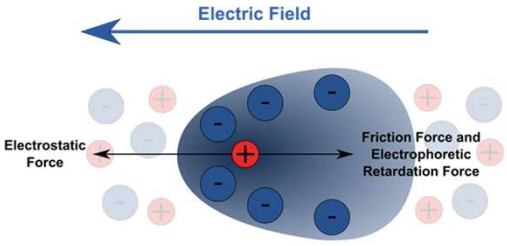
The rate of movement depends on the field strength and the number of charges. Biomolecules such as proteins possess surface charge due to the presence of acidic and basic amino acids

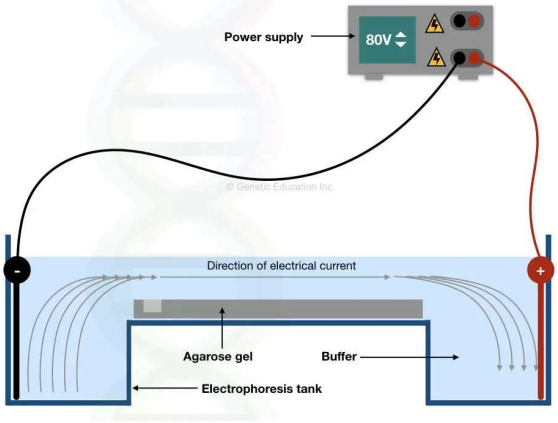
Advantages of using gels instead of paper

- **Resolution**: Gels provide higher resolution compared to paper.
- **Customization**: Gels can be customized to suit the specific needs of the experiment.
- **Durability**: Gels are more durable and less prone to tearing or damage compared to paper.
- **Visualization**: Gels provide a clear background against which separated molecules can be visualized.
- **Sample capacity**: Gels can accommodate a larger number of samples and allow for higher throughput compared to paper electrophoresis systems.
- **Compatibility with modern techniques**: Gel electrophoresis is compatible with various downstream applications such as western blotting, in-gel digestion for mass spectrometry analysis, and transfer to membranes for nucleic acid hybridization

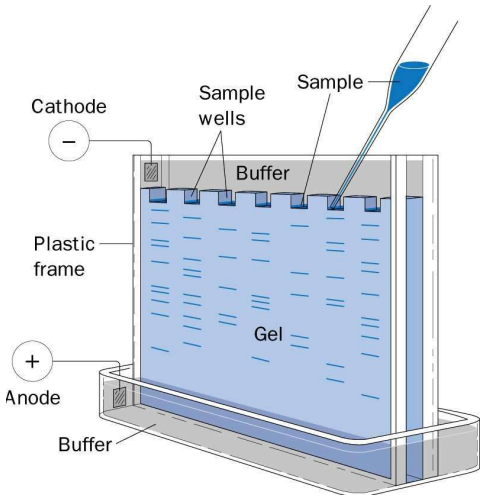
The **electrical mobility (μ),** which is the ability of a charged particle to move in response to the applied electric field (E), can be expressed with the velocity (v) of the charged particles as follows:

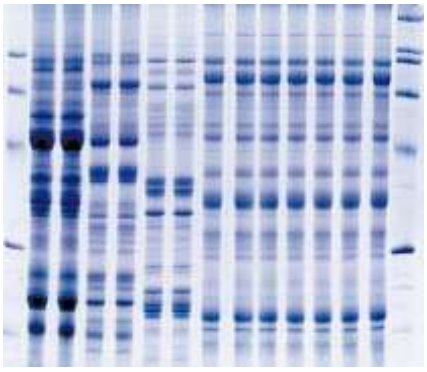
$$\mu = v/E = q/f$$





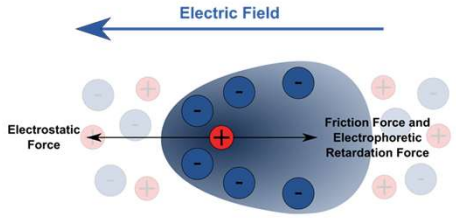
Polyacrylamide gel electrophoresis (PAGE)





For a particle possessing a total charge of *q* coulomb, the **force *(F)*** that pushes a charged particle is proportional to the net charge of that particle in that particular electric field (E):

F=qE

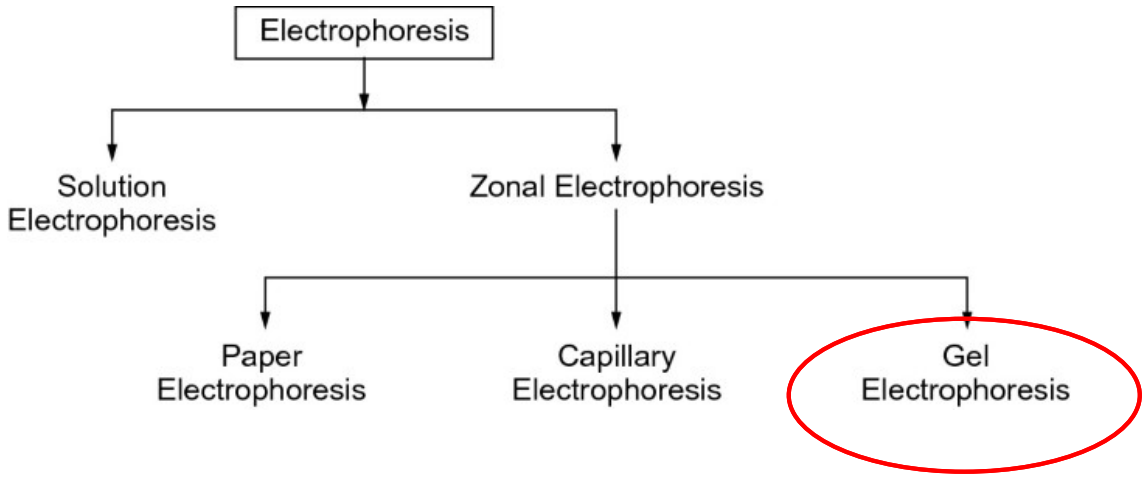


During electrophoresis, the **velocity (v)** of a charged particle, at which it moves in a particular direction in an electric field, is expressed as:

v=qE/f

where f is a frictional coefficient that is dependent on the shape and size of the particle and on the pore size of the medium and the velocity of the buffer solution used in electrophoresis.

Types of electrophoresis



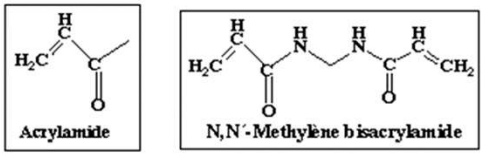
Gel electrophoresis

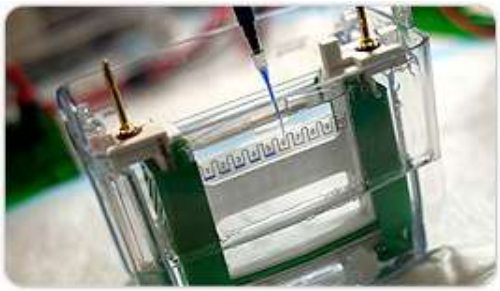
- The use of gels has replaced porous supports, they are used in thin layers, for the separation of high molecular weight substances (proteins, nucleic acids)

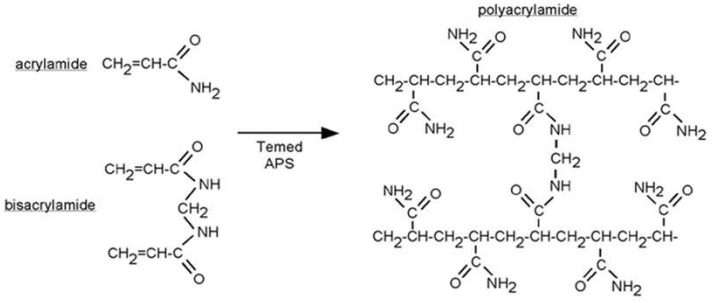
- Gels according to their physical properties, their hydrophilic, semi-colloid property and their insolubility in water allow a better resolution

Polyacrylamide is a clear and transparent gel made from the **co-polymerization** of acrylamide monomers in the presence of a crosslinking agent, N, N-methylene-bisacrylamide, (bis-acrylamide).

The polymerization reaction is catalyzed by ammonium persulphate (APS) and N, N, N', N'-tetramethylene diamine (TEMED).





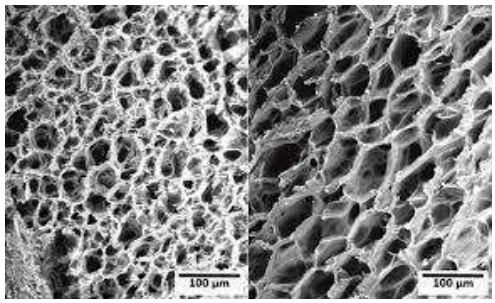


The pore size of polyacrylamide gels is determined by the concentration of acrylamide, which must be in proportion with its crosslinking agent.

Generally, a low percentage of acrylamide gel (3%-15%) is used to separate DNA and proteins.

A higher percentage of acrylamide gel (10%-20%) is commonly used in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE), in which proteins are [illegible]size,

Acrylamide percentage	Separation range
3.5	500–2000
5.0	80–500
8.0	60–400
12.0	40–200
15.0	25–150
20.0	6–100



Some PAGE types

- **SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)**: proteins are denatured and coated with SDS, a strong anionic detergent that imparts a uniform negative charge to the proteins, disrupting their native structures.
- **Native PAGE:** native PAGE preserves the native conformation of proteins without denaturation. Proteins retain their native charge, and separation occurs based on both size and charge.
- **2D-PAGE (Two-Dimensional Polyacrylamide Gel Electrophoresis)**

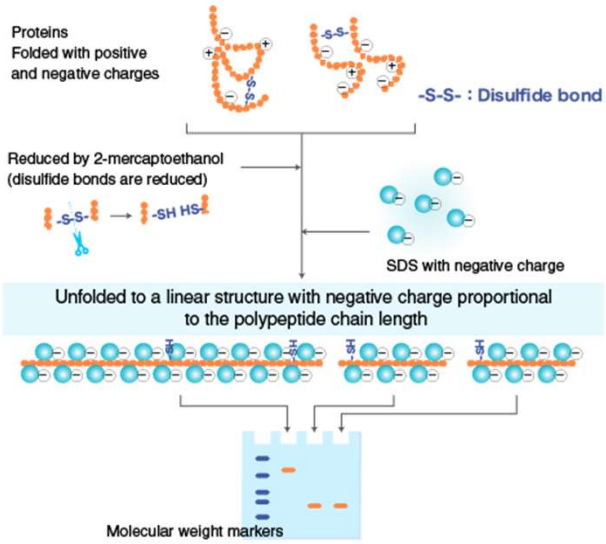
SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

In SDS-PAGE, proteins are denatured and coated with SDS, a strong anionic detergent that imparts a uniform negative charge to the proteins, disrupting their native structures.

This results in the separation of proteins primarily based on their molecular weight.

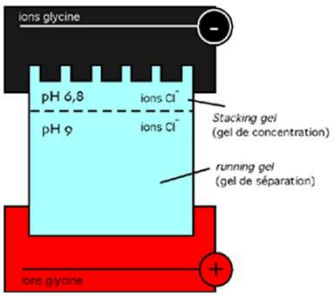
SDS-PAGE is commonly used for protein analysis, quantification, and characterization.

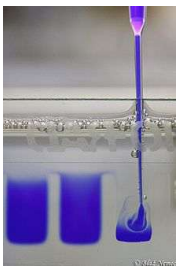
SDS : $CH_3 - (CH_2)_{10} - CH_2 - O - SO_3^-, Na^+$),



Principle and steps of SDS-PAGE

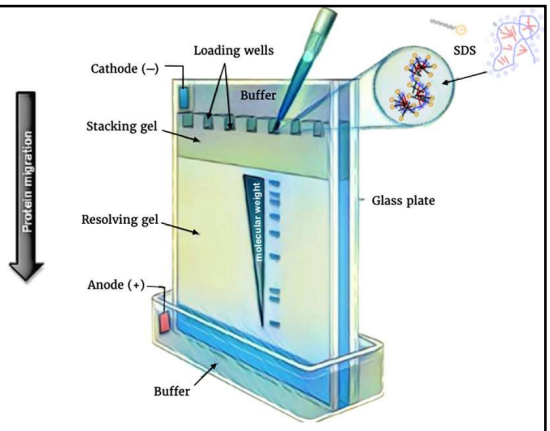
- The gel is prepared by mixing acrylamide, bisacrylamide, buffer, and a polymerization initiator.
- Resolving (running) and stacking gels are poured and allowed to polymerize.
- Samples are prepared by mixing protein samples with **SDS** and a reducing agent: **β-mercaptoethanol** which reduces disulfide bonds, the sample is **heated** to denature proteins.
- Proteins are consequently denatured: they have lost their native three-dimensional structure
- Proteins no longer have a disulfide bond: they are in a **monomeric** form
- Samples are loaded into wells on the gel along with a protein molecular weight marker.

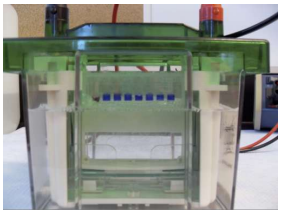


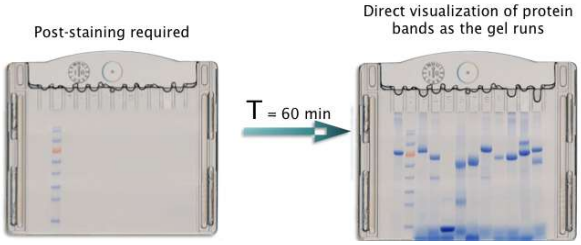


- The density of the samples is artificially increased by adding sucrose or glycerol (10 to 15%).
- An electrophoretic marker (dye) is used to detect any migration abnormalities

- Electrophoresis is conducted by submerging the gel in a buffer solution in an electrophoresis chamber and applying an electric current.
- Proteins migrate through the gel based on their molecular weight, facilitated by the presence of SDS.
- Electrophoresis is stopped once proteins have migrated sufficiently.
- The gel is stained with a **protein stain**, and excess dye is removed by destaining.
- Protein bands are visualized and analyzed based on their migration distance and intensity, often compared with a molecular weight marker,







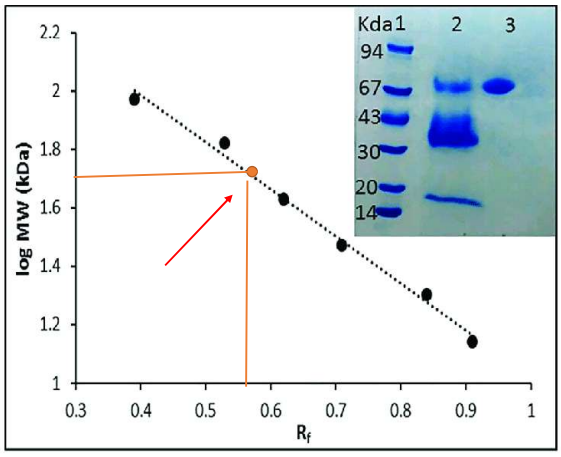
Determination of the molecular weight of proteins by SDS PAGE

Examples of markers :

- myosin (205 kDa)
- B-galactosidase (116 kDa)
- phosphorylase b (97,4 kDa)
- albumin (66 kDa)
- ovalbumin (45 kDa)
- carbonic anhydrase (29 kDa)

$$Rf = \frac{\text{distance of migration of the band}}{\text{distance of migration of the front}}$$

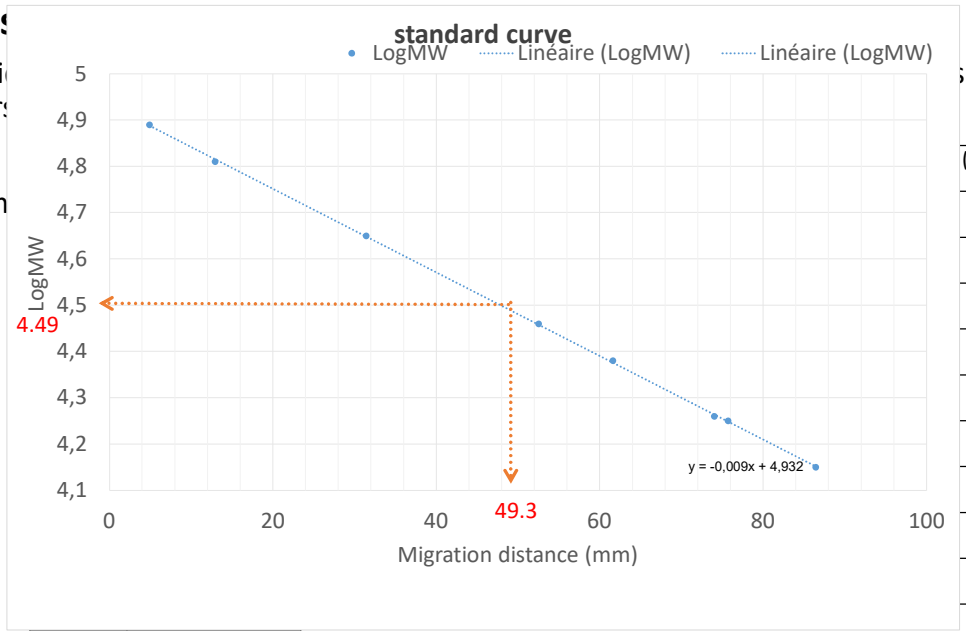
or the distance of migration only !



Exercis

A pepti
markers

Determ



(mm)

Applications

- **Protein Separation and Analysis**
- **Protein Quantification**: By comparing the intensity of protein bands against known standards or internal controls, researchers can estimate the relative concentration of proteins within a sample.
- **Quality Control in Protein Purification**: SDS-PAGE is an essential tool for assessing the purity and integrity of protein samples during purification processes.
- **Identification of Protein-Protein Interactions**: SDS-PAGE can be coupled with techniques such as co-immunoprecipitation or cross-linking to study protein-protein interactions.

- **Determination of Protein Molecular Weight**: By comparing the migration of target proteins with standards of known molecular weight, researchers can infer the size of the proteins under investigation. The number of sub-units can be determined as well.

- **Assessment of Post-Translational Modifications**: SDS-PAGE can be used to analyze post-translational modifications (PTMs) of proteins. Variations in protein mobility on the gel can indicate modifications such as phosphorylation, glycosylation, or acetylation, providing insights into the regulation and function of proteins.

- **Western blot**

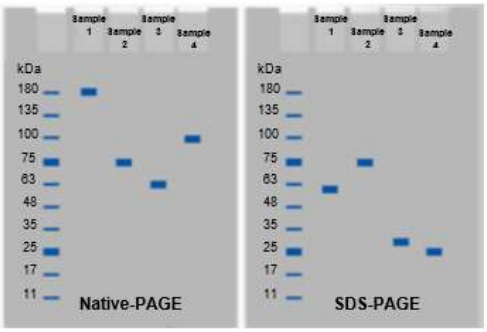
- **Proteomics** : SDS-PAGE is a key component of proteomics workflows, where it is often coupled with techniques such as mass spectrometry for comprehensive protein identification and characterization in complex biological samples.

Native PAGE (Polyacrylamide Gel Electrophoresis)

- The principle of native PAGE (Polyacrylamide Gel Electrophoresis) revolves around separating proteins based on their size and charge under native conditions, meaning that proteins retain their native conformation and charge during the electrophoretic process.

- Proteins maintain their native structure, including any tertiary and quaternary structures and post-translational modifications.

The gel matrix and running buffer are formulated to maintain protein stability and solubility under native conditions, typically using non-denaturing detergents and pH conditions that preserve protein integrity



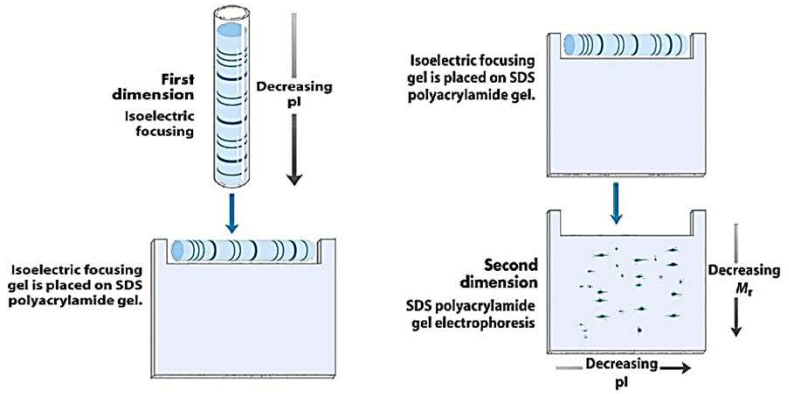
Variatons that improved the resolution:
- Blue native PAGE for (+) charged proteins
- Use of gel gradients...

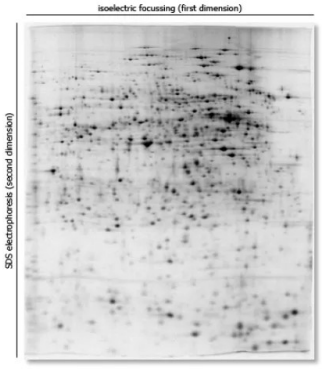
Applications:

- **Analysis of Protein Complexes:** Native PAGE is commonly used to study protein complexes, such as multimeric enzymes, protein-protein interactions, and nucleoprotein complexes.
- **Assessment of Protein Purity**
- **Determination of Protein Oligomeric States**
- **Characterization of Protein Conformational Variants**: Native PAGE can help identify conformational variants of proteins, such as different isoforms or post-translationally modified forms.
- **Study of Protein Folding and Stability**
- **Analysis of Protein-DNA and Protein-RNA Complexes**: Native PAGE is useful for studying interactions between proteins and nucleic acids, such as DNA-protein and RNA-protein complexes.
- **Enzyme Activity Assays**: Native PAGE can be coupled with enzyme activity assays to assess the activity of enzymes in their native state (zymogram)

2D-PAGE (Two-Dimensional Polyacrylamide Gel Electrophoresis)

2D-PAGE combines two separation techniques to achieve higher resolution. In the first dimension, proteins are separated based on their isoelectric point (pI) using isoelectric focusing (IEF), where proteins migrate in a pH gradient until they reach a pH matching their pI. In the second dimension, proteins are separated based on their molecular weight using SDS-PAGE. This technique allows for the separation of thousands of proteins in a single gel and is commonly used in proteomics studies. it is often coupled with techniques such as mass spectrometry

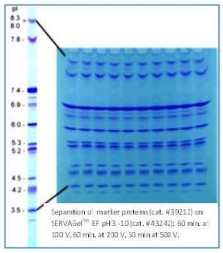




Isoelectric focusing (IEF)

Isoelectric focusing (IEF) is a technique used in biochemistry and analytical chemistry to separate molecules based on their isoelectric point (pI).

The principle behind isoelectric focusing is that molecules, such as proteins or amino acids, will migrate towards the electrode until they reach the pH at which they have no net charge.



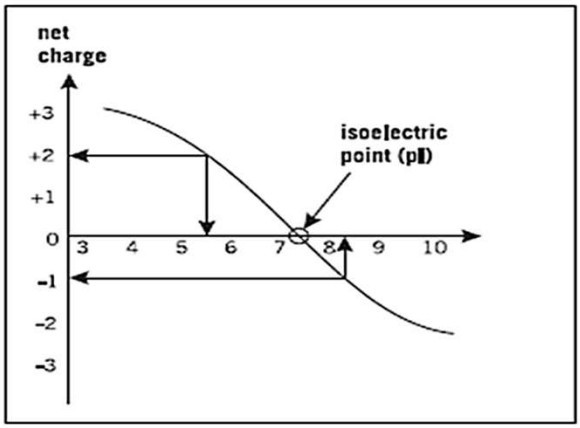


Fig 3.1. Net charge on a protein as a function of pH. In this example the protein has a net charge of +2 at pH 5.5, 0 at pH 7.5 (the isoelectric point), and -1 at pH 8.5.

Principle and steps

A gel containing an immobilized pH gradient (IPG) is prepared. This gel has a pH gradient established across its length (ampholyte molecules).

A mixture of molecules (e.g., proteins) to be separated is placed at one end of the gel.

An electric field is applied across the gel.

Due to their charges, the molecules migrate towards the electrode, but they slow down and eventually stop when they reach the region of the gel where the pH equals their pI.

At this point, the molecules have no net charge and become immobilized in the gel.

The result is a separation of molecules based on their pI values, with the molecules being arranged along the gel according to their respective pI values.

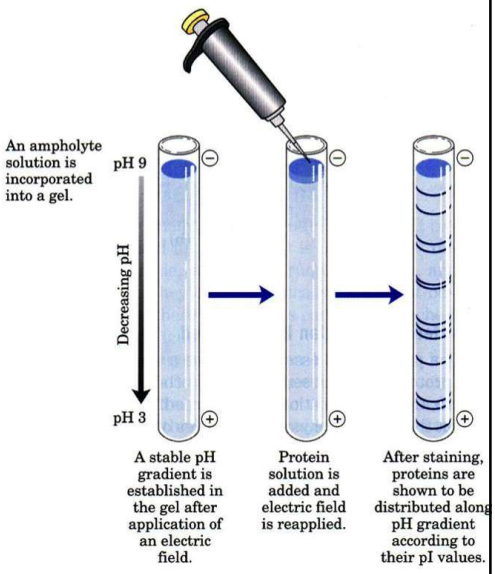


table 5–6

The Isoelectric Points of Some Proteins

Protein	pI
Pepsin	~1.0
Egg albumin	4.6
Serum albumin	4.9
Urease	5.0
β-Lactoglobulin	5.2
Hemoglobin	6.8
Myoglobin	7.0
Chymotrypsinogen	9.5
Cytochrome *c*	10.7
Lysozyme	11.0

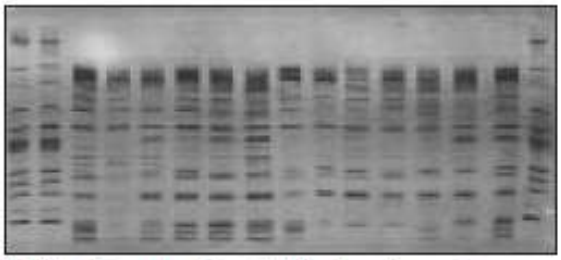
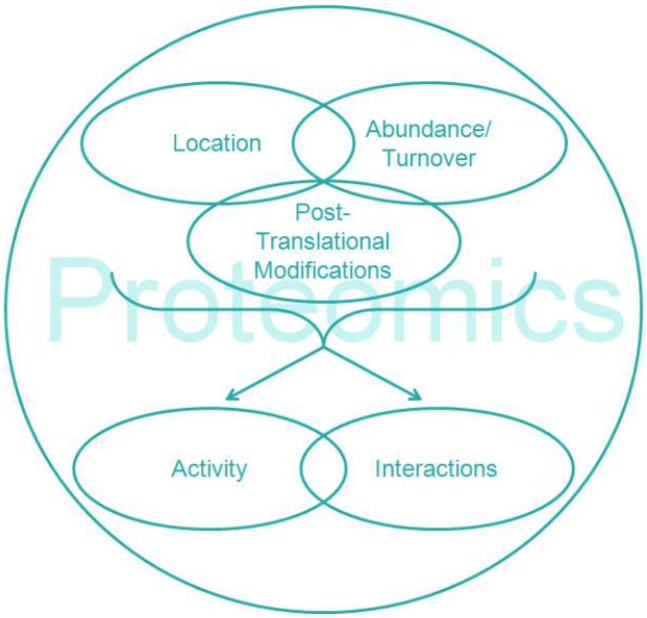


Fig 3.8. Example of isoelectric focusing using CleanGel IEF.

Proteomics

- Proteomics is the large-scale study of proteomes.
- A proteome is a set of proteins produced in an organism, system, or biological context.
- We may refer to, for instance, the proteome of a species (for example, *Homo sapiens*) or an organ (for example, the liver).
- The proteome is not constant; it differs from cell to cell and changes over time.
- To some degree, the proteome reflects the underlying transcriptome. However, protein activity (often assessed by the reaction rate of the processes in which the protein is involved) is also modulated by many factors in addition to the expression level of the relevant gene.



Areas of proteomics: Proteomic experiments generally collect data on three properties of proteins in a sample: location, abundance/turnover and post-translational modifications.

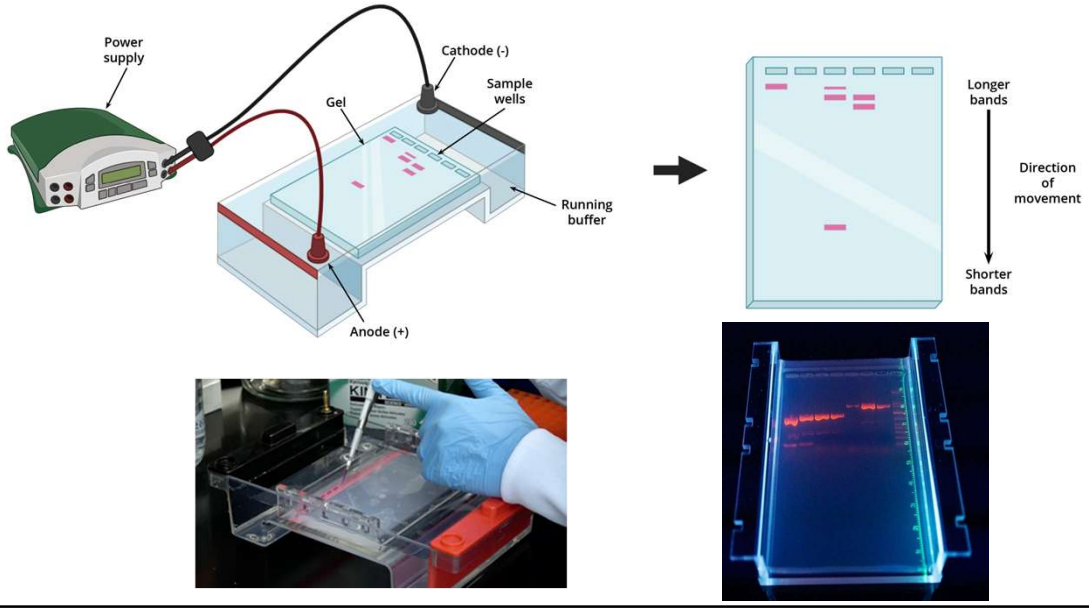
- **Proteomics is used to investigate:**
- when and where proteins are expressed
- rates of protein production, degradation, and steady-state abundance
- how proteins are modified (for example, post-translational modifications (PTMs) such as phosphorylation)
- the movement of proteins between subcellular compartments
- the involvement of proteins in metabolic pathways
- how proteins interact with one another

Methods in proteomics

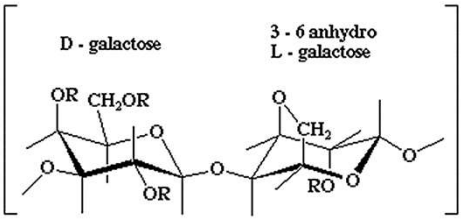
- Several high-throughput technologies have been developed to investigate proteomes in depth.
- The most commonly applied are mass spectrometry (MS)-based techniques such as Tandem-MS and gel-based techniques such as differential in-gel electrophoresis (DIGE).
- These high-throughput technologies generate huge amounts of data.
- Databases are critical for recording and carefully storing this data, allowing the researcher to make connections between their results and existing knowledge.

https://www.ebi.ac.uk/training/online/courses/proteomics-an-introduction/what-is-proteomics/methods-in-proteomics/

Agarose gel electrophoresis



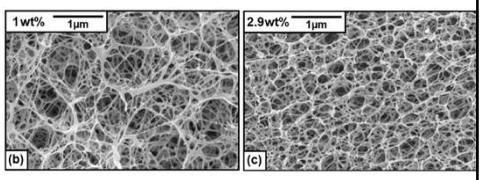
Agarose gel is a type of gel matrix commonly used in electrophoresis for the separation of nucleic acids, such as DNA and RNA.
The molecular structure of agarose consists of repeating units of agarobiose, a disaccharide composed of D-galactose and 3,6-anhydro-L-galactopyranose.
It is derived from agar, a polysaccharide extracted from seaweed, and it forms a solid matrix when cooled.



E.J. (2002) Motif de base de l'agarose

Table 1. Range of Separation in Gels Containing Different Amounts of Agarose

Amount of agarose in gel (% [w/v])	Efficient range of separation of linear DNA molecules (kilobase [Kb; 1 Kb = 1000 base pairs])
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7.0
1.2	0.4-6.0
1.5	0.2-3.0
2	0.1-2.0

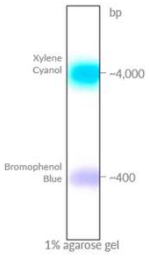


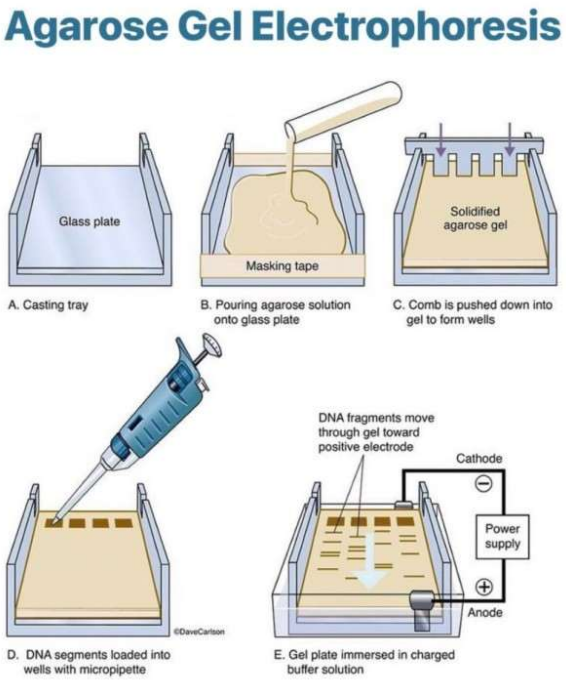
The DNA or RNA samples are mixed with a loading dye (containing tracking dyes and often glycerol) and heated to denature

Loading dye: bromophenol blue 0.02% - xylene cyanol 0.02% - glycerol 3% - TBE buffer.

The 2 dyes make it possible to follow the migration of DNA fragments:

- the migration of bromophenol blue is "comparable" to that of a DNA fragment of 300/400 base pairs
- the migration of Xylene cyanol is "comparable" to that of a DNA fragment of 4000 base pairs.

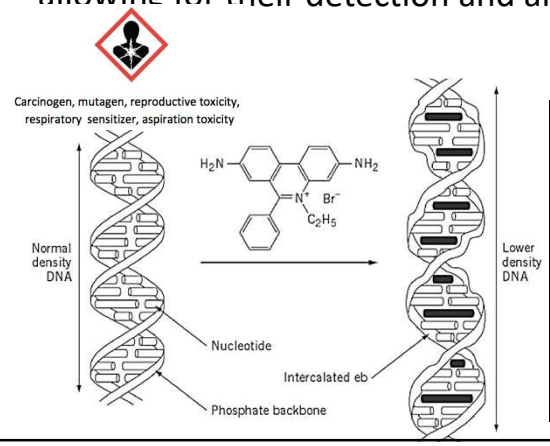


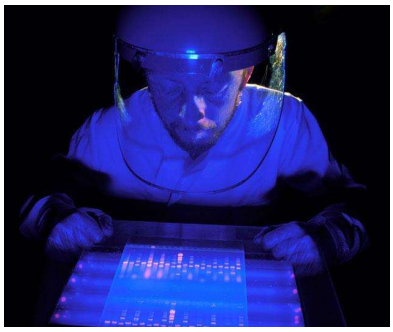


Visualization

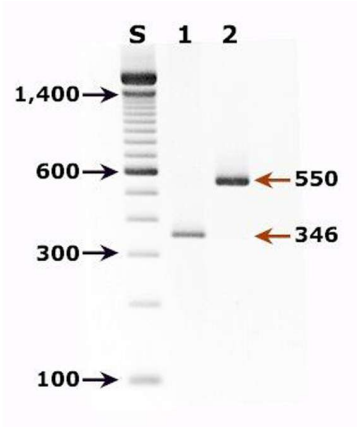
After electrophoresis, the DNA or RNA molecules are visualized by staining the gel with a fluorescent dye such as ethidium bromide or a safer alternative like SYBR Safe.

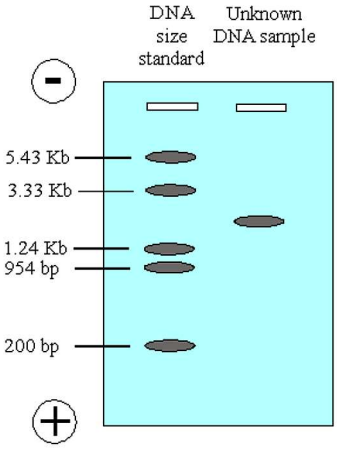
The stained nucleic acids appear as bands under ultraviolet (UV) light, allowing for their detection and analysis.

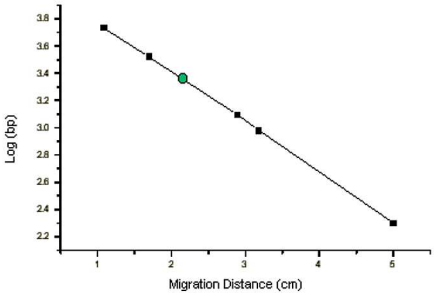




How to calculate the size of a DNA fragment







Applications

- DNA sequencing
- Determination of the size of DNA fragments
- Separation of nucleic acids to be analyzed by Northern blot (RNA) or Southern blot (DNA) techniques
- Establishment of the restriction profile (hydrolysis by restriction enzymes) of DNA fragments
- …

Other electrophoresis-based techniques

- PFGE (Pulsed field gel electrophoresis)
- TGGE (Temperature Gradient Gel Electrophoresis)
- DGGE (Denaturing Gradient Gel Electrophoresis)
- Capillary electrophoresis
- Immunoectrophoresis
- …

PFGE (Pulsed Field Gel electrophoresis)

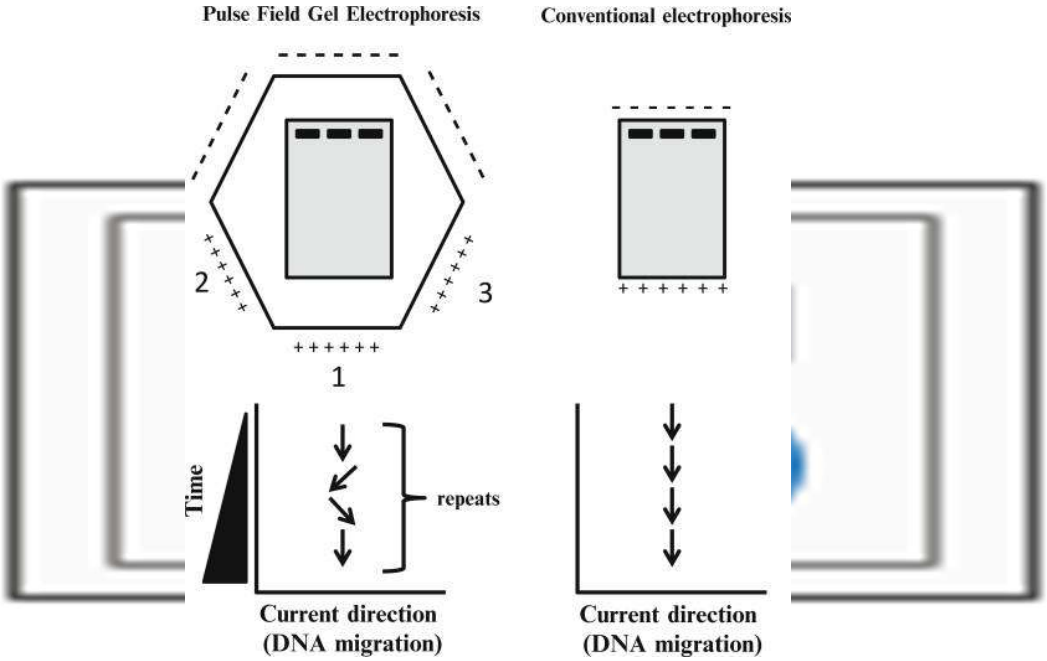
Pulse Field Gel Electrophoresis (PFGE) is a powerful genotyping technique used for the separation of **large** DNA molecules (entire genomic DNA) after **digesting** it with unique restriction enzymes and applying to a gel matrix under the electric field that **periodically changes direction**.

PFGE is a variation of agarose gel electrophoresis that permits analysis of bacterial DNA fragments over an order of magnitude (over 20kb) larger than that with conventional restriction enzyme analysis.

It provides a good representation of the entire bacterial chromosome in a single gel with a highly reproducible restriction profile, providing clearly distinct and well-resolved DNA fragments.

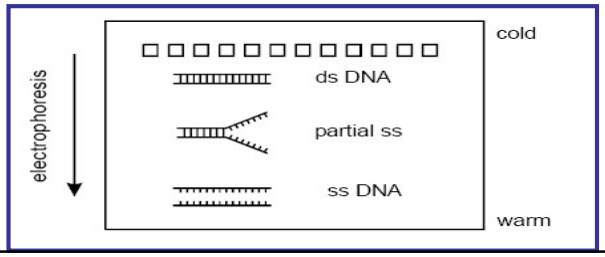
- In this size range (beyond 20 kb), molecules are no longer separated by conventional methods, since migration becomes independent of size (the movement of these cylindrical molecules, all having the same diameter, occurs through "**reptation**").
-
- The use of **two orthogonal fields applied alternately** causes DNA molecules, which require a certain time to orient themselves in the direction of the electric field, to migrate only once this orientation is achieved.
-
- The time required for orientation increases proportionally with the length of the DNA molecule.

- It thus becomes possible to separate molecules according to their length. This method proves to be highly useful in the analysis of



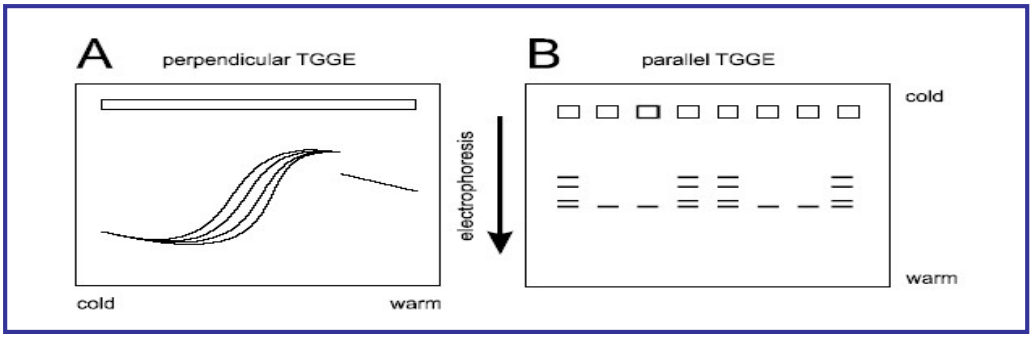
Temperature Gradient Gel Electrophoresis (TGGE)

- TGGE is a type of acrylamide gel electrophoresis which is used to detect point mutations and polymorphisms within PCR-products.
- TGGE is very fast and sensitive in detecting heterozygous sequence variations within the PCR-product.
- Conventional protein or nucleic acid electrophoresis separates molecules according to their size or charge.
- TGGE separates molecules by their melting behavior.



- TGGE separate samples either **parallel** or **perpendicular** to a temperature gradient.

- **A**: temperature gradient from left to right.
- **B**: temperature gradient from top to bottom.



Application of TGGE

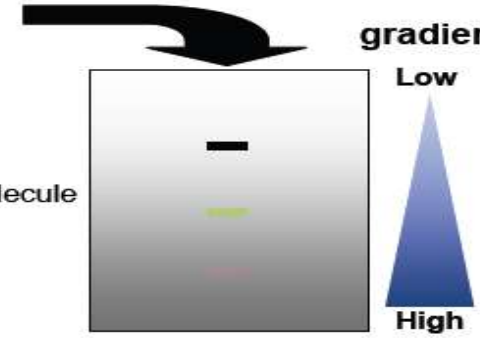
Two major fields of application for TGGE:

- **Mutation Analysis in PCR Fragment.**
- **Diversity Analysis of Complex Bacterial samples.**

Denaturing Gradient Gel Electrophoresis (DGGE)

Principle of DGGE

Separation of DNA fragments of the same length but with different base-pair sequences

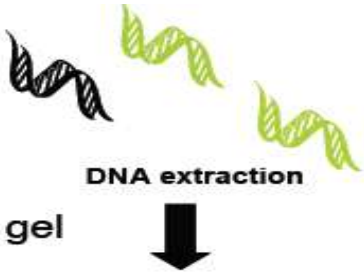


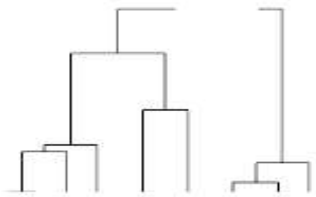
Based on the decreased electrophoretic mobility of a partially melted DNA molecule

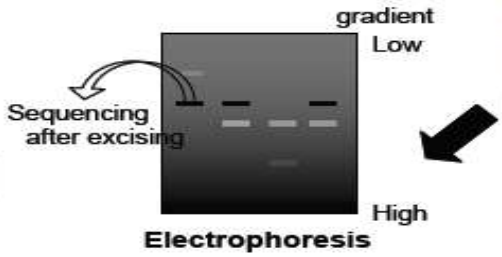
In polyacrylamide gels containing a linearly increasing gradient of DNA denaturants

Overview of DGGE analysis

1. DNA extraction
2. Polymerase chain reaction (PCR)
3. Electrophoresis in polyacrylamide gel
4. Gel image analysis
5. Excising, followed by Sequencing





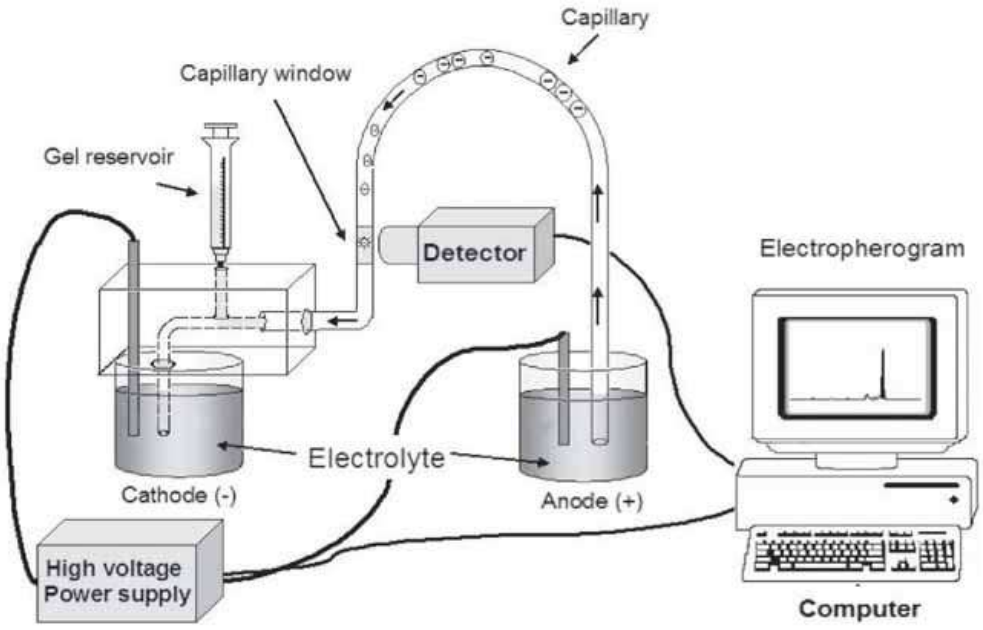


Capillary electrophoresis (CE)

Capillary electrophoresis (CE) is a family of related techniques that employ narrow-bore (20-200 μm i.d.) capillaries to perform high efficiency separa tions of both large and small molecules.

These separations are facilitated by the use of high voltages, which may generate electroosmotic and electrophoretic flow of buffer solutions and ionic species, respectively, within the capillary.

The properties of the separation and the ensuing electropherogram have characteristics resembling a cross between traditional polyacrylamide gel electrophoresis (PAGE) and modern high performance liquid chromatography (HPLC).



Its advantages are :

- requirement of very small samples,
- can be automated for precise quantitative analysis,
- User-friendly,
- use of small amounts of reagents, and
- applicability to a wide selection of analytes