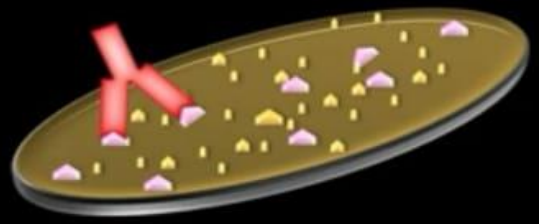
Department Of Biotechnology

- **Precipitation Reactions**
- **Immunodiffusion**
- **Immunoelectrophoresis**

By
Dr. Sapna Baghel

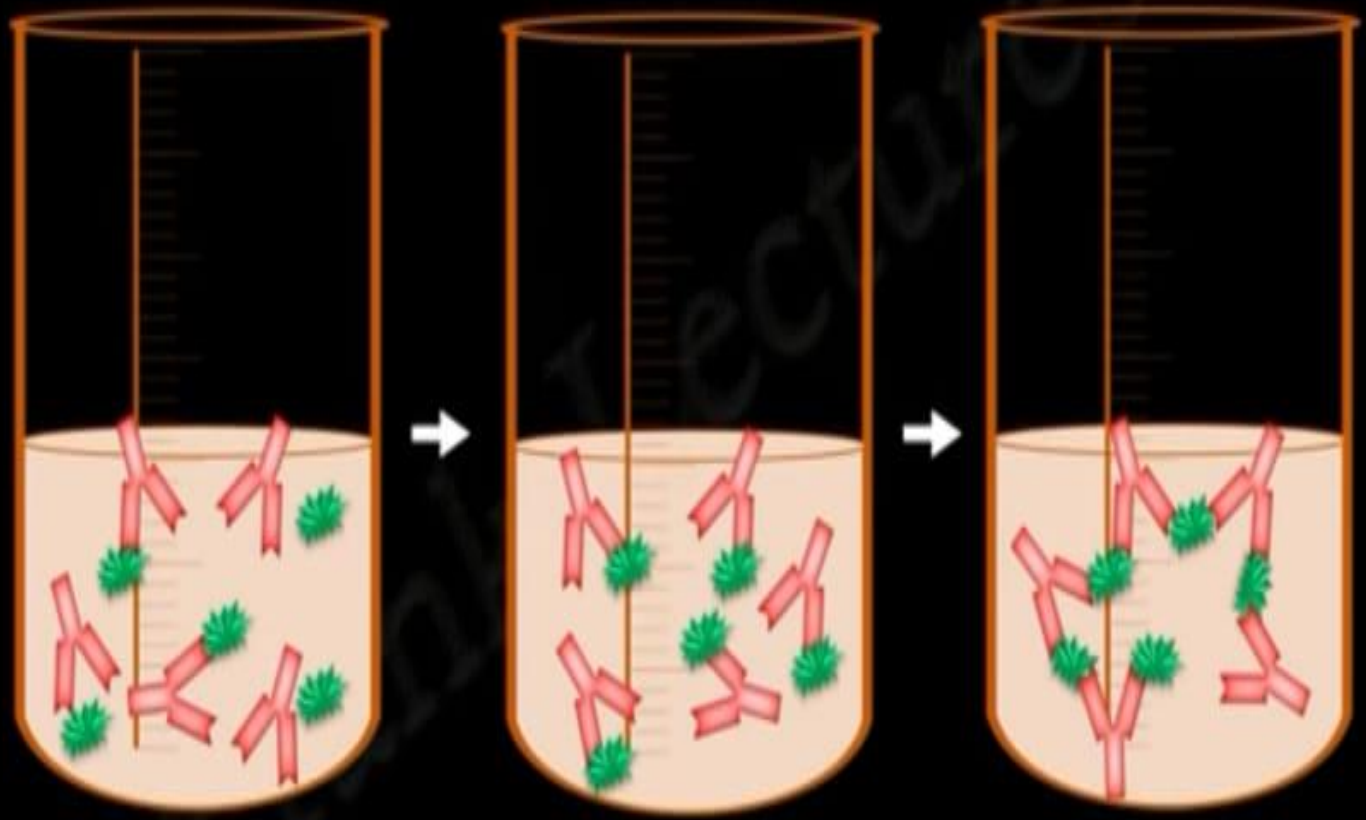
Diagnostic Immunology

Antigen-Antibody reactions are highly specific



Precipitation Reactions

Immune Complex Formation



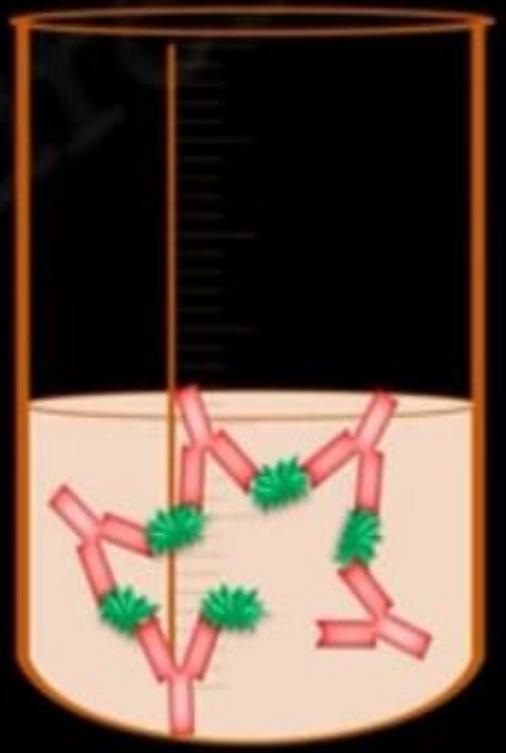
Antigen

Antibody

*Cross-linking of antibodies result in the formation of **immune complex** or lattice*

Immune Complex Formation

- *As the size of antigen-antibody lattice increases, it loses its solubility and precipitates out of the solution and thus become visible.*



Precipitation Reactions

Antibodies when mixed with soluble antigens in equal proportions

↓ *results in*

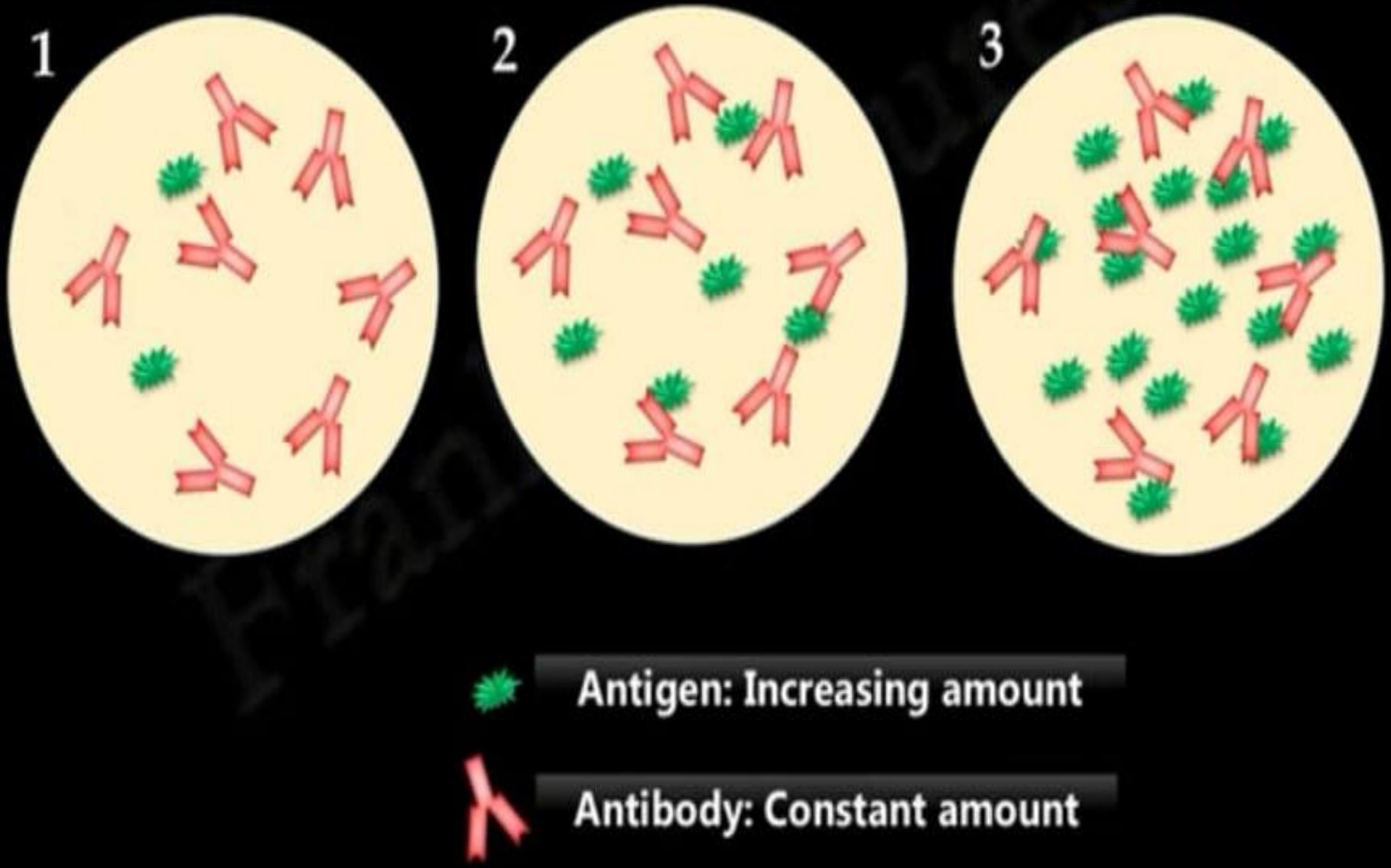
Insoluble lattice or immune complex formation.

Conditions necessary for precipitation reactions

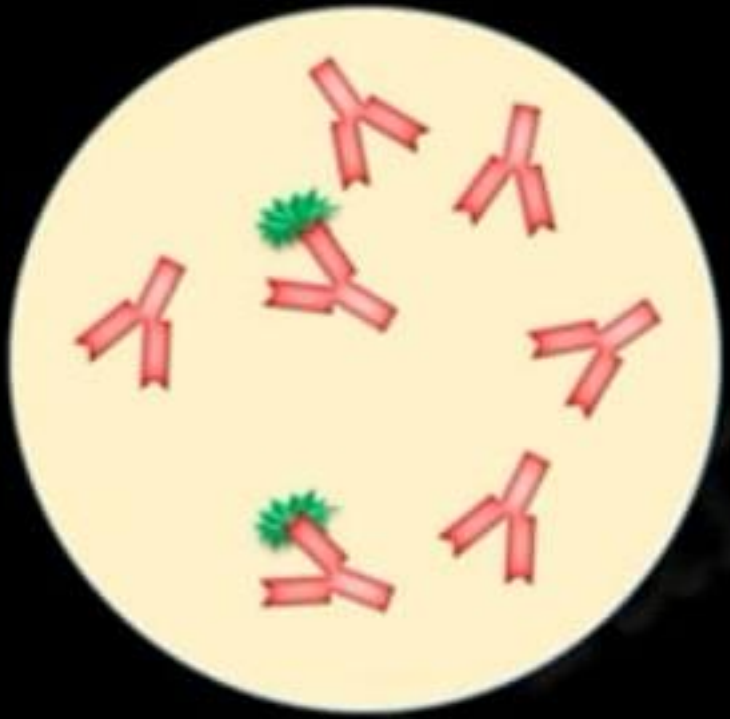
- Antibody must have atleast two antigen binding sites (bivalent).
- Antigen must be soluble, either bivalent or multivalent.
- The proportions of the antigens and antibodies must be equal.
- This reaction occurs in the presence of a electrolyte at suitable temperature and pH.
- Precipitation can take place in liquid media or in gels.

How the relative proportions of antigen and antibodies influence the formation of precipitate?

Test Samples

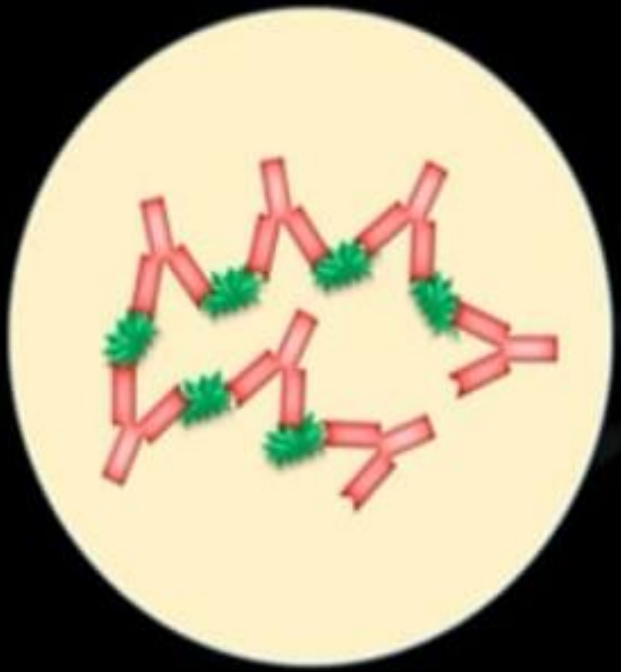


Test Sample 1



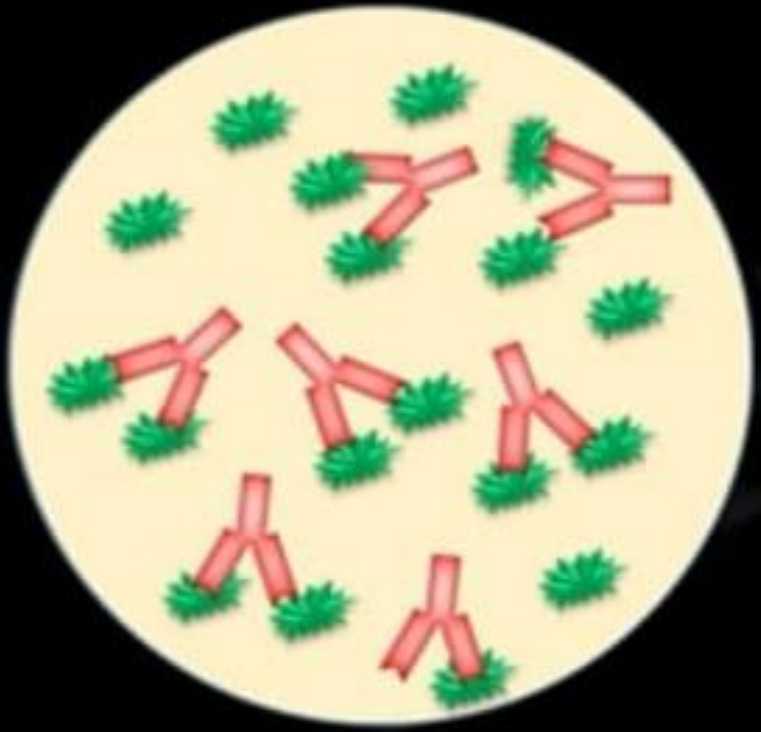
- Antibodies are in excess
- No cross-linkages
- No lattice formation

Test Sample 2



- Antibodies and antigen are in equal proportion
- Cross-linking occurs
- Lattice formation takes place
- Insoluble visible precipitate

Test Sample 3

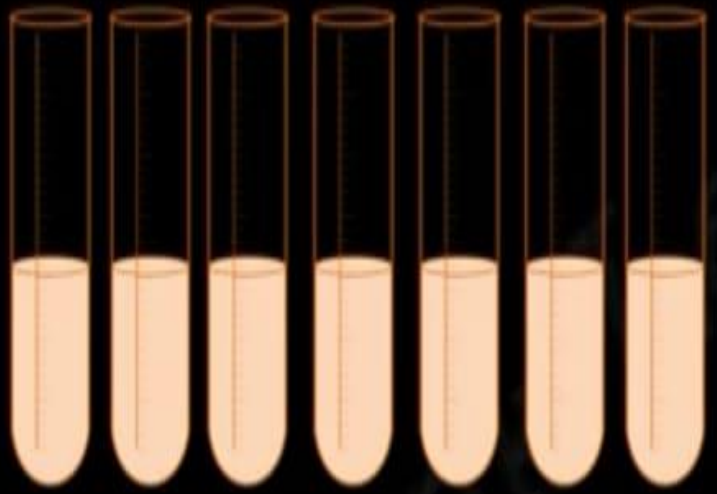


- Antigens are in excess
- No cross-linkages
- No lattice formation

Precipitation Curve

- Graphic representation of precipitation reactions
- Concentration of one reactant is kept constant
- Concentration of second reactant is increased serially

Precipitation Curve



Constant amount of antibody

+

Increasing amount of antigen

⬇

After sometime precipitation occurs

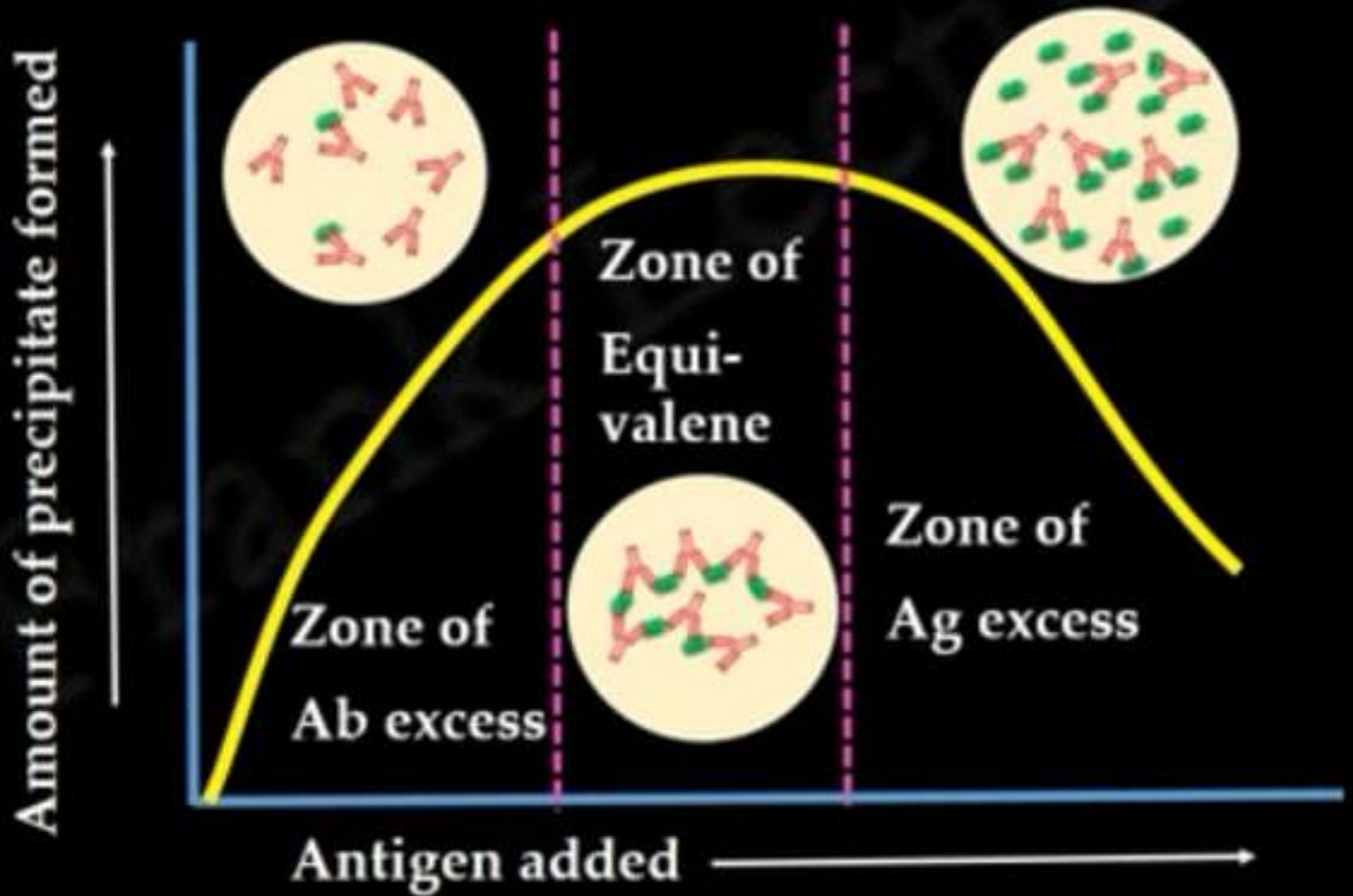
⬇

Centrifugation

⬇

Precipitate is measured

Precipitation Curve



Precipitation Reactions

When antigens (soluble) and antibody molecules are mixed in proper proportions in the presence of electrolytes at suitable temperature and pH, they form huge, insoluble, lattice-like complexes called precipitates.

PRECIPITATION REACTION

- Precipitation reactions are based on the interaction of antibodies and antigens. In this reaction, **two soluble reactants that come together to make one insoluble product**, the precipitate.
- Any antibody which reacts with an antigen to form a precipitate is called **Precipitin**.
- Precipitation assays are performed in semi-solid media such as Agar or Agarose where antibodies and antigens can diffuse toward one another and form a **visible line of precipitation**.

- Precipitation reactions are serological assays for the **detection of immunoglobulin levels from the serum** of a patient with infection.
- Precipitation reactions are widely used in Medicine for analysis of **Hormones, Enzymes, Toxins and Immune system products**.
- Some of the examples of Precipitation reactions are 1) Capillary Tube Precipitation (Ring Test), 2) Ouchterlony Double Immunodiffusion (Immunodiffusion or Agar gel Immunodiffusion or Passive Double Immunodiffusion), 3) Single Radial Immunodiffusion (SRID) (Mancini Method), 4) Immunoelectrophoresis (IEP), 5) Rocket electrophoresis and 6) Counterimmunoelectrophoresis (CIEP)

❑**Immunodiffusion** is a diagnostic technique for the detection or measurement of antibodies and antigens by their precipitation which involves diffusion through a substance such as agar or gel agarose .

ADVANTAGE

- The reaction formed from this method is stable and can be preserved for staining
- It can be used to detect identity ,non identity and cross reaction between antigens in a mixture

Classified on the basis of :-

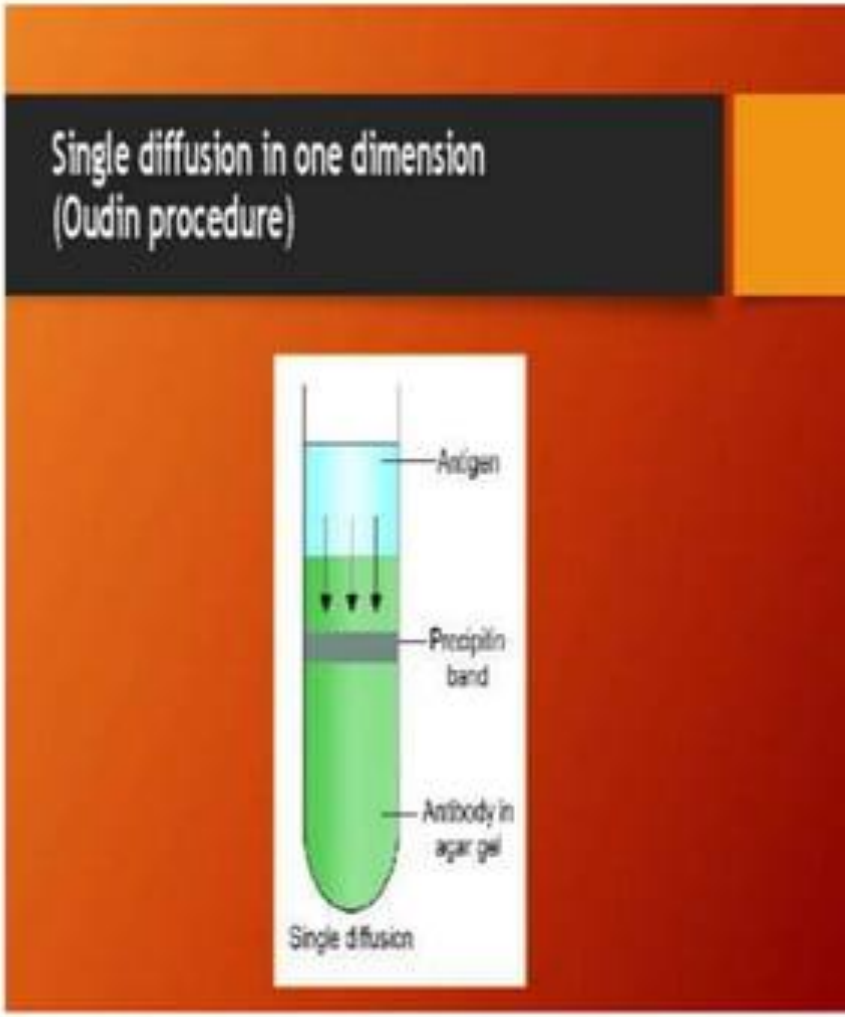
- Number of reactants diffusing
- Direction of diffusion

❖ The common types :-

- Single diffusion in one dimension (Oudin procedure)
- Double diffusion in one dimension (Oakley Fulthorpe procedure)
- Single diffusion in two dimension (radial immunodiffusion or Mancini method)
- Double diffusion in two dimensions (Ouchterlony double immunodiffusion

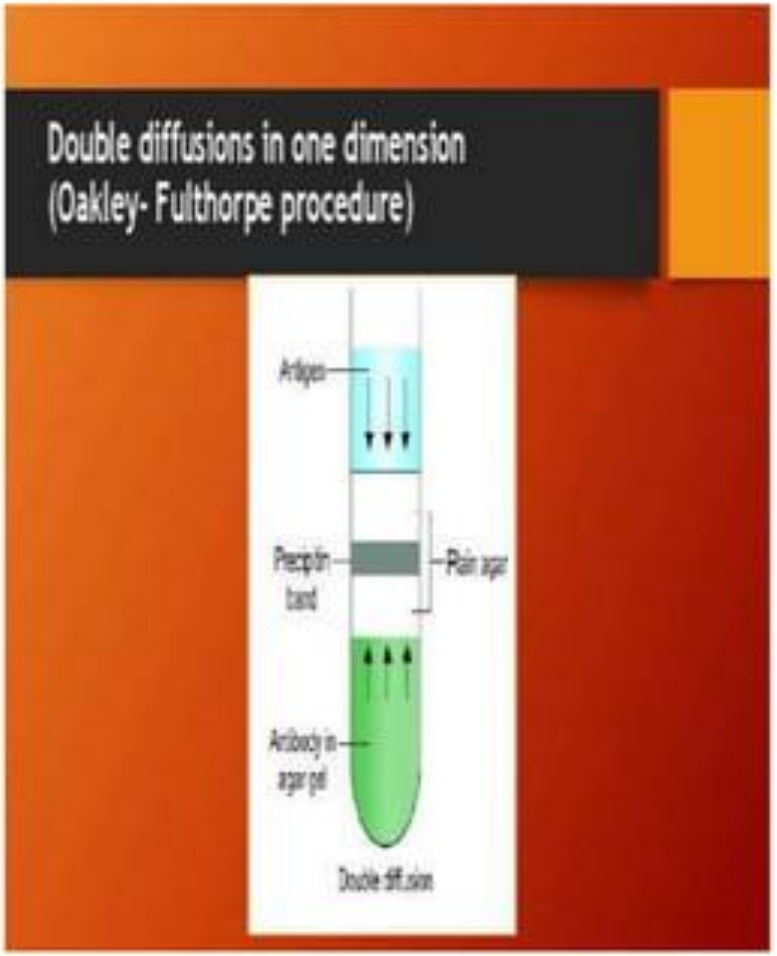
SINGLE DIFFUSION IN ONE DIMENSION (OUDIN TEST)

- The antiserum (antibody) is incorporated in melted agar and mixture is poured into a tube and allowed to solidify.
- Antigen solution is placed above the agar.
- The precipitin band appears in the agar.



DOUBLE DIFFUSION IN ONE DIMENSION (OAKLEY- FULTHORPE TEST)

- Antibody (antiserum) is incorporated in agar, poured into a tube and allowed to harden.
- A second layer of agar without antibody is placed above and allowed to solidify.
- Antigen solution is placed above the agar.
- The precipitin band appears in the plain agar column.



RADIAL IMMUNODIFFUSION/ MANCINI TECHNIQUE

Single Radial immunodiffusion is used extensively for the quantitative estimation of antigen

PRINCIPLE :- Here the antigen-antibody reaction is made more sensitive by the addition of antiserum into the agarose gel and loading the antigen sample in the well. As the antigen diffuses into the agarose radially in all directions, it's concentration continuously falls until the equivalence point is reached at which the antigen concentration is in equal proportion to that of the antibody present in the agarose gel. At this point ring of precipitation ('precipitin ring') is formed around the well. The diameter of the precipitin ring is proportional to the concentration of antigen. With increasing concentration of antigen, precipitin rings with larger diameter are formed.

➢ The size of the precipitin rings depends on:

- Antigen concentration in the sample well
- Antibody concentration in the agarose gel
- Size of the sample well
- Volume of the sample

Thus, by having various concentrations of a standard antigen, standard curve can be obtained from which one can determine the amount of an antigen in an unknown sample. Thus, this is a quantitative test. If more than one ring appears in the test, more than one antigen/antibody reaction may have occurred. This could be due to a mixture of antigens or antibodies.

This test is commonly used in the clinical laboratory for the determination of immunoglobulin levels in patient samples.

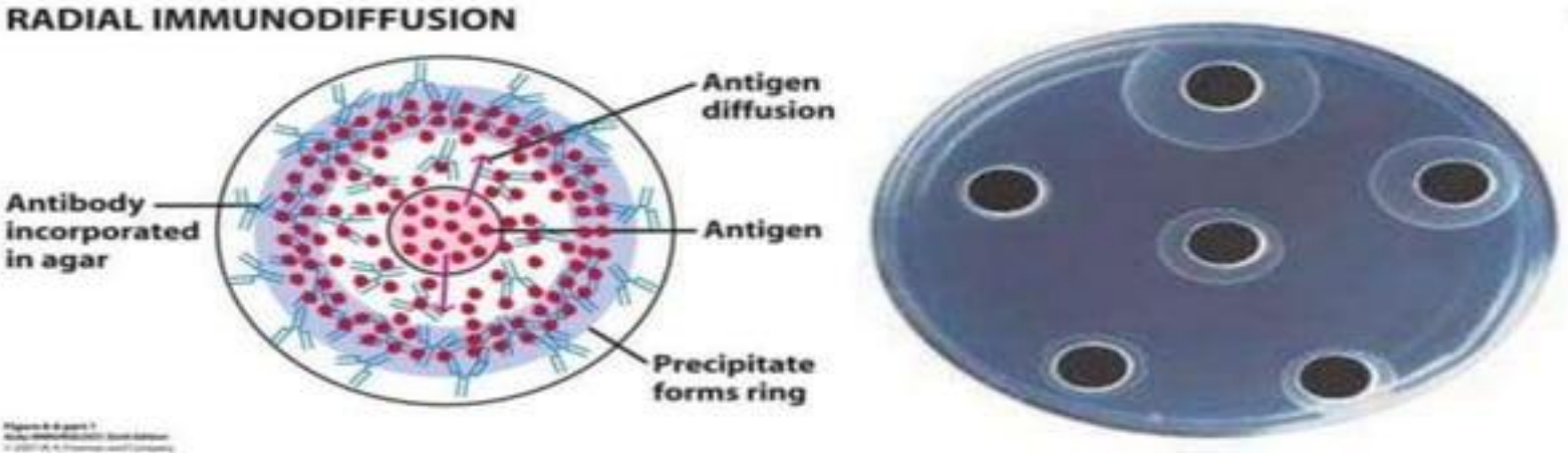
PROCEDURE

1 . An agar containing an appropriate antiserum (antibody) is poured in plates.
2 . Carefully circular wells are cut and removed from the plates.
3 . A single or series of standards containing known concentration of antigen are placed in separate wells, while control and “unknown” samples are placed in other remaining wells.
4 . As the antigen diffuses radially, a ring of precipitate will form in the area of optimal antigen – antibody concentration.
5 . The ring diameters are measured and noted.
6 . A standard curve is prepared using the ring diameters of the standards versus their concentrations. This curve is then used to determine the concentration of the control and unknown samples

Result Interpretation of Radial Immunodiffusion

1. The presence of a precipitin ring around the antigen wells indicate specific antigen-antibody interaction.
2. Absence of precipitin ring suggest absence of reaction.
3. The greater the amount of antigen in the well, the farther the ring will form from the wel

Single diffusion in two dimensions
(Radial immunodiffusion)



APPLICATIONS OF RADIAL IMMUNODIFFUSION

- Immuno-diffusion techniques are mostly used in immunology to determine the quantity or concentration of an antigen in a sample.
- Estimation of the immunoglobulin classes in sera.
- To determine relative concentrations of antibodies in serum.
- To compare properties of two different antigens.

OUCHTERLONY DOUBLE DIFFUSION

PRINCIPLE:- antigen solution placed in wells bore on gel plates while antibodies are placed in other remaining wells .on inucubation , both the antigens in the solution and the antibodies each diffuse out of their respective wells .in case of the antibodies recognizing the antigens ,they interact together to form visible immune complexes which precipitate in the gel to give a thin white line indicating a reaction

in case multiple wells are filled with different antigen mixtures and antibodies , the precipitate developed between two specific wells indicate the corresponding pair of antigen - antibodies

PROCEDURE

1.The test is performed by cutting wells in the agar gel poured on a petriplate

2. The antibodies is places in a centre well and different antigen are added in the well surrounding the centre well

3.after an incubation period in a moisture chamber ,the lines preciptins are formed at the site of the combination of antigen and antibody

The results may be either of the following:

A full identity (i.e. a continuous line): Line of precipitation at their junction forming an arc represents serologic identity or the presence of a common epitope in antigens.

Non-identity (i.e. the two lines cross completely): A pattern of crossed lines demonstrates two separate reactions and indicates that the compared antigens are unrelated and share no common epitopes.

Partial identity (i.e. a continuous line with a spur at one end): The two antigens share a common epitope, but some antibody molecules are not captured by the antigen and traverse through the initial precipitin line to combine with additional epitopes found in the more complex antigen.

The pattern of the lines that form can determine whether the antigens are the same.

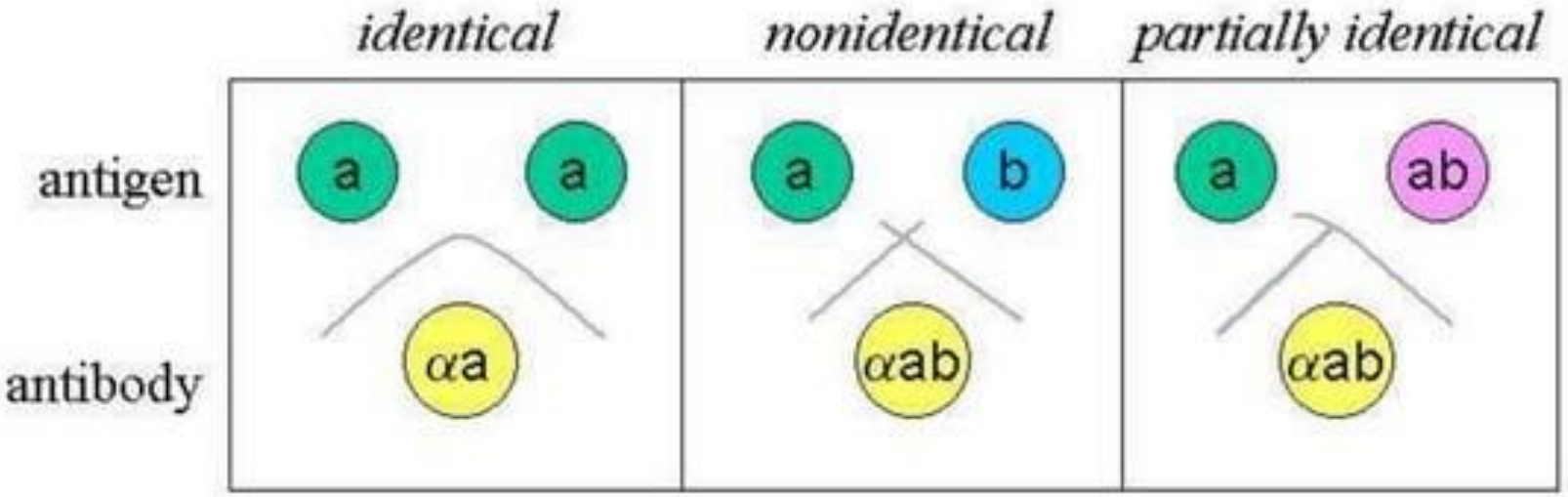
Applications

- It is useful for the analysis of antigens and antibodies.
- It is used in the detection, identification, and quantification of antibodies and antigens, such as immunoglobulins and extractable nuclear antigens.
- Agar gel immunodiffusions are used as serologic tests that historically have been reported to identify antibodies to various pathogenic organisms such as *Blastomyces*.
- Demonstration of antibodies in serodiagnosis of smallpox.
- Identification of fungal antigens.

Ouchterlony Double Immunodiffusion

- Ouchterlony Double Immunodiffusion (ODD) is also called as **Immunodiffusion or Agar gel Immunodiffusion or Passive Double Immunodiffusion**.
- ODD method was used in the detection, identification and **quantification of antibodies and antigens**, such as Immunoglobulins and extractable nuclear antigens.
- The technique is named after **Orjan Ouchterlony**, the Swedish physician who invented the test in 1948.
- In ODD method, the **pattern of lines** that form

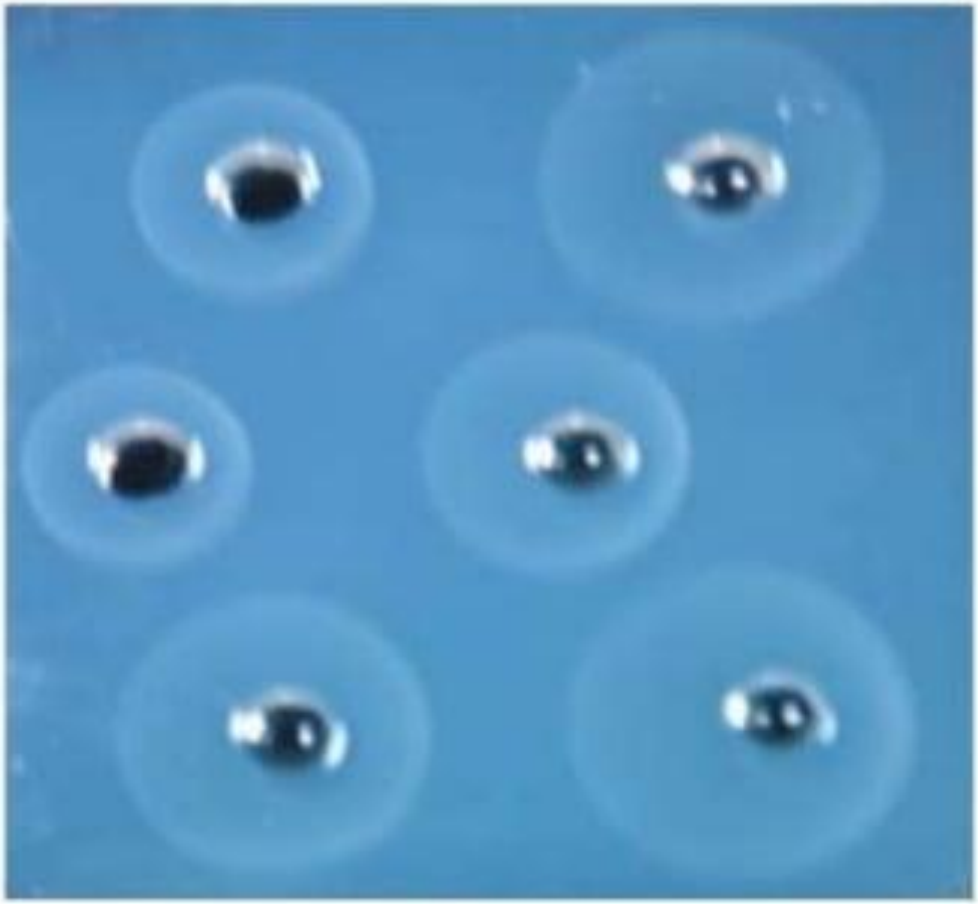
Ouchterlony Double Diffusion



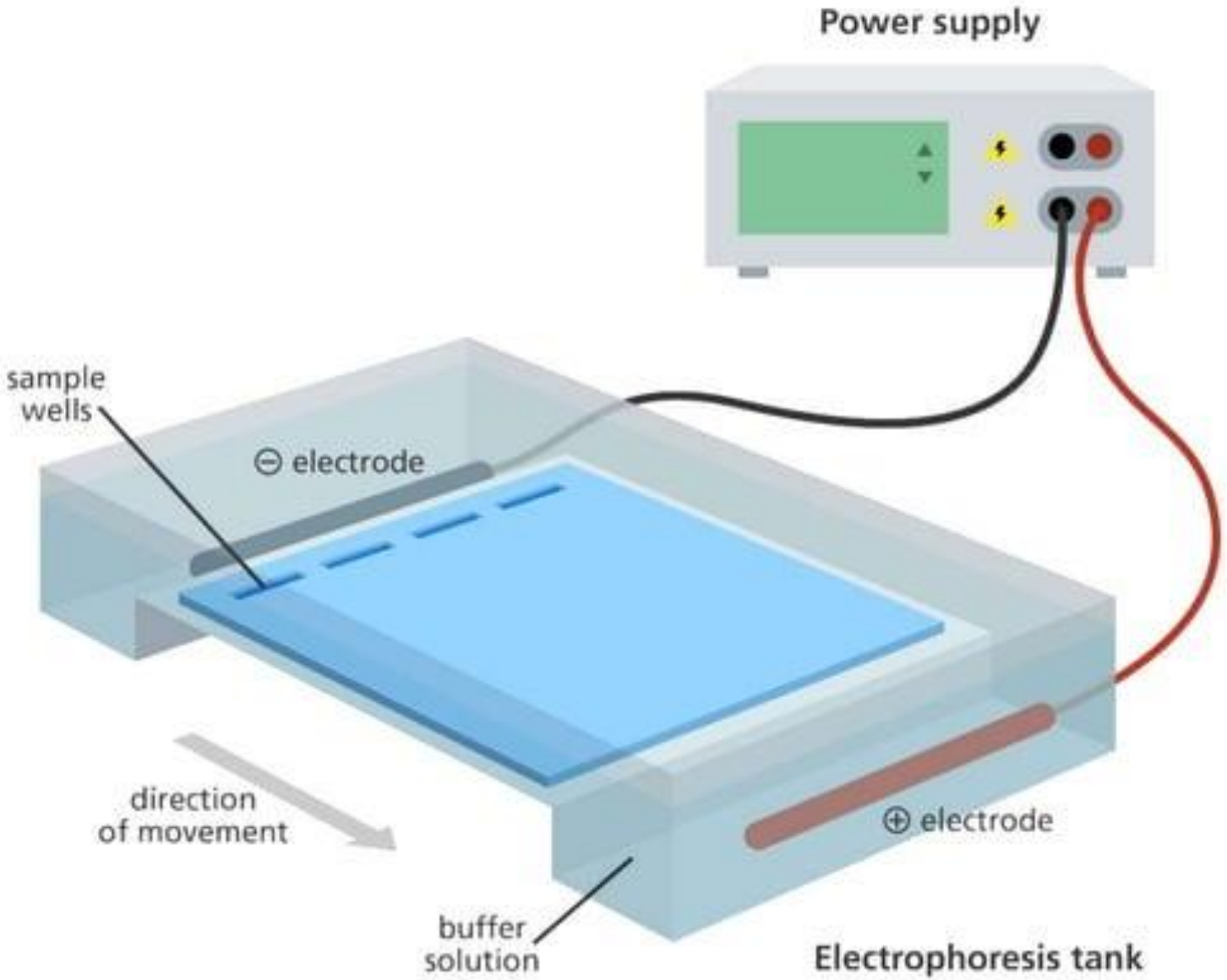
Single Radial Immunodiffusion (SRID)

- Radial Immunodiffusion or Mancini method, Mancini Immunodiffusion or Single Radial Immunodiffusion (SRID).
- SRID is an Immunodiffusion technique used to determine the **quantity or concentration of an antigen** in a sample.
- In SRID method the Antibody is incorporated into the Agarose gel whereas the Antigen diffuses into it in a radial pattern.
- SRID is an Immunodiffusion technique used to detect the **concentration of antigen by measuring the diameter of the precipitin ring** formed by the interaction of the antigen and the

Single Radial Immunodiffusion



Electrophoresis



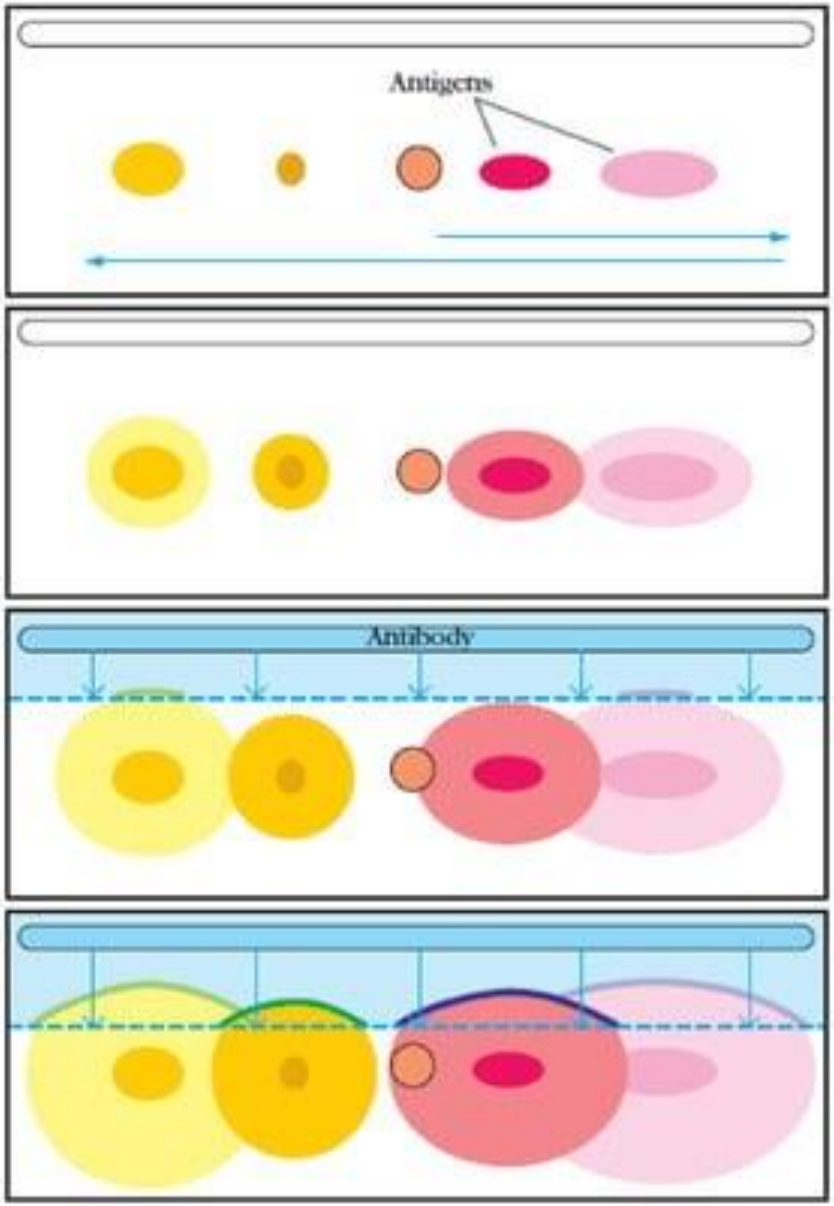
IMMUNOELECTROPHORESIS

- Immunoelectrophoresis was first coined by **Grabar and Williams** in 1953.
- Immunoelectrophoresis is a general name for a number of biochemical methods for **separation and characterization of proteins based on electrophoresis and reaction with antibodies**.
- Immunoelectrophoresis is the combination of **Immunodiffusion** (Mancini's Single Radial Immunodiffusion and Ouchterlony Double Diffusion) and **Electrophoresis**.

IMMUNOELECTROPHORESIS

- In Immunoelectrophoresis, the antigen mixture is first electrophoresed to separate its components by charge.
- Troughs are then cut into the agar gel parallel to the direction of the electric field, and antiserum is added to the troughs.
- Antibody and antigen then diffuse toward each other and produce lines of precipitation where they meet in appropriate proportions.
- Immunoelectrophoresis is a strictly Qualitative technique that only detects relatively high antibody concentrations (greater than 100 g/ml), it utility is limited to the detection of quantitative abnormalities only when the departure from

IMMUNOELECTROPHORESIS



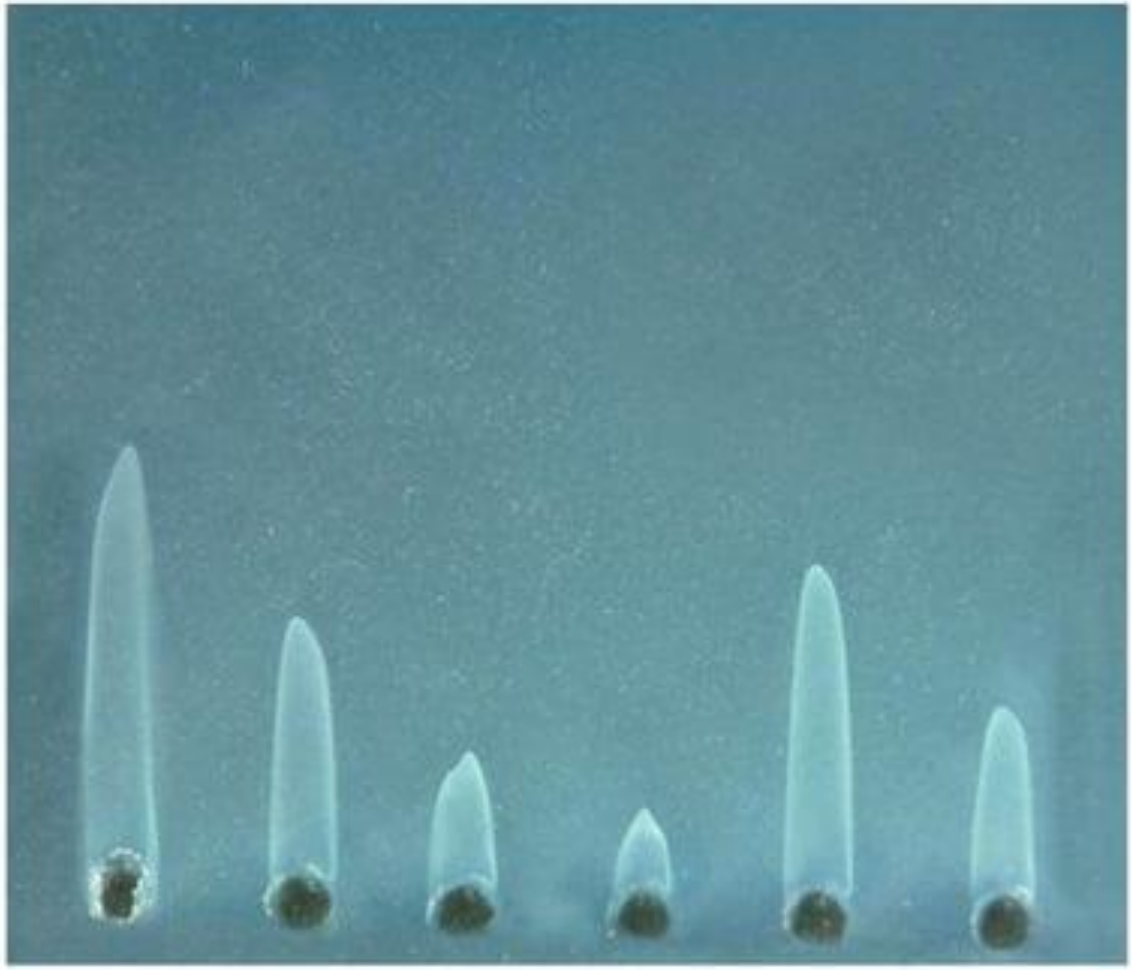
Applications of Immunoelectrophoresis

- Immunoelectrophoresis is used in clinical laboratories to detect the presence or absence of proteins in the serum.
- Detection of Immunodeficiency (In Immunodeficiency sample, no precipitin band is formed with particular antigen).
- Detection of Over production of Serum proteins (Albumin, Immunoglobulin and Transferrin).
- Detection of deficiency in Complement.
- Used to identify normal and abnormal proteins in Urine or Serum.
- Testing the purity of Antigen.

ROCKET ELECTROPHORESIS

- A related **Quantitative technique**, Rocket electrophoresis, does permit **measurement of antigen levels**.
- Also called as **Laurell Technique or One dimension electroimmunodiffusion**.
- It is called as an **adaption of SRID** and **more rapid than SRID**.
- In Rocket electrophoresis, a **negatively charged antigen** is electrophoresed in a gel containing antibody.
- The precipitate formed between antigen and antibody has the **shape of a Rocket (measured)**, the height of which is proportional

- One limitation of rocket electrophoresis is the need for the antigen to be negatively charged for electrophoretic movement within the agar matrix. Some proteins, immunoglobulins for example, are not sufficiently charged to be quantitatively analyzed by Rocket electrophoresis.



Thank You