

# **ELECTROPHORESIS**

Separation of charged molecules in a solution based on their tendency to move towards electrodes, by applying an electric field is called **Electrophoresis**. This technique was first developed by Arne Tiselius in 1930s.

Electrophoresis is a combination of two words Electro means Electricity, Phoresis means movement. Biomolecules such as nucleotides, amino acids, RNA, DNA and proteins are charged molecules. So, they can be separated by means of electrophoresis.

## **Principle:**

If a direct current is passed through a solution containing charged molecules, the positively charged cations (+) will migrate towards cathode (-) and the negatively charged anions (-) will move towards the anode (+). The mobility of the molecules, however, depends on the net charge, size and shape of the molecule, pH and composition of the suspending medium and applied current between the electrodes.

## **The Rate of Electrophoretic Migration Depends on The Following Factors:**

1. Net charge on the molecule.
2. Strength of electric field.
3. Size, mass and shape of the molecules.
4. pH of the medium.
5. Temperature.
6. Concentration of sample.

## **Electrophoresis Media:**

The solid materials and the buffer solutions which favour the separation of solute particle is called electrophoresis media. Electrophoresis has two types of media.

- i. Buffers
- ii. Supporting media

## **i. Buffers**

Buffer is a solution of a weak acid and one of its salts. It maintains a constant pH. It resists changes in  $H^+$  and  $OH^-$  ion concentrations. If the pH of the medium is changed, it affects the migration of ions. Phosphate Buffer, Tris-borate EDTA Buffer (TBE), Tris-acetate EDTA Buffer (TAE) and Tris-Glycine buffer are some of the commonly used buffers.

## **ii. Supporting Media**

A supporting medium or matrix is required to separate molecules according to their size and charge. It should be an inert, non-toxic material with specific porosity. It should have good filtering effect to separate molecules based on their size. It should not interfere with electrical conductivity of buffer. Paper strips, cellulose acetate, starch, agarose, polyacrylamide, sephadox, etc. are used as supporting medium in electrophoresis.

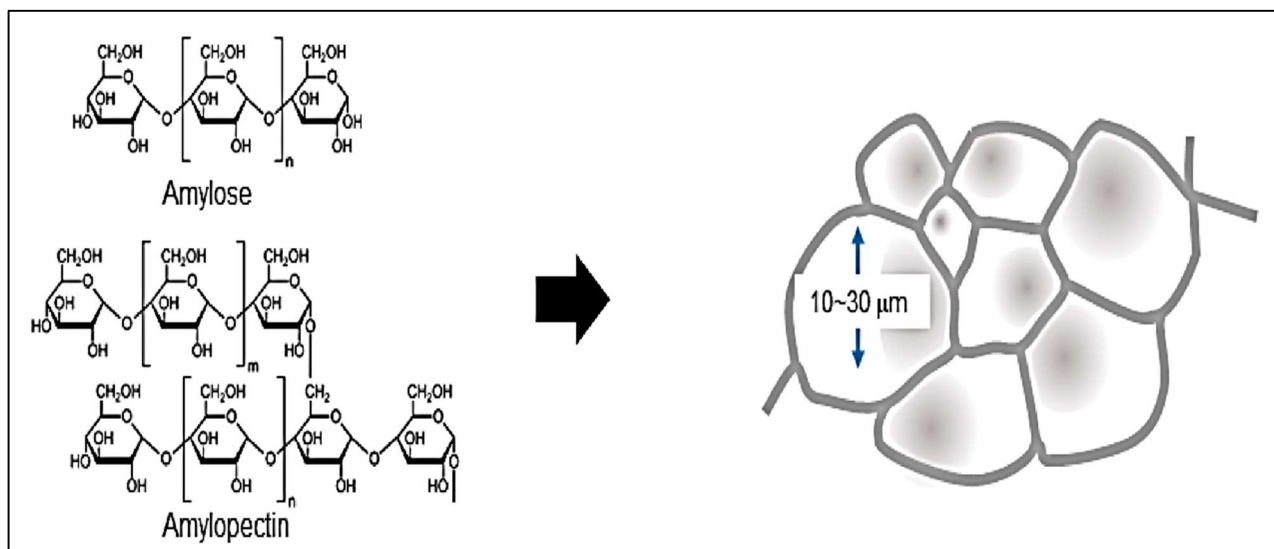
### **I. PAPER:**

Paper is a solid, fibrous macro support derived from high purity cellulose (typically Whatman No.1 or No.3). It consists of a non-uniform, coarse network of interwoven cellulose fibers with extremely large macro-pores. As it requires a long runtime and low voltage for separation, the resolution is poor due to the increased diffusion of the separated analytes. It is primarily applied in educational laboratories for demonstrating basic separation principles, such as analysing small polar molecules including free amino acids and simple sugars.

**Cellulose Acetate Membrane** is a modified chemical membrane designed to fix the surface interaction issues of standard paper. The hydroxy groups ( $-OH$ ) of cellulose are replaced with acetyl groups ( $-OCOCH_3$ ) at C-3 and C-6 glucose units positions. Manufactured by chemically acetylating cellulose fibers, resulting in a thin, uniform microporous film. The medium is

chemically inert, allowing molecules to pass strictly based on their charge-to-mass ratio without adsorption. Its mainly used for protein separation, especially serum proteins + hemoglobin variants.

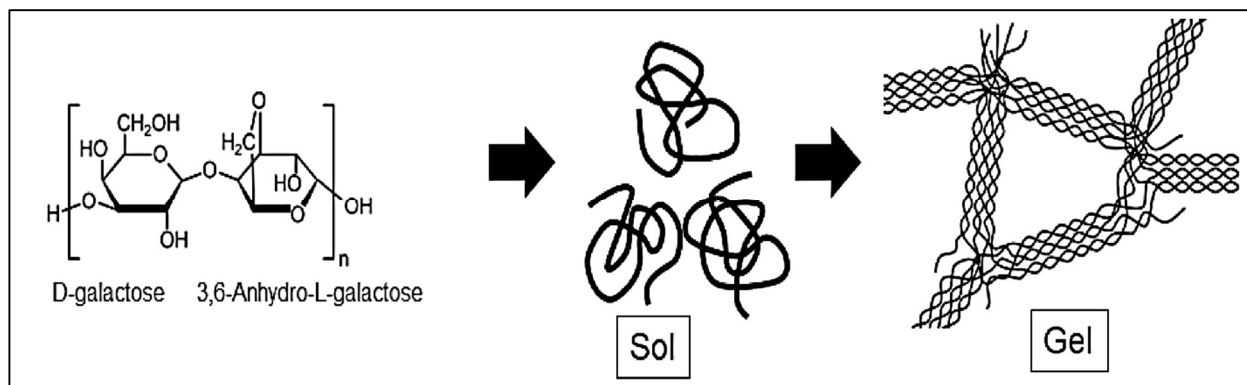
## II. STARCH:



It is the first gel medium used for electrophoresis. A colloidal suspension was prepared by boiling the suspension of starch granules in a buffer when allowed to cool sets as a semisolid gel due to the intertwining of the branched chains of amylopectin. Chemically, it consists of a mixture of two glucose polymer: linear amylose ( $\alpha$ -1,4 linkages) and branched amylopectin ( $\alpha$ -1,6 linkages). It is non-adsorbing and free of impurities. It gives better results in the separation of some isoenzymes.

The resolving power of starch gel is remarkably good, but its pore size cannot be controlled. It is often contaminated by microbes. So it becomes opaque on staining. This is not suitable for the separation of proteins. As gel preparation with reproducibility is challenging, it is not currently used.

### III. AGAROSE GELS

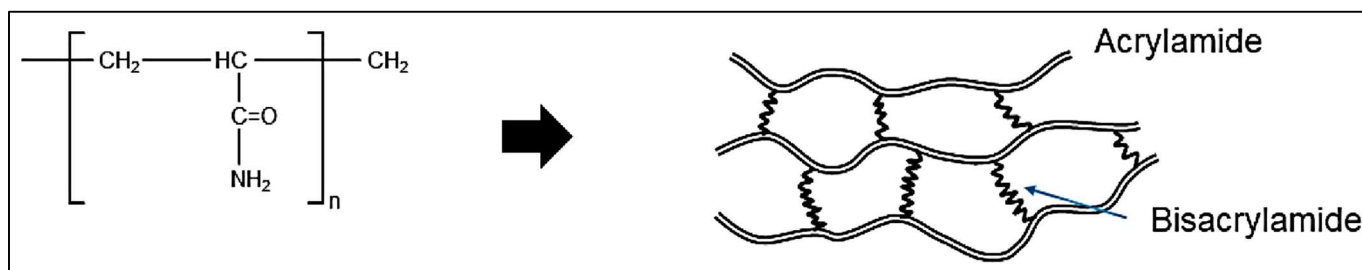


Agarose is isolated from red seaweed genera *Gelidium* and *Gracilaria*. It is a naturally occurring linear polysaccharide built from repeating agarobiose units, each a disaccharide of **D-galactose** and **3,6-anhydro-L-galactose** are linked by Glycosidic bonds.

Agarose gels are formed by suspending dry agarose in aqueous buffer, then boiling the mixture until a clear solution forms. This is poured and allowed to cool to room temperature to form a rigid gel. The gel formation is due to formation of inter- and intramolecular hydrogen bonding within and between the long agarose chains.

The pore size in the gel is controlled by the initial concentration of agarose, large pore sizes are formed from low concentrations and smaller pore sizes are formed from the higher concentrations. Agarose gels are used for the electrophoresis of both proteins and nucleic acids.

### IV. POLYACRYLAMIDE GELS:

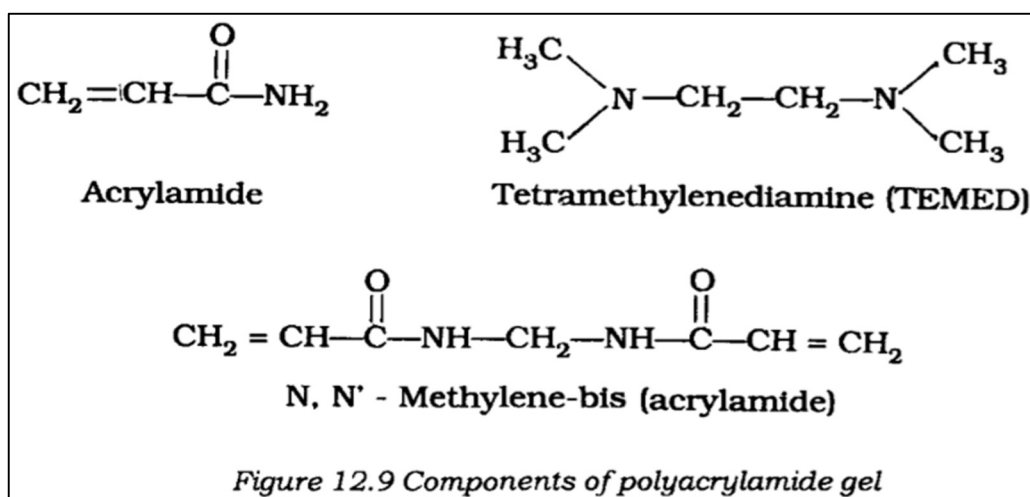


Polyacrylamide gels are chemically cross-linked gels formed by the polymerization of **acrylamide** with a cross-linking agent, usually

**N,N'-methylenebisacrylamide.** The reaction is a free radical polymerization, usually carried out with **ammonium persulfate** as the initiator and **TEMED** as the catalyst.

The acrylamide solution is poured in between the glass plate fitted into the gel caster. The linear monomers are interconnected by the cross linking with bis-acrylamide monomer to form a 3-D mesh with pores. The size of pore is controlled by the concentration of acrylamide and amount of bis-acrylamide in the gel. It is a technique widely used in biochemistry, forensic chemistry, genetics and biotechnology to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility.

### Chemistry of Polymerization of Acrylamide Gels:

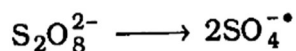


The most commonly used components to synthesize the matrix are acrylamide monomer, N, N'-methylenebisacrylamide (bis), ammonium per sulphate and tetramethylethylenediamine (TEMED). Polyacrylamide gels are formed by copolymerization of acrylamide and Bis-acrylamide (N, N' – Methylene-bisacrylamide). The reaction is a vinyl addition polymerization initiated by a free radical generating system.

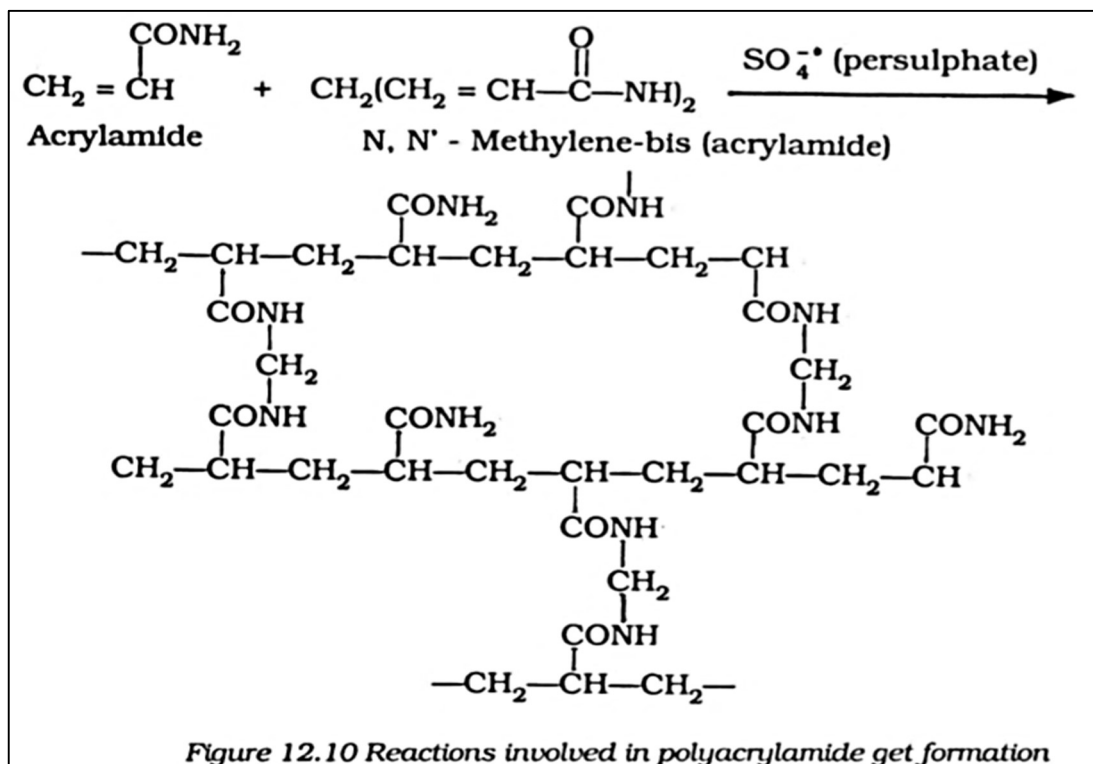
Polymerization is initiated by ammonium persulfate and TEMED (Tetramethylethylenediamine): TEMED accelerates the rate of formation of free radicals from persulfate and these in turn catalyze polymerization.

The acrylamide solution (a mixture of monomeric acrylamide and a bifunctional crosslinker bisacrylamide) is mixed with TEMED and APS and poured in between the glass plate fitted into the gel caster.

Ammonium persulphate when dissolved in water generates free radicals.

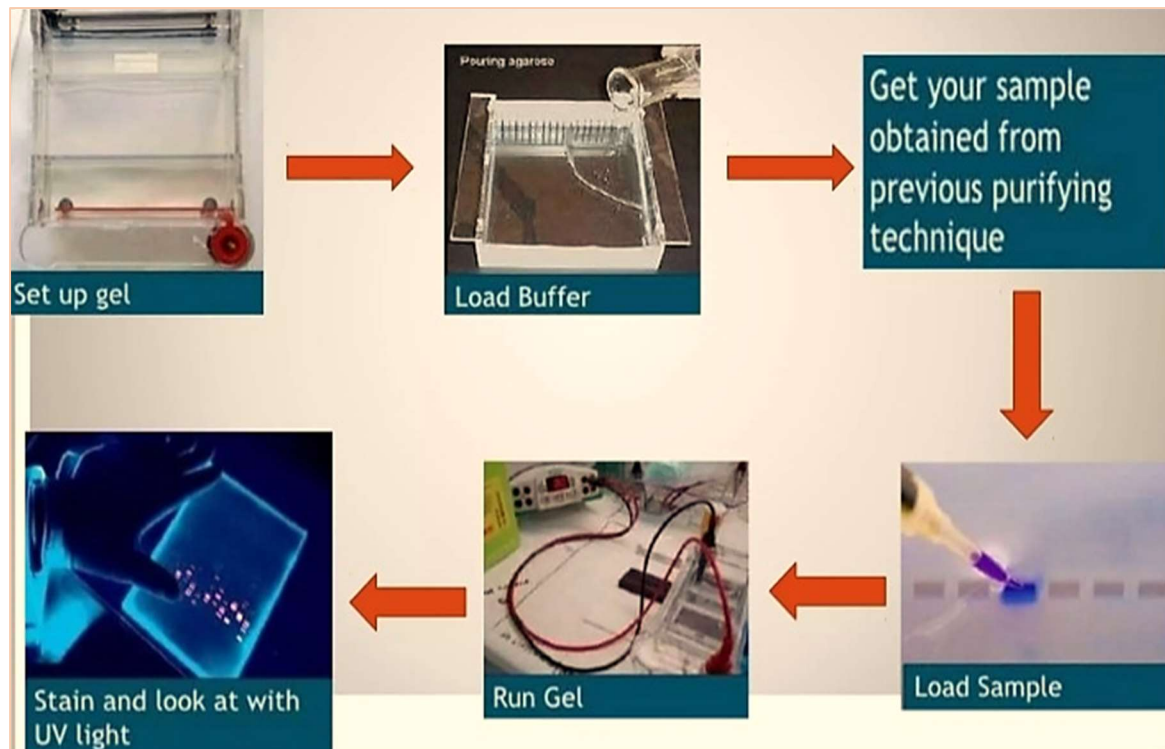


Ammonium persulfate in the presence of TEMED forms oxygen free radicals and induces the polymerization of acrylamide monomer to form a linear polymer. These linear monomers are interconnected by the cross linking with bis-acrylamide monomer to form a 3-D mesh with pores. The size of pore is controlled by the concentration of acrylamide and amount of bis-acrylamide in the gel.



# AGAROSE GEL ELECTROPHORESIS

Separation of molecules based on their charges and size using agarose gel as a supporting medium, is called agarose gel electrophoresis.

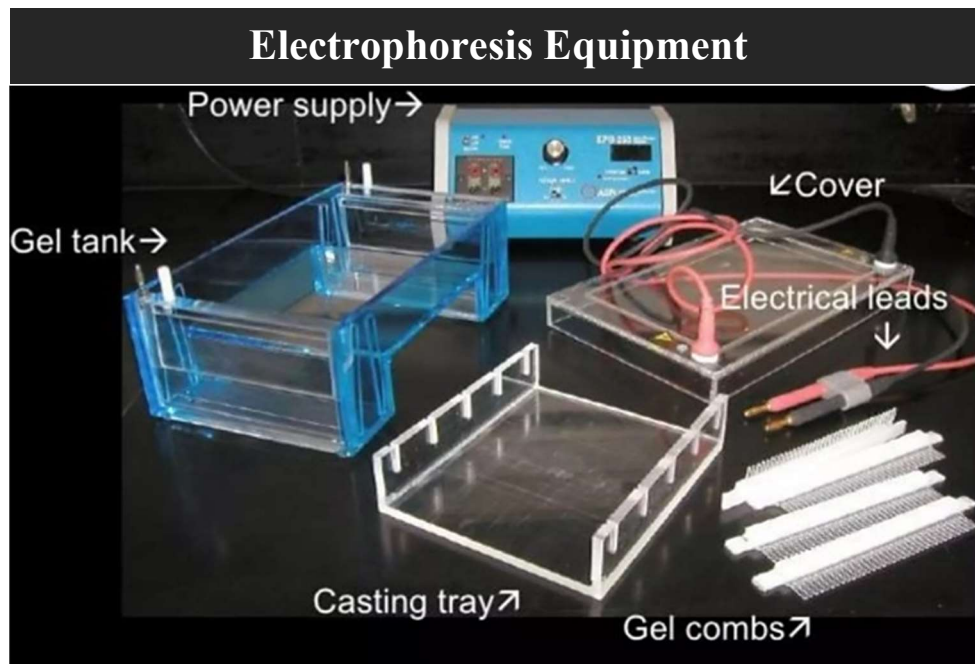


## Principle:

Agarose is a linear polysaccharide extracted from seaweed that forms a gel. Gel electrophoresis separates DNA fragments by size in a solid support medium such as an agarose gel.

During gelation, agarose polymers form a network of bundles whose pore sizes determine a gel's molecular sieving properties. In general, the higher the concentration of agarose, the smaller the pore size.

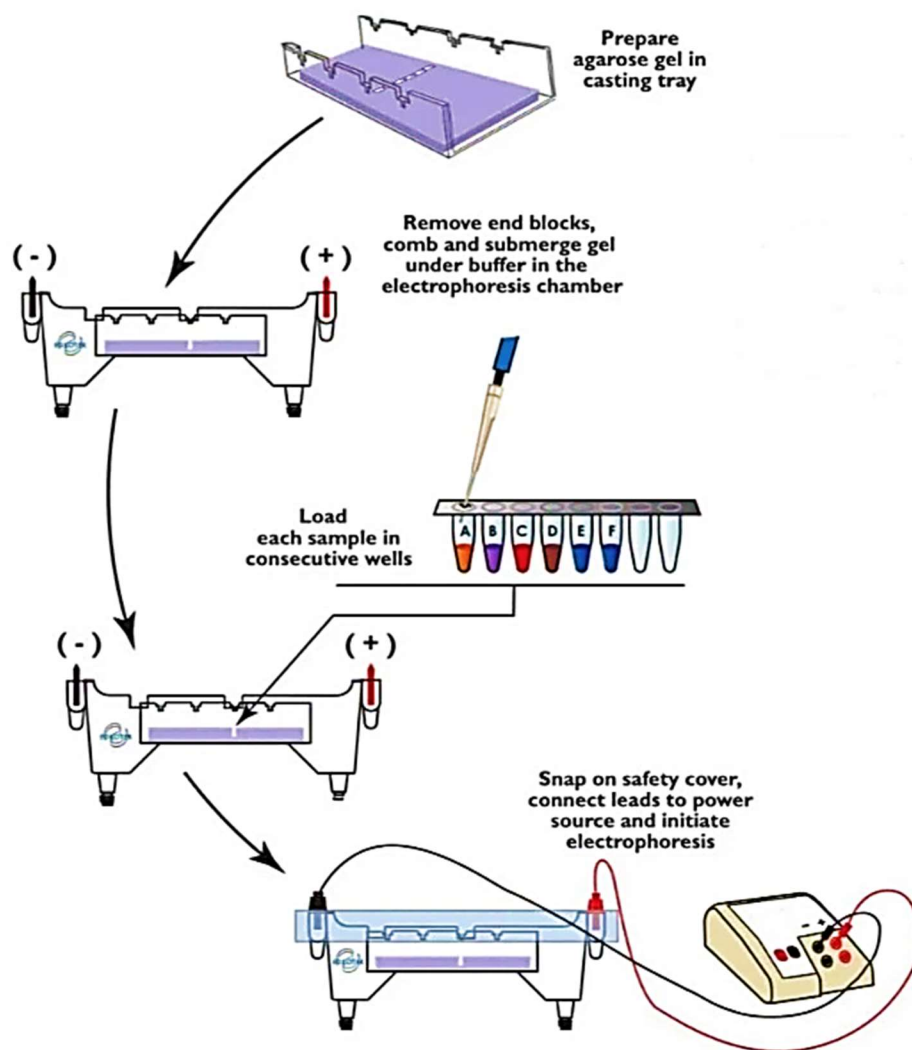
Sample (DNA) are pipetted into the sample wells, followed by the application of an electric current which causes the negatively charged DNA to migrate towards positively charged anode. The rate of migration is proportional to size of DNA fragments. Smaller fragments migrate faster than larger fragments under the influence of electric field. DNA is visualized in the gel by intercalating dye, **Ethidium Bromide** using UV transilluminator.



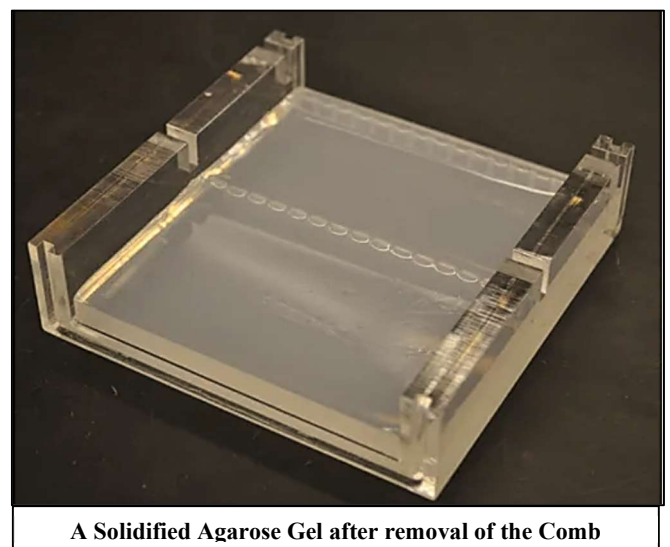
### Instrumentation of Agarose Gel Electrophoresis:

- An **Electrophoresis chamber** and **Power supply**
- **Gel casting trays**, which are available in a variety of sizes and composed of UV transparent plastic. The open ends of the tray are closed with tape while the gel is being cast, then removed prior to electrophoresis.
- **Sample combs**, around which molten medium is poured to form sample wells in the gel.
- **Electrophoresis Buffer**, usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).
- **DNA Ladder**: The DNA standard contains a mixture of DNA fragments of pre-determined sizes that can be compared against the unknown DNA samples.
- **Staining**: **Ethidium Bromide** binds to DNA and fluoresces under UV light, allowing the visualization of DNA on a gel.
- **Transilluminator** (an ultraviolet light box), which is used to visualize stained DNA in gels.

## Procedure:

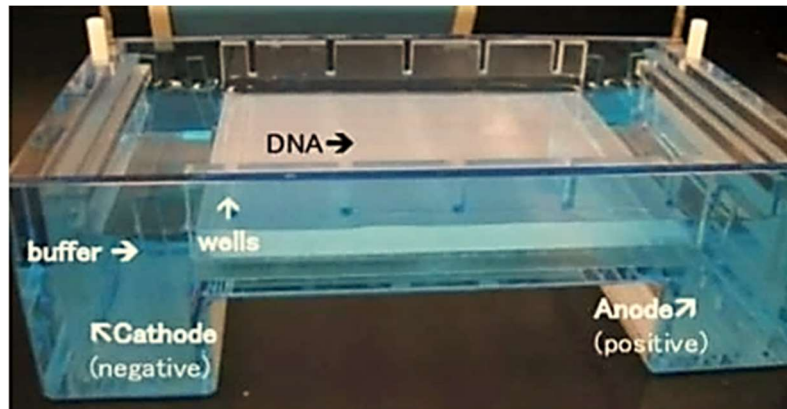


- Preparation of Agarose Gel Matrix:** The gel is made by dissolving agarose powder in a boiling buffer solution. The concentration of agarose in a gel depends on the sizes of the DNA fragments to be separated, with most gels ranging between 0.5% - 2%.
- The solution is then cooled to 55°C and poured into a casting tray and a comb is placed across the end of the casting tray to form wells when the gel solution solidifies.



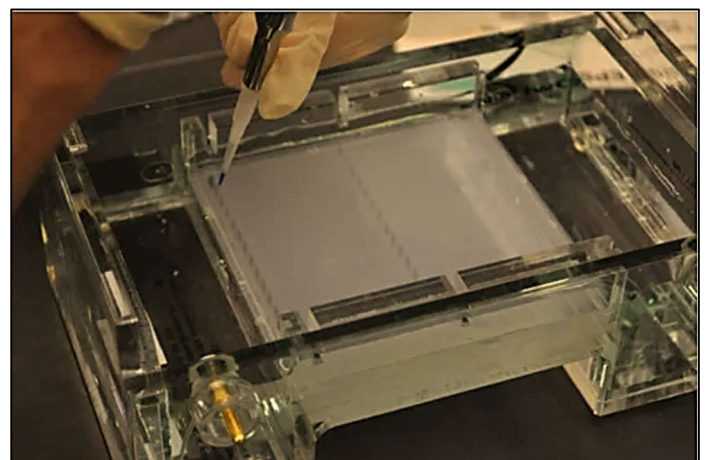
A Solidified Agarose Gel after removal of the Comb

- iii. After the gel solidifies, it is submerged in a buffer-filled electrophoresis chamber which contains a positive electrode (anode) at one end and a negative electrode (cathode) at the other.



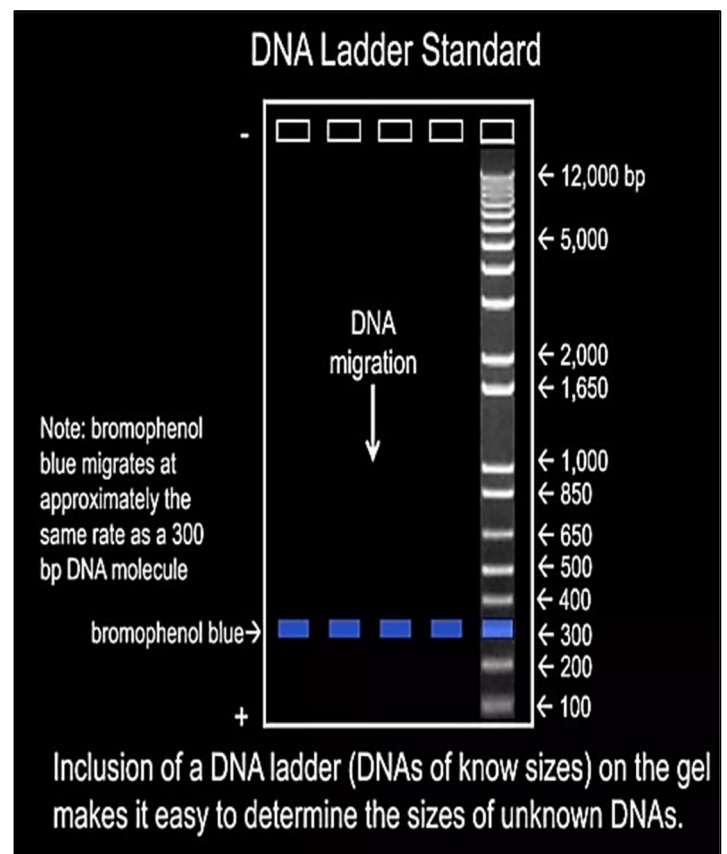
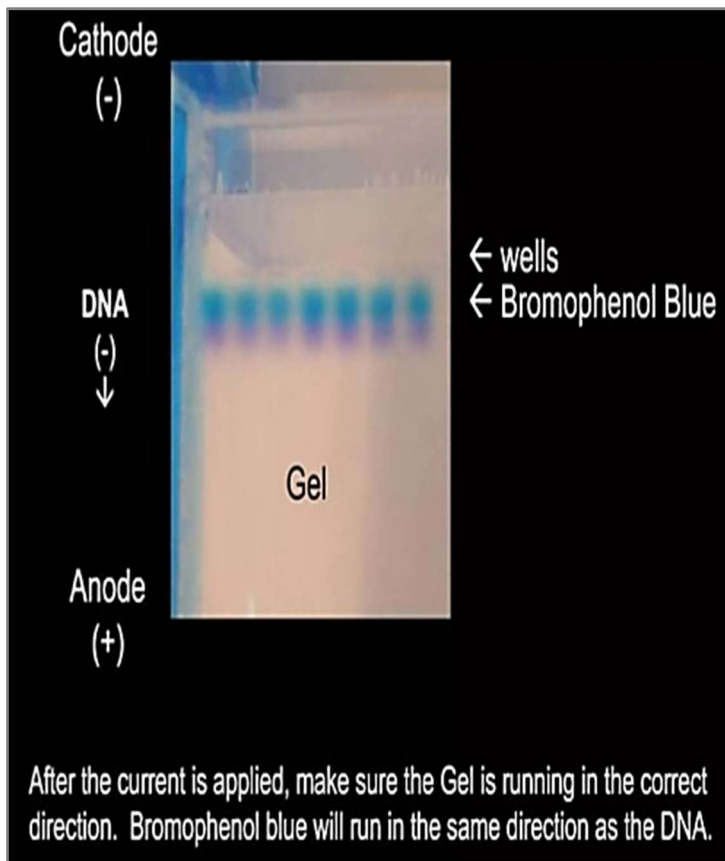
- iv. The two common running buffers are TAE (1X: 40 mM Tris-acetate, 1 mM EDTA) or TBE (0.5X: 45 mM Tris-borate, 1 mM EDTA). The buffer should cover the gel surface by roughly 2 to 5 mm. Too little buffer allows the gel to dry and overheat. Too much buffer allows current to shunt through the buffer layer above the gel rather than through the gel itself, which generates heat and blurs the bands.
- v. **Sample Preparation and Loading:** Samples (DNA) are prepared for electrophoresis by mixing them with loading dyes. These samples are delivered to the sample wells with a clean, adjustable-volume micropipette. Gel loading dye is typically supplied at 6X concentration (0.25% bromophenol blue, 30% glycerol). Loading dyes used in gel electrophoresis serve three major purposes:

- Add density to the sample, so that it sinks to the bottom of the well rather than drifting out into the running buffer.
- Provide colour and simplify the loading process.
- The dyes move at standard rates through the gel, allowing for the estimation of the distance that DNA fragments have migrated.



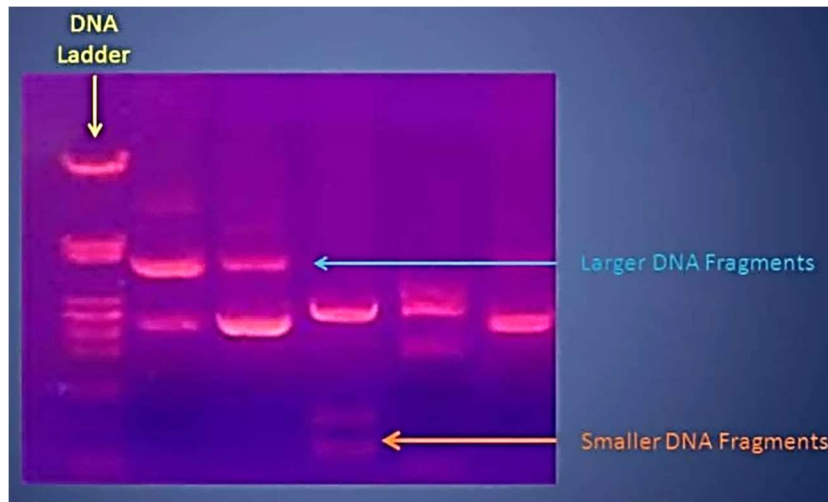
Loading the DNA sample into a well in the gel

- vi. **Applying Electric Current and Separating Biomolecules:** A power source is connected to the electrophoresis apparatus and an electrical current is applied. Charged molecules in the sample enter the gel through the walls of the wells. The buffer serves as a conductor of electricity and controls the pH, which is important to the charge and stability of biological molecules. Since **DNA has a strong negative charge** at neutral pH, it migrates through the gel towards the **positive electrode** during electrophoresis.



- vii. Ethidium bromide can be added during the gel preparation or alternatively, the gel may also be stained after electrophoresis in running buffer containing 0.5 µg/ml EtBr for 15-30 min, followed by destaining in running buffer for an equal length of time.
- viii. **Visualization:** Ethidium bromide (EtBr) works by intercalating itself in the DNA molecule in a concentration-dependent manner. When exposed to an ultraviolet light source (transilluminator), fluoresces under UV light, allowing the visualization of DNA on a Gel.

- The size of the DNA fragments can be estimated by comparing their locations in relation to the positions of the molecular markers.



### **Application of Agarose Gel Electrophoresis:**

- Estimation of the size of DNA molecules.
- It is used for the separation of DNA fragments in a sample and to detect the purity of DNA fragments.
- Agarose gels allow purification of DNA fragments.
- Analysis of PCR products, e.g. in molecular genetic diagnosis or genetic fingerprinting.
- Used to resolve circular DNA with different supercoiling topology, and to resolve fragments that differ due to DNA synthesis.
- To estimate the size of DNA fragments after digesting with restriction enzymes, e.g. in restriction mapping of cloned DNA.