Electrophoresis Theory and Basics

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Definition

As given by American Heritage Dictionaries

1. The migration of charged colloidal particles or molecules through a solution under the influence of an applied electric field usually provided by immersed electrodes. Also called cataphoresis.
2. A method of separating substances, especially proteins, and analyzing molecular structure based on the rate of movement of each component in a colloidal suspension while under the influence of an electric field

Better definition and theory

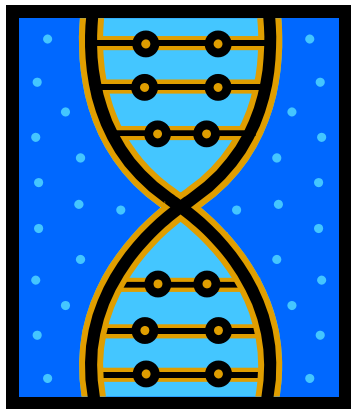
- Electrophoresis is a method where charged molecules in solution, mainly proteins and nucleic acids, migrate in response to an electrical field. The rate of migration or mobility through the electrical field depends on the strength of the charge, on the net charge of the protein/nucleic acid, the size and shape of the molecules, and also on the ionic strength, viscosity, and temperature of the medium through which the molecules are moving.

History of Electrophoresis

- Started in early 20th century
- 1930s First reports of the use of sucrose for gel electrophoresis.
- Swedish chemist *Arne Tiselius* – used electrophoresis as analytical tool in his dissertation in 1930, later in modified and improved form in 1937
- 1955 Introduction of starch gels, not very good separation
- 1959 Introduction of acrylamide gels for 1st time (Raymond and Winstraub) Accuracy of control of parameters such as pore size and stability
- 1964 Disc gel electrophoresis Ornstein and Davis
- 1969 Introduction of denaturing agents especially SDS separation of protein subunit (Beber and Osborn)
- 1970 Laemmli-T4 phage separated 28 components "stacking gel + SDS
- 1975 2 Dimensional gels O'Farrell isoelectric focussing then SDS gel electrophoresis.
- 1977 Sequencing gels
- Late 1970s agarose gels
- 1983 Pulsed field gel electrophoresis
- 1983 Capillary electrophoresis introduced

Types of materials that can be separated or detected with electrophoresis

- Proteins
  - Storage
  - Metabolically active
  - DNA
  - RNA
  - Enzymes

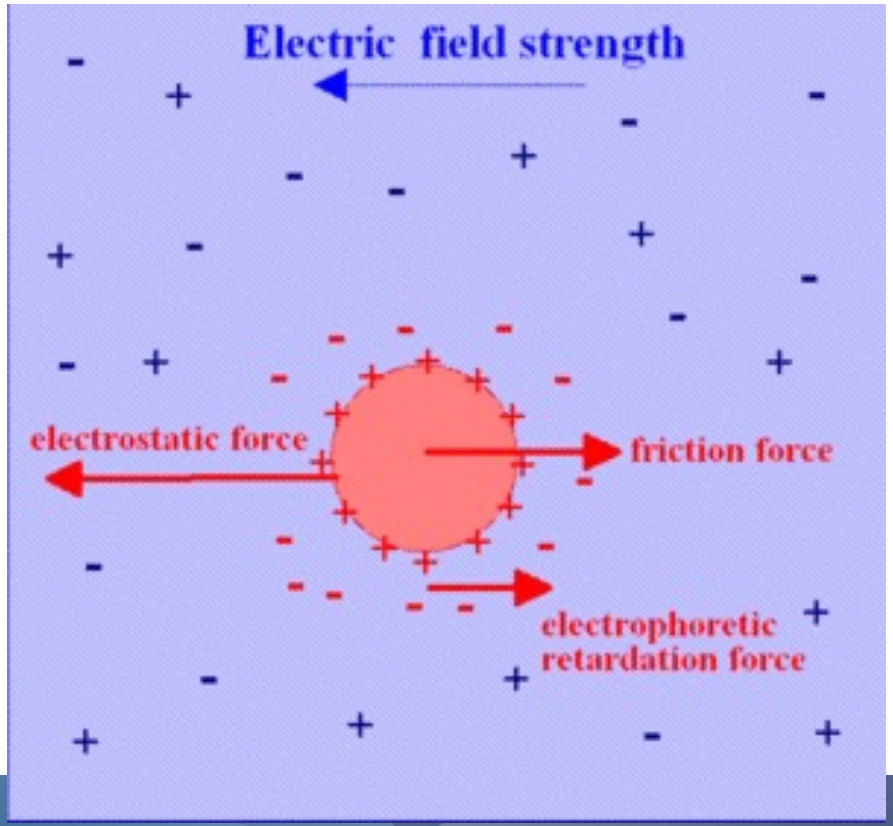


Graphic - source unknown.

- Electrophoresis can separate a wide variety of molecules of biological interest such as metabolites, drugs, amino acids, nucleic acids, carbohydrates, peptides and proteins based on their sizes and ionic properties.

Basics of Separation

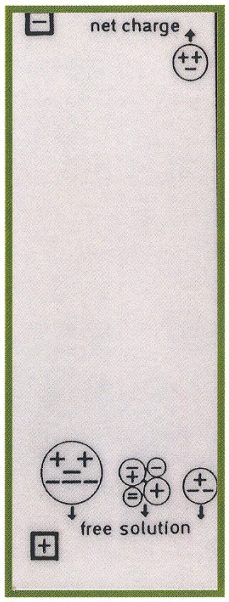
- The fundamental requirement for electrophoresis is that the molecules under study must have either a net positive or negative charge.



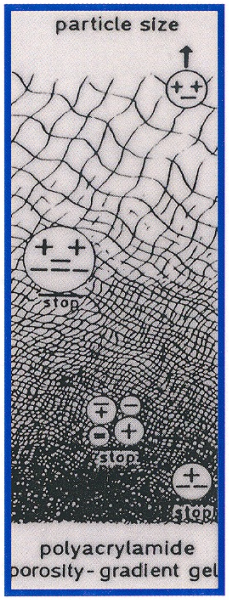
https://testbook.com/chemistry/electrophoresis

Predominant Factors in Electrophoretic Separation

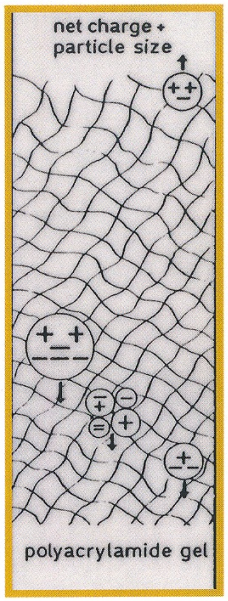
(Main parameters in electrophoretic separations. Proteins of different charge and size are compared)



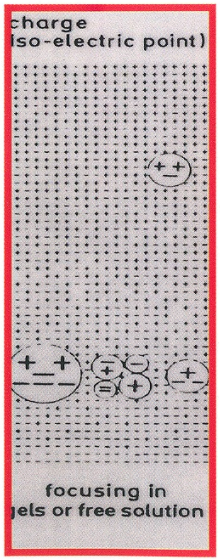
Electrophoresis *without or with little* molecular sieving (carrier-free, paper, starch agarose).



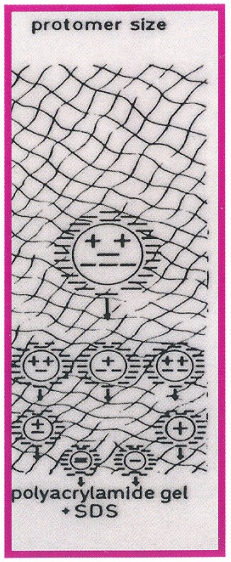
Porosity gradient: created by decreasing pore size of PAA (polyacrylamide). Proteins are finally trapped.



PAGE: both molecular sieving and charge separation affecting the migration rate.



Focusing: a pH gradient is created by small molecules of slightly different charge. Proteins focus at their isoelectric points.



SDS-PAGE: Sodium Dodecrylsulfate, highly negatively charged, unfolding proteins to a random coil and giving them a uniform load.

Source unknown

- The degree of separation and rate of molecular migration of mixtures of molecules depends upon the **size** and **shape** of the molecules, the **respective molecular charges**, the **strength of the electric field**, the **type of medium used** (e.g., cellulose acetate, starch gels, paper, agarose, polyacrylamide gel, etc.) and the **conditions of the medium** (e.g., electrolyte concentration, **pH**, ionic strength, viscosity, temperature, etc.).
- Some mediums (also known as support matrices) are porous gels that can also act as a **physical sieve for macromolecules**.

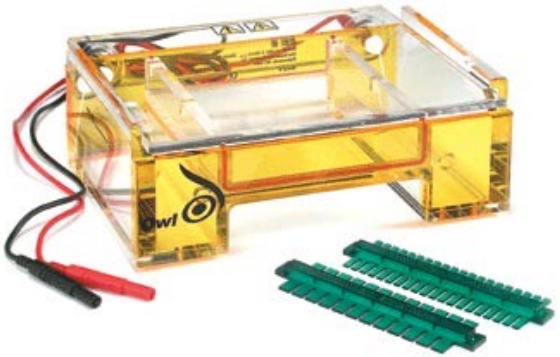
- In general, the medium is mixed with buffers needed to carry the electric charge applied to the system.
- The medium/buffer matrix is placed in a tray.
- Samples of molecules to be separated are loaded into wells at one end of the matrix.
- As electrical current is applied to the tray, the matrix takes on this charge and develops positively and negatively charged ends.
- As a result, molecules such as DNA and RNA that are negatively charged, are pulled toward the positive end of the gel.

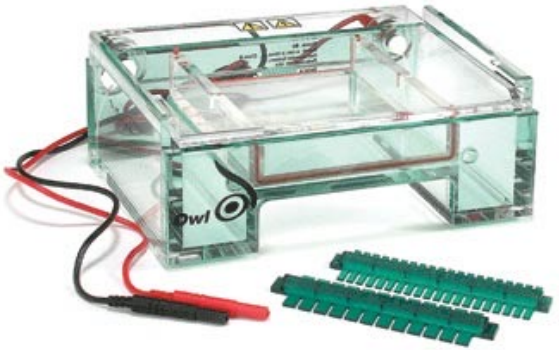
- Because molecules have differing shapes, sizes, and charges they are pulled through the matrix at different rates and this, in turn, causes a separation of the molecules.
- Generally, the smaller and more charged a molecule, the faster the molecule moves through the matrix.

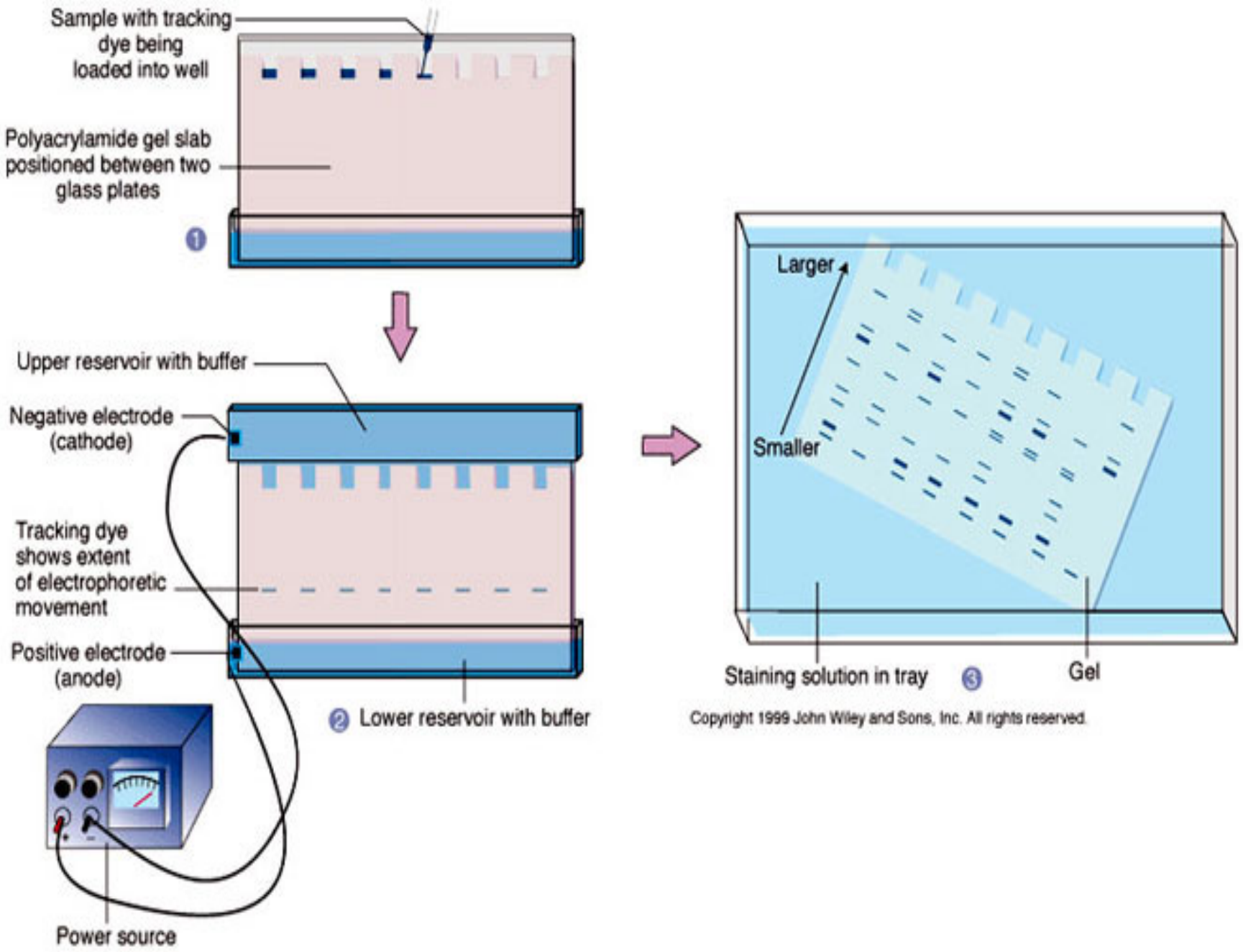
Electrophoretic Process

- Two pieces of equipment are necessary for electrophoresis: a d.c. power supply and a buffer reservoir system.
- The system consists of upper and lower buffer reservoirs with provision for suspending the gel. The only electrical connection between the reservoirs is via the gel. Platinum electrodes are positioned in each reservoir.

- The macromolecular samples are dissolved in a glycerol containing buffer to increase the density of the sample.
- Often, a “tracking dye” such as bromophenol blue (BB) is included in the sample as a reference. BB migrates faster than any of the macromolecules.







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Factors affecting electrophoresis
The electric field

- **Voltage**
- **Current**
- **Resistance**

Ohm's law expresses the relationship between current **I** (measured in amperes, A), voltage **V** (measured in volts, V) and resistance **R** (measured in ohms, W) in which:

V/I=R

- Three equations relating voltage (V), current (I), resistance (R) and power (W) describe the behavior of charges moving in a field. These are :

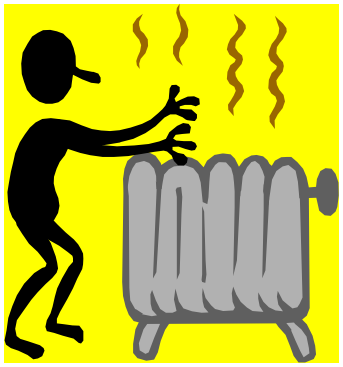
  - **$V=IR$**
  - **$I = V/R$**
  - **$W = VI = I^2R = V^2/R$**

- The current, and hence the rate of migration are inversely proportional to the resistance, which in turn is a function of the medium, the buffer and its concentration.
- Resistance will increase with the length of the supporting medium but will decrease with its cross-sectional area and with increasing buffer ion concentration.
- During electrophoresis the power dissipated in the supporting medium (W, measured in watts) is such that:
  - An increase in temperature will cause the resistance to fall.

- When constant voltage is applied, the current will increase during electrophoresis due to a decrease in resistance of the medium with the rise in temperature. Consequently, more heat will be produced resulting in more evaporation of solvent and a decrease in resistance.
- A constant current avoids these problems but may lead to a drop in voltage due to decreased resistance, resulting in reduced rate of migration.

- If a number of gels are run in parallel with one power supply, then the total current supplied must be increased in proportion to the number of gels used, assuming that they all have the same resistance.

- Gels can be run under:
  - Constant Voltage
  - Constant Current
  - Constant Power



- **Take-home message "Control the Heat"**
  - Run in cold room/refrigerator
  - Use a cool/cold water recirculator
  - Use appropriate constant power or constant current or constant voltage

The sample

- **Charge.** The rate of migration increases with an increase in the net charge. The magnitude of the charge is generally pH dependent.
- **Size.** The rate of migration decreases for larger molecules, due to the increased frictional and electrostatic forces exerted by the surrounding medium.
- **Shape.** Molecules of similar size but different shapes such as fibrous and globular proteins exhibit different migration characteristics because of the differential effect of frictional and electrostatic forces.

The buffer

- **Composition.** The buffers in common use are formate, acetate, citrate, barbitone, phosphate, Tris, EDTA and pyridine. The buffer should not bind to the sample.
- **Concentrations** As the ionic strength of the buffer increase, the proportion of current carried by the buffer will increase and the share of the current carried by the sample will decrease, thus slowing the sample migration.

The supporting medium

- **Adsorption.**
- **Electro-osmosis.**
- **Molecular Sieving.**

What concentration of gel?

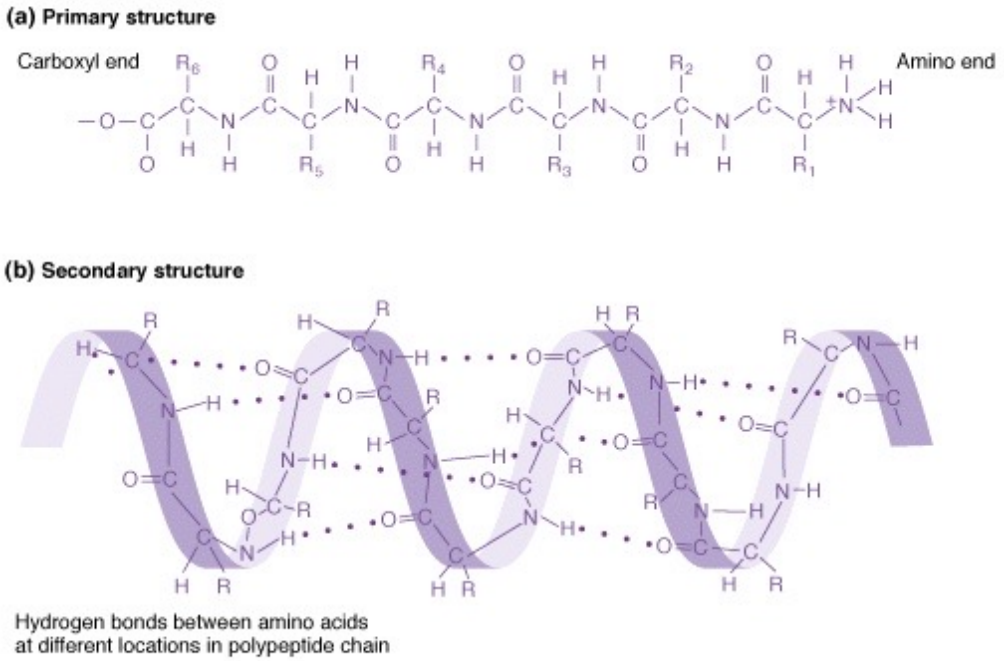
- Proteins with a wide range of molecular weights may be separated with 7 1/2% acrylamide gels.
- Proteins with higher molecular weights require lower acrylamide gel concentrations.
- Conversely, gels up to 30% have been used to separate small polypeptides.
- The percent gel to use depends on the molecular weight of the protein to be separated. Use 5% gels for proteins ranging from 60,000 to 200,000 daltons, 10% gels for a range of 16,000 to 70,000 daltons and 15% gels for a range of 12,000 to 45,000 daltons.

Proteins

- Proteins are separated on the basis of charge, and the charge of a protein can be either + or -- , depending upon the pH of the buffer.
- If the electrodes are arranged in such a way that the upper bath is -- (cathode), while the lower bath is + (anode), and -- anions are allowed to flow toward the anode, the system is known as an **anionic system**.
- Flow in the opposite direction, with + cations flowing to the cathode is a **cationic system**.

- Proteins have net charges determined by charged groups of amino acids from which they are constructed. Proteins can also be amphoteric compounds, meaning they can take on a negative or positive charge depending on the surrounding conditions.
- A protein in one solution might carry a positive charge in a particular medium and thus migrate toward the negative end of the matrix. In another solution, the same protein might carry a negative charge and migrate toward the positive end of the matrix.
- For each protein there is an isoelectric point related to a pH characteristic for that protein where the protein molecule has no net charge. Thus, by varying pH in the matrix, additional refinements in separation are possible.

Protein Structures

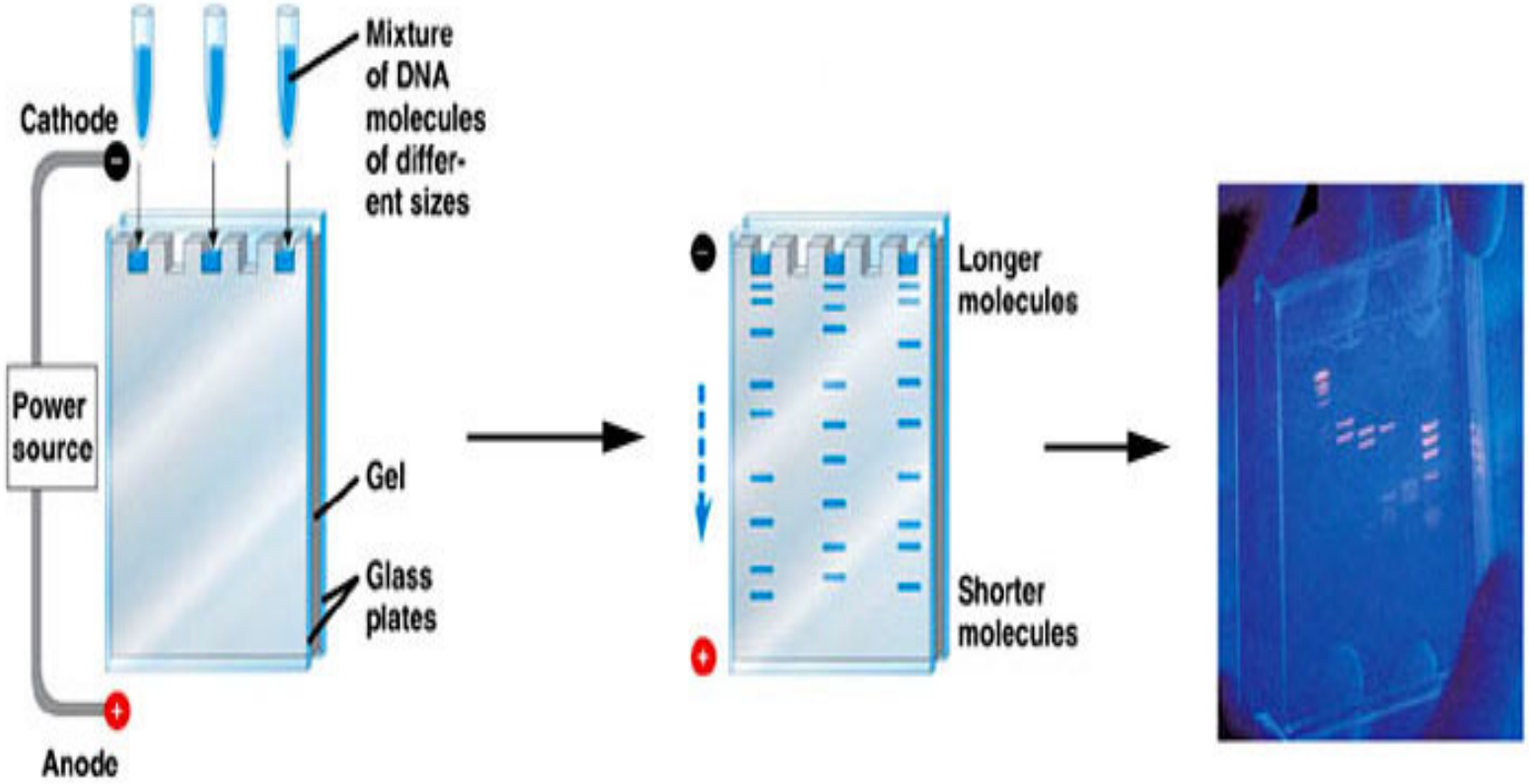


Different levels of protein structure. The amino acids in the primary structure build a simple chain held together by peptide bonds. The secondary structure of a protein is referred to amino acids interactions that are close together, created by hydrogen bonds between the CO and N H groups of different residues. The tertiary is a superstructure of the secondary, created by electrostatic, hydrogen and Van der Waals bonds. The quaternary structure is built of several separate polypeptide chains that are folded together.

Image source: Griffiths A. J. F., Miller J. H., Suzuki D. T., Lewontin R. C., Gelbart W. M., An introduction to genetic analysis, (2000) W. H. Freeman and Company.

DNA

- When DNA is subjected to electrophoresis, the DNA is first broken by what are termed **restriction enzymes** that act to cut the DNA in selected places.
- After being subjected to restriction **enzymes**, DNA molecules appear as bands (composed of similar length DNA molecules) in the electrophoresis matrix.
- Because **nucleic acids** always carry a negative charge, separation of nucleic acids occurs strictly by molecular size.



Restriction Map - a type of physical "map" of the banding pattern seen in gel electropherograms made from treating chromosomal DNA with restriction enzymes and then electrophoresing the fragments

... a kind of "fingerprint" of the DNA fragments from one restriction enzyme..

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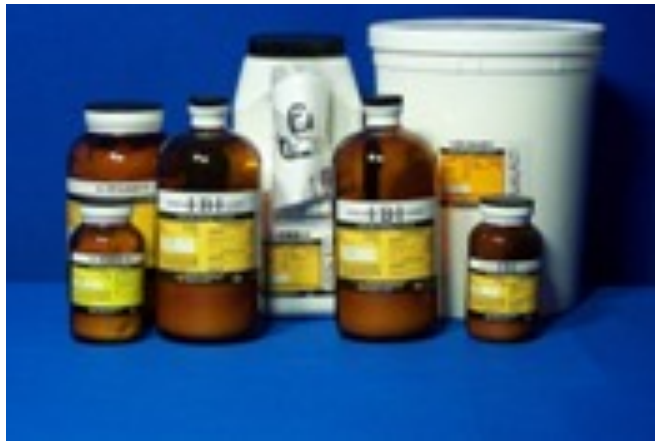
Gel Electrophoresis

The gel serves two purposes.

1. It serves to diffuse convective currents that would result in localized heating in the matrix, which would result in irregular migration patterns.
2. The gel also creates a molecular sieve that enhances the separation based on molecular weight.

- Gels may be prepared from **starch**, **agar** and **polyacrylamide**. The molecular sieving property of the semi-rigid gel helps to separate large ionic compounds such as proteins.

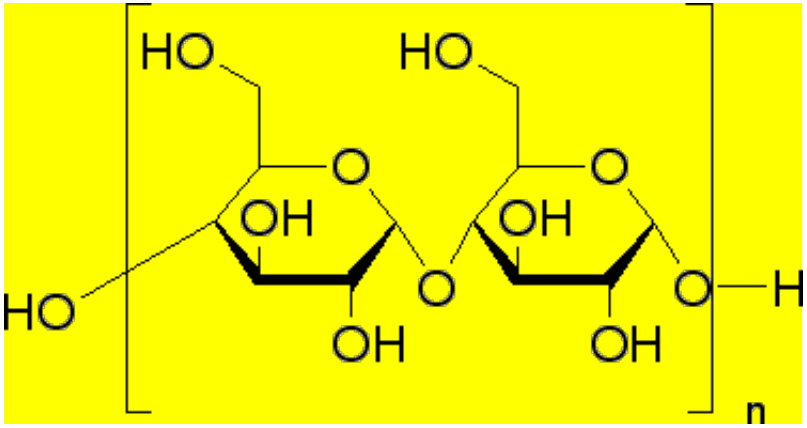


Acrylamide

Bis- Acrylamide

Acrylamide – not suitable

Agarose



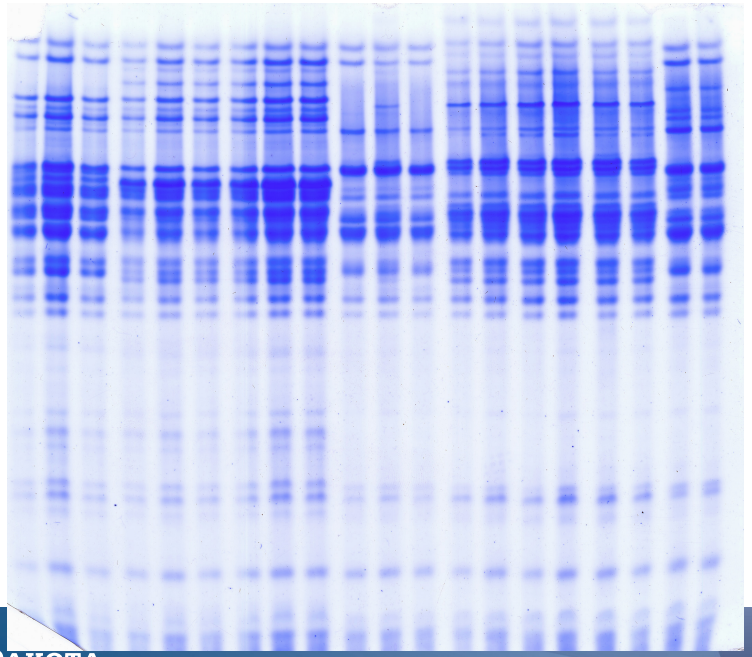
Starch from potato
for electrophoresis

- **Starch gels** are prepared by heating and cooling a mixture of partially hydrolysed starch in an appropriate buffer. This causes the branched chains of the amylopectin components of starch to intertwine and form a semi-rigid gel.

- **Agar/Agarose gels** consist of two galactose-based polymers, agarose and agaropectin.

- **Polyacrylamide** gels are made from acrylamide monomers copolymerised with the cross-linker N,N'methylenebisacrylamide in the presence of ammonium persulphate and TEMED as catalyst.

- Electrophoresis can be one dimensional (i.e. one plane of separation) or two dimensional.
  - One dimensional electrophoresis is used for most routine protein and nucleic acid separations.
  - Two dimensional separation of proteins is used for finger printing , and when properly constructed can be extremely accurate in resolving all of the proteins present within a cell (greater than 1,500).



Source – SDSU Seed Testing Lab

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Types of Electrophoresis

There are quite a number of types of electrophoresis commonly used. It is not possible to go through them all in any detail here, but a brief description of a few of the most common types follows

SDS-PAGE

- One of the most common means of analyzing proteins by electrophoresis is by using Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis. SDS is a detergent which denatures proteins by binding to the hydrophobic regions and essentially coating the linear protein sequence with a set of SDS molecules. The SDS is negatively charged and thus becomes the dominant charge of the complex.

- The number of SDS molecules that bind is simply proportional to the size of the protein. Therefore, the charge to mass ratio should not change with size. The density and pore size of this polymer can be varied by just how you make it (concentration of monomer and of cross-linking agent). Thus, the size of molecules that can pass through the matrix can be varied. This determines in what molecular weight range the gel will have the highest resolving power

- Basically get a sieving effect based on size of the protein

Native Gels

- It is also possible to run protein gels without the SDS. These are called native gels in that one does not purposely denature the protein. Here, the native charge on the protein (divided by its mass) determines how fast the protein will travel and in what direction.

Electrofocusing Gels

- Another variation of gel electrophoresis is to pour a gel that purposely has a pH gradient from one end to the other. As the protein travels through this pH gradient, its various ionizable groups with either pick up or lose protons. Eventually, it will find a pH where its charge is zero and it will get stuck (focused) at that point.

DNA Agarose Gels

- A simple way of separating fairly large fragments of DNA from one another by size is to use an agarose gel.
- DNA does not need a detergent, since it already has a large number of negative phosphate groups evenly spaced.
- Thus, as with SDS-PAGE, the charge to mass ratio is constant. Also like SDS-PAGE, the separation results from the matrix itself.
- The range of size sensitivity can be varied by changing the density of the agarose.

DNA denaturing polyacrylamide gels (often called *sequencing gels*)

- To look at smaller DNA molecules with much higher resolution, people generally denature the DNA via heat and run it through a thin polyacrylamide gel that is also kept near the denaturing temperature. These gels usually contain additional denaturing compounds such as Urea. Two pieces of DNA that differ in size by 1 base can be distinguished from each other this way

Capillary electrophoresis

- This type of electrophoresis can separate a wide variety of molecules of biological interest such as metabolites, drugs, amino acids, nucleic acids, carbohydrates, peptides and proteins based on their sizes and ionic properties. CE separations of proteins and peptides are based on charge-to-mass ratios. While PAGE separations are restricted to polyacrylamide matrices and a relatively small number of buffer systems, CE separations can be achieved in a variety of matrices and buffers. Consequently, there is much greater flexibility in the design of the optimal separation protocols.

Others

- Discontinuous (Disc) Gel Electrophoresis
- Two Dimensional Gel Electrophoresis
- Gradient Gel Electrophoresis
- Isoelectric Focusing
- Isotachophoresis
- Pulse-Field Gel Electrophoresis

Others - Types of Electrophoresis

Low voltage thin sheet electrophoresis

- The supporting medium may be paper, cellulose acetate, or a thin layer of material such as silica.
- **Paper.** Chromatography paper is suitable for electrophoresis. Low voltage paper electrophoresis has been extensively used in the past for the separation of a range of charged compounds such as amino acids, peptides, proteins, nucleotides, nucleic acids and charged carbohydrate derivatives. Considerable diffusion of small molecules occurs on paper during low voltage electrophoresis and better resolution may be obtained by applying high voltage, where the time required for separation is reduced and less diffusion of the molecules occurs.
- **Cellulose Acetate.** Cellulose acetate is a suitable medium for the separation of radiolabelled substances and for such microtechniques as immunodiffusion and immunoeletrophoresis. Cellulose acetate will, in general, separate the same range of compounds as paper but has found particular application in clinical investigations for the separation of blood proteins, including glycoproteins, lipoproteins and hemoglobins.
- **Thin Layer Electrophoresis (TLE).** Thin layers of silica, alumina or cellulose can be prepared on glass plates as for thin layer chromatography (TLC). The plates are placed horizontally into the electrophoresis unit and the thin layer is allowed to saturate with buffer by diffusion from the reservoir. TLE, like TLC, is rapid and gives good resolution and high sensitivity. High voltages may also be used in TLE.

- *There are many variations on gel electrophoresis with wide-ranging applications.*
- *Some specialized techniques include Southern, Northern, and Western Blotting. Blots are named according to the molecule under study.*
  - *In Southern blots, DNA is cut with restriction enzymes then separated.*
  - *In Northern blotting, RNA is separated.*
  - *Western blots target proteins.*

Tracking Dyes

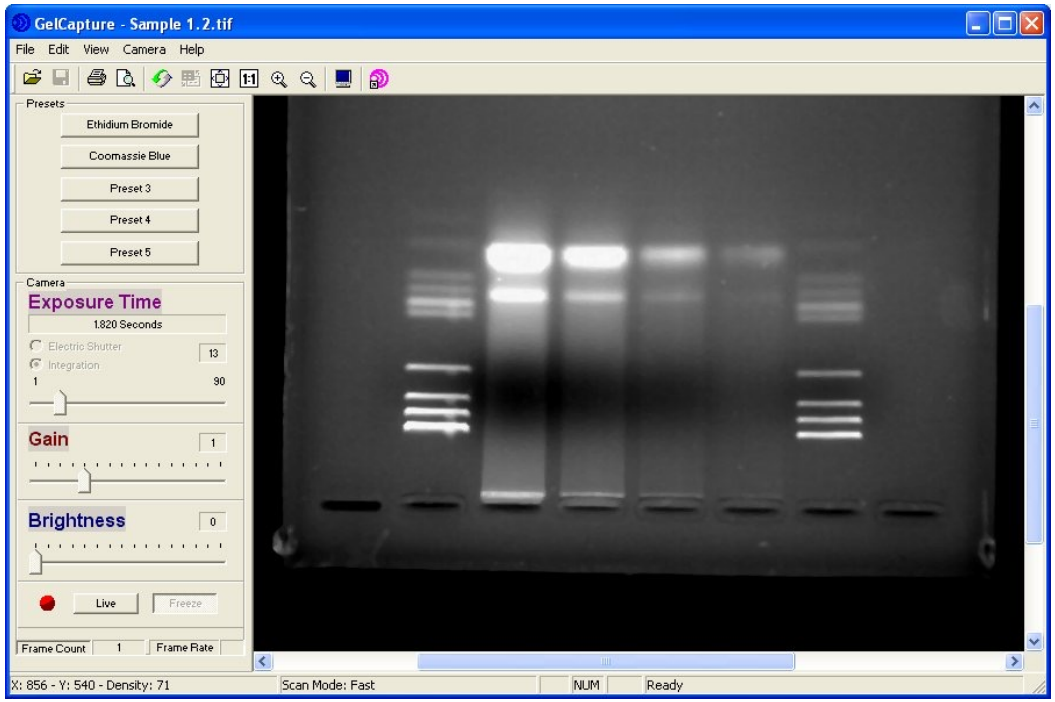
- Used when you don't know how long to run a gel. Appropriate tracking dye will run faster than your protein. Terminate the run when the tracking dye is about 5mm from the bottom of the gel.
  - Bromphenol Blue – for alkaline buffer systems
  - Methylene green, methylene blue, or Pyronine Y is appropriate for acid systems.

Detection of Proteins - Stains

- Coomassie Brilliant Blue Staining for proteins
- Silver Stain
- Copper Stain
- Alcian blue, Toluidine blue for glycoproteins
- Amido black, Wilson sudan black for lipoproteins
- Fluorescent Staining Techniques:
  - Ethidium Bromide – stains both RNA and DNA
- Specific Enzyme Visualization: lactate dehydrogenase-reduction of nitroblue tetrazolium chloride (yellow to blue); Periodic acid-Schiff for glycoproteins.
- Specific enzyme stains – peroxidase, etc.
- Detection of Radioactive Macromolecules: X-rays, liquid scintillation, sepctrophotometry

Gel Documentation

- Commercial scanners/photograph setups, with computer analysis/documentation
- Use a high quality desktop scanner, import into Powerpoint and annotate.
- Dry gels for preservation
  - Gel driers available
  - Dry between cellophane sheets
- Photograph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Ready


PENTAX

Uses of Gel Electrophoresis

- Quality control
- Complement field growouts
- Check out mixtures
- Check “rogues” in Foundation/Breeder seed fields
- PVP applications
- PVP enforcement
- Criminal court cases

- Backup test to other varietal purity tests
  - Fluorescence checks
  - Hilum checks
  - Phenol tests
  - KOH for white vs. red wheat
- Forgot to label bins
- **Corn - Check for selfs, hybrid, outcrosses, etc.**

How to Learn Electrophoresis?

- Experience
  - Work with someone already doing it
  - Trial and error
    - Patience needed
- Publications
- Equipment provider – technical assistance
- Classes – Biology and Biochemistry
- Websites – easy to find videos