Unit 3: Electrophoresis

Paper Electrophoresis

- Filter Paper:
- Act as a stabilizing medium is very popular for the study of normal and abnormal plasma proteins.
- Contain 95% cellulose
- Apparatus:
- Contains a power pack and an electrophoretic cell.

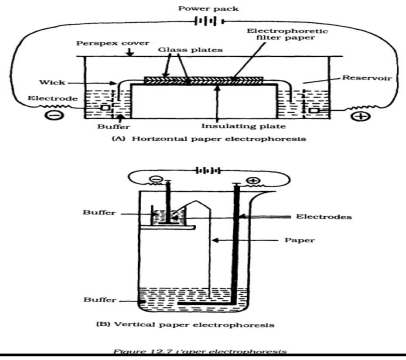


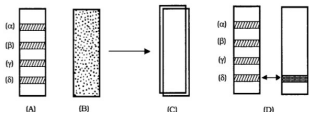
Figure 12.7 Paper electrophoresis

- Sample Application:
- This is the single most critical procedure in the whole electrophoresis process.
- The sample may be applied as a spot or as a narrow uniform streak.
- Electrophoretic run:
- Detection and quantitative Assay

1. Florescence
2. Ultraviolet absorption
3. Staining

Compound	Dye	Comments
Proteins	Bromophenol blue in acetic acid	Visual, quantitative
	Nigrosine in trichloro acetic acid/ acetic acid	Visual, very sensitive
	Lissamine green in acetic acid	Visual, quantitative
	Coomassie Brilliant Blue R-250	Visual, quantitative
	Dansyl Chloride	Fluorescent, quantitative
	Fluorescamine	Fluorescent, very sensitive
	Aqueous anilinonapthalene sulphonate (ANS)	Fluorescent, very sensitive
Nucleic Acids	Methyl green-pyronine	DNA-blue, RNA-red sensitive
	Lanthanum acetate + acridine orange in acetic acid	DNA, RNA-orange red
	Toluidine blue	Visual, sensitive
	Pyronine	RNA, sensitive
	Methylene blue	RNA, visual, sensitive
	Ethidium bromide	Fluorescent, very sensitive
Lipoproteins	Sudan Black in 60% ethanol	Visual, senstive
Glycoproteins	Periodate oxidation + treatment with Schiff's reagent	Visual, quantitative
	Alcian blue	Visual, sensitive
Polysaccharide	Iodine	Visual, sensitive
General	Stains-All	DNA-bluish purple, RNA-blue, Proteins-red, Phosphoproteins-blue, Acid mucopolysaccharides-blue to purple

4. Detection of enzymes insitu



5. Quantitative estimation

Western Blotting

- ***Introduction:***
- western blotting, is a widely used analytical technique in molecular biology and immunogenetics to detect specific proteins in a sample of tissue homogenate or extract.
- Western blot technique uses three elements to achieve its task of separating a specific protein from a complex: separation by size, transfer of protein to a solid support, and marking target protein using a primary and secondary antibody to visualize.
- the primary antibody is created that recognizes and binds to a specific target protein.
- The electrophoresis membrane is washed in a solution containing the primary antibody, before excess antibody is washed off.

- A secondary antibody is added which recognizes and binds to the primary antibody. The secondary antibody is visualized through various methods such as staining, immunofluorescence, and radioactivity, allowing indirect detection of the specific target protein.
- ***Application:***
- The western blot is extensively used in biochemistry for the qualitative detection of single proteins and protein-modifications.
- The western blot is routinely used for verification of protein production after cloning. It is also used in medical diagnostics, e.g., in the HIV test or BSE-Test.
- Procedure:
- The western blot method is composed of a gel electrophoresis to separate native proteins by 3-D structure or denatured proteins by the length of the polypeptide, followed by an electrophoretic transfer onto a membrane and an immunostaining procedure to visualize a certain protein on the blot membrane.

- SDS-PAGE is generally used for the denaturing electrophoretic separation of proteins.
- SDS is generally used as a buffer in order to give all proteins present a uniform negative charge, since proteins can be positively, negatively, or neutrally charged.
- This type of electrophoresis is known as SDS-PAGE.
- Prior to electrophoresis, protein samples are often boiled to denature the proteins present.
- This ensures that proteins are separated based on size and prevents proteases from degrading samples.
- Following electrophoretic separation, the proteins are transferred to a membrane.
- The membrane is often then stained with Ponceau S in order to visualize the proteins on the blot and ensure a proper transfer occurred.
- Next the proteins are blocked with milk to prevent non-specific antibody binding, and then stained with antibodies specific to the target protein.

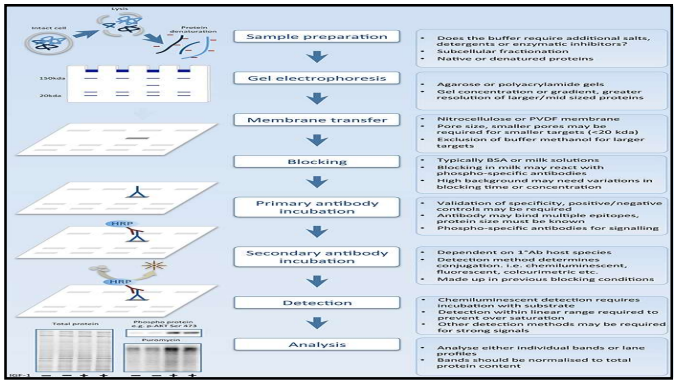
- Lastly, the membrane will be stained with a secondary antibody that recognizes the first antibody staining, which can then be used for detection by a variety of methods. The gel electrophoresis step is included in western blot analysis to resolve the issue of the cross-reactivity of antibodies.

1. Gel Electrophoresis
2. Transfer
3. Blocking
4. Incubation

i. Primary antibody
ii. Secondary antibody
iii. One step

5. Detection and visualization

i. Colorimetric detection
ii. Chemiluminescent detection



iii. Radioactive detection
iv. Fluorescent detection

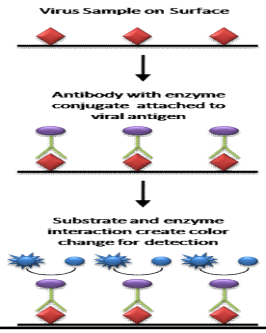
ELISA

- ***Introduction:***
- The enzyme-linked immunosorbent assay (ELISA) is a commonly used analytical biochemistry assay
- The assay uses a solid-phase type of enzyme immunoassay (EIA) to detect the presence of a ligand in a liquid sample using antibodies directed against the protein to be measured.
- ELISA has been used as a diagnostic tool in medicine, plant pathology, and biotechnology, as well as a quality control check in various industries.
- In the most simple form of an ELISA, antigens from the sample to be tested are attached to a surface. Then, a matching antibody is applied over the surface so it can bind the antigen.

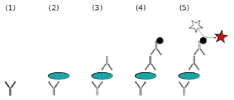
- This antibody is linked to an enzyme and then any unbound antibodies are removed.
- In the final step, a substance containing the enzyme's substrate is added.
- If there was binding ,the subsequent reaction produces a detectable signal, most commonly a color change.
- Principle:
- As an analytical biochemistry assay and a "wet lab" technique, ELISA involves detection of an analyte in a liquid sample by a method that continues to use liquid reagents during the analysis that stays liquid and remains inside a reaction chamber or well needed to keep the reactants contained.
- This is in contrast to "dry lab" techniques that use dry strips.
- Even if the sample is liquid, the final detection step in "dry" analysis involves reading of a dried strip by methods such as reflectometry and does not need a reaction containment chamber to prevent spillover or mixing between samples.

- Types:

1. Direct ELISA:



2. Sandwich ELISA:



- Application:
- Can be performed to evaluate either the presence of antigen or the presence of antibody in a sample
- It has also found applications in the food industry in detecting potential food allergens, such as milk, peanuts, walnuts, almonds, and eggs and as serological blood test for coeliac disease.
- ELISA can also be used in toxicology as a rapid presumptive screen for certain classes of drugs.

- The ELISA was the first screening test widely used for HIV because of its high sensitivity.
- There are ELISA tests to detect various kind of diseases, such as dengue, malaria, Chagas disease, and others.
- ELISA tests also are extensively employed for *in vitro* diagnostics in medical laboratories.
- The other uses of ELISA include: detection of SARS-CoV-2 antibodies in blood samples

Isoelectric Focussing

- This technique was discovered by H. Svensson in Sweden and has a high-resolution power.
- A simple comparison would help establish the method's supremacy over other methods; while paper electrophoresis resolves plasma proteins into six bands, isoelectric focussing resolves it into atleast 40 bands.
- The isoionic point of a molecule is thus determined by the number and types of protolytic groups and their dissociation constants.
- Although there is considerable variation in the isoionic point of proteins, they are generally in the pH range of 3-11.
- In isoelectric focussing a stable pH gradient is arranged; the pH increases gradually from anode to cathode.

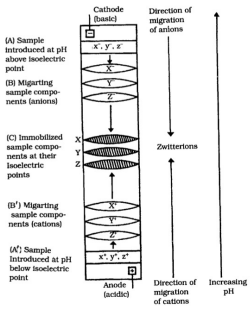


Figure 12.16 Schematic representation of the principle of isoelectric focussing

- Establishing the pH Gradient:
- Carrier Ampholytes The pH gradients may be obtained by electrofocusing special buffer substances known as carrier ampholytes. The carrier ampholytes must have the properties listed below.

(i) Since carrier ampholytes must dictate the pH course. they should have a certain buffering capacity at their isoelectric point.

(ii) They should have a conductance at their isoelectric point.

(iii) They should have low molecular weight so that macromolecules can be separated from them easily after electrofocusing.

(iv) They should be soluble in water. This hydrophilic character will also prevent their binding to hydrophobic regions of proteins.

(v) Ideally they should have a low light absorption at 280 nm.

- This would permit the detection of proteins after electrofocusing by measurements at 280 nm.
- Carrier ampholytes are isomers and homologs of aliphatic polyamino polycarboxylic acids.
- The general formula for a carrier ampholyte is:

$$R—N—(CH_2)_n—N—(CH_2)_n—COOH$$

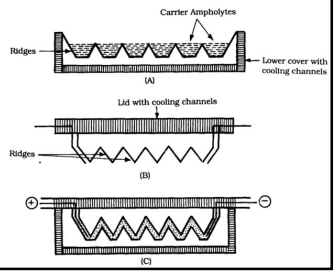
$$—(CH_2)_n—COOH,H, \text{ or } —(CH_2)_n—N—R \quad (R)$$

- Stabilization Against Convection
- As for all other electrophoretic techniques described thus far, electrofocusing also needs provision for stabilization of separating protein zones against convective flow in the solution.
- Three ways are in use:

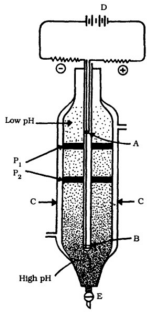
(i) density gradient.

(ii) Gel

(iii) zone convection electrofocusing.



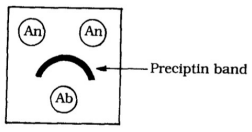
- Procedure:



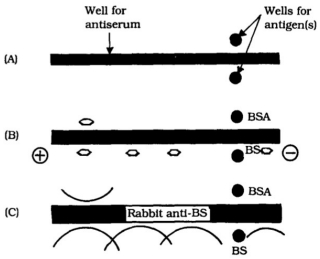
Immunoelectrophoresis

- The technique exploits the specificity of reaction between an antigen and antibody and molecular sieving of the gel in which this reaction is taking place for analysis of components of a given sample.
- The technique was developed by Grabar and Williams and is actually a modification of Ouchterlony double diffusion technique.
- The Ouchterlony method depends on the formation of a micellar structure during gelation of agar or sucrose.
- Molecular aggregates with molecular weights not exceeding 200,000 Daltons can diffuse freely through micellar channels.
- The aggregates with higher molecular weights are, however, retarded.

- Thus, the individual antigen and antibody molecules filled in adjacent wells cut into the gel can diffuse freely till they come into contact.
- On contact, if the antibody is specific for the antigen, they form complexes with larger molecular weight; these complexes are immobile.
- If the concentrations of corresponding antigen and antibody are sufficient to form a visible aggregate, a precipitin band appears.



- Immunoelectrophoresis, a powerful modification of Ouchterlony method, is performed using a double diffusion chamber



- The chamber may either be purchased from the market or prepared readily by coating a glass microscope slide with agarose/purified agar and punching appropriate holes in the supporting medium.
- The antigen is filled in the small round well (1-100 mg).
- The current is switched on (8 mA, 4-8 volts/cm) and the electrophoresis is allowed to continue for 1-2 hours.
- Immediately after disconnecting the voltage supply, the rectangular well is filled with appropriate antisera and the gel incubated overnight at room temperature in a humid chamber to permit diffusion of antigen and antibody toward each other.
- This leads to the formation of precipitin bands at the site lateral to the position where the desired component has separated during electrophoresis from the rest of the components.
- The major advantage of this method is its increased resolving ability due to the combination of electrophoresis with immune specificity.

- The technique can be made quantitative and has been used to detect particular antigens in sera, tissue or cell extracts, and culture filtrates.
- It has also been used to determine the purity of a given antigen.