Microbiology

US05EMIC26

Bioinstrumentation and Biotechniques

Unit- II Electrophoresis:

(a) Principle, Support media, Methods and applications of electrophoresis

(b) Separation of Protein and nucleic acids: PAGE,SDS-PAGE, Agarose and IEF

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Electrophoresis:

GENERAL PRINCIPLES:

- **Many important biological molecules such as amino acids, peptides, proteins, nucleotides and nucleic acids possess ionisable groups and can therefore be made to exist in solution as electrically charged species, either as cations (+) or anions (–).**
- **Even typically non-polar substances such as carbohydrates can be given weak charges by derivatisation, for example as borates or phosphates.**
- **Moreover, molecules which have a similar charge will have different molecular weight. In combination these differences form a sufficient basis for a differential migration when the ions in solution are subjected to an electric field. This is the principle of electrophoresis.**

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- **The equipment required for electrophoresis consists basically of two items,**
  (1) A power pack and
  (2) An electrophoresis unit

- **The power pack supplies a direct current between the electrodes in the electrophoresis unit. Cations move to the cathode (-) and anions move to the anode (+) at rates which depend on the balance between the impelling force of the electric field on the charged ion and the frictional and electrostatic retarding effects between the sample and the surrounding medium.**

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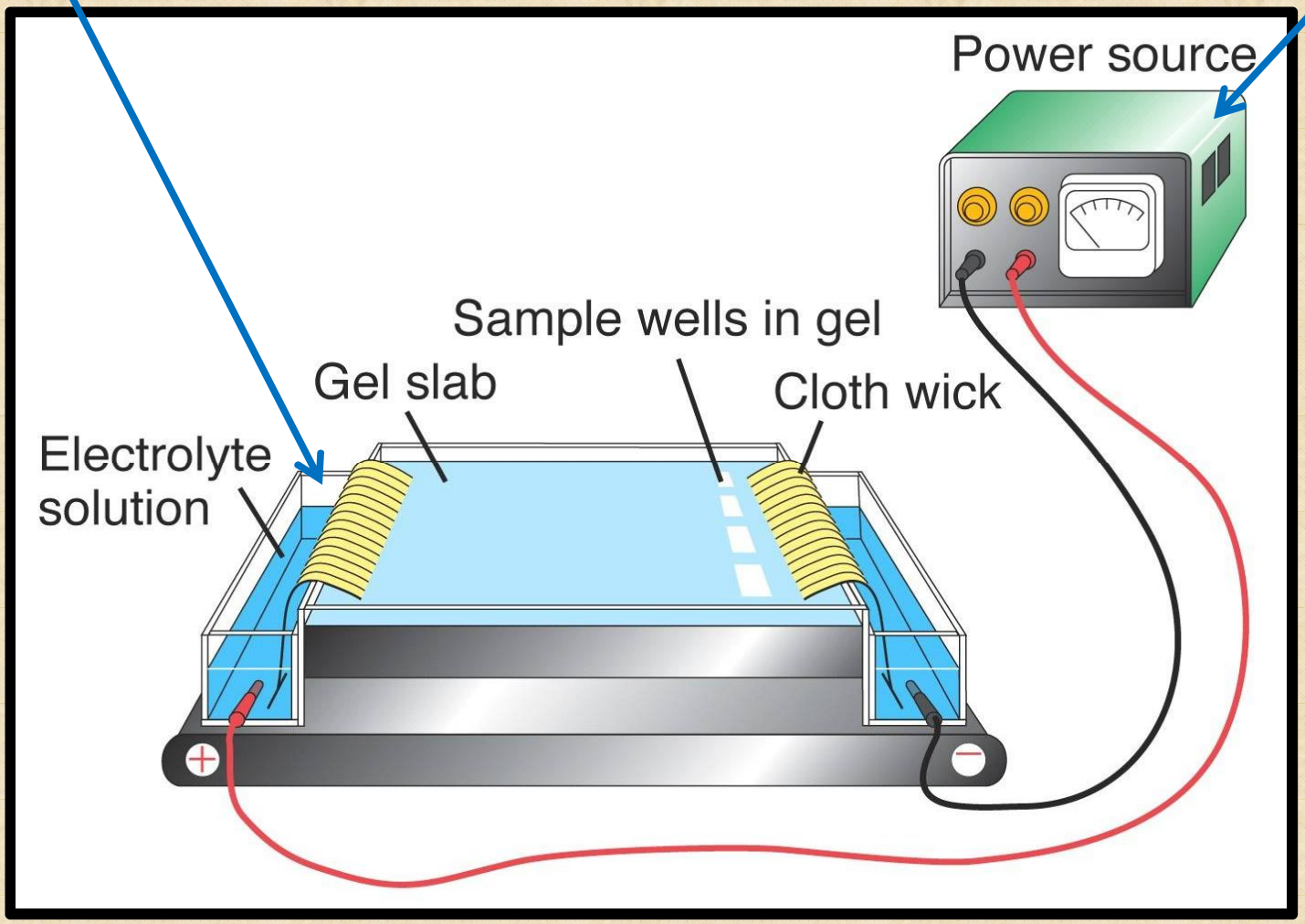
Electrophoresis unit

Power pack



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FACTORS AFFECTING ELECTROPHORETIC MOBILITY:

[a] The Sample:

Charge / mass ratio of the sample dictates its electrophoretic mobility. The mass consists of not only the size (molecular weight) but also the shape of the molecule.

(1) Charge :

The higher the charge, greater is the electrophoretic mobil-ity. The charge, however, is dependent on pH of the medium.

(2) Size :

The bigger the molecule, greater are the frictional and electro-static forces exerted upon it by the medium of suspension. Consequently, larger particles have a smaller electrophoretic mobility compared to the smaller particles.

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(3) Shape :

Rounded particles elicit lesser frictional and electrostatic retardation compared to sharp particles. As an example consider the case of globular and fibrous proteins. Given the same size (molecular weight) the globular protein will migrate faster than the fibrous protein.

[B] The Electric Field :

The current (total charge carried per second to the electrode) in the solution placed between two electrodes is carried mainly by the buffer ions, only a small proportion being carried by the sample ions. An increase in the potential difference therefore increases the current.

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[C] The Medium:

An inert supporting medium is chosen for electrophoresis. but even this inert medium can exert adsorption and/or molecular sieving effects on the particle thereby influencing its rate of migration.

(1) Adsorption:

Adsorption, here, means retention of a component on the surface of supporting medium. Such a component has two forces acting upon it;

- The applied electric field and
- Adsorption

Thus, the component is not resolved as a sharp band but as a band which has a tail rather like a comet.

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(2) Molecular Sieving :

Supporting media such as polyacrylamide, agarose, starch and sephadex have cross-linked structures giving rise to pores within the gel beads.

The smaller molecules here pass through the pores easily, but the larger molecules are retarded. The electrophoretic mobility is thus modified by molecular sieving effects of the supporting medium.

[D] The Buffer:

Apart from maintaining the pH of the supporting medium, the buffer can affect the electrophoretic mobility of the sample in various other ways.

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(1) Composition :

- Commonly used buffers are formate, citrate, phosphate, EDTA, acetate, pyridine, Tris and barbitone etc.
- The choice of buffer depends upon the type of sample being elecIrophoresced. The buffer can affect electrophoretic mobility if it is able to bind component(s) of the sample being separated

(2) pH :-

- Since pH determines the degree of ionization of organic compounds, it can also affect the rate of migration of these compounds.
- Increases in pH increase ionization of organic acids and a decrease in pH increases the ionization of organic bases. For an ampholyte such as amino acid, which has both acidic and basic properties, both the above effects apply.

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(3)Ionic Strength :

- As we have already seen above, increased ionic strength of the buffer means a larger share of the current being carried by the buffer ions and a small proportion carried by the sample ions.
- This situation gets translated into a slower migration of the sample components. Since the overall current will also increase there will be heat production.
- In low ionic strength buffers, diffusion (especially of smaller molecules) tends to be high with concomitant loss of resolution.
- The chosen ionic strength of the buffer is, therefore, a compromise. The ionic strength used is usually between 0.05 – 0.1 M.

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MATERIALS:

- Gels as supporting media is widely used for the separation of high molecular weight substances such as proteins arid nucleic acids, because of the improved resolution obtained.
- This is due to the physical properties of gels which are water insoluble, hydrophilic, semi-solid colloids.
- Suitable gels may be prepared shortly before use from a variety of powdered solids, for example: starch, agar and polyacrylamide.
- The molecular sieving property of the semi-rigid gel helps to separate large ionic compounds such as proteins which have similar charge properties but which differ in size and shape.

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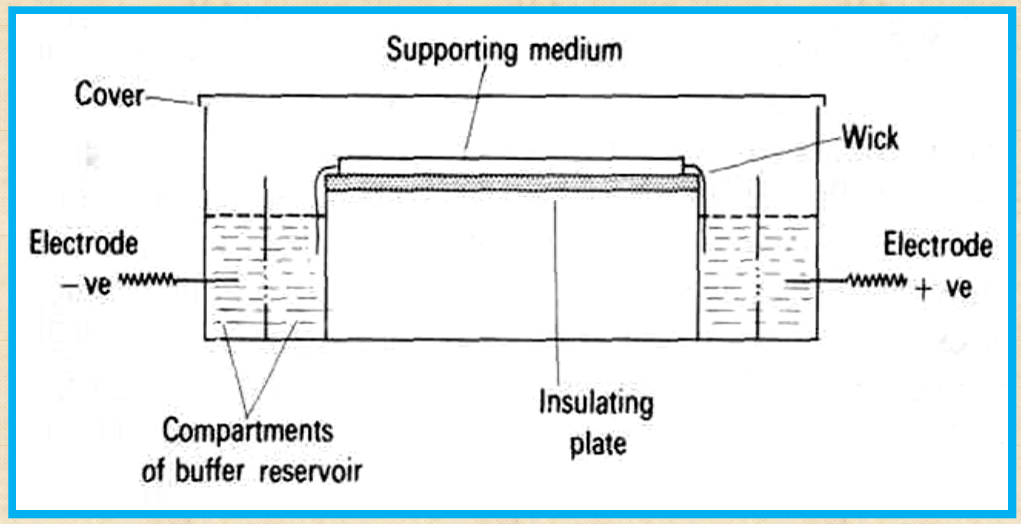
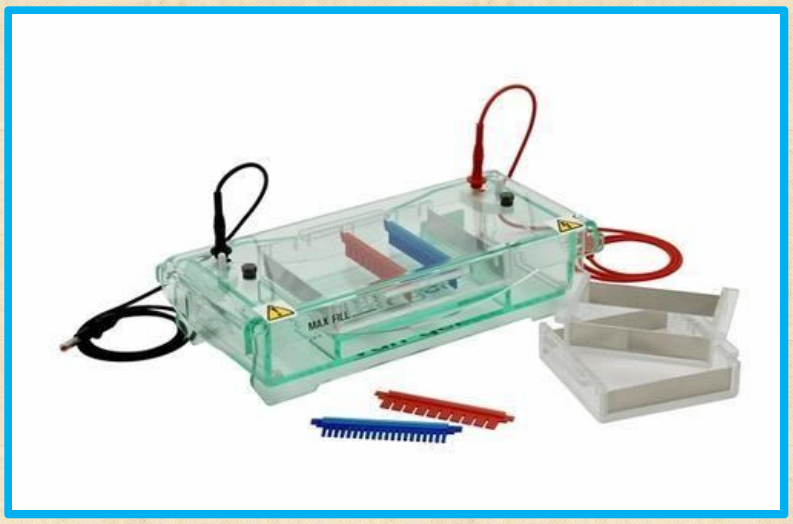
APPARATUS:

Two types : I. Horizontal slabs II. Vertical slabs

Gels can be run as horizontal slabs using equipment similar to that shown in following figures





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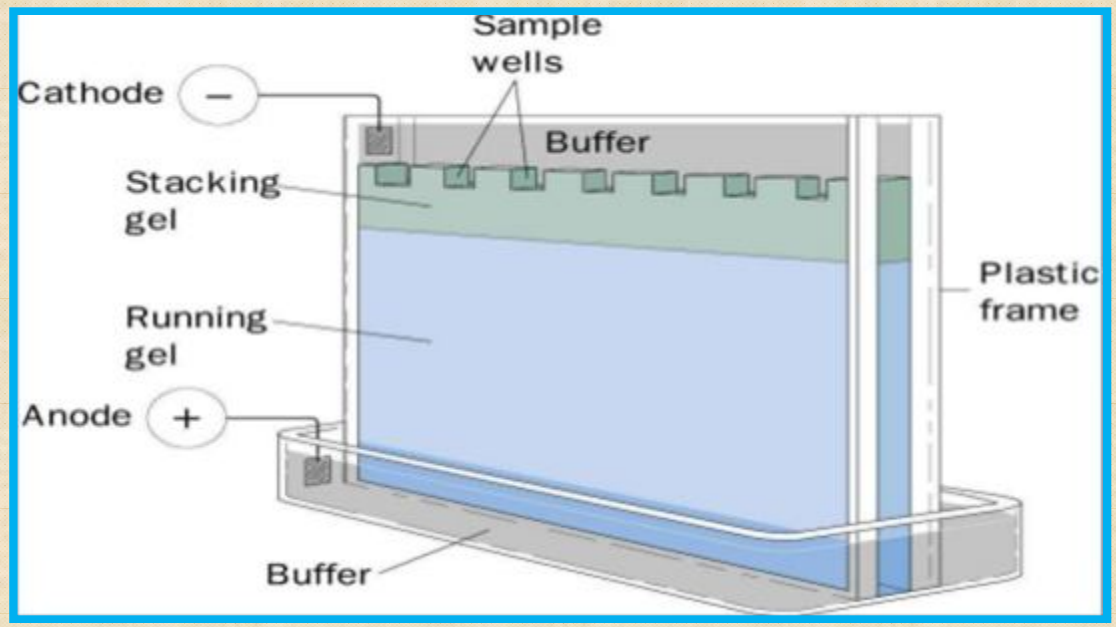
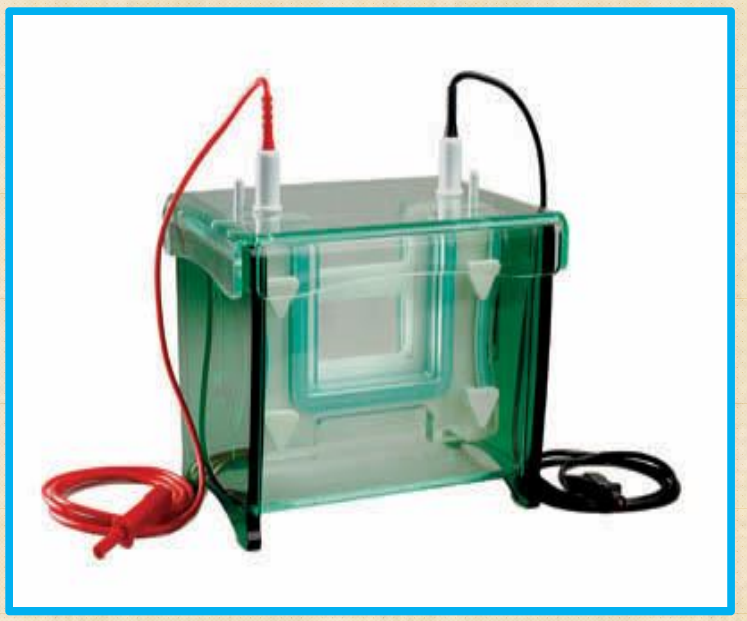
Vertical slabs are more frequently used.





Slabs of gel carry more samples (up to 25 samples can be run on a single gel plate) making them more economical to use and enabling more samples to be compared with each other when run under identical conditions.

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METHOD:

(1) Preparation of Gel:

- Gels are prepared in the glass (or Perspex) containers in which they are to be used.
- In the case of slabs, the gels are cast between two clean glass plates which are clamped together but held apart by plastic spacers.
- Ver-tical slabs are run with the glass plates left on both sides of the gel. For hori-zontal slabs, the plate above the gel is removed before the run.

(2) Sample Application:

- Dissolved samples can be applied to the surface of horizontal gel slabs via filter paper strips, but more commonly for both horizontal and vertical slabs the sample solutions are injected from a micro-syringe into slots or wells in the gel

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- The buffer in which the sample is dissolved usually contains sucrose or glycerol (10 % to 15 %) to increase its density and ensure that the solution sinks into the well.
- A marker dye such as bromophenol blue is often added to aid observation of loading and monitoring the migration.
- Urea or sodium dodecylsulphate may be added to protein samples to facilitate their solubilization and also disulphide reducing agents such as dithiothreitol or 2–mercaptoethanol.

(3)Running the Samples:

- For horizontal systems, electrical contact between the gel and the buffer in the electrical compartments can be maintained by wicks.
- For vertical systems the gel slab, sandwiched between glass plates, is placed in the lower reservoir, with the top of the gel in contact with the buffer in the upper reservoir.

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- **The gel thus completes the electrical circuit between the electrodes in the upper and lower compartments.**

- **Heat generated during electrophoresis controlled by carrying out the run in cold room or by using chilled buffers (by a suitable cooling system).**

- **The precise voltage and time required to obtain optimal separations will depend on the nature of the samples and the type of gel used.**

- **Marker dyes such as bromophenol blue for proteins and ethidium bromide for nucleic acids enable the progress of the run to be monitored.**

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POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Principle:

- Proteins are amphoteric compounds i.e. they contain both acidic and basic group .Therefore the net charge on the protein is determined by the PH of the medium.
- Most of the charges on the protein is determined by the PH dependent ionization of the side chains such as carboxyl and amino groups

$$COOH \leftrightarrows COO^- + H^+$$

$$NH_2^+ + H^+ \rightleftarrows NH_3$$

- Depending upon the number and kinds of such amino acids the charges on the protein vary and hence their mobility in the Electric field.
- However at isoelectric PH(PI) , no net negative charge present on the protein.

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- So, in solution with a PH>PI protein carries net negative charge and move towards the anode and PH< PI proteins carries net positive charge and move towards the cathode.
- Therefore in an electrophoresis ,the PH of the solution must be kept constant to maintain the charge and hence mobility of the proteins for this proteins are electrophoresed in buffer.

Support Media:

- Polyacrylamide gels are synthetic gels and are tougher than Agar and Agarose gels.
- They optically clear and electrically Neutral due to the absence of any charged groups in contrast to the presence of COO^- and SO3 groups in Agar and Agarose.
- They can be prepared with wide range of pore size, therefore polyacrylamide gels are widely used to resolve the mixture of peptide, proteins and small molecular weight nucleic acid.

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Components:

Electrophoresis in acrylamide gels is frequently referred to as PAGE [polyacrylamide gel electrophoresis].

(a)Acrylamide:

It forms linear backbone of the gel. It should be of highpurity.

(b) Bis-acrylamide: (*N,N'* – Methylene bisacrylamide)

It is a cross linking reagent.During polymerization cross links the linear molecule of acrylamide to form porous structure. Thus it is the cross linker that actually makes and hold the structure together. By varying the concentration of cross linker and monomer the pore size can be increased or decreased.

(c) Initiators:

Ammonium persulphate(APS) or riboflavin can be used to initiate the polymerization. APS gives smooth homogenous polymerization but may react with proteins to give an artifact.

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Riboflavin on other hand gives poor polymerization ,but not react with protein, however it needs fluorescent light for polymerization.

(d) Catalyst:

N,N,N',N' **– tetramethylenediamine (TEMED)a quaternary amine which serve as the catalyst for polymerization. Increase in its concentration speed up the rate of gel polymerization.**

- **Gels may be prepared containing from 3% to 30% acrylamide corresponding to pore size of 0.5 nm and 0.2nm.**
- **Gels are prepared in glass containers in which they are to be used. In the case of slabs the gels are casted between two clean glass plates which are clamped together but held apart by the plastic spacer.**

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Sample Application:

- In both horizontal and vertical slabs sample solutions are Injected from a micro syringe into the slabs or wells in the gel, which can be prepared by inserting comb like template in to the gel before it set.
- Buffer in which the sample is dissolved usually contain sucrose or glycerol (10 to 15 %) to increase the density and ensure sinking of the sample solution.
- A tracking dye/ marker dye such as bromophenol blue is often added to aid observation of loading and migration.

Running the sample:

- For horizontal systems electrical contact between the gel and Buffer is established by a wicks.

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- For vertical systems gel slabs is sandwiched, between the glass plates kept in the lower reservoir with the top of the gel in the upper reservoir thus it completes the electrical circuit between the electrodes in upper and lower compartments.
- To minimize heat generation ,run is carried out under cold conditions or by circulating buffer through the cooling system.
- The pore size voltage and time require for the separation will depend upon the nature of the sample and the type of the gel used.
- Marker dyes such as Bromophenol blue for proteins and Ethidium bromide for nucleic acid are used for tracking the sample.
- After running the sample band can be visualized by staining method. Commasie brilliant blue and silver staining is used. however silver staining is 10 times quicker.

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SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS [SDS – PAGE]

- **This form of polyacrylamide gel electrophoresis is the most widely used method for analyzing protein mixtures qualitatively.**
- **It is particularly useful for monitoring protein purification and because the method is based on the separation of proteins according to size, the method can also be used to determine the relative molecular mass of proteins.**
- **PAGE is of two types (i) denaturing (ii) non denaturing**
- **In denaturing gels method proteins sample are treated with an anionic detergent Sodium Dodecylsulphate and Sulphahydryl reducing agent [β – mercaptoethanol or Dithiothretol (DDT)]**

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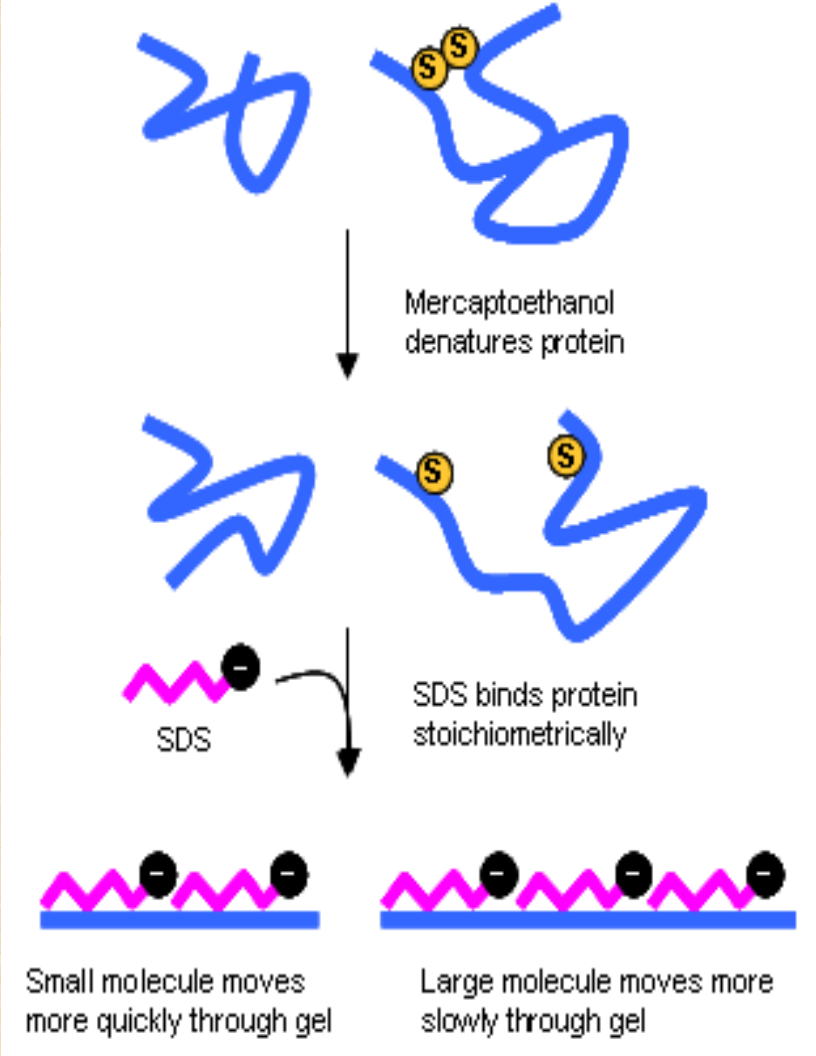
Principle:

- **The mercaptoethanol reduces any disulphide bridges present that are holding together the protein tertiary structure, and the SDS binds to protein strongly and wrap around them by strong hydrophobic interaction and denatures the protein completely.**



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- On average one SDS molecule binds for every two amino acid residues.
- The original native charge on the molecule is therefore completely swamped by the SDS molecules.

- Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod – shaped structure with a series of negatively charged SDS molecules along the polypeptide chain.
- In order to maintain proteins in the denaturing state 0.1% SDS is included in the gel as well as in the running buffer.

- Small proteins migrate faster through the gel under the influence of the applied electric field.

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- The number of SDS molecules that bind is proportional to the size of the protein, thereby in the electrical field,protein molecules move towards the anode (+) and separated only according to their molecular weight.

Support Media

- SDS-PAGE is differentiated into two systems
  1. Continuous SDS-PAGE
  2. Discontinuous SDS-PAGE

  Here, in SDS-PAG vertical slabs and discontinuous system is used.
- Discontinues SDS-PAGE consists of two different gels.

(a) Stacking gel (top gel)-4% of acrylamide

(b) Separating gel (bottom gel)- range from 5 to 15%of acrylamide

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Sample Application:

- sample solutions are injected from a micro syringe into the slabs or wells.
- Buffer in which the sample is dissolved usually contain sucrose or glycerol (10 to 15 %) to increase the density and ensure sinking of the sample solution.
- A tracking dye/ marker dye such as bromophenol blue is often added to aid observation of loading and migration.

Running the sample:
(Stacking process in discontinuous buffer system):

- Here in discontinuous buffer system two buffers with a PH difference of 2.0 is used.

1. Large pore size stacking gel with PH 6.8 and
2. Small pore separating gel with PH 8.8

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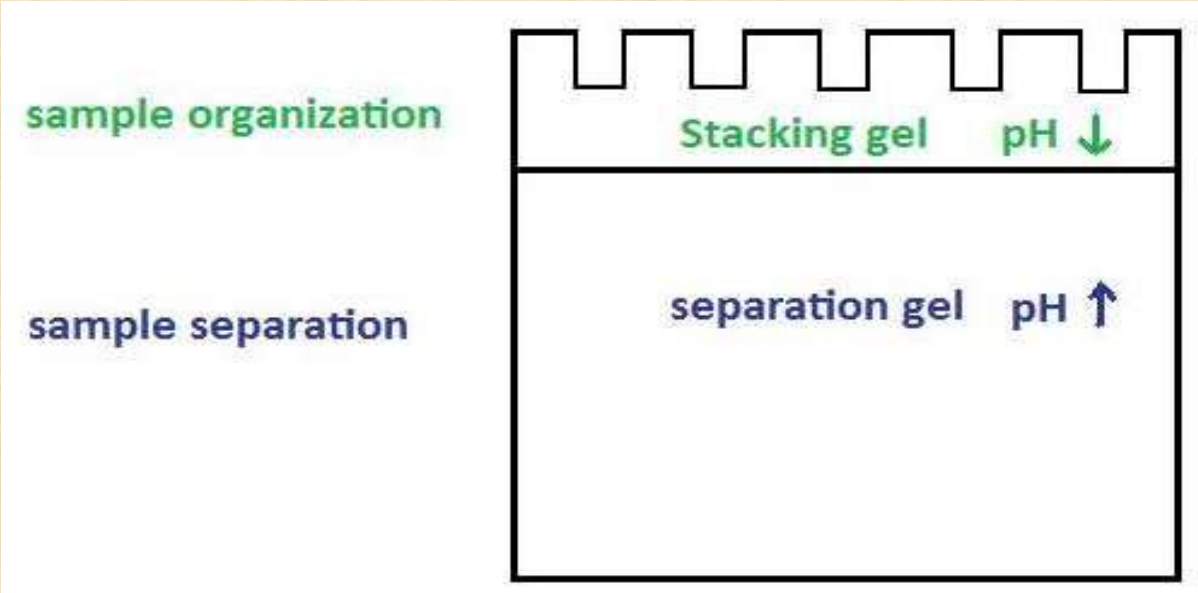

- **When electrophoresis is started the ions and proteins start migrating in to the stacking gel.**
- **The completely ionized fast moving Cl^- ions (from stacking Buffer) becomes the leading ion and its mobility is faster than fastest moving protein.**
- **But poorly ionized glacinate becomes the trailing ion and its mobility is slower that the slowest moving protein.**

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- This causes all proteins to stack over one another in order of decreasing mobility between the two ions and form a very thin sharp starting point.
- The thinner the starting zone the better is the resolution.
- When they reach the separating gel (pH 8.8) the glycinate ions are completely ionized and start moving faster whereas there will not be any charge. As a result glycine ions overtake the proteins zones and catch up with the trailing edge of cl^- ions.
- Now the proteins are separated in separating gel according to their molecular weight.

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Analysis of Gel:

- Staining with coomasie blue or silver nitrate
- Autoradiography followed by densitometry
- Immunodetection
- KCl treatment followed by visualizing the band against black background.
- Use of fluorescent dye and detect under U.V. radiation.

Applications:

- Determine purity of protein samples
- Determine molecular weight of protein
- Identifying disulfide bonds between protein
- For Quantifying proteins
- For Blotting applications
- For Peptide Mapping

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ISOELECTRIC FOCUSING (IEF) GELS

Introduction:

- This method is ideal for the separation of amphoteric substances such as proteins because it is based on the separation of molecules according to their different isoelectric points.
- The method has high resolution, being able to separate proteins that differ in their isoelectric points by as little as 0.01 of a pH unit.
- The most widely used system for IEF utilizes horizontal gels on glass plates or plastic sheets.
- ISOELECTRIC POINT (pI): The pH at which net charge on protein becomes zero.

1. Below pI – Positive charge.
2. Above pI – Negative charge.

- Proteins move toward the electrode with the opposite charge.

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- **Separation is achieved by applying a potential difference across a gel that contains a pH gradient.**

- **The pH gradient is formed by the introduction into the gel of compounds known as ampholytes, which are complex mixtures of synthetic polyamino – polycarboxylic acids.**

- **Ampholytes can be purchased in different pH ranges covering either a wide band (E.g. pH 3 – 10) or various narrow bands (E.g. pH 7 – 8), and a pH range is chosen such that the samples being separated will have their isoelectric points (pI values) within this range.**

- **Commercially available ampholytes include Bio – Lyte and Pharmalyte**

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Principle:

- All proteins have an isoelectric point pH.
- IEF is preformed in a pH gradient.
- Proteins are amphoteric molecules with acidic and basic buffering groups (side chain).
- In basic environment, the acidic groups become negatively charged.
- In acidic environment, the basic groups become positively charged.
- Isoelectric point (pI): the pH where the charge of a protein is zero.
- Proteins are positively charged in solutions at pH values below pI and migrate towards cathode.
- Proteins are negatively charged in solutions at pH values above pI and migrate towards anode.

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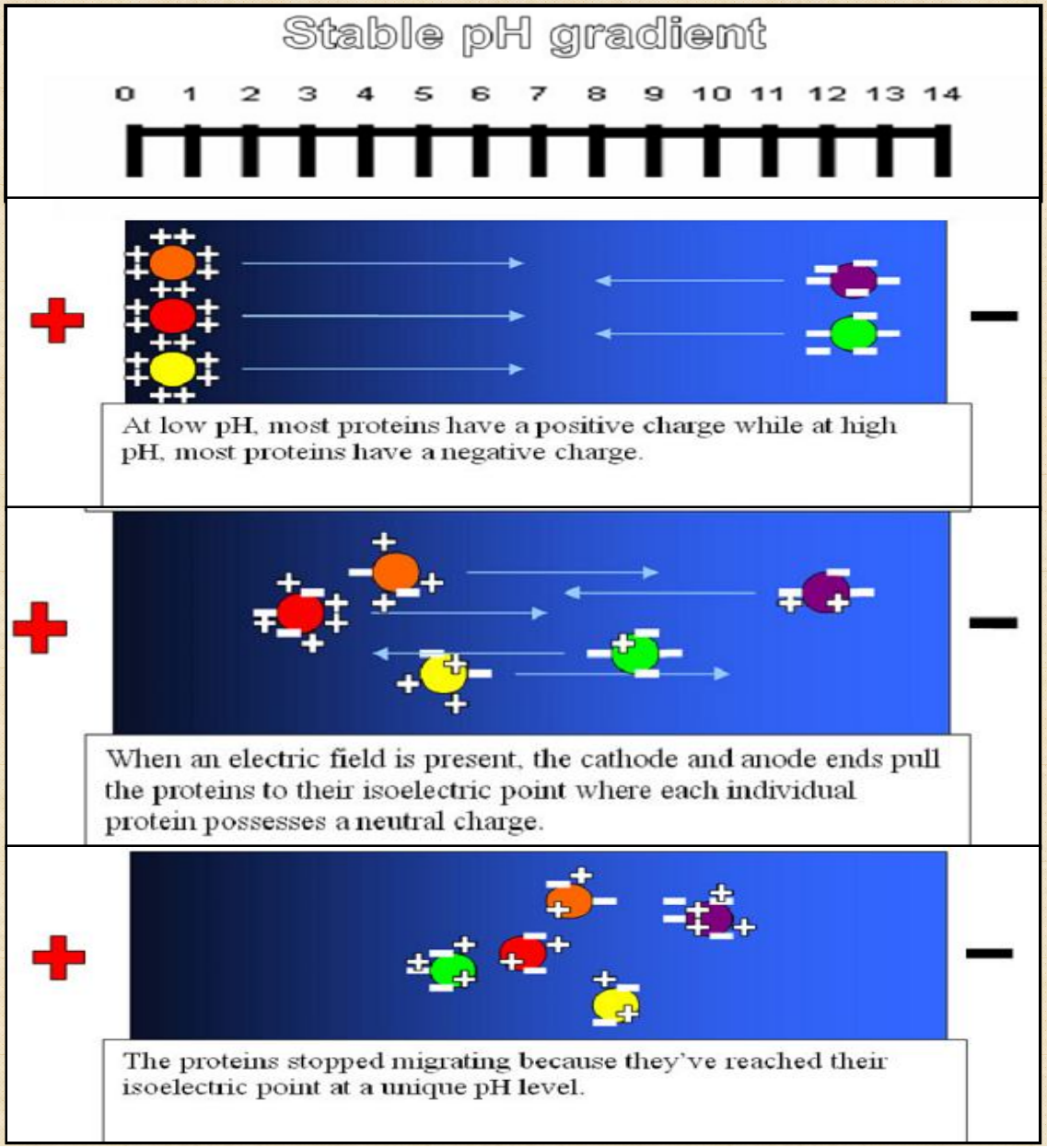

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Support Media (Gel preparation with pH gradient):

- IEF is carried out in low percentage gels to avoid any sieving effect within the gel.
- Polyacrylamide gels (4 %) are commonly used, but agarose is also used, especially for the study of high molecular weight proteins.
- To prepare a thin layer IEF gel, carrier ampholytes, covering a suitable pH range and riboflavin are mixed with the acrylamide solution.
- The mixture is then poured over a glass plate (typically 25 cm x 1O cm), which contains the spacer.

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- **The second glass plate is then placed on top of the first to form the gel cassette, and the gel polymerized by photo polymerization by placing the gel in front of a bright light. The photodecomposition of the riboflavin generates a free radical, which initiates polymerization.**
- **This takes 2 – 3 hours.**

- **Once the gel has set, the glass plates are prised apart to reveal the gel stuck to one of the glass sheets.**

- **Electrode wicks, which are thick (3mm) strips of wetted filter paper (the anode is phosphoric acid, the cathode sodium hydroxide) are laid along the long length of each side of the gel and a potential difference applied.**

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- Under the effect of this potential difference, the ampholytes form a pH gradient between the anode and cathode.

- The power is then turned off and samples applied.

Sample Application:

- Samples applied by laying on the gel small squares of filter paper soaked in the sample.

Running the sample:

- A voltage is again applied for about 30 min to allow the sample to electrophorese off the paper into the gel, at which time the paper squares can be removed from the gel.
- Depending on which point on the pH gradient the sample has been loaded, proteins that are initially at a pH region below their isoelectric point will be positively charged and will initially migrate towards the cathode.

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- As they proceed, however, the sur-rounding pH will be steadily increasing, and therefore the positive charge on the protein will decrease correspondingly until eventually the protein arrives at a point where the pH is equal to its isoelectric point.

- The protein will now be in the zwitterion form with no net charge, so further movement will cease. Likewise, substances that are initially at pH regions above their isoelectric points will be negatively charged and will migrate towards the anode until they reach their isoelectric points and become stationary.

- It can be seen that as the samples will always move towards their isoelectric points it is not critical where on the gel they are applied.

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Analysis of Gel:

- Following electrophoresis, the gel must be stained to detect the proteins. However, this cannot be done directly, because the ampholytes will stain too, giving a totally blue gel.

- The gel is therefore first washed with fixing solution (E.g. 10 % (v/v) trichloroacetic acid). This precipitates the proteins in the gel and allows the much smaller ampholytes to be washed out.

- The gel is stained with Coomassie Brilliant Blue and then destained.

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Applications:

- IEF is a highly sensitive analytical technique and is particularly useful for studying micro heterogeneity in a protein.

  For example:

  - A protein may show a single band on an SDS gel, but may show three bands on an IEF gel.
- This may occur, for example, when a protein exists in mono-, di- and tri-phosphorylated forms.
- The difference of a couple of phosphate groups has no significant effect on the overall relative molecular mass of the protein, hence a single band on SDS gels, but the small charge difference introduced on each molecule can be detected by IEF.

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- **The method is useful for separating isoenzymes (which are different forms of the same enzyme often differing by only one or two amino acid residues).**

- **2D Gel electrophoresis is an application of IEF.**
  Here, protein is first separated based on pI and then based on molecular weight using SDS-PAGE.

- **Widely used for separation and identification of serum proteins.**
  For example: Detection of oligoclonal bands in gamma-globulin with the use of IEF.

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Electrophoresis of Nucleic Acid(Agarose Gel Electrophoresis)

- **Agarose gel electrophoresis is a method 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 (electrophoresis).**
- **Shorter molecules move faster and migrate faster than longer ones .**
- **Agarose is purified from agar which is a mixture of neutral agarose and sulphated nengeling agarose pectin.**

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Principle:

- **DNA is negatively charged.**
- **When placed in an electrical field, DNA will migrate toward the positive pole (anode).**
- **An agarose gel is used to slow the movement of DNA and separate by size.**
- **Convenient analytical method for determining the size of DNA molecules in the range of 500 to 30,000 base pairs.**
- **Separates molecules from each other on the basis of**
  - **size and/or**
  - **charge and/or**
  - **shape**

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Support Media:

- Agarose is a linear polysaccharide (average relative molecular mass about 12000) made up of the basic repeat unit agarobiose, which comprises alternating units of galactose and 3,6 – anhydrogalactose.

- Agarose is one of the components of agar that is a mixture of polysaccharides isolated from certain seaweeds. Agar is usually used at concentrations of between 1 % and 3 %.

- 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.

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- **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.**

Factor affecting migration of Nucleic Acid:

Agarose concentration:

- **By changing the gel concentration the pore size can be altered. Higher concentration of gels are used for the separation of low molecular weight DNA & RNA fragments and vice versa.**

Molecular Size:

- **The largest molecule will have the most difficulty passing through the gel pores while small molecules will be relatively unhurdeled.**

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Confirmation:

- **Super coiled DNA moves fastest followed circular and linear form.**

Applied voltage:

- **As voltage increase mobility of high molecular weight DNA fragment increases.**

Base Composition:

- **Base composition and running gel between 4 ℃ to 30 ° C do not change the mobilities.**

General Procedure:

- **Agarose gel electrophoresis is most commonly done horizontally in a submarine mode whereby the slab gel is completely submerged in buffer during electrophoresis**

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Casting of gel:

- Agarose first soluballized by heating for 15 mins.
- Once the agarose is completely dissolved it is left at room temperature to cool.

- The glass platform of electrophoresis unit is washed with distilled water and wiped with the dry tissue paper. The open ends of the platform are sealed with the cello tape and comb template is placed such a way that it will not touch the surface of the glass platform.

- After cooling agarose solution to 50 °C the solution is poured gently to cover the entire surface of the platform and left undisturbed for 30 mins.

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Sample Application:

- Once the gel has set, the comb is removed, leaving wells where DNA samples can be loaded.

- Loading buffer is mixed with the DNA sample before the mixture is loaded into the wells. The loading buffer contains a dense compound, which may be glycerol, sucrose which make sample solution denser so that the DNA sample may sink to the bottom of the well.

- The loading buffer also include colored dyes such as and bromophenol blue used to monitor the progress of the electrophoresis.

- The DNA samples are loaded using a micropipette.

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Analysis of Gel:

- **DNA as well as RNA is normally visualized by staining with ethidium bromide, which intercalates into the major grooves of the DNA and fluoresces under UV light.**

- **The DNA bands of interest can be cut out of the gel and the DNA is recovered by (i) electro elution (ii) Macerating the gel piece in the buffer, centrifuge and collecting the supernatant.**

- **In each stage DNA is finally recovered by precipitation of the supernatant with ethanol.**

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Applications:

- **Estimation of the size of DNA molecules following restriction enzyme digestion, e.g. in restriction mapping of cloned DNA.**

- **Analysis of PCR products, e.g. in molecular genetic diagnosis or genetic fingerprinting separation of restricted genomic DNA prior to Southern transfer or of RNA prior to Northern transfer.**

- **Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, and clinical chemistry to separate a mixed population of DNA or proteins in a matrix of agarose**

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DNA Sequencing Gel:

- **This method is used to analyze single stranded DNA molecule shorter than about 1000 nucleotides and differing in size by only 1 nucleotide.**

- **To achieve this it is necessary to have a small pored size gel and so acrylamide gels are used e.g. 3.5% polyacrylamide gels are used to separate DNA in range of 80 to 1000 Nucleotides.**
- **Sequencing gels are run in the presence of denaturing agent Urea ,formamide.**

- **To separate DNA molecule that are similar in size, DNA sequencing gels used are very long to maximize the separation.**

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RNA Gel Electrophoresis:

- It is carried out in the agarose gel.
- In this extreme care is taken because even trace amount of enzyme RNase present in water ,Buffer and Glassware can degrade RNA molecule.
- Unlike DNA ,RNA molecules form hairpin or loop like secondary structures, therefore denaturation of secondary structure is required .
- For this variety of reagents are utilized like urea, formamide formaldehyde, glyoxal, methyl mercury hydroxide etc.
- In RNA gel electrophoresis casting of the gel and sample application is same as DNA gel electrophoresis but it is run at 75-100 v for 3-4 hours.
- Denatured RNA stains very weakly with ethidium bromide, so acridine orange is commonly used.

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