BIOINSTRUMENTATION - (E CONTENT)

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INTRODUCTION TO BIO INSTRUMENTATION

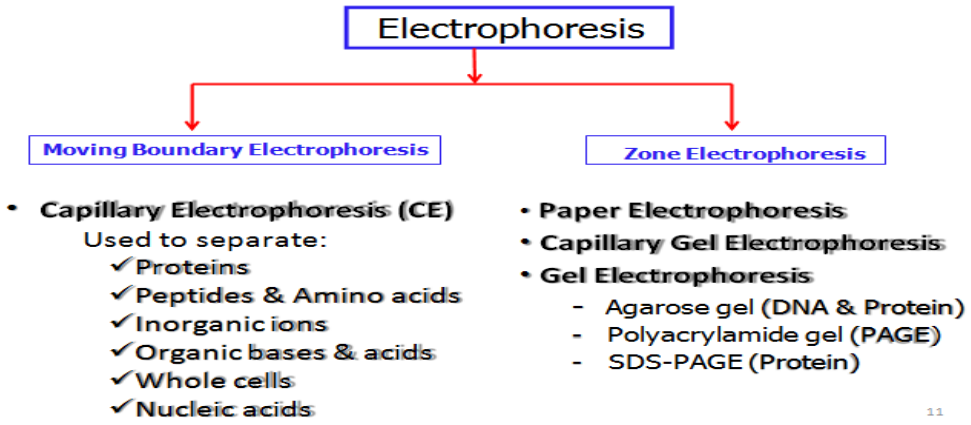
Bioinstrumentation or Biomedical instrumentation is an application of biomedical Engineering, which focuses on the devices and mechanics used to measure, evaluate, and treat biological systems. It focuses on the use of multiple sensors to monitor physiological characteristic of a human or animal.

ELECTROPHORESIS- PRINCIPLE AND INSTRUMENTATION OF ZONE ELECTROPHORESIS, AGAROSE GEL ELECTROPHORESIS

ELECTROPHORESIS

- Electrophoresis is a technique that allows us to separate DNA, RNA or Proteins according to their size.
- Differential movement or migration of charged molecules (ions) in solution, with response to an electrical current.
- Negatively charged molecules (anions) will be attracted towards anode.
- Positively charged molecules (cations) will move towards cathode.

TYPES OF ELECTROPHORESIS



GEL ELECTROPHORESIS

Gel Electrophoresis: Supporting medium is Gel. Gels are composed of polymers of sugars (Agarose or Polyacrylamide).

- **Agarose –** a complex sugar chain from red seaweed.
    - It has a large pore size good for separating large molecules.
- **Polyacrylamide –** chain of Acrylamide molecules.
    - It has a small pore size good for separating small molecules.

The kind of supporting **matrix used depends on the type of molecules** to be separated and on the desired basis for separation: charge, molecular weight, or both.

AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is a widely used procedure in various areas of Biotechnology. This simple, but precise, analytical procedure is used in research, biomedical and forensic laboratories.

It is a powerful separation method frequently used to analyze DNA fragments generated by Restriction enzymes, and it is a convenient analytical method for determining the size of DNA molecules in the range of 500 to 30,000 base pairs. It can also be used to separate other charged biomolecules such as dyes, RNA and proteins.

The separation medium is a gel made from Agarose, which is a polysaccharide derivative of agar. Originating from seaweed, Agarose is highly purified to remove impurities and charge. It is derived from the same seaweed as bacterial agar used in microbiology, as well as a food product called Agar Agar

The gel is made by dissolving Agarose powder in boiling buffer solution. The solution is then cooled to approximately 55°C and poured into a casting tray which serves as a mold. A well-former template (often called a comb) is placed across the end of the casting tray to form wells when the gel solution solidifies.

After the gel solidifies, the gel is submerged in a buffer-filled electrophoresis chamber which contains a positive electrode at one end, and a negative electrode at the other. Samples are prepared for electrophoresis by mixing them with components, such as glycerol or sucrose that will give the sample density. This makes the samples sink through the buffer and remain in the wells. These samples are delivered to the sample wells with a micropipette or transfer pipet.

A Direct Current (D.C.) power source is connected to the electrophoresis apparatus and electrical current is applied. Charged molecules in the sample enter the gel through the walls of the wells. Molecules having a net negative charge migrate towards the positive electrode (anode).while net positively charged molecules migrate towards the negative electrode (cathode).

Within a range, the higher the applied voltage, the faster the samples migrate. The buffer serves as a conductor of electricity and to control the pH, which is important to the charge and stability of biological molecules. Since DNA has a strong negative charge at neutral pH, it migrates through the gel towards the positive electrode during electrophoresis.

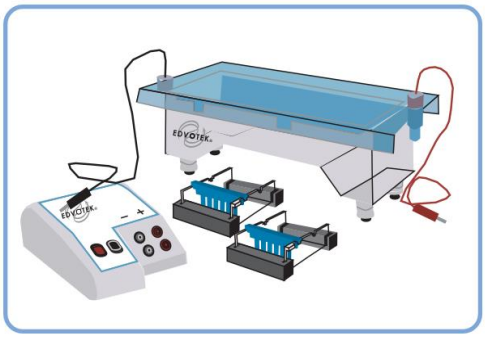
The bluish-purple dye allows for visual tracking of sample migration during the electrophoresis. In general, most DNA samples follow behind the tracking dye during electrophoresis. Thus, it is important that electrophoresis is terminated before the tracking dye runs off the end of the gel.

The most commonly used stains for visualizing DNA contain either Ethidium bromide or Methylene blue. Ethidium bromide is a mutagen and must be handled and disposed according to strict local and/or state guidelines.

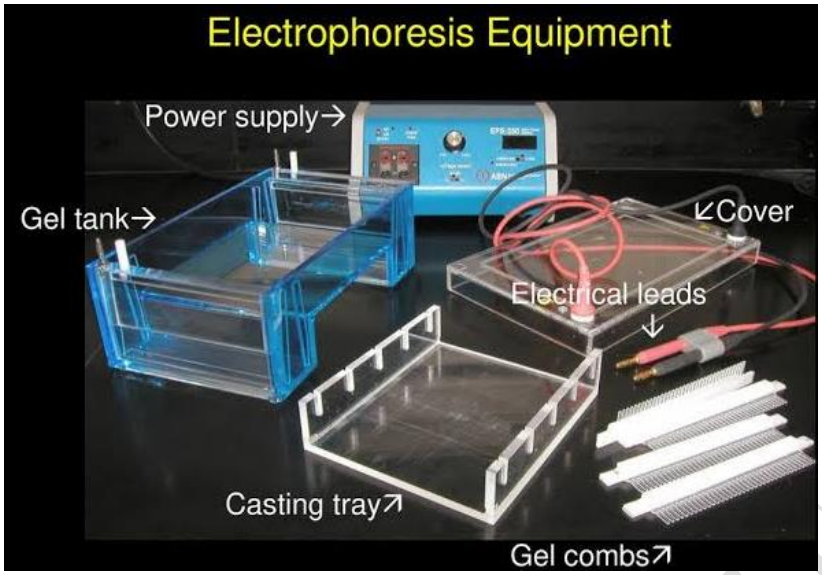
Visualization also requires a short wave ultraviolet light source (transilluminator).

MATERIALS AND REAGENT

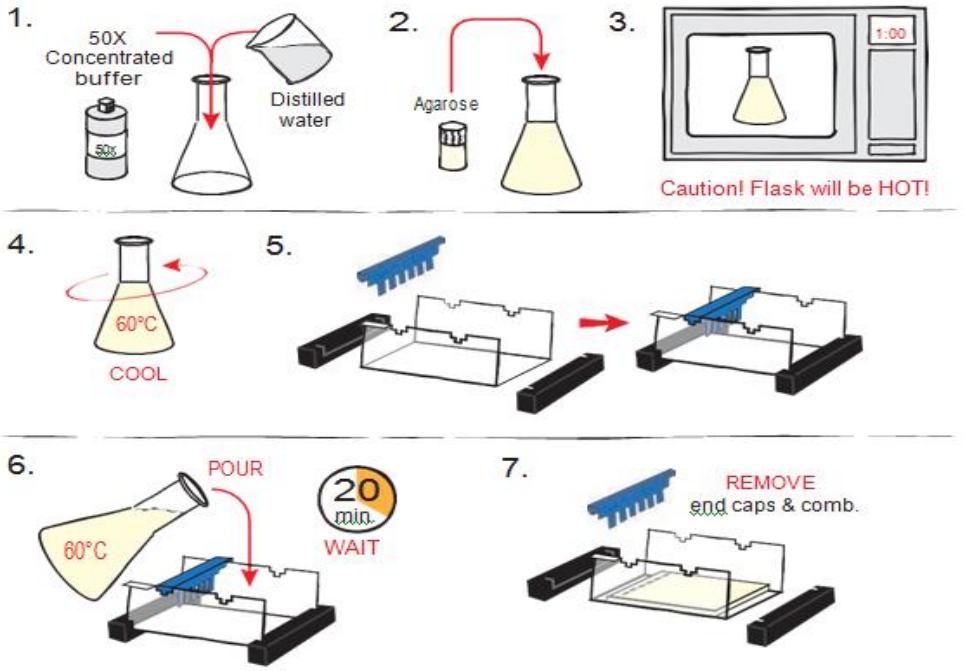
- Horizontal Gel Electrophoresis Apparatus
- Gel Casting Trays
- Well-Former Templates (Combs)
- Micropipette
- Buffer(TAE or TBE)
- Ethidium bromide
- Agarose
- Gel loading Dye



- Sample



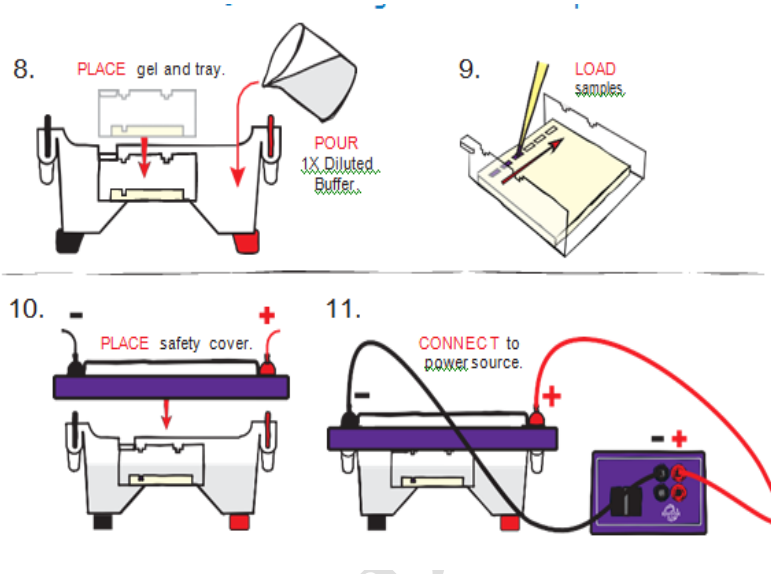
PROCEDURE

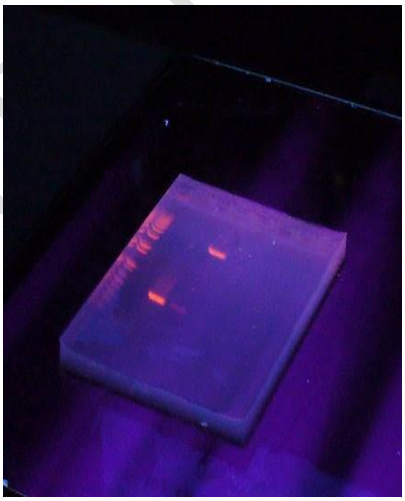


1. **DILUTE** concentrated (50X) buffer with distilled water to create 1X buffer
2. **MIX** Agarose powder with 1X buffer in a 250 ml flask
3. **DISSOLVE** Agarose powder by boiling the solution. **MICROWAVE** the solution on high for 1 minute. Carefully **REMOVE** the flask from the microwave and **MIX** by swirling the flask. Continue to **HEAT** the

solution in 15-second bursts until the Agarose is completely dissolved (the solution should be clear like water).

4. **COOL** Agarose to 60° C with careful swirling to promote even dissipation of heat.
5. While Agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the well template (comb) in the appropriate notch.
6. **POUR** the cooled Agarose solution into the prepared gel-casting tray. The gel should thoroughly solidify within 20 minutes. The gel will stiffen and become less transparent as it solidifies.
7. **REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.





8. **PLACE** gel (on the tray) into electrophoresis chamber. Completely **COVER** the gel with 1X electrophoresis buffer.
9. **LOAD** entire sample volumes into wells in consecutive order.
10. **PLACE** safety cover. **CHECK** that the gel is properly oriented. Remember, the samples will migrate toward the positive (red) electrode.
11. **CONNECT** leads to the power source and **PERFORM** electrophoresis
12. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber and proceedto Staining & Visualization.

SDS PAGE

INTRODUCTION

- SDS-PAGE, a simple and inexpensive method for resolving proteins in complex mixtures. SDS-PAGE gels provide the starting materials for western blots and for some proteomic techniques.
- Used to separate proteins based up on the molecular weight.
- SDS- Sodium Dodecyl Sulphate
- PAGE- Polyacrylamide Gel Electrophoresis

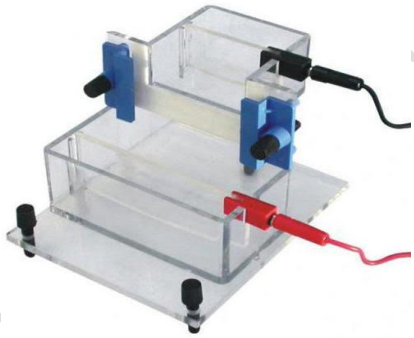
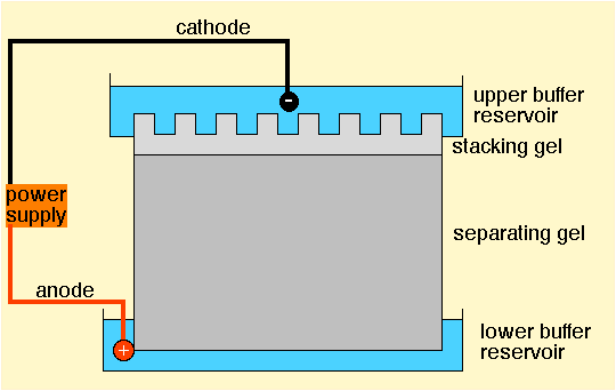


Figure 1: SDS PHAGE APPARATUS

PRINCIPLE

- SDS-PAGE is widely used to analyze the proteins in complex extracts. The most commonly used methods are derived from the discontinuous SDS-PAGE system first described by Laemmli (1970). The system actually consists of two gels - a resolving (aka running) gel in which proteins are resolved on the basis of their molecular weights (MWs) and a stacking gel in which proteins are concentrated prior to entering the resolving gel. Differences in the compositions of the stacking gel, resolving gel and electrophoresis buffer produce a system that is capable of finely resolving proteins according to their MWs.



- Protein electrophoresis is somewhat more complicated than DNA electrophoresis. Proteins are much smaller than DNA molecules, so Polyacrylamide gels are used for their separation. In addition, proteins are much more structurally diverse than DNA, so chemical treatments are used to impart a uniform geometry and charge/mass ratio to the proteins
- The Polyacrylamide gels used to separate proteins are formed by the chemical polymerization of Acrylamide and a cross-linking reagent, N, N'methylenebisacrylamide. Investigators are able to control the size of the pores in the gel by adjusting the concentration of Acrylamide, as well as the ratio of Acrylamide to bisacrylamide. Raising either the concentration of Acrylamide or bisacrylamide, while holding the other concentration constant, will decrease the pore size of the gel. Polymerization occurs because of free oxygen radicals that react with the vinyl groups in Acrylamide and Bisacrylamide, The oxygen radicals are generated from the catalyst, ammonium per sulfate (APS), when it reacts with a second catalyst, N,N,N',N'-tetramethylethylenediamine (TEMED).
- **Proteins Are Denatured Prior To Electrophoresis**

Compared to DNA molecules, proteins are structurally very diverse. Proteins show tremendous variation in their amino acid compositions and in the distribution of amino acids in their folded structures, features with important implications for electrophoresis. Recall that Proteins are mixtures of hydrophobic and hydrophilic amino acids and that the primary sequence of the protein determines its final folded form. Because of the hydrophobic effect, the surfaces Of proteins have a higher frequency of polar and charged amino acids than the interiors, where hydrophobic residues predominate Folded proteins assume many different geometries and their surfaces are mosaics with respect to the distribution of R groups with different chemistries. Because proteins are so diverse with respect to their surface charges and geometries, the molecular weights of ***folded*** proteins cannot be simply determined by their migration rate in an electric field. Positively and negatively charged proteins would migrate in different directions.

- To resolve the proteins in a sample according to their size, we must convert the proteins to a uniform geometry and impart a uniform charge/mass ratio to the proteins. In SDS- PAGE, the solution is to denature the proteins by boiling them with the anionic detergent, Sodium Dodecyl Sulfate (SDS) and 2-mercaptoethanol. The combination of heat and detergent is sufficient to break the many non-covalent bonds that stabilize protein folds, and 2-mercaptoethanol breaks any covalent bonds between cysteine residues. Like other detergents, SDS is an amphipathic molecule, consisting of a hydrophobic 12-carbon chain and a hydrophilic sulfate group. The SDS Hydrocarbon chain permeates the protein interior and binds to hydrophobic groups, reducing the protein to a random coil, coated with negatively charged detergent molecules all along its length. Denatured proteins bind quite a lot of SDS, amounting to ~1.4 g SDS/g protein, or ~one SDS molecule for every two amino acids.
- SDS-PAGE system can be considered a 3-component system. The stacking and running (resolving) gels have different pore sizes, ionic strengths and pHs. The third component is the electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH ~8.3), which contains large amounts of glycine. The ionization state of the glycine is critical to the separation. At neutral pH, glycine is a zwitterions, with a negatively charged carboxyl group and a positively charged amino group
- The sample buffer used for SDS-PAGE contains a tracking dye, Bromophenol blue (BPB), which will migrate with the leading edge of the proteins being separated on the gel. The sample buffer also contains glycerol, which allows the protein samples to settle into the bottom of the gel wells. The gel is vertically positioned in the electrophoresis apparatus and covered with chamber buffer containing glycine
- The pores in the running gel are much smaller than those of the stacking gel, so the pores present frictional resistance to the migration of proteins. Proteins begin to migrate at different rates, because of the sieving properties of the gel. Smaller protein-SDS complexes migrate more quickly than larger protein- SDS complexes.
- To visualize the positions of proteins after electrophoresis is complete, stain the gels with various dyes that bind non covalently and with very little specificity to proteins.
- Brilliant Blue G-250 binds proteins nonspecifically through a large number of ionic and Van der Waals interactions. gels are rinsed with water to remove the buffer salts used for electrophoresis and then treated with the colloidal G-250 suspension. Protein bands appear rapidly, and when necessary, the gels can be Distained with deionizer water to lower the gel background.

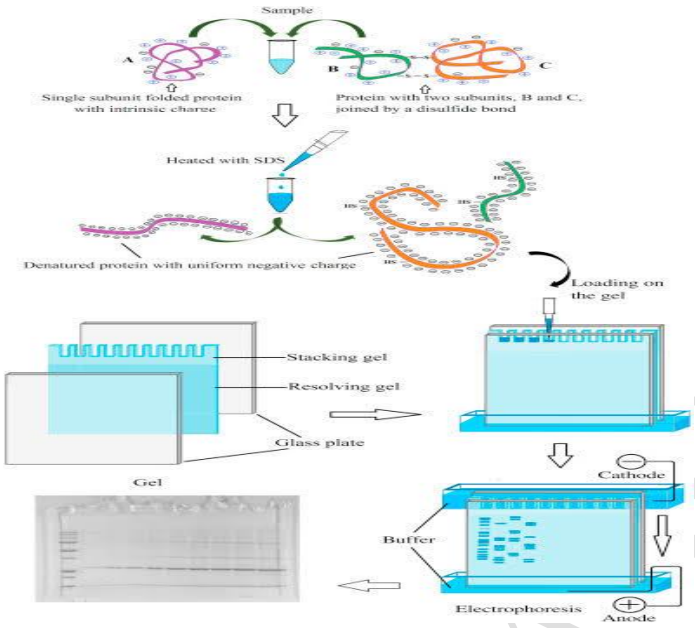


Figure 2 PROCERURE OF SDS PHAGE

ASSEMBLE THE GEL CASTING APPARATUS

- Assemble the components that you will need for casting the gel: a tall glass plate with attached 1 mm spacers, a small glass plate, casting frame and a casting stand.
- Insert the short glass plate in the front of the casting frame. ***There should be a space between the plates.***
- Check to see if the assembled plates in the casting stand are sealed properly by pipetting a small amount of Deionized water into the gap between the plates. If the glass plates hold water and don't leak, you are ready to make the gels. Pour the water out by holding the entire casting platform over a liquid waste container or sink. Use paper towels or tissues to absorb any residual water. If the gel leaks, disassemble the frame, dry the plates and go back

Preparation of two resolving gels:

Safety note: *Acrylamide and bisacrylamide monomers are weak neurotoxins.* Gloves and goggles should be used when working with Acrylamide.

- Assemble the chemicals that you will need to pour the gels. Polymerization occurs rapidly, so be sure to follow the step-by-step instructions below.

Reagent	Resolving gel	Stacking gel
Deionized water	3.5 mL	2.1 mL

30% acrylamide:bis-acrylamide (29:1)	4.0 mL	0.63 mL
1.5 M Tris-HCl, 0.4% SDS, pH 8.8	2.5 mL	------
0.5 M Tris-HCl, 0.4% SDS, pH 6.8	------	1.0 mL
10% ammonium persulfate (catalyst)	100 µL	30 µL
TEMED (catalyst)	10 µL	7.5 µL

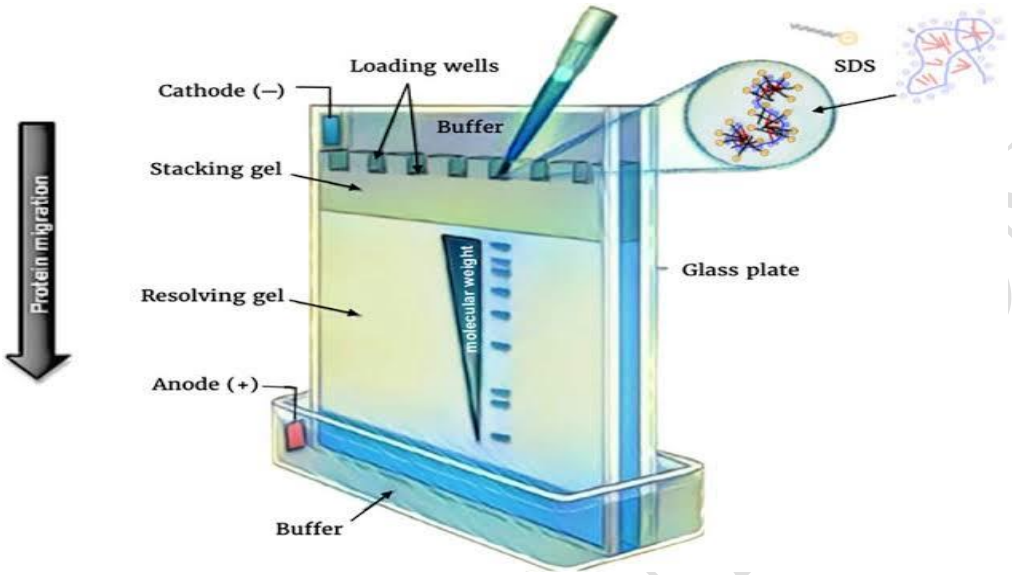
- Label two 15 mL conical tubes "Resolving gel" and "Stacking gel".
  - **Resolving gels**: Mix the Acrylamide solution, **pH 8.8** Tris buffer and water, as shown in the chart above. Add 100 µL 10% APS and 10 µL TEMED to the resolving gel Acrylamide mixture. Mix the contents by gently inverting the tube twice. Use a plastic transfer pipette to fill the space between the two plates until the resolving gel solution reaches a height (10 cm) just above the clamps on the gel casting frame. Draw up any remaining Acrylamide into the transfer pipet. Allow the gel to polymerize, which takes ~15-20 minutes. When polymerization is complete, remove the water from the top of the resolving gel by tilting the gel to the side and using a paper towel.
  - **Prepare the stacking gels**: Mix the Acrylamide solution, **pH 6.8** Tris buffer and water, as shown in the chart above. Add 30 µL 10% APS and 7.5 µL TEMED to the stacking gel Acrylamide mixture. Mix the contents by gently inverting the tube twice. Use a transfer pipette to pipette the stacking gel on top of the resolving gel (1 cm) between the two glass plates. Add enough stacking solution until it just reaches the top of the small plate.
  - Adding the comb will force some solution out of the gel, but this is fine. If air bubbles become trapped below the comb, remove the comb and reposition it.

RUNNING SDS PAGE GELS

- Set up the electrophoresis apparatus
- Carefully remove the comb from the spacer gel.
- Remove the casting frame from the gel cassette sandwich and place the sandwich against the gasket on one side of the electrode assembly, with the short plate facing inward. Place a second gel cassette or a buffer dam against the gasket in the other side of the electrode assembly.
- Fill the space between the two gels with Tris-glycine running buffer. This forms the upper chamber for electrophoresis.
- Add Tris-glycine running buffer to the outer (lower) chamber until the level is high enough

to cover the platinum wire in the electrode assembly.

- Electrode assembly: Electrode assembly with two gels is lowered into the clamping frame



- Load and run samples on the SDS-PAGE gel
- Samples have already been mixed with a tracking dye and glycerol.
- Using gel loading micropipette tips (tips have very long, thin points and fit P20s or P200s), load up to 15 µL of sample into each well. Load 5 µL of a molecular weight standard into one lane of the gel. Load samples slowly and allow the samples to settle evenly on the bottom of the well.
- Connect the tank to the power supply. Fit the tank cover onto the electrodes protruding up from the electrode assembly. Insert the electrical leads into the power supply outlets (connect black to black and red to red).
- Turn on the power supply. Run the gel at a constant voltage of 120-150 V. Run the gel until the blue dye front nearly reaches the bottom of the gel. This may take between 45-60 min.

- Turn off the power supply.
- Remove the gel apparatus from the tank. Open the clamping frame and remove the gel cassette sandwich. Carefully, remove the two plates apart with a spatula. With the spatula, remove the lower right or left corner of the gel to serve as an orientation marker.
- Place the gel in a small plastic tray and label the tray with your initials on a piece of tape. To do this, fill the tray about halfway with deionized water. Gently free the gel from the glass plate, allowing it to slide into the water. The gel should move freely in the water. Place the gel and tray on a rocking platform. Rock the gel for ~2 minutes.
- Drain the water from the gel and add enough Simply Blue to cover the gel, while allowing the gel to move freely when the tray is rocked. Cover the gel container. Make sure that the gel does not stick to the bottom of the tray.
- In the morning, drain the Simply Blue stain into an appropriately labeled waste container in the hood of the lab room.
- Destain the gel by filling the container about half full with deionized water. Shake the gel in the water for ~2 minutes. Pour off the water and add new deionized water. Repeat, if necessary, until protein bands become visible.
- When individual bands are detectable, record your data. After recording the data, dispose of the gel in the Biohazard waste container.

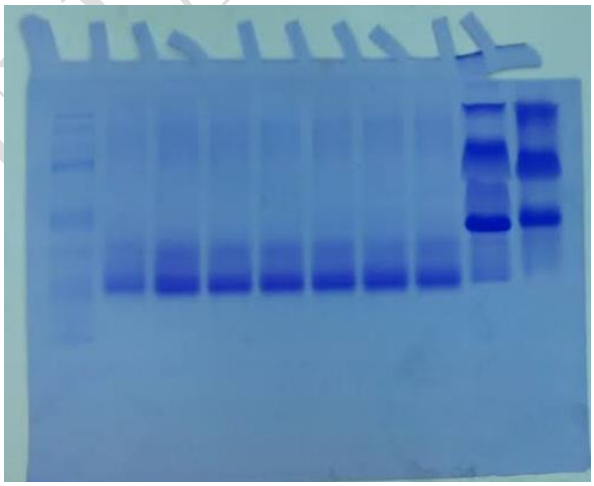


Figure 3: SDS RESULT

IMMUNO ELECTROPHORESIS

IMMUNO ELECTROPHESIS

- Technique based on the principles of electrophoresis of antigens and Immunodiffusion of the electrophoresed antigens with a specific antiserum to form precipitin bands.
- It is used to detect the presence of antibodies.
- Used mainly to determine the blood levels of three major Immunoglobulins: immunoglobulin M (IgM), immunoglobulin G (IgG), and immunoglobulin A (IgA).
- The use of Immunoelectrophoresis to separate and characterize a mixture of proteins and examine the specificity of the antigen-antibody interaction.
- **It** is used in both clinical and research laboratories for separating and identifying proteins on the basis of their electrophoretic behavior and their immunological properties. Proteins such as rabbit serum proteins, which are antigens, when injected into another animal such as a goat (the host); elicit the production of antibodies in the host. The interaction between the antigen and its antibody, which is also a protein, is both strong and highly specific. If solutions of antigen and antibody are mixed in different ratios, it is found that at a specific ratio, known as the **equivalence point**, the binding is maximized and a precipitate of antigen-antibody complex is formed.

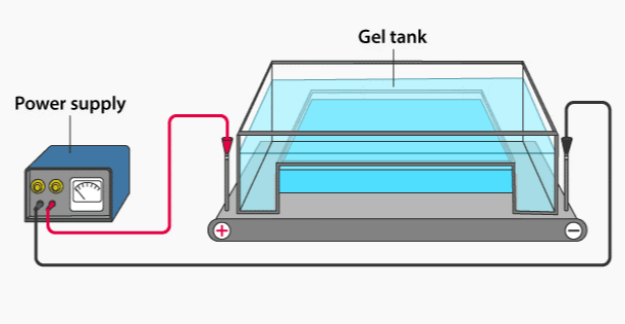


Figure 4 Immunoelectrophoresis Tank

- In the clinical laboratory, immunoelectrophoresis is used diagnostically. It is utilized in examining certain serum abnormalities, especially those involving Immunoglobulins, urine protein, cerebrospinal fluid, pleural fluids and other body fluids. In research, this procedure may be used to monitor antigen and/or antibody purifications, to detect impurities, analyze soluble antigens from plant and animal tissues, and microbial extracts.

- **Gel electrophoresis** is a widely used analytical method that separates molecules based upon charge, size and shape. It is particularly useful for determining the size of biomolecules. Samples of proteins are loaded into wells made in the gel during casting. The gel, which consists of microscopic pores that act as a molecular sieve, is placed in a chamber containing a buffer solution and electrodes. Current is applied from a Direct Current (D.C.) power source. Since biomolecules are charged, they will migrate through the gel.
- In immunoelectrophoresis, the proteins are first separated by horizontal Agarose gel electrophoresis on the basis of their different charge-to-mass ratios. The gel with the separated proteins (antigens) is then removed from the electric field and antibodies to the proteins are introduced into narrow troughs parallel to the separated antigens. Diffusion of both antigen antibodies takes place and, at a particular locus, the equivalence point is reached resulting in precipitation.

PROCEDURE

1. **Preparations Of Electrophoresis Buffer**

- Prepare electrophoresis buffer by diluting the entire contents (40 ml) of the bottle containing concentrated buffer (component G) in 960 ml distilled water for a total of 1000 ml.
- This buffer will be used to prepare Agarose as well as for electrophoresis.

2. **Preparing The Agarose Gel**

- Add 100 ml diluted electrophoresis buffer in 400 to 600 ml beaker. Heat the mixture to dissolve the Agarose powder. The final solution should be clear (like water) without any undissolved particles. Cool the Agarose to 60°C in a water bath.
- pouring the gel solution into the casting tray

3. Cut wells using well cutter. The distance between the troughs and the edge of each well should not be more than 0.5 cm.
4. Remove Agarose gel plugs with a toothpick or spatula.
5. Transfer the tray containing the gel to the electrophoresis apparatus.
6. Gently lay the filter paper wicks over the ends of the gel, (They should overlap about 3 to 4 mm) and allow them to become saturated with electrophoresis buffer.

7. The wicks should be submerged in the buffer. Press lightly on the wicks to ensure good contact between the gel and the electrophoresis buffer.
8. If necessary, add more buffers, but do not cover the gel with buffer.
9. Load 20 µL of each antigen (A, B, and C) in the wells. Change pipet tips between samples.
10. Carefully, snap on the lid of the electrophoresis apparatus, insert the leads into the power supply with the black lead in the black (negative) input and the red lead into the red (positive) input.
11. Turn on and set the power supply for the required voltage (50V to 125V). When current is flowing properly, bubbles should form on the electrodes.
12. Run the electrophoresis until the blue dye has migrated to the ends of the troughs. The exact time required is dependent upon the voltage
13. After electrophoresis is completed, turn off the power, unplug the power source, disconnect the leads, and remove the cover.
14. Discard the filter paper wicks and remove the gel tray from the apparatus. Set the tray on a level surface and proceed with the diffusion steps.

15. Diffusion Of Antibodies And Antigens

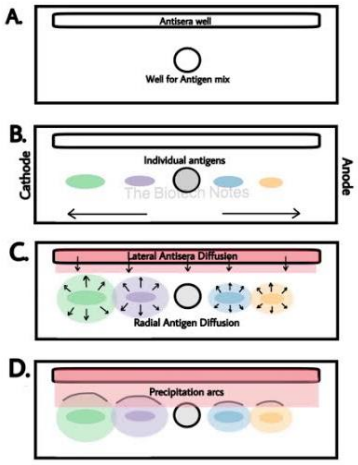


Figure 5 Antigen - Antibody Reaction

- Add 50 µL of each antibody to the appropriate trough
- Use the pipette tip to carefully spread the antibody solution along the entire length of the trough.
- Place the tray in a closed humidifying chamber containing moistened paper towels.

- Allow diffusion to take place over a 24 to 48 hour period, or until visible precipitates form in the gel. The chamber can be placed in a 37°C incubation oven or remain at room temperature.
- Finally, formation of arcs of white precipitate in the gel.

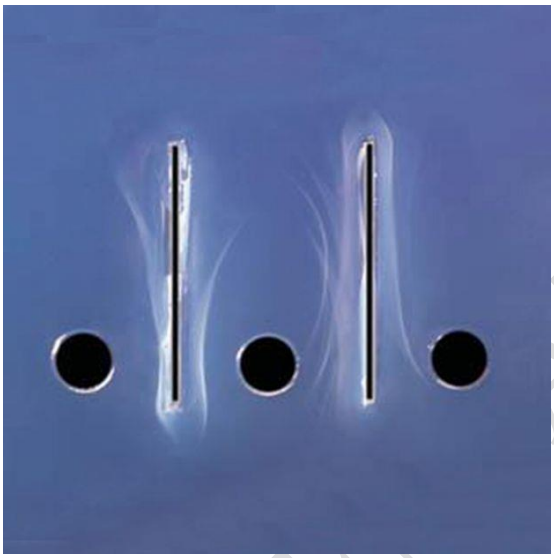


Figure 6 Immunoelectrophoresis Result

APPLICATIONS

- Immunoelectrophoresis is used in clinical laboratories to detect the presence or absence of proteins in the serum.
- This technique is useful in determining whether a patient produces abnormally low amounts of one or more isotypes of Ig, characteristic of certain immunodeficiency diseases.
- It can also show whether a patient overproduces some serum protein, such as albumin, immunoglobulin, or transferring.

ISO ELECTRIC FOCUSING

INTRODUCTION

Isoelectric focusing (IEF), also known as Electro focusing, is a technique for separating different molecules by differences in their isoelectric point (pI).It is a type of zone electrophoresis usually performed on proteins in a gel that takes advantage of the fact that overall charge on the molecule of interest is a function of the pH of its surroundings.

The separation of biomolecules, particularly proteins, in the presence of an electric field (e.g., electrophoresis) has given rise to an array of methodologies to reduce the complexity of samples to probe the physiochemical properties of such biomolecules. Proteins and peptides represent possibly the most highly studied class of molecules that are interrogated by electrophoretic methods. These methods include: Agarose and Polyacrylamide gel electrophoresis, two-dimensional gel electrophoresis (2DE), capillary electrophoresis, isotachophoresis and others.

One such electrophoretic technique is isoelectric focusing (IEF) which provides separation of ampholytic components, molecules that act as weak acids and bases, according to their isoelectric points. In IEF, ampholytes travel according to their charge under the influence of an electric field, in the presence of a pH gradient, until the net charge of the molecule is zero (e.g., isoelectric point, pI). When considering peptides and proteins, the separation is deemed according to the composition of amino acids and exposed charged residues, which behave as weak acids and bases. The migration of the ampholytic species will follow basic principles of electrophoresis; however, the mobility will change in the presence of the pH gradient by slowing down migration at values close to the pI value. Even the simplest ampholytes (e.g., amino acids) can create a pH gradient and act as an isoelectric buffer.

The history of IEF begins with early work carried out by A.J.P. Martin who made several contributions in the field of electrophoresis. Martin also contributed significantly to the field of chromatography and was awarded a Novel Prize for his efforts. The work of P.G. Righetti has been paramount in the ability to separate biomolecules electrophoretically, particularly according to isoelectric point. To fully understand these contributions, one must review the details of the experiment, particularly establishing the pH gradient. Furthermore, classical work regarding ampholytes was carried out by Svensson in the early 1960s.

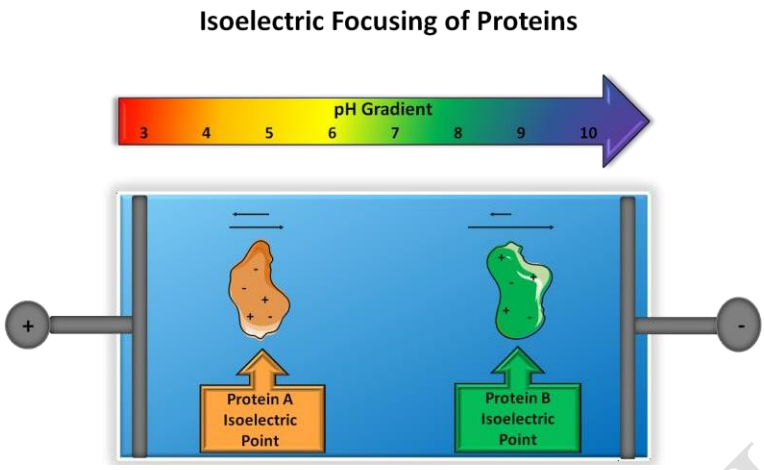


Figure1. Principle of isoelectric focusing. Two proteins with varying isoelectric points will migrate in the presence of a pH gradient and electric field until the net charge of a protein is zero, in which migration will cease.

Carrier ampholytes are the most commonly used chemical components used to generate pH gradients. The chemistry of carrier ampholytes was originally generated via pentaethylenehexamine and addition of acrylic acid. A second generation approach in carrier ampholyte synthesis was performed by Vesterberg, in which a heterogeneous mixture of amines was reacted with acrylic acid and a complex product resulted in the generation of thousands of molecules with varying pI values, yet very small changes in pI values across a pH range. Therefore, an ideal carrier ampholyte mixture is generated—a large number of components with close pI values resulting in a linear pH gradient. With regard to gels, carrier ampholytes can also be embedded into Acrylamide gels and separation carried out in slab/flatbed format.

A major achievement, which was an extension of the synthesis of carrier ampholytes, was the generation of immobilized pH gradients in 1982.utilized Acrylamide as a backbone incorporating amino and carboxyl groups via radical mediated reactions allowing for branching and cross linking with carrier ampholytes of different p*K*a values. The resulting product is a pH gradient that is immobile in an electric field and acts as a buffer. The values of pH range from 1 to 13 and can be synthesized in linear and nonlinear forms. The length of the IEF setup that is used plays a role in the desired resolution needed. This major advancement opened doors for various applications of isoelectric focusing for the separation of biological molecules, especially peptides and proteins. The resolving power of IEF (ΔpI) is determined by a series of factors in the experiment including

the diffusion coefficient, conductivity and the electric current density. Properties of the gradient include the slope and the charge curve near the focusing point.

IEF can be performed in a variety of formats, including preparative, analytical and micro scale. On the larger end, IEF has proven to be beneficial as a preparative method due to its ability to separate large amounts of samples providing high resolution with large recovery yields. Notably, this separation method is advantageous in its ability to concentrate large quantities of samples while simultaneously removing common interfering agents or unwanted analytes. Additionally, IEF can be carried out in capillaries, micro fluidic channels and multi-compartment electrolyzers (MCE) as described below. In general, IEF is an extremely powerful technique that, when used in any format, allows for the fractionation of samples resulting in reduced sample complexity and more in-depth analysis.

ESTABLISHING PH GRADIENTS

Stable, linear pH gradients are the keys to successful IEF. Establishment of such gradients is accomplished in two ways with two different types of molecules, carrier ampholytes and Acrylamide buffers.

Carrier ampholytes (*ampho*teric electro*lytes*) are mixtures of molecules containing multiple aliphatic amino and carboxylate groups. They are small (about 300-1000 Da in size) multi-charged organic buffer molecules with closely spaced p*I* values and high conductivity. Ampholytes are included directly in IEF gels. In electric fields, carrier ampholytes partition into smooth pH gradients that increase linearly from the anode to the cathode. The slope of a pH gradient is determined by the pH interval covered by the carrier ampholyte mixture and the distance between the electrodes. The use of carrier ampholytes is the most common and simplest means for forming pH gradients.

Acrylamide buffers are derivatives of Acrylamide containing both reactive double bonds and buffering groups. Their general structure is CH_2 = CH-CO-NH-R, where R contains either a carboxyl [-COOH] or a tertiary amino group [*e.g.*, -N $(CH_3)_2$]. They are covalently incorporated into Polyacrylamide gels at the time of casting. The key Acrylamide buffers have pK values at pH 1, 3.6, 4.6, 6.2, 7.0, 8.5, 9.3, 10.3, and >12.

They can be used to cast just about any conceivable pH gradients. In any given gradient, some of the Acrylamide compounds act as buffers while others serve as titrants. Published formulations and methods are available for casting the most common gradients (48, 50). Because the buffering compounds are fixed in place in the separation medium, the gels are called "immobilized pH gradients", or IPGs. IPGs offer the advantage of gradient stability over extended runs. They are, however, more cumbersome and expensive to cast than carrier ampholyte gels. IPGs are commercially available in sheet form in a few pH ranges. A greater variety of pH ranges are available in IPGs that have been cut into strips for the IEF first dimension of 2-D PAGE.

IEF is a high-resolution technique that can routinely resolve proteins differing in p*I* by less than 0.05 pH units. Antibodies, antigens, and enzymes usually retain their activities during IEF. The proper choice of ampholyte or IPG range is very important to the success of a fractionation. Ideally, the pH range covered by an IEF gel should be centered on the pI of the proteins of interest. This ensures that the proteins of interest focus in the linear part of the gradient with many extraneous proteins excluded from the separation zone.

With carrier ampholytes, concentrations of about 2% (w/v) are best. Ampholyte concentrations below 1% (w/v) often result in unstable pH gradients. At concentrations above 3% (w/v) ampholytes are difficult to remove from gels and can interfere with protein staining. When casting IPGs, follow published recipes and use buffering powers of about 3 meq throughout the gradient (method not presented).

GELS FOR ISOELECTRIC FOCUSING

As an analytical tool, IEF is carried out in large-pore Polyacrylamide gels (5%T, 3%C) which serve mainly as anticonvective matrices. Polyacrylamide IEF gels are polymerized with an initiator system including riboflavin for photo polymerization.

Photochemical initiation of polymerization with a combination of the three compounds riboflavin, ammonium persulfate, and TEMED, results in more complete polymerization of IEF gels than does chemical polymerization in gels containing low-pH ampholytes (15). Suitable initiator concentrations are 0.015% ammonium persulfate, 0.05% TEMED, and 5 µg/ml riboflavin-5'-phosphate. Photochemical polymerization is allowed to continue for 2 hr, with the second hour under direct lighting from a nearby fluorescent lamp.

The most common configuration for analytical IEF is the horizontal Polyacrylamide slab gel. Gels are cast with one exposed face on glass plates or specially treated plastic sheets. They are placed on cooling platforms and run with the exposed face upward. Electrolyte strips, saturated with 0.1-1 *M* phosphoric acid at the anode and 0.1-1 *M* sodium hydroxide at the cathode, are placed directly on the exposed surface of the IEF gel. Electrodes of platinum wire maintain contact between the electrical power supply and the electrolyte strips. In another possible configuration, the gel and its backing plate are inverted and suspended between two carbon rod electrodes without the use of electrolyte strips. IPG strips for 2-D PAGE are often run with the gel facing down in dedicated IEF cells.

PROCEDURE

Protocol 1 Casting gels for isoelectric focusing

Equipment and reagents

- Gel casting apparatus for a horizontal electrophoresis cell; *e.g.*, the GE MultiPhor
- Fluorescent lamp
- 30%T, 3%C Acrylamide stock solution
- 50% glycerol
- 0.1% riboflavin-5'-phosphate (FMN)
- Carrier ampholytes with a pH range spanning the p*I*s of the proteins of interest
- 10% APS and TEMED

Method

The formulation given here is for 12 ml of gel solution containing 5% T (3%C) Acrylamide, 2% carrier ampholytes, 5% glycerol. The volume needed depends on the casting apparatus that is used; adjust volumes accordingly. This recipe is sufficient for casting one gel of 100 x 125 x 0.8 mm (10 ml) or four 100 x 125 x 0.2 mm (10 ml total). 8 M urea can be substituted for the glycerol if desired.

1. Assemble the casting apparatus according to the manufacturer's instructions. The use of gel support film for Polyacrylamide is highly recommended.

2. Combine

30%T, 3%C Acrylamide stock	2.0 ml
Carrier ampholytes (40%)	0.6 ml
50% glycerol	1.2 ml
Water	8.2 ml
0.1% FMN	60 □l
10% APS	18□ l
TEMED	4 □l

Swirl the solution gently to mix the components.

3. Transfer the gel solution to the casting apparatus with a pipette and bulb.
4. Position a fluorescent lamp about 3-4 cm from the gel solution and illuminate the solution for about one hour.
5. Open the gel cassette or lift the gel from the casting tray to expose the face of the gel. Place the gel with the open face upward and illuminate it with the fluorescent lamp for an additional 30 min.
6. The gel may be used immediately or it can be covered with plastic wrap and stored at 4°C for several days. Best results are sometimes obtained when IEF gels are left overnight at 4°C before use.

Ultrathin gels (<0.5 mm) allow the highest field strengths and, therefore, the highest resolution of the analytical methods. Electro focusing can also be done in tubes, and this configuration once constituted the first dimension of 2-D PAGE (17). Because of difficulties in handling and reproducibility with tube gels, IPG strips have largely replaced them.

Good visualization of individual bands generally requires a minimum of 0.5 µg each with dye staining or 50 ng each with silver staining (see the Application Focus about the Detection of Proteins in Gels on this website). One of the simplest methods for applying samples to thin Polyacrylamide gels is to place filter paper strips impregnated with sample directly on the gel surface. Up to 25 µl of sample solution can be conveniently applied after absorption into 1-cm

squares of filter paper. A convenient size for applicator papers is 0.2 x 1 cm, holding 5 µl of sample solution. Alternatively, 1- to 2- µl samples can be placed directly on the surface of the gel. In most cases, IPG strips (which are provided in dehydrated form) are rehydrated in sample-containing solution prior to electrophoresis (18). Rehydration loading allows higher protein loads to be applied to gels than do other methods. It is particularly popular because of its simplicity.

There are no fixed rules regarding the positioning of the sample on the gel. In general, samples should not be applied to areas where they are expected to focus. To protect the proteins from exposure to extreme pH, the samples should not be applied closer than 1 cm from either electrode. Forming the pH gradient before sample application also limits the exposure of proteins to pH extremes.

Precast IEF mini gels (6 cm long by 8 cm wide and 1 mm thick) are available for carrying out carrier-ampholyte electro focusing. A selection of IPG sheets is also available for horizontal IEF. Vertical IEF gels have the advantage that the electrophoresis equipment for running them is available in most laboratories and they can hold relatively large sample volumes. Because vertical electrophoresis cells cannot tolerate very high voltages, this orientation is not capable of the ultrahigh resolution of horizontal cells. To protect the materials of the electrophoresis cells (mainly the gaskets) from caustic electrolytes alternative catholyte and anolyte solutions are substituted in vertical IEF runs. As catholyte, 20 m*M* arginine, 20 m*M* lysine is recommended in vertical slab systems (0.34 g arginine free base and 0.36 lysine free base in 100 ml of water). The recommended anolyte is 70 m*M* H_3PO_4, but it can be substituted with 20 m*M* aspartic acid, 20 m*M* glutamic acid (0.26 g aspartic acid and 0.29 g glutamic acid in 100 ml of water).

POWER CONDITIONS AND RESOLUTION IN ISOELECTRIC FOCUSING

The pH gradient and the applied electric field determine the resolution of an IEF run. According to theory and experiment (1, 8, and 12), the difference in p*I* between two resolved adjacent protein IEF bands (! pI) is directly proportional to the square root of the pH gradient and inversely proportional to the square root of the voltage gradient (field strength) at the position of the bands:

PI [(pH gradient)/ (voltage gradient)]¨.

Thus, narrow pH ranges and high applied-voltages give high resolution (small! pI) in IEF.

In addition to the effect on resolution, high electric fields also result in shortened run times. However, high voltages in electrophoresis are accompanied by large amounts of generated heat (see the discussion of Joule heating, in the Electrical Considerations section of the Application Focus on Gel Electrophoresis of Proteins on this website).

Thus, there are limitations on the magnitudes of the electric fields that can be applied and the ionic strengths of the solutions used in IEF. Because of their higher surface-to-volume ratio, thin gels are better able to dissipate heat than thick ones and are therefore capable of higher resolution (high voltage). Electric fields used in IEF are generally of the order of 100 *V*/cm. At focusing, currents drop to nearly zero since the current carriers have stopped moving by then.

PROTEIN SOLUBILIZATION FOR ISOELECTRIC FOCUSING

A fundamental problem with IEF is that some proteins tend to precipitate at their p*I* values. Carrier ampholytes sometimes help overcome pI precipitation and they are usually included in the sample solutions for IPG strips. In addition, nonionic detergents or urea are often included in IEF runs to minimize protein precipitation.

Urea is a common solubilizing agent in gel electrophoresis. It is particularly useful in IEF, especially for those proteins that tend to aggregate at their p*I*s. Urea disrupts hydrogen bonds and is used in situations in which hydrogen bonding can cause unwanted aggregation or formation of secondary structures that affect mobilities.

Dissociation of hydrogen bonds requires high urea concentrations (7-8 *M*). If complete denaturation of proteins is sought, samples must be treated with a thiol-reducing agent to break disulfide bridges (protein solutions in urea should not be heated above 30□C to avoid carbamylation).

High concentrations of urea make gels behave as if they had reduced pore sizes.

This is because of either viscosity effects or reductions in the effective size of water channels (pores). Urea must be present in the gels during electrophoresis, but, unlike SDS, urea does not affect the intrinsic charge of the sample polypeptides. Urea solutions should be used soon after

they are made or treated with a mixed-bed ion-exchange resin to avoid protein carbamylation by cyanate in old urea.

Some proteins, especially membrane proteins, require detergent solubilization during isolation. Ionic detergents, such as SDS, are not compatible with IEF, although nonionic detergents, such as octylglucoside, and zwiterionic detergents, such as 2-[(3- cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS) and its hydroxyl analog CHAPSO, can be used. NP-40 and Triton X-100 sometimes perform satisfactorily, but some preparations may contain charged contaminants.

Concentrations of CHAPS and CHAPSO or of octylglucoside of 1-2% in the gel are recommended. Some proteins may require as high as 4% detergent for solubility.

Even in the presence of detergents, some samples may have stringent salt requirements. Salt should be present in a sample only if it is an absolute requirement. Carrier ampholytes contribute to the ionic strength of the solution and can help to counteract a lack of salts in a sample. Small samples (1 to 10 µl) in typical biochemical buffers are

Usually tolerated, but better results can be obtained with solutions in deionized water, 2% ampholytes, or 1% glycine. Suitable samples can be prepared by dialysis or gel filtration.

Protocol 2. Isoelectric focusing

Equipment and reagents

- Flat-bed electrophoresis cell; *e.g.*, the GE Multiphor
- Power supply capable of delivering 2-3000 *V* and 6 *W* at high voltage.
- Refrigerated water circulator if required
- 1 *N* NaOH catholyte if required [a]
- 1 *N* H_3PO_4 anolyte if required
- Electrode strips if required
- Sample application strips

Method

- Set up the IEF cell as recommended by the manufacturer. This includes connecting a

water circulator, if used, and preparing the cooling platform and electrode strips, if necessary.

- Place sample application strips on a glass plate and pipette 5 □l of a protein sample to each strip. Place the application strips 1 cm from the anode end of the gel.
- Position the gel in the IEF cell and make electrode contact as specified for the particular cell.
- Close the electrophoresis cell and connect the leads to the power supply; the red lead is the anode and the black lead is the cathode.
- Set the running conditions as recommended by the manufacturer of the electrophoresis cell.

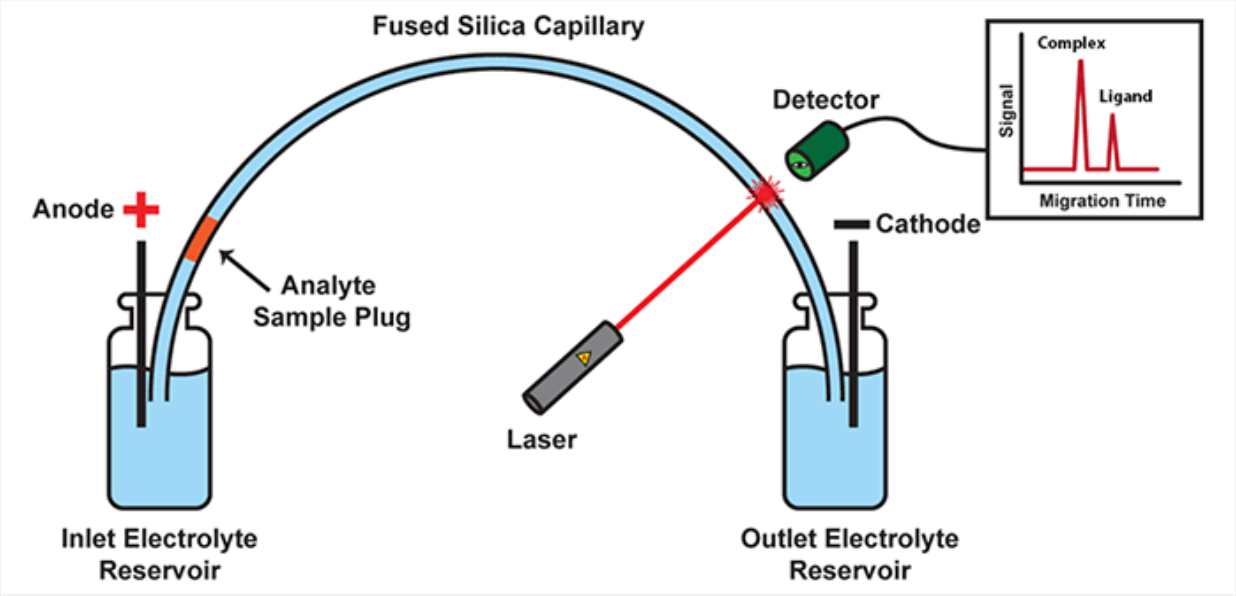
CAPILLARY ELECTROPHORESIS

INTRODUCTION

Capillary Electrophoresis (CE) is one of the possible methods to analyse complex samples. In High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) the separating force is the difference in affinity of the sample components to a stationary phase, and or difference in boiling point. With both techniques the most important factor is the polarity of a sample component. In CE the separating force is the difference in charge to size ratio. Not a flow through the column, but the electric field will do the separation.

In Capillary Electrophoresis a capillary is filled with a conductive fluid at a certain pH value. This is the buffer solution in which the sample will be separated. A sample is introduced in the capillary, either by pressure injection or by electro kinetic injection. A high voltage is generated over the capillary and due to this electric field (up to more than 300 V/cm) the sample components move (migrate) through the capillary at different speeds. Positive components migrate to the negative electrode; negative components migrate to the positive electrode. When you look at the capillary at a certain place with a detector you will first see the fast components pass, and later on the slower components.

Principle of Capillary Electrophoresis



MOBILITY

As mentioned before the speed of a component (the mobility) is dependable on size and charge. The size is a combination of the sample component and the shield of water that is bound to the

component. Even a small ion (as Fluoride, F-) can be big due to a large water shield. In general, the bigger the component, the slower it will migrate through the buffer.

The charge of ions can be strongly dependent on pH value. That is the reason why a buffer at certain pH is used for separations. For example Acetic Acid (pK value 4.756) will be almost completely negative charged at pH 7. The mobility (speed) of the acetic ions will be big. At pH 3, where about 80 % of the acetic acid is neutral, the mobility will be much lower. By changing the pH of a buffer system, the mobilities of the different components can be altered to achieve the best separation. In general the best pH for a separation is between the pK values of the sample components.

ENDO OSMOTIC FLOW (EOF)

In most applications the capillary that is being used is made of bare fused silica. This material has at its surface silanol groups (Si - O - H). These groups are slightly acidic. In buffers at higher pH value there are a lot of negative charges at the capillary wall (Si – O-). In the buffer fluid positive charges will be present because of the law of electrical neutrality. When a high voltage is generated over the capillary, these positive charges will start to migrate through the capillary towards the negative electrode. They will drag along the buffer fluid with them. This flow is called the (Electro) Endo Osmotic Flow (EOF). It is well possible to calculate a mobility of this EOF. The higher the pH, the more negative charges on the capillary wall and the more positive charges in the fluid. This will generate a stronger EOF.

Because the positive charges are all located close to the capillary wall, and there is no pressure force in the middle of the capillary, the flow profile of the EOF is completely flat. This will cause no peak broadening like the parabolic flow profile in HPLC and GC, and that is one of the reasons why such a high resolution can be achieved in CE. As mentioned before the EOF is towards the negative electrode. This flow drags along neutral components (which would not migrate without any fluid flow), and even positive components, whose mobility is lower than the mobility of the EOF, will migrate towards the negative electrode. In this way in one run negative, neutral and (slow) positive components can be separated and detected.

INSTRUMENTAL PARAMETERS

Voltage: A Joule heating plot is useful in optimizing the applied voltage and column temperature. The separation time is inversely proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and, as a result, viscosity gradients in the buffer inside the capillary, which causes band broadening and decreases resolution.

Polarity: Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the electroosmotic flow will move toward the cathode. If the electrode polarity is reversed the electroosmotic flow is away from the outlet and only charged analytes with electrophoretic mobilities greater than the electroosmotic flow will pass to the outlet.

Temperature: The main effect of temperature is observed on buffer viscosity and electrical conductivity, thus affecting migration velocity. In some cases, an increase in capillary temperature can cause a conformational change of some proteins, modifying their migration time and the efficiency of the separation.

Capillary: The length and internal diameter of the capillary affects the analysis time, the efficiency of separations and the load capacity. Increasing both effective length and total length can decrease the electric fields, at a constant voltage, which increases migration time. For a given buffer and electric field, heat dissipation (thus, sample band broadening) depends on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected into the capillary and the detection system used.

The adsorption of sample components on the capillary wall limits efficiency; therefore, methods to avoid these interactions should be considered in the development of a separation method. In the specific case of proteins, several strategies have been devised to avoid adsorption on the capillary wall. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer composition to prevent protein adsorption. Other strategies include the coating of the internal wall of the capillary with a polymer covalently bonded to the silica that prevents interaction between the proteins and the negatively charged silica surface. For this purpose, ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic and anionic polymers are commercially available.

ELECTROLYTE SOLUTION PARAMETERS

Buffer type and concentrations: Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimize current generation. To minimize band distortion, it is important to match buffer-ion mobility to solute mobility whenever possible. The type of sample solvent used is important to achieve on-column sample focusing, which increases separation efficiency and improves detection. Also, an increase in buffer concentration at a given pH decreases electroosmotic flow and solute velocity.

Buffer pH: The pH of the buffer can affect separation by modifying the charge of the analyte or additives and by changing the electroosmotic flow. For protein and peptide separation, a change in the pH of the buffer from above the isoelectric point (pI) to below the pI changes the net charge of the solute from negative to positive. An increase in the buffer pH generally increases the electroosmotic flow.

Organic solvents: Organic modifiers, such as methanol, acetonitrile and others may be added to the aqueous buffer to increase the solubility of the solute or other additives and/or to affect the ionization degree of the sample components. The addition of these organic modifiers to the buffer generally causes a decrease in the electroosmotic flow.

Additives for chiral separations: To separate optical isomers, a chiral selector is added to the separation buffer. The most commonly used chiral selectors are cyclodextrins, although in some cases crown ethers, certain polysaccharides or even proteins can be used. Because chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. While developing a given separation it may be useful to test cyclodextrins having a different cavity size (α-, β-, or -cyclodextrin) or modified cyclodextrins with neutral (methyl, ethyl, hydroxyalkyl, etc.) or ionizable (aminomethyl, carboxymethyl, sulfobutylether, etc.) moities. When using modified cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins must be taken into account because it will influence the selectivity. The resolution of chiral separations is also controlled by the concentration of the chiral selector, the composition and pH of the buffer and the separation temperature. Organic additives, such as methanol or urea, can also affect the resolution.

DIFFERENT MODES OF CAPILLARY ELECTROPHORESIS

1. **Capillary Zone Electrophoresis (CZE or FSCE)**

Capillary Zone Electrophoresis (CZE), also known as free-solution CE, is the most standard form of CE. Buffer is flushed through the capillary by pressure, sample is injected and high voltage is applied. Dependable on the polarity the EOF is towards the inlet or the outlet. Each sample component will migrate through the capillary at its own speed. Only the difference in mobility will cause the separation.

2. **Capillary Gel Electrophoresis (CGE)**

With this technique there is a gel matrix inside the capillary. Components with different size but the same mobility are separated with this technique. Components of bigger size will be slowed down more by the gel, and will migrate later through the capillary. Especially with protein and DNA separations this technique is frequently used.

3. **Micellar ElectoKinetic Chromatography (MECC or MEKC)**

In this way of electrophoresis micelles are generated in the buffer. These micelles have a non polar inside, and a polar (or charged) surface. Sample components in the buffer solution will be divided over the micelles and the buffer solution, dependable on the affinity to the micelles. Just like in HPLC and GC, there will be a certain stable diversion between buffer and micelles. When the migration speed of the buffer differs from the speed of the micelles, it is possible to separate different components on the fact that there is a different affinity for the micelles. In this way, there is a lot of synergy with HPLC and GC.

4. **Non-Aqueous Capillary Electrophoresis (NACE)**

With this technique components that are insoluble in water are separated, mainly depending on the use of organic solvents. The viscosity and dielectric constants of organic solvents affect both sample ion mobility and the level of electroosmotic flow.

5. **Iso Electric Focussing (IEF)**

When a pH gradient is applied across the capillary, and a voltage is applied from positive voltage at low pH to negative voltage at high pH, components will migrate to the pH value that equals their pI value. At lower pH value, the components are positively charged; at higher pH values the

components are negatively charged. In this way, each component migrates to a different position in the capillary. When pressure is applied, the complete pH gradient moves through the capillary, and subsequently the components will pass the detection window.

6. Capillary Electro Chromatography (CEC)

With this technique a capillary is partly packed with silica based particles with a stationary phase. When high voltage is applied over the capillary, the buffer fluid will start to migrate due to the EOF that is present because of the silica. The sample will have, just as in HPLC, more or less affinity for the stationary phase. This is the separating force in this technique. The only difference between HPLC and CEC is that not a pressure pump is being used to force the mobile phase through the packed bed (HPLC), but a high voltage is used for this purpose.

7. Electro Chromatography (EKC)

With this technique there is a differential interaction of enantiomers with the cyclodextrins, which allows the separation of chiral compounds. This enantiomer analysis is used for the analyses of natural products, such as pharmaceutical/herbal products, toxicology compounds, food and food contaminants, forensic, fingerprinting and many more.

8. Capillary IsoTechoPhoresis (ITP)

With this technique two kinds of buffers are used. One buffer with high mobility as a leading buffer and one buffer with very low mobility as a terminating buffer. The mobility of the sample components must be between the leading and terminating. In the stable situation, all components migrate through the capillary at the same velocity (hence the name itsotacho=same speed). In the figure, two components A and B are positioned between L (eading) and T (erminating). For the mobilities: m (L)> m (A)> m (B)> m (T).

Because all components have the same speed, there will be different electric fields in each zone, as the law v=m*E is everywhere valid. The electric field in the Terminating zone will be highest. These differences in electric field result in the self-correcting behaviour. If a component (say B) is for some reason (diffusion) in zone A, it will be under a higher electric field giving it a higher speed. The result is that the ion migrates back into its own zone. The same if it would be in the

Leading zone, where it is in a lower electric field. The velocity is lower, and it will be caught by the quicker moving zone of B.

In addition, because of electrical laws, the concentrations are related to the concentration of the Leading electrolyte. For this reason, small concentration samples are strongly concentrated into very narrow zones. Especially this effect is used quite often to concentrate large volume injections.

CENTRIFUGE- PRINCIPLE, INSTRUMENTATION AND APPLICATIONS OF DIFFERENTIAL, ZONAL, DENSITY GRADIENT AND ULTRA CENTRIFUGATION

INTRODUCTION

Centrifugation is a technique of separating substances which involves the application of centrifugal force.

The particles are separated from a solution according to their size, shape, density, the viscosity of the medium and rotor speed.

A centrifuge is a device used to separate components of a mixture on the basis of their size, density, the viscosity of the medium, and the rotor speed.

DEFINITION

- Centrifugation is the technique of separating components where the centrifugal force/ acceleration causes the denser molecules to move towards the periphery while the less dense particles move to the center.
- The process of centrifugation relies on the perpendicular force created when a sample is rotated about a fixed point.
- The rate of centrifugation is dependent on the size and density of the particles present in the solution.

Relative Centrifugal Force (RCF)

- Relative centrifugal force is the measure of the strength of rotors of different types and sizes.
- This is the force exerted on the contents of the rotor as a result of the rotation.

- RCF is the perpendicular force acting on the sample that is always relative to the gravity of the earth.
- The RCF of the different centrifuge can be used for the comparison of rotors, allowing the selection of the best centrifuge for a particular function.
- The formula to calculate the relative centrifugal force (RCF) can be written as:
- **RCF (g Force)= $1.118 \times 10^{-5} \times r \times (RPM)^2$**
- Where **r** is the radius of the rotor (in centimeters), and **RPM** is the speed of the rotor in rotation per minute.

CENTRIFUGE ROTORS

Rotors in centrifuges are the motor devices that house the tubes with the samples. Centrifuge rotors are designed to generate rotation speed that can bring about the separation of components in a sample. There are three main types of rotors used in a centrifuge, which are:

1. Fixed angle rotors

- These rotors hold the sample tubes at an angle of 45° in relation to the axis of the rotor.
- In this type of rotor, the particles strike the opposite side of the tube where the particles finally slide down and are collected at the bottom.
- These are faster than other types of rotors as the path length of the tubes increases.
- However, as the direction of the force is different from the position of the tube, some particles might remain at the sides of the tubes.

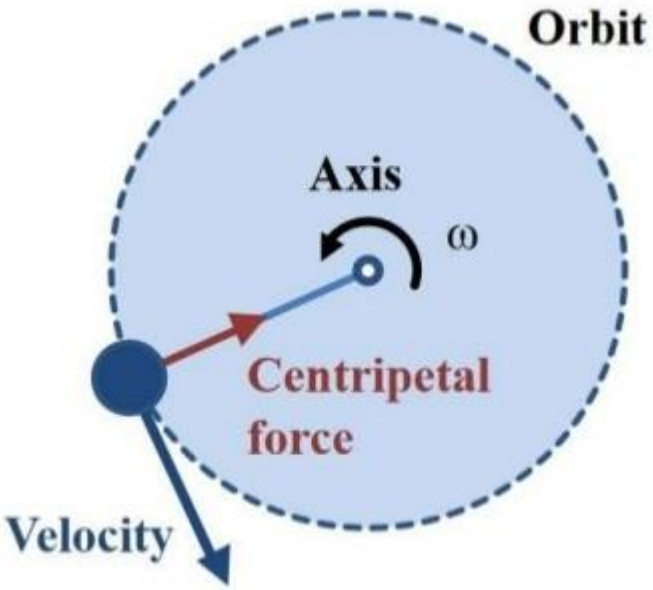
2. Swinging bucket rotors/ Horizontal rotors

- Swinging bucket rotors hold the tubes at an angle of 90° as the rotor swings as the process is started.
- In this rotor, the tubes are suspended in the racks that allow the tubes to be moved enough to acquire the horizontal position.
- In this type of rotors, the particles are present along the direction or the path of the force that allows the particles to be moved away from the rotor towards the bottom of the tubes.
- Because the tubes remain horizontal, the supernatant remains as a flat surface allowing the deposited particles to be separated from the supernatant.

3. Vertical rotors

- Vertical rotors provide the shortest path length, fastest run time, and the highest resolution of all the rotors.
- In vertical rotors, the tubes are vertical during the operation of the centrifuge.
- The yield of the rotor is not as ideal as the position of the tube doesn't align with the direction of the centrifugal force.
- As a result, instead of settling down, particles tend o spread towards the outer wall of the tubes.
- These are commonly used in isopycnic and density gradient centrifugation.

PRINCIPLE OF CENTRIFUGATION



- In a solution, a particle whose density is higher than that of the solvent sink (sediment), and particles that are lighter than it floats to the top.
- The greater the difference in density, the faster they move. If there is no difference in density (isopycnic conditions), the particles stay steady.
- To take advantage of even tiny differences in density to separate various particles in a solution, gravity can be replaced with the much more powerful "centrifugal force" provided by a centrifuge.
- A centrifuge is a piece of equipment that puts an object in rotation around a fixed axis (spins it in a circle), applying a potentially strong force perpendicular to the axis of spin (outward).
- The centrifuge works using the sedimentation principle, where the centripetal acceleration causes denser substances and particles to move outward in the radial direction.
- At the same time, objects that are less dense are displaced and move to the center.
- In a laboratory centrifuge that uses sample tubes, the radial acceleration causes denser particles to settle to the bottom of the tube, while low- density substances rise to the top.

TYPES OF CENTRIFUGE

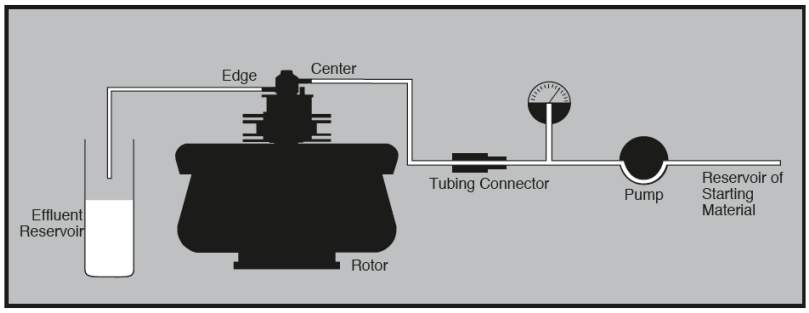
1. Bench top centrifuge

- Bench top centrifuge is a compact centrifuge that is commonly used in clinical and research laboratories.
- It is driven by an electric motor where the tubes are rotated about a fixed axis, resulting in force perpendicular to the tubes.
- Because these are very compact, they are useful in smaller laboratories with smaller spaces.
- Different variations of bench top centrifuges are available in the market for various purposes.
- A bench top centrifuge has a rotor with racks for the sample tubes and a lid that closes the working unit of the centrifuge.

2. Continuous flow centrifuge

- Continuous flow centrifuge is a rapid centrifuge that allows the centrifugation of large volumes of samples without affecting the sedimentation rates.
- This type of centrifuge allows the separation of a large volume of samples at high centrifugal force, thus removing the tedious part of emptying and filling the tubes with each cycle.

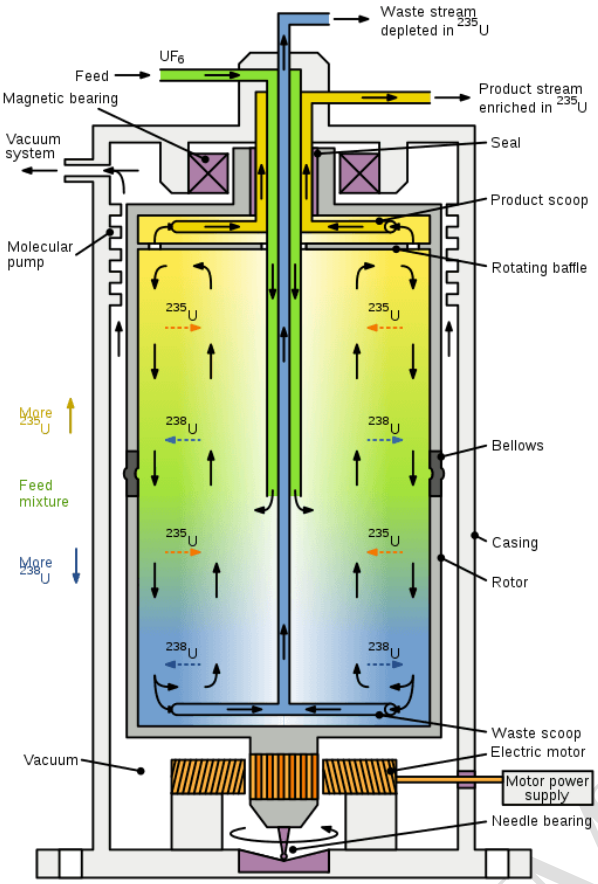
They have a shorter path length which facilitates the process of pelleting out the solid part out of the supernatant, thus maintaining the speed of the process.



- They also have larger capacities which save time as the sample doesn't have to be load and unloaded over and over again like in traditional centrifuges.
- Up to 1 liter of samples can be centrifuged by this centrifuge at a time period of 4 hours or less.

3. Gas centrifuge

- A gas centrifuge is a centrifuge explicitly used for the separation of gases based on heir isotopes.
- This centrifuge is based on the same principle of centrifugal force as all other centrifuges where the molecules are separated on the basis of their masses.
- This centrifuge is used mainly for the extraction and separation of uranium -235 and uranium-238.
- The gas centrifuge works on eh design of the continuous flow of gas in and out of the centrifuge, unlike other centrifuge working on batch processing.
- These centrifuges are arranged in cascades so that the gases are separated into two units based on their isotopes and then are passed onto the next centrifuge for further processing.
- Gas centrifuges have replaced other gaseous diffusion methods as they provide a yield of higher concentration of the gases than the previous techniques.



GAS CENTRIFUGE **HEMATOCRIT CENTRIFUGE**

4. Hematocrit centrifuge

- Hematocrit centrifuges are specialized centrifuges used for the determination of volume fraction of erythrocytes (RBCs) in a given blood sample.
- This centrifuge provides Hematocrit values that can be used for testing in biochemistry, immunity, blood test, and other general clinical tests.
- Hematocrit centrifuges may be used to help diagnose blood loss, polycythemia (an elevation of the erythrocyte count to above-normal levels), anemia, bone marrow failure, leukemia, and multiple myeloma.
- The microhematocrit centrifuge quickly attains speeds of 11,000 rpm and RCFs of up to 15,000 g to spin tube samples.
- The components of a Hematocrit centrifuge are similar to that of the bench top centrifuge, but this centrifuge is specialized for the use of blood samples.

5. Low-speed centrifuge

- Low-speed centrifuges are the traditional centrifuges that are commonly used in laboratories for the routine separation of particles.
- These centrifuges operate at the maximum speed of 4000-5000 rpm.
- These are usually operated under room temperature as they are not provided with a system for controlling the speed or temperature of the operation.
- Swinging bucket and fixed angle type of rotors can be used in these centrifuges.
- These are easy and compact centrifuges that are ideal for the analysis of blood samples and other biological samples.
- The low-speed centrifuge works on the same principle as all other centrifuges, but the application is limited to the separation of simpler solutions.

6. High-Speed Centrifuges

- High-speed centrifuge, as the name suggests, is the centrifuge that can be operated at somewhat larger speeds.
- The speed of the high-speed centrifuge can range from 15,000 to 30,000 rpm.
- The high-speed centrifuge is commonly used in more sophisticated laboratories with the biochemical application and requires a high speed of operations.
- High-speed centrifuges are provided with a system for controlling the speed and temperature of the process, which is necessary for the analysis of sensitive biological molecules.

- The high-speed centrifuges come with different adapters to accommodate the sample tubes of various sizes and volumes.
- All three types of rotors can be used for the centrifugation process in these centrifuges.

HIGH SPEED CENTRIFUGE

MICROCENTRIFUGE

7. Micro centrifuge

- Microcentrifuges are the centrifuges used for the separation of samples with smaller volumes ranging from 0.5 to 2 µl.
- Micro centrifuges are usually operated at a speed of about 12,000-13,000 rpm.
- This is used for the molecular separation of cell organelles like nuclei and DNA and phenol extraction.
- Micro centrifuges, also termed, microfuge, use sample tubes that are smaller in size when compared to the standard test tubes used in larger centrifuges.
- Some micro centrifuges come with adapters that facilitate the use of larger tubes along with the smaller ones.

- Micro centrifuges with temperature controls are available for the operation of temperature-sensitive samples.

8. Refrigerated centrifuges

- Refrigerated centrifuges are the centrifuges that are provided with temperature control ranging from -20°C to -30°C.
- A different variation of centrifuges is available that has the system of temperature control which is essential for various processes requiring lower temperatures.
- Refrigerated centrifuges have a temperature control unit in addition to the rotors and racks for the sample tubes.
- These centrifuges provide the RCF of up to 60,000 xg that is ideal for the separation of various biological molecules.
- These are typically used for collecting substances that separate rapidly like yeast cells, chloroplasts, and erythrocytes.
- The chamber of refrigerated centrifuge is sealed off from the outside to meet the conditions of the operations.

9. Ultracentrifuges

- It is the most sophisticated instrument.

- Ultracentrifuge has a maximum speed of 65,000 RPM (100,000's x g).
- Intense heat is generated due to high speed thus the spinning chambers must be refrigerated and kept at a high vacuum.
- It is used for both preparative work and analytical work.

TYPES OF CENTRIFUGATION

1. ANALYTICAL CENTRIFUGATION

Analytical centrifugation is a separation method where the particles in a sample are separated on the basis of their density and the centrifugal force they experience. Analytical ultracentrifugation (AUC) is a versatile and robust method for the quantitative analysis of macromolecules in solution.

Principle of Analytical Centrifugation

Analytical centrifugation is based on the principle that particles that are denser than others settle down faster. Similarly, the larger molecules move more quickly in the centrifugal force than the smaller ones.

Analytical ultracentrifugation for the determination of the relative molecular mass of a macromolecule can be performed by a sedimentation velocity approach or sedimentation equilibrium methodology.

The hydrodynamic properties of macromolecules are described by their sedimentation coefficients. They can be determined from the rate that a concentration boundary of the particular biomolecules moves in the gravitational field.

The sedimentation coefficient can be used to characterize changes in the size and shape of macromolecules with changing experimental conditions.

Three optical systems are available for the analytical ultracentrifuge (absorbance, interference, and fluorescence) that permit precise and selective observation of sedimentation in real-time.

Steps of Analytical Centrifugation

Small sample sizes (20-120 mm^3) are taken in analytical cells to be placed inside the ultracentrifuge.

The ultracentrifuge is then operated so that the centrifugal force causes a migration of the randomly distributed biomolecules through the solvent radially outwards from the center of rotation.

The distance of the molecules from the center is determined through the Schlieren optical system.

A graph is drawn from the solute concentration versus the squared radial distance from the center of rotation, based on which the molecular mass is determined.

Uses of Analytical Centrifugation

Analytical centrifugation can be used for the determination of the purity of macromolecules.

It can also be used for the examination of changes in the molecular mass of supramolecular complexes.

Besides, it allows the determination of the relative molecular mass of solutes in their native state.

2. DENSITY GRADIENT CENTRIFUGATION

Density gradient centrifugation is the separation of molecules where the separation is based on the density of the molecules as they pass through a density gradient under a centrifugal force.

Principle of Density gradient centrifugation

Density gradient centrifugation is based on the principle that molecules settle down under a centrifugal force until they reach a medium with the density the same as theirs.

In this case, a medium with a density gradient is employed, which either has to decrease density or increasing density.

Molecules in a sample move through the medium as the sample are rotated creating a centrifugal force.

The more dense molecules begin to move towards the bottom as they move through the density gradient.

The molecules then become suspended at a point in which the density of the particles equals the surrounding medium.

In this way, molecules with different densities are separated at different layers which can then be recovered by various processes.

Steps of Density gradient centrifugation

A density gradient of a medium is created by gently laying the lower concentration over the higher concentrations in a centrifuge tube.

The sample is then placed over the gradient, and the tubes are placed in an ultracentrifuge.

The particles travel through the gradient until they reach a point at which their density matches the density of the surrounding medium.

The fractions are removed and separated, obtaining the particles as isolated units.

Uses of Density gradient centrifugation

Density gradient centrifugation can be applied for the purification of large volumes of biomolecules.

It can even be used for the purification of different viruses which aids their further studies.

This technique can be used both as a separation technique and the technique for the determination of densities of various particles.

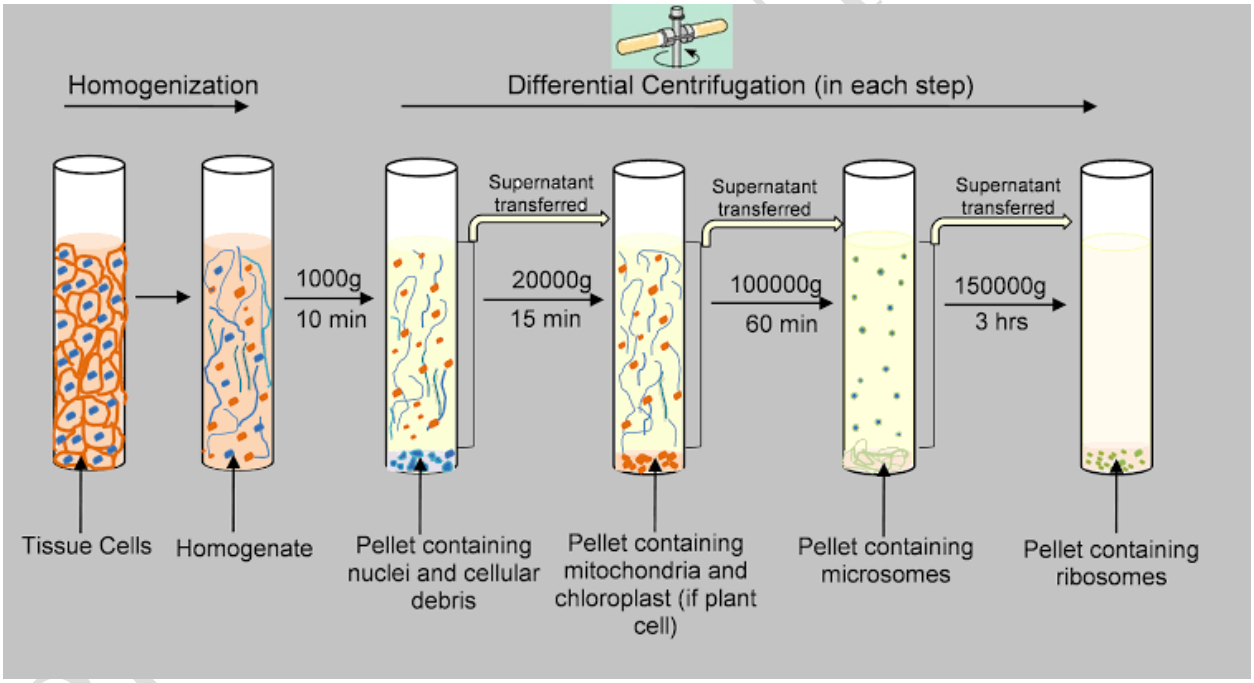
Examples of Density gradient centrifugation

This method was used in the famous experiment, which proved that DNA is semi-conservative by using different isotopes of nitrogen.

Another example is the use of this technique for the isolation of the microsomal fraction from muscle homogenates and subsequent separation of membrane vesicles with a differing density.

3. DIFFERENTIAL CENTRIFUGATION

Differential centrifugation is a type of centrifugation process in which components are separately settled down a centrifuge tube by applying a series of increasing centrifugal force.



Principle of Differential centrifugation

Differential centrifugation is based upon the differences in the sedimentation rate of biological particles of different size and density.

As the increasing centrifugal force is applied, initial sedimentation of the larger molecules takes place.

Further particles settle down depending upon the speed and time of individual centrifugation steps and the density and relative size of the particles.

The largest class of particles forms a pellet on the bottom of the centrifuge tube, leaving smaller-sized structures within the supernatant.

Thus, larger molecules sediment quickly and at lower centrifugal forces whereas the smaller molecules take longer time and higher forces.

In the case of particles that are less dense than the medium, the particles will float instead of settling.

Steps of Differential centrifugation

The sample solution is homogenized in the medium containing buffer.

The sample is then placed in the centrifuge tube, which is operated at a particular centrifugal force for a specific time at a particular temperature.

By the end of this operation, a pellet will be formed at the bottom of the tube, which is separated from the supernatant.

The supernatant is added to a new centrifuge tube where it is centrifuged at another speed for a particular time and particular temperature.

Again, the supernatant is separated from the pellets formed.

These steps are continued until all particles are separated from each other.

The particles can then be identified by testing for indicators that are unique to the specific particles.

Uses of Differential centrifugation

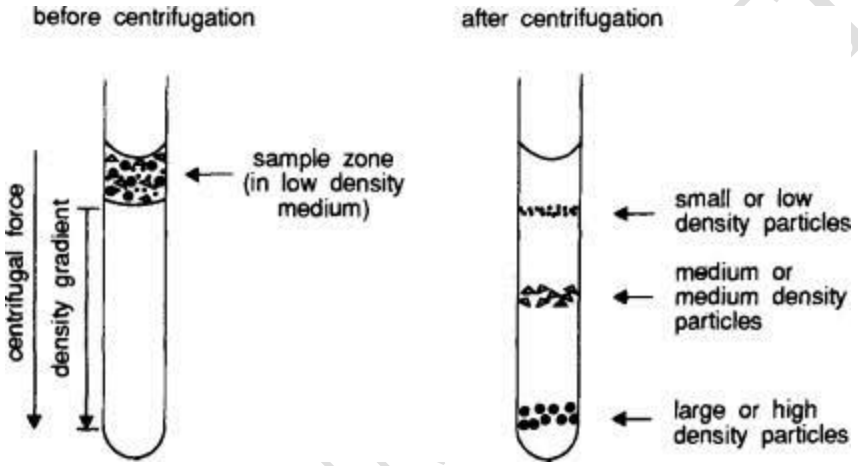
Differential centrifugation is commonly used for the separation of cell organelles and membranes found in the cell.

It can also be used for low-resolution separation of the nucleus.

As this technique separates particles based on their sizes, this can be used for the purification of extracts containing larger-sized impurities.

4. RATE-ZONAL DENSITY GRADIENT CENTRIFUGATION/ MOVING ZONE CENTRIFUGATION

Rate-zonal density gradient centrifugation is a type of centrifugation that separates particles on the basis of their shape as size and works on the same principle of density gradient centrifugation but works in a different way. It is also called the moving zone centrifugation.



Principle of Rate-zonal density gradient centrifugation

Rate zonal centrifugation fractionates particles by both size and shape.

The procedure is to layer a sample in a restricted zone on top of a pre-poured density gradient. The density gradient is then centrifuged.

All particles migrate into the density gradient because the density gradient has only densities much lower than the densities of the particles being centrifuged.

The particles are fractionated primarily by size and shape. The larger a particle is, the more rapidly it sediments.

The more spherically symmetrical a particle is, the more rapidly it sediments.

The particles sediment through the gradient at a rate that is a function of their sedimentation coefficient.

Unlike differential centrifugation where the sample is distributed throughout the medium, in rate-zonal centrifugation, the sample is initially present only on top of the gradient as a narrow band.

Steps of Rate-zonal density gradient centrifugation

A density gradient is prepared in a centrifuge tube before applying the sample.

The same is then layered on the top of the gradient in the form of a band.

During centrifugation, fast-moving particles (larger in size and circular in shape) move ahead of slower particles so that different particles are separated as various bands on different parts of the gradient.

The particles are separated on the basis of their sedimentation coefficients, and they are obtained from the bottom of the tube through a perforation.

Uses of Rate-zonal density gradient centrifugation

Rate-zonal differential centrifugation has been used for the separation of viruses as they have components that are of different size and density that are unique to each virus.

This method has been employed for the fractionation of RNA on sucrose gradients.

Besides, rate-zonal differential centrifugation has also been used for the separation, purification and fractionation of DNA molecules from both viruses and bacteria.

The fractionation of polysomes and ribosome subunits has been one of the earliest applications of this method.

5. DIFFERENTIAL VELOCITY (MOVING BOUNDARY) CENTRIFUGATION

Differential velocity centrifugation is a type of centrifugation process in which components are separately settled down a centrifuge tube by applying a series of increasing velocities.

Principle of Differential velocity (Moving Boundary) centrifugation

Differential centrifugation is based upon the differences in the rate of sedimentation of biological particles of different size and density.

As the increasing speed of the rotors is applied, initial sedimentation of the larger molecules takes place.

Further particles settle down depending upon the speed and time of individual centrifugation steps and the density and relative size of the particles.

The largest class of particles forms a pellet on the bottom of the centrifuge tube, leaving smaller-sized structures within the supernatant.

The pellet is then removed, and the supernatant is further centrifuged to obtain smaller particles.

Thus, larger molecules sediment quickly and at lower velocities, whereas the smaller molecules take longer time and higher velocities.

In the case of particles that are less dense than the medium, the particles will float instead of settling.

Steps of Differential velocity (Moving Boundary) centrifugation

The sample solution is homogenized in the medium containing buffer.

The sample is then placed in the centrifuge tube, which is operated at a lower rotor speed for a particular time at a particular temperature.

By the end of this operation, a pellet will be formed at the bottom of the tube, which is separated from the supernatant.

The supernatant is added to a new centrifuge tube where it is centrifuged at another speed for a particular time and particular temperature.

Again, the supernatant is separated from the pellets formed.

These steps are continued until all particles are separated from each other.

The particles can then be identified by testing for indicators that are unique to the specific particles.

Uses of Differential velocity (Moving Boundary) centrifugation

Differential centrifugation is commonly used for the separation of cell organelles and membranes found in the cell.

It can also be used for low-resolution separation of the nucleus.

As this technique separates particles based on their sizes, this can be used for the identification and comparison of particles of different sizes.

APPLICATIONS OF CENTRIFUGATION

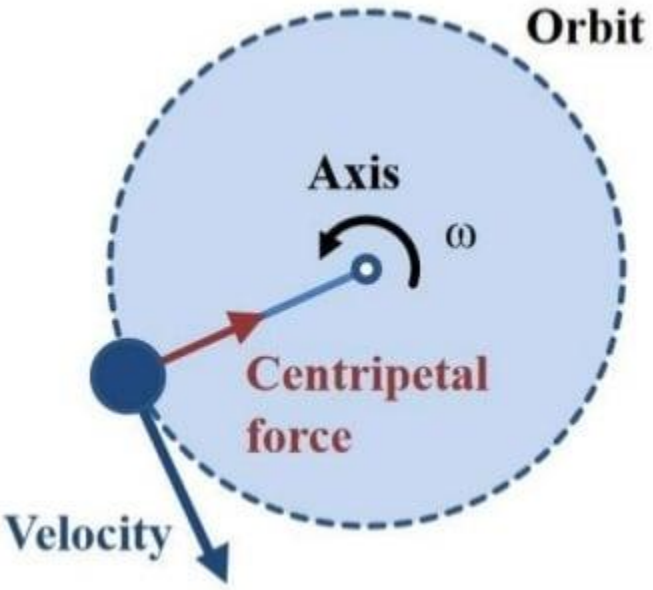
- To separate two miscible substances
- To analyze the hydrodynamic properties of macromolecules
- Purification of mammalian cells
- Fractionation of sub cellular organelles (including membranes/membrane fractions) Fractionation of membrane vesicles
- Separating chalk powder from water
- Removing fat from milk to produce skimmed milk
- Separating particles from an air-flow using cyclonic separation
- The clarification and stabilization of wine
- Separation of urine components and blood components in forensic and research laboratories
- Aids in the separation of proteins using purification techniques such as salting out, e.g. ammonium sulfate precipitation.

CENTRIFUGATION

Centrifugation is the technique of separating components where the centrifugal force/ acceleration causes the denser molecules to move toward the periphery while the less dense particles move to the center.

- The process of centrifugation relies on the perpendicular force created when a sample is rotated about a fixed point.
- The rate of centrifugation is dependent on the size and density of the particles present in the solution.

Principle of Centrifugation



- In a solution, particles whose density is higher than that of the solvent sink (sediment), and particles that are lighter than it floats to the top.
- The greater the difference in density, the faster they move. If there is no difference in density (isopycnic conditions), the particles stay steady.
- To take advantage of even tiny differences in density to separate various particles in a solution, gravity can be replaced with the much more powerful "centrifugal force" provided by a centrifuge.
- A centrifuge is a piece of equipment that puts an object in rotation around a fixed axis (spins it in a circle), applying a potentially strong force perpendicular to the axis of spin (outward).
- The centrifuge works using the sedimentation principle, where the centripetal acceleration causes denser substances and particles to move outward in the radial direction.
- At the same time, objects that are less dense are displaced and move to the center.
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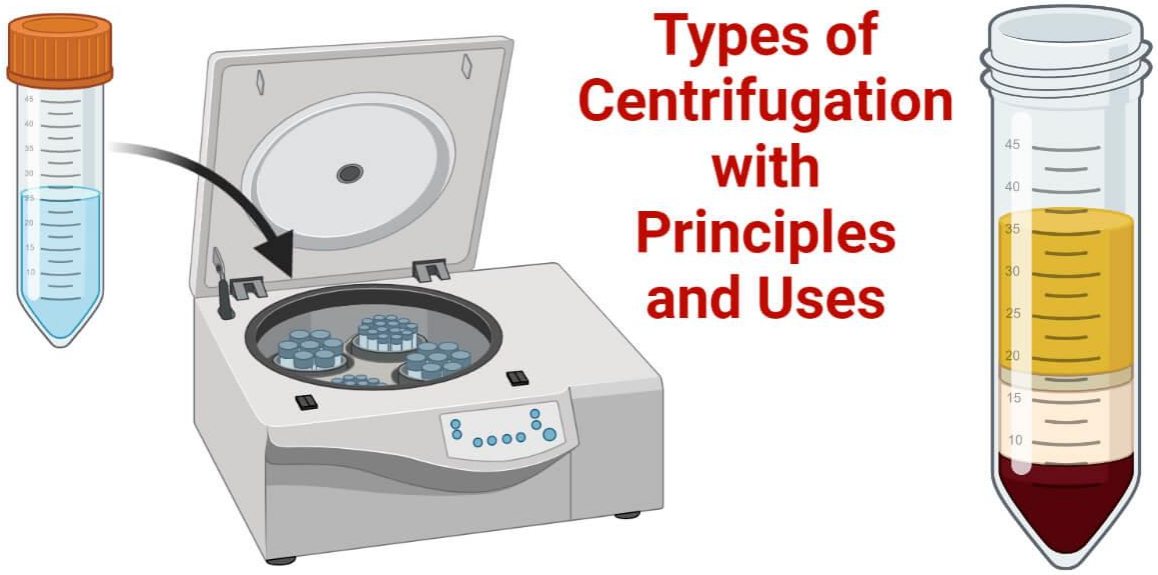
Types of Centrifugation

- Types of Centrifugation



Analytical Centrifugation

Analytical centrifugation is a separation method where the particles in a sample are separated on the basis of their density and the centrifugal force they experience. Analytical ultracentrifugation (AUC) is a versatile and robust method for the quantitative analysis of macromolecules in solution.

Principle of Analytical Centrifugation

- Analytical centrifugation is based on the principle that particles that are denser than others settle down faster. Similarly, the larger molecules move more quickly in the centrifugal force than the smaller ones.
- Analytical ultracentrifugation for the determination of the relative molecular mass of a macromolecule can be performed by a sedimentation velocity approach or sedimentation equilibrium methodology.
- The hydrodynamic properties of macromolecules are described by their sedimentation coefficients. They can be determined from the rate that a concentration boundary of the particular biomolecules moves in the gravitational field.

- The sedimentation coefficient can be used to characterize changes in the size and shape of macromolecules with changing experimental conditions.
- Three optical systems are available for the analytical ultracentrifuge (absorbance, interference, and fluorescence) that permit precise and selective observation of sedimentation in real-time.

Steps of Analytical Centrifugation

- Small sample sizes (20-120 mm^3) are taken in analytical cells to be placed inside the ultracentrifuge.
- The ultracentrifuge is then operated so that the centrifugal force causes a migration of the randomly distributed biomolecules through the solvent radially outwards from the center of rotation.
- The distance of the molecules from the center is determined through the Schlieren optical system.
- A graph is drawn from the solute concentration versus the squared radial distance from the center of rotation, based on which the molecular mass is determined.

Uses of Analytical Centrifugation

- Analytical centrifugation can be used for the determination of the purity of macromolecules.
- It can also be used for the examination of changes in the molecular mass of supramolecular complexes.
- Besides, it allows the determination of the relative molecular mass of solutes in their native state.

Density gradient centrifugation

Density gradient centrifugation is the separation of molecules where the separation is based on the density of the molecules as they pass through a density gradient under a centrifugal force.

Principle of Density gradient centrifugation

- Density gradient centrifugation is based on the principle that molecules settle down under a centrifugal force until they reach a medium with the density the same as theirs.

- In this case, a medium with a density gradient is employed, which either has to decrease density or increasing density.
- Molecules in a sample move through the medium as the sample is rotated creating a centrifugal force.
- The more dense molecules begin to move towards the bottom as they move through the density gradient.
- The molecules then become suspended at a point in which the density of the particles equals the surrounding medium.
- In this way, molecules with different densities are separated at different layers which can then be recovered by various processes.

Steps of Density gradient centrifugation

- A density gradient of a medium is created by gently laying the lower concentration over the higher concentrations in a centrifuge tube.
- The sample is then placed over the gradient, and the tubes are placed in an ultracentrifuge.
- The particles travel through the gradient until they reach a point at which their density matches the density of the surrounding medium.
- The fractions are removed and separated, obtaining the particles as isolated units.

Uses of Density gradient centrifugation

- Density gradient centrifugation can be applied for the purification of large volumes of biomolecules.
- It can even be used for the purification of different viruses which aids their further studies.
- This technique can be used both as a separation technique and the technique for the determination of densities of various particles.

Examples of Density gradient centrifugation

- This method was used in the famous experiment, which proved that DNA is semi-conservative by using different isotopes of nitrogen.
- Another example is the use of this technique for the isolation of the microsomal fraction from muscle homogenates and subsequent separation of membrane vesicles with a differing density.

Differential centrifugation

Differential centrifugation is a type of centrifugation process in which components are separately settled down a centrifuge tube by applying a series of increasing centrifugal force.

Principle of Differential centrifugation

- Differential centrifugation is based upon the differences in the sedimentation rate of biological particles of different size and density.
- As the increasing centrifugal force is applied, initial sedimentation of the larger molecules takes place.
- Further particles settle down depending upon the speed and time of individual centrifugation steps and the density and relative size of the particles.
- The largest class of particles forms a pellet on the bottom of the centrifuge tube, leaving smaller-sized structures within the supernatant.
- Thus, larger molecules sediment quickly and at lower centrifugal forces whereas the smaller molecules take longer time and higher forces.
- In the case of particles that are less dense than the medium, the particles will float instead of settling.

Steps of Differential centrifugation

- The sample solution is homogenized in the medium containing buffer.
- The sample is then placed in the centrifuge tube, which is operated at a particular centrifugal force for a specific time at a particular temperature.
- By the end of this operation, a pellet will be formed at the bottom of the tube, which is separated from the supernatant.
- The supernatant is added to a new centrifuge tube where it is centrifuged at another speed for a particular time and particular temperature.
- Again, the supernatant is separated from the pellets formed.
- These steps are continued until all particles are separated from each other.

- The particles can then be identified by testing for indicators that are unique to the specific particles.

Uses of Differential centrifugation

- Differential centrifugation is commonly used for the separation of cell organelles and membranes found in the cell.
- It can also be used for low-resolution separation of the nucleus.
- As this technique separates particles based on their sizes, this can be used for the purification of extracts containing larger-sized impurities.

Isopycnic centrifugation

Isopycnic centrifugation is a type of centrifugation where the particles in a sample are separated on the basis of their densities as centrifugal force is applied to the sample.

Principle of Isopycnic centrifugation

- Isopycnic centrifugation is also termed the equilibrium centrifugation as the separation of particles takes place solely on the basis of their densities and not on their sizes.
- The particles move towards the bottom, and the movement is based on the size of the particles. And, the flow ceases once the density of the particle becomes equal to the density of the surrounding medium.
- The density in the gradient increases as we move down the tube towards the bottom. As a result, the particles with higher densities settle down at the bottom, followed by less dense particles that form bands above the denser particles.
- It is considered as a true equilibrium as this depends directly on the buoyant densities and not the sizes of the particles.

Steps of Isopycnic centrifugation

- A gradient prepared with an increasing density towards the bottom of the tube is prepared. A pre-performed gradient can also be used.
- The solution of the biological sample and salt is uniformly distributed in the centrifuge tube and placed inside the centrifuge.
- Once the centrifuge is operated, a density gradient of the salt is formed in the tube.
- The particles move down the tube and settle down as they reach the region with their respective densities.
- The particles are then separated and identified using different other processes.

Uses of Isopycnic centrifugation

- Isopycnic centrifugation can be applied for the purification of large volumes of biomolecules.
- This technique can be used as a technique for the determination of densities of various particles.

Rate-zonal density gradient centrifugation/ Moving Zone Centrifugation

Rate-zonal density gradient centrifugation is a type of centrifugation that separates particles on the basis of their shape as size and works on the same principle of density gradient centrifugation but works in a different way. It is also called the moving zone centrifugation.

Principle of Rate-zonal density gradient centrifugation

- Rate zonal centrifugation fractionates particles by both size and shape.
- The procedure is to layer a sample in a restricted zone on top of a pre-poured density gradient. The density gradient is then centrifuged.
- All particles migrate into the density gradient because the density gradient has only densities much lower than the densities of the particles being centrifuged.
- The particles are fractionated primarily by size and shape. The larger a particle is, the more rapidly it sediments.
- The more spherically symmetrical a particle is, the more rapidly it sediments.
- The particles sediment through the gradient at a rate that is a function of their sedimentation coefficient.

- Unlike differential centrifugation where the sample is distributed throughout the medium, in rate-zonal centrifugation, the sample is initially present only on top of the gradient as a narrow band.
-

Steps of Rate-zonal density gradient centrifugation

- A density gradient is prepared in a centrifuge tube before applying the sample.
- The same is then layered on the top of the gradient in the form of a band.
- During centrifugation, fast-moving particles (larger in size and circular in shape) move ahead of slower particles so that different particles are separated as various bands on different parts of the gradient.
- The particles are separated on the basis of their sedimentation coefficients, and they are obtained from the bottom of the tube through a perforation.
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Uses of Rate-zonal density gradient centrifugation

- Rate-zonal differential centrifugation has been used for the separation of viruses as they have components that are of different size and density that are unique to each virus.
- This method has been employed for the fractionation of RNA on sucrose gradients.
- Besides, rate-zonal differential centrifugation has also been used for the separation, purification and fractionation of DNA molecules from both viruses and bacteria.
- The fractionation of polysomes and ribosome subunits has been one of the earliest applications of this method.

Differential velocity (Moving Boundary) centrifugation

Differential velocity centrifugation is a type of centrifugation process in which components are separately settled down a centrifuge tube by applying a series of increasing velocities.

Principle of Differential velocity (Moving Boundary) centrifugation

- Differential centrifugation is based upon the differences in the rate of sedimentation of biological particles of different size and density.
- As the increasing speed of the rotors is applied, initial sedimentation of the larger molecules takes place.

- Further particles settle down depending upon the speed and time of individual centrifugation steps and the density and relative size of the particles.
- The largest class of particles forms a pellet on the bottom of the centrifuge tube, leaving smaller-sized structures within the supernatant.
- The pellet is then removed, and the supernatant is further centrifuged to obtain smaller particles.
- Thus, larger molecules sediment quickly and at lower velocities, whereas the smaller molecules take longer time and higher velocities.
- In the case of particles that are less dense than the medium, the particles will float instead of settling.

Steps of Differential velocity (Moving Boundary) centrifugation

- The sample solution is homogenized in the medium containing buffer.
- The sample is then placed in the centrifuge tube, which is operated at a lower rotor speed for a particular time at a particular temperature.
- By the end of this operation, a pellet will be formed at the bottom of the tube, which is separated from the supernatant.
- The supernatant is added to a new centrifuge tube where it is centrifuged at another speed for a particular time and particular temperature.
- Again, the supernatant is separated from the pellets formed.
- These steps are continued until all particles are separated from each other.
- The particles can then be identified by testing for indicators that are unique to the specific particles.

Uses of Differential velocity (Moving Boundary) centrifugation

- Differential centrifugation is commonly used for the separation of cell organelles and membranes found in the cell.
- It can also be used for low-resolution separation of the nucleus.
- As this technique separates particles based on their sizes, this can be used for the identification and comparison of particles of different sizes.
-

Equilibrium density gradient centrifugation

Equilibrium density gradient centrifugation is a modified and specialized form of density gradient centrifugation.

Principle of Equilibrium density gradient centrifugation

- Equilibrium density gradient centrifugation is based on the principle that particles in a solution are separated on the basis of their densities.
- In this case, the particles move through the density gradient and stop in a region where the density of the medium is equal to the density of the particle.
- At this point, the centrifugal force acting on the particle is equal to the buoyant force pushing the particles up. As a result, the particles cease to move and can be separated into different layers.
- The density in the gradient increases as we move down the tube towards the bottom. As a result, the particles with higher densities settle down at the bottom, followed by less dense particles that form bands above the denser particles.
-

Steps of Equilibrium density gradient centrifugation

- A gradient prepared with an increasing density towards the bottom of the tube is prepared. A pre-performed gradient can also be used.
- The solution of the biological sample and salt is uniformly distributed in the centrifuge tube and placed inside the centrifuge.
- Once the centrifuge is operated, a density gradient of the salt is formed in the tube.
- The particles move down the tube and settle down as they reach the region with their respective densities.
- The particles are then separated and identified using different other processes.
-

Uses of Equilibrium density gradient centrifugation

- Equilibrium density gradient centrifugation can be applied for the purification of large volumes of biomolecules.
- This technique can be used as a technique for the determination of densities of various particles.

Examples of Equilibrium density gradient centrifugation

- This has been used in experiments performed by Meelson and Stahl to determine the densities of different DNA molecules based on where they reached on the density gradient.

Sucrose gradient centrifugation

Sucrose gradient centrifugation is a type of density gradient centrifugation where the density gradient is formed of sucrose by changing the concentration of sucrose.

Principle of Sucrose gradient centrifugation

- Sucrose gradient centrifugation is based on the principle that molecules settle down under a centrifugal force until they reach a medium with the density the same as theirs.
- In this case, a medium with sucrose gradient is employed, which either has a lower density at the top and higher density at the bottom.
- Molecules in a sample move through the medium as the sample is rotated creating a centrifugal force.
- The more dense molecules begin to move towards the bottom as they move through the density gradient.
- The molecules then become suspended at a point in which the density of the particles equals the surrounding medium.
- In this way, molecules with different densities are separated at different layers which can then be recovered by various processes.
-

Steps of Sucrose gradient centrifugation

- A density gradient of sucrose is created by gently laying the lower concentration of sucrose over the higher concentrations in a centrifuge tube.
- The sample is then placed over the gradient, and the tubes are placed in an ultracentrifuge.
- The particles travel through the gradient until they reach a point at which their density matches the density of the surrounding medium.
- The fractions are removed and separated, obtaining the particles as separated units.
-

Uses of Sucrose gradient centrifugation

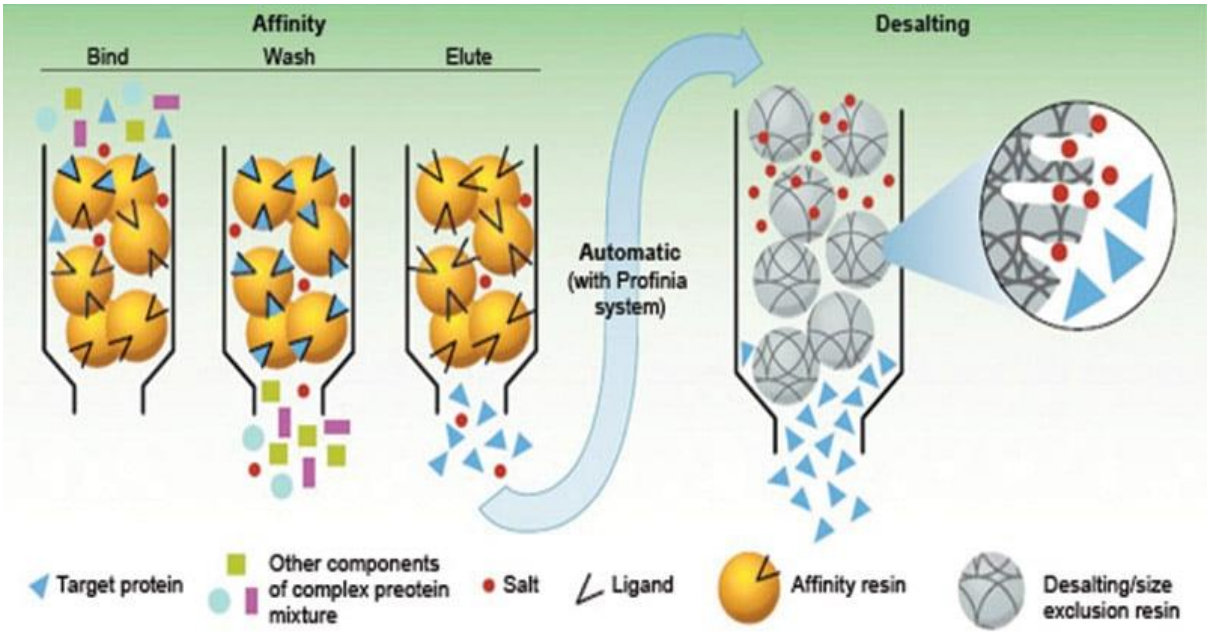
- Sucrose gradient centrifugation is a powerful technique for the separation of macromolecules like DNA and RNA.
- This has also been used for the analysis of protein complexes and to determine the density as well as the size of various other macromolecules.

Affinity Chromatography

- Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.
- It is a separation technique in which a mobile phase carrying a mixture is caused to move in contact with a selectively absorbent stationary phase.
- Affinity chromatography is a type of liquid **chromatography** for the separation, purification or specific analysis of sample components.
- It utilizes the reversible biological interaction or molecular recognition called affinity which refers to the attracting forced exerted in different degrees between atoms which cause them to remain in combination.

Example: Enzyme with an inhibitor, antigen with an antibody, etc.

- It was discovered by Pedro Cuatrecasas and Meir Wilcheck.



Principle of Affinity Chromatography

- The stationary phase consists of a support medium, on which the substrate (ligand) is bound covalently, in such a way that the reactive groups that are essential for binding of the target molecule are exposed.
- As the crude mixture of the substances is passed through the chromatography column, substances with binding site for the immobilized substrate bind to the stationary phase, while all other substances is eluted in the void volume of the column.
- Once the other substances are eluted, the bound target molecules can be eluted by methods such as including a competing ligand in the mobile phase or changing the pH, ionic strength or polarity conditions.
-

Components of Affinity Chromatography

1. Matrix

- The matrix is an inert support to which a ligand can be directly or indirectly coupled.
- In order to for the matrix to be effective it must have certain characters:
- Matrix should be chemically and physically inert.
- It must be insoluble in solvents and buffers employed in the process
- It must be chemically and mechanically stable.

- It must be easily coupled to a ligand or spacer arm onto which the ligand can be attached.
- It must exhibit good flow properties and have a relatively large surface area for attachment.
- The most useful matrix materials are agarose and polyacrylamide.

2. Spacer arm

- It is used to improve binding between ligand and target molecule by overcoming any effects of steric hindrance.

3. Ligand

- It refers to the molecule that binds reversibly to a specific target molecule.
- The ligand can be selected only after the nature of the macromolecule to be isolated is known.
- When a hormone receptor protein is to be purified by affinity chromatography, the hormone itself is an ideal candidate for the ligand.
- For antibody isolation, an antigen or hapten may be used as ligand.
- If an enzyme is to be purified,a substrate analog, inhibitor, cofactor, or effector may be used as a the immobilized ligand.

Steps in Affinity Chromatography

- Affinity medium is equilibrated in binding buffer.
- Sample is applied under conditions that favor specific binding of the target molecule(s) to a complementary binding substance (the ligand). Target substances bind specifically, but reversibly, to the ligand and unbound material washes through the column.
- Elution is performed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Target protein is collected in a purified, concentrated form.
- Affinity medium is re-equilibrated with binding buffer.

These events can be summarized into the following three major steps:

1. Preparation of Column

- The column is loaded with solid support such as sepharose, agarose, cellulose etc.
- Ligand is selected according to the desired isolate.
- Spacer arm is attached between the ligand and solid support.

2. Loading of Sample

- Solution containing a mixture of substances is poured into the elution column and allowed to run at a controlled rate.

3. Elution of Ligand-Molecule Complex

- Target substance is recovered by changing conditions to favor elution of the bound molecules.

Applications of Affinity Chromatography

- Affinity chromatography is one of the most useful methods for the separation and purification of specific products.
- It is essentially a sample purification technique, used primarily for biological molecules such as proteins.

Its major application includes:

- Separation of mixture of compounds.
- Removal of impurities or in purification process.
- In enzyme assays
- Detection of substrates
- Investigation of binding sites of enzymes
- In in vitro antigen-antibody reactions
- Detection of Single Nuceotide polymorphisms and mutations in nucleic acids

Advantages of Affinity Chromatography

- High specificity
- Target molecules can be obtained in a highly pure state
- Single step purification
- The matrix can be reused rapidly.
- The matrix is a solid, can be easily washed and dried.
- Give purified product with high yield.
- Affinity chromatography can also be used to remove specific contaminants, such as proteases.

Limitations of Affinity Chromatography

- Time consuming method.
- More amounts of solvents are required which may be expensive.
- Intense labour
- Non-specific adsorption cannot be totally eliminated, it can only be minimized.

- Limited availability and high cost of immobilized ligands.
- Proteins get denatured if required pH is not adjusted.

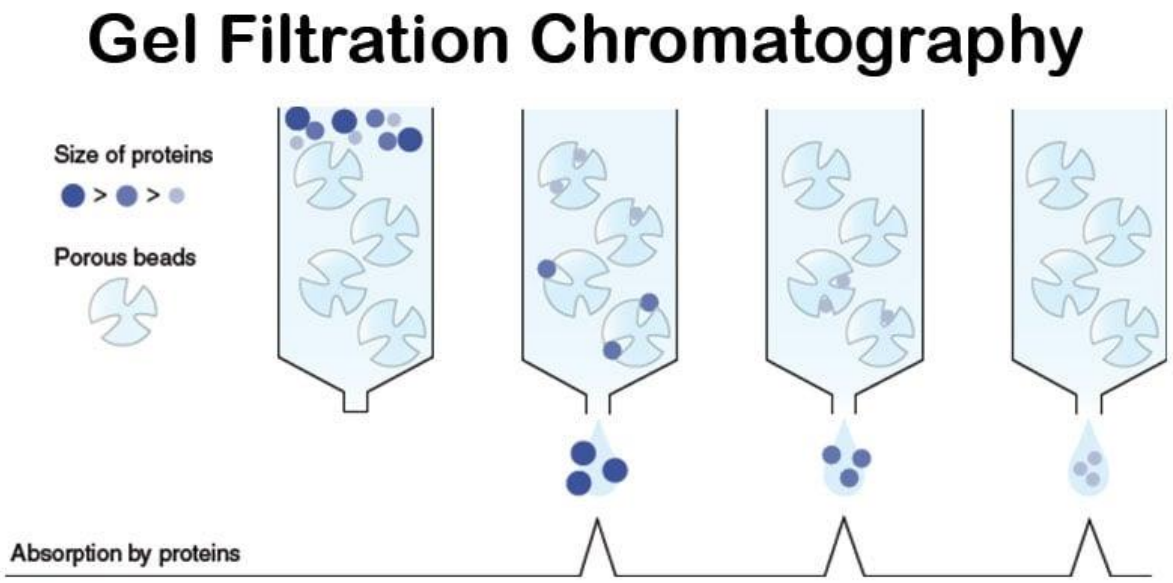
Gel Filtration Chromatography- Definition, Principle, Types, Parts, Steps, Uses

Biomolecules are purified using different techniques that separate them according to the differences in their specific properties such as size, hydrophobicity, biorecognition, charge, etc.

Gel filtration is a technique in which the separation of components is based on the difference in molecular weight or size.

It is the simplest and mildest of all the **chromatography** techniques and separates molecules on the basis of differences in size.

Principle of Gel Filtration Chromatography



o perform a separation, the gel filtration medium is packed into a column to form a packed bed. The medium is a porous matrix in the form of spherical particles that have been chosen for their chemical and physical stability, and inertness (lack of reactivity and adsorptive properties). The packed bed is equilibrated with a buffer which fills the pores of the matrix and the space in between the particles. The liquid inside the pores is sometimes referred to as the stationary phase and this liquid is in equilibrium with the liquid outside the particles, referred to as the mobile phase.

- The stationary phase used is a porous polymer matrix whose pores are completely filled with the solvent to be used as the mobile phase.
- The molecules in the sample are pumped through specialized columns containing such microporous packing material (gel).
- The basis of the separation is that molecules above a certain size are totally excluded from the pores, while smaller molecules access the interior of the pores partly or wholly.
- The flow of the mobile phase hence will cause larger molecules to pass through the column unhindered, without penetrating the gel matrix, whereas smaller molecules will be retarded according to their penetration of the gel.

Types of Gel Filtration Chromatography

Group Separations

- The components of a sample are separated into two major groups according to the size range.
- A group separation can be used to remove high or low molecular weight contaminants (such as phenol red from culture fluids) or to desalt and exchange buffers.

High-resolution fractionation of biomolecules

- The components of a sample are separated according to differences in their molecular size.
- High-resolution fractionation can be used to isolate one or more components, to separate monomers from aggregates, to determine the molecular weight or to perform a molecular weight distribution analysis.

Steps in Gel Filtration Chromatography

1. Spherical particles of gel filtration medium are packed into a column.
2. The sample is applied to the column.
3. Buffer (mobile phase) and sample move through the column.
4. Molecules diffuse in and out of the pores of the matrix (also described as the partitioning of the sample between the mobile phase and the stationary phase).
5. Smaller molecules move further into the matrix and so stay longer on the column.
6. As buffer passes continuously through the column, molecules that are larger than the pores of the matrix are unable to diffuse into the pores and pass through the column.
7. Smaller molecules diffuse into the pores and are delayed in their passage down the column.
8. Separation occurs at different intervals which are followed by detection of components.

Applications of Gel Filtration Chromatography

- Gel filtration plays a key role in the purification of enzymes, polysaccharides, nucleic acids, proteins, and other biological macromolecules.
- Gel filtration can also be used to facilitate the refolding of denatured proteins by careful control of changing buffer conditions.
- It is used in protein fractionation experiments.
- Gel filtration technique is also used in molecular weight determination.
- Separation of sugar, proteins, peptides, rubbers, and others on the basis of their size.

- Can be used to determine the quaternary structure of purified proteins.

Advantages of Gel Filtration Chromatography

- Gel filtration is a robust technique that is well suited to handling biomolecules that are sensitive to changes in pH, the concentration of metal ions or co-factors and harsh environmental conditions.
- A significant advantage of gel filtration is that conditions can be varied to suit the type of sample or the requirements for further purification, analysis or storage without altering the separation.
- Separations can be performed in the presence of essential ions or cofactors, detergents, urea, guanidine hydrochloride, at high or low ionic strength, at 37 °C or in the cold room according to the requirements of the experiment.
- Unlike ion exchange or affinity chromatography, molecules do not bind to the chromatography medium so buffer composition does not directly affect resolution (the degree of separation between peaks).
- Short analysis time.
- Well defined separation.
- Narrow bands and good sensitivity.
- There is no sample loss.
- The small amount of mobile phase required.
- The flow rate can be set.

Limitations of Gel Filtration Chromatography

- The limited number of peaks that can be resolved within the short time scale of the run.
- Filtrations must be performed before using the instrument to prevent dust and other particulates from ruining the columns and interfering with the detectors.
- The molecular masses of most of the chains will be too close for the separation to show anything more than broad peaks.

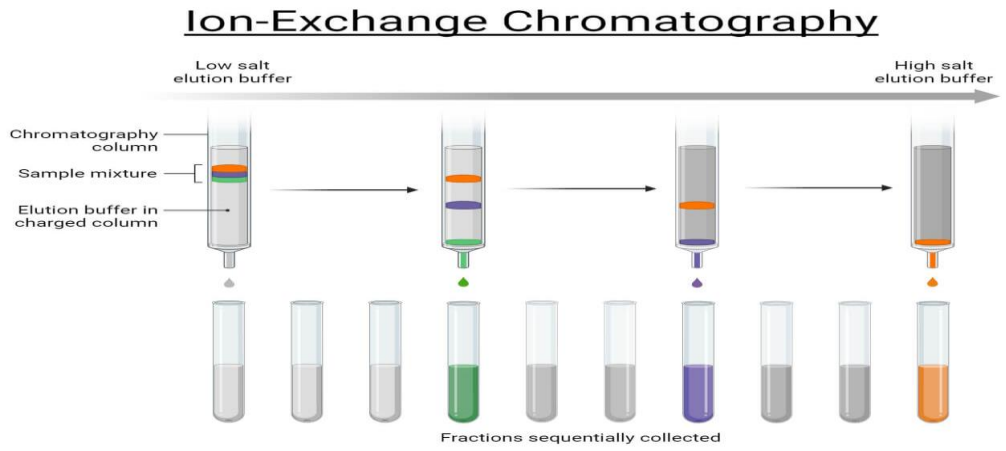
Ion Exchange Chromatography:

Chromatography is the separation of a mixture of compounds into their individual components based on their relative interactions with an inert matrix.

What is Ion Exchange Chromatography?

Ion exchange chromatography (or ion chromatography) is a process that allows the separation of ions and polar molecules based on their affinity to ion exchangers.

The principle of separation is thus by reversible exchange of ions between the target ions present in the sample solution to the ions present on ion exchangers.

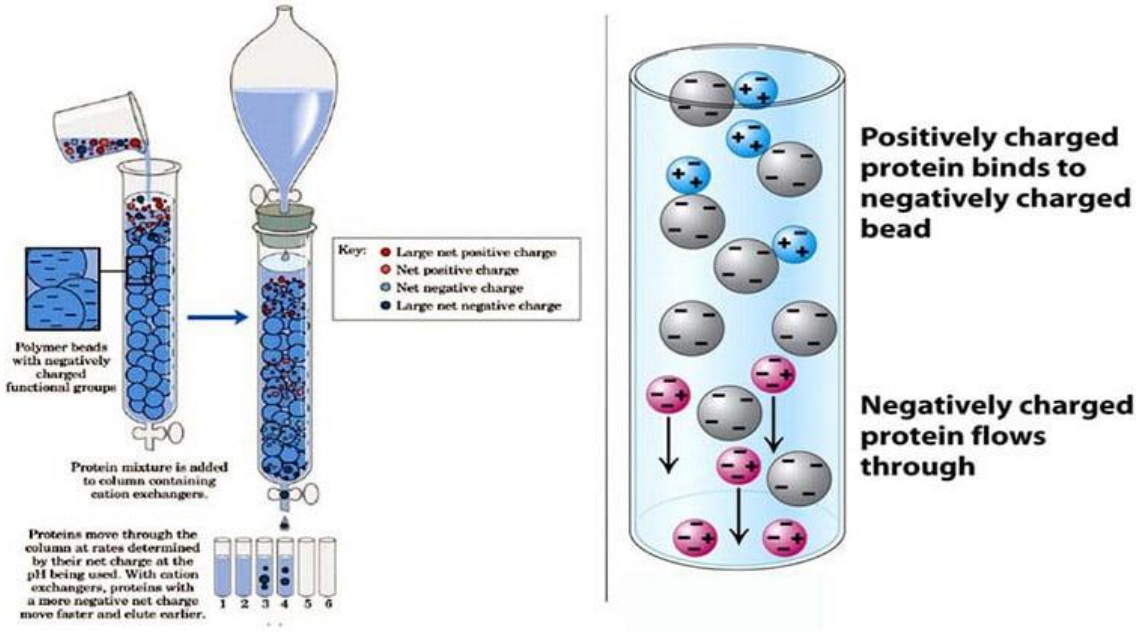


In this process, two types of exchangers i.e., cationic and anionic exchangers can be used.

1. **Cationic exchangers** possess negatively charged group, and these will attract positively charged cations. These exchangers are also called "Acidic ion exchange" materials, because their negative charges result from the ionization of acidic group.

2. **Anionic exchangers** have positively charged groups that will attract negatively charged anions. These are also called "Basic ion exchange" materials.

- Ion exchange chromatography is most often performed in the form of column chromatography. However, there are also thin-layer chromatographic methods that work basically based on the principle of ion exchange.



Working Principle of ion exchange chromatography

This form of chromatography relies on the attraction between oppositely charged stationary phase, known as an ion exchanger, and analyte.

- The ion exchangers basically contain charged groups covalently linked to the surface of an insoluble matrix.
- The charged groups of the matrix can be positively or negatively charged.
- When suspended in an aqueous solution, the charged groups of the matrix will be surrounded by ions of the opposite charge.
- In this "ion cloud", ions can be reversibly exchanged without changing the nature and the properties of the matrix.

Instrumentation of ion exchange chromatography

Typical IC instrumentation includes: pump, injector, column, suppressor, detector and recorder or data system.

1. **Pump**

The IC pump is considered to be one of the most important components in the system which has to provide a continuous constant flow of the eluent through the IC injector, column, and detector.

2. **Injector**

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. Liquid samples may be injected directly and solid samples need only to be dissolved in an appropriate solvent. Injectors should provide the possibility of injecting the liquid sample within the range of 0.1 to 100 ml of volume with high reproducibility and under high pressure (up to the 4000 psi).

3. **Columns**

Depending on its ultimate use and area of application, the column material may be stainless steel, titanium, glass or an inert plastic such as PEEK. The column can vary in diameter from about 2mm to 5 cm and in length from 3 cm to 50 cm depending on whether it is to be used for normal analytical purposes, microanalysis, high speed analyses or preparative work.

Guard column is placed anterior to the separating column. This serves as a protective factor that prolongs the life and usefulness of the separation column. They are dependable columns designed to filter or remove particles that clog the separation column

4. **Suppressor**

The suppressor reduces the background conductivity of the chemicals used to elute samples from the ion-exchange column which improves the conductivity measurement of the ions being tested. IC suppressors are membrane-based devices which are designed to convert the ionic eluent to water as a means of enhancing the sensitivity.

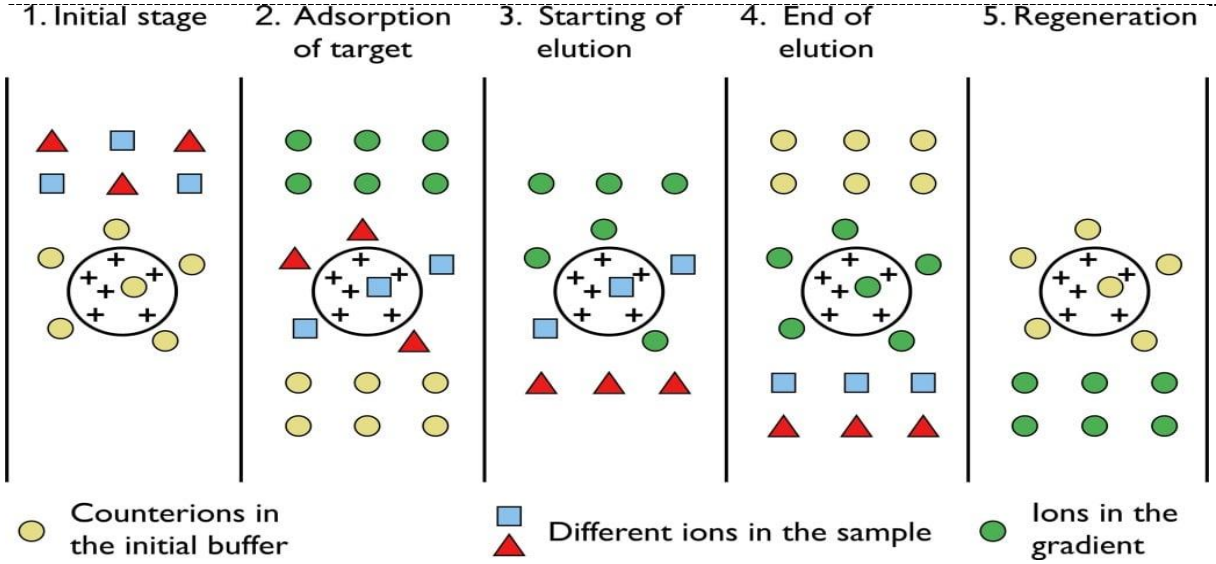
5. **Detectors**

Electrical conductivity detector is commonly use.

6. **Data system**

In routine analysis, where no automation is needed, a pre-programmed computing integrator may be sufficient. For higher control levels, a more intelligent device is necessary, such as a data station or minicomputer

Procedure of ion exchange chromatography



- Ion exchange separations are carried out mainly in columns packed with an ion-exchanger.
- These ionic exchangers are commercially available. They are made up of styrene and divinyl benzene. Example. DEAE-cellulose is an anionic exchanger, CM-cellulose is a cationic exchanger.
- The choice of the exchanger depends upon the charge of particle to be separated. To separate anions "Anionic exchanger" is used, to separate cations "Cationic exchanger" is used.
- First the column is filled with ion exchanger then the sample is applied followed by the buffer. The tris-buffer, pyridine buffer, acetate buffer, citrate and phosphate buffers are widely used.
- The particles which have high affinity for ion exchanger will come down the column along with buffers.

- In next step using corresponding buffer separates the tightly bound particles.
- Then these particles are analyzed spectroscopically.

Applications of ion exchange chromatography

- An important use of ion-exchange chromatography is in the routine analysis of **amino acid** mixtures.
- The 20 principal amino acids from blood serum or from the hydrolysis of proteins are separated and used in clinical diagnosis.
- This is most effective method for water purification. Complete deionization of water (or) a non-electrolyte solution is performed by exchanging solute cations for hydrogen ions and solute anions for hydroxyl ions. This is usually achieved by method is used for softening of drinking water.
- In the analysis of products of hydrolysis of nucleic acids. In this way, information is gained about the structure of these molecules and how it relates to their biological function as carriers of hereditary information.
- Chelating resins are used to collect trace metals from seawater.
- To analyze lunar rocks and rare trace elements on Earth.

Advantages of ion exchange chromatography

1. It is one of the most efficient methods for the separation of charged particles.
2. It can be used for almost any kind of charged molecule including large proteins, small nucleotides and amino acids.
3. Ion exchange is used for both analytical and preparative purposes in the laboratory, the analytical uses being the more common.
4. Inorganic ions also can be separated by ion-exchange chromatograph.y

Limitations of ion exchange chromatography

- Only charged molecules can be separated.
- Buffer Requirement

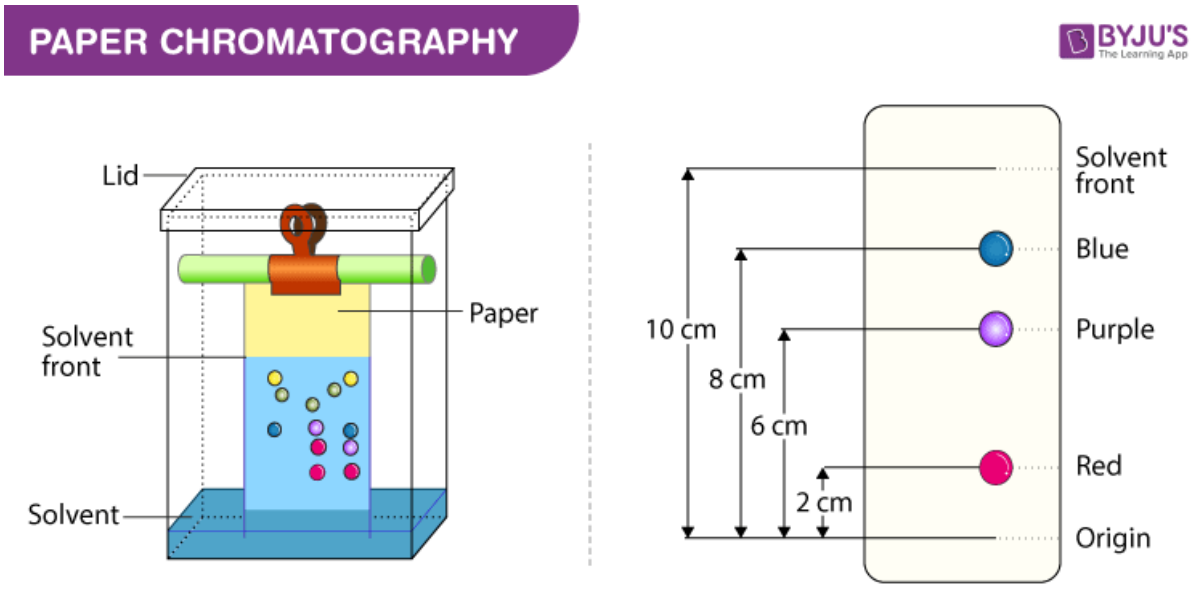
What Is Paper Chromatography?

Chromatography technique that uses paper sheets or strips as the adsorbent being the stationary phase through which a solution is made to pass is called paper chromatography. It is an inexpensive method of separating dissolved chemical substances by their different migration rates across the sheets of paper. It is a powerful analytical tool that uses very small quantities of material. Paper chromatography was discovered by Synge and Martin in the year 1943.

Paper Chromatography Principle

The principle involved can be partition chromatography or adsorption chromatography. Partition chromatography because the substances are partitioned or distributed between liquid phases. The two phases are water held in pores of the filter paper and the other phase is a mobile phase which passes through the paper. When the mobile phase moves, the separation of the mixture takes place. The compounds in the mixture separate themselves based on the differences in their affinity towards stationary and mobile phase solvents under the capillary action of pores in the paper. Adsorption chromatography between solid and liquid phases, wherein the solid surface of the paper is the stationary phase and the liquid phase is the mobile phase.

Paper Chromatography Diagram



Paper Chromatography Procedure

Below we have explained the procedure to conduct Paper Chromatography Experiment for easy understanding of students.

1. **Selecting a suitable type of development:** It is decided based on the complexity of the solvent, paper, mixture, etc. Usually ascending type or radial paper chromatography is used as they are easy to perform. Also, it is easy to handle, the chromatogram obtained is faster and the process is less time-consuming.

2. **Selecting a suitable filter paper**: Selection of filter paper is done based on the size of the pores and the sample quality.
3. **Prepare the sample:** Sample preparation includes the dissolution of the sample in a suitable solvent (inert with the sample under analysis) used in making the mobile phase.
4. **Spot the sample on the paper:** Samples should be spotted at a proper position on the paper by using a capillary tube.
5. **Chromatogram development:** Chromatogram development is spotted by immersing the paper in the mobile phase. Due to the capillary action of paper, the mobile phase moves over the sample on the paper.
6. **Paper drying and compound detection**: Once the chromatogram is developed, the paper is dried using an air drier. Also, detecting solution can be sprayed on the chromatogram developed paper and dried to identify the sample chromatogram spots.

Paper Chromatography Applications

There are various applications of paper chromatography. Some of the uses of Paper Chromatography in different fields are discussed below:

- To study the process of fermentation and ripening.
- To check the purity of pharmaceuticals.
- To inspect cosmetics.
- To detect the adulterants.
- To detect the contaminants in drinks and foods.
- To examine the reaction mixtures in biochemical laboratories.
- To determine dopes and drugs in humans and animals.

Types of paper chromatography:

1. Ascending Paper Chromatography – The techniques goes with its name as the solvent moves in an upward direction.

2. Descending Paper Chromatography – The movement of the flow of solvent due to gravitational pull and capillary action is downwards, hence the name descending paper chromatography.
3. Ascending – Descending Paper Chromatography – In this version of paper chromatography, movement of solvent occurs in two directions after a particular point. Initially, the solvent travels upwards on the paper which is folded over a rod and after crossing the rod it continues with its travel in the downward direction.
4. Radial or Circular Paper Chromatography – The sample is deposited at the centre of the circular filter paper. Once the spot is dried, the filter paper is tied horizontally on a Petri dish which contains the solvent.
5. Two Dimensional Paper Chromatography – Substances which have the same r_f values can be resolved with the help of two-dimensional paper chromatography.

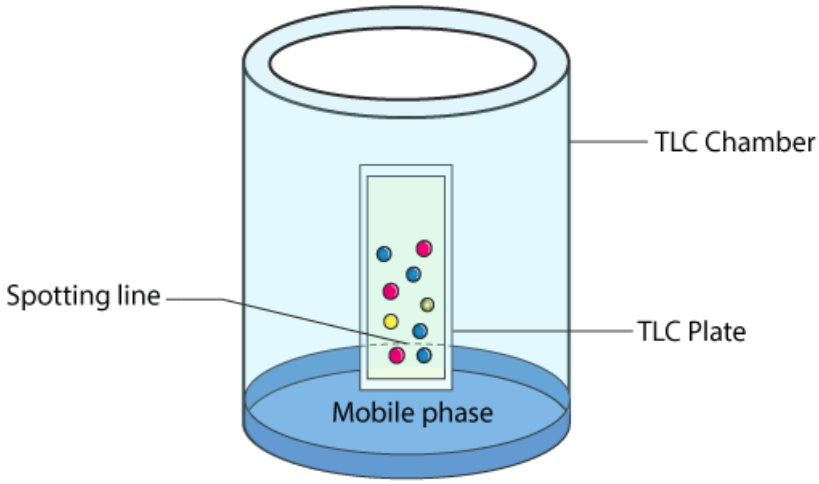
What Is Thin Layer Chromatography?

Thin Layer Chromatography is a technique used to isolate non-volatile mixtures. The experiment is conducted on a sheet of aluminium foil, plastic, or glass which is coated with a thin layer of adsorbent material. The material usually used is aluminium oxide, cellulose, or silica gel.

On completion of the separation, each component appears as spots separated vertically. Each spot has a retention factor (R_f) expressed as:

R_f = dist. travelled by sample / dist. travelled by solvent

The factors affecting retardation factor are the solvent system, amount of material spotted, adsorbent and temperature. TLC is one of the fastest, least expensive, simplest and easiest chromatography technique.



Thin Layer Chromatography Procedure

Before starting with the Thin Layer Chromatography Experiment, let us understand the different components required to conduct the procedure along with the phases involved.

1. Thin Layer Chromatography Plates – ready-made plates are used which are chemically inert and stable. The stationary phase is applied on its surface in the form of a thin layer. The stationary phase on the plate has a fine particle size and also has a uniform thickness.
2. Thin Layer Chromatography Chamber – Chamber is used to develop plates. It is responsible to keep a steady environment inside which will help in developing spots. Also, it prevents the solvent evaporation and keeps the entire process dust-free.
3. Thin Layer Chromatography Mobile phase – Mobile phase is the one that moves and consists of a solvent mixture or a solvent. This phase should be particulate-free. The higher the quality of purity the development of spots is better.
4. Thin Layer Chromatography Filter Paper – It has to be placed inside the chamber. It is moistened in the mobile phase.

Thin Layer Chromatography Experiment

The stationary phase that is applied to the plate is made to dry and stabilize.

- To apply sample spots, thin marks are made at the bottom of the plate with the help of a pencil.
- Apply sample solutions to the marked spots.
- Pour the mobile phase into the TLC chamber and to maintain equal humidity, place a moistened filter paper in the mobile phase.
- Place the plate in the TLC chamber and close it with a lid. It is kept in such a way that the sample faces the mobile phase.
- Immerse the plate for development. Remember to keep the sample spots well above the level of the mobile phase. Do not immerse it in the solvent.
- Wait till the development of spots. Once the spots are developed, take out the plates and dry them. The sample spots can be observed under a UV light chamber.

Thin Layer Chromatography Applications

- The qualitative testing of Various medicines such as sedatives, local anaesthetics, anticonvulsant tranquilisers, analgesics, antihistamines, steroids, hypnotics is done by TLC.
- TLC is extremely useful in Biochemical analysis such as separation or isolation of biochemical metabolites from its blood plasma, urine, body fluids, serum, etc.
- Thin layer chromatography can be used to identify natural products like essential oils or volatile oil, fixed oil, glycosides, waxes, alkaloids, etc.
- It is widely used in separating multicomponent pharmaceutical formulations.
- It is used for the purification of samples and direct comparison is done between the sample and the authentic sample.
- It is used in the food industry, to separate and identify colours, sweetening agent, and preservatives
- It is used in the cosmetic industry.
- It is used to study if a reaction is complete.

Disadvantages Of Thin Layer Chromatography:

1. Thin Layer Chromatography plates do not have longer stationary phase.
2. When compared to other chromatographic techniques the length of separation is limited.
3. The results generated from TLC are difficult to reproduce.
4. Since TLC operates as an open system, some factors such as humidity and temperature can be can affect the final outcome of the chromatogram.
5. The detection limit is high and therefore if you want a lower detection limit, you cannot use TLC.
6. It is only a qualitative analysis technique and not quantitative.

GAS CHROMATOGRAPHY

- Gas chromatography differs from other forms of **chromatography** in that the mobile phase is a gas and the components are separated as vapors.
- It is thus used to separate and detect small molecular weight compounds in the gas phase.
- The sample is either a gas or a liquid that is vaporized in the injection port. The mobile phase for gas chromatography is a carrier gas, typically helium because of its low molecular weight and being chemically inert.
- The pressure is applied and the mobile phase moves the analyte through the column. The separation is accomplished using a column coated with a stationary phase.

Principle of Gas chromatography (how does gas chromatography work)

The equilibrium for gas chromatography is partitioning, and the components of the sample will partition (i.e. distribute) between the two phases: the stationary phase and the mobile phase.

Compounds that have a greater affinity for the stationary phase spend more time in the column and thus elute later and have a longer **retention time (Rt)** than samples that have a higher affinity for the mobile phase.

Affinity for the stationary phase is driven mainly by intermolecular interactions and the polarity of the stationary phase can be chosen to maximize interactions and thus the separation.

Ideal peaks are Gaussian distributions and symmetrical, because of the random nature of the analyte interactions with the column.

- The separation is hence accomplished by partitioning the sample between the gas and a thin layer of a nonvolatile liquid held on a solid support.
- A sample containing the solutes is injected into a heated block where it is immediately vaporized and swept as a plug of vapor by the carrier gas stream into the column inlet.
- The solutes are adsorbed by the stationary phase and then desorbed by a fresh carrier gas.
- The process is repeated in each plate as the sample is moved toward the outlet.
- Each solute will travel at its own rate through the column.
- Their bands will separate into distinct zones depending on the partition coefficients, and band spreading.
- The solutes are eluted one after another in the increasing order of their kd, and enter into a detector attached to the exit end of the column.
- Here they register a series of signals resulting from concentration changes and rates of elution on the recorder as a plot of time versus the composition of carrier gas stream.
- The appearance time, height, width, and area of these peaks can be measured to yield quantitative data.

as chromatography is mainly composed of the following parts:

1. **Carrier gas in a high-pressure cylinder with attendant pressure regulators and flow meters**

- Helium, N_2, H, Argon are used as carrier gases.

- Helium is preferred for thermal conductivity detectors because of its high thermal conductivity relative to that of most organic vapors.
- N_2 is preferable when a large consumption of carrier gas is employed.
- Carrier gas from the tank passes through a toggle valve, a flow meter, (1-1000 ml/min), capillary restrictors, and a pressure gauge (1-4 atm).
- Flow rate is adjusted by means of a needle valve mounted on the base of the flow meter and controlled by capillary restrictors.
- The operating efficiency of the gas chromatograph is directly dependant on the maintenance of constant gas flow.

2. **Sample injection system**

- Liquid samples are injected by a microsyringe with a needle inserted through a self-scaling, silicon-rubber septum into a heated metal block by a resistance heater.
- Gaseous samples are injected by a gas-tight syringe or through a by-pass loop and valves.
- Typical sample volumes range from 0.1 to 0.2 ml.

3. **The separation column**

- The heart of the gas chromatography is the column which is made of metals bent in U shape or coiled into an open spiral or a flat pancake shape.
- Copper is useful up to 250^0
- Swege lock fittings make column insertion easy.
- Several sizes of columns are used depending upon the requirements.

4. **Liquid phases**

- An infinite variety of liquid phases are available limited only by their volatility, thermal stability and ability to wet the support.
- No single phase will serve for all separation problems at all temperatures.

Non-Polar – Parafin, squalane, silicone greases, apiezon L, silicone gum rubber. These materials separate the components in order of their boiling points.

Intermediate Polarity – These materials contain a polar or polarizable group on a long non-polar skeleton which can dissolve both polar and non-polar solutes. For example. diethyl hexyl phthalate is used for the separation of high boiling alcohols.

Polar – Carbowaxes – Liquid phases with a large proportion of polar groups. Separation of polar and non-polar substances.

Hydrogen bonding – Polar liquid phases with high hydrogen bonding e.g. Glycol.

Specific purpose phases – Relying on a chemical reaction with solute to achieve separations. e.g AgNO3 in glycol separates unsaturated hydrocarbons.

5. **Supports**

- The structure and surface characteristics of the support materials are important parameters, which determine the efficiency of the support and the degree of separation respectively.
- The support should be inert but capable of immobilizing a large volume of liquid phase as a thin film over its surface.
- The surface area should be large to ensure the rapid attainment of equilibrium between stationary and mobile phases.
- Support should be strong enough to resist breakdown in handling and be capable of packed into a uniform bed.
- Diatomaceous earth, kieselguhr treated with Na 2CO 3 for 900^0 C causes the particle fusion into coarser aggregates.
- Glass beads with a low surface area and low porosity can be used to coat up to 3% stationary phases.
- Porous polymer beads differing in the degree of cross-linking of styrene with alkyl-vinyl benzene are also used which are stable up to 250^0

6. **Detector**

- Detectors sense the arrival of the separated components and provide a signal.
- These are either concentration-dependent or mass dependant.
- The detector should be close to the column exit and the correct temperature to prevent decomposition.

7. **Recorder**

- The recorder should be generally 10 mv (full scale) fitted with a fast response pen (1 sec or less). The recorder should be connected with a series of good quality resistances connected across the input to attenuate the large signals.
- An integrator may be a good addition.

The procedure of Gas Chromatography

Step 1: Sample Injection and Vapourization

1. A small amount of liquid sample to be analyzed is drawn up into a syringe.
2. The syringe needle is positioned in the hot injection port of the gas chromatograph and the sample is injected quickly.
3. The injection of the sample is considered to be a "point" in time, that is, it is assumed that the entire sample enters the gas chromatograph at the same time, so the sample must be injected quickly.
4. The temperature is set to be higher than the boiling points of the components of the mixture so that the components will vaporize.
5. The vaporized components then mix with the inert gas mobile phase to be carried to the gas chromatography column to be separated.
6.

Step 2: Separation in the Column

- Components in the mixture are separated based on their abilities to adsorb on or bind to, the stationary phase.
- A component that adsorbs most strongly to the stationary phase will spend the most time in the column (will be retained in the column for the longest time) and will, therefore, have the longest retention time (Rt). It will emerge from the gas chromatograph last.
- A component that adsorbs the least strongly to the stationary phase will spend the least time in the column (will be retained in the column for the shortest time) and will, therefore, have the shortest retention time (Rt). It will emerge from the gas chromatograph first.
- If we consider a 2 component mixture in which component A is more polar than component B then:

1. component A will have a **longer retention time** in a polar column than component B

2. component A will have a **shorter retention time** in a non-polar column than component B

Step 3: Detecting and Recording Results

1. The components of the mixture reach the detector at different times due to differences in the time they are retained in the column.
2. The component that is retained the shortest time in the column is detected first. The component that is retained the longest time in the column is detected last.
3. The detector sends a signal to the chart recorder which results in a peak on the chart paper. The component that is detected first is recorded first. The component that is detected last is **recorded last.**

Applications

- GC analysis is used to calculate the content of a chemical product, for example in assuring the quality of products in the chemical industry; or measuring toxic substances in soil, air or water.
- Gas chromatography is used in the analysis of:

  (a) air-borne pollutants

  (b) performance-enhancing drugs in athlete's urine samples

  (c) oil spills

  (d) essential oils in perfume preparation
- GC is very accurate if used properly and can measure picomoles of a substance in a 1 ml liquid sample, or parts-per-billion concentrations in gaseous samples.
- Gas Chromatography is used extensively in forensic science. Disciplines as diverse as solid drug dose (pre-consumption form) identification and quantification, arson investigation, paint chip analysis, and toxicology cases, employ GC to identify and quantify various biological specimens and crime-scene evidence.

Advantages

- The use of longer columns and higher velocity of carrier gas permits the fast separation in a matter of a few minutes.
- Higher working temperatures up to 5000C and the possibility of converting any material into a volatile component make gas chromatography one of the most versatile techniques.

- GC is popular for environmental monitoring and industrial applications because it is very reliable and can be run nearly continuously.
- GC is typically used in applications where small, volatile molecules are detected and with non-aqueous solutions.
- GC is favored for non-polar molecules.

Limitations

- Compound to be analyzed should be stable under GC operation conditions.
- They should have a vapor pressure significantly greater than zero.
- Typically, the compounds analyzed are less than 1,000 Da, because it is difficult to vaporize larger compounds.
- The samples are also required to be salt-free; they should not contain ions.
- Very minute amounts of a substance can be measured, but it is often required that the sample must be measured in comparison to a sample containing the pure, suspected substance known as a reference standard.

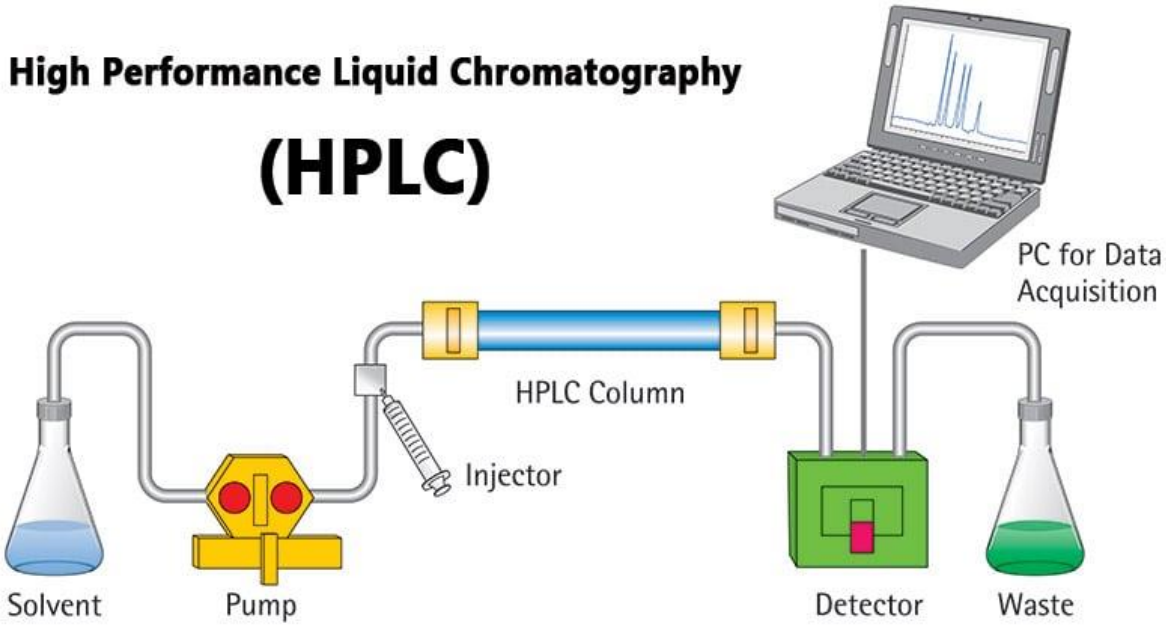
HPLC

High-performance liquid chromatography or commonly known as HPLC, is an analytical technique used to separate, identify or quantify each component in a mixture.

The mixture is separated using the basic principle of column **chromatography** and then identified and quantified by spectroscopy.

In the 1960s, the column chromatography LC with its low-pressure suitable glass columns was further developed to the HPLC with its high-pressure adapted metal columns.

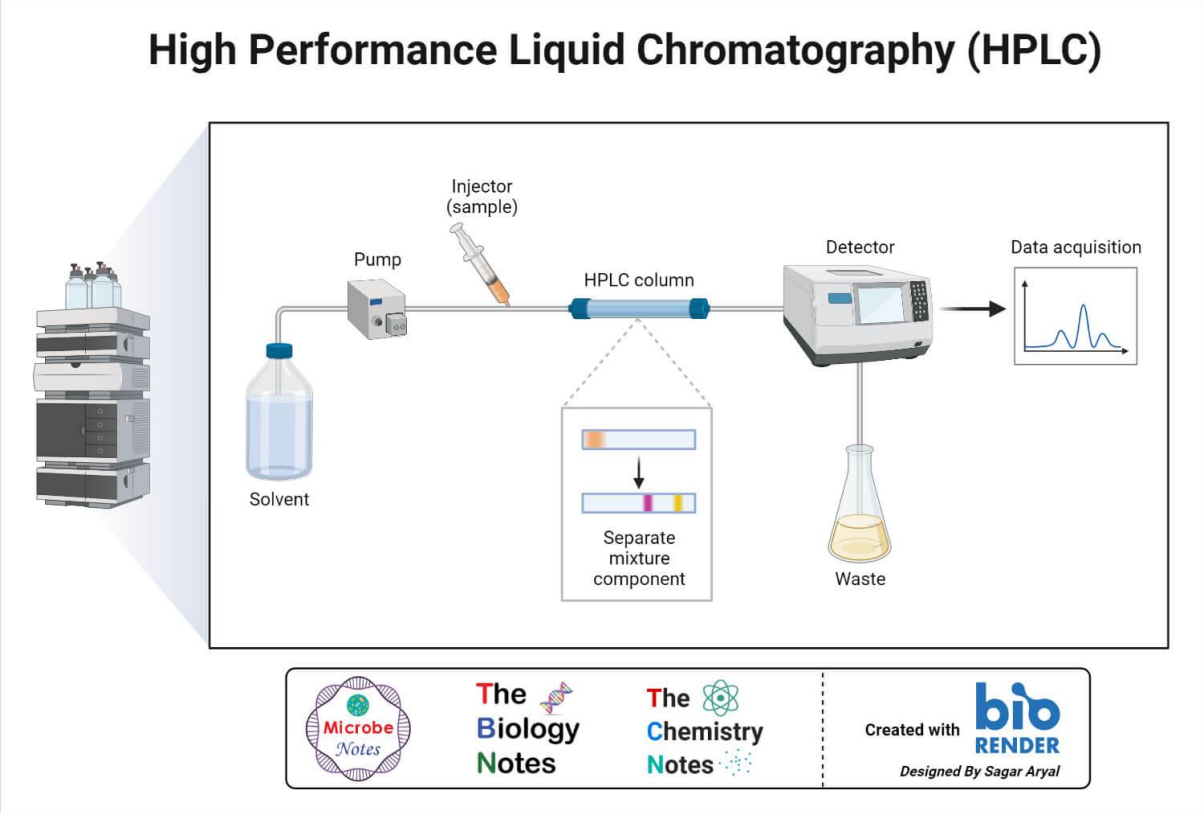
HPLC is thus basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres.



HPLC Principle

- The purification takes place in a separation column between a stationary and a mobile phase.
- The stationary phase is a granular material with very small porous particles in a separation column.
- The mobile phase, on the other hand, is a solvent or solvent mixture which is forced at high pressure through the separation column.
- Via a valve with a connected sample loop, i.e. a small tube or a capillary made of stainless steel, the sample is injected into the mobile phase flow from the pump to the separation column using a syringe.
- Subsequently, the individual components of the sample migrate through the column at different rates because they are retained to a varying degree by interactions with the stationary phase.
- After leaving the column, the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer.
- At the end of this operation/run, a chromatogram in the HPLC software on the computer is obtained.
- The chromatogram allows the identification and quantification of the different substances.

Instrumentation of HPLC



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Pump

- The development of HPLC led to the development of the pump system.
- The pump is positioned in the most upper stream of the liquid chromatography system and generates a flow of eluent from the solvent reservoir into the system.
- High-pressure generation is a "standard" requirement of pumps besides which, it should also to be able to provide a consistent pressure at any condition and a controllable and reproducible flow rate.
- Most pumps used in current LC systems generate the flow by back-and-forth motion of a motor-driven piston (reciprocating pumps). Because of this piston motion, it produces "pulses".

Injector

- An injector is placed next to the pump.
- The simplest method is to use a syringe, and the sample is introduced to the flow of eluent.
- The most widely used injection method is based on sampling loops.
- The use of the autosampler (auto-injector) system is also widely used that allows repeated injections in a set scheduled-timing.

Column

- The separation is performed inside the column.
- The recent columns are often prepared in a stainless steel housing, instead of glass columns.
- The packing material generally used is silica or polymer gels compared to calcium carbonate. The eluent used for LC varies from acidic to basic solvents.
- Most column housing is made of stainless steel since stainless is tolerant towards a large variety of solvents.

Detector

- Separation of analytes is performed inside the column, whereas a detector is used to observe the obtained separation.
- The composition of the eluent is consistent when no analyte is present. While the presence of analyte changes the composition of the eluent. What detector does is to measure these differences.
- This difference is monitored as a form of an electronic signal. There are different types of detectors available.

Recorder

- The change in eluent detected by a detector is in the form of an electronic signal, and thus it is still not visible to our eyes.
- In older days, the pen (paper)-chart recorder was popularly used. Nowadays, a computer-based data processor (integrator) is more common.
- There are various types of data processors; from a simple system consisting of the in-built printer and word processor while those with software that are specifically designed for an LC system which not only data acquisition but features like peak-fitting, baseline correction, automatic concentration calculation, molecular weight determination, etc.

Degasser

The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes.

- When gas is present in the eluent, this is detected as noise and causes an unstable baseline.
- Degasser uses special polymer membrane tubing to remove gases.
- The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go through the pore.

Column Heater

The LC separation is often largely influenced by the column temperature.

- In order to obtain repeatable results, it is important to keep consistent temperature conditions.
- Also for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperatures (50 to 80°C).
- Thus columns are generally kept inside the column oven (column heater).

Types of HPLC

1. **Normal phase:**

Column packing is polar (e.g silica) and the mobile phase is non-polar. It is used for water-sensitive compounds, geometric isomers, cis-trans isomers, and chiral compounds.

2. **Reverse phase:**

The column packing is non-polar (e.g C18), the mobile phase is water+ miscible solvent (e.g methanol). It can be used for polar, non-polar, ionizable, and ionic samples.

3. **Ion exchange:**

Column packing contains ionic groups and the mobile phase is buffer. It is used to separate anions and cations.

4. **Size exclusion:**

Molecules diffuse into pores of a porous medium and are separated according to their relative size to the pore size. Large molecules elute first and smaller molecules elute later.

Applications of HPLC

The HPLC has developed into a universally applicable method so that it finds its use in almost all areas of chemistry, biochemistry, and pharmacy.

- Analysis of drugs
- Analysis of synthetic polymers
- Analysis of pollutants in environmental analytics
- Determination of drugs in biological matrices
- Isolation of valuable products
- Product purity and quality control of industrial products and fine chemicals
- Separation and purification of biopolymers such as enzymes or nucleic acids
- Water purification
- Pre-concentration of trace components

- Ligand-exchange chromatography
- Ion-exchange chromatography of proteins
- High-pH anion-exchange chromatography of carbohydrates and oligosaccharides

Advantages of HPLC

1. Speed
2. Efficiency
3. Accuracy
4. Versatile and extremely precise when it comes to identifying and quantifying chemical components.

Limitations of HPLC

1. **Cost:** Despite its advantages, HPLC can be costly, requiring large quantities of expensive organics.
2. **Complexity**
3. HPLC does have **low sensitivity** for certain compounds, and some cannot be detected as they are irreversibly adsorbed.
4. Volatile substances are better separated by gas chromatography.

LIGHT MICROSCOPE

A light microscope is a biological laboratory equipment, which uses visible light to detect and magnify very small objects.

A light microscope uses a lens to focus light on a specimen, magnifying it to produce an image. The specimen is usually placed close to a microscopic lens.

Microscopic magnification varies greatly depending on the type and number of lenses that make up the microscope. Depending also on the number of lenses, there are two types of microscope:

- Simple light microscope: it has a low magnification because it uses a single lens.
- Compound light microscope: has a higher magnification than a simple microscope because it uses at least two sets of lenses, an objective lens and an eyepiece.

Light Microscope Function

The function of the light microscope is based on its ability to focus a beam of light through a very small and transparent specimen, to produce an image.

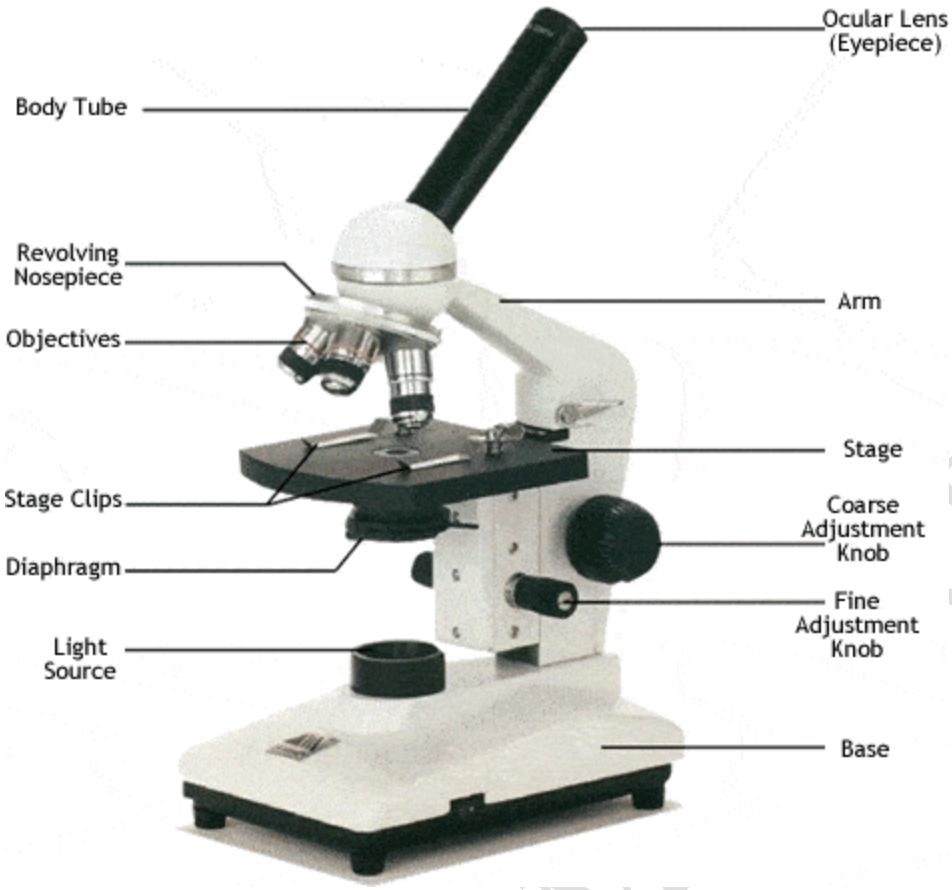
The image is then passed through one or two lenses for magnification to view. The transparency of the specimen allows for easy and fast light penetration. Specimens can vary from bacteria to cells and other microbial particles.

The principle of a light microscope is to visualize an image by using the ability of the lens to bend light and focus it on the specimen is what makes up the image.

When a ray of light passes from one medium to another, it is bent at the interface causing refraction.

The bending of light is determined by its index of refraction, which is a measure of how much a substance slows down the speed of light. The direction and magnitude of the bending of light is determined by the refractive index of the two mediums that make up the interface.

Light Microscope Parts



- **Ocular**

The ocular lens is part of the optical system, which is directed at the user of the microscope. This is the part that has at least one or more lenses.

The function of the ocular lens on a microscope is to convert the enlarged real-middle image from the objective into an enlarged virtual-image. The size of the incoming light cone will be adjusted to the size of the human eye.

- **Body Tube**

Body tube is part of the microscope that serves to accommodate the lens system that enlarges the specimen.

- **Objectives**

Objective revolvers are used in microscopes with multiple objective lenses, which have different magnification factors. By rotating the revolver, you can select the lens with the desired level of magnification.

- **Arm**

Arm is a holder connected to all components that function as a support for the microscope so that the microscope can be used properly.

- **Stage Clip**

The stage clip acts as a holder for the object plate and ensures that it doesn't fall out of place accidentally.

- **Microscope Stage / Cross Table**

The microscope stage is the part that serves to place the object plate with a cover glass on it. By sliding the platen, you can select the part of the object you want to view.

Higher quality microscopes sometimes use a cross table as a table. This makes it possible to slide the object plate through the adjusting screw.

Cross table is a technical term which is a table construction that is mounted into a rail system. There is an adjustment screw, which can be used to move the table with great ease and precision.

The screw is equipped with a measuring scale, so you can easily find a specific point of the object again.

- **Condenser**

Condenser serves to bind the rays from the light source, so that the light can be projected on the object. Thus, every part of the object will be illuminated at the same brightness level.

- **Diaphragm**

The diaphragm usually consists of one or two lenses. These lenses fractionate the light and all the rays leave as parallel beams. Aspherical lenses ensure that no aberrations occur.

This will guarantee better image quality. The production cost of the diaphragm is more expensive than the normal lens. You can tell which microscope is better by looking at it from this diaphragm.

Another way to reflect light perfectly is to use an optical filter with a dielectric surface. Bragg mirror which is a kind of thin mirror with a refractive index that can be high and low.

This mirror allows for very high-quality light beam reflection, which makes the image sharper later on and proactively reduces lens error.

- **Fine Adjustment Knop & Coarse Adjustment Knop**

With fine focus you can adjust the distance between the object and the objective, to achieve the required sharpness. Fine focus works by moving the lens smoothly.

Like fine focus, coarse focus also moves the stage to adjust the difference between the object and the objective. The function of the coarse focus is to capture the exact distance roughly and quickly. Optimal sharpness can be adjusted to finer roughness afterwards.

- **Light Source**

Early microscopes used concave mirrors to reflect light on objects. Then, they used light bulbs. Most microscopes operate with an LED light. The job of the light source is to illuminate the object evenly.

- **Base**

Base is the very bottom. Base serves to accommodate all parts of the light microscope.

How to Use a Light Microscope

To use a light microscope, you can follow the steps below carefully.

- Start with a low lens and a clean slide. The microscope stage should be lowered as low as possible.
- Center the slide so that the specimen is under the objective lens.
- Use the coarse adjustment knob to get a general focus. Then slowly move up the stage until focus is achieved.
- Use the fine adjustment knob to get clear focus.
- Center the specimen on a low plane before moving it to a medium plane.
- Switch the objective lens to medium and focus using a fine adjustment. If you can't see the specimen at this point, return to the low plane and re-center.
- Once you've focused and centered on the medium power, you can move it to even greater magnification.

Types of light microscopes (optical microscope)

With the evolved field of Microbiology, the microscopes
used to view specimens are both simple and compound light microscopes, all using lenses. The difference is simple light microscopes use a single lens for magnification while compound lenses use two or more lenses for magnifications. This means, that a series of lenses are placed in an order such that, one lens magnifies the image further than the initial lens.
The modern types of Light Microscopes include:

1. Bright field Light Microscope
2. Phase Contrast Light Microscope
3. Dark-Field Light Microscope
4. Fluorescence Light Microscope

BRIGHTFIELD MICROSCOPE

Brightfield Microscope is also known as the **Compound Light Microscope**. It is an optical microscope that uses light rays to produce a dark image against a bright background. It is the standard microscope that is used in Biology, Cellular Biology, and Microbiological Laboratory studies.

This microscope is used to view fixed and live specimens, that have been stained with basic stains which gives a contrast between the image and the image background. It is specially designed with magnifying glasses known as lenses that modify the specimen to produce an image seen through the eyepiece.

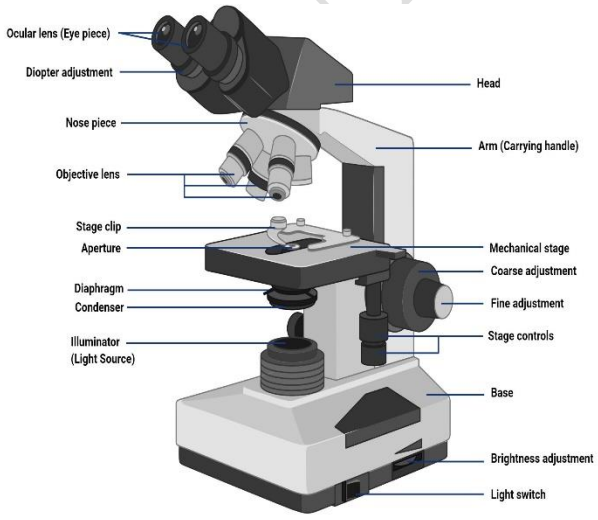
Principle of Brightfield Microscope

For a specimen to be the focus and produce an image under the Brightfield Microscope, the specimen must pass through a uniform beam of the illuminating light. Through differential absorption and differential refraction, the microscope will produce a contrasting image.

The specimens used are prepared initially by staining to introduce color for easy contracting characterization. The colored specimens will have a refractive index that will differentiate it from the surrounding, presenting a combination of absorption and refractive contrast.

The functioning of the microscope is based on its ability to produce a high-resolution image from an adequately provided light source, focused on the image, producing a high-quality image.

The specimen which is placed on a microscopic slide is viewed under oil immersion or/and covered with a coverslip.



The brightfield microscope is made up of various parts, including

- **Eyepiece (Ocular lens)** – it has two eyepiece lenses at the top of the microscope which focuses the image from the objective lenses. this is where you see the formed image from, with your eyes.
- **The objective lenses** which are made up of six or more glass lenses, which make a clear image clear from the specimen or the object that is being focused.
- **Two focusing knobs** i.e the fine adjustment knob and the coarse adjustment knob, found on the microscopes' arm, which can move the stage or the nosepiece to focus on the image. Their function is to ensure the production of a sharp image with clarity.
- **The stage** is found just below the objectives and this is where the specimen is placed, allowing movement of the specimen around for better viewing with the flexible knobs and it is where the light is focused on.
- **The condenser**: It is mounted below the stage which focuses a beam of light onto the specimen. It can be fixed or movable, to adjust the quality of light, but this entirely depends on the microscope.
- **The arm**: This is a sturdy metallic backbone of the microscope, used to carry and move the microscope from one place to another. They also hold the microscope **base** which is the stand of the microscope. The arm and the base hold all the microscopic parts.
- It has a **light illuminator** or a **mirror** found at the base or on the microscope's nosepiece.
- The **nosepiece** has about two to five objective lenses with different magnifying power. It can move round to any position depending on the objective lens to focus on the image.
- **An aperture diaphragm (contrast)**: It controls the diameter of the beam of light that passes through the condenser. When the condenser is almost closed, the light comes through to the center of the condenser creating high contrast and when the condenser is widely open, the image is very bright with very low contrast.

Magnification by Brightfield Microscope

- The objective lenses are the main lenses used for focusing the image, on the condenser. This produces an enlarged clear image that is then magnified again by the eyepiece to form the primary image that is seen by the eyes.
- During imaging, the objective lenses remain parfocal in that, even when the objective lens has changed the image still remains focused. The image seen at the eyepiece is the enlarged clear image of the specimen, known as the virtual image.
- The magnification of the image is determined by the magnification of the objective against the magnification of the eyepiece lens. The objectives have a magnification power of 40x-1000x depending on the type of brightfield microscope while the eyepiece lens has a standard magnification power of 10x.
- Therefore to calculate:

Total Magnification power = Magnification of the objective lens x Magnification of the eyepiece

- For example: if the magnification of the objective is 45x and that of the eyepiece is 10x, the total magnification of the specimen will be 450x.
- The magnification is standard, i.e. not too high nor too low, and therefore depending on the magnification power of the lenses, it will range between 40X and 100oX.
- The objective lens enlarges the image which can be viewed, a characteristic known as resolution. Resolution according to Prescott, is the ability of a lens to separate or distinguish between small objects closely linked together.
- Whereas the eyepiece magnifies the image at the end of the viewing, its magnification range is lower than that of the objective lens at 8X-12X (10X standard) and that of the objective lens at 40X-100X, magnification, and resolution of the microscope is highly dependent on the objective lens.

Applications of Brightfield microscope

Brightfield Microscope is used in several fields, from basic biology to understanding cell structures in cell Biology, Microbiology, Bacteriology to visualizing parasitic organisms in

Parasitology. Most of the specimens to be viewed are stained using special staining to enable visualization. Some of the staining techniques used include Negative staining and Gram staining.

Some of its applications include:

1. Used to visualize and study the animal cells
2. Used to visualize and study plant cells.
3. Used to visualize and study the morphologies of bacterial cells
4. Used to identify parasitic protozoans such as *Paramecium.*

Advantages of Brightfield Microscope

1. It is simple to use with few adjustments involved while viewing the image.
2. It can be used to view both stained and unstained.
3. The optics of the microscope do not alter the color of the specimen.
4. The microscope can be adjusted and modified for better viewing such as installing a camera, to form a digital microscope or in the way image illumination is done such as by use of fluorochromes on the specimen and viewing under a dark environment, forming a darkfield microscope.

Disadvantages of Brightfield microscope

1. The aperture diaphragm may cause great contrast which may distort the outcome of the image, therefore iris diaphragm is preferred.
2. It cannot be used to view live specimens such as bacterial cells. Only fixed specimens can be viewed under the brightfield microscope.
3. The maximum magnification of the brightfield microscope is 100x but modification can readjust the magnification to 1000x which is the optimum magnification of bacterial cells.
4. It has low contrast hence most specimens must be stained for them to be visualized.
5. The use of oil immersion may distort the image
6. The use of a coverslip may damage the specimen

7. Staining may introduce extraneously unwanted details into the specimen or contaminate the specimen.
8. It is tedious to stain the specimen before visualizing it under the brightfield microscope.
9. The microscope needs a strong light source for magnification and sometimes the light source may produce a lot of heat which may damage or kill the specimen.

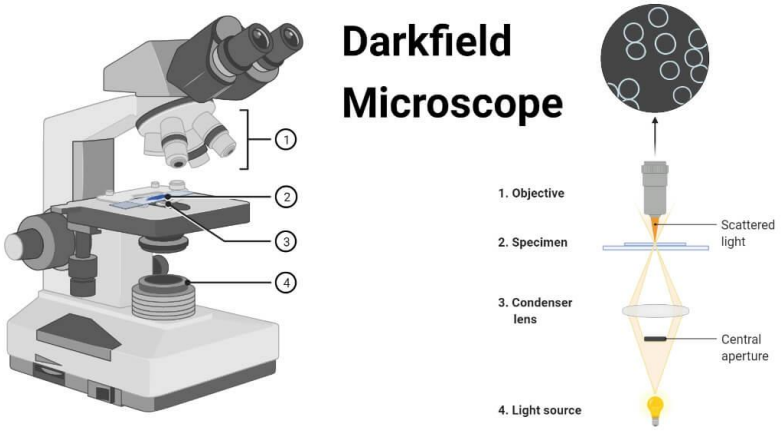
DARKFIELD MICROSCOPE

- Microbiology, the branch of science that has so vastly extended and expanded our knowledge of the living world, owes its existence to Antoni van Leeuwenhoek.
- In 1673, with the aid of a crude microscope consisting of a biconcave lens enclosed in two metal plates, Leeuwenhoek introduced the world to the existence of microbial forms of life.
- Over the years, microscopes have evolved from the simple, single-lens instrument of Leeuwenhoek, with a magnification of 300 X, to the present-day electron microscopes capable of magnifications greater than 250,000X.
- Microscopes are designated as either light microscopes or electron microscopes.
- Light microscopes use visible light or ultraviolet rays to illuminate specimens. They include **brightfield**, darkfield, phase-contrast, and fluorescent instruments.
- This is similar to the ordinary light microscope; however, the condenser system is modified so that the specimen is not illuminated directly.
- The condenser directs the light obliquely so that the light is deflected or scattered from the specimen, which then appears bright against a dark background.

- Living specimens may be observed more readily with darkfield than with brightfield microscopy.

Principle of the Darkfield Microscope

- A dark field microscope is arranged so that the light source is blocked off, causing light to scatter as it hits the specimen.
- This is ideal for making objects with refractive values similar to the background appear bright against a dark background.
- When light hits an object, rays are scattered in all azimuths or directions. The design of the dark field microscope is such that it removes the dispersed light, or zeroth order, so that only the scattered beams hit the sample.
- The introduction of a condenser and/or stop below the stage ensures that these light rays will hit the specimen at different angles, rather than as a direct light source above/below the object.
- The result is a "cone of light" where rays are diffracted, reflected and/or refracted off the object, ultimately, allowing the individual to view a specimen in dark field.



This is to say:

1. The dark-ground microscopy makes use of the dark-ground microscope, a special type of compound light microscope.
2. The dark-field condenser with a central circular stop, which illuminates the object with a cone of light, is the most essential part of the dark-ground microscope.
3. This microscope uses reflected light instead of transmitted light used in the ordinary light microscope.
4. It prevents light from falling directly on the objective lens.
5. Light rays falling on the object are reflected or scattered onto the objective lens with the result that the microorganisms appear brightly stained against a dark background.

Uses of Darkfield Microscope

The dark ground microscopy has the following uses:

- It is useful for the demonstration of very thin bacteria not visible under ordinary illumination since the reflection of the light makes them appear larger.
- This is a frequently used method for rapid demonstration of *Treponema pallidum* in clinical specimens.
- It is also useful for the demonstration of the motility of flagellated bacteria and protozoa.
- Darkfield is used to study marine organisms such as algae, plankton, diatoms, insects, fibres, hairs, yeast and protozoa as well as some minerals and crystals, thin polymers and some ceramics.
- Darkfield is used to study mounted cells and tissues.
- It is more useful in examining external details, such as outlines, edges, grain boundaries and surface defects than internal structure.

Advantages of Darkfield Microscope

- Dark-field microscopy is a very simple yet effective technique.
- It is well suited for uses involving live and unstained biological samples, such as a smear from a tissue culture or individual, water-borne, single-celled organisms.

- Considering the simplicity of the setup, the quality of images obtained from this technique is impressive.
- Dark-field microscopy techniques are almost entirely free of artifacts, due to the nature of the process.
- A researcher can achieve a dark field by making modifications to his/her microscope.

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Limitations of Darkfield Microscope

- The main limitation of dark-field microscopy is the low light levels seen in the final image.
- The sample must be very strongly illuminated, which can cause damage to the sample.

FLUORESCENCE MICROSCOPE

A **fluorescence microscope** is an optical microscope that uses fluorescence and phosphorescence instead of, or in addition to, reflection and absorption to study the properties of organic or inorganic substances. Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation while phosphorescence is a specific type of photoluminescence related to fluorescence. Unlike fluorescence, a phosphorescent material does not immediately re-emit the radiation it absorbs. The fluorescence microscope was devised in the early part of the twentieth century by August Köhler, Carl Reichert, and Heinrich Lehmann, among others.

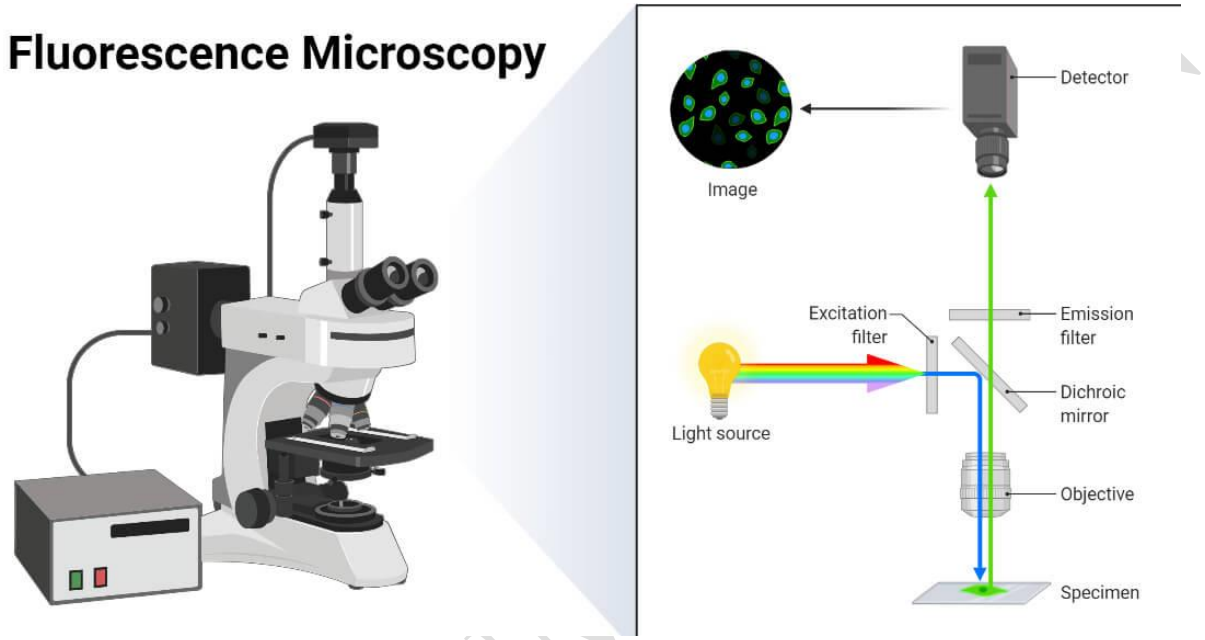
Principle of Fluorescence Microscope

Most cellular components are colorless and cannot be clearly distinguished under a microscope. The basic premise of fluorescence microscopy is to stain the components with dyes.

Fluorescent dyes, also known as fluorophores or fluorochromes, are molecules that absorb excitation light at a given wavelength (generally UV), and after a short delay emit light at a

longer wavelength. The delay between absorption and emission is negligible, generally on the order of nanoseconds.

The emission light can then be filtered from the excitation light to reveal the location of the fluorophores.



- Fluorescence microscopy uses a much higher intensity light to illuminate the sample. This light excites fluorescence species in the sample, which then emits light of a longer wavelength.
- The image produced is based on the second light source or the emission wavelength of the fluorescent species rather than from the light originally used to illuminate, and excite, the sample.

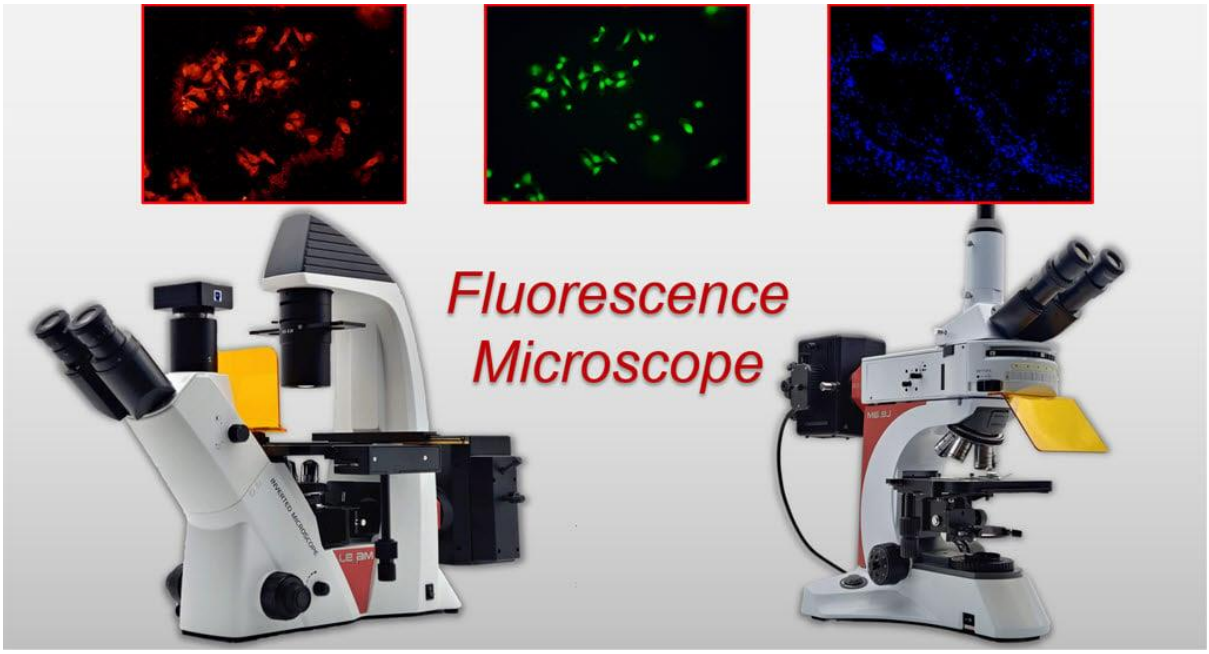
Working

Light of the excitation wavelength is focused on the specimen through the objective lens. The fluorescence emitted by the specimen is focused on the detector by the objective. Since most of the excitation light is transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light.

Forms

The “fluorescence microscope” refers to any microscope that uses fluorescence to generate an image, whether it is a more simple set up like an epifluorescence microscope, or a more complicated design such as a confocal microscope, which uses optical sectioning to get better resolution of the fluorescent image.

Most fluorescence microscopes in use are epifluorescence microscopes, where excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective).



Typical components of a fluorescence microscope are:

- **Fluorescent dyes (Fluorophore)**
  - A fluorophore is a fluorescent chemical compound that can re-emit light upon light excitation.
  - Fluorophores typically contain several combined aromatic groups, or plane or cyclic molecules with several π bonds.

- Many fluorescent stains have been designed for a range of biological molecules.
- Some of these are small molecules that are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains like DAPI and Hoechst, phalloidin which is used to stain actin fibers in mammalian cells.

- **A light source**
  - Four main types of light sources are used, including xenon arc lamps or mercury-vapor lamps with an excitation filter, lasers, and high- power LEDs.
  - Lasers are mostly used for complex fluorescence microscopy techniques, while xenon lamps, and mercury lamps, and LEDs with a dichroic excitation filter are commonly used for wide-field epifluorescence microscopes.
- **The excitation filter**
  - The exciter is typically a bandpass filter that passes only the wavelengths absorbed by the fluorophore, thus minimizing the excitation of other sources of fluorescence and blocking excitation light in the fluorescence emission band.
- **The dichroic mirror**
  - A dichroic filter or thin-film filter is a very accurate color filter used to selectively pass light of a small range of colors while reflecting other colors.
- **The emission filter.**
  - The emitter is typically a bandpass filter that passes only the wavelengths emitted by the fluorophore and blocks all undesired light outside this band – especially the excitation light.
  - By blocking unwanted excitation energy (including UV and IR) or sample and system autofluorescence, optical filters ensure the darkest background.

Applications of Fluorescence Microscope

- To identify structures in fixed and live biological samples.
- Fluorescence microscopy is a common tool for today's life science research because it allows the use of multicolor staining, labeling of structures within cells, and the measurement of the physiological state of a cell.

Advantages of Fluorescence Microscope

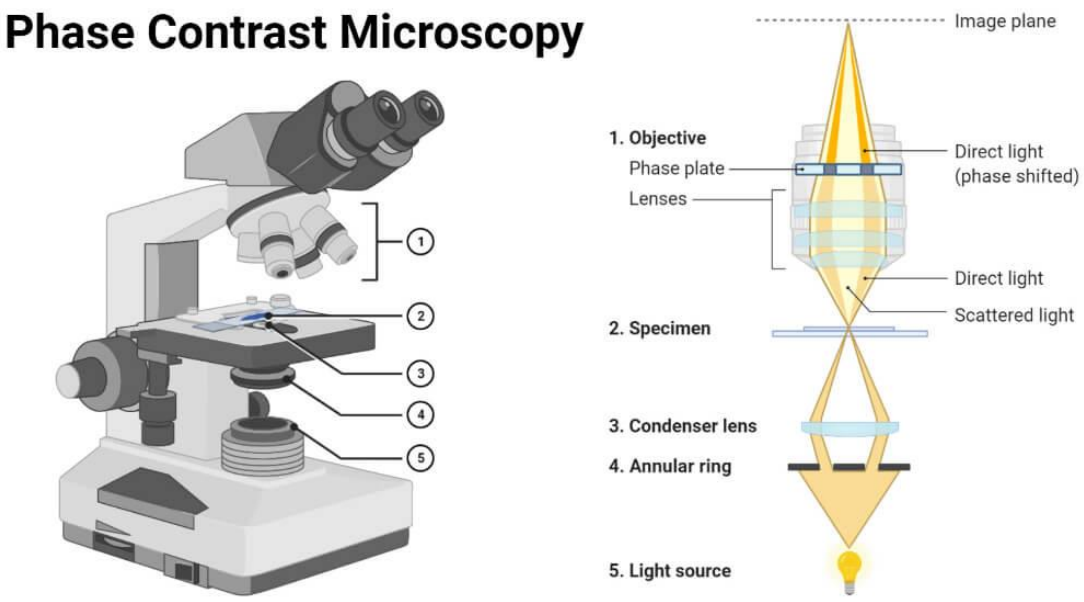
1. Fluorescence microscopy is the most popular method for studying the dynamic behavior exhibited in live-cell imaging.
2. This stems from its ability to isolate individual proteins with a high degree of specificity amidst non-fluorescing material.
3. The sensitivity is high enough to detect as few as 50 molecules per cubic micrometer.
4. Different molecules can now be stained with different colors, allowing multiple types of the molecule to be tracked simultaneously.
5. These factors combine to give fluorescence microscopy a clear advantage over other optical imaging techniques, for both in vitro and in vivo imaging.

Limitations of Fluorescence Microscope

- Fluorophores lose their ability to fluoresce as they are illuminated in a process called photobleaching. Photobleaching occurs as the fluorescent molecules accumulate chemical damage from the electrons excited during fluorescence.
- Cells are susceptible to phototoxicity, particularly with short-wavelength light. Furthermore, fluorescent molecules have a tendency to generate reactive chemical species when under illumination which enhances the phototoxic effect.
- Unlike transmitted and reflected light microscopy techniques fluorescence microscopy only allows observation of the specific structures which have been labeled for fluorescence

PHASE CONTRAST MICROSCOPY

Phase-contrast microscopy is an optical microscopy technique that converts phase shifts in the light passing through a transparent specimen to brightness changes in the image. It was first described in 1934 by Dutch physicist Frits Zernike.



When light passes through cells, small phase shifts occur, which are invisible to the human eye. In a phase-contrast microscope, these phase shifts are converted into changes in amplitude, which can be observed as differences in image contrast.

The Working of Phase contrast Microscopy

1. Partially coherent illumination produced by the tungsten-halogen lamp is directed through a collector lens and focused on a specialized annulus (labeled condenser annulus) positioned in the substage condenser front focal plane.
2. Wavefronts passing through the annulus illuminate the specimen and either pass through undeviated or are diffracted and retarded in phase by structures and phase gradients present in the specimen.
3. Undeviated and diffracted light collected by the objective is segregated at the rear focal plane by a phase plate and focused at the intermediate image plane to form the final phase-contrast image observed in the eyepieces.

Parts of Phase contrast Microscopy

Phase-contrast microscopy is basically a specially designed light microscope with all the basic parts in addition to which an annular phase plate and annular diaphragm are fitted.

The annular diaphragm

- It is situated below the condenser.
- It is made up of a circular disc having a circular annular groove.
- The light rays are allowed to pass through the annular groove.
- Through the annular groove of the annular diaphragm, the light rays fall on the specimen or object to be studied.
- At the back focal plane of the objective develops an image.
- The annular phase plate is placed at this back focal plane.
-

The phase plate

- It is either a negative phase plate having a thick circular area or a positive phase plate having a thin circular groove.
- This thick or thin area in the phase plate is called the conjugate area.
- The phase plate is a transparent disc.
- With the help of the annular diaphragm and the phase plate, the phase contrast is obtained in this microscope.
- This is obtained by separating the direct rays from the diffracted rays.
- The direct light rays pass through the annular groove whereas the diffracted light rays pass through the region outside the groove.
- Depending upon the different refractive indices of different cell components, the object to be studied shows a different degree of contrast in this microscope.

Applications of Phase contrast Microscopy

To produce high-contrast images of transparent specimens, such as

1. living cells (usually in culture),
2. microorganisms,
3. thin tissue slices,
4. lithographic patterns,

5. fibers,
6. latex dispersions,
7. glass fragments, and
8. subcellular particles (including nuclei and other organelles).

Advantages of Phase contrast Microscopy

- Living cells can be observed in their natural state without previous fixation or labeling.
- It makes a highly transparent object more visible.
- No special preparation of fixation or staining etc. is needed to study an object under a phase-contrast microscope which saves a lot of time.
- Examining intracellular components of living cells at relatively high resolution. eg: The dynamic motility of **mitochondria**, mitotic chromosomes & vacuoles.
- It made it possible for biologists to study living cells and how they proliferate through cell division.
- Phase-contrast optical components can be added to virtually any brightfield microscope, provided the specialized phase objectives conform to the tube length parameters, and the condenser will accept an annular phase ring of the correct size.

Limitations of Phase contrast Microscopy

- Phase-contrast condensers and objective lenses add considerable cost to a microscope, and so phase contrast is often not used in teaching labs except perhaps in classes in the health professions.
- To use phase-contrast the light path must be aligned.

- Generally, more light is needed for phase contrast than for corresponding bright-field viewing, since the technique is based on the diminishment of the brightness of most objects.

STEREO MICROSCOPE

A stereo microscope is a type of optical microscope that allows the user to see a three-dimensional view of a specimen. Otherwise known as a dissecting microscope or stereo zoom microscope, the stereo microscope differs from the compound light microscope by having separate objective lenses and eyepieces. This results in two separate optical paths for each eye. Three-dimensional visuals are produced by the different angling views for the left and right eye.

Stereo microscopes use reflected light from the object being studied, compared to the transmitted light that is used by compound light microscopes. Magnification ranges from 7.5 to 75x. Opaque, thick, solid objects are ideal for study with these tools.

Most, but not all, stereo microscopes have two light sources: one above the sample, which is reflected in the eyepieces, and one below the sample for illumination through thinner samples. Resolution is determined by the wavelength of light and numerical aperture of the objective, the same as any other form of optical light microscopy.

Uses of stereo microscopes

There are many uses of stereo microscopes across a diverse range of industries. Some uses include:

- Surgery – The operating microscope, a variant of the stereo microscope, is used during microsurgery in many hospitals
- Paleontology – Paleontologists use stereo microscopes when they clean and analyze fossils.
- Biological research – Stereo microscopes are used by biologists to aid in dissections.
- Entomology – Used in the study of insects without having to dissect them.
- Botany – Botanists study flowers and other plant structures using a stereo microscope
- Technicians use stereo microscopes when they repair circuit boards
- Quality control – Stereo microscopes are used across all types of industry to check the quality of products, including looking for microfractures
- Pathology – Pathologists use stereo microscopes to examine skin conditions amongst other uses
- Watchmaking – Stereo microscopes are useful in watchmaking due to the delicate nature and small size of components.

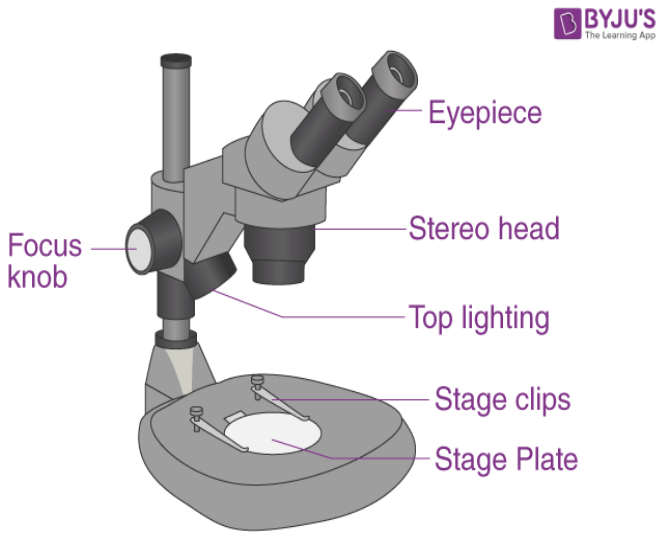
Stereo microscopes: observing the microscopic world in three-dimensions.

A mainstay of modern scientific research, industry, and medical practice, the advantages of stereomicroscopy over standard compound microscopy are notable. Incredibly versatile, they remain one of the most useful pieces of scientific equipment nearly two centuries after the first useable examples were designed, and with further technological developments will no doubt be used for the foreseeable future.

Stereo Microscope

A stereo microscope is defined as a type of microscope that provides a three-dimensional view of a specimen. It is also known as a dissecting microscope. In a stereo microscope, there are separate objective lenses and eyepiece such that there are two separate optical paths for each eye.

Stereo Microscope Diagram



Stereo microscope

Principle of Stereo Microscope

A stereo microscope works on the reflected light from the sample. The magnification of the microscope takes place at low power and hence, it is suitable for magnifying opaque objects. It is suitable for thick and solid samples because it uses light reflected from the sample. The magnification of the stereo microscope is between 20x and 50x.

Applications of Stereo Microscope

- Examination of historic coins and artefacts is possible with the help of the stereomicroscope.
- It finds application in microsurgery.
- Viewing of crystals became easy with the use of a stereomicroscope.

TRANSMISSION ELECTRON MICROSCOPE

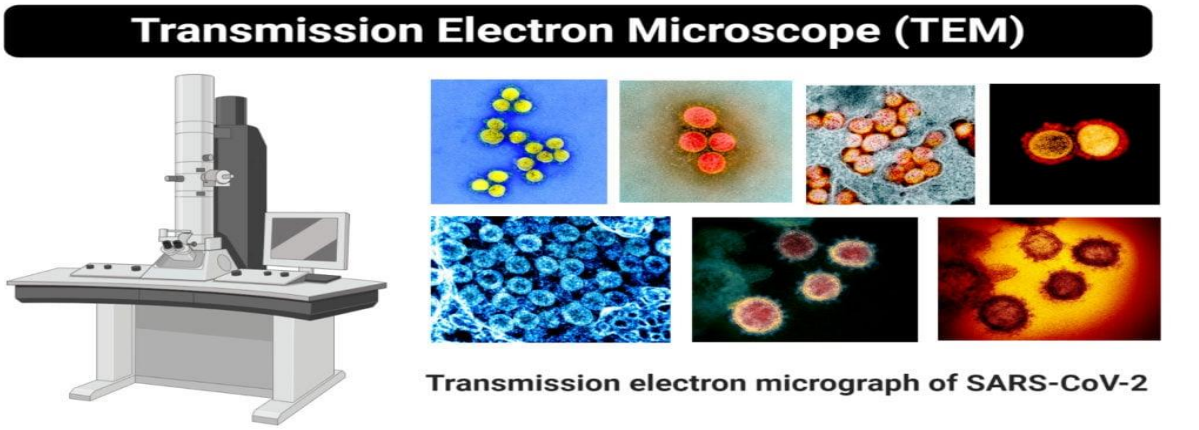
- This is a powerful electron microscope that uses a beam of electrons to focus on a specimen producing a highly magnified and detailed image of the specimen.
- The magnification power is over 2 million times better than that of the **light microscope**, producing the image of the specimen which enables easy characterization of the image in its morphological features, compositions and crystallization information is also detailed.
- Early discovery of cathode rays like electrons by Louis de Broglie in the early 1920s, paved way into the development of an electron microscope where they used a beam of electrons creating a form of wave motion.
- Magnetic fields were used as lenses for the electrons. With these discoveries, the first electron microscope was later developed by Ernst Ruska and Max Knolls in 1931 and modified into a Transmission Electron Microscope (TEM) by Ernst Ruska along with the Sieman's company, in 1933.
- This TEM microscope has several advantages compared to the light microscope with its efficiency also being very high.
- Among all microscopes both light and electron microscopes, TEM are the most powerful microscopes used in laboratories. It can magnify a mall particle of about 2nm, and therefore they have a resolution limit of 0.2um.

Principle of Transmission Electron Microscope (TEM)

The working principle of the Transmission Electron Microscope (TEM) is similar to the light microscope. The major difference is that light microscopes use light rays to focus and produce an image while the TEM uses a beam of electrons to focus on the specimen, to produce an image.

Electrons have a shorter wavelength in comparison to light which has a long wavelength. The mechanism of a light microscope is that an increase in resolution power decreases the wavelength of the light, but in the TEM, when the electron illuminates the specimen, the resolution power increases increasing the wavelength of the electron transmission. The wavelength of the electrons is about 0.005nm which is 100,000X shorter than that of light, hence TEM has better resolution than that of the light microscope, of about 1000times.

This can accurately be stated that the TEM can be used to detail the internal structures of the smallest particles like a virion particle.



Parts of Transmission Electron Microscope (TEM)

Their working mechanism is enabled by the high-resolution power they produce which allows it to be used in a wide variety of fields. It has three working parts which include:

1. Electron gun
2. Image producing system
3. Image recording system

Electron gun

- This is the part of the Transmission Electron Microscope responsible for producing electron beams.
- Electrons are produced by a cathode that is a tungsten filament that is V-shaped and it is normally heated. The tungsten filament is covered by a control grid known as a Wehnelt cylinder made up of a central hole which lies columnar to the tube. The

cathode lies on top of or below the cylindrical column hole. The cathode and the control grid are negatively charged with an end of the anode which is disk-shaped that also has an axial hole.

- When electrons are transmitted from the cathode, they pass through the columnar aperture (hole) to the anode at high voltage with constant energy, which is efficient for focusing the specimen to produce an accurately defined image.
- It also has the condenser lens system which works to focus the electron beam on the specimen by controlling the energy intensity and the column hole of the electron gun. The TEM uses two condenser lenses to converge the beam of electrons to the specimen. The two condenser lens each function to produce an image i.e the first lens which has strong magnification, produces a smaller image of the specimen, to the second condenser lens, directing the image to the objectives.

Image- Producing system

- Its made up of the objective lens, a movable stage or holding the specimen, intermediate and projector lenses. They function by focusing the passing electrons through the specimen forming a highly magnified image.
- The objective has a short focal length of about 1-5mm and it produces an intermediate image from the condenser which are transmitted to the projector lenses for magnification.
- The projector lenses are of two types, i.e the intermediate lens which allows great magnification of the image and the projector lens which gives a generally greater magnification over the intermediate lens.
- To produce efficient high standard images, the objectives and the projector lenses need high power supplies with high stability for the highest standard of resolution.

Image-Recording System

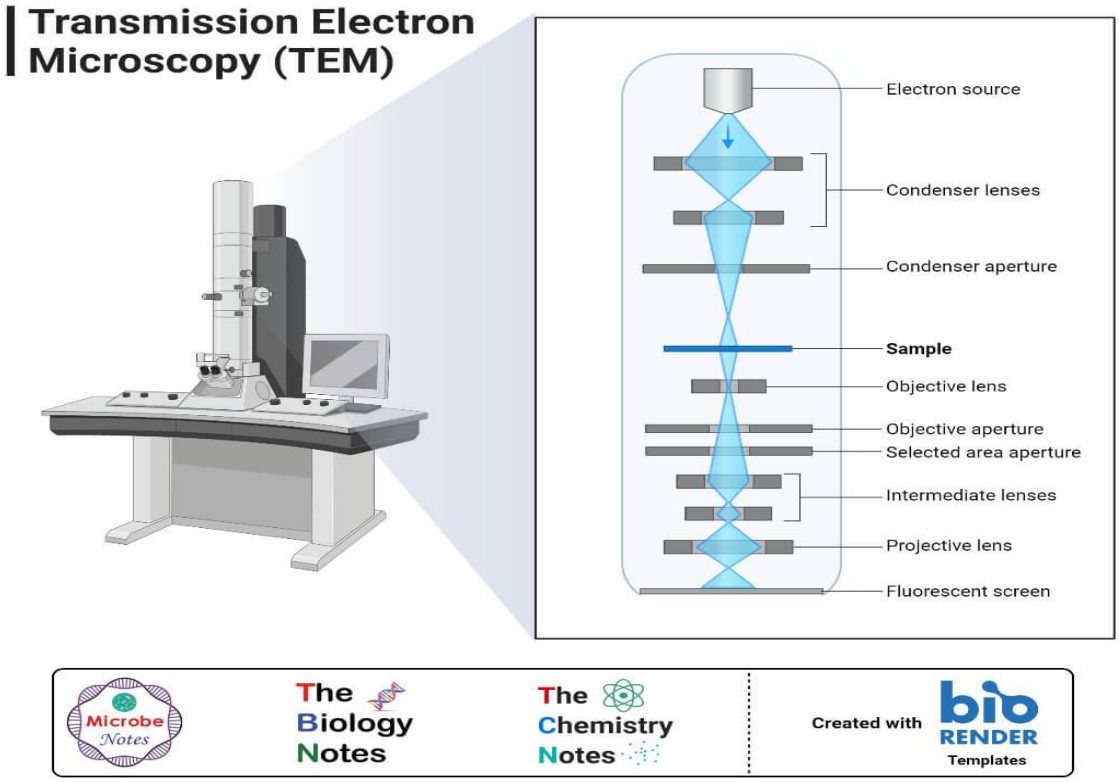
- Its made up of the fluorescent screen used to view and to focus on the image. They also have a digital camera that permanently records the images captured after viewing.

- They have a vacuum system that prevents the bombardment or collision of electrons with air molecules disrupting their movement and ability to focus. A vacuumed system facilitates the straight movement of electrons to the image.
- The vacuumed system is made up of a pump, gauge, valves and a power supply.
- The image that is formed is called a monochromatic image, which is greyish or black and white. The image must be visible to the human eye, and therefore, the electrons are allowed to pass through a fluorescent screen fixed at the base of the microscope.
- The image can also be captured digitally and displayed on a computer and stored in a JPEG or TIFF format. During the storage, the image can be manipulated from its monochromatic state to a colored image depending on the recording apparatus eg use of pixel cameras can store the image in color.
- The presence of colored images allows easy visualization, identification, and characterization of the images.

How does a Transmission Electron Microscope (TEM) work?

From the instrumentation described, the working mechanism is a sequential process of the parts of the TEM mentioned above. To mean:

- A heated tungsten filament in the electron gun produces electrons that get focus on the specimen by the condenser lenses.
- Magnetic lenses are used to focus the beam of electrons of the specimen. By the assistance offered by the column tube of the condenser lens into the vacuum creating a clear image, the vacuum allows electrons to produce a clear image without collision with any air molecules which may deflect them.
- On reaching the specimen, the specimen scatters the electrons focusing them on the magnetic lenses forming a large clear image, and if it passes through a fluorescent screen it forms a polychromatic image.
- The denser the specimen, the more the electrons are scattered forming a darker image because fewer electron reaches the screen for visualization while thinner, more transparent specimens appear brighter.



Preparation of specimen for visualization by TEM

The specimen to be viewed under the TEM must undergo a special preparation technique to enable visualization and creation of a clear image.

- Electrons are easily absorbed and easily scattered on solid elements, showing poor visualization for thick specimens. And therefore, very thin specimens are used for accurate and clear visualization forming a clear image as well. The specimen should be about 20-100nm thin and 0.025-0.1nm diameter, as small as that of a bacterial cell. Thin specimens allow interaction with electrons in a vacuumed space, are able to maintain their innate structure.
- To get thin slice specimens, the specimen is first fixed on a plastic material with glutaraldehyde or osmium tetraoxide. These chemical agents stabilize the structure of the cell and maintain its originality. The addition of an organic solvent like alcohol

such as ethanol will dehydrate the cell completely for embedding the specimen to the plastics.

- The specimen is then permeated by adding an unpolymerized liquid epoxy plastic making it hardened like a solid block. This is where thin sections are cut from using a glass knife with a piece of special equipment known as an ultramicrotome.
- The specimen is then stained appropriately (with the appropriate stain) for the uniform scattering of electrons. The thin sections are then soaked in heavy metallic elements such as lead citrate and uranyl acetate allowing the lean and aluminum ions to bind to the cell structures. This forms an opaque layer against the electrons on the cell structures to increase contrast.
- The stained thin sections are then mounted on copper grids for viewing.
- The primary staining techniques that are applied for viewing under the TEM is Negative staining coupled with heavy metallic elements coating. The metallic coating scatters electrons which appears on the photographic film while uncoated sections and used to study bacterial, viral cell morphologies and structures.

Freeze-itching treatment:

To reduce the possible dangers of artifacts, freeze-itching is used especially for the treatment of microbial cells, unlike chemical fixation, dehydration, and embedding, where most specimens get contaminated.

- Microbial cell organelles undergo special treatment known as Freeze-itching whereby the specimens are prepared with liquid nitrogen and then warmed at -100°C in a vacuum chamber.
- The sections are then cut with a precooled knife in liquid nitrogen at -196°C. After warming up the sectioned specimen in a high vacuum for about 2 minutes, it can then coated ith platinum and carbon layer forming replicas.
- These are then be viewed under the TEM displaying more detailed internal structures of the cell in 3D.
- This step of treatment with Liquid nitrogen is known as freeze-itching.

Applications of Transmission Electron Microscope (TEM)

TEM is used in a wide variety of fields From Biology, Microbiology, Nanotechnology, forensic studies, etc. Some of these applications include:

1. To visualize and study cell structures of bacteria, viruses, and fungi
2. To view bacteria flagella and plasmids
3. To view the shapes and sizes of microbial cell organelles
4. To study and differentiate between plant and animal cells.
5. Its also used in nanotechnology to study nanoparticles such as ZnO nanoparticles
6. It is used to detect and identify fractures, damaged microparticles which further enable repair mechanisms of the particles.

Advantages of Transmission Electron Microscope (TEM)

1. It has a very powerful magnification of about 2 million times that of the Light microscope.
2. It can be used for a variety of applications ranging from basic Biology to Nanotechnology, to education and industrial uses.
3. It can be used to acquire vast information on compounds and their structures.
4. It produces very efficient, high-quality images with high clarity.
5. It can produce permanent images.
6. It is easy to train and use the Transmission Electron Microscope

Limitations of Transmission Electron Microscope (TEM)

1. Generally, the TEMs are very expensive to purchase
2. They are very big to handle.
3. The preparation of specimens to be viewed under the TEM is very tedious.
4. The use of chemical fixations, dehydrators, and embedments can cause the dangers of artifacts.
5. They are laborious to maintain.
6. It requires a constant inflow of voltage to operate.

7. They are extremely sensitive to vibrations and electro-magnetic movements hence they are used in isolated areas, where they are not exposed.
8. It produces monochromatic images, unless they use a fluorescent screen at the end of visualization.

SCANNING ELECTRON M,ICROSCOPE

The first Scanning Electron Microscope was initially made by Mafred von Ardenne in 1937 with an aim to surpass the transmission electron Microscope. He used high-resolution power to scan a small raster using a beam of electrons that were focused on the raster. He also aimed at reducing the problems of chromatic aberrations images produced by the Transmission electron Microscopes. More studies followed by scientists and research institutions such as Cambridge Scientific Instrument Company who eventually developed a fully constructed Scanning electron Microscope, in 1965 and named it a Stereoscan. *The*

price of the Scanning Electron Microscope (SEM) is approximately $1 million. **Scanning Electron Microscope**

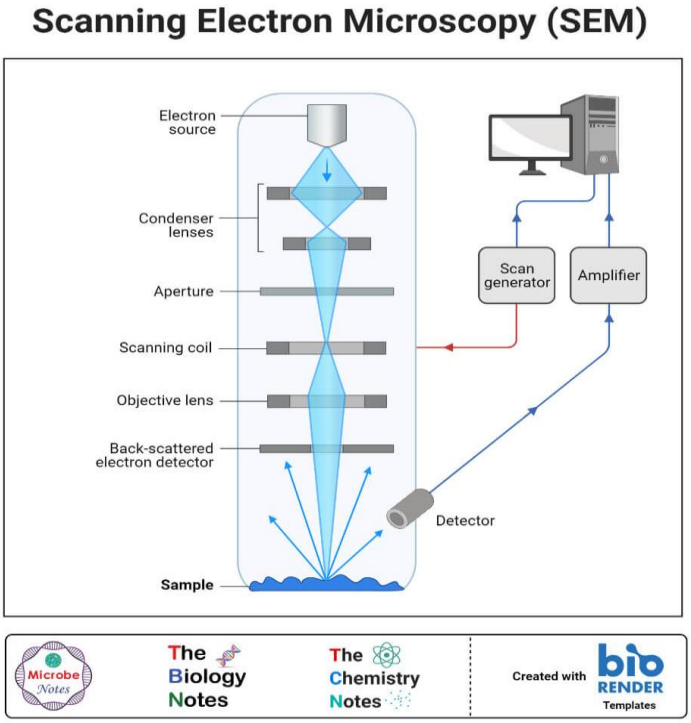
Scanning Electron Microscope (SEM) is a type of electron microscope that scans surfaces of microorganisms that uses a beam of electrons moving at low energy to focus and scan specimens. The development of electron microscopes was due to the inefficiency of the wavelength of light microscopes. electron microscopes have very short wavelengths in comparison to the light microscope which enables better resolution power.

Principle of Scanning Electron Microscope (SEM)

Unlike the Transmission Electron Microscope which uses transmitted electrons, the scanning electron Microscope uses emitted electrons. The Scanning electron microscope works on the principle of applying kinetic energy to produce signals on the interaction of the electrons. These electrons are secondary electrons, backscattered electrons, and diffracted backscattered electrons which are used to view crystallized elements and photons. Secondary and backscattered electrons are used to produce an image. The secondary electrons are emitted from the specimen play the

primary role of detecting the morphology and topography of the specimen while the backscattered electrons show contrast in the composition of the elements of the specimen.

How does the Scanning Electron Microscope (SEM) work?



- The source of the electrons and the electromagnetic lenses are from tungsten filament lamps that are placed at the top of the column and it is similar to those of the transmission electron Microscope.
- The electrons are emitted after thermal energy is applied to the electron source and allowed to move in a fast motion to the anode, which has a positive charge.
- The beam of electrons activates the emission of primary scattered (Primary) electrons at high energy levels and secondary electrons at low-energy levels from the specimen

surface. The beam of electrons interacts with the specimen to produce signals that give information about the surface topography and composition of the specimen.

- The specimen does not need special treatment for visualization under the SEM, even air-dried samples can be examined directly. However, microbial specimens need fixation, dehydration, and drying in order to maintain the structural features of the cells and to prevent collapsing of the cells when exposed to the high vacuum of the microscope.
- The samples are mounted and coated with thin layer of heavy metal elements to allow spatial scattering of electric charges on the surface of the specimen allowing better image production, with high clarity.
- Scanning by this microscope is attained by tapering a beam of electrons back and forth over a thin section of the microscope. When the electrons reach the specimen, the surface releases a tiny staw of electrons known as secondary electrons which are then trapped by a special detector apparatus.
- When the secondary electrons reach and enter the detector, they strike a scintillator (a luminescence material that fluoresces when struck by a charged particle or high-energy photon). This emits flashes of light which get converted into an electric current by a photomultiplier, sending a signal to the cathode ray tube. This produces an image that looks like a television picture that can be viewed and photographed.
- The quantity of secondary electrons that enter the detector is highly defined by the nature of the specimen i.e raised surfaces to receive high quantities of electrons, entering the detector while depressed surfaces have fewer electrons reaching the surface and hence fewer electrons enter the detector.
- Therefore raised surfaces will appear brighter on the screen while depressed surfaces appear darker.

Parts of a Scanning Electron Microscope (SEM)

The major components of the Scanning Electron Microscope include;

- Electron Source – This is where electrons are produced under thermal heat at a voltage of 1-40kV. the electrons condense into a beam that is used for the creation of an image

and analysis. There are three types of electron sources that can be used i. e Tungsten filament, Lanthanum hexaboride, and Field emission gun (FEG)

- Lenses – it has several condenser lenses that focus the beam of electrons from the source through the column forming a narrow beam of electrons that form a spot called a spot size.
- Scanning Coil – they are used to deflect the beam over the specimen surface.
- Detector – It's made up of several detectors that are able to differentiate the secondary electrons, backscattered electrons, and diffracted backscattered electrons. The functioning of the detectors highly depends on the voltage speed, the density of the specimen.
- The display device (data output devices)
- Power supply
- Vacuum system

Like the transmission electron Microscope, the Scanning electron microscope should be free from vibrations and any electromagnetic elements.

Applications of the Scanning Electron Microscope (SEM)

It is used in a variety of fields including Industrial uses, nanoscience studies, Biomedical studies, Microbiology

1. Used for spot chemical analysis in energy-Dispersive X-ray Spectroscopy.
2. Used in the analysis of cosmetic components which are very tiny in size.
3. Used to study the filament structures of microorganisms.
4. Used to study the topography of elements used in industries.

Advantages of the Scanning Electron Microscope (SEM)

- They are easy to operate and have user-friendly interfaces.
- They are used in a variety of industrial applications to analyze surfaces of solid objects.
- Some modern SEMs are able to generate digital data that can be portable.
- It is easy to acquire data from the SEM, within a short period of time of about 5 minutes.

Limitations

- They are very expensive to purchase
- They are bulky to carry
- They must be used in rooms that are free of vibrations and free of electromagnetic elements
- They must be maintained with a consistent voltage
- They should be maintained with access to cooling systems

The combination of the working principles of the Scanning Electron Microscope (SEM) and the Transmission Electron Microscope (TEM) formed the **Scanning-Transmission Electron Microscope (STEM).** The Scanning- Transmission Electron Microscope (STEM), uses a convergent beam of electrons to focus on a probe on the specimen, and the probe is then scanned on its surface collecting signals which are then collected as point-to-point to form an image.

GEIGER COUNTER

What is a Geiger Counter?

A lot of modern applications require working in the presence of high radioactive substances. If adequate safety measures are not taken, then radiation can have a very detrimental effect on the health of people. The horrors of Chernobyl will always be a reminder for humanity that radioactive energy is a force to be reckoned with.

Therefore, to assess levels of radiation so it does not cause us harm, a device called a Geiger Counter is used. This device is used in the detection and measurement of radiation in ionized substances. This has numerous applications in the field of research and medicine.**History of Geiger Counter**

Hans Geiger in 1908, under the supervision of Ernest Rutherford, developed an experimental technique for detecting alpha particles which became the basis for developing the Geiger–Müller

tube in 1928. The basic ionization mechanism used was discovered by John Sealy Townsend between 1897 and 1901 and is known as the Townsend discharge.

Geiger and Walther in 1928 developed the sealed Geiger–Müller tube which they used. It was so small, rugged that not only could it detect alpha-beta radiation both, but also gamma radiation. So radiation-instruments could be produced relatively cheaply, and so the Geiger counter came into existence.

What is the use of Geiger Counter?

A Geiger Counter is an instrument which is used for detecting and measuring ionizing radiation. It is also known as a Geiger–Muller counter; this is widely used in many applications like experimental physics, radiological protection, radiation dosimetry, nuclear industry and nuclear-industry.

It detects ionizing-radiation such as alpha particles, beta particles and gamma particles using the ionization effect produced in the Geiger Muller tube by which the name of the instrument is known.

What Principle does a Geiger Counter Work on?

This device consists of a Geiger-Muller tube, which contains a noble gas (usually helium or argon) at a very low pressure, possibly the lowest. Under the application of a relatively high voltage, the electrical change would indicate radiation if the gas becomes conducive. It can be used to detect all kinds of radiation- alpha, beta and gamma.

The modern type of Geiger Counter uses the halogen tube, and these lightweight instruments offer an improvement. Better range of detection of multiple types of ionizing radiation, i.e. alpha, beta, X-rays, and gamma, all are available even in the same unit. Their features are easy to use, common-interface and compatible with a catalogue of optional accessories for wireless reach back, rechargeable batteries, and extension poles to maintain good ALARA (as low as reasonably achievable) principles.

Types and Applications of Geiger Counter

An intended-detection application of a Geiger Counter explains the tube design being used. Subsequently, there are many designs which may be generally categorized as end-window, or windowless also as thin-walled or thick-walled, and sometimes hybrids of these types.

Historical uses of the Geiger principle was for the alpha and beta particles detection. However, this instrument is still being used for this purpose today. Geiger Counter shares wide applications as they are used as handheld radiation survey instruments and is probably one of the world's best instruments known for radiation detection.

Some applications of a Geiger Counter are as follows:

- Detection of radioactive rocks and minerals in mining.
- For first responders such as firemen and hazard management personnel to ensure that the site is clear of radiation.
- Ensuring that levels of radiation are within permissible levels around nuclear power plants.
- Detection of radiation in scrap metal processing industries.
- Detection of radiation in erstwhile warzones.
- Ensuring that patients undergoing radiation therapy are not overexposed to radiation.
- Ensuring that uranium mines and surrounding areas do not become overly radioactive.

The "end-window" type of a GM tube has to be used for alpha particles, and low energy beta particles as the particles show a limited range and are easily stopped by the solid-material. Therefore, this tube requires a window which is thin enough to allow as many as possible of these particles. The window is usually made of mica with1.5 - 2.0 mg/cm2 density.

Alpha particles contain the shortest range, and to detect these the window should ideally be less than 10 mm of the radiation source. Geiger–Müller tube generates a pulse output that is the same magnitude for all radiations detected, hence the Geiger counter with an end window tube is not able to distinguish the alpha or beta particles.

The "pancake" GM tube is a type of end-window probe and designed with a larger detection area for quick checking. However, Atmospheric pressure against fill gas's low pressure minimizes the window size because of the limited strength of the membrane.

Some beta-particles can also be detected by thin-walled "windowless" GM tubes that have no end-window, however, allow high energy beta-particles to pass through the tube. The tube walls have more stopping power in comparison to a thin end-window; they even allow these energetic particles to reach the fill gas.

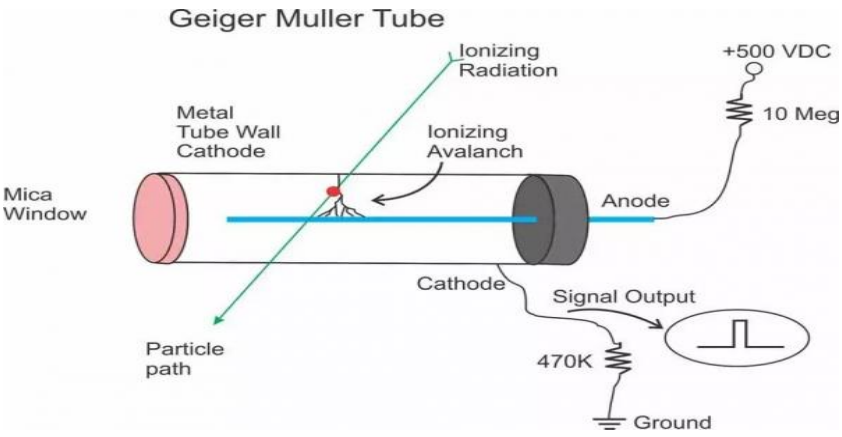
‘End-window’ GM counters are still being used as a portable, general-purpose, radioactive-contamination measurement and detection-instruments, due to their less cost, robustness and high detection-efficiency especially with energy-rich beta particles.

For differentiation between alpha and beta particles and to know the particle's energy information, the scintillation counters are used.

Points To Note:

- Detected radiation readout method in Geiger counter are of two types, i.e. Counts and Radiation dose. There is a simple type of display unit which shows the number of ionizing events detected displayed as a count rate, like "counts per minute or seconds" or as the total number of counts over the set period.
- This count readout is usually taken when alpha particles or beta particles are being detected normally. To achieve a display of radiation-dose rate is more complicated. It displays in Sievert, normally used to measure gamma or X-ray dose rates.
- Presence of radiation can be detected by a GM tube, not its energy, that influences the radiation's ionizing effect. The electronic processor will apply only known factors to make this conversion, i.e., specific to every instrument and is determined by its design & calibrations.

The readout may be analogue or digital, and the modern instruments provide serial communications with a host network or computer. The option is there to generate audible clicks that represent ionization events. It is the distinctive sound normally seen with handheld or portable Geiger counters.



Advantages of a Geiger Counter

The benefits of using these devices are mentioned as follows:

- They can prevent nuclear accidents by always giving a reading of radiation levels. Since radiation cannot be seen, it is otherwise impossible to know if the levels at a place have become hazardous.
- They are used to ensure safety in all operations that require working with radioactive material.
- They are highly sensitive devices, therefore the readings are usually accurate.
- They can be very useful in expanding the scope of nuclear energy to greater levels in order to fully harness it for the benefit of mankind.

Disadvantages of GM Counter

Below is the list of demerits as discovered by working with Geiger-Muller counter:

- GM counters can not measure energy due to a lack of differentiating abilities.Uncharged particles like neutrons cannot be detected.
- GM counters are less efficient due to its large paralysis time limits and also large dead-time.
- Quenching agents used in GM counters often decompose, which leads to the reduction in a lifetime.

Thus, GM Counter is primarily used due to its advantages. However, GM counters are not free from disadvantages, its uses make it preferable over other radiation counters.

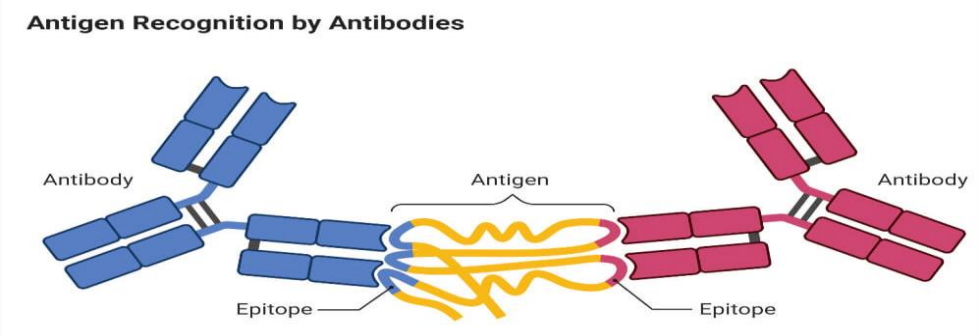
RADIOIMMUNOASSAY(RIA)

Radioimmunoassay is one of the sensitive immunoassay techniques which helps in the determination of antigens or antibodies in a sample with the use of radioisotopes.

It is an in vitro type of antigen-antibody interaction.

When radioisotopes instead of enzymes are used as labels to be conjugated with antigens or antibodies, the technique of detection of the antigen-antibody complex is called radioimmunoassay (RIA). Radioimmunoassay (RIA) is an *in vitro* assay that measures the presence of an antigen with very high sensitivity. RIA was first described in 1960 for the measurement of endogenous plasma insulin by **Solomon Berson and Rosalyn Yalow** of the Veterans Administration Hospital in New York.

The classical RIA methods are based on the principle of competitive binding. In this method, an unlabeled antigen competes with a radiolabeled antigen for binding to an antibody with the appropriate specificity. Thus, when mixtures of radiolabeled and unlabeled antigen are incubated with the corresponding antibody, the amount of free (not bound to antibody) radiolabeled antigen is directly proportional to the quantity of unlabeled antigen in the mixture.



Radioimmunoassay (RIA) Requirements

Radiolabeled antigens: The antigens are generally labeled with gamma-ray emitting isotopes such as I-125 and beta-ray emitting isotopes such as Tritium. They are also called hot antigens.

S**pecific Antibodies:** They are required in smaller amounts than antigens.

Unlabeled antigens (sample antigens): They are also called cold antigens.

M**icrotitre plates:** 96 wells microtitre plate

Washing Buffer solutions: Wash buffer such as 1% Trifluoroacetic acid is used.

Radioimmunoassay (RIA) Principle

Antigens and antibodies bind specifically to form the Ag-Ab complex. The antigen can be labeled or conjugated with radioisotopes. The unlabeled antigens from the sample compete with radiolabeled antigens to bind on paratopes of specific antibodies. The unlabeled antigens replace labeled antigens that are already linked with the antibodies. The unlabeled antigens when bind with antibodies, increases the amount of free radiolabeled antigens in the solution. Hence the concentration of free labeled antigens is directly proportional to the bound unlabeled antigens.

It involves a combination of three principles.

1. An immune reaction i.e. antigen, antibody binding.
2. A competitive binding or competitive displacement reaction. (It gives specificity)
3. Measurement of radio emission. (It gives sensitivity)

Immune Reaction

When a foreign biological substance enters the body's bloodstream through a non-oral route, the body recognizes the specific chemistry on the surface of the foreign substance as antigen and produces specific antibodies against the antigen so as nullify the effects and keep the body safe. The antibodies are produced by the body's immune system so, it is an immune reaction. Here the antibodies or antigens bind and move due to chemical influence. This is different from the principle of electrophoresis where proteins are separated due to charge.

Competitive binding or competitive displacement reaction

This is a phenomenon wherein when there are two antigens that can bind to the same antibody, the antigen with more concentration binds extensively with the limited antibody displacing others. So here in the experiment, a radiolabelled antigen is allowed to bind to a high-affinity antibody. Then when the patient serum is added to unlabeled antigens it starts binding to the antibody displacing the labeled antigen.

Measurement of radio emission

Once the incubation is over, then washings are done to remove any unbound antigens. Then radio emission of the antigen-antibody complex is taken, and the gamma rays from the radiolabeled antigen are measured.

The target antigen is labeled radioactively and bound to its specific antibodies (a limited and known amount of the specific antibody has to be added). A sample, for e.g. blood serum, is added in order to initiate a competitive reaction of the labeled antigens from the preparation, and the unlabeled antigens from the serum sample, with the specific antibodies. The competition for

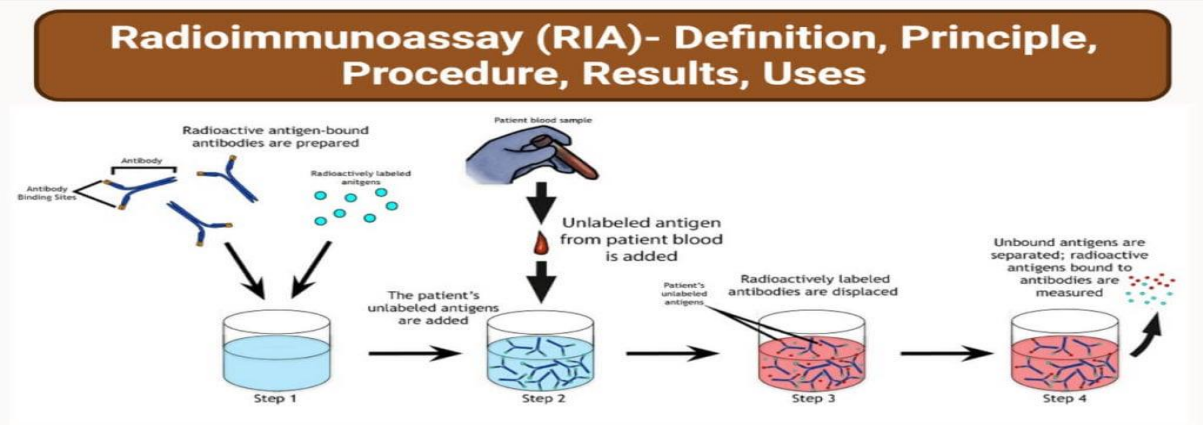
the antibodies will release a certain amount of labeled antigen. This amount is proportional to the ratio of labeled to an unlabeled antigen. A binding curve can then be generated which allows the amount of antigen in the patient's serum to be derived. That means as the concentration of unlabeled antigen is increased, more of it binds to the antibody, displacing the labeled variant. The bound antigens are then separated from the unbound ones, and the radioactivity of the free antigens remaining in the supernatant is measured.

Antigen-antibody complexes are precipitated either by crosslinking with a second antibody or by means of the addition of reagents that promote the precipitation of antigen-antibody complexes. Counting radioactivity in the precipitates allows the determination of the amount of radiolabeled antigen precipitated with the antibody. A standard curve is constructed by plotting the percentage of antibody-bound radiolabeled antigen against known concentrations of a standardized unlabeled antigen, and the concentrations of antigen in patient samples are extrapolated from that curve.

The **extremely high sensitivity** of RIA is its **major advantage**.

Radioimmunoassay (RIA) Procedure

1. Specific antibodies of known concentration are fixed in the microtitre well.
2. A known amount of hot antigens is then added to the well
3. Washed carefully to remove any unbound antigens
4. At this point, the radioactivity of the well will be maximum.
5. Unlabeled antigens are then added to the well
6. The unlabeled antigens will bind to the antibodies and there will be free labeled antigens in the well.
7. Again washed carefully to remove the free labeled antigens.
8. Radioactivity of wells is then measured by gamma-counter.



Radioimmunoassay (RIA) Result Interpretation

At first, the labeled antigens will bind to the antibodies hence radioactivity will be maximum.

If the sample contains specific antigens of interest, it will bind to the antibodies releasing labeled antigens and hence the radioactivity of the solution will decrease.

So by observation of decreasing radioactivity, it can be confirmed that the antigen of interest is present in the sample. And if the radioactivity remains the same, it can be called a negative test.

With the increasing concentration of unlabeled antigens, the radioactivity decreases. By plotting a graph of radioactivity(in percentage) vs concentration of unlabeled antigens, a standard curve is obtained.

The sample to be assayed is run parallel following a similar procedure and the radioactivity measured is calibrated with the standard curve to determine the concentration of the antigen.

Radioimmunoassay (RIA) Applications

- It was first used for the detection of peptide hormones.
- Detection of different viral antigens
- Detection of many hormones and drugs
- Detection of Hepatitis B surface antigens
- Detection of mycotoxins
- Detection of the early stage of cancer

Radioimmunoassay (RIA) Advantages

- High specificity

- High sensitivity
- Can detect a very small amount (nanograms) of antigen or antibodies.

Radioimmunoassay (RIA) Limitations

- Working with radioactive substances makes it a bit risky.
- Disposal of radioactive substances can be problematic.
- Equipment and reagents are expensive.
- Radiolabeled substances used have a short shelf-life.

HEALTH EFFECTS OF RADIATION

Radiation can be defined as

The transmission or emission of energy in the form of particles or waves through a material medium or space.

Radiation includes:

- ***Electromagnetic radiation*** such as radio waves, heat, x-rays, gamma radiation, and even visible light.
- ***Particle radiation*** such as alpha radiation, neutron radiation, and beta radiation
- ***Acoustic radiation*** such as seismic waves, sound, and ultrasound
- ***Gravitational radiation***

Radiation poisoning occurs when a radioactive material releases particles which enter a person's body and cause damage. The different characteristics of the radioactive substances are different. In different ways they can harm and help people and some are more dangerous than others.

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Harmful Effects of Radiation

Radiation can be categorized into non-ionizing or ionizing depending on the radiated particle's energy. Radioactive materials that emit α, β, or γ radiation are the common sources of ionizing radiation. Visible light, microwaves, infrared light, are the common sources of non-ionizing radiation. Both types of radiation are harmful to health even though they have few advantages.

Radiation exposure can have different effects depending on the dose received. A key factor in determining the health effects is whether it is chronic or acute. When a dose of radiation is received at once, then it can be called an acute exposure, and when a small dose of radiation is exposed for a long period then it is chronic exposure.

Health effects of radiation can be classified into two categories: **threshold effects** and **non-threshold effects**. Threshold effects appear after a certain level of radiation exposure is reached

and enough cells have been damaged to make the effect apparent. Non-threshold effects can occur at lower levels of radiation exposure.

When considering radiation ‘s health effects on the human body, one method is to consider the deterministic effects and stochastic effects separately. Those two effects are compiled in the figure above. Deterministic effects do not appear unless the radiation exceeds a certain level has been exposed. Most of the deterministic effects are classified into acute disorders, the symptoms of which appear within several weeks of exposure.

Here are a few common health effects or ***harmful effects of radiation*** on the human body.

1. Hair

Loss of hair fall occurs when exposure to radiation is higher than 200 rems.

2. Heart and Brain

Intense exposure to radiation from 1000 to 5000 rems will affect the functioning of the heart. Radiation kills nerve cells and small blood vessels of heart which may cause immediate death. Brain cells are affected if the radiation exposure is greater than 5000 rems.

3. Thyroid

Certain body parts are affected specifically when exposed to different types of radiation sources. The thyroid gland may be affected when exposed to radioactive iodine. If exposed to a considerable amount of radioactive iodine, whole or part of the thyroid can be affected.

4. Blood System

A number of lymphocytic cells present in the blood will be reduced if a person is exposed to 100 rems. This may cause several immune problems. This is termed as mild radiation sickness. As per the reports from Nagasaki and Hiroshima, symptoms may be present more than ten years from that exposure.

5. Reproductive Tract

As the cells of the reproductive tract divide fastly, these are more prone to be affected even if the exposure is not more than 200 rems.

Facts on Effects of Radiation

- Radiation is around us, and is used in many applications safely.
- The radiation effects can be mild or life-threatening, depending on the dose. All sources of radiation poisoning can be nuclear accidents, the work environment, and some medical treatment.
- There is no cure, but barriers can prevent exposure and some medicines can remove some of the body's radiation.
- Anyone who thinks they are radiation sensitive will seek medical attention as soon as possible.

Effects of Radiation Pollution

In reproductive cells, ionizing radiation damages the genetic material and results in mutations that are transmitted from generation to generation. The mutagenic effects of radiation were first recognized in the 1920s, and since that time radiation has been used as an important means in genetic research to obtain new mutations in experimental organisms.

The radiation 's genetic effects are reflected not in individuals irradiated but in their immediate or distant offspring. Due to the duration of the human life cycle, the time lag is great, and massive epidemiological studies with long-term follow-up are needed to gather sufficient data for statistical analysis.

Protective Measures

The objective of radiation protection is to protect people and the environment against the harmful effects of ionizing radiation. This is achieved through a process called risk assessment which involves

1. Identifying the hazard
2. estimating the size of the risk and
3. Assessing its importance in comparison with other risks.

The results of the risk assessment should be recorded appropriately and used as the basis for making decisions about how to manage the risk. Finally, each risk assessment needs to be reviewed and updated periodically and when new equipment or work practices are introduced.

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