Antigen–Antibody Reaction

Dr. Tanmay Mehta

Learning objectives

By the end of this session students should be able to know

- General properties of antigen antibody reactions
- Types of antigen antibody reactions
- Conventional immunoassays
- Newer techniques

Antigen-antibody reaction

- Bimolecular association where the antigen and antibody combine with each other specifically and in an observable manner .
  - Similar to an enzyme-substrate interaction.
  - Only difference is, it does not lead to an irreversible alteration in either antibody or in antigen.

PROPERTIES OF ANTIGEN ANTIBODY REACTIONS

1. **Specific**:
   - Ag-Ab reaction involves specific interaction of epitope of an antigen with the corresponding paratope of its homologous antibody.
   - Exception is the cross reactions which may occur due to sharing of epitopes among different antigens.
   - In such case, antibody against one antigen can cross react with a similar epitope of a different antigen.

PROPERTIES OF ANTIGEN ANTIBODY REACTIONS

2. **Non-covalent interactions:**

- Union of antigen and antibody requires formation of large number of non-covalent interactions between them such as:
  - Hydrogen bonds
  - Electrostatic interactions
  - Hydrophobic interactions
  - van der Waals forces

PROPERTIES OF ANTIGEN ANTIBODY REACTIONS

- 3. Strength- The strength or the firmness of the association is influenced by the affinity and avidity of the antigen-antibody interaction.
  - Affinity
    - Refers to sum total of non covalent interactions between a single epitope of an antigen with its corresponding paratope present on antibody.
    - Affinity can be measured by two methods- i) by equilibrium dialysis and ii) by surface plasmon resonance metho

PROPERTIES OF ANTIGEN ANTIBODY REACTIONS

- Avidity: Term used to describe the affinities of all the binding sites when multivalent antibody reacts with a complex antigen carrying multiple epitopes.
  - When a complex antigen carrying multiple epitopes reacts with a multivalent antibody; the total strength (i.e. avidity) would be much higher than the individual affinity at each binding site.
  - Avidity is not exactly equal but lower than the sum of all affinities.
  - This difference is primarily due to geometry of antigen antibody binding.

PROPERTIES OF ANTIGEN ANTIBODY REACTIONS

- Avidity is a better indicator of strength of an antigen antibody reaction.
- Avidity of an antibody can compensate for its low affinity.
  - For example, IgM has a low affinity than IgG but it is multivalent (10 valencies), therefore has a much higher avidity.It can bind to an antigen more effectively than IgG.

Diagnostic Use of Ag-Ab reactions

- Specific and observable
- The diagnostic tests based on antigen-antibody reactions are called as immunoassays
- Immunoassays can be broadly categorized into two types-
  - Antigen detection assays
  - Antibody detection assays

Qualitative assays

- Result is read as ‘positive’ or ‘negative’ based on presence or absence of antigen or antibody in the clinical specimen.

Disadvantages

- Number of antigen or antibody molecules in the reaction are disproportionate to each other.
  - If either antigen or antibody are present in higher quantity - false negative.
    - To rule out a false negative result, it is ideal to test the series of diluted sera (quantitative test) instead of just testing the one specimen of undiluted serum.

Quantitative assays

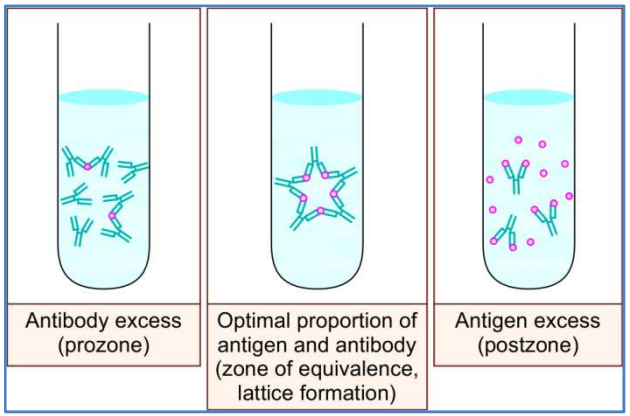
- When the qualitative test turns positive, the exact amount of antibody in serum can be known by serial dilution of the patient's serum and mixing each dilution of the serum with a known quantity of antigen.
- The measurement of antibody is expressed in terms of units or more commonly titer.

Quantitative assays

- Quantitative tests are more reliable as they can differentiate between true negative and false negative results.

Marrack's or Lattice hypothesis

- Ag-Ab reaction is weak or fails to occur when the number of antigen and antibodies are not proportionate to each other.
- Prozone phenomenon - In the earlier test tubes, antibodies are excess, hence the Ag-Ab reaction does not occur.
- Post zone phenomenon - In the later test tubes, antigen is excess, hence the Ag-Ab reaction fails to occur.



Marrack's or Lattice hypothesis

- In prozone/post zone - Lattice does not enlarge, due to inhibition of lattice formation by the excess antibody or antigen respectively, as the valencies of the antibody and the antigen respectively, are fully satisfied.

Evaluation of Immunoassays

- Sensitivity and specificity are the two most important statistical parameters.
- Sensitivity is defined as ability of a test to identify correctly all those who have the disease i.e. true positives.
- Sensitivity is calculated as = $\frac{\text{True positives}}{\text{True positives + False negatives}}$

Evaluation of Immunoassays

- Specificity is defined as ability of a test to identify correctly all those who do not have disease i.e. true negatives.
- Specificity is calculated as = $\frac{\text{True negatives}}{\text{True negatives + False positives}}$

Types of antigen-antibody reactions

Conventional techniques -

- Precipitation reaction
- Agglutination reaction
- Complement fixation test
- Neutralization test

Newer techniques -

- Enzyme linked immunosorbent assay (ELISA)
- Enzyme linked fluorescent assay (ELISA)
- Immunofluorescence Assay (IFA)
- Radioimmunoassay (RIA)
- Chemiluminescence-linked immunoassay (CLIA)
- Immunohistochemistry
- Rapid tests-
  - Lateral flow assay (Immunochromatographic test)
  - Flow through assay
- Western blot
- Immunoassays using electron microscope

PRECIPITATION REACTION

- **Definition-**When a *soluble antigen* reacts with its antibody in the presence of optimal temperature, pH and electrolytes (NaCl), it leads to formation of the antigen-antibody complex in the form of:
  - Insoluble precipitate band when gel containing medium is used or
  - Insoluble floccules when liquid medium is used (precipitate remains suspended as floccules)

Precipitation in liquid medium

- **Ring test**: In a narrow tube (e.g. capillary tube), antigen solution is layered over an antiserum;
  - Precipitate ring appears at the junction of two liquids.
  - Example: Streptococcal grouping by Lancefield technique, and Ascoli's thermoprecipitin test done for anthrax.

Precipitation in liquid medium

- **Flocculation test**- When a drop of antigen is mixed with a drop of patient's serum, then floccules appear.
  - Examples of slide flocculation test- VDRL and RPR tests used for diagnosis of syphilis
  - Examples of tube flocculation test- Kahn test used previously for syphilis

Precipitation in gel (Immunodiffusion)

- Using 1% soft agarose gel for precipitation reaction has many advantages over liquid medium-
  - Results in formation of clearly visible bands instead of floccules that can be preserved for longer time.
  - Can differentiate individual antigens from a mixture as each antigen forms a separate band after reacting with specific antibody.

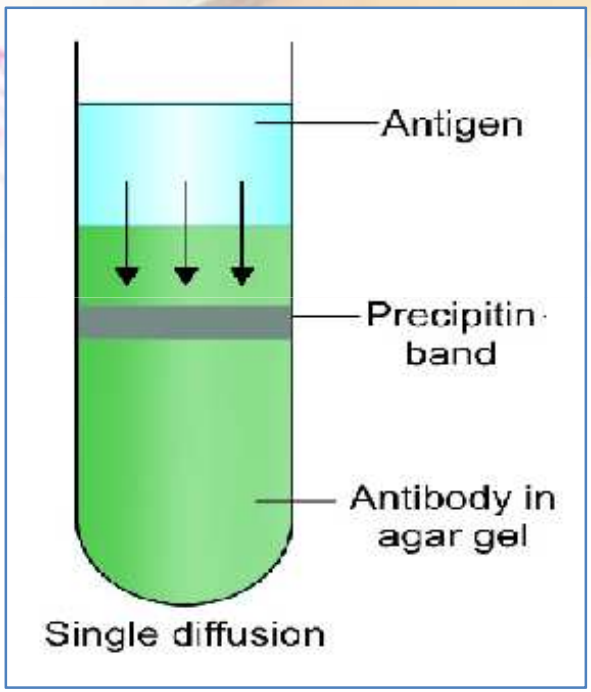
Principle of Immunodiffusion

- Whether only Ag diffuses (single diffusion) or both Ag and Ab diffuse (double diffusion)
- Whether Ag or Ab diffuses in *one dimension* (i.e. vertical diffusion when test is done on a tube layered with gel) or *two dimensions* (i.e. diffusion in both X and Y axis when test is done on a slide or a petri dish layered with gel)

Types of immunodiffusions in gel

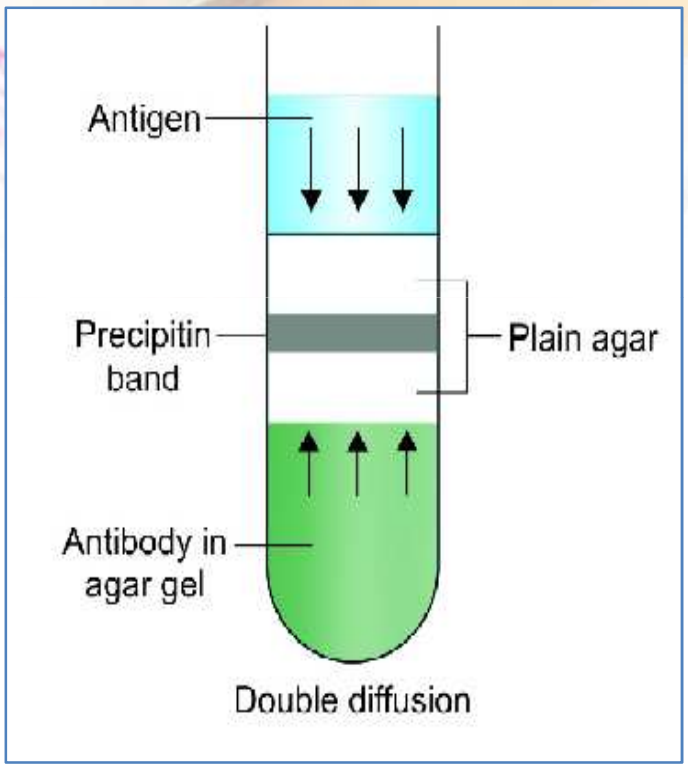
1. Single diffusion in one dimension (Oudin procedure)
2. Double diffusions in one dimension (Oakley- Fulthorpe procedure)
3. Single diffusion in two dimensions (Radial immunodiffusion)
4. Double diffusions in two dimensions (Ouchterlony procedure)

Single diffusion in one dimension (Oudin procedure)

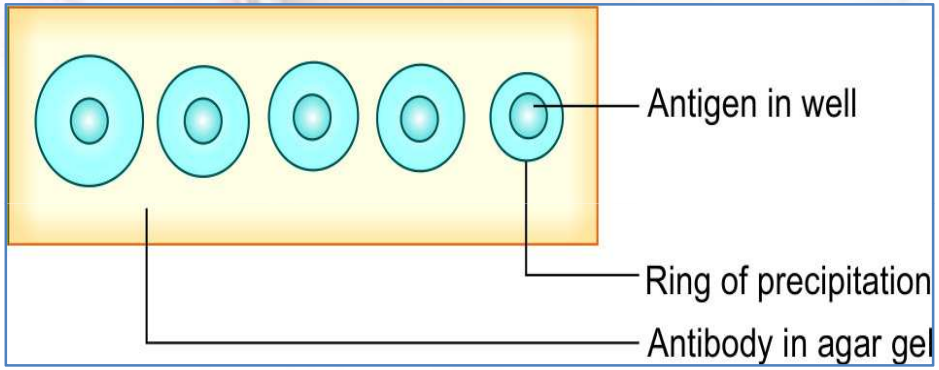


Single diffusion

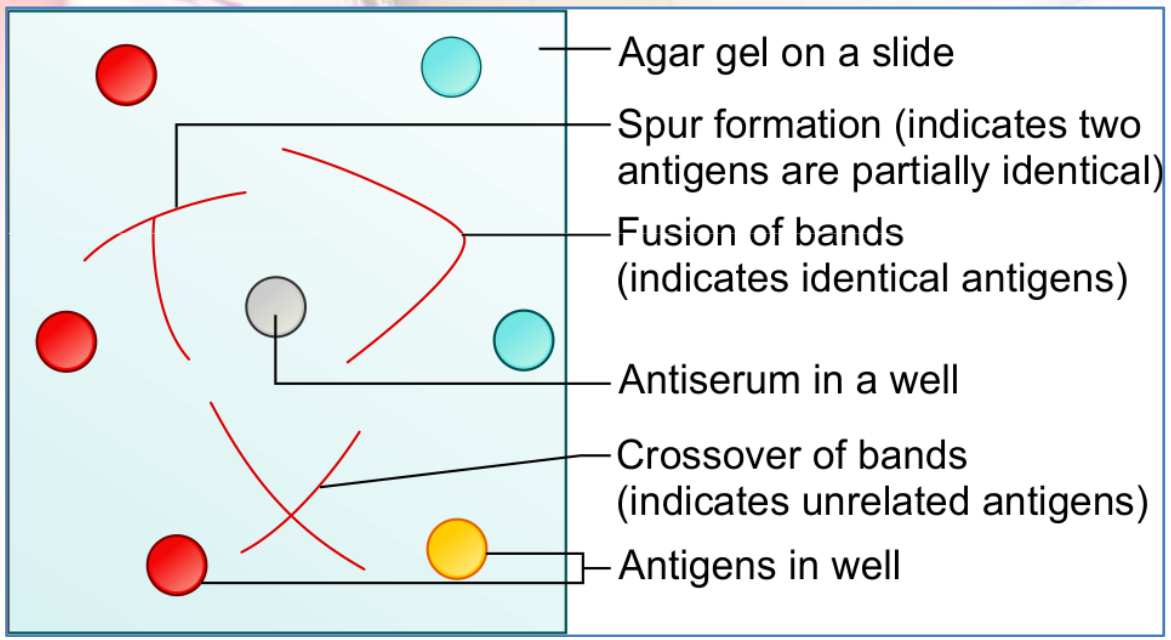
Double diffusions in one dimension (Oakley- Fulthorpe procedure)



Single diffusion in two dimensions (Radial immunodiffusion)



Double diffusions in two dimensions (Ouchterlony procedure)



Double diffusions in two dimensions (Ouchterlony procedure)

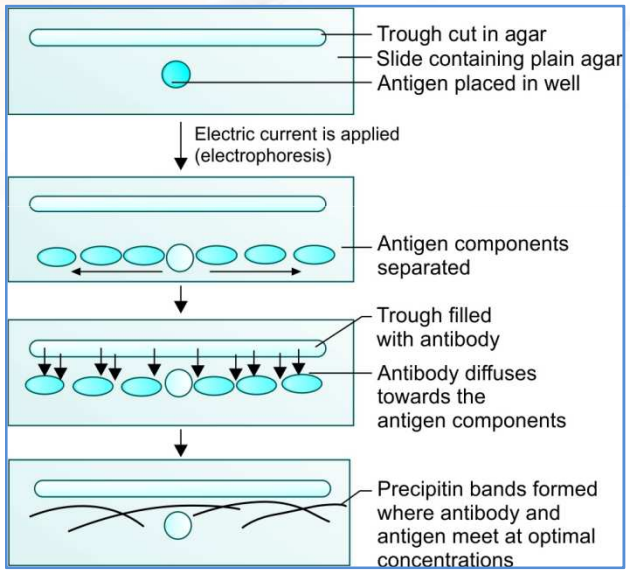
- Examples of double diffusions in two dimensions include:
  - **Elek's test** for detecting toxin of *Corynebacterium diphtheria*.
  - **Eiken test** to detect toxin of *Escherichia coli*

Precipitation in gel in presence of electric current

1. Electroimmunodiffusion (EID)
2. CIEP (Countercurrent immunoelectrophoresis)
3. Rocket electrophoresis

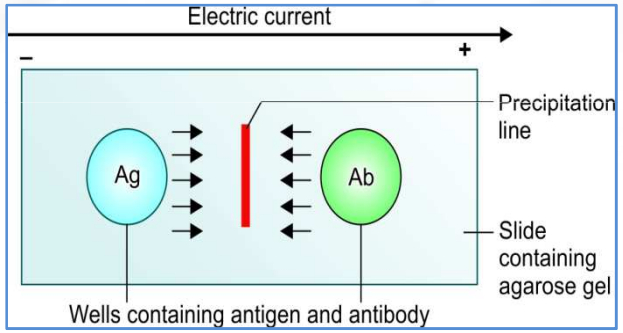
Electroimmunodiffusion

- Electric current is applied to a slide layered with gel.
- Helps in identification and approximate quantitation of various proteins present in the serum.



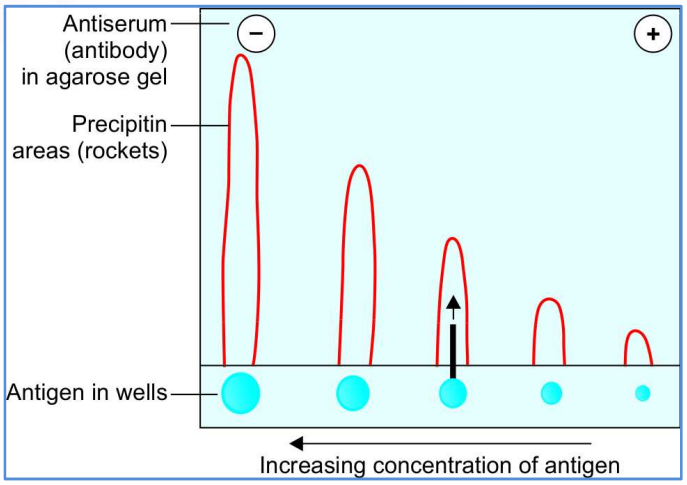
Counter-current immunoelectrophoresis

- Test is even faster (takes 30 minutes) and more sensitive than EID.
- Popular in the past - detecting alpha fetoprotein in serum and capsular antigens of *Cryptococcus* and meningococcus in the cerebrospinal fluid.



Rocket electrophoresis

- One-dimensional single electroimmunodiffusion test.
- Was mainly used in the past for quantitative estimation of antigens.



AGGLUTINATION REACTION

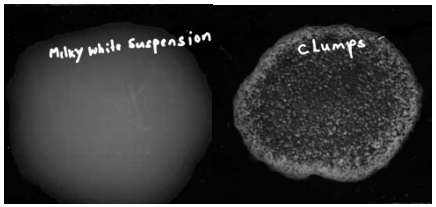
- **Definition-**When a **particulate** or **insoluble** antigen is mixed with its antibody in the presence of electrolytes at a suitable temperature and pH, the particles are clumped or agglutinated.
- **Advantage**:
  - More sensitive than precipitation test.
  - Clumps are better visualized and interpreted than bands or floccules.

AGGLUTINATION REACTION

- Wide diagnostic applications of agglutination reactions.
- Classified as:
  - Direct agglutination reactions.
  - Indirect (passive) agglutination reactions.
  - Reverse passive agglutination reactions.
- Performed either on a slide, or in tube or in card or some time in microtiter plates.

Direct agglutination test

- Antigen directly agglutinates with the antibody.
- ***Slide agglutination*:**
  - Performed to confirm the identification and serotyping of bacterial colonies grown in culture.
  - Method used for blood grouping and cross matching.



Tube agglutination

- Standard quantitative test for estimating antibody in serum.
- ***Antibody titer*** can be estimated as the highest dilution of the serum which produces a visible agglutination.

Tube agglutination- Applications

- Typhoid fever (Widal test)
- Acute brucellosis (Standard agglutination test)
- Coombs Antiglobulin test (see below)
- Heterophile agglutination tests:
  - Typhus fever (Weil Felix reaction)
  - Infectious mononucleosis (Paul Bunnell test)
  - *Mycoplasma* pneumonia (Cold agglutination test)

Microscopic agglutination test (MAT)

- Agglutination test is performed on a microtiter plate.
- Result is read under a microscope.
- MAT is done for leptospirosis.

Indirect or passive agglutination test (for antibody detection)

- Indirect hemagglutination test (IHA)
- Latex agglutination test (LAT)

Indirect hemagglutination test (IHA)

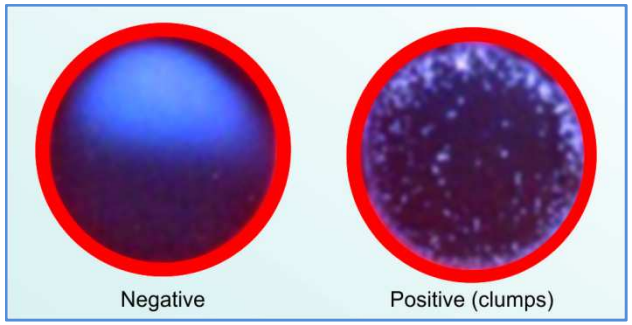
- Passive agglutination test where RBCs are used as carrier molecules.
- IHA was used widely in the past, but is less popular at present.

Latex agglutination test (LAT) for antibody detection

- Polystyrene latex particles (0.8 – 1 µm in diameter) are used as carrier molecules which are capable of adsorbing several types of antigens.
- For better interpretation of result, the test is performed on a black color card.
- Drop of patient's serum (containing antibody) is added to a drop of latex solution coated with the antigen and the card is rocked.

Latex agglutination test (LAT) for antibody detection

- Positive result is indicated by formation of visible clumps.
- LAT is one of the most widely used tests at present as it is very simple and rapid.
- Used for detection of ASO (antistreptolysin O antibody).



Reverse passive agglutination test (for antigen detection)

- Antibody is coated on a carrier molecule which detects antigen in the patient's serum.

Test	Carrier molecule	Clinical applications
RPHA (Reverse passive hemagglutination assay)	RBCs	• Detection of hepatitis B surface antigen (HBsAg) – not used now.
Latex agglutination test	Latex particles	• Detection of CRP (C reactive protein), RA (rheumatoid arthritis factor), capsular antigen detection in CSF (for pneumococcus, meningococcus and *Cryptococcus*) and streptococcal grouping.

Reverse passive agglutination test (for antigen detection)

Test	Carrier molecule	Clinical applications
Coagglutination test	*Staphylococcus aureus*	• Used in past for antigen detection test in the past (e.g. *Salmonella* antigen detection from blood and urine). • Obsolete at present.

Hemagglutination test

- Refers to the agglutination tests that use red blood cells (RBCs) as source of antigen.
- Various types of hemagglutination tests include:
  - ***Direct hemagglutination test***
  - ***Indirect hemagglutination test***

Direct hemagglutination test

- Serum antibodies directly agglutinate with surface antigens of RBCs to produce a matt. Examples include-
  - Paul Bunnell test
  - Cold agglutination test
  - Blood grouping
  - Coombs test or Antiglobulin test

Coombs test or **Antiglobulin test**

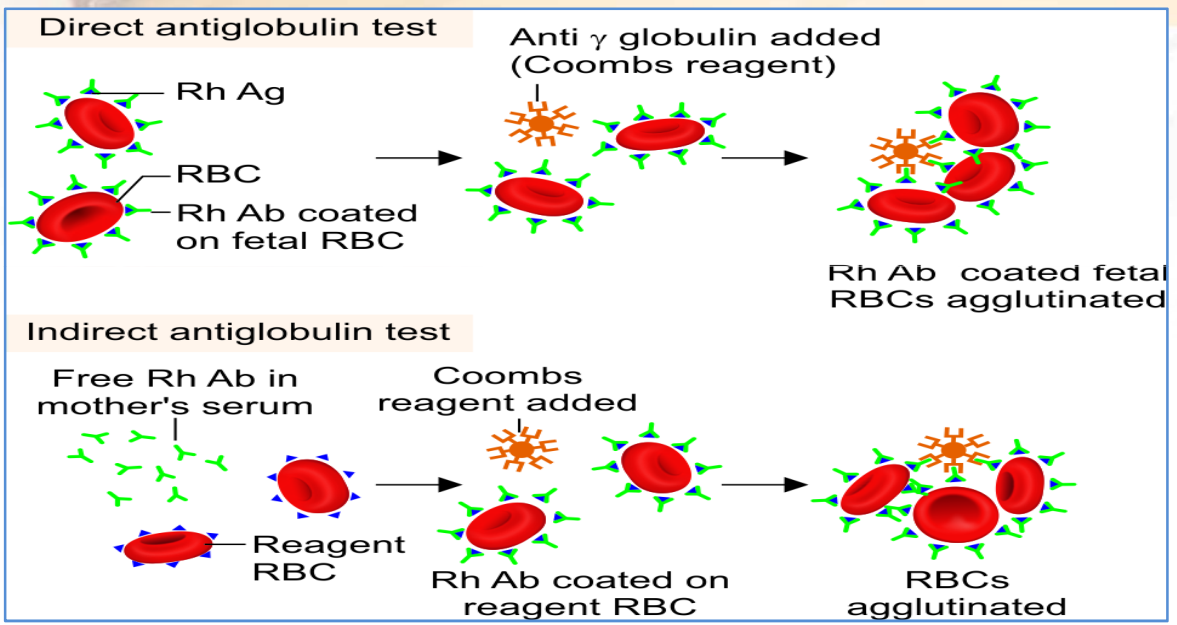
- Performed to diagnose Rh incompatibility by detecting Rh antibody from mother's and baby's serum.
- *Rh incompatibility* is a condition when an Rh negative mother (Rh Ag -ve and Rh Ab -ve) delivers a Rh positive baby (Rh Ag +ve and Rh Ab -ve).
- During birth, some Rh Ag +ve RBCs may pass from fetus to the maternal circulation and may induce Rh Ab formation in mother and affect future Rh positive pregnancies.

Coombs test or **Antiglobulin test**

- Rh antibodies are ***incomplete*** or ***blocking*** antibodies of IgG type.
- They can cross placenta and bind to Rh Ag on fetal RBCs.
- Does not result in agglutination; instead they block the sites on fetal RBCs.
- Such reaction can be visualized by adding ***Coombs reagent*** *(antiglobulin* or *antibody to human IgG*) which can bind to Fc portion of Rh Ab bound on RBCs, resulting in visible agglutination.

Coombs test or Antiglobulin test - Types

- *Direct Coombs test*
- *Indirect Coombs test*



Viral hemagglutination test

- In strict sense, it is not an antigen antibody reaction. The hemagglutinin antigens (HA) present on surface of some viruses
- Hemagglutinating viruses (e.g. influenza virus) can agglutinate with the receptors present on the surface of RBCs.

Technical issues in agglutination reactions

- Two main problems pertaining to agglutination - can cause **false negative** agglutination test.
  - *Prozone phenomenon*
  - *Blocking antibodies*

COMPLEMENT FIXATION TEST

- Detects the antibodies in patient's serum that are capable of fixing with complements. It was once very popular, now is almost obsolete.
- **Applications:**
  - **Wasserman test**
  - Was also widely used for detection of complement fixing antibodies in *Rickettsia, Chlamydia, Brucella, Mycoplasma infections and some viral* infections, such as arboviruses, rabies, etc.
  - Complements are also used for various serological tests other than CFT, such as:
    - *Treponema pallidum immobilization test*
    - Sabin-feldman dye test for *Toxoplasma*
    - Vibriocidal antibody test.

NEUTRALIZATION TEST

- Neutralization tests are also less commonly used in modern days. Various examples are as follows:
  - **Viral neutralization test**
  - **Plaque inhibition test**

NEUTRALIZATION TEST

- **Toxin–antitoxin neutralization test: Examples include**
  - **Schick test**
  - **Nagler's reaction**
  - **ASO test**

NEUTRALIZATION TEST

- **Hemagglutination inhibition (HAI) test:**
  - Antibodies in patient's sera can agglutinate with the hemagglutinin antigens present on the surfaces of some viruses.
  - Was used in past for the diagnosis of various viral diseases, e.g. influenza.

NEWER TECHNIQUES

- Uses a detector molecule to label antibody or antigen which in turn detects the corresponding antigen or the antibody in the sample by producing a visible effect.
- Most of the newer techniques use the same principle, but they differ from each other by the type of labelled molecule used and the type of visible effect produced.

Immunoassays and the types of molecule used for labeling

Abbreviation	Immunoassay method	Molecules used for labeling	Type of visible effect
ELISA	Enzyme linked immunosorbent assay	Enzyme- substrate-chromogen complex	Color change is detected by spectrophotometer
ELFA	Enzyme linked fluorescent assay	Enzyme- substrate	Fluorometric detection
IFA	Immunofluorescence Assay	Fluorescent dye	Emits light, detected by fluorescence microscope
RIA	Radioimmunoassay	Radioactive isotope	Emits β and γ radiations, detected by β and γ counters
CLIA	Chemiluminescence-linked immunoassay	Chemiluminescent compounds	Emits light, detected by luminometer

Immunoassays and the types of molecule used for labeling

Abbreviation	Immunoassay method	Molecules used for labeling	Type of visible effect
IHC	Immunohistochemistry	Enzyme or Fluorescent dye	Color change (naked eye) or Fluorescence microscope
WB	Western blot	Enzyme	Color band (naked eye)
Rapid test	Immunochromatographic test	Colloidal gold or silver	Color band, (naked eye)
	Flow through assay	Protein A conjugate	Color band, (naked eye)
IEM	Immunoferritin electron microscopy	Electron dense molecules (e.g ferritin)	Appears as black dot under electron microscope

ENzYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

- Enzyme immunoassay (EIA) is a term used to describe all the tests that detect either antigen or antibodies or haptens in the specimen, by using enzyme–substrate system for detection.
- They can be classified as below:
  - **Homogenous EIA**
  - **Heterogeneous EIA**

ENzYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

- **Homogenous EIA**
  - All reagents are added at one step.
  - Used for detection of haptens such as drugs (e.g. opiates, cocaine), but not for detection of microbial antigens and antibodies
- **Heterogeneous EIA**
  - **Involves multiple steps with** different reagents being added at every step.
  - Used for detection of antigens and antibodies.
  - ELISA is a classical example.

ELISA (ENZYME LINKED IMMUNOSORBENT ASSAY)

- So named because of two of its components-
  - Immunosorbent- Absorbing material used (e.g. polystyrene, polyvinyl) that specifically absorbs the antigen or antibody present in serum.
  - Enzyme is used to label one of the components of immunoassay (i.e. antigen or antibody).

Substrate-chromogen system

- Substrate- chromogen system is added at the final step of ELISA.
- Enzyme-substrate reaction in turn activates the chromogen to produce a color.
- Sometime, the substrate is chromogenic in nature (e.g. pNPP), on reaction with the enzyme, it changes its color.

Substrate-chromogen system

- Color change - detected by spectrophotometry in an ELISA reader.
- Intensity of the color is directly proportional to the amount of detection molecule (Ag or Ab) present in test serum.

Enzyme	Substrate	Chromogen
Horseradish Peroxidase	Hydrogen peroxide	Tetramethyl benzidine (TMB)
Urease	Urea	Bromocresol
β-Galactosidase	ONPG	ONPG
Alkaline Phosphatase	pNPP*	pNPP*

ELISA

- (Ag-Ab complex)-enzyme + substrate → activates the chromogen → color change → detected by spectrophotometry

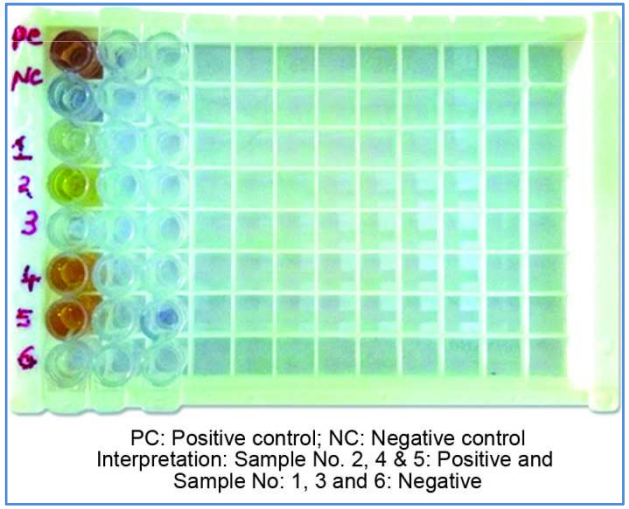
ELISA reader (Biorad)

ELISA washer

Automated ELISA
(EVOLIS system, Biorad)

ELISA

- Performed on a microtiter plate containing 96 wells (micro-ELISA) or less commonly performed in tubes (macro-ELISA).
- The microtiter plate or the tubes are made up of polystyrene, polyvinyl or polycarbonate material.



PC: Positive control; NC: Negative control
Interpretation: Sample No. 2, 4 & 5: Positive and Sample No: 1, 3 and 6: Negative

ELISA

- ELISA kits are commercially available; contain all the necessary reagents (such as enzyme conjugate, dilution buffer, substrate/chromogen etc).
- Procedure involves a series of steps done sequentially; at each step, a reagent is being added, and then incubated followed by washing of the wells (manually or by automated ELISA washer).

Types of ELISA

ELISA type	Used for detection of	Enzyme is labeled with
Direct ELISA	Antigen	Primary antibody
Indirect ELISA	Antibody or antigen	Secondary antibody
Sandwich ELISA	Antigen	Primary antibody in sandwich direct ELISA Secondary antibody in sandwich indirect ELISA
Competitive ELISA	Antigen or antibody	Secondary antibody
ELISPOT	Cells producing antibody or cytokine	Primary antibody

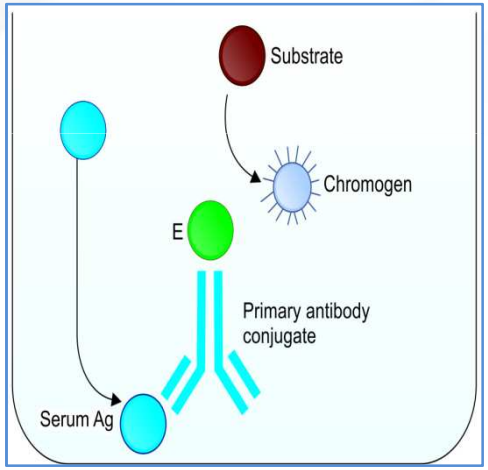
Direct ELISA

- Used for detection of antigen in test serum.
- Primary antibody (targeted against the serum antigen) is labeled with the enzyme.

Steps	Explanation
Step 1	Wells of microtiter plate are empty, not precoated with Ag or Ab.
Step-2	Test serum (containing antigen) is added into the wells. Antigen becomes attached to the solid phase by passive adsorption.
Step-3	After washing, the enzyme-labeled primary antibodies (raised in rabbits) are added.
Step-4	After washing, a substrate- chromogen system is added and color is measured.

Direct ELISA

- Well + Ag (test serum) + primary Ab-Enzyme + substrate-chromogen → Color change.



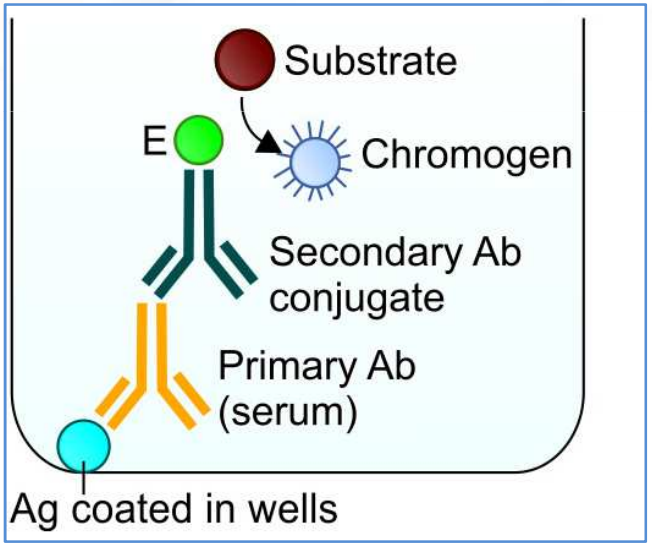
Indirect ELISA – Antibody detection

- It differs from the direct ELISA in that the secondary antibody (Ab targeted to Fc region of any human Ig) is labeled with enzyme instead of primary antibody.

Steps	**Explanation**
Step 1	Solid phase of the wells of microtiter plates are precoated with the Ag.
Step-2	Test serum (containing primary Ab specific to the Ag) is added to the wells. Ab gets attached to the Ag coated on the well.
Step-3	After washing, enzyme-labeled secondary Ab (anti-human immunoglobulin) is added.
Step-4	After washing, a substrate-chromogen system is added and color is developed.

Direct ELISA

- Wells are coated with Ag + primary Ab (test serum) + secondary Ab-Enzyme + substrate- chromogen → development of color.



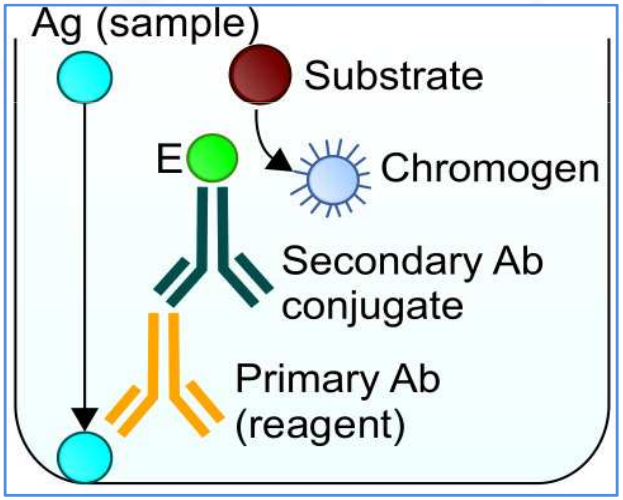
Indirect ELISA – Antigen detection

- Wells are empty, not precoated with Ag or Ab.

Steps	**Explanation**
Step 1	Test antigen (serum) is added to the well. The Ag gets absorbed onto the well.
Step-2	Primary antibody raised in rabbits (reagent) is added. The Ag binds to the primary antibody.
Step-3	After washing, enzyme-labeled secondary Ab (anti-rabbit Ab) is added.
Step-4	After washing, a substrate- chromogen system is added and color is developed.

Direct ELISA

- Ag (test serum) added, gets adsorbed to well + primary Ab + secondary Ab-Enzyme + substrate- chromogen → development of color.



Sandwich ELISA

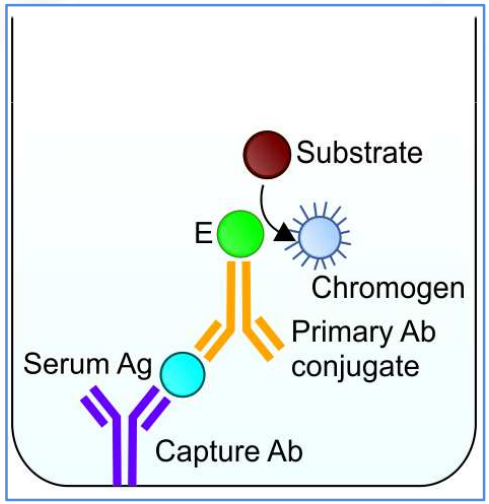
- Detects the antigen in test serum.
- So named because the antigen gets sandwiched between a capture antibody and a detector antibody.
- There are two types of sandwich ELISA:
  - Direct - detector antibody is a primary antibody.
  - Indirect - detector antibody is a secondary antibody.

Direct sandwich ELISA

Steps	Explanation
Step 1	Microtiter well is precoated with the capture antibody (monoclonal Ab raised in rabbit) targeted against the test antigen.
Step-2	Test serum (containing antigen) is added to the wells. Ag gets attached to the capture antibody coated on the well.
Step-3	After washing, an enzyme labeled primary 'detector antibody' specific for the antigen is added. The detector antibody can be same as the capture antibody.
Step-4	After washing, a substrate-chromogen system is added and color is developed.

Direct sandwich ELISA

- Wells coated with capture Ab + Ag (test serum) + primary Ab-enzyme + substrate- chromogen → color

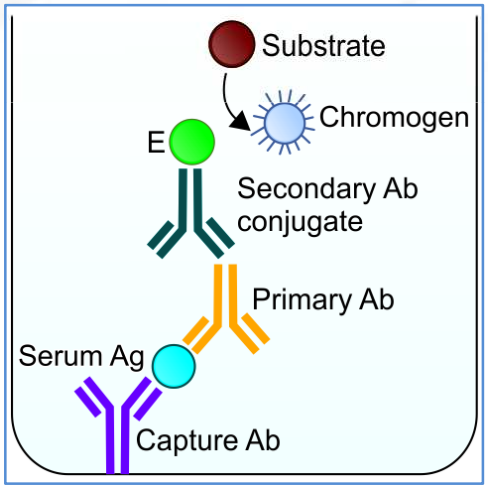


Indirect sandwich ELISA

- Primary antibody and the capture antibody belong to different species. More so, the primary antibody is not labelled with enzyme.
- Another enzyme-labelled secondary antibody targeted against the primary antibody is added.
- More specific than direct sandwich ELISA.

Direct sandwich ELISA

- Wells coated with capture Ab + Ag (test serum) + primary Ab + secondary Ab- enzyme + substrate- chromogen → color.



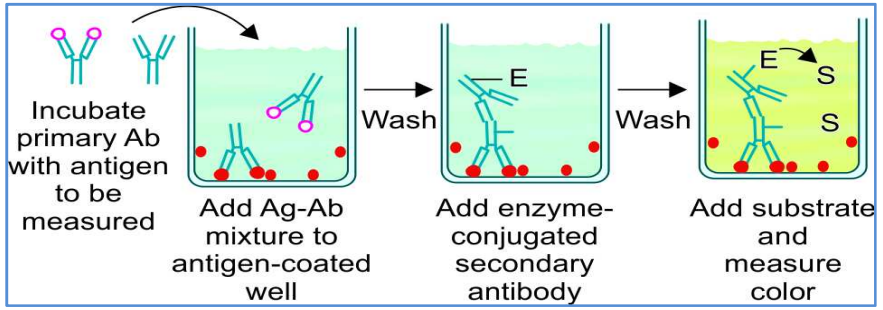
Competitive ELISA

- Example: Indirect competitive ELISA format used for antigen detection.

Steps	Explanation
Step 1	Primary antibody is first incubated in a solution with a serum sample containing the test antigen.
Step-2	Antigen-antibody mixture is then added to the microtiter well precoated with the same type of antigen.
Step-3	Free antibodies bind to the antigen coated on the well. More the test antigens present in the sample, lesser free antibodies will be available to bind to the antigens coated onto well.

Competitive ELISA

Steps	Explanation
Step-4	After washing (to remove free antibodies and antigens), enzyme-conjugated secondary antibody is added.
Step-5-	After washing, a substrate- chromogen system is added and color is developed. Intensity of the color is ***inversely proportional*** to the amount of antigen present in the test serum.



Competitive ELISA

- Can also be used for the detection of antibody in serum.
- Different formats are available such as direct, indirect and sandwich formats.

ELISPOT test

- Modification of ELISA that allows the quantitative detection of cells producing antibodies (plasma cells) or cytokines (e.g. macrophage).
- Procedure:
  - Microtiter well is coated with the capture antibody specific for the cytokine.
  - A suspension of the cell population under investigation is then added to the coated wells and incubated.
  - Sensitized cells capable of producing the cytokines settle onto the surface of the well.

ELISPOT test

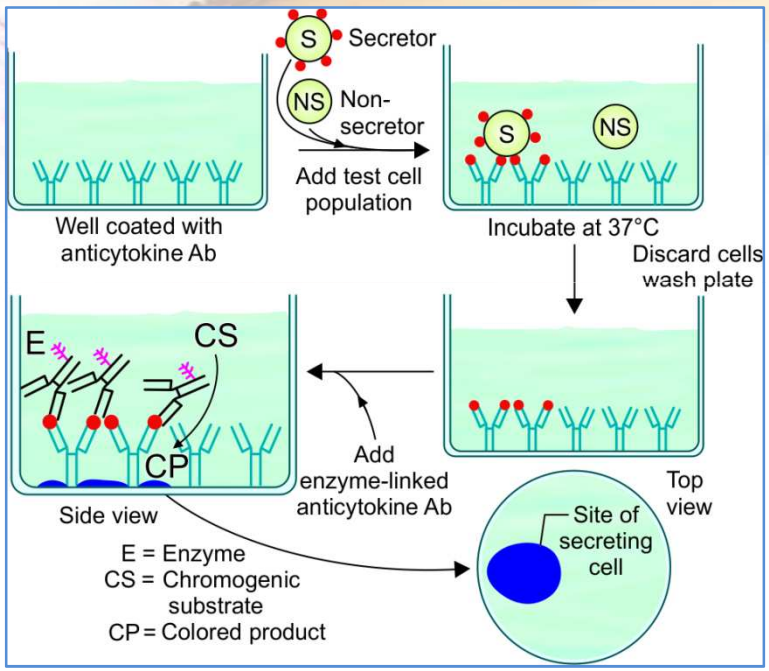
- Released cytokines are bound by the capture antibodies in the vicinity of the secreting cells, producing a ring of antigen-antibody complexes around each cell that is producing the cytokine of interest.
- After the incubation period, the well is washed and an enzyme-labeled anti-cytokine antibody is added.
- After washing away unbound antibody, a chromogenic substrate that forms an insoluble colored product is added.
- Colored product (purple) precipitates and forms a spot only on the areas of the well where cytokine-secreting cells have been deposited.

ELISPOT test

- Quantitation-The number of colored spots corresponds to the number of cytokine producing cells present in the added cell suspension.
- For quantitative detection of antibody producing cells, the procedure is similar except that the wells are coated with capture antigen which would bind to the antibodies produced by the cells.

- ELISPOT test is currently used in IGRA (interferon gamma assay), for diagnosis of latent tuberculosis.

ELISPOT test



IgG Avidity ELISA

- **Principle:** Avidity of IgG indicates how firmly the IgG antibody is bound with its antigen. Avidity reflect the maturity of the antibodies; which usually increases with time.
  - Low avidity antibodies are synthetized during the primary infection. it also occurs in immunosuppression.
  - High avidity antibodies are produced by memory B-cells during the secondary infection, or reactivation, recovery or in vaccinated people.

IgG Avidity ELISA

- **Procedure:**
  - ELISA is performed in two parallel wells of microtiter plate; one well with untreated patient serum and a second well with serum sample after treating with dissociating agents such as urea.
    - Low avidity antibodies dissociate from complexes and are washed away during the washing step of ELISA;
    - High avidity antibodies withstand urea treatment and remain bound to the antigens.

IgG Avidity ELISA

- **Results:** Avidity (%) is expressed as absorbance of the well with urea to absorbance of the well without urea.
  - Avidity <40%- interpreted as low avidity
  - Avidity >60%- interpreted as high avidity
  - Avidity 40-60% - interpreted as grey zone (inconclusive result); test has to be repeated.

IgG Avidity ELISA

- **Uses: IgG avidity ELISA is useful in:**
  - Differentiating recent and past infection- in situations where IgM disappears very soon in primary infection; or IgM persists long time even after resolution of primary infection.
  - Diagnosing atypical or sub clinical course of the disease.
  - For diagnosing congenital infection.
- **Applications:** IgG avidity test is available for : rubella, CMV, VZV, toxoplasmosis, EBV, HIV, viral hepatitis and West Nile virus infection.

Advantage of ELISA

- Method of choice for detection of antigens/antibodies in serum - in big laboratories as large number of samples can be tested together using the 96 well microtiter plate.
- Economical, takes 2-3 hours for performing the assay.
- Most sensitive immunoassay - commonly used for performing screening test at blood banks and tertiary care sites.
- Specificity used to be low - But now, with use of more purified recombinant and synthetic antigens, and monoclonal antibodies, ELISA has become more specific.

Disadvantage of ELISA

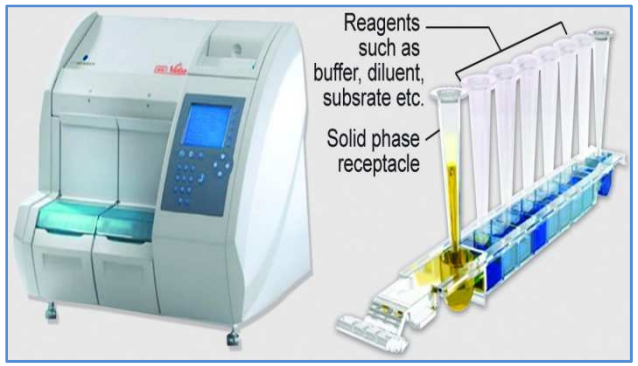
- Small laboratories with less sample load - ELISA is less preferred than rapid tests as the later can be done on individual samples.
- More time (2-3 hours) compared to rapid tests which take 10-20 min.
- Needs expensive equipments such as ELISA washer and reader.

Applications of ELISA

- **ELISA used for antigen detection** -hepatitis B [hepatitis B surface antigen (HBsAg) and precore antigen (HBeAg)], NS1 antigen for dengue etc.
- **ELISA can also be used for antibody detection** against hepatitis B, hepatitis C, HIV, dengue, EBV, HSV, toxoplasmosis, leishmaniasis, etc.

ENZYME-LINKED FLUORESCENT ASSAY (ELFA)

- Modification of ELISA, differs from ELISA in two ways:
  - Automated system, all steps are performed by the instrument.
  - Ag-Ab-enzyme complex is detected by fluorometric method.
- VIDAS and mini VIDAS (bioMérieux) are commercially available systems based on ELFA technology.



ELFA – Advantages over ELISA

- Automated system
- Easy to perform and user friendly
- Less contamination
- Gives quantitative results
- More sensitive and specific

ELFA – Disadvantages over ELISA

- Expensive
- Can run only 12–24 number of tests at a time
- Can run 2–4 types of tests at a time

ELFA – Uses

- **Can be used to detect more than 100 parameters.**
  - *Infectious diseases: Markers of hepatitis viruses and* HIV (Ag and Ab), Ab to TORCH infection, measles, mumps, varicella, *H. pylori and antigen to C. difficile,* Rotavirus, etc.
  - *Other use: Biomarkers (e.g. procalcitonin), hormones* (e.g. thyroid), tumor markers, cardiac markers and screening for allergy.

IMMUNOFLUORESCENCE ASSAY

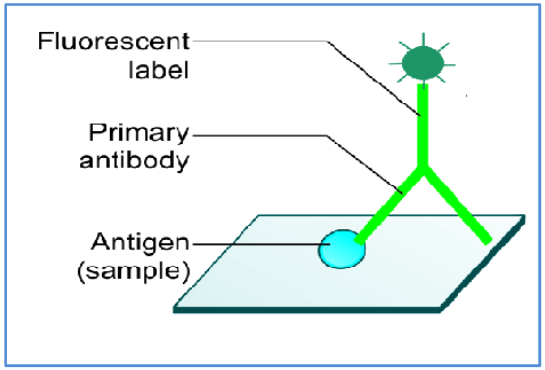
- Technique similar to ELISA, but differs by some important features:
  - Fluorescent dye is used instead of enzyme for labelling of antibody.
  - Detects cell surface antigens or antibodies bound to cell surface antigens, unlike ELISA which detects free antigen or antibody.

IMMUNOFLUORESCENCE ASSAY - Principle

- Fluorescence refers to absorbing high energy-shorter wavelength ultraviolet light rays by the fluorescent compounds and in turn emitting visible light rays with a low energy-longer wavelength.
- Fluorescent dye (FITC) is used to conjugate the antibody and such labeled antibody can be used to detect the antigens or antigen-antibody complex on the cell surface.
- **Types of Immunofluorescence Assays :** *Direct and Indirect*.

Direct Immunofluorescence Assay

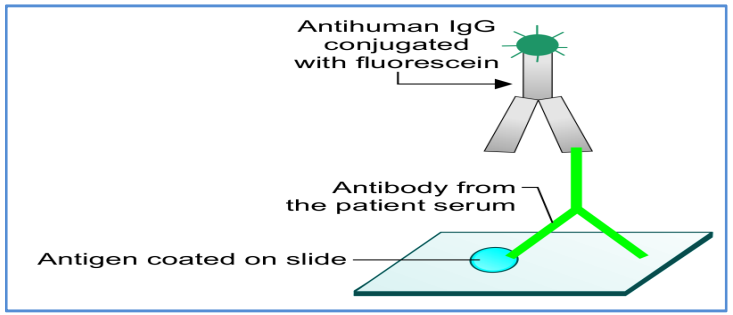
Steps	Explanation
Step-1	Sample containing cells carrying surface antigens is smeared on a slide.
Step-2	Primary antibody specific to the antigen, tagged with fluorescent dye is added.
Step-3	Slide is washed to remove the unbound antibodies and then viewed under a fluorescence microscope.



Indirect Immunofluorescence Assay

- Detects antibodies in sample. Slides smeared with cells carrying known antigens are commercially available.

Steps	Explanation
Step-1	Test serum containing primary antibody is added to the slide.
Step-2	Slide is washed to remove the unbound antibodies. A secondary antibody (anti human antibody conjugated with fluorescent dye) is added.
Step-3	Slide is washed and then viewed under a fluorescence microscope

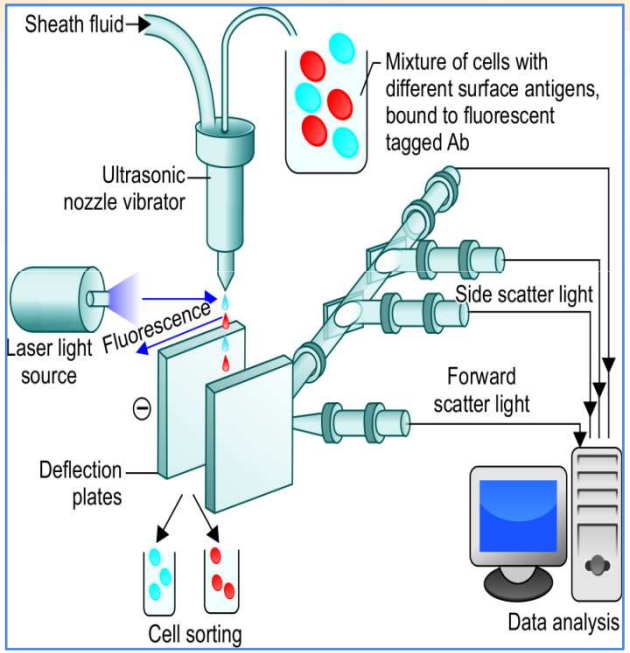


Applications

- Detection of autoantibodies (e.g. antinuclear antibody) in autoimmune diseases.
- Detecting microbial antigens, e.g. Rabies antigen in corneal smear
- Detection of viral antigens in cell lines inoculated with the specimens

Flow Cytometry

- Laser-based technology that quantitatively analyses and separates the cells as they pass through the laser beam.
- Advanced and upgraded form is called as fluorescence-activated cell sorting (FACS).



Properties can be determined during the flow of cells

- *Cell Counting*:
- *Differentiating between two cells*

Properties can be determined during the flow of cells

- ***Scattering***
- ***Sorting out of cells***

Flow Cytometry- Applications

- Flow cytometry can be used to analyse multiple parameters of cells (e.g. leukocytes) such as cell counting, cell sorting, analysis of size, shape, granularity, DNA or RNA content of a cell, etc. Important applications include-
  - CD4 T cell count in HIV infected patients
  - Detection of leukocyte with specific markers for the diagnosis of various lymphomas.

IMMUNOHISTOCHEMISTRY

- Process of detecting antigens (e.g. proteins) in cells of a tissue section by exploiting the principle of using labelled antibodies binding specifically to the antigens in biological tissues.
- Immunohistochemical staining can be ELISA or IFA based
- Widely used in the diagnosis of abnormal cells (e.g. tumor cells).

Radioimmunoassay (RIA)

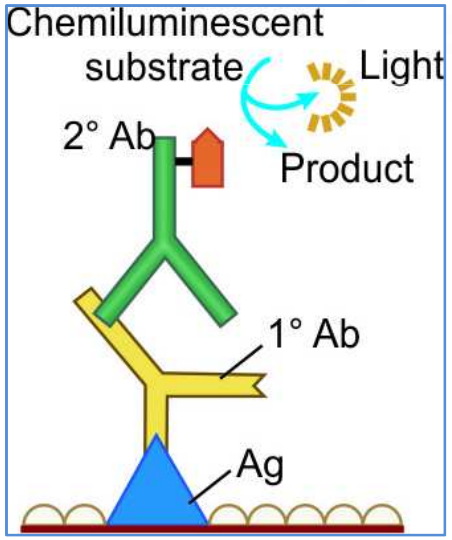
- Very sensitive and specific technique that is used for quantitative detection of antigens such as hormones, proteins, drugs, vitamins and microbial antigens (e.g. HBsAg) at a concentration of <0.01 µg/mL.

CHEMILUMINESCENCE-LINKED IMMUNOASSAY

- Emission of light (luminescence), as a result of a chemical reaction.
- Principle: similar to that of ELISA; however the chromogenic substance is replaced by chemiluminescent compounds (e.g. luminol and acridinium ester) that generate light during a chemical reaction (luxogenic).
- Light (photons) can be detected by a photomultiplier, also called as luminometer.

CLIA

- Ag-Ab-[Luminol] + [hydrogen peroxide] → Ag-Ab-[Products] + light(photons) → detected by luminometer.



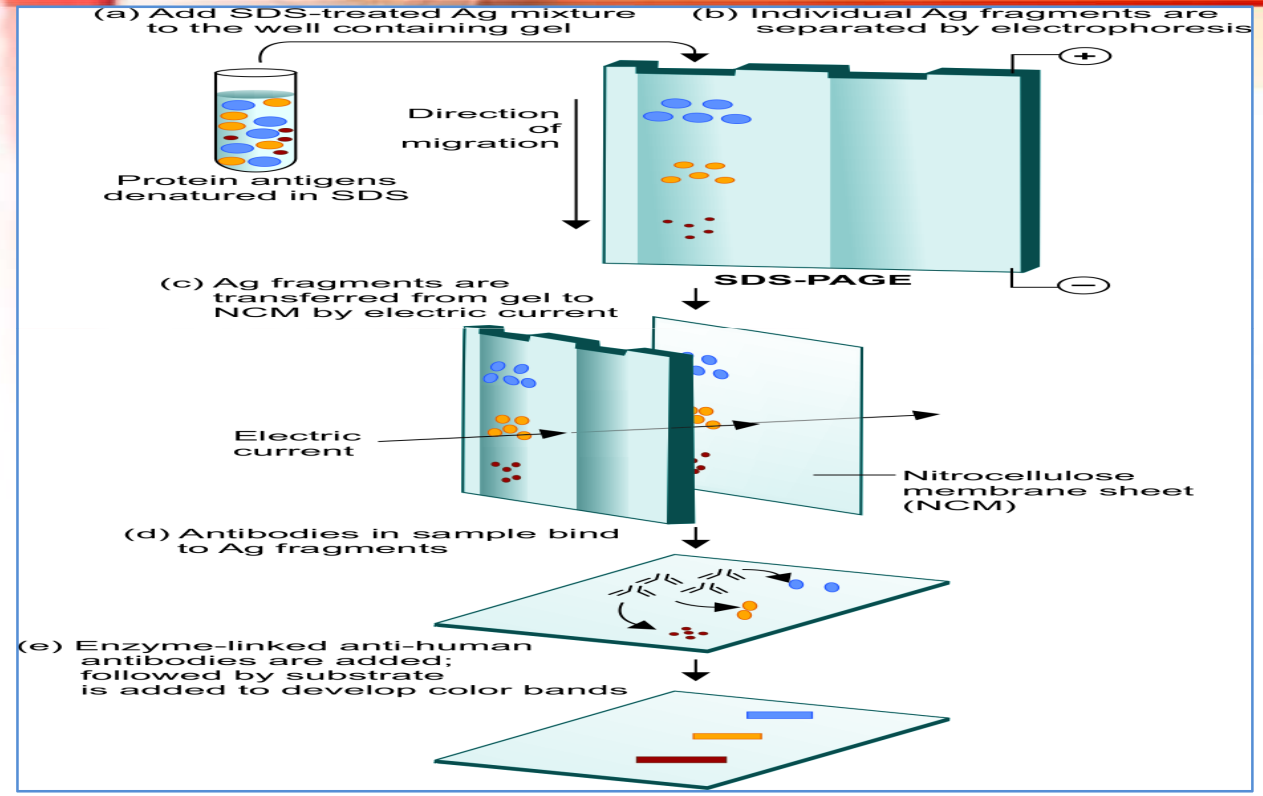
Advantage of CLIA

- 10 times more sensitive than ELISA.
- Can be further modified by using an enhancer that potentiates the chemical reaction. This gives CLIA an overall improvement of 200 folds over ELISA.
- Most samples have no 'background' signal, i.e. luminol compounds do not themselves emit light.
- Measurement of chemiluminescence is not a ratio unlike the measurement of fluorescence (IFA) and or color (ELISA).
- Applications of CLIA are similar to those of ELISA.

WESTERN BLOT

- Western blot detects specific proteins (antibodies) in a sample containing mixture of antibodies each targeted against different antigens of same microbe.
- Southern blot - detects DNA fragments.
- Northern blot - detects mRNAs.
- Eastern blot - detects the carbohydrate epitopes present on proteins or lipids.

Western blot - Procedure



Western blot - Applications

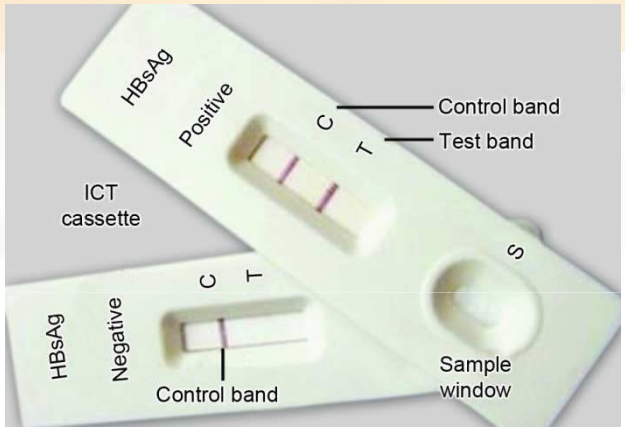
- Excellent specificity.
- Used as a supplementary test to confirm the result of ELISA or other immunoassays having higher sensitivity.
- Western blot formats are available to detect antibody in various diseases such as HIV, Lyme's disease, Herpes simplex virus infection, cysticercosis, hydatid disease and toxoplasmosis.

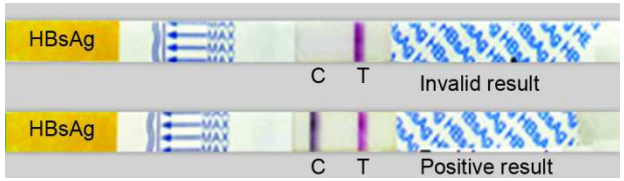
RAPID TEST

- **Point of care** (POC) tests, because unlike ELISA and other immunoassays, the Point of Care tests can be performed independent of laboratory equipment and deliver instant results.
- Two principles of rapid tests are available:
  - Lateral flow assay
  - Flow through assay.
- Both the formats are available for the diagnosis of various diseases such as malaria, hepatitis B, hepatitis C, HIV, leptospirosis, *Helicobacter pylori*, syphilis, etc.

Immunochromatographic Test (Lateral Flow Assay)

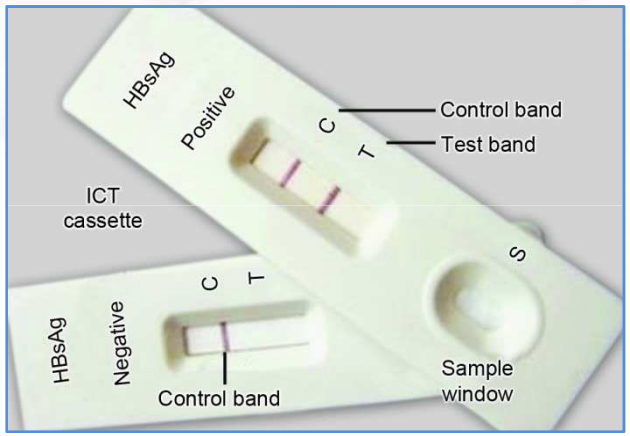
- **Principle (antigen detection):**
  - Test system consists of a nitrocellulose membrane (NCM) and an absorbent pad.
  - Two formats are available- cassette or strip.





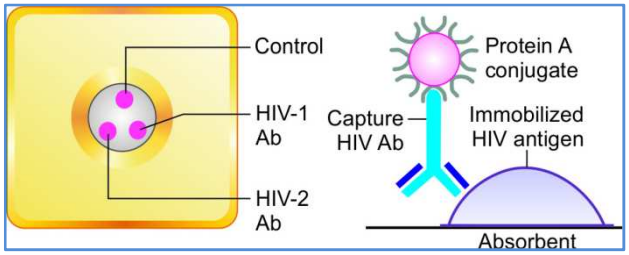
Immunochromatographic Test (Lateral Flow Assay)

- **Test band**-At the test line, the Ag-labeled Ab complex is immobilized by binding to the monoclonal Ab in the test line to form a colored band.
- **Control band**- The free colloidal gold labeled Ab can move further and binds to the anti-human Ig to form a color control band.
  - If the control band is not formed, then the test is considered invalid irrespective of whether the test band is formed or not.



Flow Through Assay

- Flow-through tests are another type of rapid diagnostic assays which differ from ICT in two aspects:
  - Protein A is used for labeling antibody instead of gold conjugate.
  - Sample flows *vertically* through the nitrocellulose membrane (NCM) as compared to lateral flow in ICT.
- Flow-through tests can be used for both antigen and antibody detection. HIV TRIDOT test is a classical example.
- Detects antibodies to HIV-I and II separately in patient's serum.
- Test system is in a cassette format, consisting of a NCM and an absorbent pad.

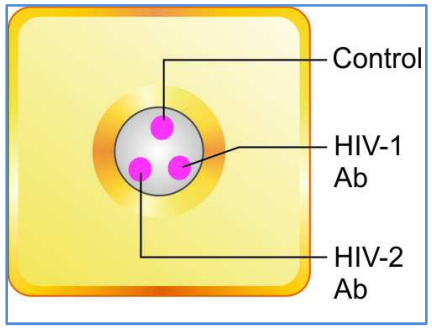


Flow Through Assay

- The NCM is coated at three regions- two test regions coated with HIV-1 & 2 antigens and a third control region coated with antihuman Ig.
- Sample and buffer reagents are added sequentially from the top following which they pass through the membrane and excess fluid is absorbed into the underlying absorbent pad.
- As the patient's sample passes through the membrane, HIV antibodies, if present bind to the immobilized antigens.

Flow Through Assay

- **Test dots**- Protein-A conjugate (present in buffer) binds to the Fc portion of the HIV antibodies to give distinct pinkish purple DOT(s), separately for HIV-1 and 2 antibodies.
- **Control dot**- Irrespective of whether the HIV antibodies are present or not, Protein-A can bind to any IgG present in serum and the IgG-protein A complex can further bind to the antihuman Ig at the control line to give a pinkish purple DOT.



TECHNIQUES USING ELECTRON MICROSCOPE

- **Immunoferritin test**:
  - In electron microscopy, the electron dense areas appear darker than other areas.
  - Electron dense molecules such as ferritin particle can be used to label an antibody which can bind to surface antigens of cells and tissues.
  - When visualized under electron microscope, the electron dense label absorbs more electrons and appears as small black dot, thus confirming the presence of antigen.

TECHNIQUES USING ELECTRON MICROSCOPE

- **Immunoelectron microscopy**
  - Viral particles appear to be clumped when mixed with specific antisera and observed under the electron microscope.
  - Used for finding hepatitis A virus and rotavirus particles from stool specimen.