Gel electrophoresis

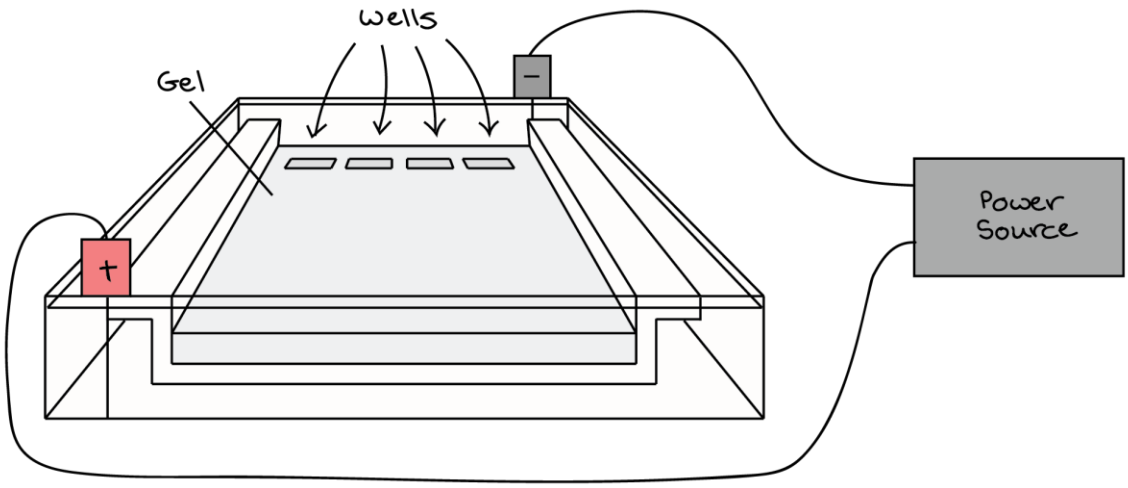
Gel electrophoresis is a technique used to separate DNA fragments (or other macromolecules, such as RNA and proteins) based on their size and charge. Electrophoresis involves running a current through a gel containing the molecules of interest. Based on their size and charge, the molecules will travel through the gel in different directions or at different speeds, allowing them to be separated from one another.

All DNA molecules have the same amount of charge per mass. Because of this, gel electrophoresis of DNA fragments separates them based on size only. Using electrophoresis, we can see how many different DNA fragments are present in a sample and how large they are relative to one another. We can also determine the absolute size of a piece of DNA by examining it next to a standard "yardstick" made up of DNA fragments of known sizes.

What is a gel?

As the name suggests, gel electrophoresis involves a gel: a slab of Jello-like material. Gels for DNA separation are often made out of a polysaccharide called **agarose**, which comes as dry, powdered flakes. When the agarose is heated in a buffer (water with some salts in it) and allowed to cool, it will form a solid, slightly squishy gel. At the molecular level, the gel is a matrix of agarose molecules that are held together by hydrogen bonds and form tiny pores.

At one end, the gel has pocket-like indentations called **wells**, which are where the DNA samples will be placed:



Before the DNA samples are added, the gel must be placed in a **gel box**. One end of the box is hooked to a positive electrode, while the other end is hooked to a negative electrode. The main body of the box, where the gel is placed, is filled with a salt-containing buffer solution that can conduct current. Although you may not be able to see in the image above (thanks to

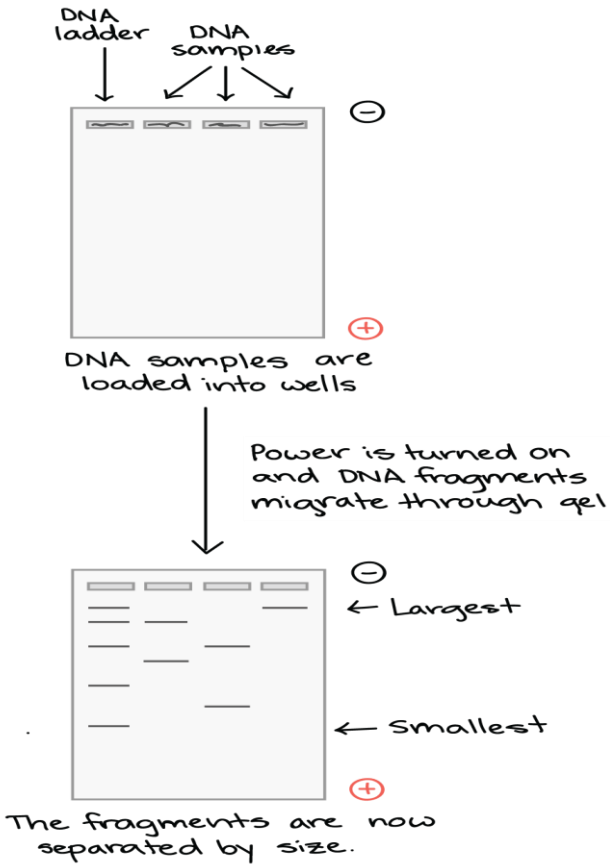
my amazing artistic skills), the buffer fills the gel box to a level where it just barely covers the gel.

The end of the gel with the wells is positioned towards the negative electrode. The end without wells (towards which the DNA fragments will migrate) is positioned towards the positive electrode.

How do DNA fragments move through the gel?

Once the gel is in the box, each of the DNA samples we want to examine (for instance, each PCR reaction or each restriction-digested plasmid) is carefully transferred into one of the wells. One well is reserved for a **DNA ladder**, a standard reference that contains DNA fragments of known lengths. Commercial DNA ladders come in different size ranges, so we would want to pick one with good "coverage" of the size range of our expected fragments.

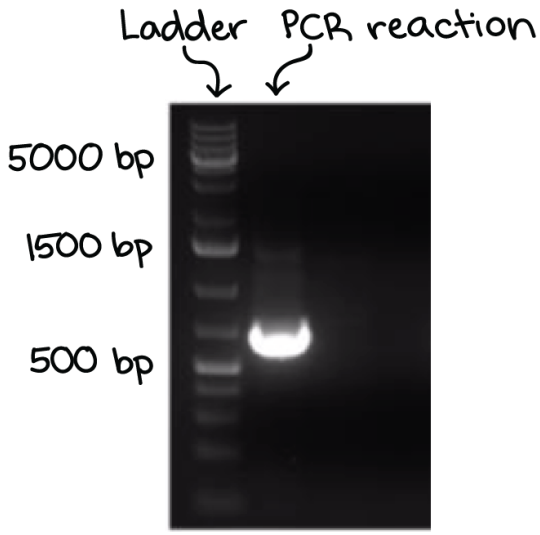
Next, the power to the gel box is turned on, and current begins to flow through the gel. The DNA molecules have a negative charge because of the phosphate groups in their sugar-phosphate backbone, so they start moving through the matrix of the gel towards the positive pole. When the power is turned on and current is passing through the gel, the gel is said to be **running**.



As the gel runs, shorter pieces of DNA will travel through the pores of the gel matrix faster than longer ones. After the gel has run for awhile, the shortest pieces of DNA will be close to the positive end of the gel, while the longest pieces of DNA will remain near the wells. Very short pieces of DNA may have run right off the end of the gel if we left it on for too long.

Visualizing the DNA fragments

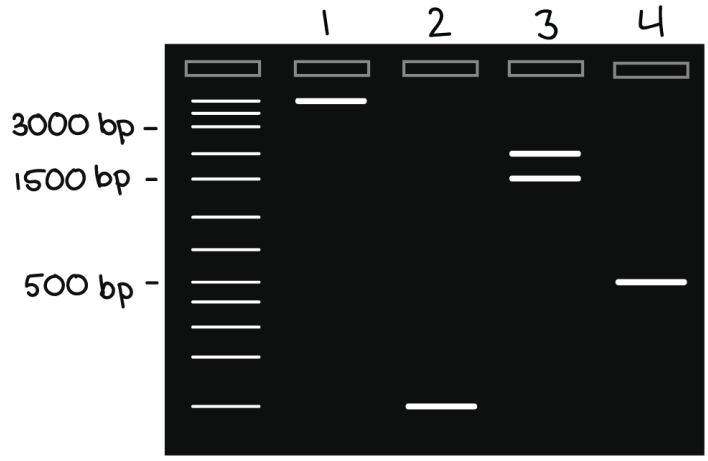
Once the fragments have been separated, we can examine the gel and see what sizes of bands are found on it. When a gel is stained with a DNA-binding dye and placed under UV light, the DNA fragments will glow, allowing us to see the DNA present at different locations along the length of the gel.



A well-defined "line" of DNA on a gel is called a **band**. Each band contains a large number of DNA fragments of the same size that have all travelled as a group to the same position. A single DNA fragment (or even a small group of DNA fragments) would not be visible by itself on a gel.

By comparing the bands in a sample to the DNA ladder, we can determine their approximate sizes. For instance, the bright band on the gel above is roughly 700700700 base pairs (bp) in size.

Check your understanding:



Four lanes are numbered on the gel above. (A **lane** is a corridor through which DNA passes as it leaves a well.)

Which lane matches each description below?

a) This lane contains the longest DNA fragment.

b) This lane contains the shortest DNA fragment.

c) This lane contains a 150015001500 base pair (bp) DNA fragment.

HINT:

In a DNA gel, the longest DNA fragments migrate most slowly through the gel and stay closest to the wells (where they are initially loaded into the gel). The shortest DNA fragments migrate most quickly through the gel and get the closest to its far end (away from the wells).

Thus, the band representing the longest fragment will be closest to the top of the gel. This band is found in lane 111.

Similarly, the band representing the shortest fragment will be closest to the bottom of the gel. This band is found in lane 222.

We can use the DNA ladder (in the leftmost lane) to figure out which lane contains a 150015001500 bp band. If we trace the 150015001500 bp band of the ladder rightwards across the other lanes, we find a band of matching size in lane 333.

Gel Electrophoresis:

Gel electrophoresis 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 immuno-blotting.

This is the most commonly used electrophoresis in biotechnology laboratories and is used for almost all types of experiments in RD.

Principle:

The electromotive force (EMF) generated across the electrodes pushes or pulls the molecules (nucleic acids or proteins) through the gel matrix. The molecules move towards the anode if negatively charged or towards the cathode if positively charged.

A typical gel electrophoresis apparatus is of two kinds:

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

Types of Gel-Electrophoresis:

Agarose Gel Electrophoresis:

The supporting medium in this type of electrophoresis is agarose gel. This is used for the electrophoresis of Nucleic acids like DNA and RNA.

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:

1. Physical Apparatus:

This includes the physical body of the experimental set-up.

It is of following three types:

Electrophoretic 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, a wick poured from the agarose, and flat horizontal bed of agarose upon which small wells for sample loading are located.

Power Supply:

For a standard agarose 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).

Trans illuminator:

This is an ultraviolet light box which is used to visualize ethidium bromide-stained DNA in gels.

2. Chemical Components:

This includes all the chemical components which are involved in the electrophoresis of the biomolecules.

Supporting Media:

Agarose is a polysaccharide extracted from seaweed. 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 (Fig. 3.14). This gelation results in a mesh of channels with a diameter varying form 50-200nm. The diameters of these channels determine the final porosity of the gel and acts as the sieve.

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%.

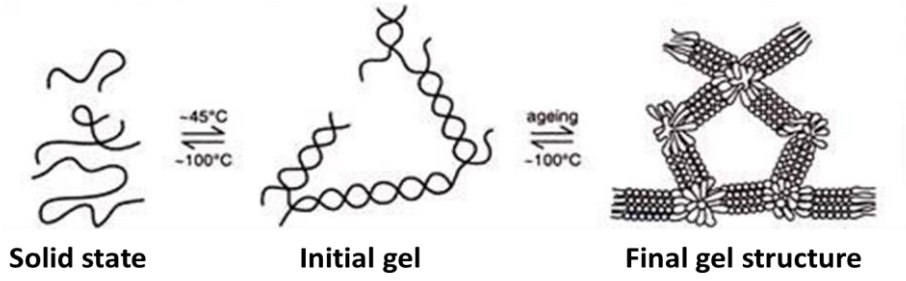


Fig: Gel structure of Agarose

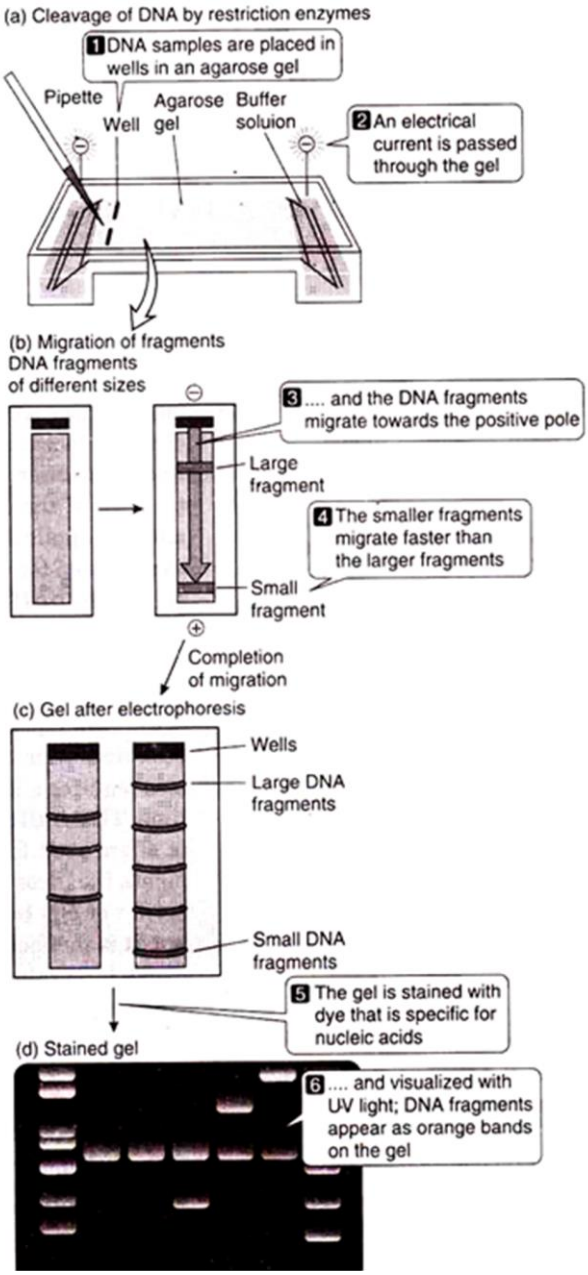


Fig: Agarose gel electrophoresis

Buffer:

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. Depending on the size of the DNA electrophoresed and the application, different buffers can be used for agarose electrophoresis.

TAE buffer (or Tris Acetate EDTA) is the most commonly used agarose gel electrophoresis buffer. TAE has the lowest buffering capacity of the buffers; however, TAE offers the best resolution for larger DNA. TAE also requires a lower voltage and more time.

TBE buffer (Tris/Borate/EDTA) 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. Two types of buffers are used in the process of agarose electrophoresis.

Electrophoresis Buffer:

This type of buffer is used to bathe the gel placed on horizontal tank. Usually Tris-ace- tate-EDTA (TAE) or Tris-borate- EDTA (TBE) is taken as electrophoresis buffer.

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.

Dye:

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.

When irradiated with UV light of a wavelength of 302 nm, ethidium bromide will emit fluorescence light of a wavelength of 590 nm (orange). After the process of electrophoresis the gel is observed under the beam of UV light produced by the trans-illuminator. The dye is added to the gel during its preparation only.

Procedure:

First of all agarose powder is mixed with electrophoresis buffer to the desired concentration, and then heated in a microwave oven until completely melted. Most commonly, ethidium bromide is added to the gel (final concentration 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 pipette

into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied.

You 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 trans-illuminator. 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:

1. 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.
2. Analysis of PCR products after polymerase chain reaction to assess for target DNA amplification.
3. Allowing estimation of the size of DNA molecules using a DNA marker or ladder which contains DNA fragments of various known sizes.
4. Allows the rough estimation of DNA quantity and quality.
5. Quantity is assessed using lambda DNA ladder which contains specific amounts of DNA in different bands.
6. Quality of DNA is assessed by observing the absence of streaking or fragments (or contaminating DNA bands).
7. Other techniques rely on agarose gel electrophoresis for DNA separation including DNA fingerprinting.

Advantages and Disadvantages of Agarose Gel Electrophoresis:

The advantages are that the gel is easily poured, and does not denature the samples. The samples can also be recovered.

The disadvantages are that gels can melt during electrophoresis, the buffer can become exhausted, and different forms of genetic material may run in unpredictable forms.

SDS-PAGE:

Sodium Dodecyl Sulfate (SDS) polyacrylamide gel electrophoresis is mostly used to separate proteins accordingly by size. This is one of the most powerful techniques to separate proteins on the basis of their molecular weight.

Principle:

This technique uses anionic detergent Sodium Dodecyl Sulfate (SDS) which dissociates proteins into their individual polypeptide subunits and gives a uniform negative charge along each denatured polypeptide. SDS also performs another important task.

It forces polypeptides to extend their conformations to achieve similar charge: mass ratio. SDS treatment therefore eliminates the effects of differences in shape so that chain length, which reflects their molecular mass, is the sole determinant of migration rate of proteins in the process of electrophoresis.

When these denatured polypeptides are loaded at the cathode end of an electrophoretic tank having polyacrylamide gel (as the supporting media) and subjected to an electric field, then we get clear bands of proteins arranged in an decreasing order of their molecular mass from the cathode to anode.

The rate of movement is influenced by the gel's pore size and the strength of electric field. In SDS- PAGE the vertical gel apparatus is mostly used. Although it is used to separate proteins on a routine basis, SDS-PAGE can also be used to separate DNA and RNA molecules.

Instrumentation:

Physical Apparatus:

This includes the physical body of the experimental set-up. It is of following two types:

Electrophoretic Apparatus:

- ✓ Vertical horizontal tank with electrodes,
- ✓ Gel cassettes,
- ✓ Teflon spacers,
- ✓ Clips,
- ✓ Pipette or syringe,
- ✓ Comb,
- ✓ Acrylic cover.

Power Supply:

A power supply of 100-200 volts is needed. This is ideal for running and transferring protein resolving gels.

Staining Box:

These are trays in which the gels are stained and made up of clear plastics. These are resistant to most organic dyes, silver and other stains.

Chemical Components:

This includes the following:

Supporting Media:

SDS-PAGE acrylamide is used as the supporting medium. It is a white crystalline powder, when acrylamide dissolves in water; it undergoes polymerization reaction to form a net-like structure called polyacrylamide. Polyacrylamide is a polymer ($CF_2CHCONH_2$-) formed from acrylamide subunits that can also be readily cross-linked.

This type of electrophoresis has a discontinuous system of gel, i.e., we have two different systems of gels present in the electrophoretic tank physically placed one over another.

These are as follows:

Resolving Gel:

This is also called separating or running gel. The separating gel constitutes about 2/3rd of the length of gel plate and is prepared by 5-10% of acrylamide. The pores in this gel (which is formed after the polyacrylamide is cross- linked) are numerous and smaller in diameter which impacts sieving property to this gel.

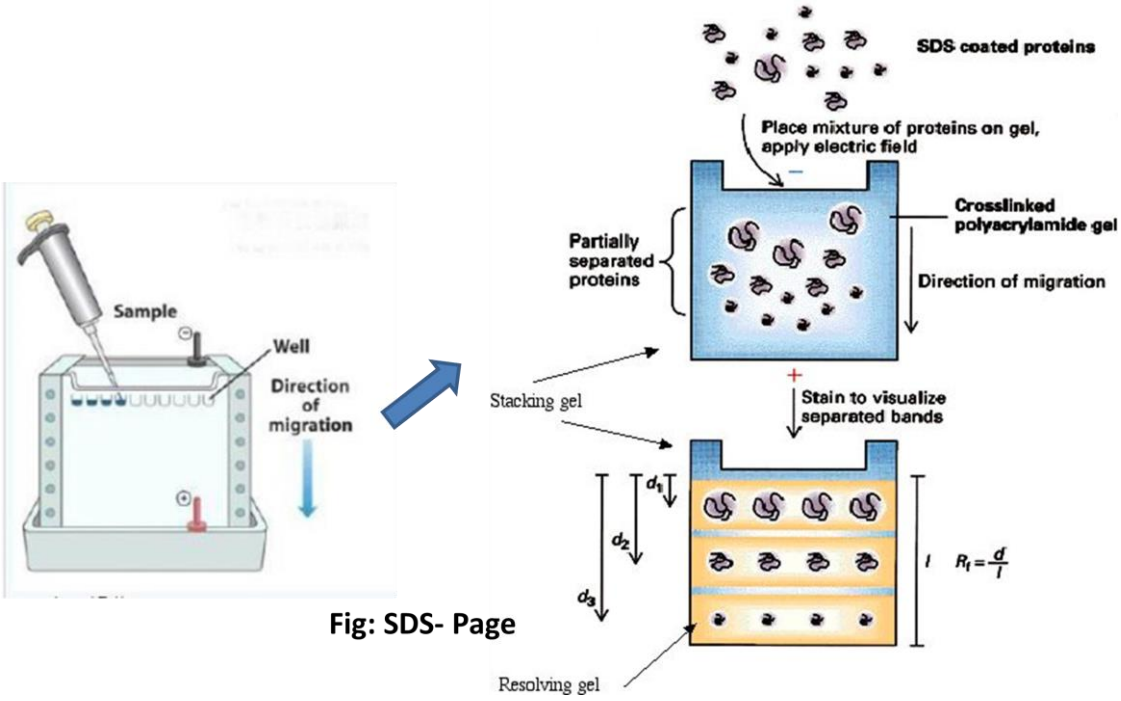


Fig: SDS- Page

Stacking gel:

Stacking gel is poured on the top of resolving gel and a gel comb is inserted which forms the well. It is the upper layer of gel and constitutes 1/3rd of the gel plate. The percentage of

acrylamide is chosen depending on the size of protein that one wishes to identify or probe in the sample.

The smaller the known weight, the higher the percentage that should be used. Generally, the percentage of acrylamide in stacking gel is 2-5%. It is highly porous and devoid of molecular sieving action.

Buffer:

Two types of buffers are used in SDS-PAGE. The lower reservoir (which has the running gel) has amine buffers. It is adjusted by using HCl. The upper reservoir (which has stacking gel) also has amine buffers but its pH is slightly above that of running gel buffer and is adjusted with glycine instead of HCl.

Dissociating Agent:

SDS is the most common dissociating agent used to denature native proteins to individual polypeptides. When a protein mixture is heated to 100°C in presence of SDS, the detergent wraps around the polypeptide backbone.

It binds to polypeptides in a constant weight ratio of 1.4 g/g of polypeptide. In this process, the intrinsic charges of polypeptides become negligible when compared to the negative charges contributed by SDS. Thus, polypeptides after treatment become rod-like structure possessing uniform charge density, which is the same net negative charge per unit length.

Stains:

The stains are used to see the bands of separated proteins after the process of electrophoresis. Coomassie Brilliant Blue R-250 (CBB) is the most popular protein stain. It is an anionic dye, which binds with proteins non-specifically.

Proteins in the gel are fixed by acetic acid and simultaneously stained. The excess dye incorporated in the gel can be removed by de-staining with the same solution but without the dye. The proteins are detected as blue bands on a clear background.

Procedure:

The solution of proteins to be analysed is first mixed with SDS, an anionic detergent, an anionic detergent which denatures secondary structure. Besides addition of SDS, proteins may optionally be boiled in the presence of a reducing agent, such as Di-Thio-Threitol (DTT) or 2-mercaptoethanol, which further denatures the proteins by reducing disulfide linkages, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure (Oligomeric subunits).

This is known as reducing SDS-PAGE, and is most commonly used. Non-reducing SDS-PAGE (no boiling and no reducing agent) may be used when native structure is important in further analysis (e.g., enzyme activity, shown by the use of zymograms). The denatured proteins are subsequently loaded into the wells of stacking gel flooded with stacking buffer.

This end is connected with the cathode of power supply. Then an electric current is applied across the gel, causing negatively charged proteins to migrate across the gel towards anode. After crossing the stacking gel, denatured proteins enter the running gel which has its own buffer system (running buffer).

Depending on their size, each protein will move differently through the gel matrix: short proteins will more easily fit through the pores in the gel, while larger ones will have more difficulty.

After the separation is over the gel is gently taken out and transferred to the staining box and treated with the staining dye, e.g., CBB R-250. Excess of stains are removed by de-staining using acetic acid solution. The bands appear to be blue stained which are then analysed according to the need of the experiment.

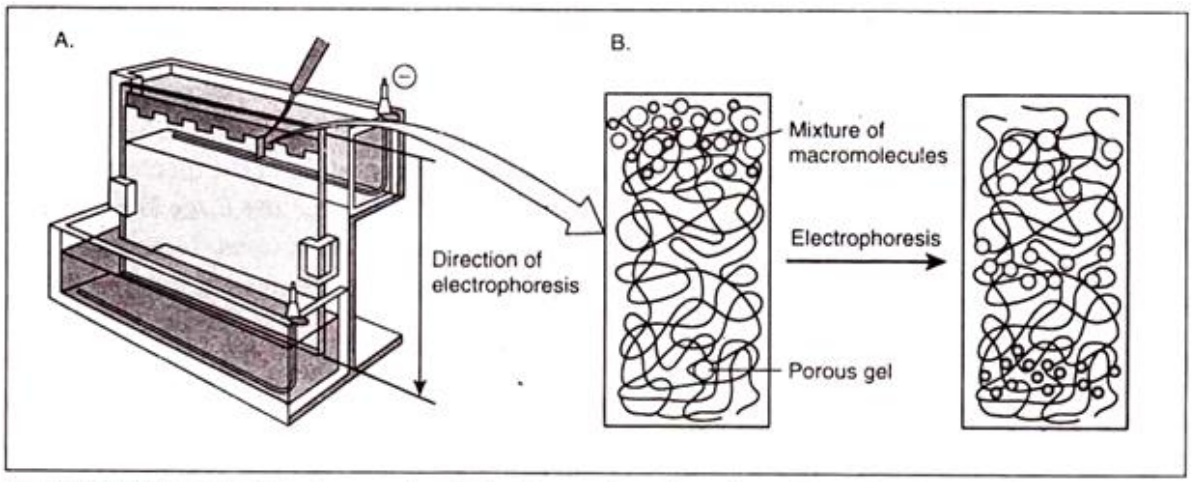


Fig. 3.16: **(A) Gel electrophoresis apparatus**: Typically, several samples undergo electrophoresis on one flat polyacrylamide gel. A microlitre pipette is used to place solutions of proteins in the wells of the slab. A cover is then placed over the gel chamber and voltage is applied. The negatively charged SDS (Sodium Dodecyl Sulfate)-protein complexes migrate in the direction of the anode, at the bottom of the gel. (**B**) The sieving action of a porous polyacrylamide gel separates proteins according to size, with the smallest moving most rapidly (*Source*: Biochemistry, Stryer et al.)

Application:

SDS-PAGE has many applications. It is mostly used for following purposes:

1. Establishing protein size
2. Protein identification
3. Determining sample purity
4. Identifying disulfide bonds

5. Quantifying proteins
6. Blotting applications

Advantages of SDS-PAGE:

SDS-PAGE has following advantages:

1. Mobility of the molecules is high and separation is rapid.
2. All the proteins are negatively charged; therefore, all migrate towards anode.
3. The proteins treated with SDS fixed dyes are better than the native proteins.
4. SDS solubilises all proteins, including very hydrophobic and even denatured proteins.

Pulse Field Gel Electrophoresis (PFGE):

Conventional methods of gel electrophoresis are carried out by placing DNA samples in a solid matrix (agarose or polyacrylamide) and inducing the molecules to migrate through the gel under a static electric field. When DNA molecules are under the influence of this electric field, they elongate and align themselves with the field.

In DNA electrophoresis by the standard method, however, DNA molecules which are larger than 20kb cannot be separated either by agarose gel electrophoresis or by SDS-PAGE. This is done by pulse field gel electrophoresis.

In this technique the periodic changing of orientation of the electric field is carried out. This forces the large DNA molecules in the gel to relax upon removal of the first and elongate to align with the new field.

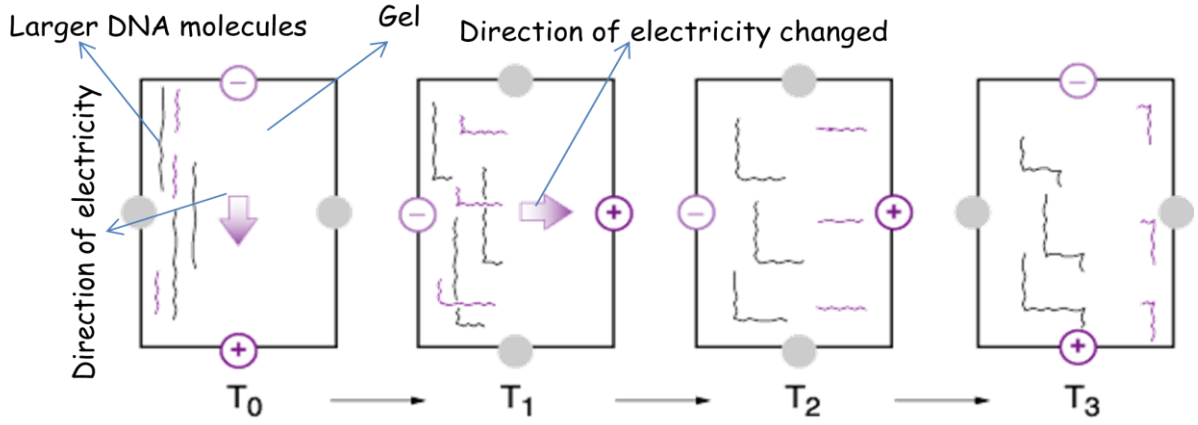


Fig: Pulse Field Gel Electrophoresis (PFGE)

Two-Dimensional Electrophoresis:

This is a very sensitive technique to purify a mixture of polypeptides.

It is carried out in two dimensions:

1st Dimension Separation:

It is where we carry out a process called isoelectric focusing. The net charge of any protein is the sum total of all positive and negative charges in it. These charges are determined by ionisable acidic and basic side chains and prosthetic groups of the proteins.

The isolectric point (pi) of a polypeptide is that pH value at which its net charge is zero. At this point the polypeptides cannot move anywhere in the gel and makes bands there. Isoelectric focusing is a procedure used to determine the isoelectric point (pi) of a protein.

A pH gradient is established by allowing a mixture of low molecular weight organic acids and bases called ampholytes to distribute themselves in an electric field generated across the gel. When a protein mixture is applied, each protein migrates until it reaches the pH that matches its pi. Proteins with different isoelectric points are thus distributed differently throughout the gel.

2nd Dimension Separation:

Isoelectric focusing is followed by SDS-PAGE as discussed above. In this process the proteins will move from cathode to anode in the decreasing order of their molecular mass (M_r).

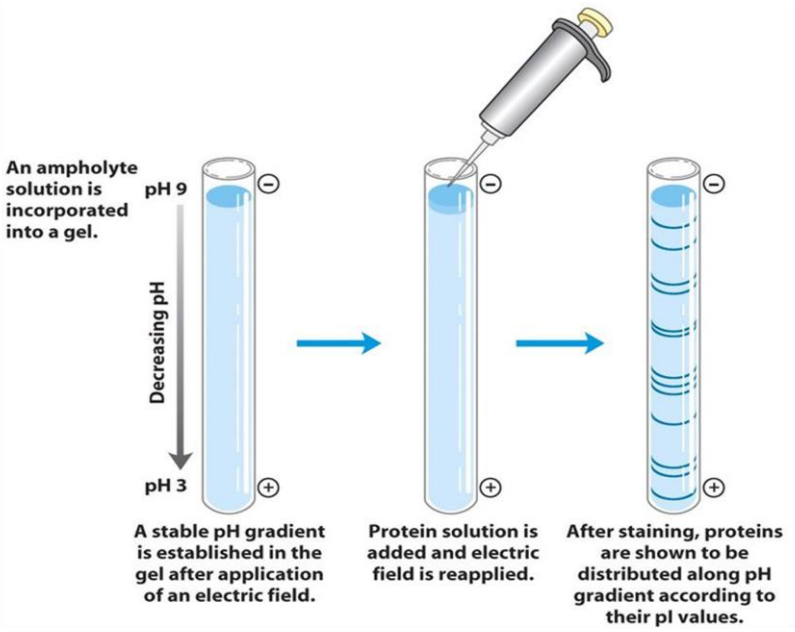


Fig: Isoelectric focussing is the 1st dimension separation in Two-Dimensional Electrophoresis

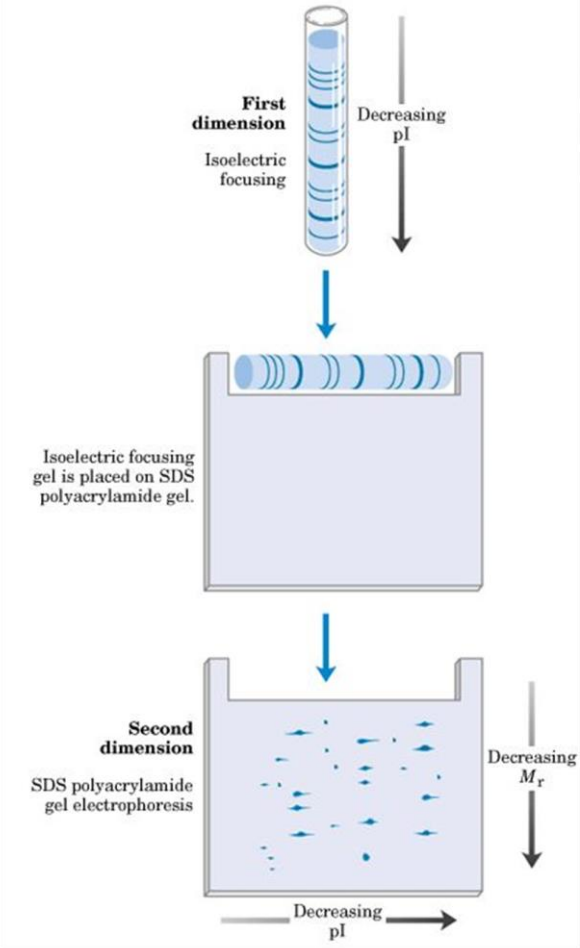


Fig: The isoelectric focussing is followed by SDS-PAGE