ANTIGEN – ANTIBODY REACTIONS

- Antigens & antibodies combine specifically with each other. This interaction between them is called 'Antigen-Antibody reaction'
- Abbreviated as Ag – Ab reaction
- When Ag – Ab reactions occur invitro, they are known as Serological reactions
- The reactions between Ag and Ab occur in three stages
  1. *Stage – 1:* Reaction involves formation of Ag-Ab complex
  2. *Stage – 2:* Leads to visible events like precipitation, agglutination etc
  3. *Stage – 3:* Includes destruction of Ag or its neutralization

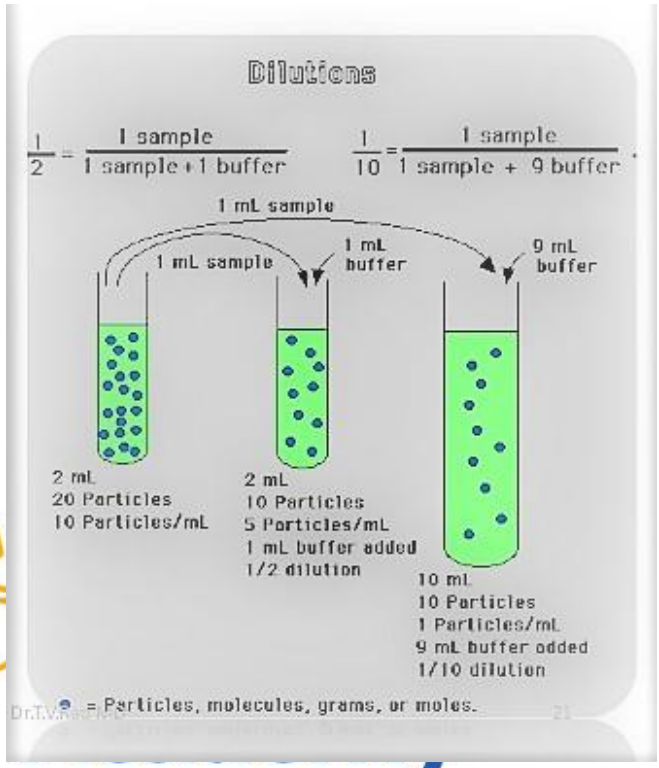
GENERAL PROPERTIES

1. *The reaction is specific.* An antigen combines only with its homologous antibody and vice versa. The specificity however is not absolute and cross reactions may occur due to antigenic similarity or relatedness
2. *Entire molecules react and not fragment*
3. There is *no denaturation of the antigen* or the antibody during the reaction
4. The *combination occurs at the surface;* therefore, it is the surface antigens that are immunologically relevant
5. The *combination is firm and irreversible.* The firmness of the union is influenced by the affinity and avidity of the reaction.
   - *Affinity:* Refers to the intensity of attraction between the antigen and antibody molecules. It is a function of the closeness of fit between an epitope and the paratope
   - *Avidity:* Refers to the strength of the bond after the formation of the antigen antibody complexes. It reflects the overall combining property of the various antibody molecules in an antiserum, possessing different affinity constants with the multiple epitopes of the antigen
6. *Antigens and antibodies can combine in varying proportions,* unlike chemicals with fixed valencies. Both antigens and antibody are multivalent, antibodies are generally bivalent, though IgM molecules may have five or ten combining sites. Antigens may have valencies up to hundreds.

MEASUREMENTS

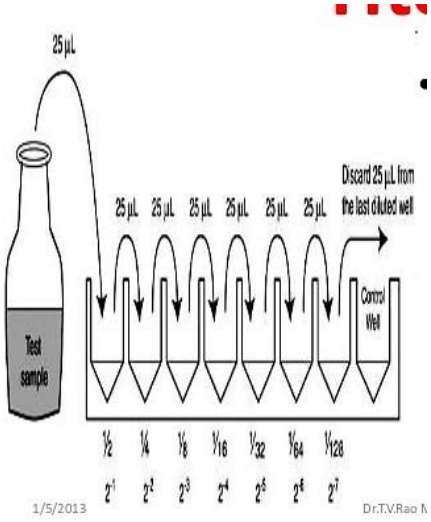
Dilution

- Estimating the antibody by determining the greatest degree to which the serum may be diluted without losing the power to give an observable effect in a mixture with specific antigen



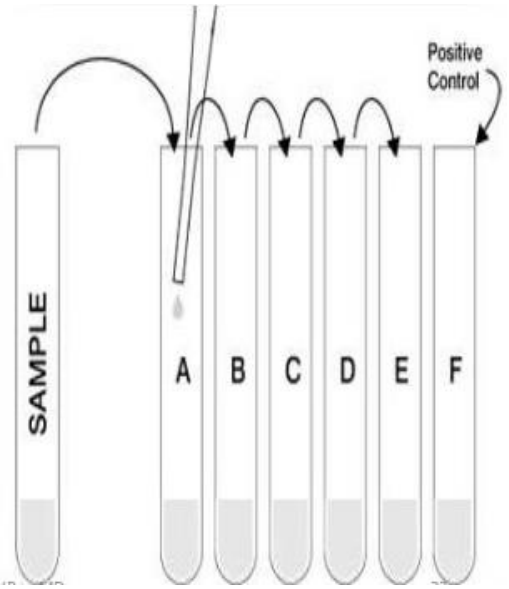
Titer

- Different dilutions of serum are tested in mixture with a constant amount of antigen and greatest reacting dilution is taken as the measure or Titer



Expression of Titers

- Expressed in term of the way in which they are made
- Dilution 1 in 8 is a dilution made by mixing one volume of serum with seven volumes of diluents (Normal Saline)
- It is Incorrect to express dilution as 1/8



TYPES OF ANTIGEN–ANTIBODY REACTIONS

Conventional Techniques

1. Precipitation
2. Agglutination
3. Complement-dependent serological tests
4. Neutralization test
5. Opsonization

Newer Techniques

6. Immunofluorescence
7. Enzyme immunoassay
8. Radioimmunoassay
9. Western blotting

PRECIPITATION REACTIONS

Principle

- When a soluble Ag combines with its Ab in the presence of electrolytes (NaCl) at a suitable temperature & pH, the Ag-Ab complex forms an insoluble precipitate
- When instead of sedimenting, the precipitate remains suspended as floccules, the reaction is called *Flocculation*
- It can take place in liquid media or in gels such as agar, agarose or polyacrylamide

Mechanism

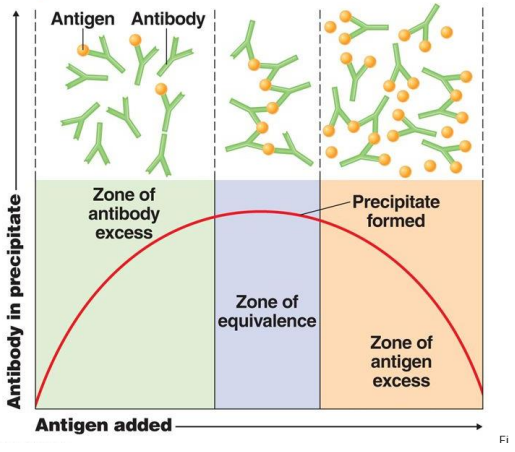
- Marrack (1934) proposed the lattice hypothesis – mechanism of precipitation
- The multivalent antigens combine with bivalent Abs in varying proportions, depending on the Ag – Ab ratio on the reacting mixture.
- Precipitation results when a large lattice is formed consisting of alternating Ag & Ab

Zone Phenomenon

- The amount of precipitate formed is greatly influenced by the relative proportions of Ags & Abs
- If increasing quantities of Ags are added to the same amount of antiserum in different tubes, precipitation is found to occur most rapidly & abundantly in the middle tubes

1. *Prozone Phenomenon*
   - Zone of Antibody excess
   - Failure of a visible reaction due to inhibition of lattice formation by excess antibody
2. *Zone of Equivalence*
   - Reaction is best when both antigens & antibodies are in equal proportion
   - Lattice formation and visible reactions are enhanced
3. *Post-zone Phenomenon*
   - Zone of antigen excess
   - No visible reaction occurs

A Precipitation Curve



Precipitation in Liquid Medium

Ring Test
• A clear solution of test antigen is layered slowly over clear solution of antiserum in narrow test tube

- Following a period of incubation, precipitation between antigen and antibodies in antiserum solution is marked by the appearance of a white ring at the junction of two liquid layers
- *Examples*
  1. C-reactive protein (CRP)
  2. Lancefield grouping of β-haemolytic streptococci
  3. Ascoli's thermoprecipitin test

Flocculation Test

- Can be performed in a Slide or Tube
- A drop of antigen solution is added to drop of serum solution in slide and mixed well
- Visible clumps appear in positive cases

Slide Test	*Tube Test*
E.g.: VDRL test, RPR test	*E.g.:* Kahn test used previously for Syphilis

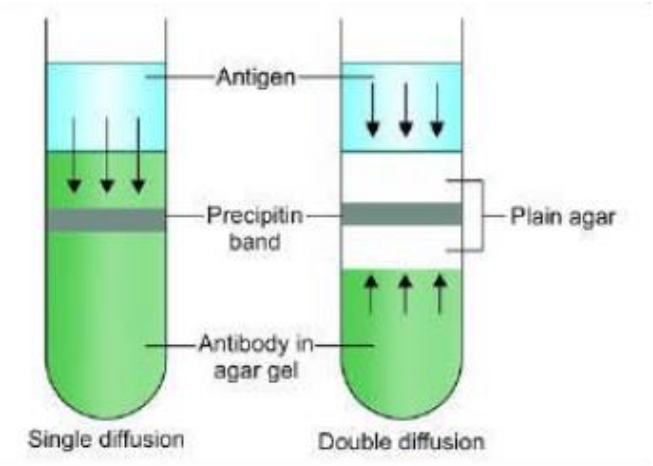
Precipitation in Gel (Immunodiffusion)

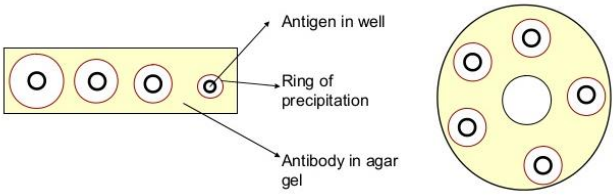
- 1% soft agarose gel is incorporated in precipitation reaction
- *Advantages*
  1. Results in formation of clearly visible bands instead of floccules, which can be preserved for longer time
  2. Used to differentiate individual antigens from a mixture, as each antigen forms a separate band after reacting with specific antibody
- Works on Two Principles
  1. *Diffusion*
     - If only Ag diffuses → Single diffusion
     - If both Ag and Ab diffuse → Double diffusion
  2. *Dimension*
     - *One dimension* → when Ag or Ab diffuses in vertical direction → seen when test is done on a tube layered with gel
     - *Two dimensions* → when Ag or Ab diffuse in both X and Y axis → seen when die test is done on a slide or a petri dish layered with gel)

Types of Immunodiffusion

Oudin Immunodiffusion	• *Single diffusion of antigen* in agar in one dimension • Here, antibody is incorporated in agar gel in test tube and antigen is layered over it • Antigen diffuses and form line of precipitate
Oakley Fulthorpe Immunodiffusion	• *Double diffusion in one dimension* • Here antibody is incorporated in agar gel in a test tube, above which a layer of plain agar is placed • Antigen is then layered on top of this plain agar • During time, the antigen and antibody move toward each other through the intervening layer of plain agar and forms band of precipitate
Radial Immunodiffusion	• *Single diffusion in two dimensions* • Here antiserum solution containing antibody is incorporated in agar gel on a slide or Petri dish • Antigen is then applied to a well cut into the gel • When antibody present in the gel reacts with the antigen, which diffuses out of the well, a ring of precipitation is formed around the wells • Diameter of the precipitin ring formed is directly proportional to the concentration of antigen
Ouchterlony Immunodiffusion	• *Double diffusion in two dimensions* • Both antigen and antibody diffuse independently through agar gel in two dimensions, horizontally and vertically • In this method wells are cut in agar gel prepared in slide • Antibody is placed in central well and antigen is placed on wells surrounding the central well • Antigen and antibody diffuse, and precipitation band is formed

	where they meet in optimal conditions





A single diffusion in two dimensions

Precipitation in Gel in Presence of Electric Current

- Ag and Ab movement can be made faster if immunodiffusion is carried out in the presence of electric current

1. *Electro Immuno Diffusion (EID)*

- When an electric current is applied to a slide layered with gel, the antigen is separated into individual antigen components
- Antiserum present in a trough moves towards the antigen components resulting in formation of separate precipitate lines in 18-24 hrs, each indicating reaction between individual proteins with its antibody
- This test helps in identification and approximate quantitation of various proteins present in the serum

2. *Counter Current Immuno Electro Phoresis (CIEP)*

- Faster and more sensitive than EID
- Antigen and antibody are placed in separate wells (opposite directions) cut in agar gel as the test relies in movement of antigen and antibody in opposite direction
- On electrophoresis, antigen (Negatively charged) moves towards positive pole and antibody (Positively charged) moves towards negative pole
- Line of precipitation appears at point where they two meets

3. *Rocket Electrophoresis*

- Various concentrations of antigens are loaded side by side in small circular wells along edge of agarose gel incorporated with specific antibody
- On electrophoresis antigens begins to migrate towards anode and interact with antibody to form complex
- As electrophoresis continues more antigen moves toward anode and at equivalence point antigen-antibody complex precipitates in form of rocket
- The height of rocket is proportional to amount of antigen loaded

AGGLUTINATION REACTIONS

- When particulate antigens react with specific antibody, antigen antibody complex forms visible clumping under optimum pH and temperature. Such reaction is called Agglutination
- Antibodies that produce such reactions are called *Agglutinins*
- The particulate antigens that are aggregated are termed *Agglutinogens*
- Agglutination is the visible expression of the aggregation of antigens and antibodies
- Agglutination occurs optimally when antigens and antibodies react in equivalent proportions

Steps

1. ***Primary Phenomenon (Sensitization)***
   - First reaction involving Ag-Ab combination
   - Single antigenic determinant on the surface particle
   - Initial reaction: rapid and reversible
   - Cross link formation
   - Visible aggregates (stabilization)

2. ***Secondary Phenomenon (Lattice Formation)***
   - Ab + multivalent Ag
   - Stable network (visible reaction)

- Governed by physiochemical factors
  - Ionic strength of milieu
  - pH
  - Temperature
- The Fab portion of the Ig molecule attaches to antigens on 2 adjacent cells-visible results in agglutination
- If both antigen and antibody are soluble, reaction will become visible over time i.e., precipitation

Types

1. Direct
2. Indirect (Passive)
3. Reverse Passive

Direct Agglutination Tests

- Antigen directly agglutinates with the antibody

Slide Agglutination

- Performed on a slide

Place a bacterial sample mixed with a drop of saline on a slide (becomes uniform smooth milky white suspension)

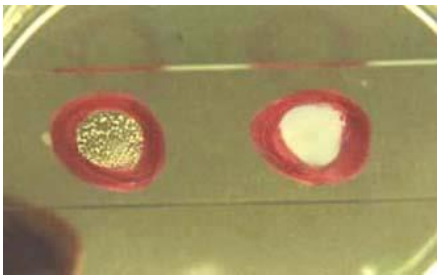
↓

Add a drop of antiserum, mix and rock slide 1 min approximately

↓

Examine for agglutination

- Positive Result: Visible clumping
- E.g.:
  1. To identify bacterial strains, such as Salmonella, Shigella, Vibrio
  2. For blood grouping and cross-matching



Tube Agglutination

- *Synonyms:* Standard Agglutination Test or Serum Agglutination Test (SAT)
- Test serum is diluted in a series of tubes (doubling dilutions)
- Constant defined amount of antigen is then added to each tube and tubes incubated for 20 hours @37°C
- Antigen clumps at the bottom of the test tube
- Test is read at 50% agglutination
- Quantitative test
- E.g.:
  1. Typhoid fever (Widal test)
     - It detects antibodies against both H (flagellar) and O (somatic) antigens of Salmonella Typhi
     - H antigen- antibody clumps appear as loose fluffy clumps
     - O antigen- antibody clumps appear as chalky white granular dense deposits
  2. Acute brucellosis (Standard agglutination test)
  3. Coombs antiglobulin test
  4. Heterophile agglutination tests
     i. Typhus fever (Weil Felix reaction)
     ii. Infectious mononucleosis (Paul Bunnell test)
     iii. Mycoplasma pneumonia (Cold agglutination test)

Microscopic Agglutination

- Agglutination test is performed on a microliter plate and the result is read under a microscope
- E.g.: Leptospirosis

Hemagglutination Test

Agglutination tests that use RBCs as source of antigen

Paul Bunnell Test	• Employs sheep RBCs as antigen to detect Epstein Barr virus antibodies in serum • Test is performed in tubes
Cold Agglutination Test	• Uses human RBCs as antigens to detect Mycoplasma antibodies in serum • Test is performed in tubes

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
- When serum containing incomplete anti-Rh antibodies is mixed with Rh+ erythrocytes in saline, incomplete antibody antiglobulin coats the surface of erythrocytes but does not cause any agglutination. When such erythrocytes are treated with antiglobulin or Coombs'

serum (rabbit antiserum against human γ, IgG globulin), then the cells are agglutinated	
Direct Coombs test ▪ Detects Rh antibodies bound to RBCs in vivo, present in fetus/baby's serum by directly adding Coombs reagent	*Indirect Coombs test* ▪ Detects free Rh antibodies present in maternal serum after the first delivery of an Rh +ve fetus/baby. The free Rh Abs are mixed with the reagent containing Rh +ve RBCs (in vitro), following which the Coombs reagent is added

Indirect Agglutination Tests (for Antibody Detection)

- Employs carrier particles that are coated with soluble antigens. This is usually done to convert precipitation reactions into agglutination reactions, since the latter are easier to perform and interpret and are more sensitive than precipitation reactions for detection of antibodies

Indirect Hem Agglutination Test (IHA)

- RBCs are used as carrier molecules
- It results in formation of matt or button which indicate presence or absence of agglutination respectively
- Test is performed on a microliter plate
- Not used now a days

Latex Agglutination Test (LAT) for Antibody Detection

- Polystyrene latex particles (0.8- 1 μm in diameter) are used as carrier molecules
- They act as adsorbents of several types of antigens
- Test is performed on a black color card for better interpretation
- 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)

3. Reverse Passive Agglutination Test (for Antigen Detection)

- When the antibody instead of antigens is adsorbed on the carrier particle for detection of antigens, it is called reverse passive agglutination

Reverse Passive Hemagglutination Assay (RPHA)
• RBCs are used as carrier molecules • RPHA was used in the past for detection of Hepatitis B surface antigen (HBsAg)
Latex agglutination test for antigen detection
• Used widely for detection of CRP (C reactive protein), RA (rheumatoid arthritis factor), capsular antigen detection in CSF (for Pneumococcus, Meningococcus and Cryptococcus) and streptococcal grouping
Coagglutination Test
Staphylococcus aureus acts as carrier molecule

COMPLEMENT-DEPENDENT SEROLOGICAL TESTS

- When antigen and antibodies of the IgM or the IgG classes are mixed, complement is "fixed" to the antigen–antibody complex
- If this occurs on the surface of RBCs, the complement cascade will be activated, and hemolysis will occur
- Were used earlier for diagnosis of many infections, such as
  - Wassermann test for syphilis
  - Tests for demonstration of antibodies to M. pneumoniae, Bordetella pertussis, many different viruses, and to fungi (such as Cryptococcus spp., Histoplasma, and Coccidioides immitis).
- Since this test is technically very cumbersome, and often difficult, it is no longer used now-a-days

NEUTRALIZATION TEST

- An antigen–antibody reaction in which the biological effects of viruses and toxins are neutralized by homologous antibodies

Types

1. *Virus Neutralization Tests*
   - Neutralization of viruses by their specific antibodies
   - When virus-specific neutralizing antibodies are injected into these systems, replication and growth of viruses is inhibited

2. *Toxin Neutralization Tests*
   - Biological action of toxin is neutralized on reacting with specific neutralizing antibodies called antitoxins

- E.g.:
    1. Schick test to demonstrate immunity against Diphtheria
    2. Clostridium welchii toxin neutralization test in guinea pig or mice antistreptolysin O test
    3. Nagler reaction used for rapid detection of C. welchii

OPSONIZATION

- A process by which a particulate antigen becomes more susceptible to phagocytosis when it combines with opsonin
- Opsonin is a heat-labile substance present in fresh normal sera
- Opsonic index: the ratio of the phagocytic activity of patient's blood for a bacterium to the phagocytic activity of blood from a normal individual. It is used to study the progress of resistance during the course of disease

IMMUNOFLUORESCENCE

- Property of certain dyes absorbing light rays at one wavelength (ultraviolet light) and emitting them at a different wavelength (visible light) is known as Fluorescence
- In immunofluorescence test, fluorescent dye which illuminates in UV light are used to detect/show the specific combination of an antigen and antibody
- The dye usually used is fluorescein isothiocynate, which gives yellow-green fluorescence
- Fluorescent dyes, such as fluorescein isothiocyanate and lissamine rhodamine, can be tagged with antibody molecules. They emit blue-green and orange-red fluorescence, respectively under ultraviolet (UV) rays in the fluorescence microscope. This forms the basis of the immunological test

Types

1. Direct Immunofluorescence Test
2. Indirect Immunofluorescence Test

1. Direct Immunofluorescence Test

- Used to detect unknown antigen in a cell or tissue by employing a known labeled antibody that interacts directly with unknown antigen
- If antigen is present, it reacts with labeled antibody and the antibody coated antigen is observed under UV light of the fluorescence microscope
- *Applications*
    1. Detection of rabies virus antigen in the skin smear collected from the nape in humans and in the saliva of dogs
    2. Also used for detection of N. gonorrhoeae, C. diphtheriae, T. pallidum, etc. directly in appropriate clinical specimens

2. Indirect Immunofluorescence Test

- Used for detection of specific antibodies in the serum and other body fluids for serodiagnosis of many infectious diseases
- Indirect immunofluorescence is a two-stage process
- *Stage-01*
    - A known antigen is fixed on a slide
    - Then the patient's serum to be tested is applied to the slide, followed by careful washing
    - If the patient's serum contains antibody against the antigen, it will combine with antigen on the slide
- *Stage-02*
    - Antibody and antigen reaction can be detected by addition of a fluorescent dye-labeled antibody to human IgG, which is examined by a fluorescence microscope

Applications

1. Detect specific antibodies for serodiagnosis of syphilis, leptospirosis, amoebiasis, toxoplasmosis
2. Identify the class of a given antibody by using fluorescent antibodies specific for different immunoglobulin isotypes
3. Identify and enumerate lymphocyte subpopulations by employing monoclonal antibodies and cytofluorographs
4. Detect autoantibodies, such as antinuclear antibodies in autoimmune diseases

ENZYME IMMUNOASSAY

- 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

Types

1. *Homogeneous EIA*

- Done for detection of haptens such as drugs (e.g. opiates, cocaine), but not for microbial antigens and antibodies

- Test can be completed in one step, with all the reagents added simultaneously
- There is no need to separate the bound and free fractions of haptens, hence washing step is not needed
- *E.g.:* Enzyme Multiplied Immunoassay Techniques (EMIT)

2. *Heterogeneous Assays*

- Done for detection of antigens and antibodies
- Multiple steps are needed
- Requires the separation of the free and bound fractions which is carried out by absorption of the test Ag or Ab on to the solid surface followed by washing
- Different reagents are added at every step
- *E.g.:* ELISA

Enzyme Linked Immunosorbent Assay (ELISA)

- Term was coined by Engvall and Pearlmann in 1971
- Similar to Radio Immuno Assay, except no radiolabel
- Can be used to detect both antibody and antigen
- Very sensitive
- Relies on monoclonal abs
- Most commonly, ELISAs are performed in 96-well (or 384-well) usually polystyrene microtiter plates, which will passively bind antibodies and proteins

Advantages

1. Economical and safest.
2. Easy visualization of results with high level of accuracy
3. Specific and highly sensitive assay that can detect protein at the picomolar to nanomolar range
4. Easily automated for performance of large numbers of tests
5. Require minimal reagents
6. Qualitative detection or Quantitative measurement of either antigen or antibody
7. Wells can be coated with antigens or antibodies.
8. Can be done by personnel with only minimal training

Principle

- ELISA is a plate-based assay technique. Along with the enzyme-labelling of antigens or antibodies, the technique involves following three principles in combination which make it one of the most specific and sensitive than other immunoassays to detect the biological molecule

1. *An immune reaction*: antigen-antibody reaction.
2. *Enzymatic chemical reaction*: enzyme catalyses the formation of colored (chromogenic) product from colorless substrate.
3. *Signal detection and Quantification*: detection and measurement of color intensity of the colored products generated by the enzyme and added substrate.

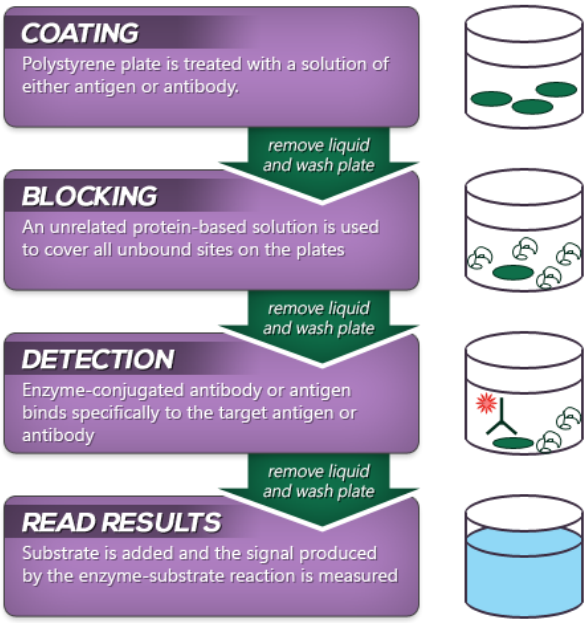
Enzymes used in ELISA and their substrate chromogen system

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

Types of ELISA

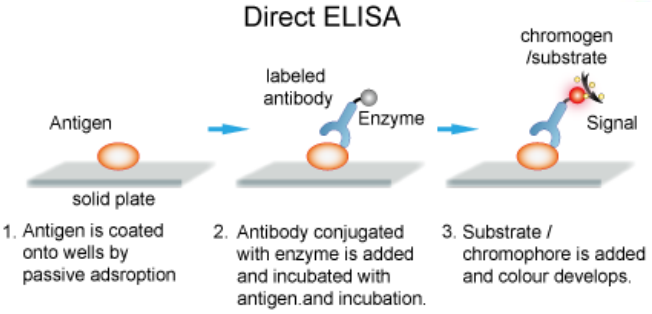
ELISA type	***Used for detection of***	***Enzyme is labeled with***
Direct ELISA	Antigen	Primary antibody
Indirect ELISA	Antigen or Antibody	Secondary antibody
Sandwich ELISA	Antigen	Primary antibody in direct sandwich ELISA
		Secondary antibody in indirect sandwich ELISA
Competitive ELISA	Antigen or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

General Procedure



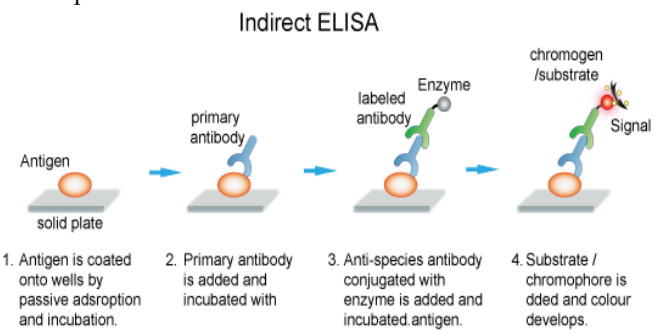
Direct ELISA

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



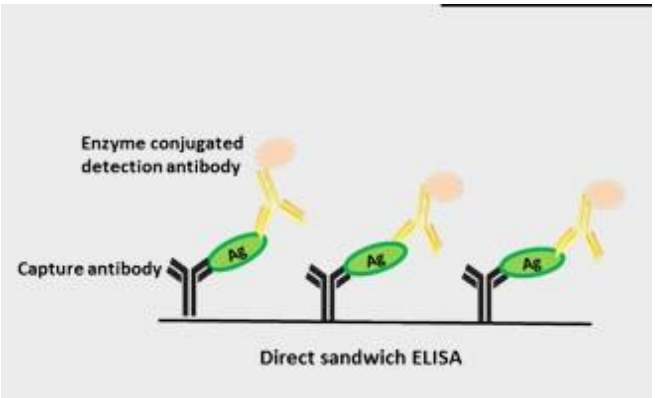
Indirect ELISA

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



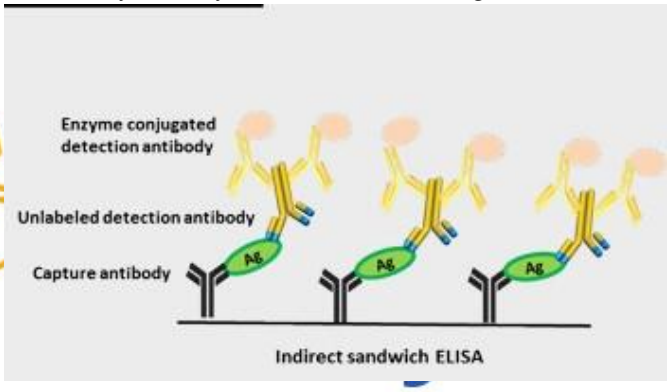
Direct Sandwich ELISA

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



Indirect Sandwich ELISA

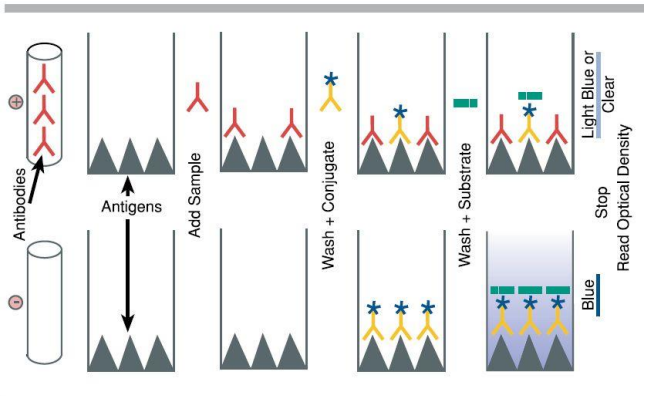
Wells coated with capture Ab + Ag (test serum) + primary Ab + secondary Ab-enzyme + substrate-chromogen → color



Competitive ELISA

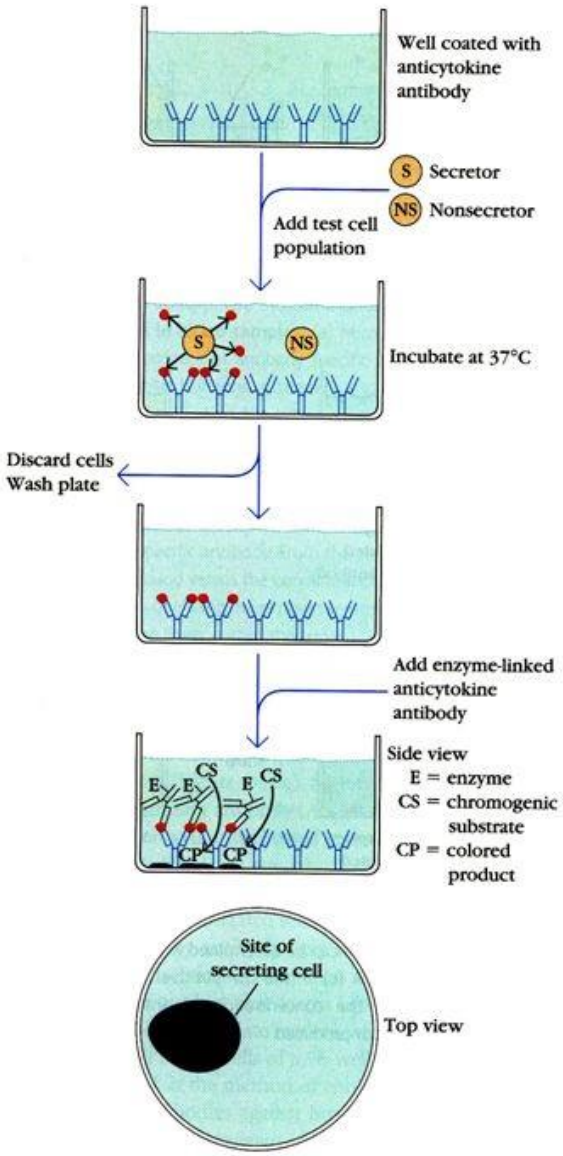
- Antigen in test serum competes with another antigen of same type coated on well to bind to the primary antibody

Incubate primary antibody with antigen to be measures + Add Ag-Ab mixture to antigen-coated well + Add enzyme-conjugated secondary antibody + Add substrate → color



ELISPOT Test

- Variation of ELISA test
- A petri plate is coated with specific capture antibody
- Cells are added to the plate and allowed to settle for a period
- Cells produce a specific cytokine that binds to the specific antibody in the area where the cells settled
- Cells are washed away
- Detection antibody bearing an enzyme is added and unbound excess antibody is washed away
- An appropriate substrate is added
- An area of the petri plate where the cytokine was produced turns color



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
- Was developed by Berson and Yalow (l 960)
- Principle of RIA is similar to that of competitive ELISA for antigen detection, except that in RIA, the radioactive molecules are used for labeling and the test is done in a Liquid medium
- Because of the radio hazard associated, the use of RIA is reduced

BLOTTING

- Blotting is a method of transferring proteins, DNA or RNA onto a carrier (nitrocellulose or PVDF or nylon membrane)

Techniques

- Southern Blotting → DNA
- Northern Blotting → mRNA
- Western Blotting → Proteins
- Eastern Blotting → Carbohydrate epitopes

Western Blotting

- Detects specific proteins (antibodies) in a sample containing mixture of antibodies each targeted against different antigens of same microbe
- It works on the principle of gel electrophoresis
- Proteins are separated based on their size on polyacrylamide gel

Procedure

Sample preparation
↓
Gel electrophoresing the protein sample
↓
Protein transfer
↓
Protein staining
↓
Blocking non-specific antibody
↓
Antibody probing
↓
Washing
↓
Protein detection
↓
Digital imaging

Applications

1. Analysis of IgG Fractions purified from human plasma
2. To detect some forms of Lyme Disease
3. Used in definitive test for BSE, which is commonly known as Mad cow disease
4. Confirmatory test for Hepatitis-B
5. Used in the analysis of biomarkers such as hormones, growth factors & cytokines
6. Gene expression studies
7. Diagnosis of HIV by ELISA, involves the western blotting technique

*****