TEJASVI NAVADHITAMASTU

"Let our (the teacher and the taught) learning be radiant"

Let our efforts at learning be luminous and filled with joy, and endowed with the force of purpose

Dr. Prabhakar Singh
Department of Biochemistry
Faculty of Science, V.B.S. Purvanchal University, Jaunpur
PIN-222 003, (U.P.), INDIA.
Mobile No.: +91-9454695363
E-Mail: pruebiochem@gmail.com

VEER BAHADUR SINGH PURVANCHAL UNIVERSITY JAUNPUR-222003

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Prepared by: Dr. Prabhakar Singh
Department : Biochemistry
Faculty : Science
Email: pruebiochem@gmail.com
Contact: +91-9454695363

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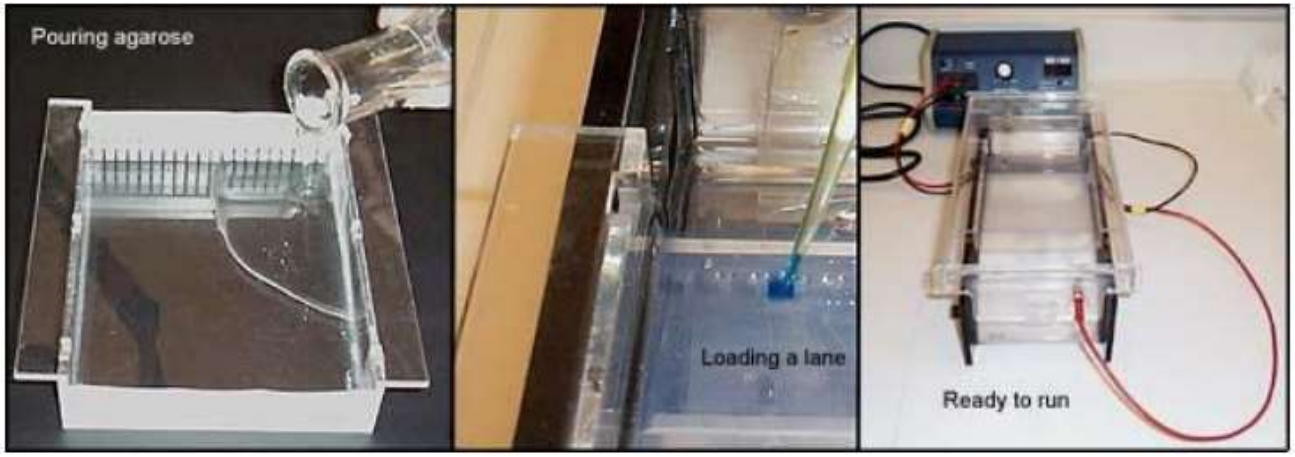
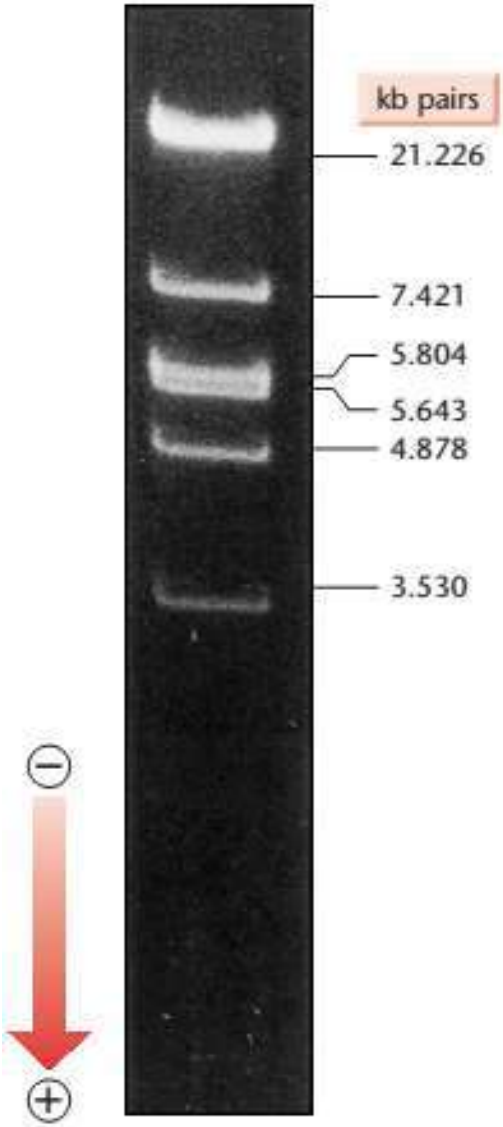
ELECTROPHORESIS





ELECTROPHORESIS

- The movement of charged particles(ions) in an electric field resulting in their migration towards the oppositely charged electrode is known as electrophoresis.
- Molecules with a net positive charge (cations) move towards the negative cathode while those with net negative charge (anions) migrate towards positive anode.
- Electrophoresis is a widely used analytical technique for the separation of biological molecules such as plasma proteins, lipoproteins and immunoglobulins.
- The rate of migration of ions in an electric field depends on several factors that include shape, size, net charge and solvation of the ions, viscosity of the solution and magnitude of the current employed.

Principle of Electrophoresis

1. Under the influence of an electric field these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge. When a potential difference (voltage) is applied across the electrodes, it generates a potential gradient, **E = V/d** (V= Applied Voltage, d = Distance between the electrodes).

2. When this potential gradient E is applied, the force on a molecule bearing a charge of q coulombs is, **F = Eq** newtons

3. It is this force that drives a charged molecule towards an electrode. However, there is also a frictional resistance that retards the movement of this charged molecule. This frictional force is a measure of the **hydrodynamic size of the molecule**, the **shape of the molecule**, the **pore size of the medium** in which electrophoresis is taking place and the viscosity of the buffer. Velocity will be $\nu = \frac{Eq}{f}$

4. Where f is the frictional coefficient. More commonly the term **electrophoretic mobility** (m) of an ion is used, which is the ratio of the velocity of the ion to field strength (n/E).

5. When a potential difference is applied, therefore, molecules with different overall charges will begin to separate based on different electrophoretic mobilities. Even molecules with similar charges will begin to separate if they have different molecular sizes, since they will experience different frictional forces.

DIFFERENT TYPES OF ELECTROPHORESIS

Among the electrophoretic techniques, **zone electrophoresis** (paper, gel), **isoelectric focusing** and **immunoelectrophoresis** are important and commonly employed in the laboratory.

1- MOVING BOUNDARY ELECTROPHORESIS

2- ZONE ELECTROPHORESIS : [A]- Paper Electrophoresis , [B] Gel Electrophoresis

3- ISOELECTRIC FOCUSSING

4- IMMUNOELECTROPHORESIS

1- MOVING BOUNDARY ELECTROPHORESIS

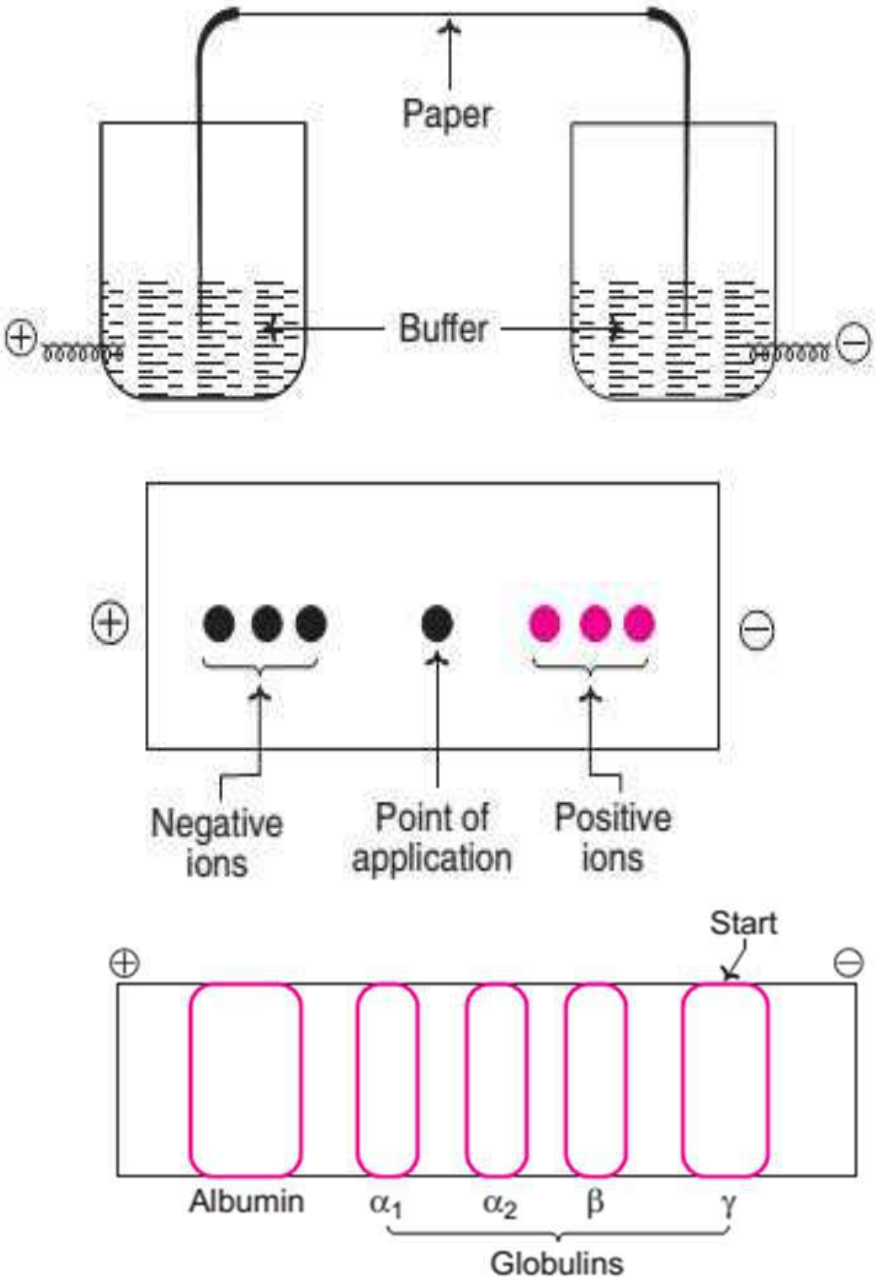
- The original moving boundary electrophoresis, developed by Tiselius (1933), is less frequently used these days.
- In this technique, the U-tube is filled with protein solution overlaid by a buffer solution.
- As the proteins move in solution during electrophoresis, they form boundaries which can be identified by refractive index.

2- ZONE ELECTROPHORESIS :

- A simple and modified method of moving boundary electrophoresis is the zone electrophoresis.
- An inert supporting material such as paper or gel are used.
- It is geneally of two type based on supportive material –
  [A]- Paper Electrophoresis , [B] Gel Electrophoresis

[A]-PAPER ELECTROPHORESIS

- In this technique, the sample is applied on a strip of filter paper wetted with desired buffer solution. The ends of the strip are dipped into the buffer reservoirs having electrodes.
- The electric current is applied allowing the molecules to migrate sufficiently.
- The paper is removed, dried and stained with a dye that specifically colours the substances to be detected.
- The coloured spots can be identified by comparing with a set of standards run simultaneously.
- For the separation of serum proteins, Whatman No. 1 filter paper, veronal or tris buffer at pH 8.6 and the stains amido black or bromophenol blue are employed.
- The serum proteins are separated into five distinct bands—albumin, D1-, D2-, E- and J-globulins.



Electrophoresis of plasma proteins

[B]-GEL ELECTROPHORESIS

- This technique involves the separation of molecules based on their size, in addition to the electrical charge.
- The movement of large molecules is slow in gel electrophoresis (this is in contrast to gel filtration).
- The resolution is much higher in this technique.
- Thus, serum proteins can be separated to about 15 bands, instead of 5 bands on paper electrophoresis.
- The gels commonly used in gel electrophoresis are agarose and polyacrylamide, sodium dodecyl sulfate (SDS).
- Polyacrylamide is employed for the determination of molecular weights of proteins in a popularly known electrophoresis technique known as SDS-PAGE

AGAROSE GEL ELECTROPHORESIS

- The migration of the DNA molecules through the pores of the matrix must play an important role in molecular-weight separations since the electrophoretic mobility of DNA in free solution is independent of molecular weight.

- An agarose gel is a complex network of polymeric molecules whose average pore size depends on the buffer composition and the type and concentration of agarose used. DNA movement through the gel was originally thought to resemble the motion of a snake (reptation). However, real-time fluorescence microscopy of stained molecules undergoing electrophoresis has revealed more subtle dynamics (Schwartz & Koval 1989, Smith et al. 1989).

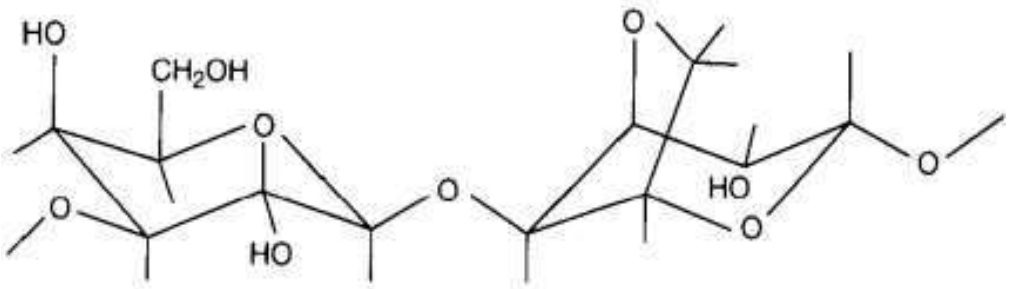
- DNA molecules display elastic behavior by stretching in the direction of the applied field and then contracting into dense balls. The larger the pore size of the gel, the greater the ball of DNA which can pass through and hence the larger the molecules which can be separated.

- Once the globular volume of the DNA molecule exceeds the pore size, the DNA molecule can only pass through by reptation. This occurs with molecules about 20 kb in size and it is difficult to separate molecules larger than this without recourse to pulsed electrical fields

AGAROSE

AGAROSE IS A LINEAR POLYMER COMPOSED OF ALTERNATING RESIDUES of D- and L-galactose joined by α-(1→3) and β-(1→4) glycosidic linkages. The L-galactose residue has an anhydro bridge between the three and six positions (please see Figure 5-1). Chains of agarose form helical fibers that aggregate into supercoiled structures with a radius of 20–30 nm. Gelation of agarose results in a three-dimensional mesh of channels whose diameters range from 50 nm to >200 nm (Norton et al. 1986; for review, please see Kirkpatrick 1990).

Commercially prepared agarose polymers are believed to contain ~800 galactose residues per chain. However, agarose is not homogeneous: The average length of the polysaccharide chains varies from batch to batch and from manufacturer to manufacturer. In addition, lower grades of agarose may be contaminated with other polysaccharides, as well as salts and proteins. This variability can affect the gelling/melting temperature of agarose solutions, the sieving of DNA, and the ability of the DNA recovered from the gel to serve as a substrate in enzymatic reactions. These potential problems can be minimized by using special grades of agarose that are screened for the presence of inhibitors and nucleases and for minimal background fluorescence after staining with ethidium bromide.



D-galactose

3,6-anhydro
L-galactose

Properties of agarose gel

1. Agarose gel is a three-dimensional matrix formed of helical agarose molecules in supercoiled bundles that are aggregated into three-dimensional structures with channels and pores through which biomolecules can pass.

2. The 3-D structure is held together with hydrogen bonds and can therefore be disrupted by heating back to a liquid state. The melting temperature is different from the gelling temperature, depending on the sources, agarose gel has a gelling temperature of 35-42 °C and a melting temperature of 85-95 °C. Low-melting and low-gelling agaroses made through chemical modifications are also available.

3. The pore size of a 1% gel has been estimated from 100 nm to 200-500 nm, and its gel strength allows gels as dilute as 0.15% to form a slab for gel electrophoresis. Low-concentration gels (0.1 - 0.2%) however are fragile and therefore hard to handle.

4. Agarose gel has lower resolving power than polyacrylamide gel for DNA but has a greater range of separation, and is therefore used for DNA fragments of usually 50-20,000 bp in size. The limit of resolution for standard agarose gel electrophoresis is around 750 kb, but resolution of over 6 Mb is possible with pulsed field gel electrophoresis (PFGE) A 0.9% agarose gel has pores large enough for the entry of bacteriophage T4.

THE RATE OF MIGRATION OF DNA THROUGH AGAROSE GELS

- ***The molecular size of the DNA.*** Molecules of double-stranded DNA migrate through gel matrices at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs (Helling et al. 1974). Larger molecules migrate more slowly because of greater frictional drag and because they worm their way through the pores of the gel less efficiently than smaller molecules.

- ***The concentration of agarose.*** A linear DNA fragment of a given size migrates at different rates through gels containing different concentrations of agarose (please see Figure 5-2). There is a linear relationship between the logarithm of the electrophoretic mobility of the DNA (μ) and the gel concentration (ι) that is described by the equation:

  $$\log \mu = \log \mu_o - K_r \iota$$

  where μ_o is the free electrophoretic mobility of DNA and K_r is the retardation coefficient, a constant related to the properties of the gel and the size and shape of the migrating molecules.

- ***The conformation of the DNA.*** Superhelical circular (form I), nicked circular (form II), and linear (form III) DNAs migrate through agarose gels at different rates (Thorne 1966, 1967). The relative mobilities of the three forms depend primarily on the concentration and type of agarose used to make the gel, but they are also influenced by the strength of the applied current, the ionic strength of the buffer, and the density of superhelical twists in the form I DNA (Johnson and Grossman 1977). Under some conditions, form I DNA migrates faster than form III DNA; under other conditions, the order is reversed. In most cases, the best way to distinguish between the different conformational forms of DNA is simply to include in the gel a sample of untreated circular DNA and a sample of the same DNA that has been linearized by digestion with a restriction enzyme that cleaves the DNA in only one place.

- ***The presence of ethidium bromide in the gel and electrophoresis buffer.*** Intercalation of ethidium bromide causes a decrease in the negative charge of the double-stranded DNA and an increase in both its stiffness and length. The rate of migration of the linear DNA-dye complex through gels is consequently retarded by a factor of ~15% (Sharp et al. 1973).

- ***The applied voltage.*** At low voltages, the rate of migration of linear DNA fragments is proportional to the voltage applied. However, as the strength of the electric field is raised, the mobility of high-molecular-weight fragments increases differentially. Thus, the effective range of separation in agarose gels decreases as the voltage is increased. To obtain maximum resolution of DNA fragments >2 kb in size, agarose gels should be run at no more than 5–8 V/cm.

- ***The type of agarose.*** The two major classes of agarose are standard agaroses and low-melting-temperature agaroses (Kirkpatrick 1990). A third and growing class consists of intermediate melting/gelling temperature agaroses, exhibiting properties of each of the two major classes. Within each class are various types of agaroses that are used for specialized applications, please

- ***The electrophoresis buffer.*** The electrophoretic mobility of DNA is affected by the composition and ionic strength of the electrophoresis buffer. In the absence of ions (e.g., if water is substituted for electrophoresis buffer in the gel or in the reservoirs), electrical conductivity is minimal and DNA migrates slowly, if at all. In buffer of high ionic strength (e.g., if 10x electrophoresis buffer is mistakenly used), electrical conductance is very efficient and significant amounts of heat are generated, even when moderate voltages are applied. In the worst case, the gel melts and the DNA denatures. For details of commonly used electrophoresis buffers, please see Table 5-3.

CLASSES OF AGAROSE AND THEIR PROPERTIES

- ***Standard (high-melting-temperature) agaroses*** are manufactured from two species of seaweed: *Gelidium* and *Gracilaria*. These agaroses differ in their gelling and melting temperatures, but, for practical purposes, agaroses from either source can be used to analyze and isolate fragments of DNA ranging in size from 1 kb to 25 kb. Several commercial grades of agaroses have been tested that (1) display minimal background fluorescence after staining with ethidium bromide, (2) are free of DNase and RNase, (3) display minimal inhibition of restriction endonucleases and ligase, and (4) generate modest amounts of electroendo-osmotic flow (EEO; please see below).

- ***Low melting/gelling temperature agaroses*** have been modified by hydroxyethylation and therefore melt at temperatures lower than those of standard agaroses. The degree of substitution determines the exact melting and gelling temperature. Low melting/gelling temperature agaroses are used chiefly for rapid recovery of DNA, as most agaroses of this type melt at temperatures (~65°C) that are significantly lower than the melting temperature of duplex DNA. This feature allows for simple purification, enzymatic processing (restriction endonuclease digestion/ligation) of DNA; and allows bacterial transformation with nucleic acids directly in the remelted gel. As is the case with standard agaroses, manufacturers provide grades of low-melting-temperature agaroses that have been tested to display minimal background fluorescence after staining with ethidium bromide, to be free of DNase and RNase activity, and to display minimal inhibition of restriction endonucleases and ligase. Low-melting-temperature agaroses not only melt, but also gel at low temperatures. This property allows them to be held as liquids in the 30–35°C range, so that cells can be embedded without damage. This treatment is useful in preparing and embedding chromosomal DNA in agarose blocks before analysis by PFGE (please see Protocols 13 and 14).

- ***Electroendo-osmosis.*** In agarose gels, the speed at which nucleic acids migrate toward the positive electrode is affected by a electroendo-osmosis. This process is due to ionized acidic groups (usually sulfate) attached to the polysaccharide matrix of the agarose gel. The acidic groups induce positively charged counterions in the buffer that migrate through the gel toward the negative electrode, causing a bulk flow of liquid that migrates in a direction opposite to that of the DNA.

  The higher the density of negative charge on the agarose, the greater the EEO flow and the poorer the separation of nucleic acid fragments. Retardation of small DNA fragments (<10 kb) is minor, but larger DNA molecules can be significantly retarded, especially in PFGE. To avoid problems, it is best to purchase agarose from reputable merchants and to use types of agarose that display low levels of EEO. Agaroses that are sold as "zero" EEO are undesirable for two reasons: They have been chemically modified by adding positively charged groups, which neutralize the sulfated polysaccharides in the gel but may inhibit subsequent enzyme reactions, and they have been adulterated by adding locust bean gum, which retards expulsion of water from the gel (Kirkpatrick 1990).

ELECTROENDOSMOSIS (EEO)

- The agarose polymer contains charged groups, in particular pyruvate and sulphate. These negatively charged groups create a flow of water in the opposite direction to the movement of DNA in a process called electroendosmosis (EEO), and can therefore retard the movement of DNA and cause blurring of bands.

- Higher concentration gel would have higher electroosmotic flow. Low EEO agarose is therefore generally preferred for use in agarose gel electrophoresis of nucleic acids, but high EEO agarose may be used for other purposes.

- The lower sulphate content of low EEO agarose, particularly low-melting point (LMP) agarose, is also beneficial in cases where the DNA extracted from gel is to be used for further manipulation as the presence of contaminating sulphates may affect some subsequent procedures, such as ligation and PCR.

- Zero EEO agaroses however are undesirable for some applications as they may be made by adding positively charged group and such groups can affect subsequent enzyme reactions

MIGRATION OF NUCLEIC ACIDS IN AGAROSE GEL

- A number of factors can affect the migration of nucleic acids:
    1. the dimension of the gel pores (gel concentration),
    2. size of DNA being electrophoresed,
    3. the voltage used,
    4. the ionic strength of the buffer
    5. the concentration of intercalating dye such as ethidium bromide if used during electrophoresis

- For standard agarose gel electrophoresis, larger molecules are resolved better using a low concentration gel while smaller molecules separate better at high concentration gel. High concentrations gel however requires longer run times

- The movement of the DNA may be affected by the conformation of the DNA molecule, for example, supercoiled DNA usually moves faster than relaxed DNA because it is tightly coiled and hence more compact. In a normal plasmid DNA preparation, multiple forms of DNA may be present

- Ethidium bromide which intercalates into circular DNA can change the charge, length, as well as the superhelicity of the DNA molecule, therefore its presence in gel during electrophoresis can affect its movement.

Migration anomalies

- **"Smiley" gels** - this edge effect is caused when the voltage applied is too high for the gel concentration used.
- **Overloading of DNA** - slows down the migration of DNA fragments.
- **Contamination** - presence of impurities, such as salts or proteins can affect the movement of the DNA
- **Thermal effects:** Most of the power generated during electrophoresis is dissipated as heat. Heating of electrophoretic medium has effects as:

1. An increased rate of diffusion of sample and buffer ions leading to broadening of the separated samples i.e. smiley band of DNA
2. The formation of convection currents, which leads to mixing of separated samples.
3. Thermal instability of samples that are rather sensitive to heat. This may include denaturation of proteins (and thus the loss of enzyme activity).
4. A decrease of buffer viscosity, and hence a reduction in the resistance of the medium.

Mechanism of migration and separation

- The negative charge of its phosphate backbone moves the DNA towards the positively charged anode during electrophoresis.
- However, the migration of DNA molecules in solution, in the absence of a gel matrix, is independent of molecular weight during electrophoresis.
- The gel matrix is therefore responsible for the separation of DNA by size during electrophoresis
- A widely accepted one is the Ogston model which treats the polymer matrix as a sieve. A globular protein or a random coil DNA moves through the interconnected pores, and the movement of larger molecules is more likely to be impeded and slowed down by collisions with the gel matrix, and the molecules of different sizes can therefore be separated in this sieving process

General procedure

1. **Casting of gel:** The concentration of gel affects the resolution of DNA separation. For a standard agarose gel electrophoresis, a 0.8% gives good separation or resolution of large 5–10kb DNA fragments, while 2% gel gives good resolution for small 0.2–1kb fragments.
2. **Loading of samples:** The loading buffer contains a dense compound, which may **be glycerol, sucrose, or Ficoll**, that raises the density of the sample so that the DNA sample may sink to the bottom of the well. If the DNA sample contains residual ethanol after its preparation, it may float out of the well. The loading buffer also include colored dyes such as xylene cyanol and bromophenol blue used to monitor the progress of the electrophoresis.
3. **Electrophoresis:** Agarose gel electrophoresis is most commonly done horizontally in a submarine mode whereby the slab gel is completely submerged in buffer during electrophoresis.
4. Since DNA is not visible in natural light, the progress of the electrophoresis is monitored using colored dyes. Xylene cyanol (light blue color) comigrates large DNA fragments, while Bromophenol blue (dark blue) comigrates with the smaller fragments. Less commonly used dyes include Cresol Red and Orange G which migrate ahead of bromophenol blue.
5. A DNA marker is also run together for the estimation of the molecular weight of the DNA fragments. Note however that the size of a circular DNA like plasmids cannot be accurately gauged using standard markers unless it has been linearized by restriction digest, alternatively a supercoiled DNA marker may be used.
6. **Staining and visualization:** DNA as well as RNA are normally visualized by staining with ethidium bromide, which intercalates into the major grooves of the DNA and fluoresces under UV light. The ethidium bromide may be added to the agarose solution before it gels, or the DNA gel may be stained later after electrophoresis. Destaining of the gel is not necessary but may produce better images. Other methods of staining are available; examples are SYBR Green, GelRed, methylene blue, brilliant cresyl blue, Nile blue sulphate, and crystal violet

Buffers

- In general, the ideal buffer should have good conductivity, produce less heat and have a long life.[29] There are a number of buffers used for agarose electrophoresis; common ones for nucleic acids include Tris/Acetate/EDTA (TAE) and Tris/Borate/EDTA (TBE).

- Tris-phosphate buffer has high buffering capacity but cannot be used if DNA extracted is to be used in phosphate sensitive reaction.

- TAE has the lowest buffering capacity but provides the best resolution for larger DNA. This means a lower voltage and more time, but a better product.

Application of agarose gel electrophoresis

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

2. Analysis of PCR products, e.g. in molecular genetic diagnosis or genetic fingerprinting

3. Separation of DNA fragments for extraction and purification.

4. Separation of restricted genomic DNA prior to Southern transfer, or of RNA prior to Northern transfer.

5. Agarose gels are easily cast and handled compared to other matrices and nucleic acids are not chemically altered during electrophoresis. Samples are also easily recovered. After the experiment is finished, the resulting gel can be stored in a plastic bag in a refrigerator.

6. Electrophoresis is performed in buffer solutions to reduce pH changes due to the electric field, which is important because the charge of DNA and RNA depends on pH, but running for too long can exhaust the buffering capacity of the solution. Further, different preparations of genetic material may not migrate consistently with each other, for morphological or other reasons.

POLYACRYLAMIDE GEL ELECTROPHORESIS

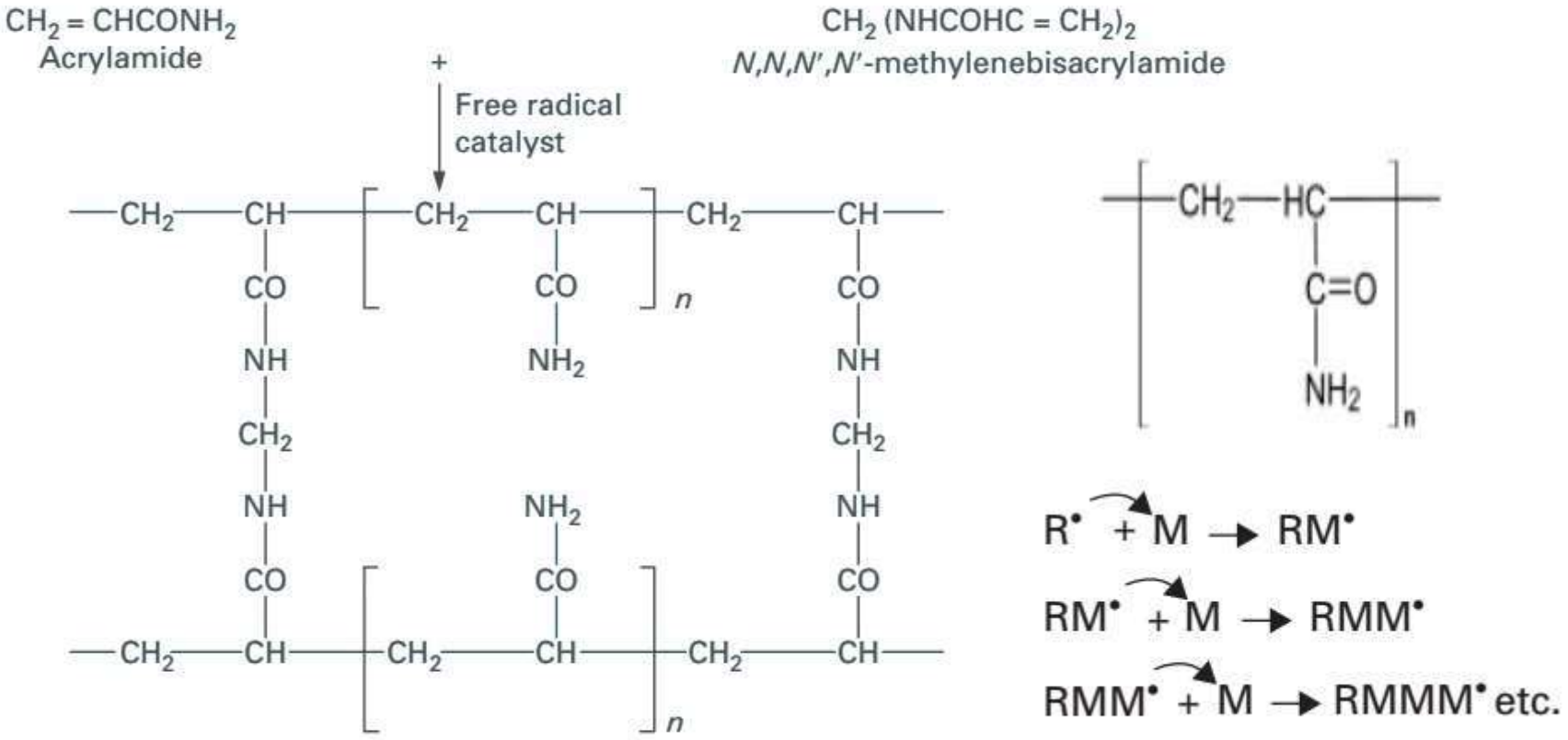
- PAGE is used for separating proteins in size from 5 to 2,000 kDa due to the uniform pore size in polyacrylamide gel. Pore size is controlled by modulating the concentrations of acrylamide and bis-acrylamide powder used in creating a gel.

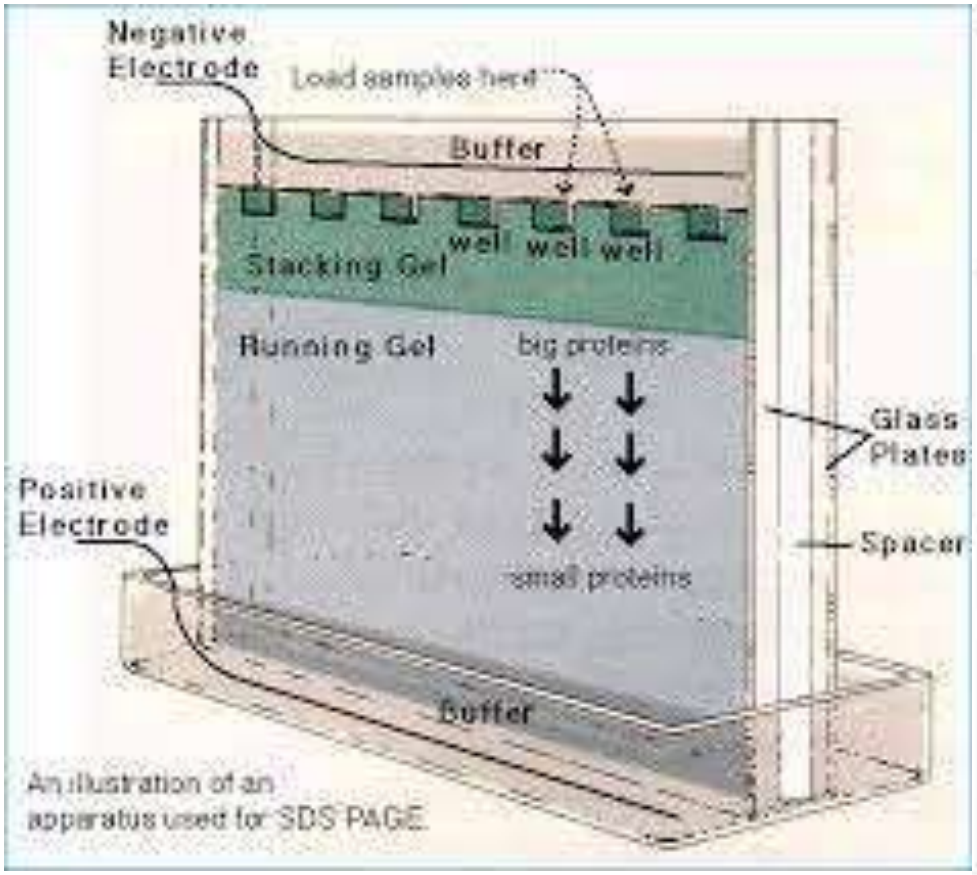
- Traditional DNA sequencing techniques such as Maxam-Gilbert or Sanger methods used polyacrylamide gels to separate DNA fragments differing by a single base-pair in length so the sequence could be read. Most modern DNA separation methods now use agarose gels, except for particularly small DNA fragments. It is currently most often used in the field of immunology and protein analysis, often used to separate different proteins or isoforms of the same protein into separate bands.

- Typically resolving gels are made in 6%, 8%, 10%, 12% or 15%. Stacking gel (5%) is poured on top of the resolving gel and a gel comb (which forms the wells and defines the lanes where proteins, sample buffer and ladders will be placed) is inserted. The percentage chosen depends on the size of the protein that one wishes to identify or probe in the sample. The smaller the known weight, the higher the percentage that should be used. Changes on the buffer system of the gel can help to further resolve proteins of very small sizes

Polyacrylamide:

- It can be synthesized as a simple linear-chain structure or cross-linked. Polyacrylamide is not toxic. However, unpolymerized acrylamide, which is a neurotoxin, can be present in very small amounts in the polymerized acrylamide





An illustration of an apparatus used for SDS PAGE

Stacking gel

0. 125 M Tris-HCl, pH 6.8 5% acrylamide*

Larger pores, lower ionic strength

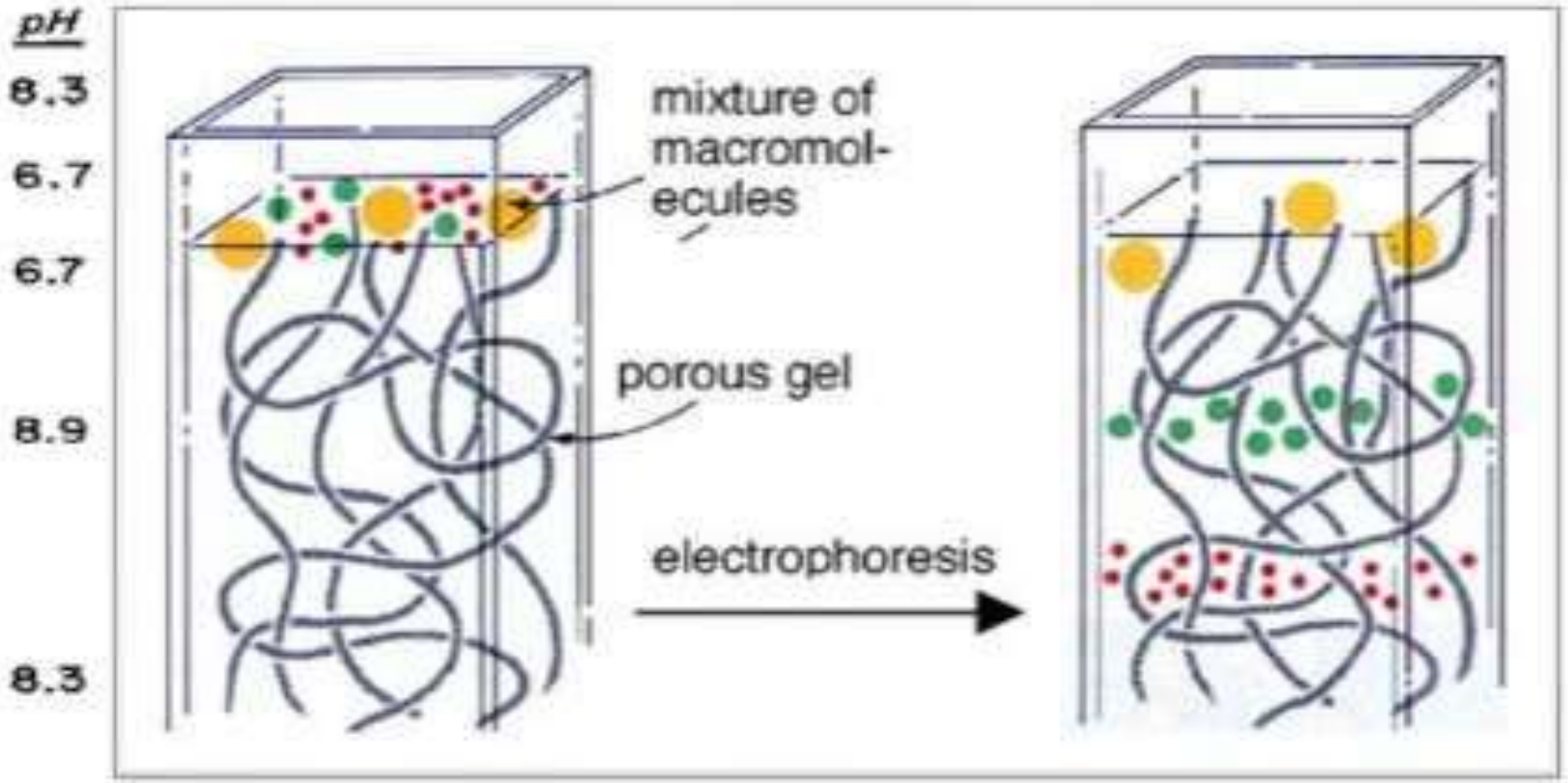
Running (resolving) gel

0. 375 M Tris-HCl, pH 8.8 12% acrylamide*

Smaller pores, higher ionic strength

*Investigators adjust the acrylamide concentration to manipulate the gel pore sizes

Movement of particle



[Cl^-] >[protein–SDS] >[glycinate]

[Cl^-] >[glycinate] >[protein–SDS]


Stacking gel
Resolving gel
It occupies 20-25% length of the entire unit
Occupies 75-80% of the entire unit
It is made of 4-6% of Acrylamide gel
It is made of 8-10% acrylamide gel
It is more porous
It is less porous
pH=6.8
pH=8.8
Band sharpening is less
Band sharpening is more

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) is the most widely used method for analysing 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, it can also be used to determine the relative molecular mass of proteins. SDS($CH_3-(CH_2)_{10}-CH_2OSO_3^-Na^+$) is an anionic detergent. Samples to be run on SDS–PAGE are firstly boiled for 5 min in sample buffer containing β-mercaptoethanol and SDS. The mercaptoethanol reduces any disulphide bridges present that are holding together the protein tertiary structure, and the SDS binds strongly to, and denatures, the protein. 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. 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 negatively charged SDS molecules.

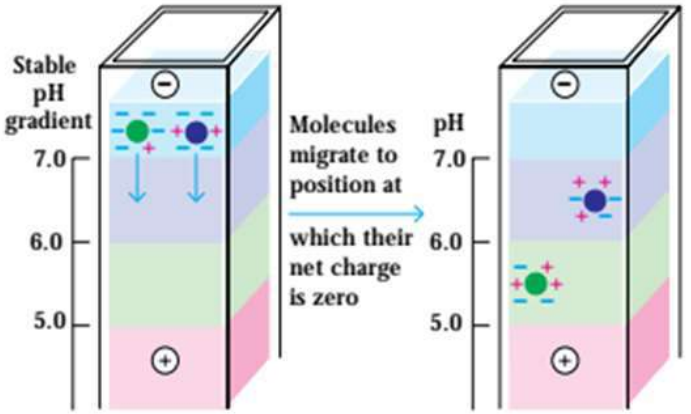
Acrylamide concentration (%)	Protein fractionation range ($M_r \times 10^{-3}$)
5	60–350
10	15–200
15	10–100

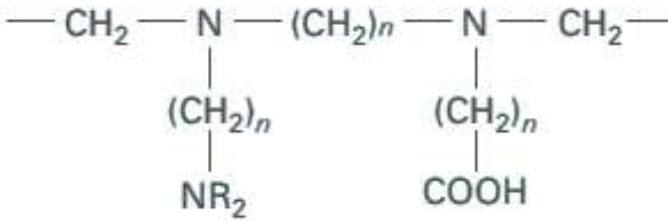
The rod-like structure remains, as any rotation that tends to fold up the protein chain would result in repulsion between negative charges on different parts of the protein chain, returning the conformation back to the rod shape. The sample buffer also contains an ionisable tracking dye, usually bromophenol blue, that allows the electrophoretic run to be monitored, and sucrose or glycerol, which gives the sample solution density thus allowing the sample to settle easily through the electrophoresis buffer to the bottom when injected into the loading well (see Fig. 10.1). Once the samples are all loaded, a current is passed through the gel. The samples to be separated are not in fact loaded directly into the main separating gel. When the main separating gel (normally about 5 cm long) has been poured between the glass plates and allowed to set, a shorter (approximately 0.8 cm) stacking gel is poured on top of the separating gel and it is into this gel that the wells are formed and the proteins loaded. The purpose of this stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel. This is achieved by utilising differences in ionic strength and pH between the electrophoresis buffer and the stacking gel buffer and involves a phenomenon known as isotachophoresis. The stacking gel has a very large pore size (4% acrylamide), which allows the proteins to move freely and concentrate, or stack, under the effect of the electric field. The band-sharpening effect relies on the fact that negatively charged glycinate ions (in the electrophoresis buffer) have a lower electrophoretic mobility than do the protein–SDS complexes, which, in turn, have lower mobility than the chloride ions (Cl^-) of the loading buffer and the stacking gel

When the current is switched on, all the ionic species have to migrate at the same speed otherwise there would be a break in the electrical circuit. The glycinate ions can move at the same speed as Cl^- only if they are in a region of higher field strength. Field strength is inversely proportional to conductivity, which is proportional to concentration. The result is that the three species of interest adjust their concentrations so that [Cl^-] > [protein–SDS] > [glycinate]. There is only a small quantity of protein–SDS complexes, so they concentrate in a very tight band between glycinate and Cl^- boundaries. Once the glycinate reaches the separating gel it becomes more fully ionised in the higher pH environment and its mobility increases. (The pH of the stacking gel is 6.8, that of the separating gel is 8.8.) Thus, the interface between glycinate and Cl^- leaves behind the protein–SDS complexes, which are left to electrophorese at their own rates. The negatively charged protein–SDS complexes now continue to move towards the anode, and, because they have the same charge per unit length, they travel into the separating gel under the applied electric field with the same mobility. However, as they pass through the separating gel the proteins separate, owing to the molecular sieving properties of the gel. Quite simply, the smaller the protein the more easily it can pass through the pores of the gel, whereas large proteins are successively retarded by frictional resistance due to the sieving effect of the gels. Being a small molecule, the bromophenol blue dye is totally unretarded and therefore indicates the electrophoresis front.

ISOELECTRIC FOCUSSING GELS

Another electrophoretic technique, isoelectric focusing (IEF), separates proteins solely on the basis of their charge. This method is based on the fact that a molecule will move in an electric field as long as it has a net positive or negative charge; molecules that bear equal numbers of positive and negative charges and therefore have a net charge of zero will not move. At most pH values, proteins (which characteristically bear a number of both positive and negative charges) have either a net negative or a net positive charge. However, for each protein there is a particular pH, called its isoelectric point (pI), at which that protein has equal numbers of positive and negative charges. Isoelectric focusing makes use of a gel containing substances, called carrier ampholytes, that arrange themselves into a continuous pH gradient when subjected to an electric field. When a mixture of proteins is applied to such a gel and subjected to electrophoresis, each protein moves until it reaches that point in the gradient where the pH of the gel is equal to its isoelectric point. It then stops moving because it has a net charge of zero. Isoelectric focusing is an extremely gentle and effective way of separating different proteins





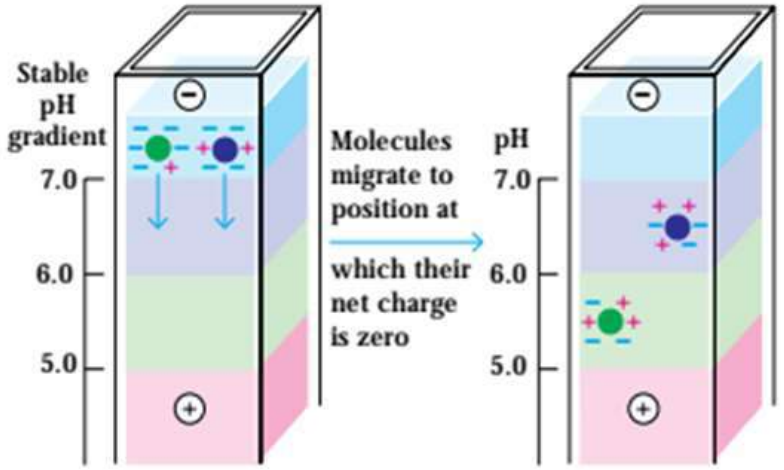
where R = H or — $(CH_2)_n$ —COOH

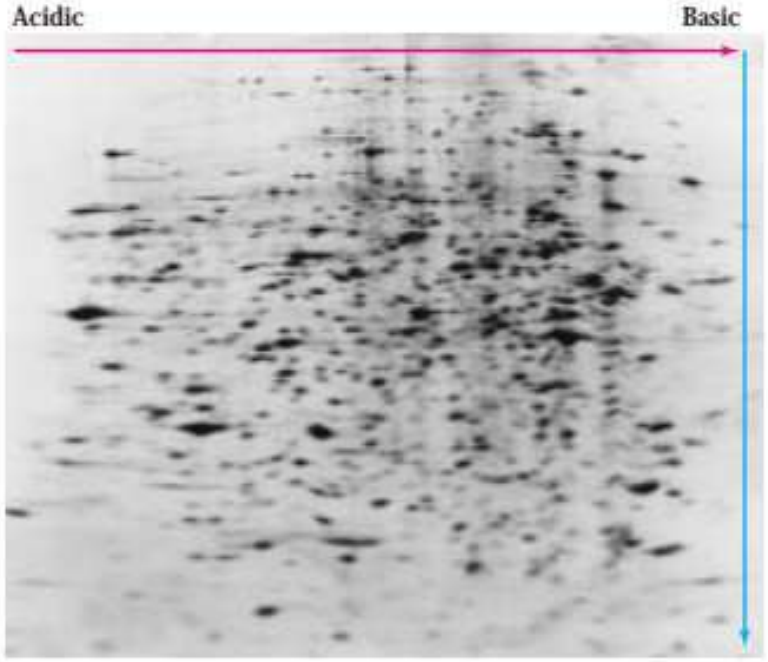
n = 2 or 3

The general formula for ampholytes.

2 D Gel Electrophoresis

A method known as two-dimensional gel electrophoresis (2D gel electrophoresis) combines the advantages of SDS-PAGE and isoelectric focusing in one of the most sensitive and discriminating ways of analyzing a mixture of proteins. In this method, one first subjects the mixture to isoelectric focusing on an IEF tube gel, which separates the molecules on the basis of their isoelectric points without regard to molecular weight. This is the first dimension. In the next step, one places the IEF gel lengthwise across the top of an SDS-polyacrylamide slab (that is, in place of the sample wells in Figure 23-4a) and runs SDS-PAGE. Preparatory to this step, all proteins have been reacted with SDS and therefore migrate out of the IEF gel and through the SDS-PAGE slab according to their molecular weights. This is the second dimension. The position of the proteins in the resulting 2D gel can be visualized in a number of ways. In the least sensitive the gel is stained with a protein-binding dye (such as Coomassie blue). If the proteins have been radiolabeled, the more sensitive method of autoradiography can be used. Alternatively, silver staining is a method of great sensitivity that takes advantage of the capacity of proteins to reduce silver ions to an easily visualized deposit of metallic silver. Finally, immunoblotting—blotting of proteins onto a membrane and detection with antibody (see Figure 6-13)—can be used as a way of locating the position of specific proteins on 2D gels if an appropriate antibody is available. Figure 23-5 shows an autoradiograph of a two-dimensional gel of labeled proteins from murine thymocytes.



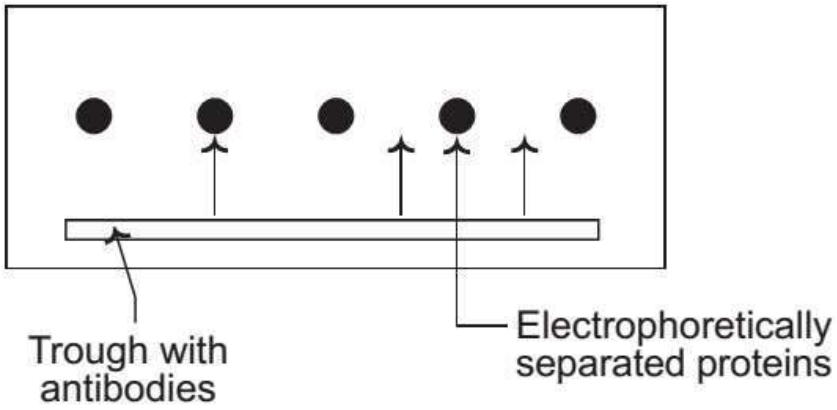


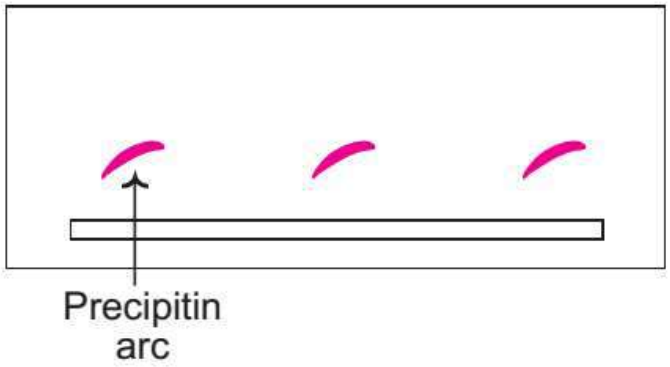
IMMUNOELECTROPHORESIS

- This technique involves combination of the principles of electrophoresis and immunological reactions. Immunoelectrophoresis is useful for the analysis of complex mixtures of antigens and antibodies.

- The complex proteins of biological samples (say human serum) are subjected to electrophoresis. The antibody (antihuman immune serum from rabbit or horse) is thenapplied in a trough parallel to the electrophoretic separation.

- The antibodies diffuse and, when they come in contact with antigens, precipitation occurs, resulting in the formation of precipitin bands which can be identified





ISOTACHOPHORESIS

Isotachophoresis (ITP) is a technique in analytical chemistry used for selective separation and concentration of ionic analytes. It is a form of electrophoresis: charged analytes are separated based on ionic mobility, a factor which tells how fast an ion migrates through an electric field.

Isotachophoresis means migration with the same speed. Many "non physisists" complain, that they do not understand isotachophoresis as easily as other biophysical methods. The reason: several things occur simultaneously.

To understand the effects and the features of the technique, one should imagine the four facts of isotachophoresis, which are equally important and which happen at the same time:

1. Migration of all ions with the same speed
2. Separation of components as an "Ion train"
3. Zone sharpening effect
4. Concentration regulating effect

- In isotachophoresis (ITP), the separation is carried out in a discontinuous buffer system. The ionized sample migrates between a leading electrolyte with a high mobility and a terminating - sometimes called trailing - ion with a low mobility, all of them migrating with the same speed.

- The different components are separated according to their electrophoretic mobilities and form stacks: the substance with the highest mobility directly follows the leading ion, the one with the lowest mobility migrates directly in front of the terminating electrolyte.

- In ITP there is a concentration regulating effect which works against diffusion.

- In an anionic separation, the leading electrolyte will be at the anodal, and the terminating electrolyte at the cathodal side. The sample is applied between the two. The system also contains a common cationic counter-ion.

- A practical example: Chloride is the leading ion, Glycine is the terminating ion, Tris is the counter ion.

- ITP is mostly applied for stacking of the samples during the first phase of disc electrophoresis.

PULSED-FIELD GEL ELECTROPHORESIS

- At Columbia University in 1984, David C. Schwartz and Charles Cantor developed a variation on the standard protocol by introducing an alternating voltage gradient to improve the resolution of larger molecules. This technique became known as pulsed-field gel electrophoresis (PFGE).

- Pulsed field gel electrophoresis is a technique used for the separation of large deoxyribonucleic acid (DNA) molecules by applying to a gel matrix an electric field that periodically changes direction.

- Standard gel electrophoresis techniques for separation of DNA molecules provided huge advantages for molecular biology research. However, it was unable to separate very large molecules of DNA effectively.

- DNA molecules larger than 15-20 kb migrating through a gel will essentially move together in a size-independent manner. The development of PFGE expanded the range of resolution for DNA fragments by as much as two orders of magnitude.

PROCEDURE

- The procedure for this technique is relatively similar to performing a standard gel electrophoresis except that instead of constantly running the voltage in one direction, the voltage is periodically switched among three directions; one that runs through the central axis of the gel and two that run at an angle of 60 degrees either side.

- The pulse times are equal for each direction resulting in a net forward migration of the DNA. For extremely large molecules (up to around 2 Mb), switching-interval ramps can be used that increases the pulse time for each direction over the course of a number of hours—take, for instance, increasing the pulse linearly from 10 seconds at 0 hours to 60 seconds at 18 hours.

- This procedure takes longer than normal gel electrophoresis due to the size of the fragments being resolved and the fact that the DNA does not move in a straight line through the gel.

THEORY

- While in general small fragments can find their way through the gel matrix more easily than large DNA fragments, a threshold length exists above 30–50 kb where all large fragments will run at the same rate, and appear in a gel as a single large diffuse band.

- However, with periodic changing of field direction, the various lengths of DNA react to the change at differing rates. That is, larger pieces of DNA will be slower to realign their charge when field direction is changed, while smaller pieces will be quicker. Over the course of time with the consistent changing of directions, each band will begin to separate more and more even at very large lengths. Thus separation of very large DNA pieces using PFGE is made possible

APPLICATIONS

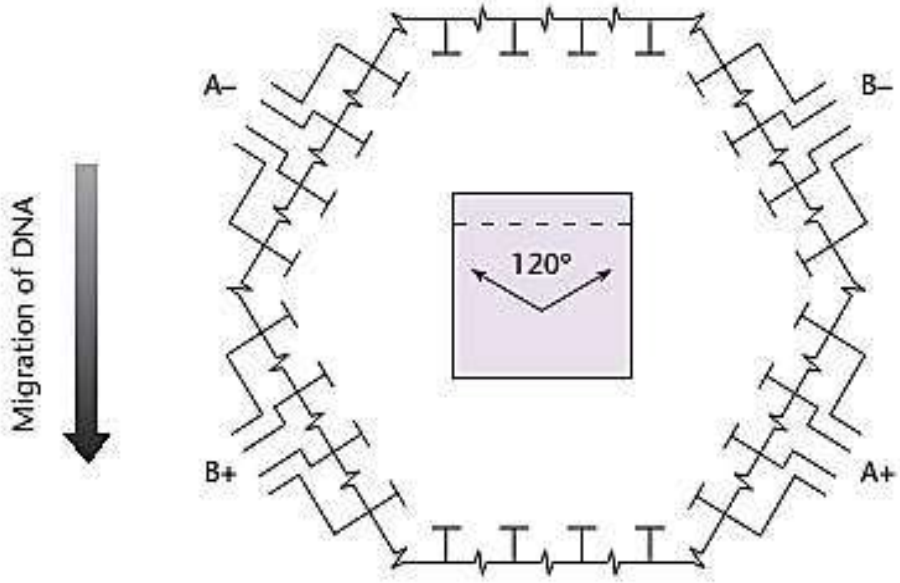
1. PFGE may be used for genotyping or genetic fingerprinting. It is commonly considered a gold standard in epidemiological studies of pathogenic organisms. Subtyping has made it easier to discriminate among strains of Listeria monocytogenes and thus to link environmental or food isolates with clinical infections.

2. PFGE allows investigators to separate much larger pieces of DNA than conventional agarose gel electrophoresis. In conventional gels, the current is applied in a single direction (from top to bottom). But in PFGE, the direction of the current is altered at a regular interval as shown in the animated gif below.

3. The gray box is the gel, the six sets of 4 black lines represent the 3 pairs of electrodes. Initially, the gel is empty but soon Whole chromosomes mixed with blue loading dye will be placed into the wells. Then the current will be turned on and the direction of the current will change in a regular pattern. This is repeated until the loading dye reaches near the end of the gel Then the gel is soaked in a solution containing ethidium bromide which fluoresces orange when bound to DNA.

CONTOUR-CLAMPED HOMOGENEOUS ELECTRICAL-FIELD (CHEF) ELECTROPHORESIS

- A major disadvantage of PFGE, as originally described, is that the samples do not run in straight lines. This makes subsequent analysis difficult. This problem has been overcome by the development of improved methods for alternating the electrical field. The most popular of these is contour-clamped homogeneous electrical-field (CHEF) electrophoresis (Chu et al.1986).
- In early CHEF-type systems the reorientation angle was fixed at 120°. However, in newer systems, the reorientation angle can be varied and it has been found that for whole-yeast chromosomes the migration rate is much faster with an angle of 106°.
- Fragments of DNA as large as 200–300 kb are routinely handled in genomics work and these can be separated in a matter of hours using CHEF systems with a reorientation angle of 90° or less.



Schematic representation of CHEF (contour-clamped homogeneous electrical field) pulsed-field gel electrophoresis.

OFFGEL electrophoresis

- OFFGEL electrophoresis separates proteins or peptides according to their isoelectric points, whereby the separated components are recovered in liquid phase. Up- or downstream sample processing steps such as immune depletion, protein digestion and liquid chromatography can be easily interfaced with this technique for multi-dimensional separations of complex samples.

- With its new state-of-the-art web-based user interface OFFGEL electrophoresis has just become even more intuitive to use. Directly connect it to the supplied Netbook or connect to your local area network to operate from any PC* or iPhone

OFFGEL ELECTROPHORESIS – High resolution pI fractionation based on proven IPG technology

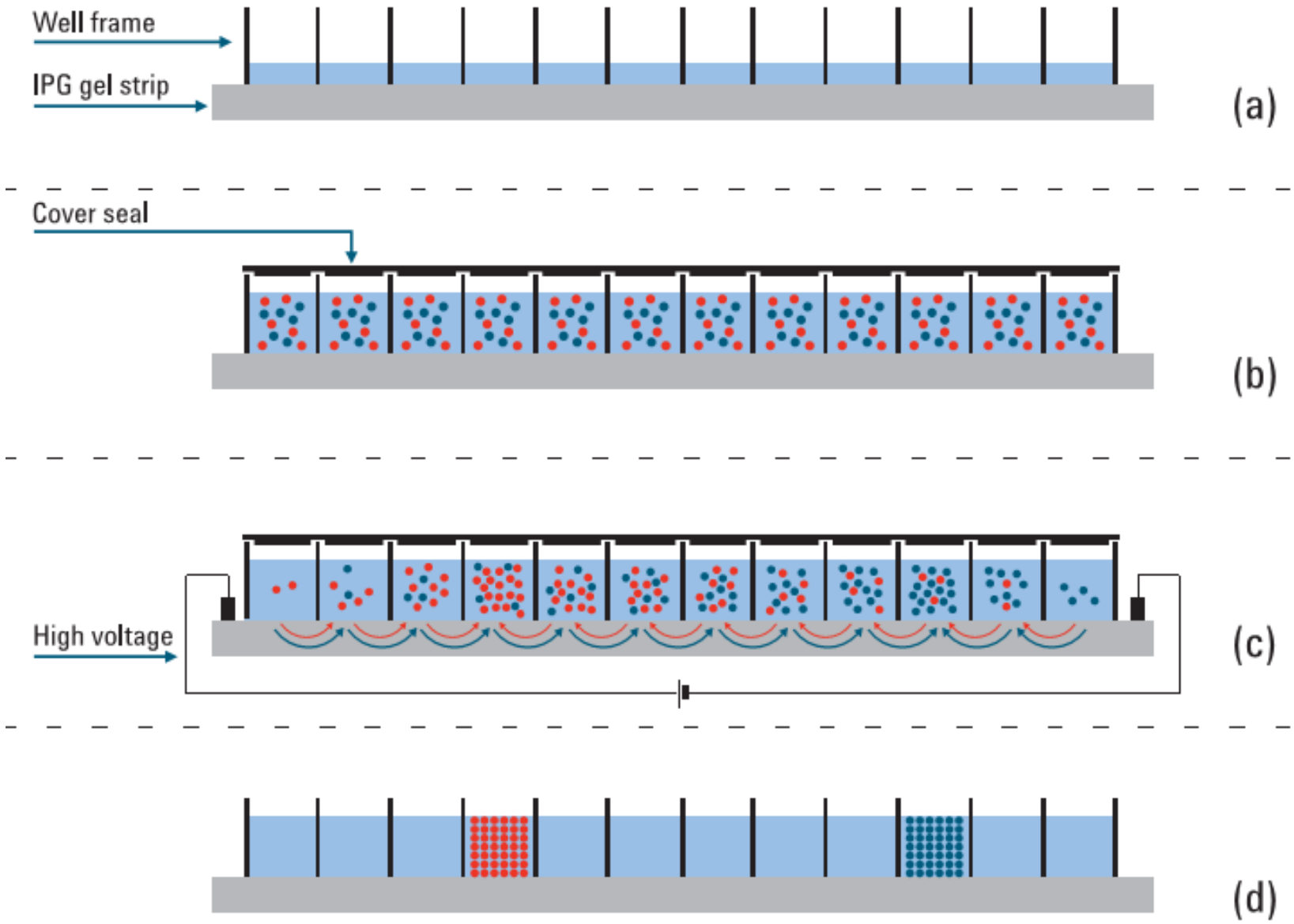
The Agilent 3100 OFFGEL Fractionator performs isoelectric focusing of proteins or peptides in immobilized pH gradient (IPG) gel strips. The technique applied by the 3100 OFFGEL Fractionator differs from conventional gel electrophoresis in that sample components are recovered from liquid phase. This makesthe technology directly compatible with liquid-phase workflows, for example LC-MS.

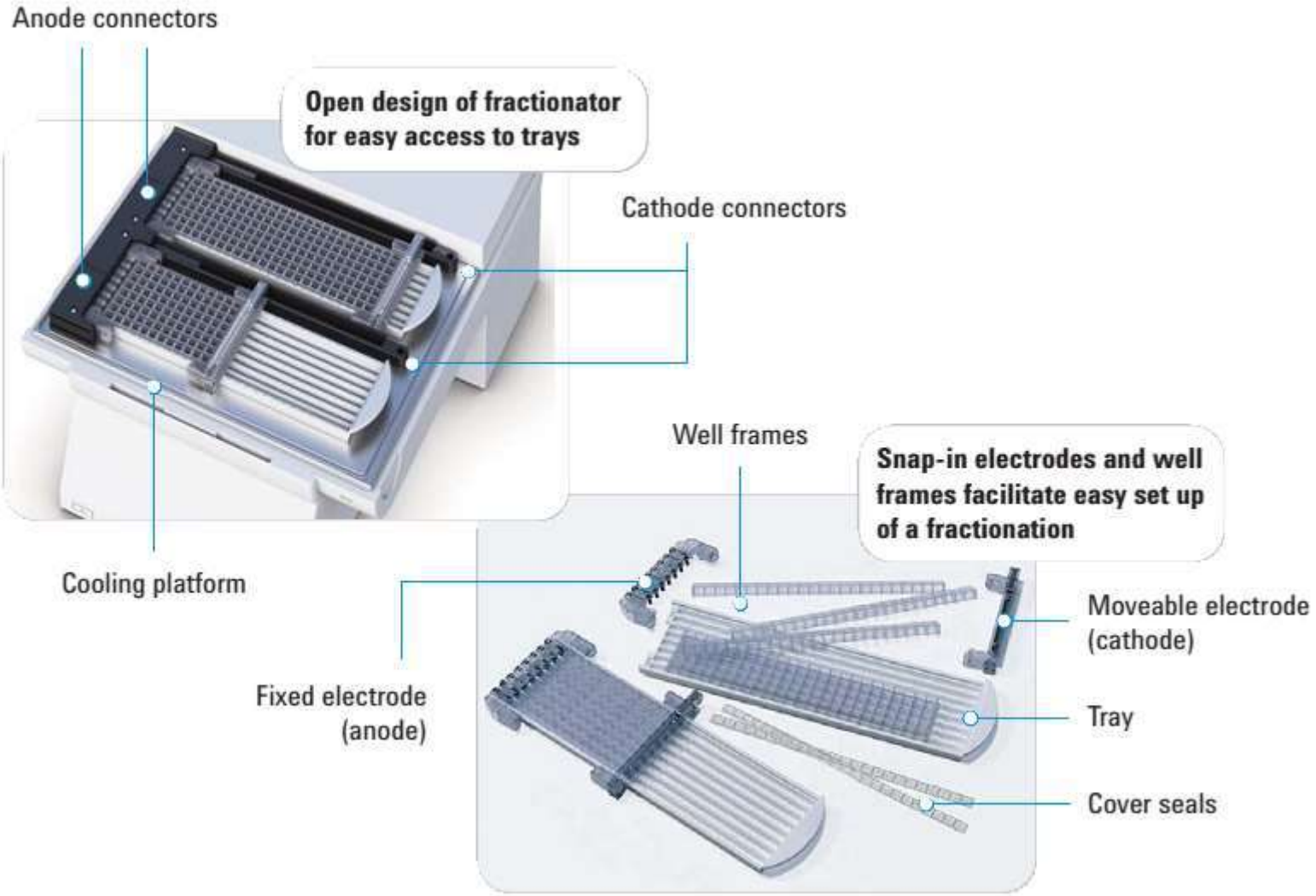
1. The IPG gel strip is rehydrated and seals tightly against the well frame.

2. The diluted sample is equally distributed into all wells in the frame, and a cover seal is placed on top of the frame to prevent sample evaporation.

3. A high voltage is applied to the ends of the gel strip and the protein or peptide molecules migrate through the gel until they reach a position where the pH equals the pI of the molecule.

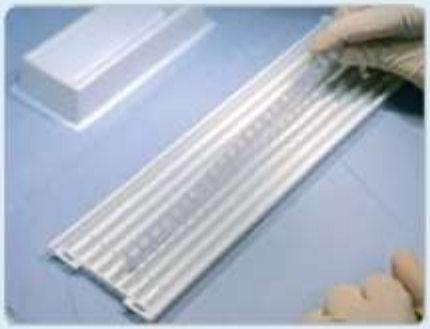
4. After fractionation, the separated proteins or peptides remain in the buffer solution and can be easily removed using a pipette for subsequent downstream processing.


Well frame
IPG gel strip
(a)
Cover seal
(b)
High voltage
(c)
(d)


Anode connectors
Open design of fractionator for easy access to trays
Cathode connectors
Cooling platform
Well frames
Snap-in electrodes and well frames facilitate easy set up of a fractionation
Fixed electrode (anode)
Moveable electrode (cathode)
Tray
Cover seals

Five easy steps to set up and run a fractionation

1. Place a dry IPG gel strip in the tray.



2. Place a well frame over the IPG gel strip, pipette 40 µl rehydration solution into each well and allow the IPG gel to swell.

3. Pipette 150 µl of the diluted sample into each well and close the frame with a cover seal.

4. Attach the electrodes to the tray.

5. Place the loaded tray into the fractionator and press “Start”.

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