# S. S. College, Jehanabad

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**Topic:** Separation techniques - Agarose Gel Electrophoresis

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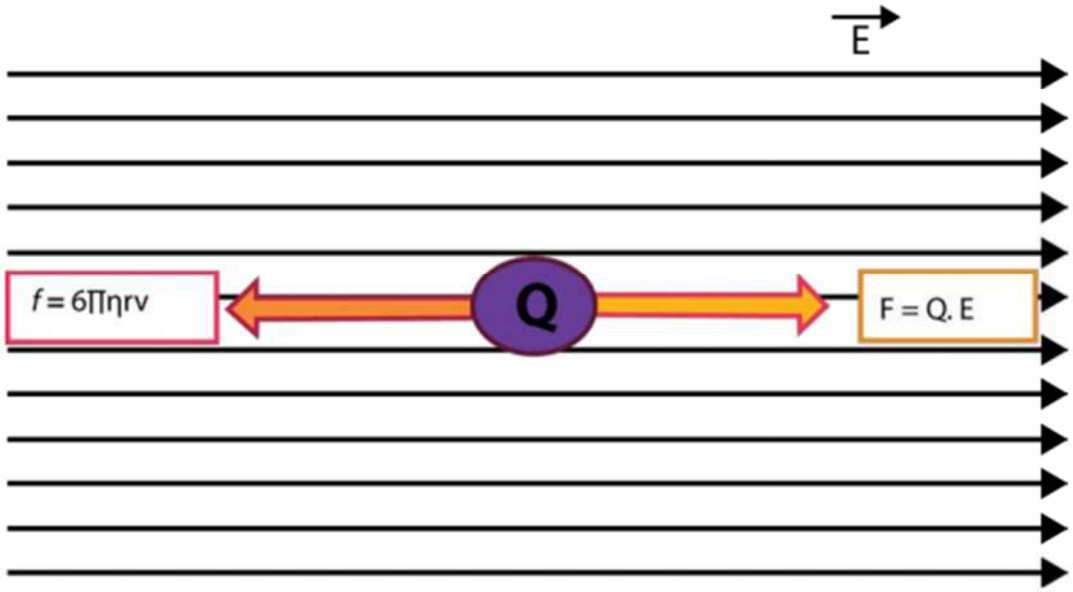
# SEPARATION TECHNIQUES - AGAROSE GEL ELECTROPHORESIS

Electrophoresis refers to the migration of a charged particle under the influence of electric field (-phoresis in suffix is indicative of transmission; from Greek *phorēsīs* being carried, from *pherein* to bear). Therefore, electrophoresis literally means running in the electric field. Charged molecules moves from one electrode to the counter electrode (from cathode to anode or from anode to cathode according to the net charge present on the molecules) in the buffer but before reaching to the counter electrode, electric field is removed and molecules are stacked before the counter electrode. The molecules move according to the charged strength and size of the molecule and thus different molecules stopped at different distance from the electrode after removing the electric field. To slow the movement so that a better resolution of molecules in the mix can be achieved, polymeric gel is used. The polymeric gel is inert, uncharged and does not cause retardation by binding the molecule. Instead, it forms pores of different sizes (depending on the concentration of polymer) and sample passes through these pores and as a result their electrophoretic mobility is reduced. In biological system, most of the biomolecules, such as amino acids, peptides, proteins, nucleotides, nucleic acids, etc. possess charged groups, and therefore, exist in solution as electrically charged species either cations or anions at any given pH.




**Movement of charged particles in the electric field**

Suppose a charged particle has net charge **'Q'** and the external electric field is **'E'**, then the force **'F'** responsible for giving electrophoretic mobility is:

$$\mathbf{F = Q \times E} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (\boldsymbol{i})$$

However, movement of molecules is not freely occurring, and frictional force hampers the movement of molecules. Therefore, frictional force '***F***' that impedes electrophoretic mobility is:

$$\boldsymbol{F = f \times v} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (\boldsymbol{ii})$$

where, '$\boldsymbol{f}$' is the friction coefficient and the '$\boldsymbol{v}$' is the velocity of the electrophoretic mobility. The movement of a spherical particle through a liquid medium (gel) of the viscosity '**η**', the friction coefficient 'f' is given by;

$$\boldsymbol{f = 6\Pi\eta r v} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (\boldsymbol{iii})$$

Therefore, by equilibrating the equation (i), (ii) and (iii), we get;

$$\mathbf{F} = \boldsymbol{F \ or \ QE = 6\Pi\eta r v}$$

and therefore electrophoretic mobility '**v**' is given by:

$$\mathbf{v} = \frac{\boldsymbol{Q}}{\boldsymbol{6\Pi\eta r}}$$

As $Q = ze$, where, '**z**' is the valency and '**e**' is the electronic charge, the electrophoretic mobility can be expressed as

$$\mathbf{v} = \frac{\boldsymbol{ze}}{\boldsymbol{6\Pi\eta r}}$$

Hence, electrophoretic mobility '**v**' is directly proportional to the charge and inversely proportional to the viscosity of the medium, size and shape of the molecule. In the case of relative mobility, it is directly related to the charge/radius of the molecule. For globular proteins, the radius '**r**' of the molecule is related to the molecular mass of the macromolecule. Therefore, the relative mobility '**v'**' will be as follows;

$$\mathbf{v} = \frac{\boldsymbol{Charge}}{\boldsymbol{Mass}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (\boldsymbol{iv})$$

Therefore, the rate of migration of charged molecules depend upon following factors;

- The strength of electric field, size and shape;
- Relative hydrophobicity of the sample;
- Ionic strength and temperature of the buffer;
- Molecular size of the taken biomolecule;
- Net charge density of the taken bio molecule; and
- Shape of the taken biomolecule.

In the process of electrophoresis large molecules have more difficulty in moving through the supporting medium (i.e., gel) whereas the smaller medium has more mobility through it.

## Types of electrophoresis

All modern electrophoretic apparatus have supporting media these days. A supporting medium is a physical support through which the charged molecules get separated. It has two primary

functions; adsorption and molecular sieving of the taken molecules which are intended to be separated. Need of supporting medium is felt due to the fact that during electrophoresis, increase in temperature due to convection current[1], liquid, solid, or gas expand, which makes the separated molecules distorted and arranged as dome shape at the latent phase. This distortion can be minimized by stabilizing the medium, which was achieved by carrying out electrophoresis on a porous mechanical support. Due to its viscosity (absent in free solution), the supporting media cut down the convection current gives the molecules separated in sharp zones. Some of the frequently used supporting media are starch, agar, polyacrylamide and agarose.

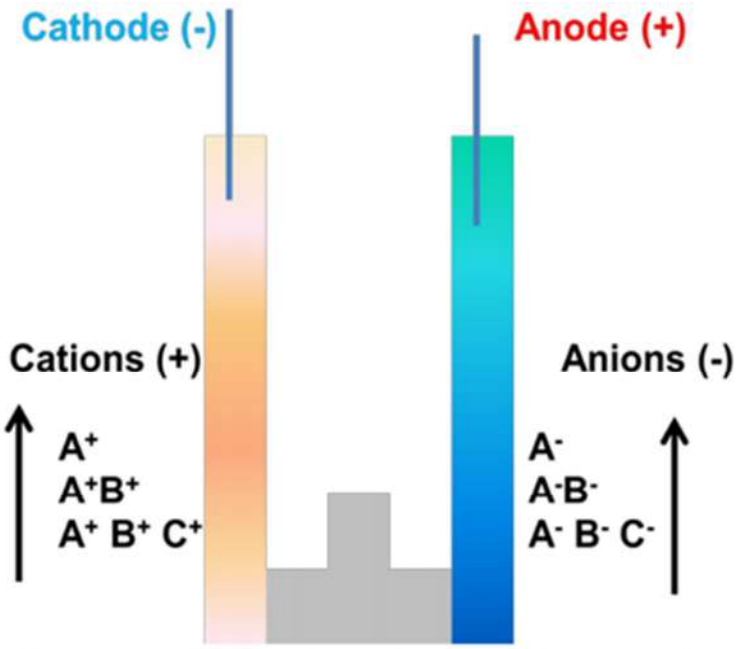
Depending upon the presence or absence of supporting media, the electrophoresis can be classified as free electrophoresis and zone electrophoresis. These are as follows;

**Free electrophoresis**

In this type of electrophoresis, a free electrolyte is taken in place of supporting media. Now-a-days, this type of electrophoresis has become obsolete and thus it has been discontinued. This type of electrophoresis was mostly used in non-biological experiments. It is also of two types; micro electrophoresis and moving boundary electrophoresis.

***Microelectrophoresis (ME):*** This is a method of studying electrophoresis of various dispersed particles using optical microscopy. This method provides image of moving particles, which is its unique advantage, e.g. observation of RBCs, neutrophils and bacteria. This type of electrophoresis is carried out in a closed medium with critical observations by focusing and adjusting the lens of microscope. Now, it is mostly used in the calculation of Zeta potential[2] (a colloidal property of cells in a liquid medium) of the cells.

***Moving boundary electrophoresis (MBE):*** It is also known as free-boundary electrophoresis. It is a technique used for separation of chemical compounds by electrophoresis in a free solution. It was developed by Arne Tiselius in 1930 for the separation of colloid through electrophoresis, for which he was awarded with Nobel Prize in Chemistry in 1949. It has been for



Movement of the charged particle in a moving boundary electrophoresis.

[1] Convection current is a process which involves the movement of energy from one place to another. They tend to move a fluid or gas particles from one place to another.

[2] Zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the particle and it is measured by a zeta potential analyzer.

many years then used for quantitative analysis of complex mixtures of macromolecules especially proteins.

**Zone Electrophoresis**

It is the widely used electrophoretic techniques mostly practiced these days. In this type of electrophoresis, the separation process is carried out on a stabilizing media as discussed above. Zone electrophoresis is further divided into following types;

- Paper electrophoresis
- Cellulose acetate electrophoresis
- Capillary electrophoresis
- Gel electrophoresis

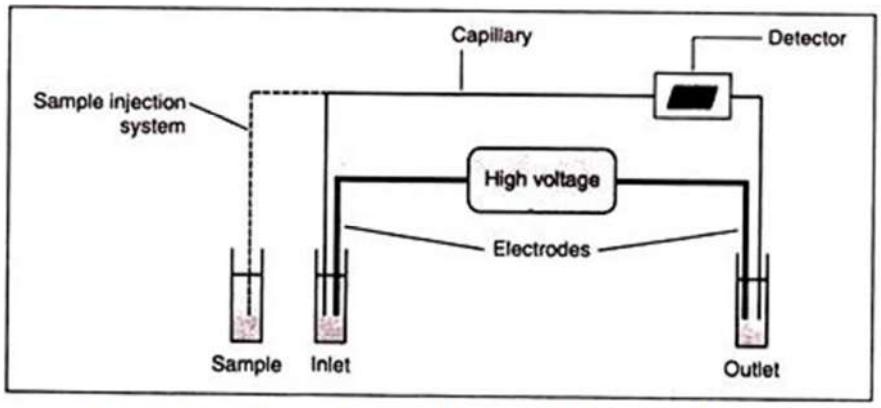
***Paper electrophoresis:*** In this type of electrophoresis, a filter paper (like chromatography paper) having slight adsorption capacity and uniform pore size is used as the supporting medium for separation of samples under the influence of an applied electric field. While carrying out paper electrophoresis, a strip of filter paper is moistened with buffer and ends of the strip are immersed into buffer reservoirs containing the electrodes.

The samples are spotted in the center of the paper, high voltage is applied, and the spots migrate according to their charges. After electrophoresis, the separated components can be detected by a variety of staining techniques, depending upon their chemical identity. It is applied mostly in diagnostic purpose like serum analysis, muscle protein (myosin) analysis, egg protein (albumin) analysis, ilk protein (casein) analysis, snake and venom analysis, etc. However, it is very time consuming process, needs about 14-16 h to complete.

***Cellulose acetate electrophoresis:*** It is a modified version of paper electrophoresis and was developed by Kohn in 1958. In this type of electrophoresis, bacteriological acetate membrane filters are taken in place of regular chromatography paper. However, it is some advantages over the paper electrophoresis, such as the cellulose acetate strips are chemically paper and free of lignin as well as hemicellulose, and due to low sugar content, it is fit for electrophoresis of polysaccharides also. Further, cellulose acetate is not hydrophilic and this holds very little buffer which further helps for a better resolution in a short time. It is especially used for clinical investigation such as separation of glycoproteins, lipoproteins and hemoglobin from blood.

***Capillary electrophoresis:*** Capillary of narrow bore tube is employed to separate the samples based on their size: charge ratio. Capillary electrophoresis (CE) is relatively new separation technique compared to the traditional techniques such as agarose gel electrophoresis or SDS-PAGE. It provides attractive features which make it both competitive and a good alternative. One of the major advantages of this technique over other separation technique is the ability to separate both charged and non-charged molecules. In CE, separation of analyte ions is performed in an electrolyte solution (background electrolyte) present in a narrow fused-silica capillary. The ends of the capillary are immersed into vials (inlet and outlet) filled with electrolyte solution, which also contain electrodes connected to a high voltage supply (schematic diagram given

below). The sample solution is introduced in the capillary as a small plug by applying pressure (hydrodynamic injection) or voltage (electrokinetic injection).



**Typical capillary electrophoresis instrumentation**

With the application of high voltage (5 – 30 kV) across the capillary, zones of analyte are formed due to different electrophoretic mobilities of ionic species and migrate towards the outlet side of the capillary. In fact, different ions can be separated when their charge/size ratio differs. Before reaching the end of the capillary, the separated analyte bands are detected directly through the capillary wall.

This technique of electrophoresis is commonly applied in the analysis of food, pharmaceutical products and environmental pollutants. It has following advantages;

- High separation efficiency
- Short analysis time
- Low sample and electrolyte consumption
- Low waste generation
- Ease of operation

In spite of these several advantages, it has some disadvantages too. In this electrophoresis technique, heat is dissipated due to small diameter that causes increased diffusion which ultimately leads to improper resolution of molecules.

***Gel electrophoresis:*** This technique involves the use of gel as supporting media for separation of DNA, RNA or proteins under the influence of electric charge. It is usually performed for analytical purposes but may be used as a preparative technique to partially purify molecules prior to use for other methods such as mass spectrometry, PCR, cloning, DNA sequencing and immunoblotting. It is the most commonly used electrophoresis technique in biotechnology laboratories and is used for almost all types of experiments in RD.

It works on the principle of electromotive force (EMF) generated across the electrodes that pushes or pulls the molecules (DNA, RNA or proteins) through the gel matrix. The molecules move towards the anode if negatively charged or towards the cathode if positively charged. This electrophoresis technique is of several types, such as agarose gel electrophoresis, sodium dodecyl sulfate polyacrylamide electrophoresis (SDS PAGE), pulsed-field gel electrophoresis (PFGE) and two-dimensional electrophoresis.

A typical gel electrophoresis apparatus is of two kinds, which are as follows;

- **Vertical Gel Apparatus –** It is used for the separation of proteins in SDS-PAGE.
- **Horizontal Gel Electrophoresis –** It is used for immune electrophoresis, isoelectric focusing and electrophoresis of DNA and RNA in the agarose gel

***We need to study only Agarose Gel Electrophoresis and SDS-PAGE in detail as given in the syllabus.***

## Agarose Gel Electrophoresis

It is a specialized method of electrophoresis that is used in biochemistry and molecular biology to separate DNA or RNA molecules by size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field. Shorter molecules move faster and migrate farther than longer ones.

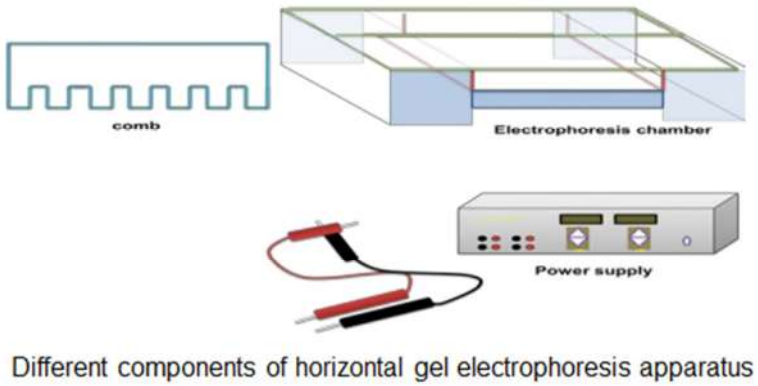
### Principle

When a potential difference is applied across the electrodes of a horizontal electrophoretic tank containing agarose gel and biomolecules (such as nucleic acids) are loaded, then they get separated according to their molecular size (bigger molecules have more molecular size and smaller molecules have small molecular size) and move to their respective electrodes. Here the agarose gel acts as a sieve.

As in a sieve the large particles stay above and the particles which are smaller than the pore size passes through it. Similarly in the gel, the larger and the bulky molecules stay behind whereas the smaller molecules move faster and quickly towards their respective electrodes. This process may be imagined like a running competition. The one who is thinner and have a flexible body will be at the ending point sooner than the one who is fat and bulky.

### Instrumentation

The electrophoresis is performed in a continuous fashion with both electrodes and gel cassette submersed within the buffer. The electrophoresis chamber has two platinum electrodes placed on the both ends are connected to the external power supply from a power pack which supplies a direct current or DC voltage. The tank, which is generally horizontal, filled with the running buffer and the gel casted is submerged inside the buffer. There are additional accessories needed for casting the agarose gel such as comb (to prepare different well), spacer, gel caster etc. The system consists of three components, which are as follows;



Different components of horizontal gel electrophoresis apparatus

**Electrophoresis apparatus:** Horizontal gel electrophoresis system is designed for very fast and clear separation of DNA restriction fragments in agarose

gels. Gel apparatus vary according to manufacturer. The simplest apparatus is called a flatbed horizontal apparatus. This apparatus consists of two buffer chambers; running buffer is poured from the agarose in flatbed horizontal tank, and sample buffer is poured in small wells in which sample loading is located.

**Power supply:** For a standard aga rose gel electrophoresis a voltage of 5 volts per cm of gel is applied (the cm value is the distance between the two electrodes, not the length of the gel).

**Transilluminator*:*** This is an ultra violet light box which is used to visualize ethidium bromide-stained DNA in gels.

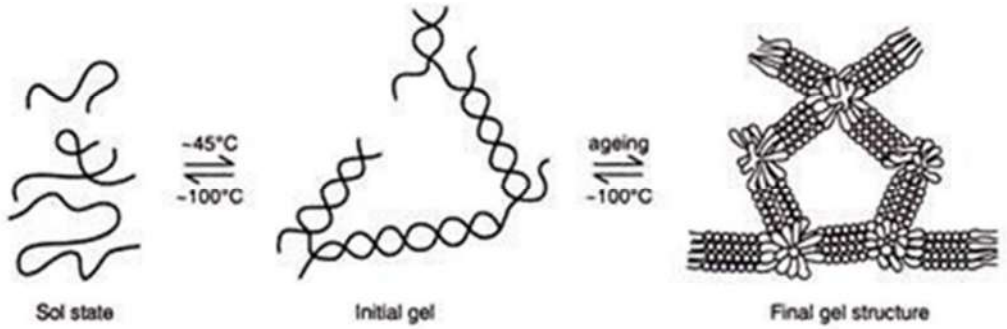
**Buffer and Reagents**

Buffer and reagents required for agarose gel horizontal electrophoresis and its purpose are as follows;

- ***Agarose:*** It is a polymeric sugar which is used to prepare horizontal gel for DNA analysis.
- ***Ethidium bromide:*** It is to stain the agarose gel to visualize the DNA under transilluminator.
- ***Sucrose:*** It is used for preparation of loading dye for horizontal gel.
- ***Tris-HCl:*** It is a major component of the running buffer.
- ***Bromophenol blue:*** It is a tracking dye to monitor the progress of the electrophoresis.

All these chemical components can be classified into three major components, which are as follows;

**Supporting media:** Agarose is a polysaccharide extracted from sea weed. It is a linear polymer composed of alternating isomers of the sugar D- and L-galactose. Agarose melts approximately at 90°C and gels approximately at 40°C. This gelation leads to the formation of a mesh of channels with a diameter which vary from 50-200nm. It is the diameters of these channels which determine the final porosity of the gel.



Gel structure in agarose gel

By using gels with different concentrations of agarose, one can resolve different sizes of DNA fragments. Higher concentrations of agarose facilitate separation of small DNAs, while low

agarose concentration is maintained during the separation of DNA having higher molecular weight. It is typically used at concentrations of 0.5 to 2%.

**Buffer:** As described above, the gel is immersed within an electrophoresis buffer that provides ions to carry a current and some type of buffer to maintain the pH at a relatively constant value. There are two types of buffer which is used in the agarose gel electrophoresis, which are electrophoresis buffer and loading buffer.

***Electrophoresis buffer:*** Depending on the size of the DNA to be electrophoresed and the application, either TAE (Tris-Acetate-EDTA) or TBE (Tris-Borate-EDTA) buffer is used as an electrophoresis buffer.

- *TAE buffer:* It is the most commonly used agarose gel electrophoresis buffer. TAE has the lowest buffering capacity; however, it offers the best resolution for larger DNA. TAE also requires a lower voltage and more time.
- *TBE buffer:* TBE buffer is often used for smaller DNA fragments (i.e., less than 500bp). Sodium borate or SB buffer is a new buffer but it is ineffective for resolving fragments larger than 5 kb. SB has advantages in its low conductivity, allowing higher voltages (up to 35 V/cm). This could allow a shorter analysis time for routine electrophoresis.

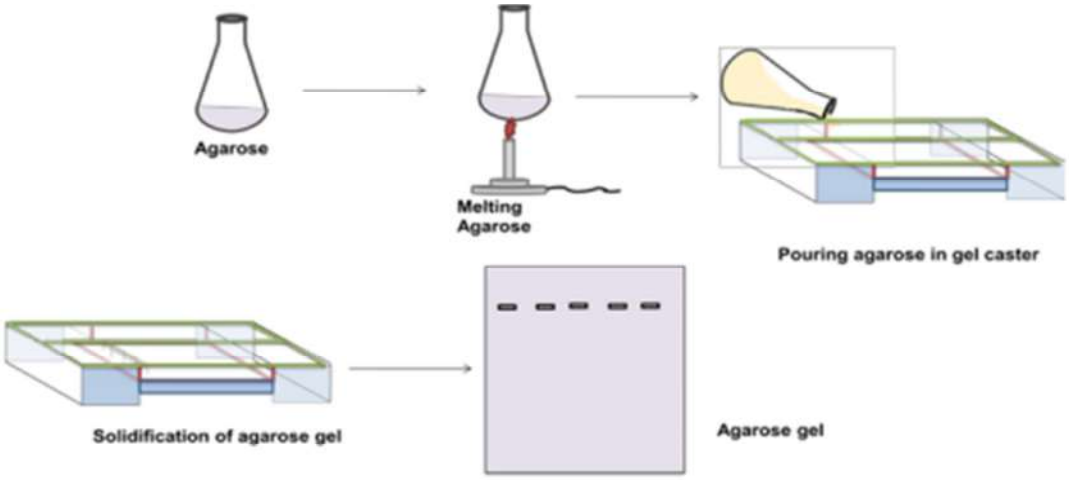
***Loading buffer:*** This contains a dense medium (e.g. glycerol) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring of the extent of electrophoresis.

***Dyes:*** This is a tracking agent that aims at visualizing the separated nucleic acid after the process of electrophoresis. In the agarose gel electrophoresis, ethidium bromide is used as a dye. Ethidium bromide is a fluorescent dye that binds to DNA and intercalates between the stacked bases.

**Procedure**

It starts with casting of agarose gel, loading of samples in the gel and running the samples in horizontal electrophoresis system. The different step of the agarose gel electrophoresis is as follows;

***Casting of the Agarose Gel:*** For casting agarose gel, the agarose powder is dissolved in a buffer which may be either Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE) and heated to melt the agarose. Hot agarose is poured into the gel cassette and allowed it to set. A comb can be inserted into the hot agarose to cast the well for loading the sample. In few cases, we can add ethidium bromide within the gel so that it stains the DNA while electrophoresis.




Different steps in casting of the agarose gel for horizontal gel electrophoresis apparatus

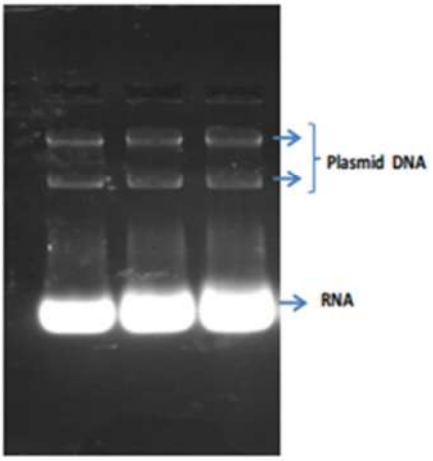
***Running and Staining:*** The detail step by step procedure is as follows;

- First of all agarose powder is mixed with electrophoresis buffer to the desired concentration, then heated in a microwave oven until completely melted. Most commonly, ethidium bromide (EtBr) is added to the gel (final concentration at 0.5 mg/ml) at this point to facilitate visualization of DNA after electrophoresis.
- After cooling the solution to about 60°C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature. After the gel has solidified, the comb is removed, using care not to rip the bottom of the wells.
- The gel, still in its plastic tray, is inserted horizontally into the electrophoresis chamber and just covered with buffer. Samples containing DNA mixed with loading buffer are then pipetted into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied.
- It should be confirmed that current is flowing by observing bubbles coming off the electrodes. DNA will migrate towards the positive electrode, which is usually coloured red. When adequate migration has occurred, DNA fragments are visualized by staining with ethidium bromide.
- This fluorescent dye intercalates between bases of DNA and RNA. It is often incorporated into the gel so that staining occurs during electrophoresis, but the gel can also be stained after electrophoresis by soaking in a dilute solution of ethidium bromide.
- To visualize DNA or RNA, the gel is placed on a ultraviolet transilluminator. It should be aware that DNA will diffuse within the gel over time, and examination or photography should take place shortly after cessation of electrophoresis.

**Application of Agarose Gel Electrophoresis**

- Separation of restriction enzyme digested DNA including genomic DNA, prior to Southern Blot transfer. It is often used for separating RNA prior to Northern transfer.
- Analysis of PCR products after polymerase chain reaction to assess for target DNA amplification.

- Allowing estimation of the size of DNA molecules using a DNA marker or ladder which contains DNA fragments of various known sizes.
- Allows the rough estimation of DNA quantity and quality.
- Assessing quantity of DNA using lambda DNA ladder which contains specific amounts of DNA in different bands.
- Assessing the quality of DNA by observing the absence of streaking or fragments (or contaminating DNA bands).
- Other techniques rely on agarose gel electrophoresis for DNA separation including DNA fingerprinting.



Observation of DNA stained with EtBr in a UV chamber

**Reference**

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2. https://people.wou.edu/~courtna/ch462/Gel%20Electrophoresis.pdf
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