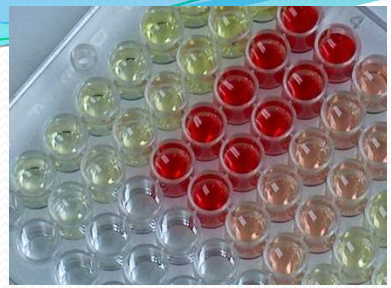
INTRODUCTION TO

ELISA

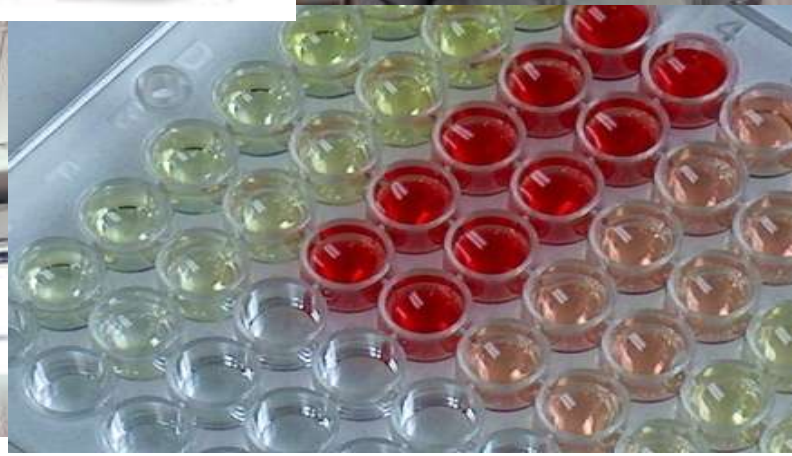
ENZYME LINKED IMMUNOSORBENT ASSAY

INTRODUCTION TO ELISA



- ELISA, or enzyme-linked immunosorbent assay, are quantitative immunological procedures in which the Ag- Ab reaction is monitored by enzyme measurements.
- The term ELISA was first used by Engvall & Perlma in 1971.
- The ELISA test, or the enzyme immunoassay (EIA), was the first screening test commonly employed for HIV. It has a high sensitivity.

Enzyme Linked Immunosorbant Assay



RML MEHROTRA PATHOLOGY

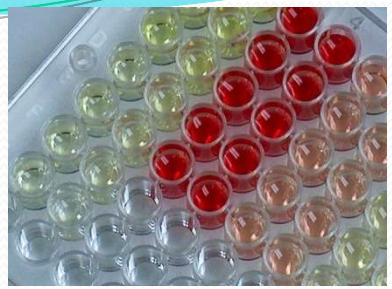
Committed to diagnostic excellence

Clinical Reference Laboratory

ELISA is the abbreviation of **ENZYME-LINKED IMMUNOSORBENT ASSAY**

It is useful & powerful method in estimating ng/mL to pg/mL ordered materials in the solution .

Why known as ……?

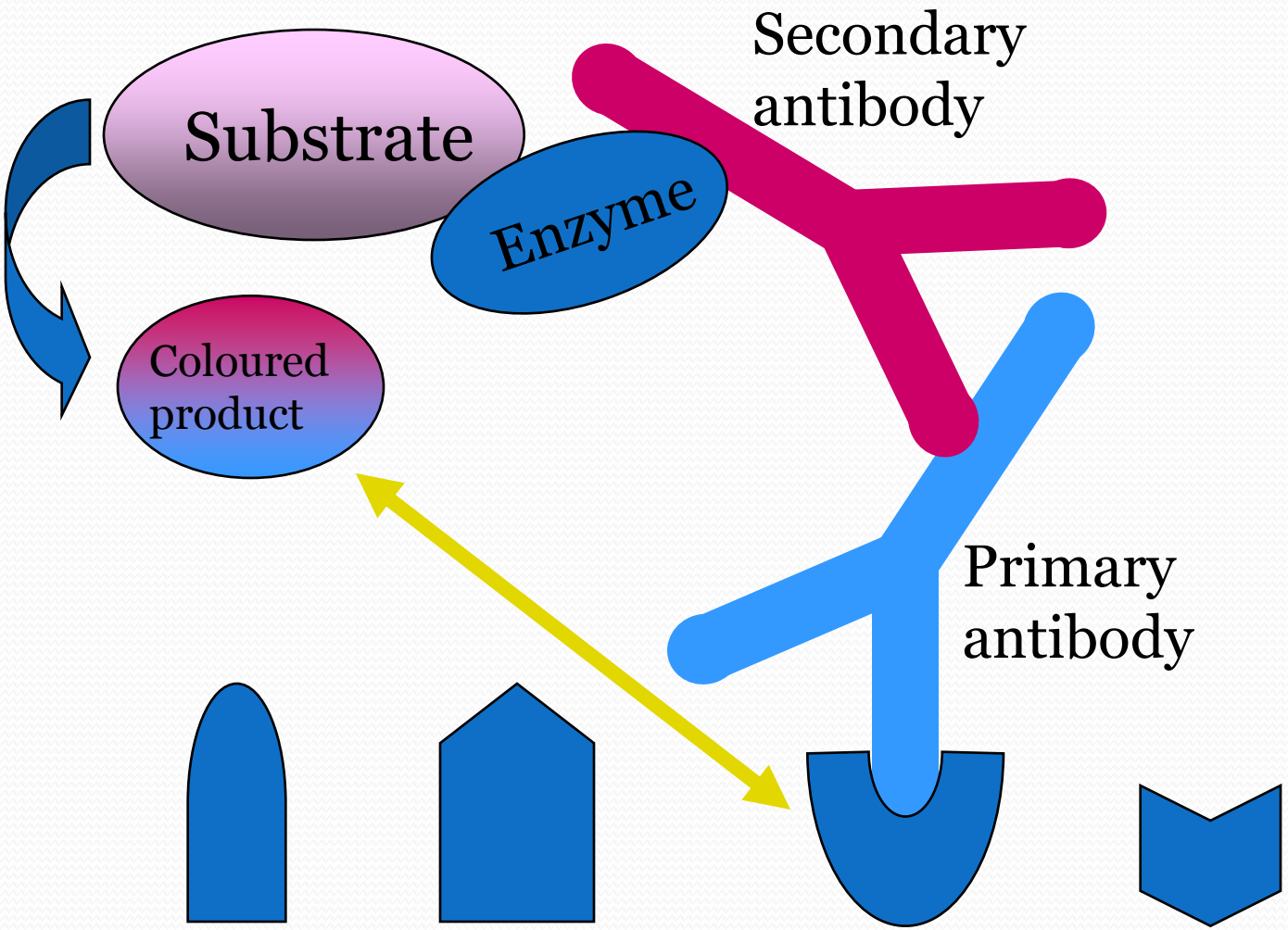


Enzyme Linked Immunosorbent Assay

1. Antigen of interest is absorbed on to plastic surface (‘*sorbent*’).
2. Antigen is recognised by specific antibody (‘*immuno*’).
3. This antibody is recognised by second antibody (‘*immuno*’) which has enzyme attached (‘*enzyme-linked*’).
4. Substrate reacts with enzyme to produce product, usually coloured.

BASIC PRINCIPLE OF ELISA

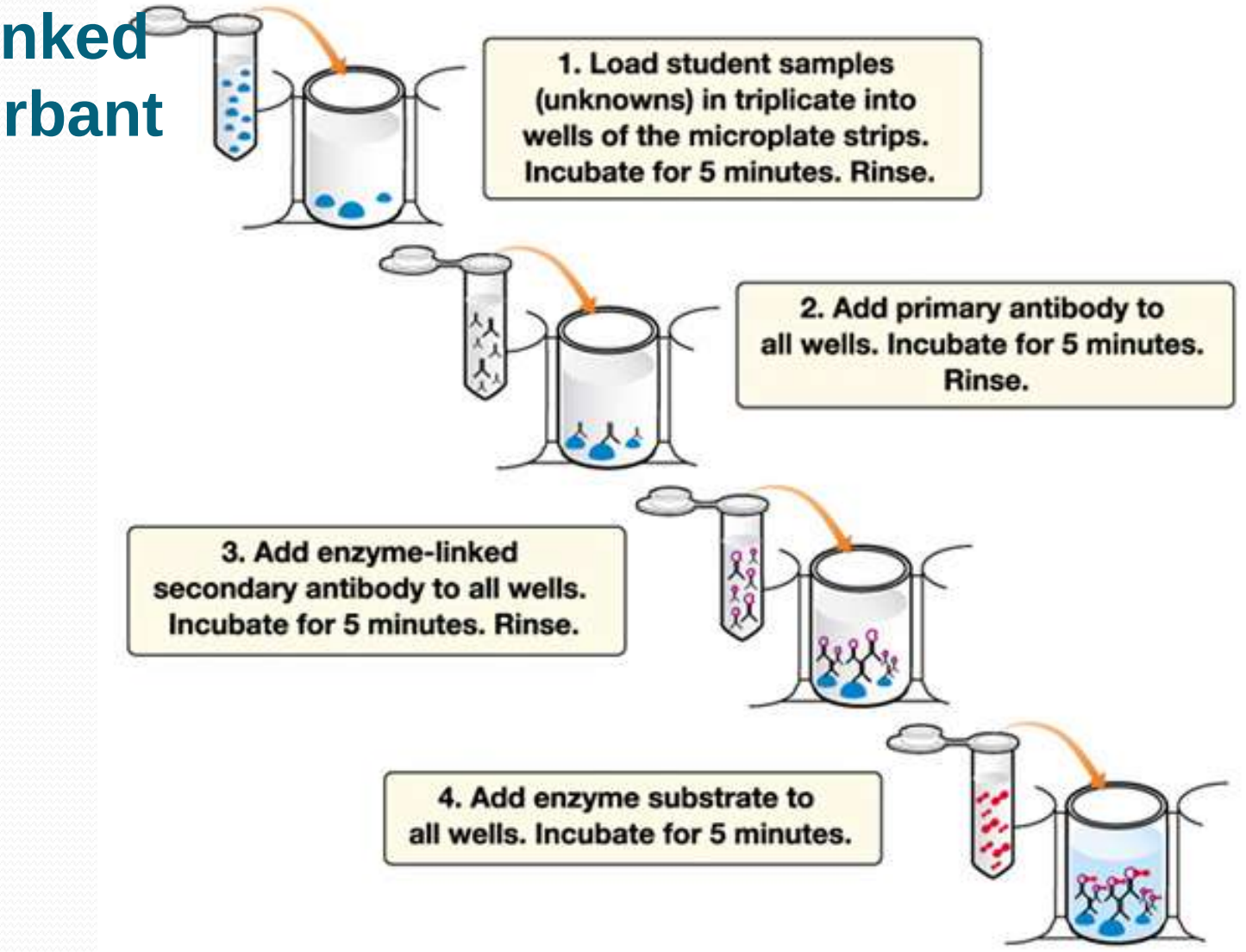
- Use an enzyme to detect the binding of antigen (Ag) antibody (Ab).
- The enzyme converts a colorless substrate (chromogen) to a colored product, indicating the presence of Ag : Ab binding.
- An ELISA can be used to detect either the presence of Antigens or antibodies in a sample depending how the test is designed.
- ELISA was dveloped in 1970 and became rapidly accepted


Secondary antibody
Substrate
Enzyme
Coloured product
Primary antibody
Different antigens in sample

ELISA Qualitative/Quantitative

- Qualitative
  - determines antigen or antibody is present or absent
- Quantitative
  - determines the quantity of the antibody
  - Titer
  - The highest dilution of the specimen usually serum which gives a positive reaction in the test

Basic Steps Of Enzyme-Linked Immunosorbant Assay



ANTIGEN (Ag)

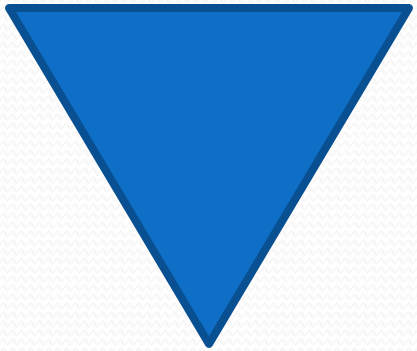
- Any molecule that induces production of antibodies when introduced in the body of an animal is called antigen.

OR

- any “thing”, foreign to the immune system. e.g. bacteria, viruses, (or their parts), pollen, etc.

- Protein molecule
- Carbohydrate molecule.
- Microorganisms
- Allergens.
- Viruses Etc.

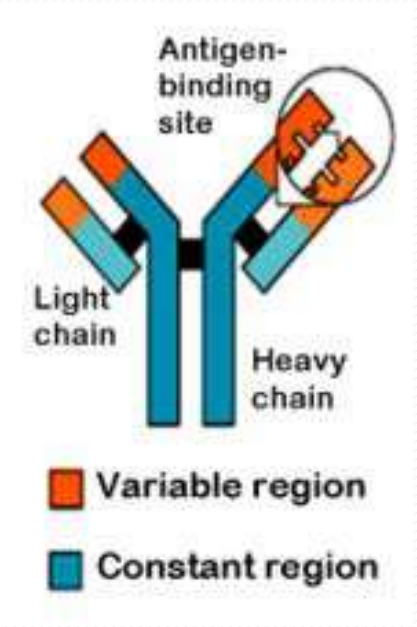
SYMBOL FOR ANTIGEN



- ANTIBODY (Ab)
- Antibody: proteins produced by the immune system which help defend against antigens

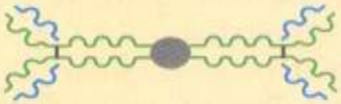
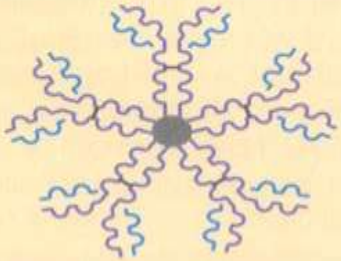
SYMBOL FOR ANTIBODY

Y



Antibodies (Immunoglobulins)

CLASSES OF IMMUNOGLOBULINS (Igs)

Name and Structure		Characteristics and Functions
IgG		Most abundant, about 75% of all antibodies in the body; found in blood, lymph, and the intestines; monomer (one unit) structure. Protect against bacteria and viruses by enhancing phagocytosis, neutralizing toxins, and triggering the complement system. The only antibodies to pass the placenta from mother to fetus and thereby confer some immune protection in newborns.
IgA		Make up about 15% of all antibodies in the body; occur as monomers and dimers (two units). Found in tears, saliva, mucus, milk, gastrointestinal secretions, blood, and lymph. Levels decrease during stress, lowering resistance to infection. Provide localized protection on mucous membranes.
IgM		About 5 to 10% of all antibodies; occur as pentamers (five units); first antibodies to be secreted by plasma cells after an initial exposure to any antigen; found in blood and lymph. Cause agglutination and lysis of microbes. Also present as monomers on the surfaces of B cells, where they serve as antigen receptors. A and B agglutinins, which bind to A and B agglutinogens on the surface of red blood cells, are IgM antibodies.
IgD		Less than 1% of all antibodies; occur as monomers; found in blood, in lymph, and on the surfaces of B cells as antigen receptors. Involved in activation of B cells.
IgE		Less than 0.1% of all antibodies; occur as monomers; located on mast cells and basophils and are involved in allergic reactions.

Materials Needed

- Testing sample
- Antibody (1^{st}, 2^{nd}) / Antigen
- Polystyrene microtiter plate
- Blocking buffer
- Washing buffer
- Substrate
- Enzyme

Specimen Sample For ELISA

SERUM
CSF
SPUTUM
URINE
SEMEN
SUPERNATANT OF CULUTRE
STOOL

Workstation Inventory

Lab Equipment and Supplies:

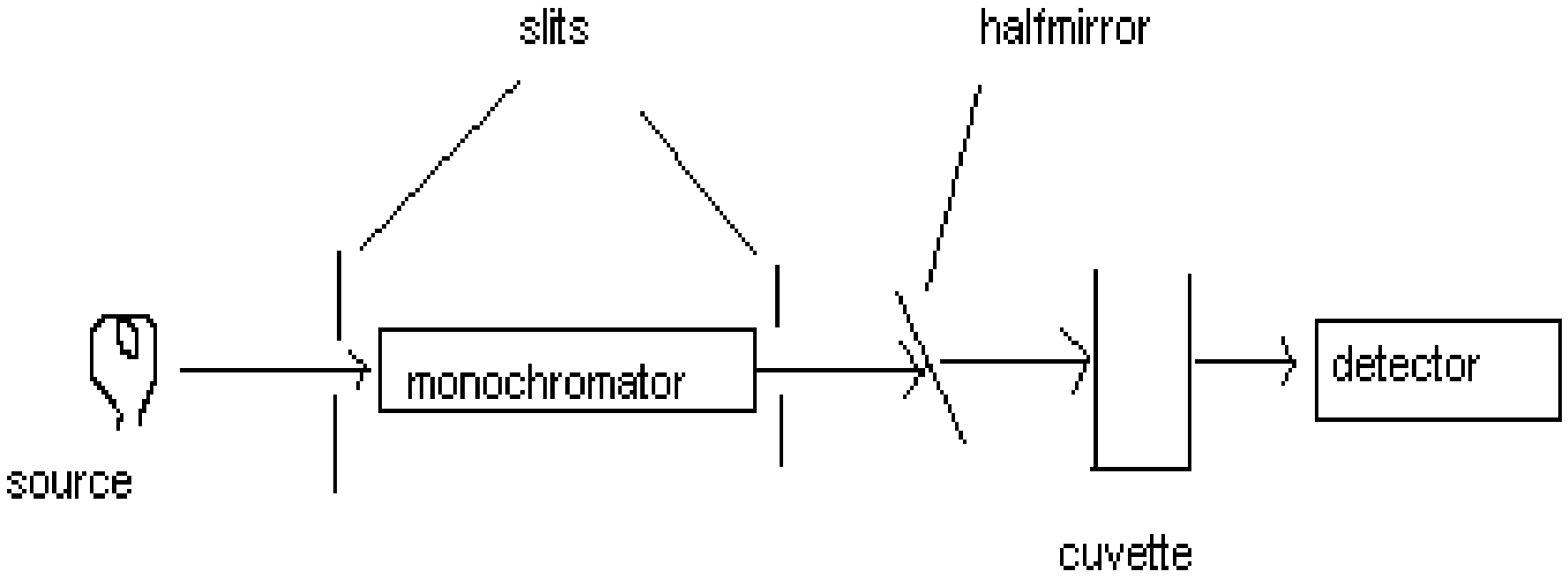
Micro plate strips, pipettes, pipette tips,
transfer pipette, wash buffer, paper towels,
marking pen

Equipment for performing the ELISA test

ELISA READER

THERMOLAB SYSTEM
(USA)

PRINCIPLE OF INSTRUMENT



TYPES OF ELISA

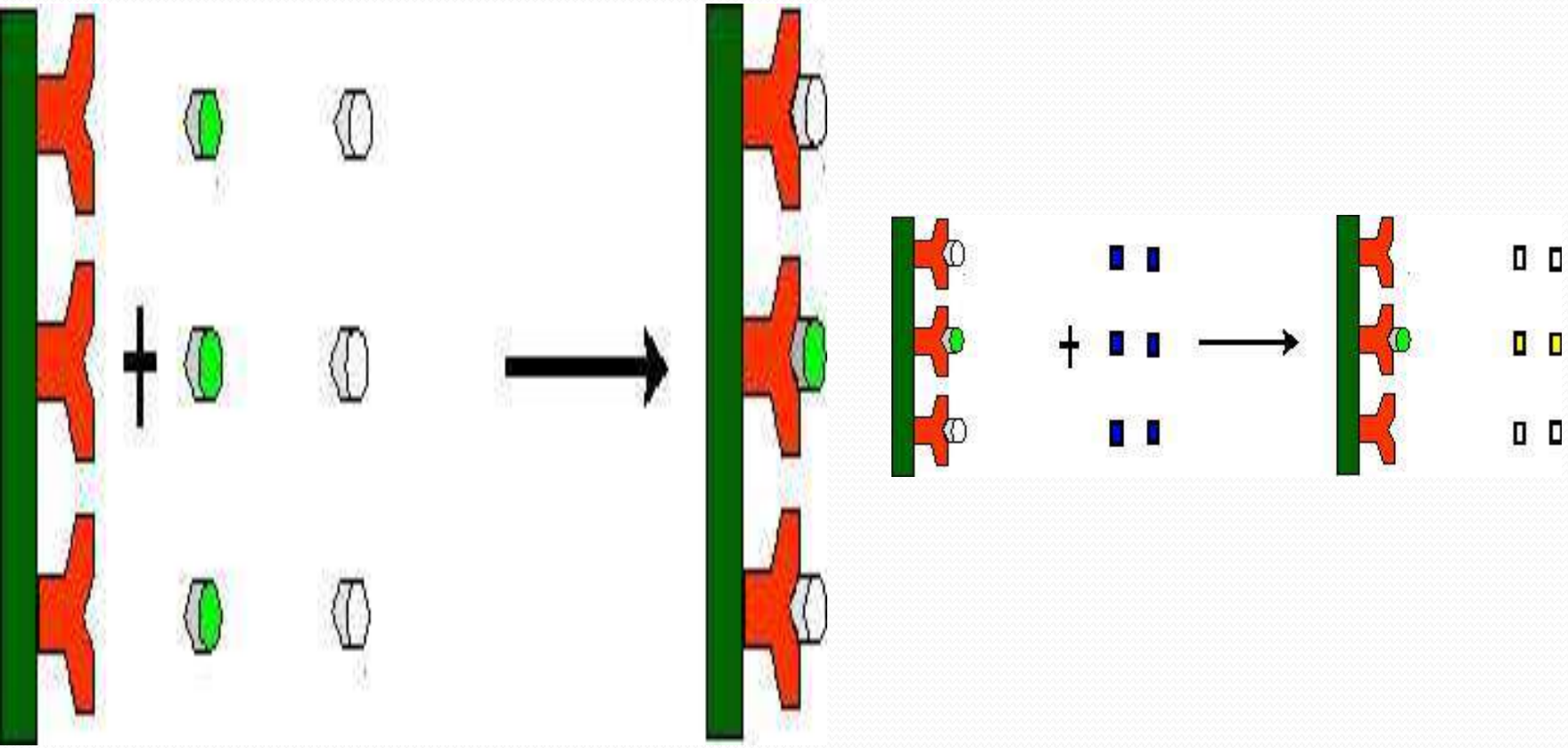
- Solid phase immunoassay
- Enzyme Label
  - Competitive assay
  - Non-competitive assay

Competitive Elisa

- Used to determine small molecule antigens.(T3,T4,progesterone etc.)
- antibody coated microwell
- serum antigen and labelled antigen added together--competition.
- antibody-antigen-enzyme complex bound is inversely related to the concentration of antigen present in the sample.
- The bound enzyme conjugate reacts with the chromogenic substrate added to produce a color reaction (blue to yellow color). .
- Increased serum antigen results in reduced binding of the antigen-enzyme conjugate with the capture antibody producing less enzyme activity and color (yellow) formation

Substrate product concentration is inversely proportional to the concentration of standard or test antigen added

DIAGRAMMATIC



Noncompetitive

- Sandwich Assay
- Direct Assay
  - Antigen capture ELISA Antigen adsorbed directly detected by labeled enzyme
  - Antibody capture ELISA Antibody adsorbed directly by labeled enzyme.
- Indirect Assay
  - Antigen directly adsorbed onto the solid phase is first incubated with patient serum, and then with a labeled antibody specific for human immunoglobulin.
  - Detection of infectious agents(HIV,HBV,HCV) and auto antibodies

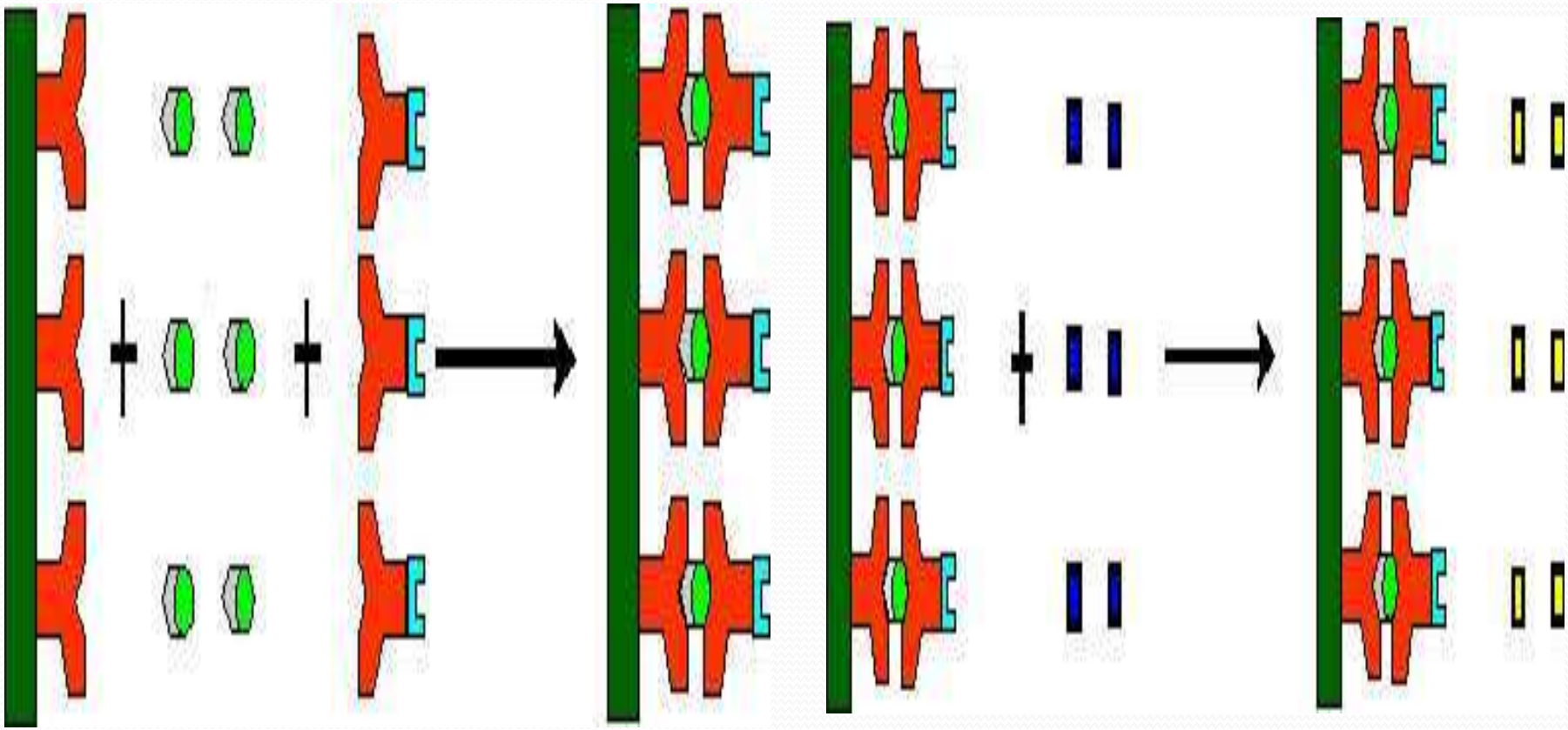
In Indirect ELISA color change is directly proportional to the concentration of specific antibodies in specimen

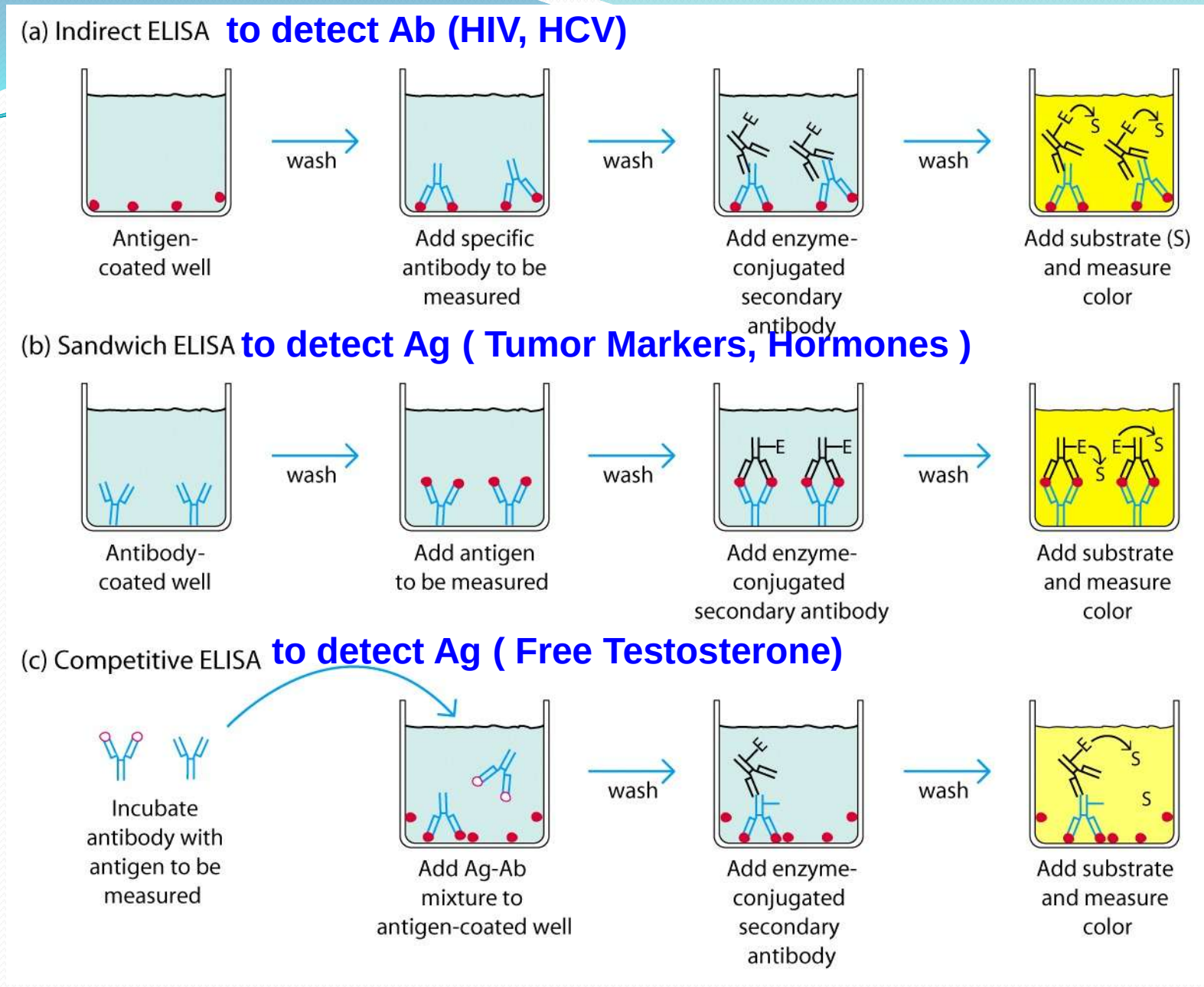
Sandwich Assay

- Antigens such as tumor markers, hormones and serum proteins may be determined
- Antigen in the sample binds with the capture antibody on the microwell and becomes immobilized.
- The antibody of the enzyme conjugate binds with the immobilized antigen to form a sandwich of antibody-antigen-antibody/enzyme bound to the microwell.

Enzyme reaction product is directly proportional to concentration of standard or analytical antigen

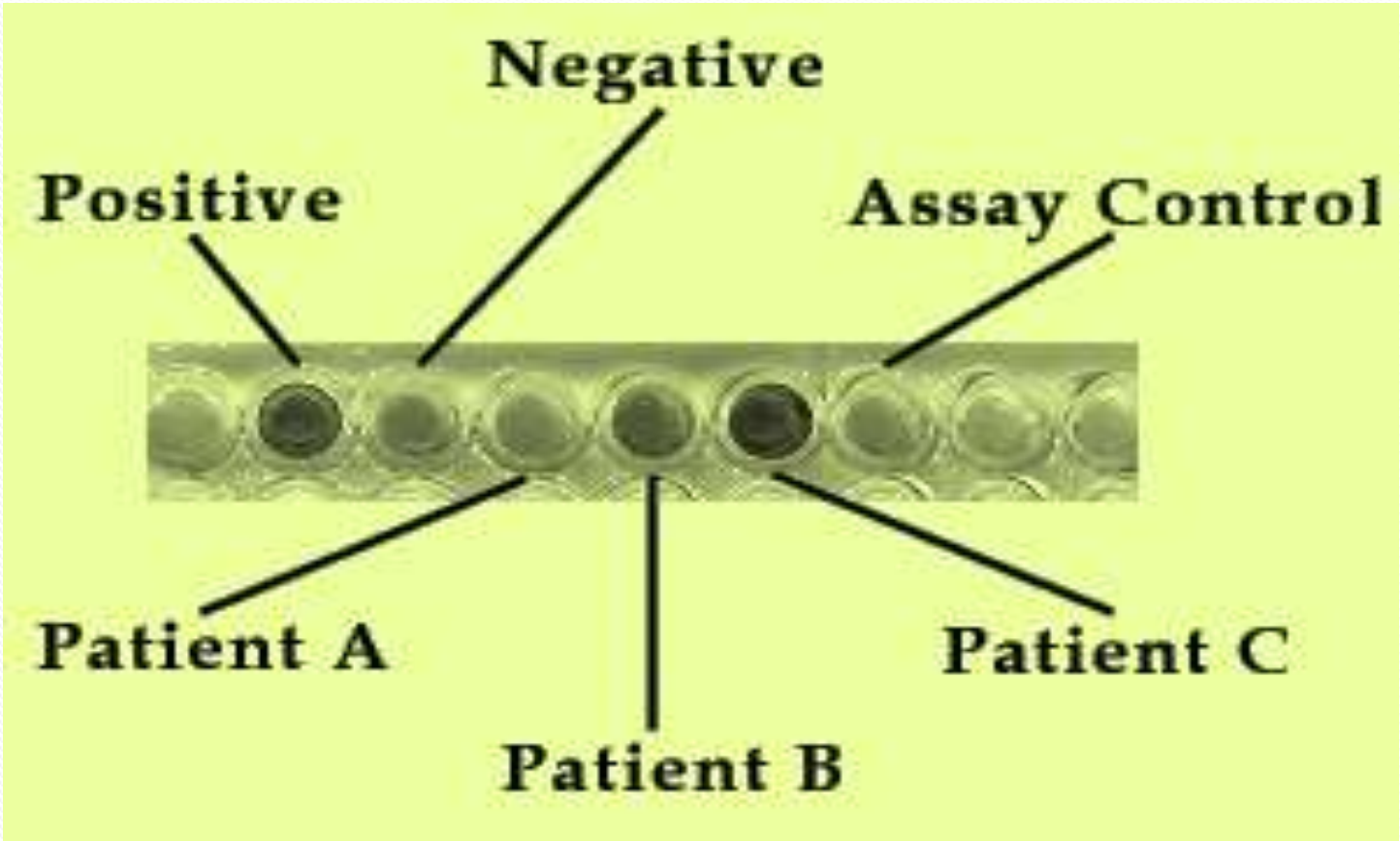
Diagrammatic





Comparison between Indirect Sandwich & Competitive ELISA

Results


Negative
Positive
Assay Control
Patient A
Patient C
Patient B

Importance of incubation step:-

- During the test performance incubation time and mentioned temperature is must required For the proper binding between antigen and antibody and also binding with conjugate and color development of substrate.
- Importance of Washing :- For the removal of any unbound Antibody/Antigen proper washing and taping is required other wise we get the incorrect result.
- So incubation & washing is much important for good results.

Enzymes Used in Elisa

- Horseradish peroxidase (most commonly used)
- Alkaline Phosphatase
- β-galactosidase
- Lactoperoxidase
- Tetra Methyl benzidine
- In case of peroxidase, the substrate hydrogen peroxide is converted into water and o2 in the presence of electron donors . (like diaminobenzidine or 4-chloronaphthol which themselves oxidized in the reaction).
- Oxidation of diaminobenzidine produces dark brown color while that of 4-chlorornaphthol yields purple color which is the basis of ELISA

ENZYME SUBSTRATE

- Initially the substrate should be colorless
- After degradation by the enzyme it should be strongly colored or fluorescent.

ENZYME	SUBSTRATE	CHROMOGEN	STOPPING
Alkaline Phosphatase	p-NPP	p-NPP+ diethandamine+Mg Cl2	1 M NaOH
Horse radish Peroxidase	H2O2	Tetramethylbenzidine + Phosphate – Citrate buffer	1 M H2SO4
Horse radish Peroxidase	H2O2	O – Phenylenediamine + HCl	1 M HCl

ELISA KIT FOR DETERMINATION OF IgA, IgG or IgM anti-Mycobacteria antibodies in human serum

ANDA-TB DETECTION OF MYCOBACTERIA

***In vitro* diagnostic test for the determination of IgA, IgG and IgM antibodies against mycobacteria in human liquid (serum, CSF, pleural fluid, sputum, saliva, etc...)**

Components of Kit

- **Pre-Coated, Stabilized 96-well Microtiter Plate.**
- **Sample Diluent**
- **Standards and controls**
- **Conjugated Detection Antibody**
- **10X Wash Solution**
- **Substrate**
- **Stop Solution**

What Are The Reagents? And What Function Do They Perform?

Antigen: **Elisa plate coated with the A60 antigen-antibodies complexes.**

Primary antibody: **Human Serum IgG, IgA, & IgM**

Secondary antibody: **Peroxidase-labelled anti-human IgA, IgG, or IgM antibodies that bind to the antibobdy complexes .**

Enzyme substrate: **3,3',5,5' – tetramethylbenzidine (TMB) – a colorless solution that when oxidized by HRP turns blue.**

Stop Solution**: Sulphuric Acid 0.5N (H_2So_4)**

Elisa Plate

- Microtitre wells
- Generally 96 wells
- Marked on one side alphabetically
- Numerically on the other side
- Comes with the kit

Complete forms and specimen identification

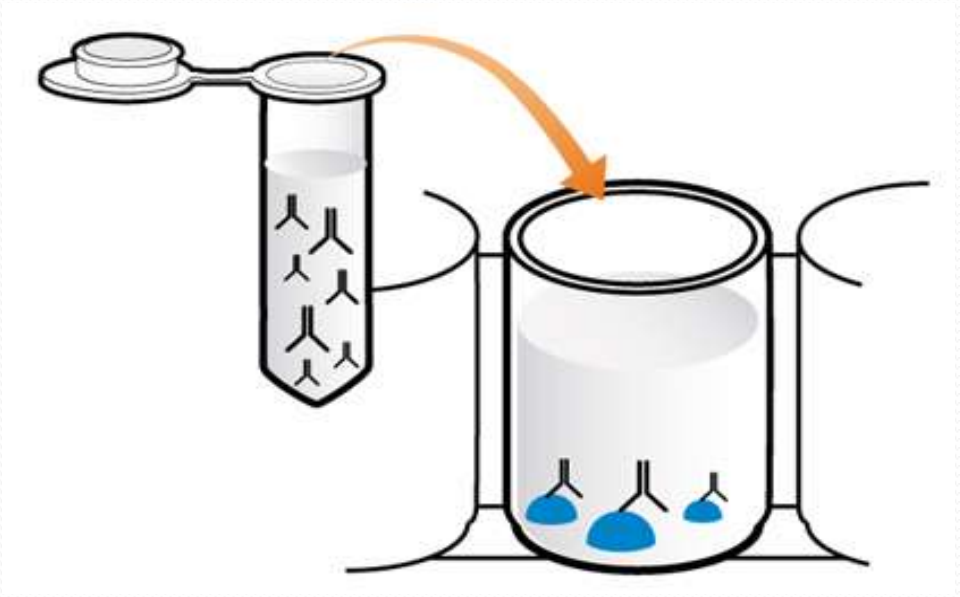
- Fill in the information on tube (identifying information, date of collection, and other information as required).
- Fill in the laboratory form that will accompany specimens.
- For TB Gold used heparin Tube

Collection and processing of serum

- Collect blood in a tube that does not contain any chemicals or anticoagulants.
- Collect 5mL of whole blood (for very small children collect 1mL).
- Place tube upright for 30-60 minutes then when firm clot has formed, centrifuge tube for 20 minutes at 2500rpm.
- Remove serum with a pipette and place in a plastic storage tube (2-3mL microtube or cryovial).
- If 5mL of blood was collected it will result in about 2mL of serum.

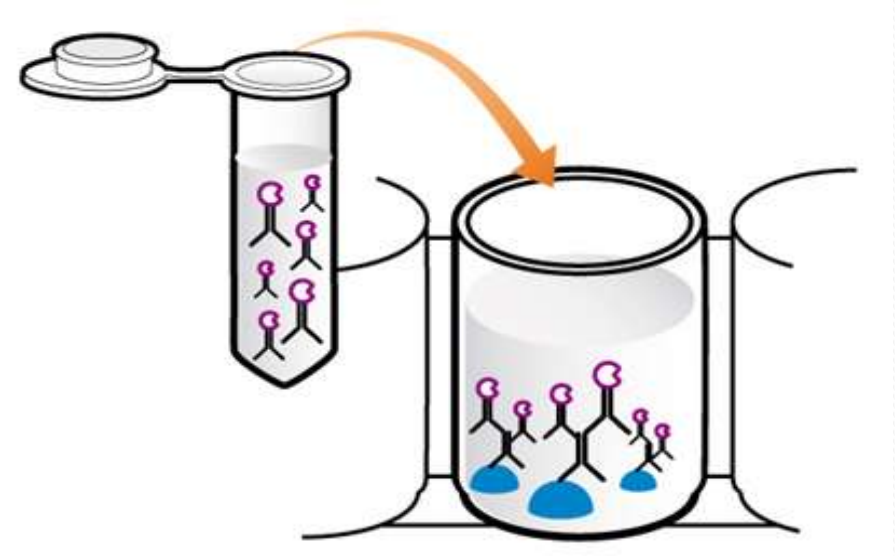
TEST PERFORMANCE

- Using a clean Pipette , add 100 µL of diluted serum sample (Dilute the sera to be tested 1:100 in the sample diluents) to each well.
- Incubate 1 hour at 37°C .

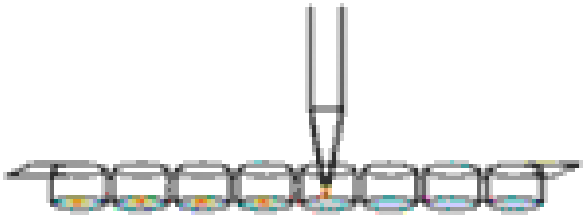


- After incubation empty out contents of wells into waste container.
- Using pipette, fill wells with washing buffer then empty out.
- Tap wells upside down on paper towel.
- Wash the wells 5 times. At the end of the washing process, the wells must be entirely dry after the last wash.

- Distribute 100µL of anti-human immunoglobulin-POD conjugate in each well. Incubate 30 minutes at 37°C.

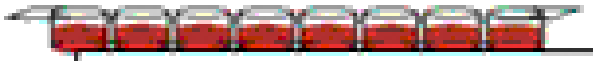


ASSAY PROCEDURE

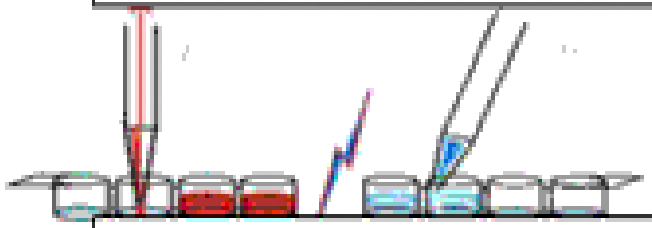


1. Dispense diluted sample

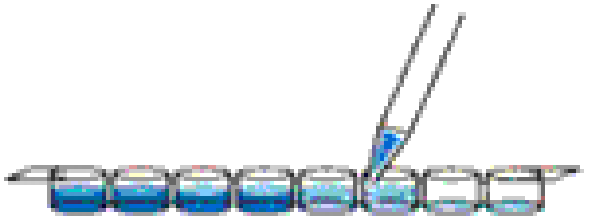
2. Add 100 ul of enzyme conjugate



3. Incubate for 1 hour at room temperature



4. Wash plates 6 times with deionized water



5. Add TMB colour developer substrate and incubate for 30 min

6. Add dilute acid stop solution and read at 450nm

ELISA PLATE READY FOR READING

Measures the absorbance at 450nm With the help of ELISA READER.

Calculate the absorbance for each sample and reference.

We used Ascent Software for Calculation of the result

ELISA READER

THERMOLAB SYSTEM
(USA)

RESULT DETERMINATION:-

A) IgA and IgG Tests:-

Plot the O.D. result of each reference , except for the negative reference on the vertical axis (Y-axis) in relation to the number of corresponding units on the horizontal axis (X-axis).
Using the absorbance value for each sample , determine the corresponding concentration of antibodies expressed in units/ml from the reference curve.

B) IgM Tests :-

We can calculate the cut off value A450nm sample / A450nm Positive limit reference
the normalized value of the positive reference is 1 .
All samples whose value is comprised between 0.8 and 1.0 are considered dubious and all samples whose normalized value is above 1.0 are considered positive for IgM antibodies.

What is Cut-off Value ……?

Cut-off: provided in the kits by the manufacturer. The cut-off value defines a range in which 90% of the normal population is negative below the cut-off value and 10% of the normal population is positive above the cut-off value.

ELISA is semiquantitative method. The calculation is done as follows. The units of ELISA is OD ratio:

Sample value= sample OD/cut-off OD

RANGE OF IgM IgG & IgA

IgM- < 0.8 Negative , 1.0 Borderline, > 1.0 Positive
IgG - < 125 Negative 125-225 Borderline, > 225 Positive
IgA - < 200 Negative, 201 – 300 Borderline – Negative, 301 – 350 Borderline – positive, > 350 Positive

Advantages of ELISA

- Reagents are relatively cheap & have a long shelf life
- ELISA is highly specific and sensitive
- No radiation hazards occur during labelling or disposal of waste.
- Easy to perform and quick procedures
- Equipment can be inexpensive and widely available.
- ELISA can be used to a variety of infections.

Disadvantages of ELISA

- Measurement of enzyme activity can be more complex than measurement of activity of some type of radioisotopes.
- Enzyme activity may be affected by plasma constituents.
- Kits are commercially available, but not cheap
- Very specific to a particular antigen. Won't recognize any other antigen
- False positives/negatives possible, especially with mutated/altered antigen

Limitations

- Results may not be absolute
- Antibody must be available
- Concentration may be unclear
- False positive possible
- False negative possible

APPLICATIONS OF ELISA

1- Hormones	**7- Vaccine Quality Control**
2- Proteins	8- FOR GMO (Genetically modified organism)
3- Infectious Agent (Viral, Bacterial, Parasitic, Fungal)	9- For Rapid Test
4- Drug Markers	10- IgG, IgM, IgA
5- Tumor Markers	11- In New Born Screening
6- Serum Proteins	12- In Clinical Research

Sensitivity of various immunoassays

Assay	Sensitivity* (μg antibody/ml)
Precipitation reaction in fluids	20–200
Precipitation reactions in gels	
Mancini radial immunodiffusion	10–50
Ouchterlony double immunodiffusion	20–200
Immunoelectrophoresis	20–200
Rocket electrophoresis	2
Agglutination reactions	
Direct	0.3
Passive agglutination	0.006–0.06
Agglutination inhibition	0.006–0.06
Radioimmunoassay	0.0006–0.006
Enzyme-linked immunosorbent assay (ELISA)	<0.0001–0.01
ELISA using chemiluminescence	<0.0001–0.01†
Immunofluorescence	1.0
Flow cytometry	0.06–0.006

*The sensitivity depends upon the affinity of the antibody as well as the epitope density and distribution.

†Note that the sensitivity of chemiluminescence-based ELISA assays can be made to match that of RIA.

SOURCE: Adapted from N. R. Rose et al., eds., 1997, *Manual of Clinical Laboratory Immunology*, 5th ed., American Society for Microbiology, Washington, D.C.

A New technique:- Reverse ELISA

- A new technique uses a solid phase made up of an immunosorbent polystyrene rod with 4-12 protruding ogives. The entire device is immersed in a test tube containing the collected sample and the following steps (washing,incubation in conjugate and incubation in chromogenous) are carried out by dipping the ogives in microwells of standard microplates pre-filled with reagents.
- Advantage of this technique:
- 1- The ogives can each be sensitized to a different reagent, allowing the simultaneous detection of different antibodies and different antigens for multi-target assays.
- 2- The sample volume can be increased to improve the test sensitivity in clinical, food and environmental samples.
- 3- One ogive is left unsensitized to measure the non-specific reactions of the sample.
- 4- The use of laboratory supplies for dispensing sample aliquots, washing solution and reagents in microwells is not required, facilitating ready-to-use lab kits and on-site kits.

SUMAN KUMAR MEKAP

Questions & Answers