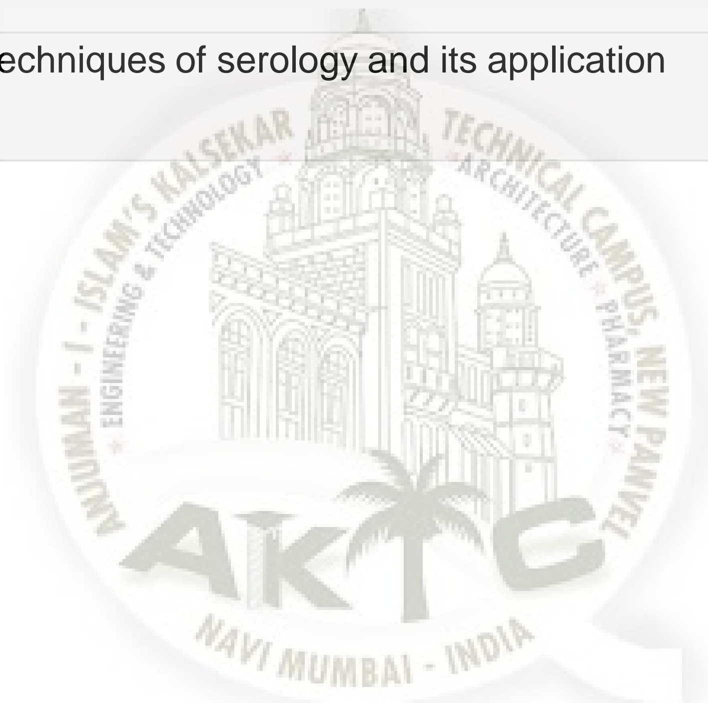
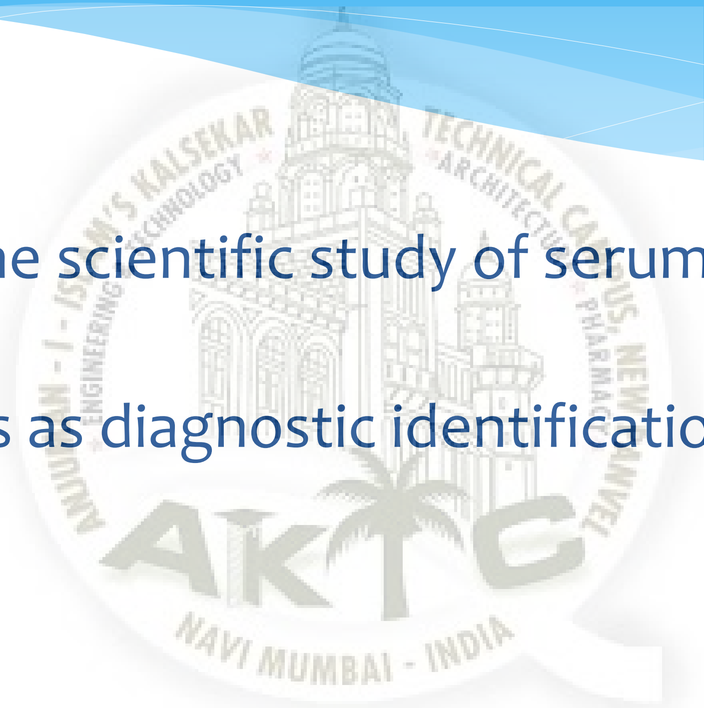
Topic: Serology
Subject: Pharmaceutical Biotechnology
Class: T. Y. B. Pharm. (Sem.- V)
Academic Year: 2018-19
Programme: 2016-2020

Priyanka Sanap
Assistant Professor
AIKTC, School of Pharmacy, New Panvel.

	Topic Learning Outcomes	COs	BL
1	Describe different techniques of serology and its application	CO2	L2



SEROLOGY



* Serology is the scientific study of serum and other body fluids.
* Usually refers as diagnostic identification of Ab in the serum

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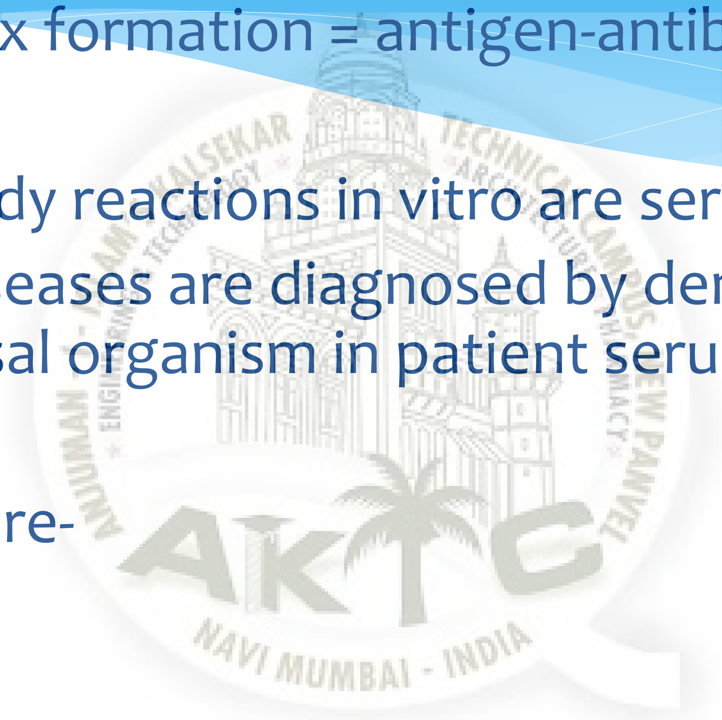
Antibody Targets & Functions

* Immune complex formation = antigen-antibody binding.

* Antigen –antibody reactions in vitro are serological reactions
* In vitro many diseases are diagnosed by demonstrating antibody to causal organism in patient serum



* Main reactions are-

1. Precipitation
2. Agglutination
3. Complement fixation
4. Neutralization

Antibody Targets & Functions

* Complement fixation: cells & bacteria.
  * Immune complex formation exposes a complement binding site on the C region of the Ig.

  Complement fixation results in cell lysis.

* Neutralization: immune complex formation blocks specific sites on virus or toxin & prohibit binding to tissues

* Agglutination: cells are cross-linked by immune complexes & clump together

* Precipitation: soluble molecules (such as toxins) are cross-linked, become insoluble, & precipitate out of the solution.

  Inflammation & phagocytosis prompted by debris

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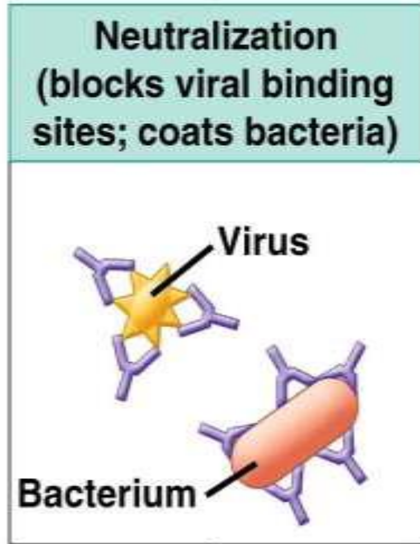
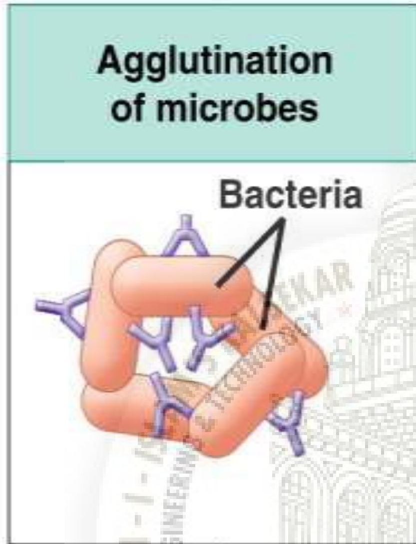
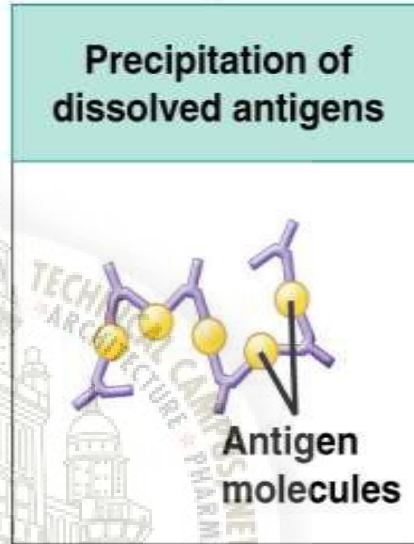
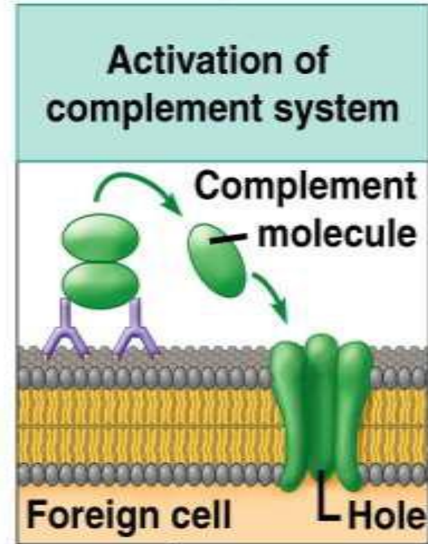
Binding of antibodies to antigens inactivates antigens by



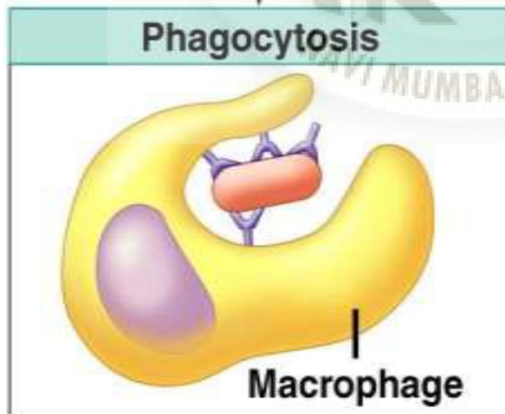


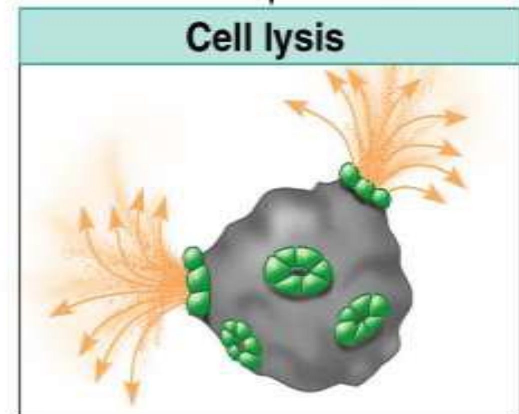




Enhances

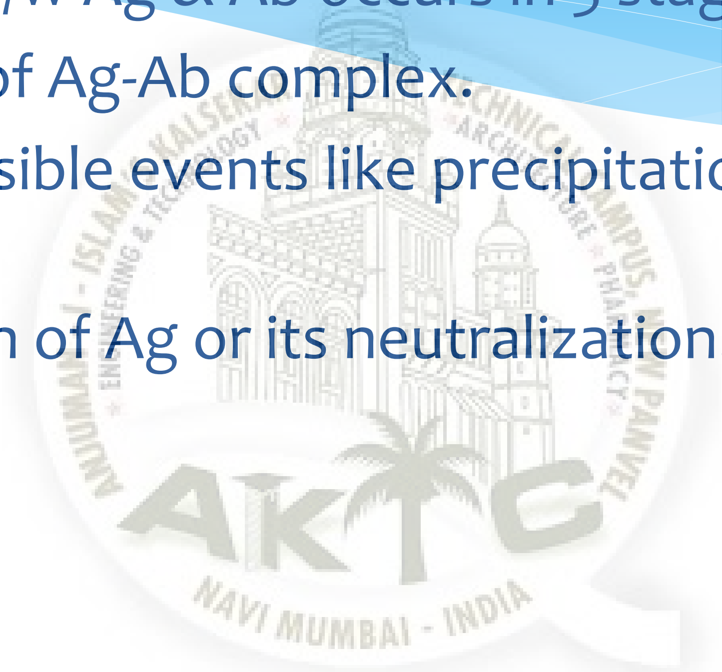
Leads to





Characteristics of antigen- antibody reactions

1. The reactions are highly specific
2. There is no denaturation of antigen or antibody during the reactions
3. Combination occurs at surface, hence surface antigen are immunological relevant
4. Combination is firm but reversible. It is influenced by affinity and avidity
5. Entire molecule should react not fragment
6. Antigen and antibody forms the ppt , they may combine in different ratio



- - The reactions b/w Ag & Ab occurs in 3 stages:
- 1st = formation of Ag-Ab complex.
- 2nd = leads to visible events like precipitation, agglutination etc.
- 3rd = destruction of Ag or its neutralization.

Antigen- antibody reactions

* Two imp parameter-

1. Specificity
2. Sensitivity

* Two stages of antigen- antibody reactions-

1. Primary interaction- interaction between two without any visible effect. Involves the use of one or other reactant labeled with suitable marker such as fluorescent dye or radioactive isotope
2. Secondary interaction-visible result of antigen antibody reactions e.g. ppt, agglutination

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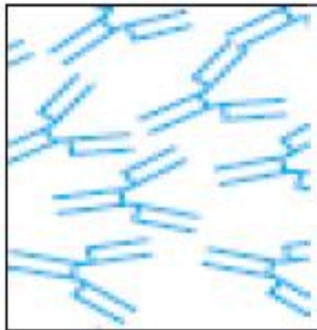
Classification of antigen-antibody interactions:

1. Primary serological tests: (Marker techniques) e.g.
   * Enzyme linked immuno sorbent assay (ELISA)
   * Immuno florescent antibody technique (IFAT)
   * Radio immuno assay (RIA)

2. Secondary serological tests: e.g.
   * Agglutination tests
   * Complement fixation tests (CFT)
   * Precipitation tests
   * Serum neutralization tests (SNT)
   * Toxin-antitoxin test

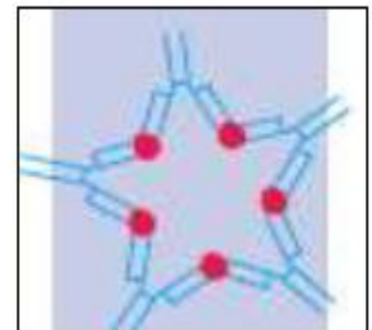
Precipitation Reaction:

When a soluble Ag combines with its Ab in the presence of an electrolyte (NaCl) at a particular temperature and pH, it forms an insoluble precipitate of Ag-Ab complex. The Ab causing precipitation is called Precipitin and the reaction is called as precipitation reaction.



Antibodies

Antigens



Ag-Ab complex

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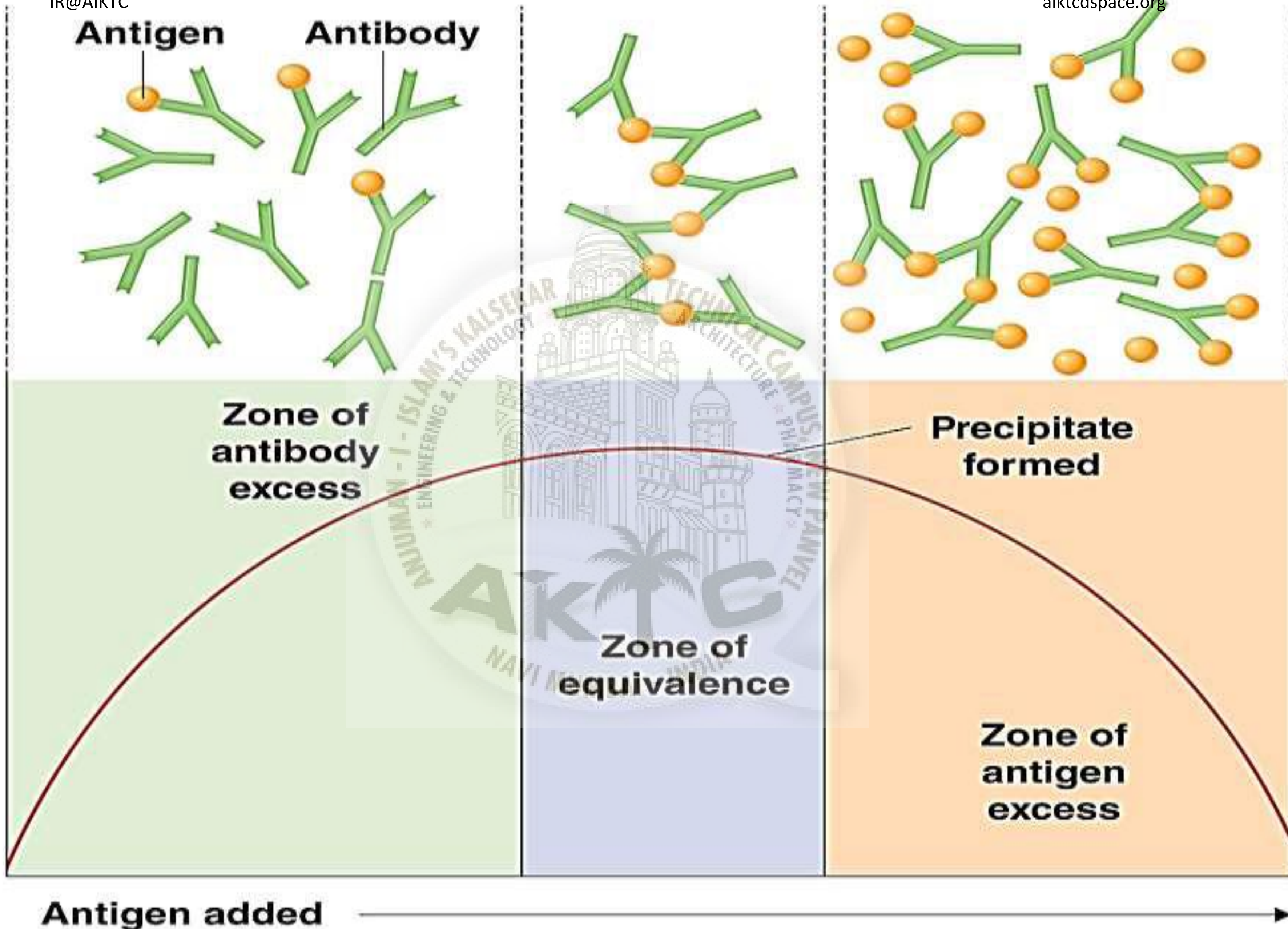
- **Function of precipitation reaction:** Precipitation occurs in two media:

➢ Liquid.
➢ Gel.

➢ **Precipitation in Liquid:**

Antigen – Antibody reaction perform by placing a constant amount of antibody in a series of tubes and adding increased amount of antigen. Antigen – Antibody reacts together resulting in precipitation. Plotting the amount of precipitate against increasing antigen conc. Yeilds a precipitation curve.



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Equivalence zone

Antibody in excess

Antigen in excess

Amount of ppt

Conc. Of antigen

YYYYYY……….

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Precipitation curve shows three zones:

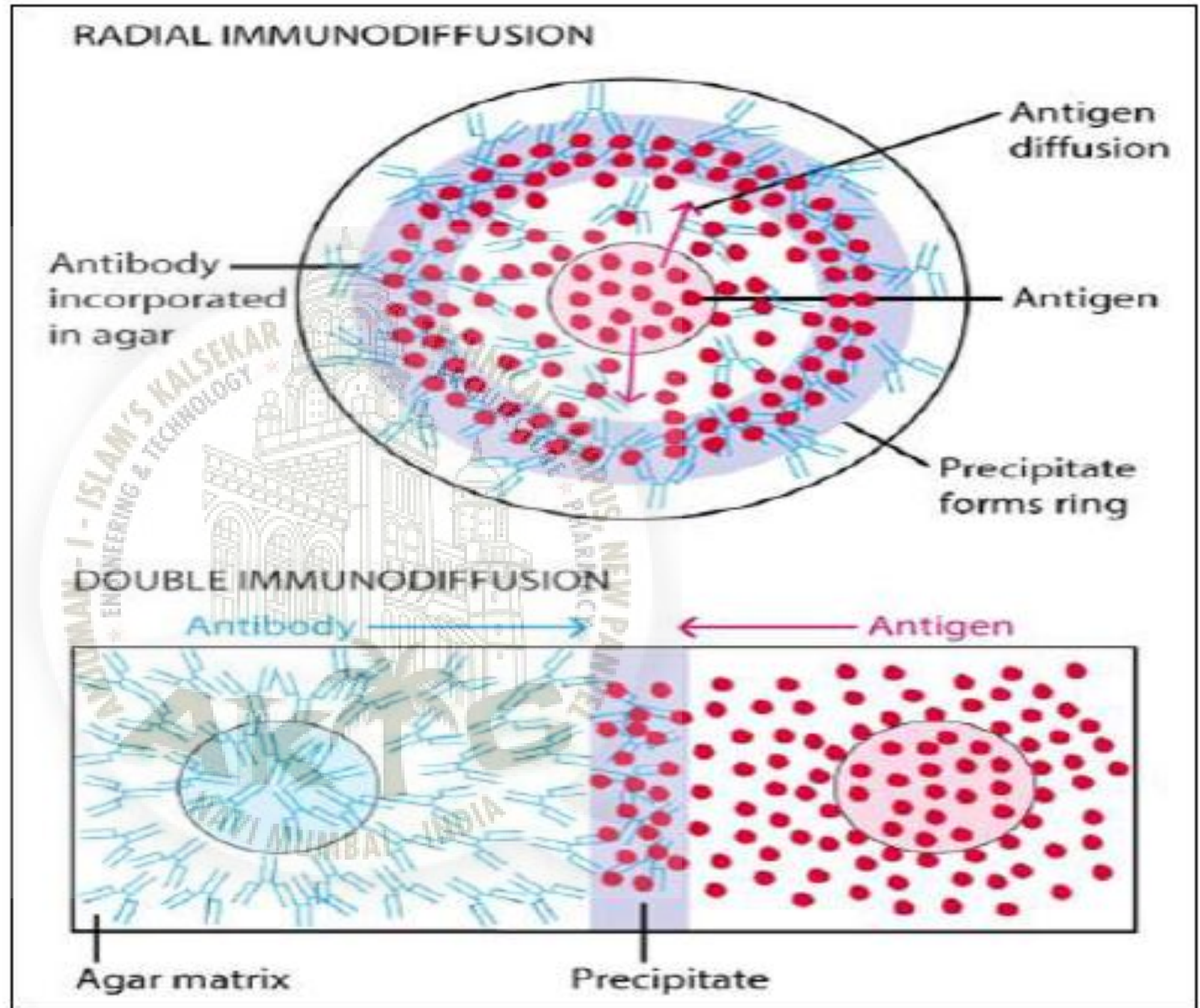
1. Zone of Ab axis.
2. Zone of equivalence.
3. Zone of Ag axis.

Precipitation in gel:

Radial Immunodiffusion (Mancini) :

In these methods agar gel or similar gels are used on plates or petriplates. Both Ag and Ab diffuse freely in the gel system in all directions. At a certain point depending on the rate of diffusion and concentration of the reactants, a zone of equivalence will be formed, which is seen as a visible precipitation.

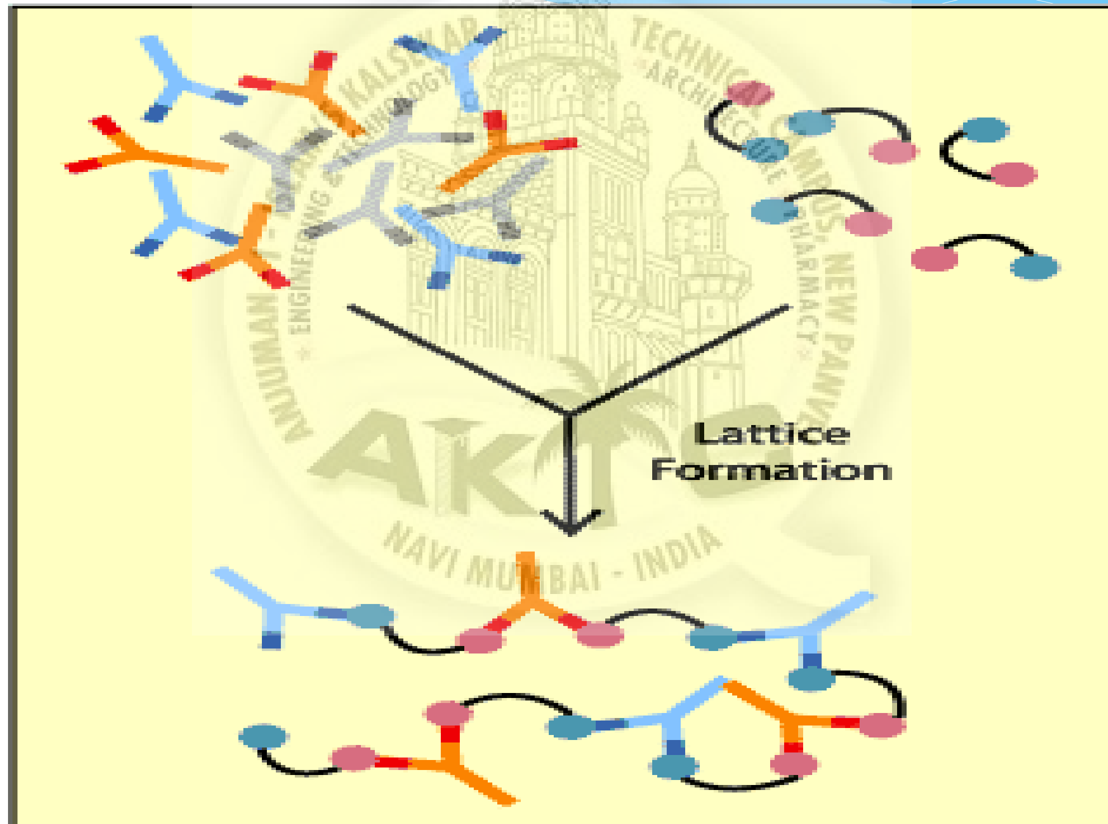
If Ag or Ab preparations are complex, multiple bands form. These are again of 2 types- Single diffusion methods and double diffusion methods.



Precipitation reactions in gels

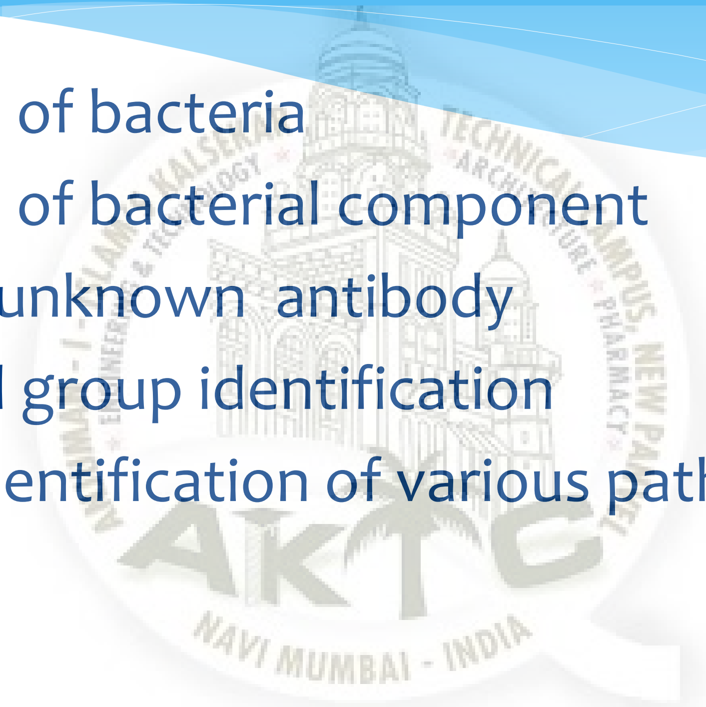
PRINCIPLE

- - Soluble antigen + antibody (in proper proportions) -
- visible precipitate
- - Lattice formation (Ag binds with Fab sites of 2 Ab's)



* Ppt reactions are very sensitive and can detect as little as 1 mcg of protein antigen
* Can be qualitative (presence of antigen) and quantitative
* Imp in –

1. Identification of bacteria
2. Identification of bacterial component
3. Detection of unknown antibody
4. Human blood group identification
5. Serological identification of various pathogens



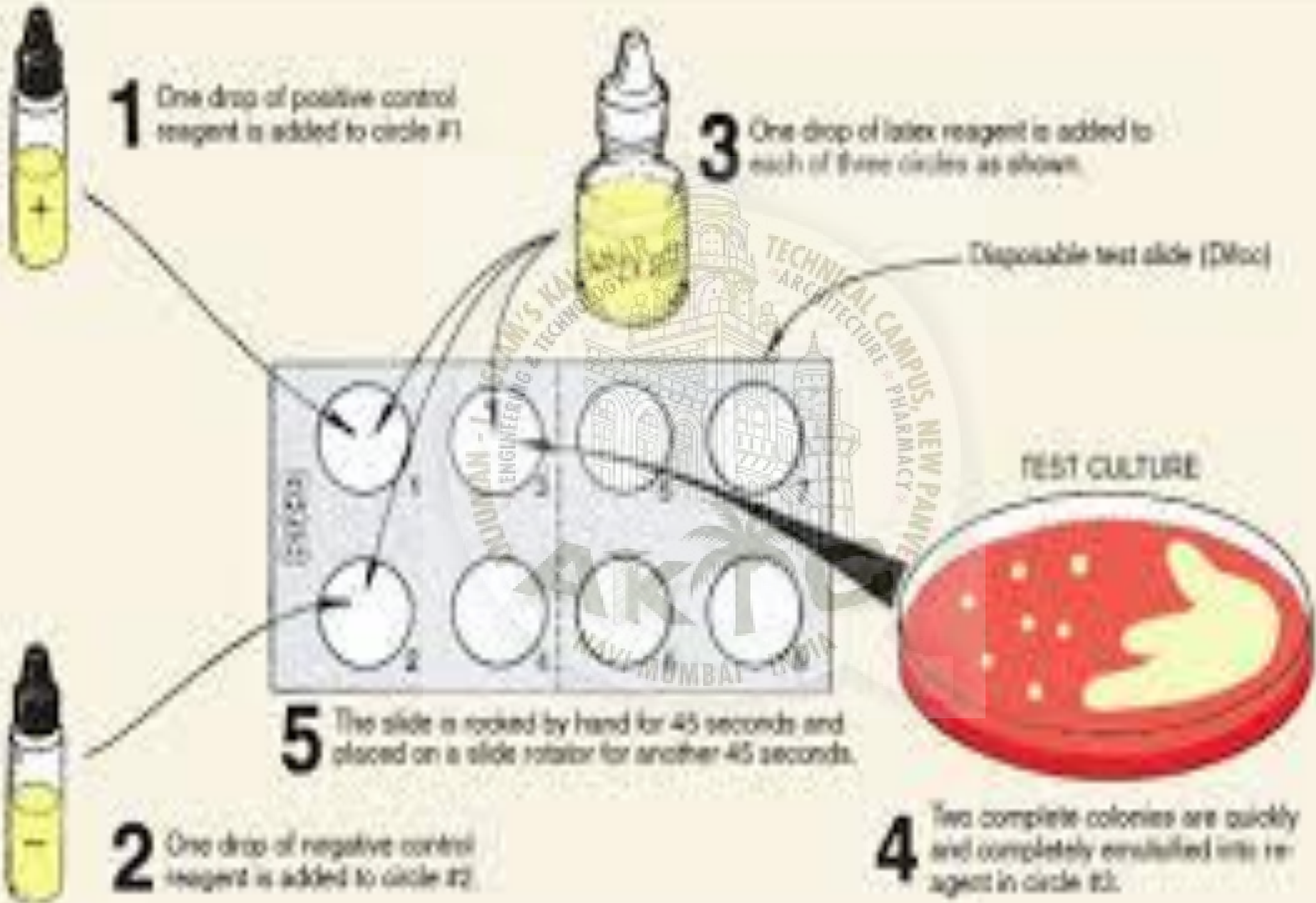
Agglutination Reaction:

- When a particular Ag is mixed with its Ab's in the presence of electrolytes at a suitable temperature and pH, the particles are clumped or agglutinated.

- The Ab of the serum causes the cellular Ag's to form clumps and these are called Agglutinins.

- The particulate antigens that are aggregated are termed Agglutinogens.

➢ **Slide agglutination:** This is a rapid method to determine the presence of agglutinating antibodies.


1 One drop of positive control reagent is added to circle #1
3 One drop of latex reagent is added to each of three circles as shown.
Disposable test slide (Difco)
TEST CULTURE
5 The slide is rocked by hand for 45 seconds and placed on a slide rotator for another 45 seconds.
2 One drop of negative control reagent is added to circle #2.
4 Two complete colonies are quickly and completely emulsified into re- agent in circle #3.

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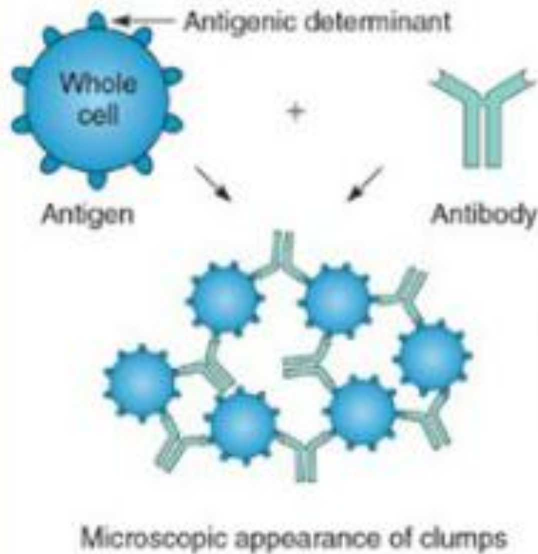
To a uniform suspension of particulate Ag, a drop of saline is added and then a drop of antiserum is added.

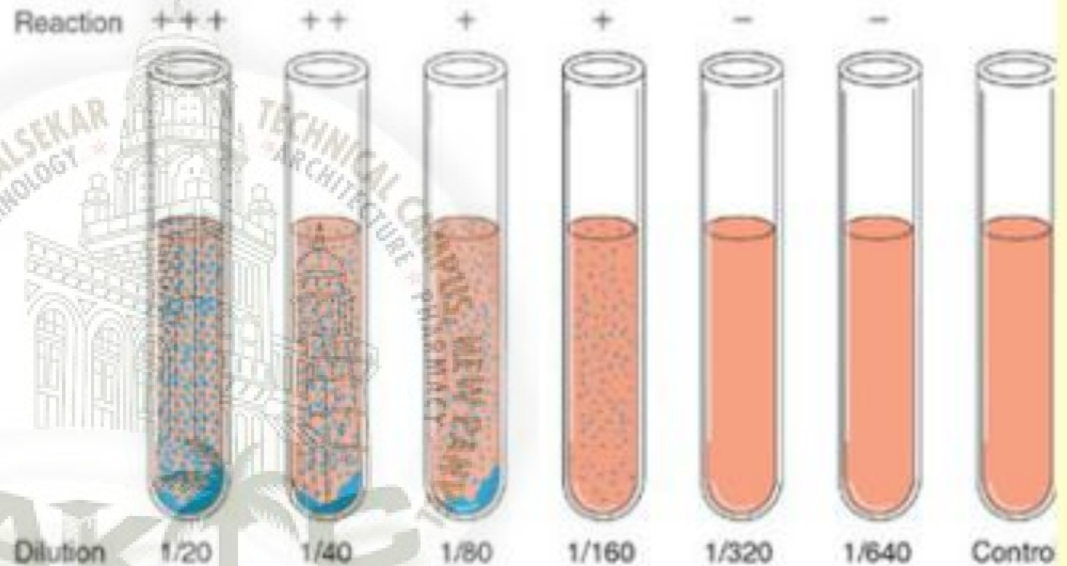
The slide is gently rocked or a fine loop is used to mix the contents. If granulation occurs the test is Positive.

It takes a minute for the test to complete and is visible to the naked eye. Some times confirmation may be done by observing slide under microscope.

This test is used for blood grouping (Haemagglutination) and cross matching.

Tube agglutinations test - Serological reaction in Serological method





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Components:

1. *Serum from patient (unknown Ab).*
2. *Specific diagnosticum – killed known pure culture of bacteria (known Ag).*
3. *NaCl solution.*

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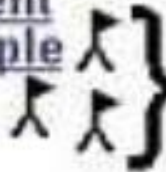
HEMAGGLUTINATION INHIBITION

Patient Sample



Human IgG expressing the G 1 epitope +

(anti- Hu IgG) G1 epitope specific

+

= NO Agglutination

All available anti-G1 epitope is bound by free G1- expressing Ab in the patient's serum sample

THIS IS A POSITIVE REACTION

Human IgG with other heavy chain isotope +

G1 epitope specific

+

=

Patient Sample

Agglutination

THIS IS A NEGATIVE REACTION

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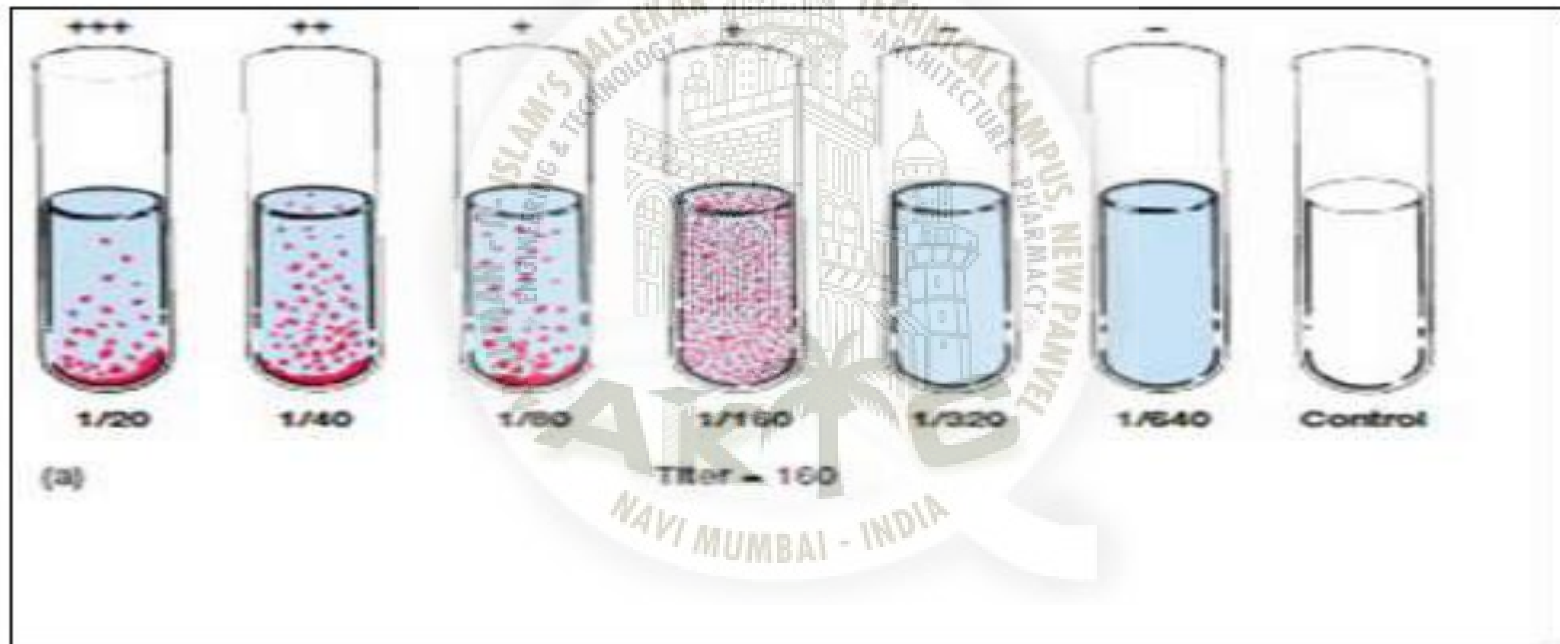
- **Tube agglutination:** This is a standard method for quantitative estimation of Ab. The serum containing Ab is diluted serially with saline in several small test tubes, to which a constant volume of Ag suspension is added.

A control tube is kept which has no antiserum. The tubes are incubated until visible agglutination is observed. The tube showing highest agglutination is referred to as the titre.

Tube agglutination is employed for the serological diagnosis of typhoid, brucellosis and typhus fever. **Widal test** is used for the estimation of typhoid fever.

In this test Ab content of the patient's serum, is measured by adding a constant amount of antigen (Solmonella typhi) to the serially diluted serum.

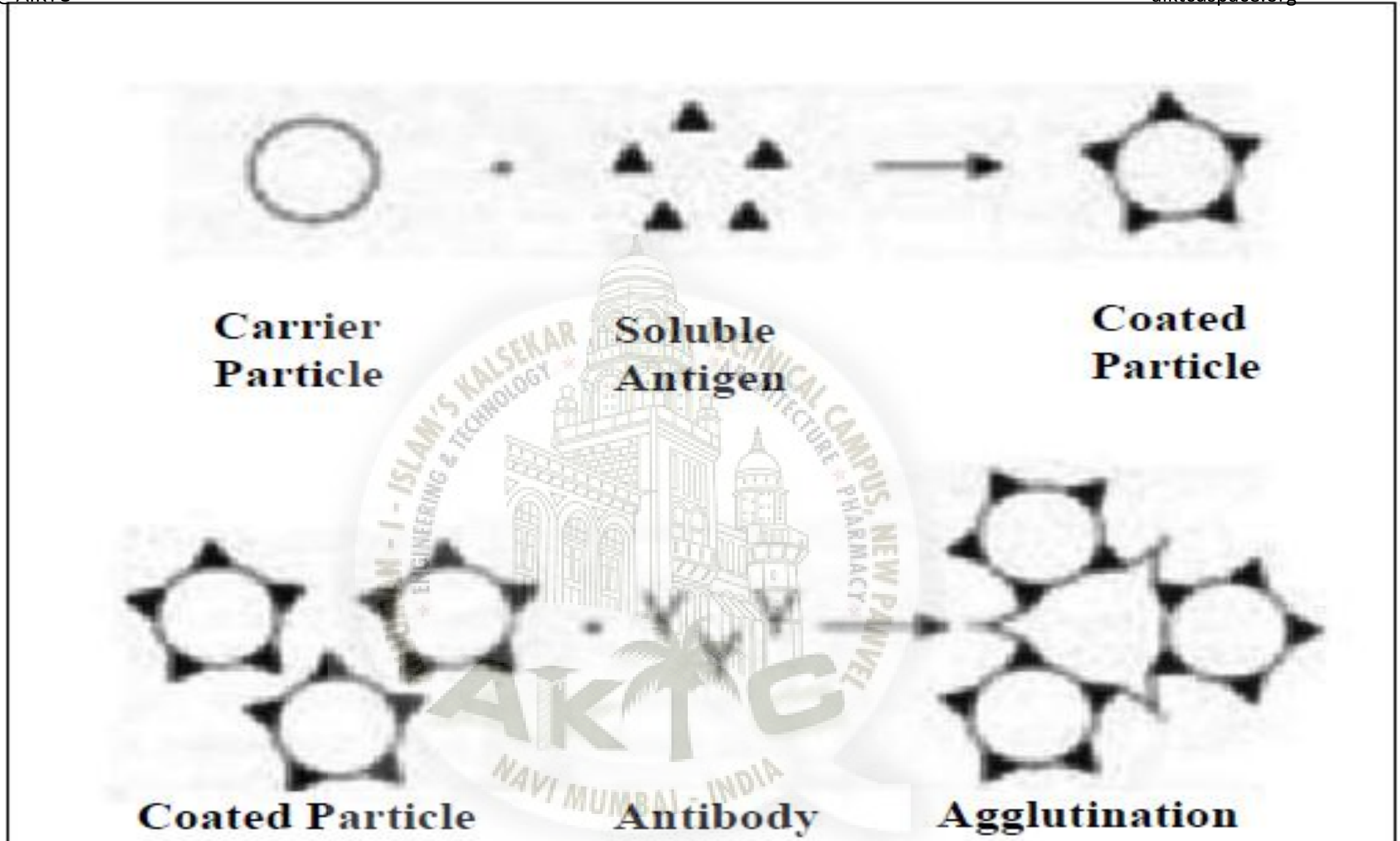


Tube Agglutination

➢ **Passive agglutination test:** It is similar to haemagglutination test but the physical nature of the reaction is altered.

The Ag is coated on the surface of a carrier particle and thereby helps to convert a precipitation reaction into an agglutination reaction making the reaction more sensitive. The carrier particles used can be RBC, latex particles or bentonite. Some times RBC coated with polystyrene (tanned RBC) can be used.

When patients serum is mixed with these, it leads to agglutination. This test is used for the diagnosis of Rheumatoid arthritis.



Passive Agglutination

Agglutination Inhibition:

Provides a highly sensitive assay for small quantities of an Antigen.

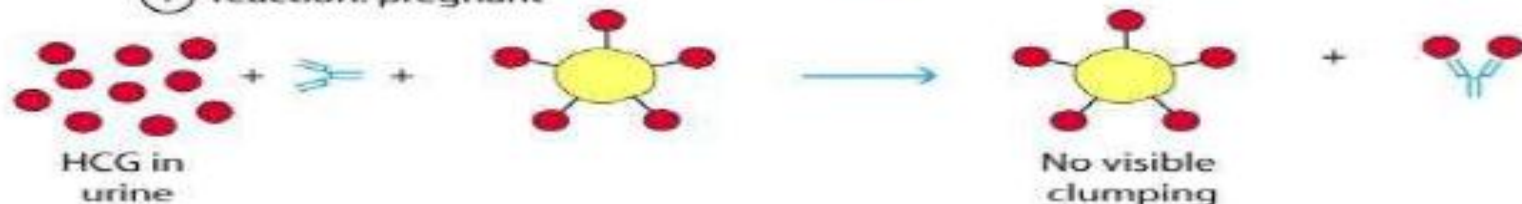
Example: First home pregnancy test

KIT REAGENTS

TEST PROCEDURE

Urine + Anti–HCG —Incubate→ + HCG carrier conjugate → Observe for visible clumping

POSSIBLE REACTIONS

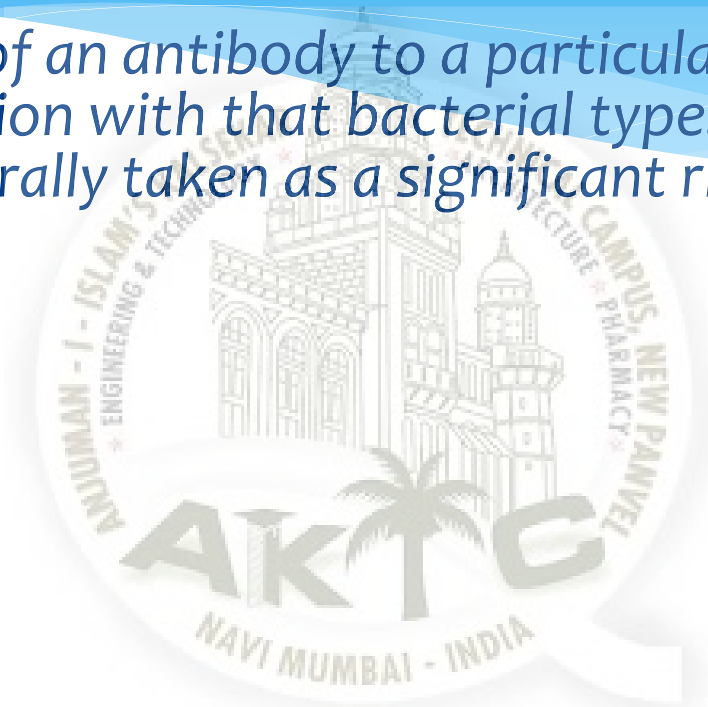


Applications of agglutination tests

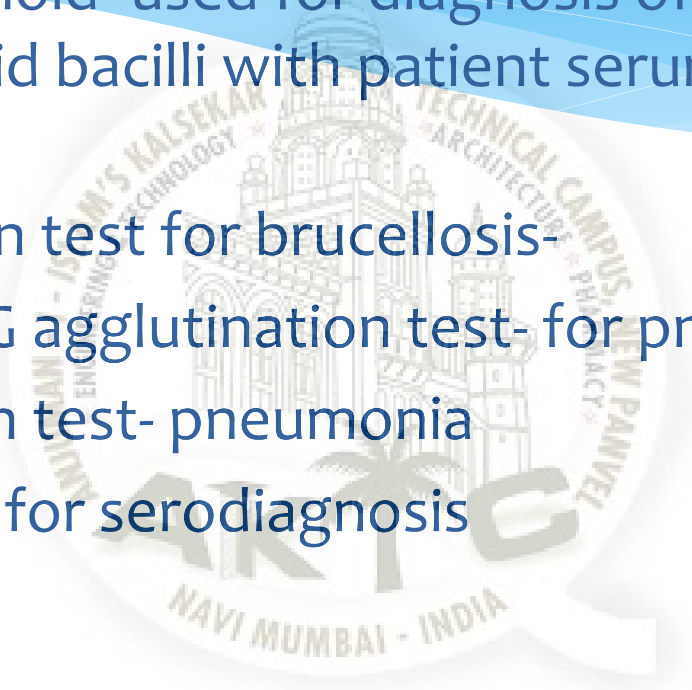
i. Determination of blood types or antibodies to blood group antigens.

ii. To assess bacterial infections

e.g. A rise in titer of an antibody to a particular bacterium indicates an infection with that bacterial type. N.B. a fourfold rise in titer is generally taken as a significant rise in antibody titer.



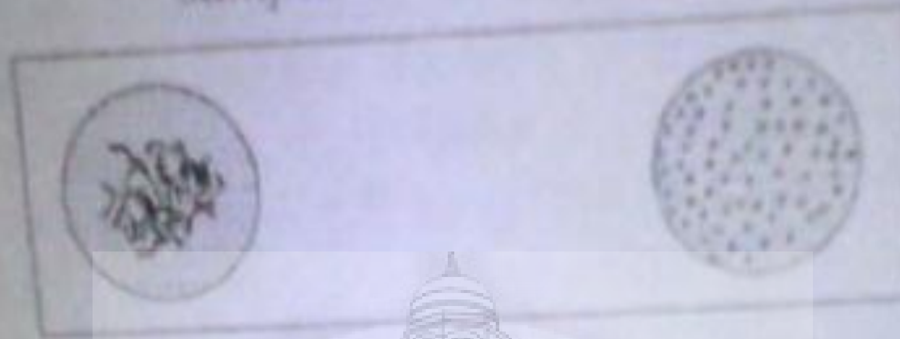
Examples of agglutination test



1. Widal test for typhoid- used for diagnosis of typhoid fever- agglutinating typhoid bacilli with patient serum containing antibody
2. Tube agglutination test for brucellosis-
3. Streptococcus MG agglutination test- for pneumonia
4. Cold agglutination test- pneumonia
5. Weilfelix reaction for serodiagnosis

- **EXAMPLE**
- **ABO BLOOD GROUP –**
- The typing of blood , for ABO groups or Rh groups, involves
- agglutination reaction.
- For typing blood , a drop of the blood sample is mixed with a drop of antiserum A & another drop of the blood sample is mixed with a drop of antiserum B on a glass slide.
- If belongs to A is clumped with antiserum A , the sample belongs to belongs to B is clumped with antiserum B , if the sample is clumped with both antiserum A & antiserum B , the blood sample belongs to group AB .
- If there is no agglutination the blood sample belongs to group O.

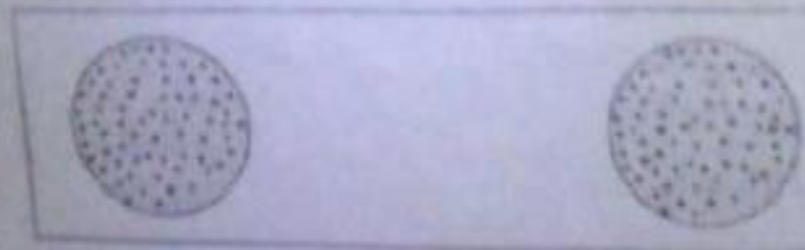
sample



A group
Agglutination with anti-A

B group
Agglutination with anti-B

AB group
Agglutination with anti-A and anti-B



O group. No agglutination

Complement Fixation:

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- Lysis of RBC or bacteria requires some non-specific unstable components of fresh serum which are called complement.

- This complement system comprises of 11 proteins and are present in ever individual. They bind to Fc component of Ab involved in Ag-Ab complex. This ability of the Ag-Ab complex to fix complement is used in complement Fixation tests.

- In the first stage, the test Ag and the antiserum (heated to 56°C to inactivate complement) are mixed in the presence of known amount of complement. This is incubated at 4°C for 18h.

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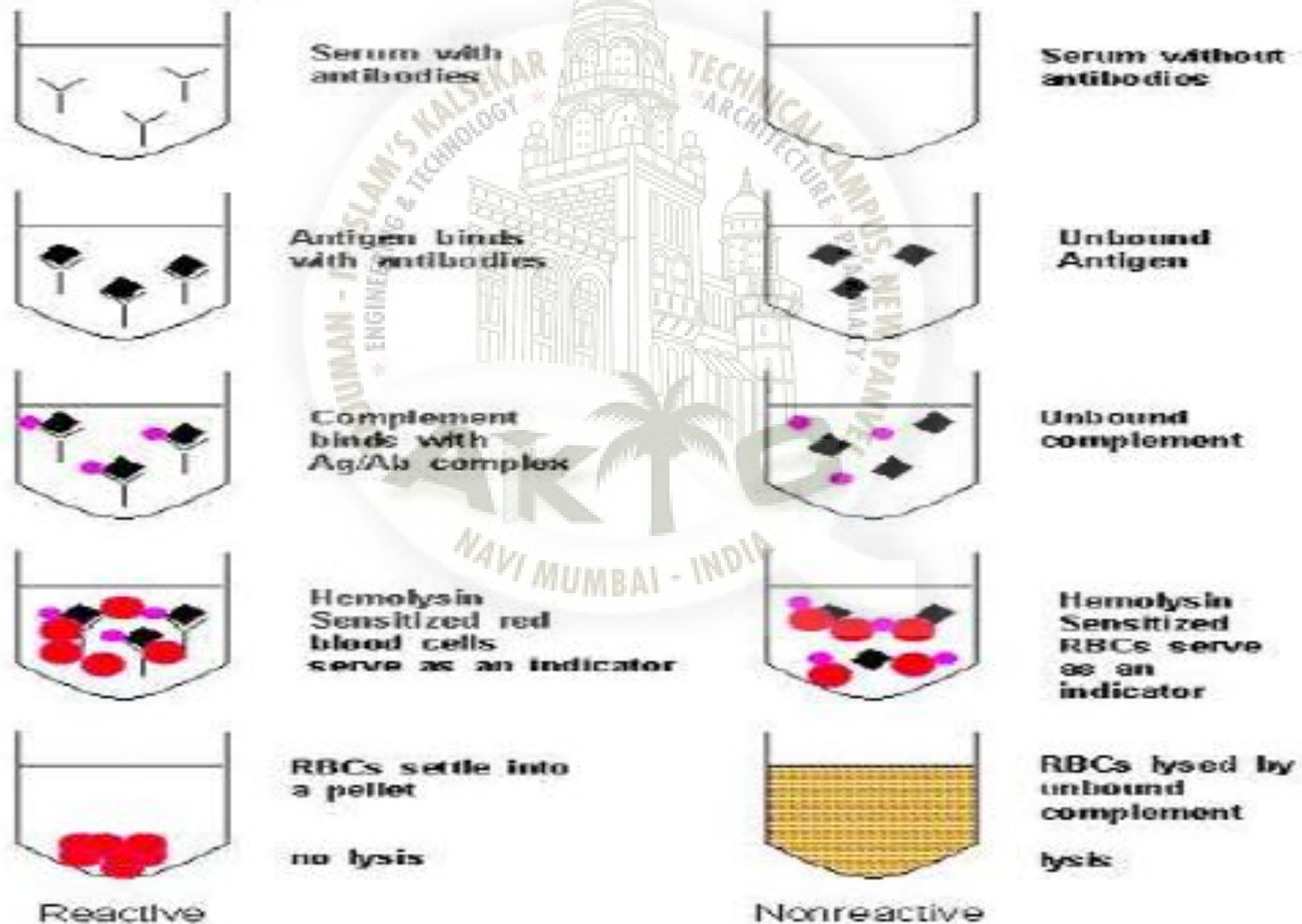
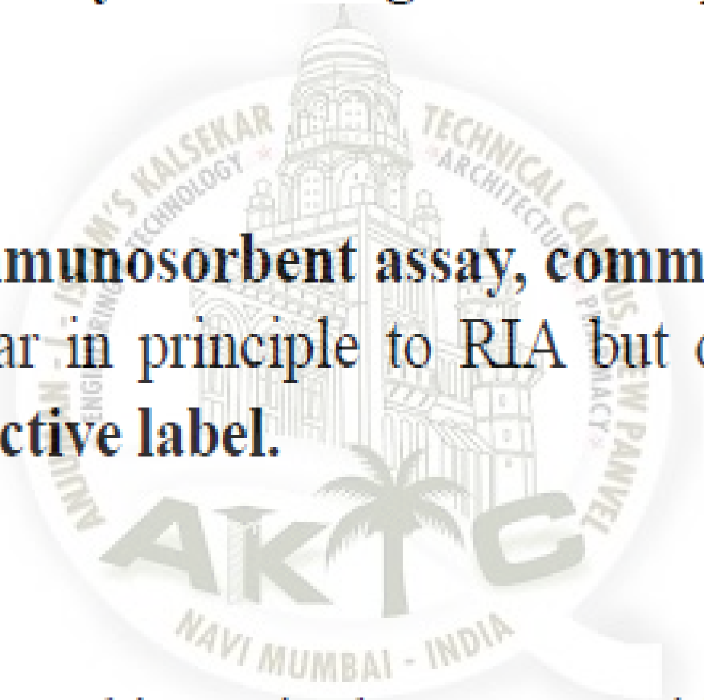
- If Ab specific for the Ag is present in the serum, Ag-Ab complex will be formed that will fix the complement.



- **ELISA** also known as an **enzyme linked immunosorbent assay** is a biochemical Techniques used mainly in immunology to detected the Presence Of an **antibody** or an **antigen** in a sample.



- **Enzyme-linked immunosorbent assay, commonly known as ELISA or EIA**), is similar in principle to RIA but depends on an enzyme rather than **a radioactive label.**

- **ELISA** can also be used in toxicology as a rapid presumptive screen for certain classes of drug.

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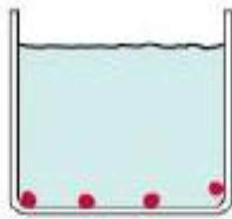
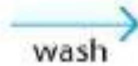
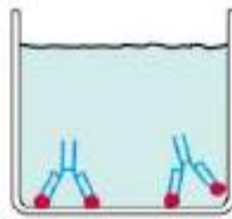
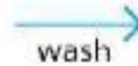
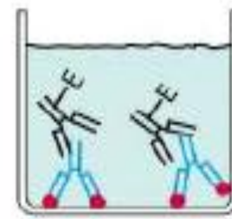
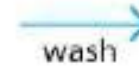
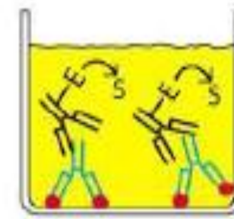
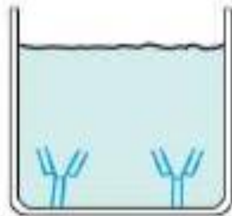
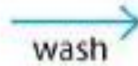
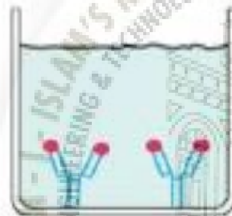
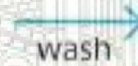
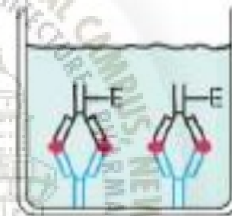
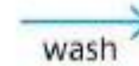
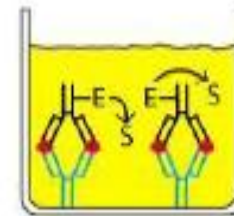
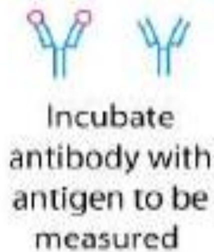
(a) Indirect ELISA

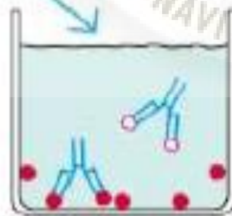
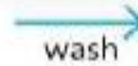








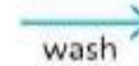
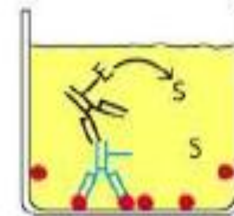
(b) Sandwich ELISA

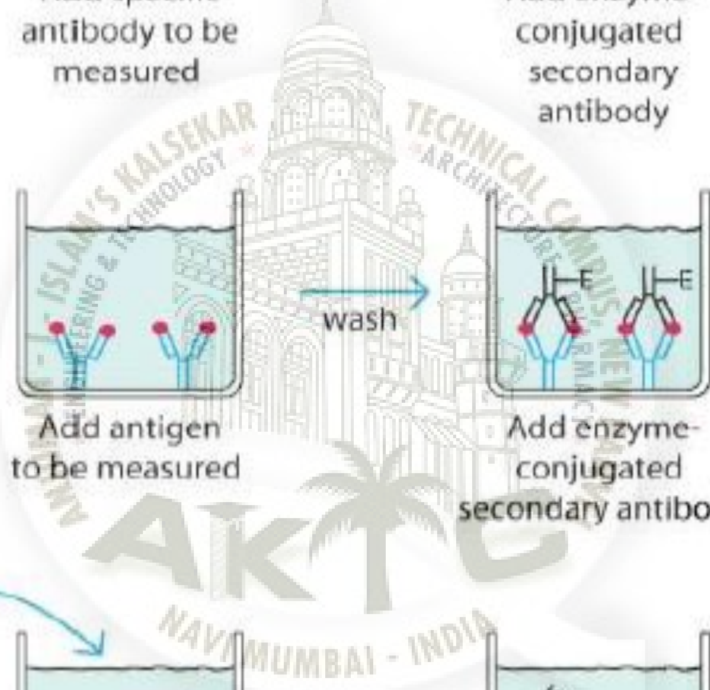








(c) Competitive ELISA




ANJUMAN-I-ISLAM'S KALSEKAR TECHNICAL CAMPUS NEW PANVEL
AIKTC
NAVI MUMBAI - INDIA

Enzyme Linked Immunosorbent Assay (ELISA)

- Term Was Coined By ***Engvall*** and ***Pearlmann*** in **1971**

- **Different Type**
  - Indirect ELISA
  - Sandwich ELISA
  - Competitive ELISA

- **ELISA** used in the detection and quantization of several antigen as well as antibodies.

- Indirect **ELISA** method to detect the presence of serum antibody against HIV. The causative agent of AIDS.

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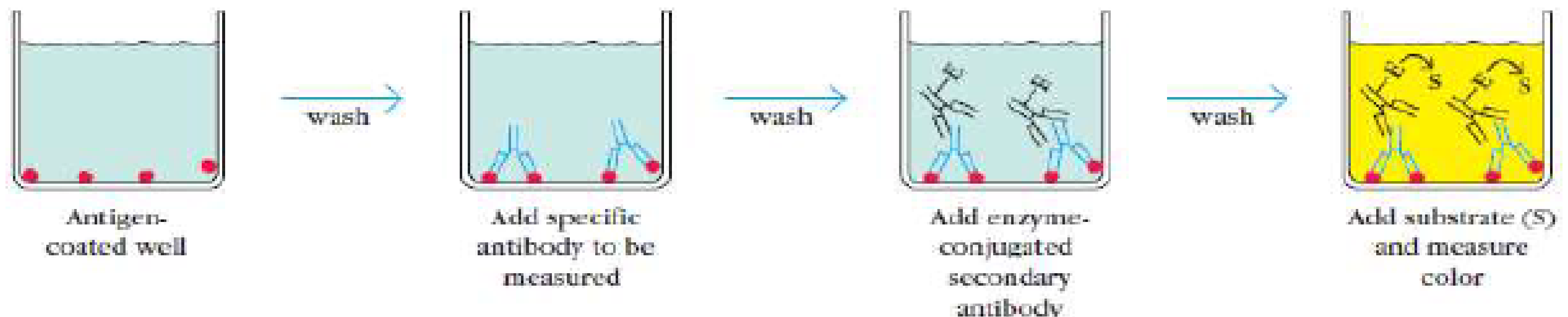
INDIRECT ELISA-

- Antibody can be detected or quantitatively determined with an indire ELISA .
- Serum or some other sample containing primary antibody (Ab1) added to an antigen-coated microtiter well .
- After any free Ab1 is washed away, the presence of antibody bound t the antigen is detected by adding an enzyme-conjugated secondar antibody (Ab2).
- Any free Ab2 then is washed away, and a substrate for the enzyme added.
- The amount of colour reaction product that forms is measured b specialized spectrophotometric plate readers.



1. Indirect ELISA

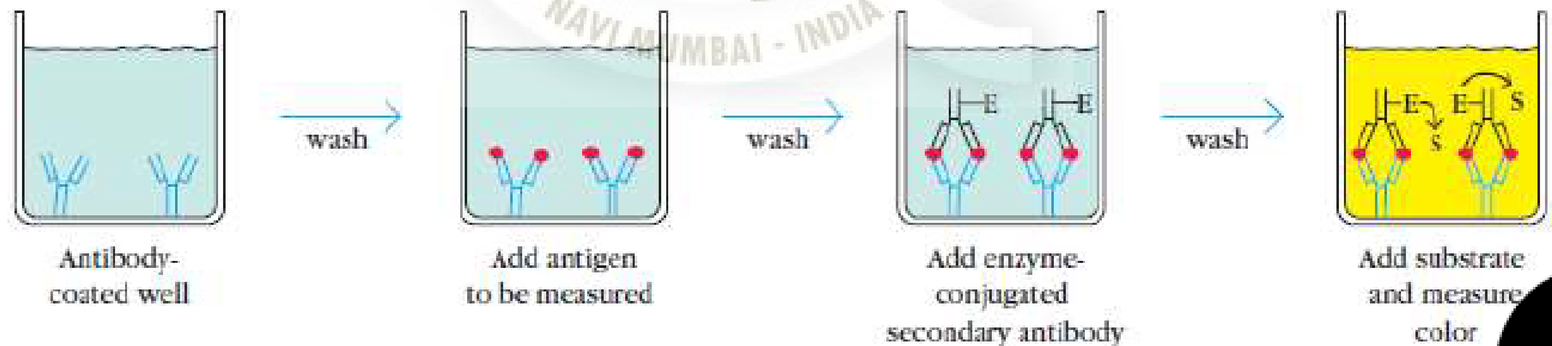
* The steps of the general, "indirect," ELISA for determining serum antibody concentrations are:

1. Apply a sample of known antigen of known concentration to a surface, often the well of a microtiter plate. The antigen is fixed to the surface to render it immobile. Simple adsorption of the protein to the plastic surface is usually sufficient. These samples of known antigen concentrations will constitute a standard curve used to calculate antigen concentrations of unknown samples. Note that the antigen itself may be an antibody.
2. The plate wells or other surface are then coated with serum samples of unknown antigen concentration, diluted into the same buffer used for the antigen standards. Since antigen immobilization in this step is due to non-specific adsorption, it is important for the total protein concentration to be similar to that of the antigen standards.
3. A concentrated solution of non-interacting protein, such as Bovine Serum Albumin (BSA) or casein, is added to all plate wells. This step is known as blocking, because the serum proteins block non-specific adsorption of other proteins to the plate.

4. The plate is washed, and a detection antibody specific to the antigen of interest is applied to all plate wells. This antibody will only bind to immobilized antigen on the well surface, not to other serum proteins or the blocking proteins.
5. The plate is washed to remove any unbound detection antibody. After this wash, only the antibody-antigen complexes remain attached to the well.
6. Secondary antibodies, which will bind to any remaining detection antibodies, are added to the wells. These secondary antibodies are conjugated to the substrate-specific enzyme. This step may be skipped if the detection antibody is conjugated to an enzyme.
7. Wash the plate, so that excess unbound enzyme-antibody conjugates are removed.
8. Apply a substrate which is converted by the enzyme to elicit a chromogenic or fluorogenic or electrochemical signal.
9. View/quantify the result using a spectrophotometer, spectrofluorometer, or other optical/electrochemical device.

SANDWICH ELISA-

- In this technique, the antibody (rather than the antigen) is immobilized on a microtiter well.
- A sample containing antigen is added and allowed to react with the immobilized antibody.
- After the well is washed, a second enzyme-linked antibody specific for a different epitope on the Antigen is added and allowed to react with the bound antigen.
- After any free second antibody is removed by washing, substrate is added, and the colour reaction product is measured.

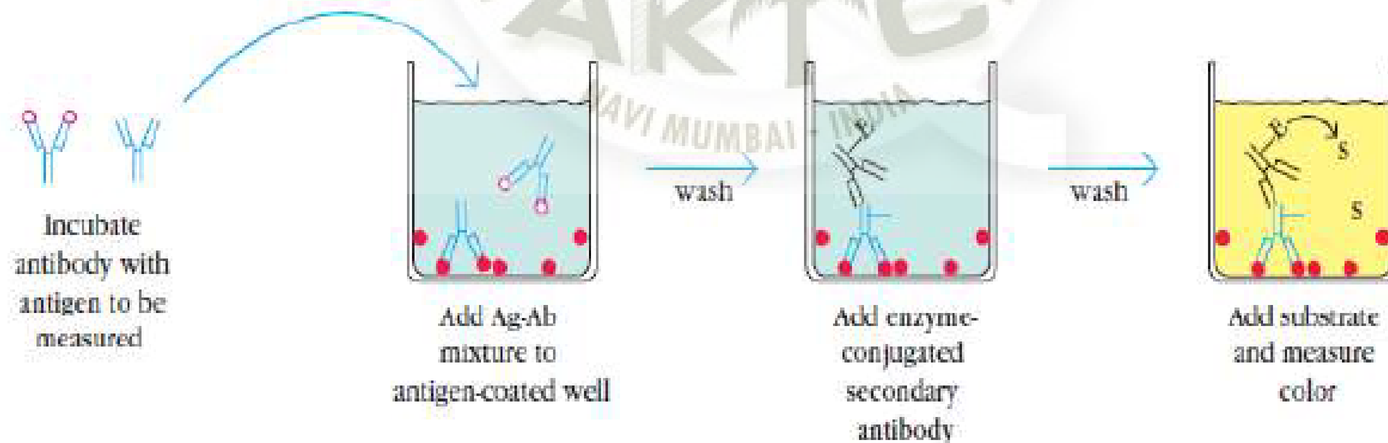


A less-common variant of this technique, called "sandwich" ELISA, is used to detect sample antigen. The steps are as follows:

1. Prepare a surface to which a known quantity of capture antibody is bound.
2. Block any non specific binding sites on the surface.
3. Apply the antigen-containing sample to the plate.
4. Wash the plate, so that unbound antigen is removed.
5. Apply primary antibodies that bind specifically to the antigen.
6. Apply enzyme-linked secondary antibodies which are specific to the primary antibodies.
7. Wash the plate, so that the unbound antibody-enzyme conjugates are removed.
8. Apply a chemical which is converted by the enzyme into a color or fluorescent or electrochemical signal.
9. Measure the absorbance or fluorescence or electrochemical signal (e.g., current) of the plate wells to determine the presence and quantity of antigen.

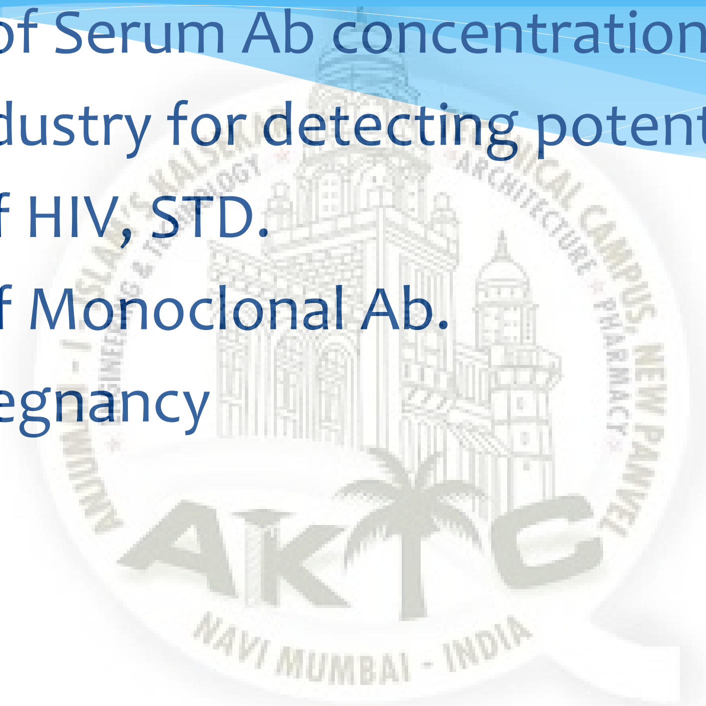
COMPETITIVE ELISA

- In this technique, antibody is first incubated in solution with a sample containing antigen.
- The antigen-antibody mixture is then added to an antigen coated microtiter well.
- The more antigen present in the sample, the less free antibody will be available to bind to the antigen-coated well.
- In the competitive assay, however, the higher the concentration of antigen.



Applications:

- Presence of antigen or antibody in sample
- Determination of Serum Ab concentrations in virus test.
- Used in food industry for detecting potential food allergen.
- For detection of HIV, STD.
- For screening of Monoclonal Ab.
- Detection of pregnancy



Immunoassay

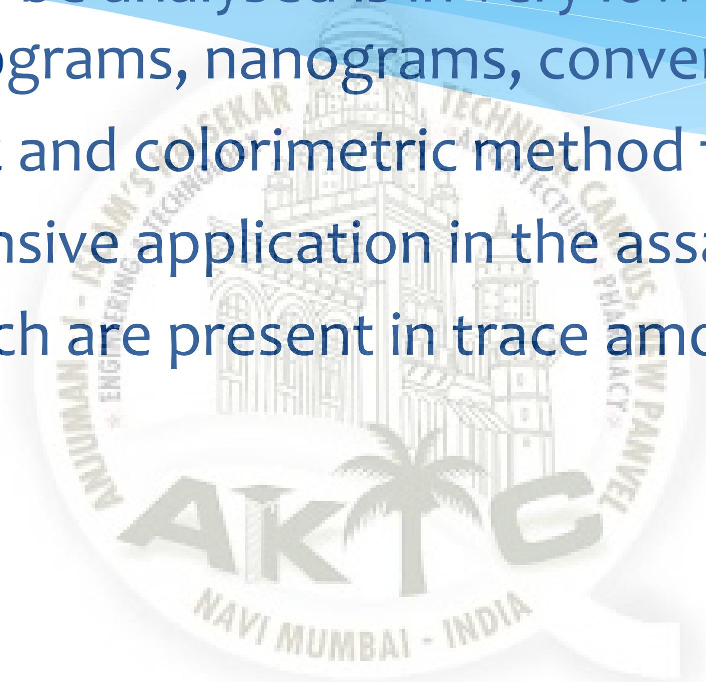
- An immunoassay is a test that uses antibody and antigen complexes.
- An antibody: antigen complex is also known as an immune complex.
- "Immuno" refers to an immune response that causes the body to generate antibodies "assay" refers to a test immunoassay is a test that utilizes immuno complexing when antibodies and antigens are brought together.

* **An antibody** is a protein that is produced by the body in response to an "invading" (foreign) substance.
* Antibodies are produced as part of the body's immune response to protect itself.
* **An antigen** is the substance that the body is trying to "fight off" by mounting an immune response.
  e.g. the drug is the antigen that binds to the antibody.
* **An immunogen** is a substance that elicits immune response.
  e.g. drug-protein conjugate.

- **An analyte** is anything measured by a laboratory test.
- In immunoassay testing, the analyte may be either an antibody, or an antigen.
- Immunoassays utilize one or more selected antibodies to detect analytes of interest.
- The analytes being measured may be:-

1. That are naturally present in the body (such as a thyroid hormone)
2. The body produces but are not typically present (such as a cancer antigen)
3. Do not naturally occur in the body (such as an abused drug)

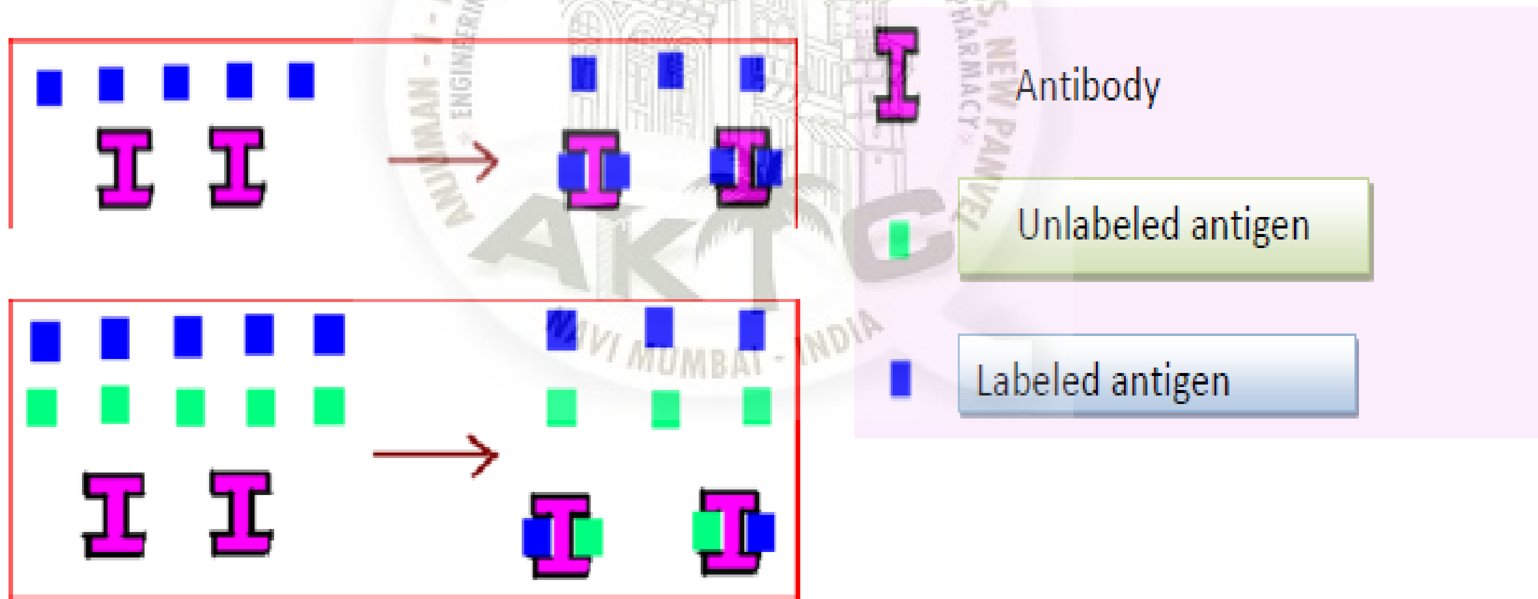
* Radio Immuno Assay (RIA) is an elegant tech. in analytical chemistry.
* If substance to be analysed is in very low quantities, in the orders of micrograms, nanograms, conventional methods like gravimetric and colorimetric method fail.
* RIA finds extensive application in the assay of many substances which are present in trace amount in blood.



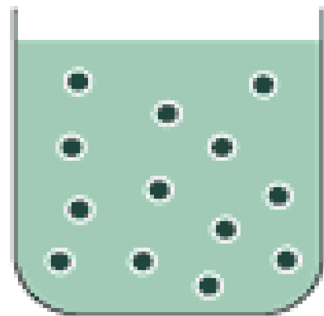
Principle Of RIA-

- The amount of **Ab per tube is kept constant, the amount of** antigen added (known or unknown) is the variable parameter.
- The added antigen will be distributed between a bound (B) and a free (F) fraction.
- This distribution is governed by the association constant (KA) of the Ab:
- Ab + Ag ⟶ AgAb
- K = [AbAg] /[Ab][Ag]
- Competitive binding of radiolabelled antigen and unlabelled antigen to a high affinity antibody.
- The labelled antigen is mixed with the antibody at a concentration that saturates the antigen –binding sites of the antibody.
- As the concentration of the unlabelled antigen increases more labelled antigen will be replaced from the binding site

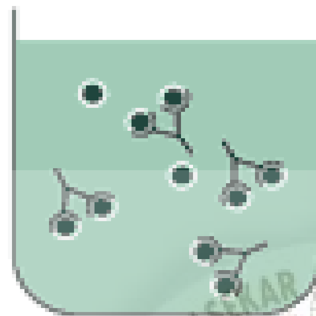
* The decrease in the amount of radiolabelled antigen bound specific antibody in the presence of the test samples is measured to determine the amount of antigen Present in the test sample.
* In std Condition, amount of labelled antigen bound to the antibody decreases as the amount of unlabelled antigen increases in sample



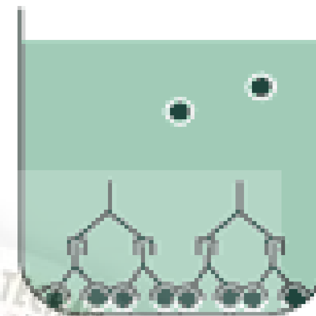
CONTROL



Known amount of radioactively labeled antigen



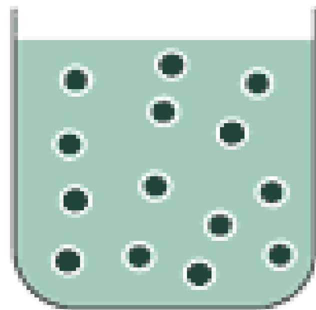
Fixed amount of specific antibody is added



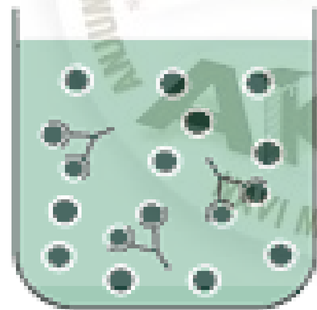
Addition of anti-immunoglobulin antibodies precipitates the antibody-labeled antigen complex

Radioactivity in precipitate is measured

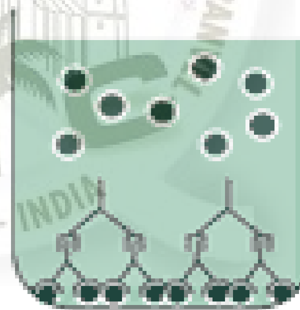
UNKNOWN SAMPLE



Known amount of radioactively labeled antigen mixed with unknown amount of unlabeled antigen



Unlabeled antigen competes with labeled antigen for the antibody

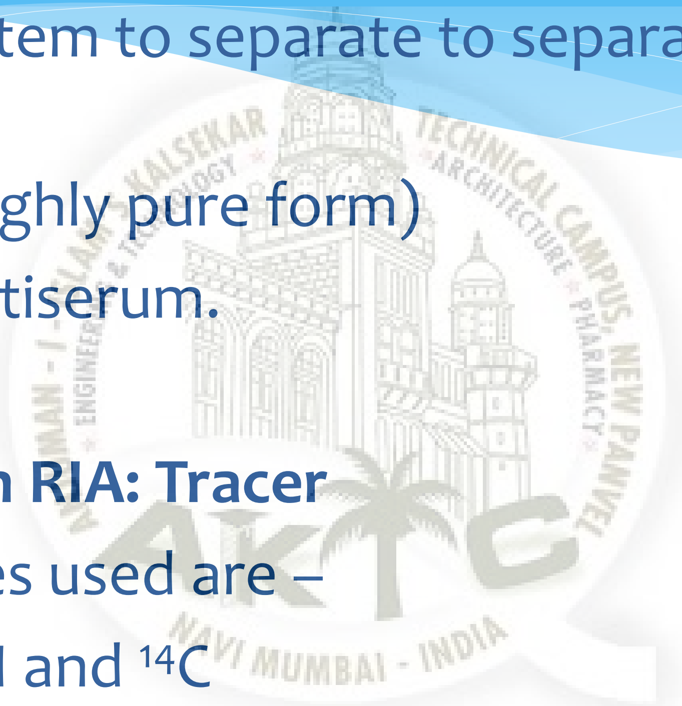


Addition of anti-immunoglobulin precipitates the antibody–antigen complex containing labeled and unlabeled antigen

The difference between the radioactivities in the control and the unknown sample is proportional to the amount of unlabeled antigen in the unknown sample

* **Reagents used in RIA:**

1. A tracer i.e. a labelled ligand.
2. A binder (Antibody) which is the specific antiserum.
3. A separation system to separate to separate the ‘bound’ and ‘free’ phases.
4. A standard (in highly pure form)
5. A free human antiserum.



* **Reagents used in RIA: Tracer**
* The radioisotopes used are –
* Beta emitters- ^{3}H and ^{14}C
* Gamma emitters- ^{125}I

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* **^{125}I is most favoured because-**
* It can be obtained with specific activity and almost 100%
* isotopic abundance, thus reducing counting time and being economic.
* Convenient half-life (60.2 days) hence shelf life for labelled antigen is long.
* Iodine is natural constituent of thyroxine and triiodothyronine.
* It can be easily introduced into peptide molecules, steroids.
* Gamma emission permits the use of simple inexpensive equipment for counting radioactivity.

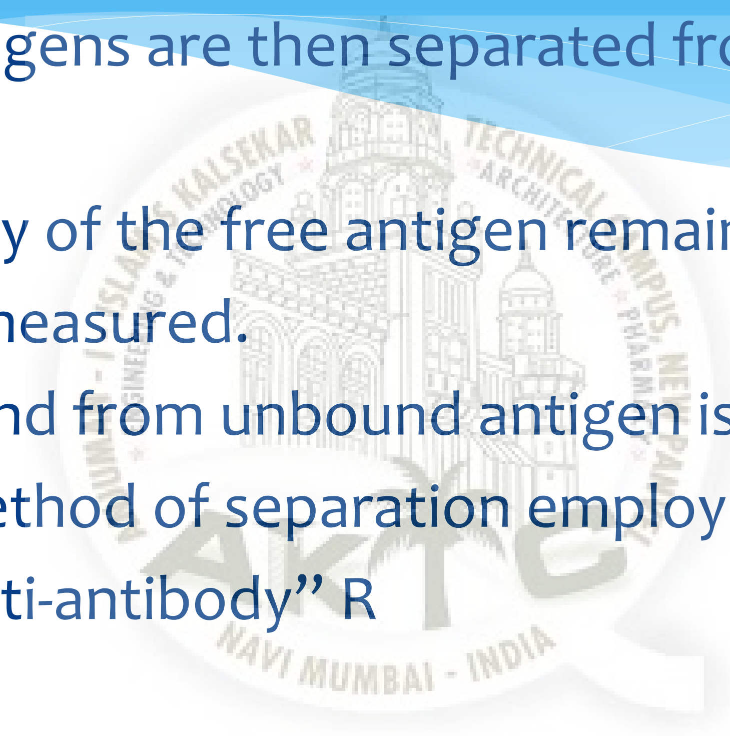
* **Disadvantages**-
* The damage to the ligand may occur during storage.
* Health hazards are more.

- **^{3}H is more efficient when relatively small sample is to be determined.**
- Advantages-
- Long shelf life (12.3 yrs).
- Higher affinity and no necessity for derivative preparation.
- Minimal health hazards.

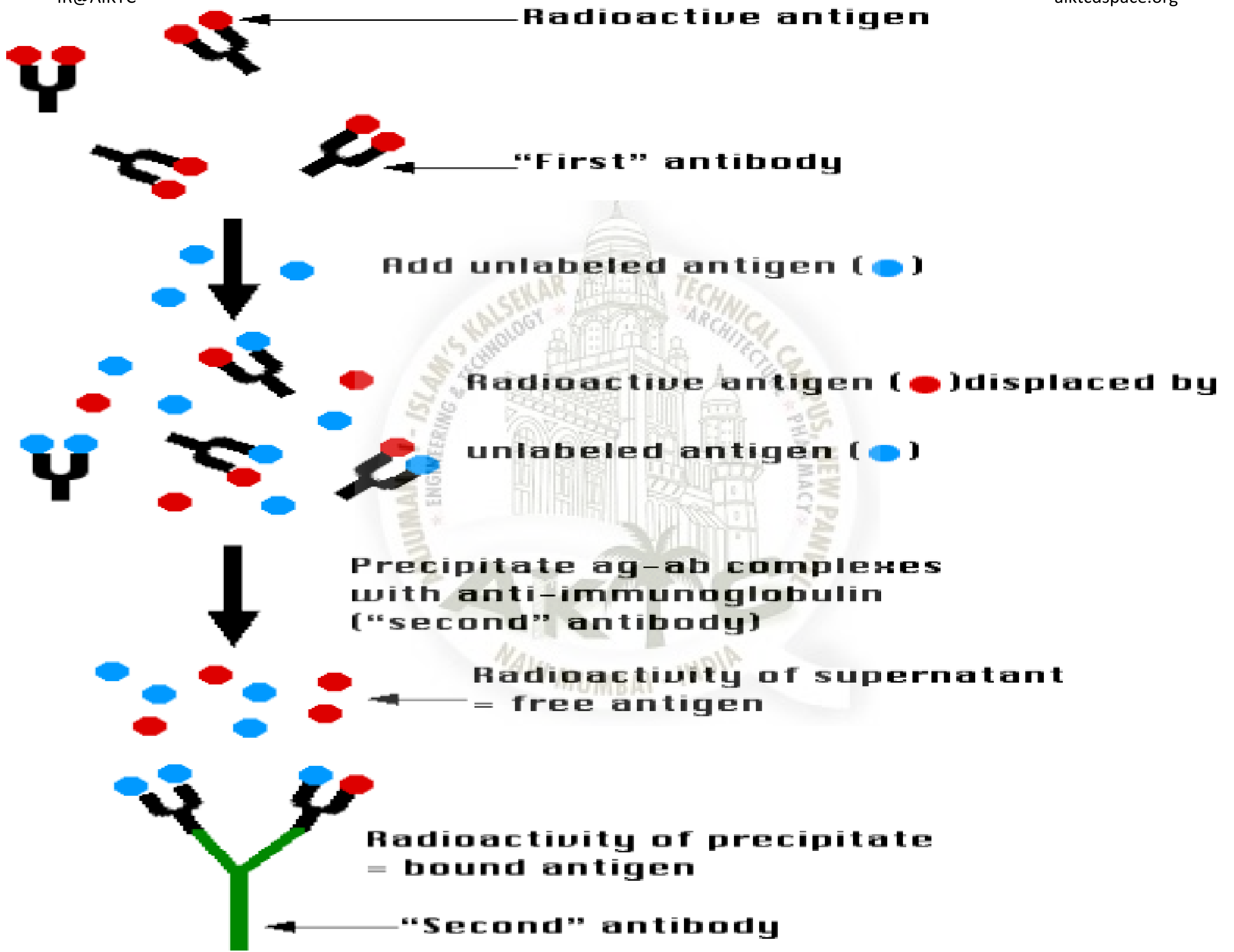
- Disadvantages-
- Requires scintillation counter which is costly
- Low specific activity (10000 ^{14}C = 100 ^{3}H = 1 ^{125}I).

General Procedure for Performing a RIA Analysis

* A known quantity of an antigen is made radioactive.
* This radiolabeled antigen is then mixed with a known amount of antibody for that antigen, and as a result, the two chemically bind to one another.
* a sample of serum from a patient containing an unknown quantity of that same antigen is added.
* This causes the unlabelled (or "cold") antigen from the serum to compete with the radiolabeled antigen for antibody binding sites.
* As the concentration of "cold" antigen is increase, more of it binds to the antibody.

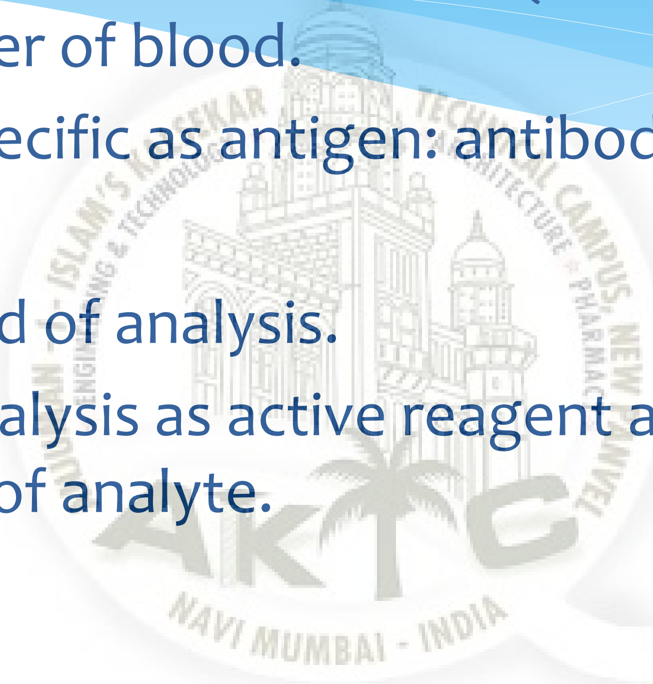


* And by displacing the radio labelled variant and reduces the ratio of antibody-bound radio labelled antigen to free
* radio labelled antigen.
* The bound antigens are then separated from the unbound ones.
* The radioactivity of the free antigen remaining in the supernatant is measured.
* separating bound from unbound antigen is crucial.
* Initially, the method of separation employed was the use of a second "anti-antibody" R

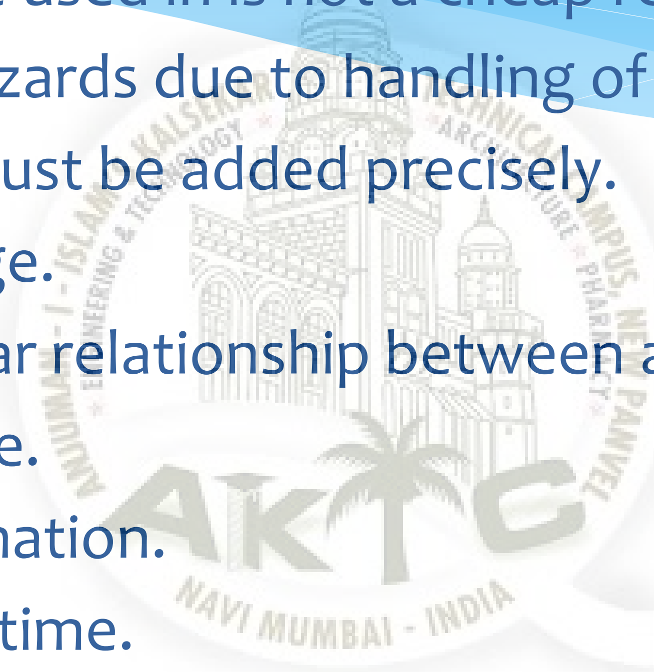

Radioactive antigen
"First" antibody
Add unlabeled antigen ( )
Radioactive antigen ( )displaced by
unlabeled antigen ( )
Precipitate ag-ab complexes
with anti-immunoglobulin
("second" antibody)
Radioactivity of supernatant
= free antigen
Radioactivity of precipitate
= bound antigen
"Second" antibody

Advantages of RIA

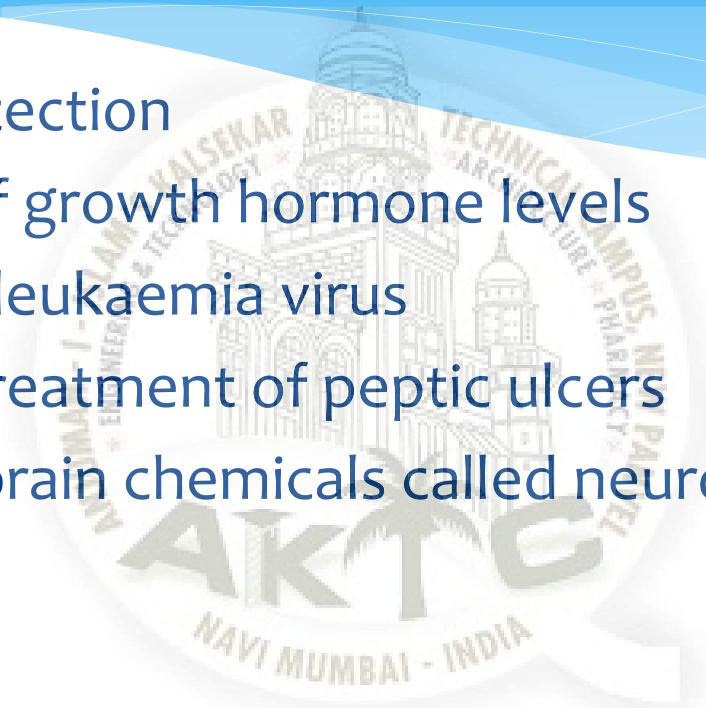
* Radio immuno assay is very sensitive technique used to measure concentrations of antigen without the need to use a bioassay. It can measure one trillionth (10-12) of a gram of material per milliliter of blood.
* It is structurally specific as antigen: antibody reaction are highly specific.
* It is indirect method of analysis.
* It is a saturation analysis as active reagent added in smaller quantity than that of analyte.



* **Disadvantages Of RIA**
* Prolonged reaction time (in days) as a consequence highly diluted reagent is used.
* Radioactive Iodine used in is not a cheap reagent.
* Possible health hazards due to handling of radioisotopes.
* All the reagents must be added precisely.
* Limited assay range.
* Lack of direct linear relationship between analyte concentration and signal response.
* Difficulty of automation.
* Lengthy counting time.

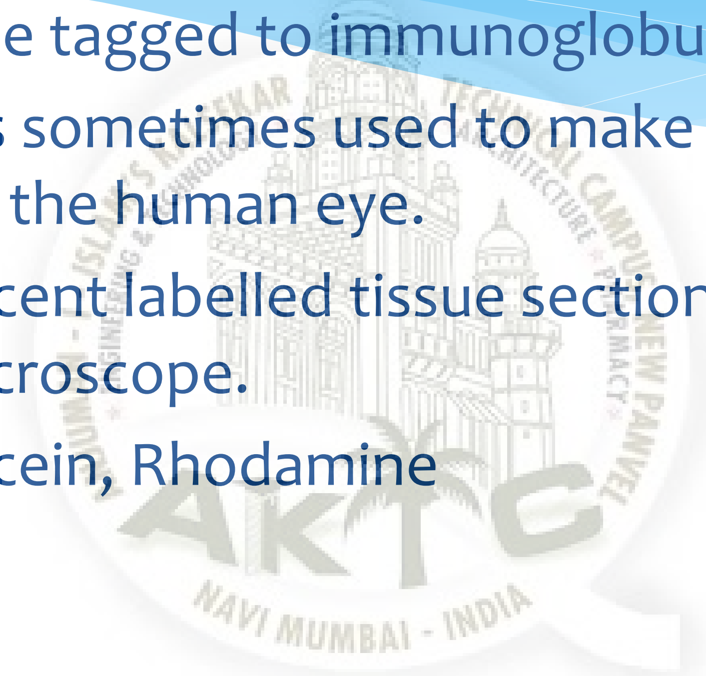


* **Applications for RIA-**
* Narcotics (drug) detection
* Blood bank screening for the hepatitis (a highly contagious condition) virus
* Early cancer detection
* Measurement of growth hormone levels
* Tracking of the leukaemia virus
* Diagnosis and treatment of peptic ulcers
* Research with brain chemicals called neurotransmitters

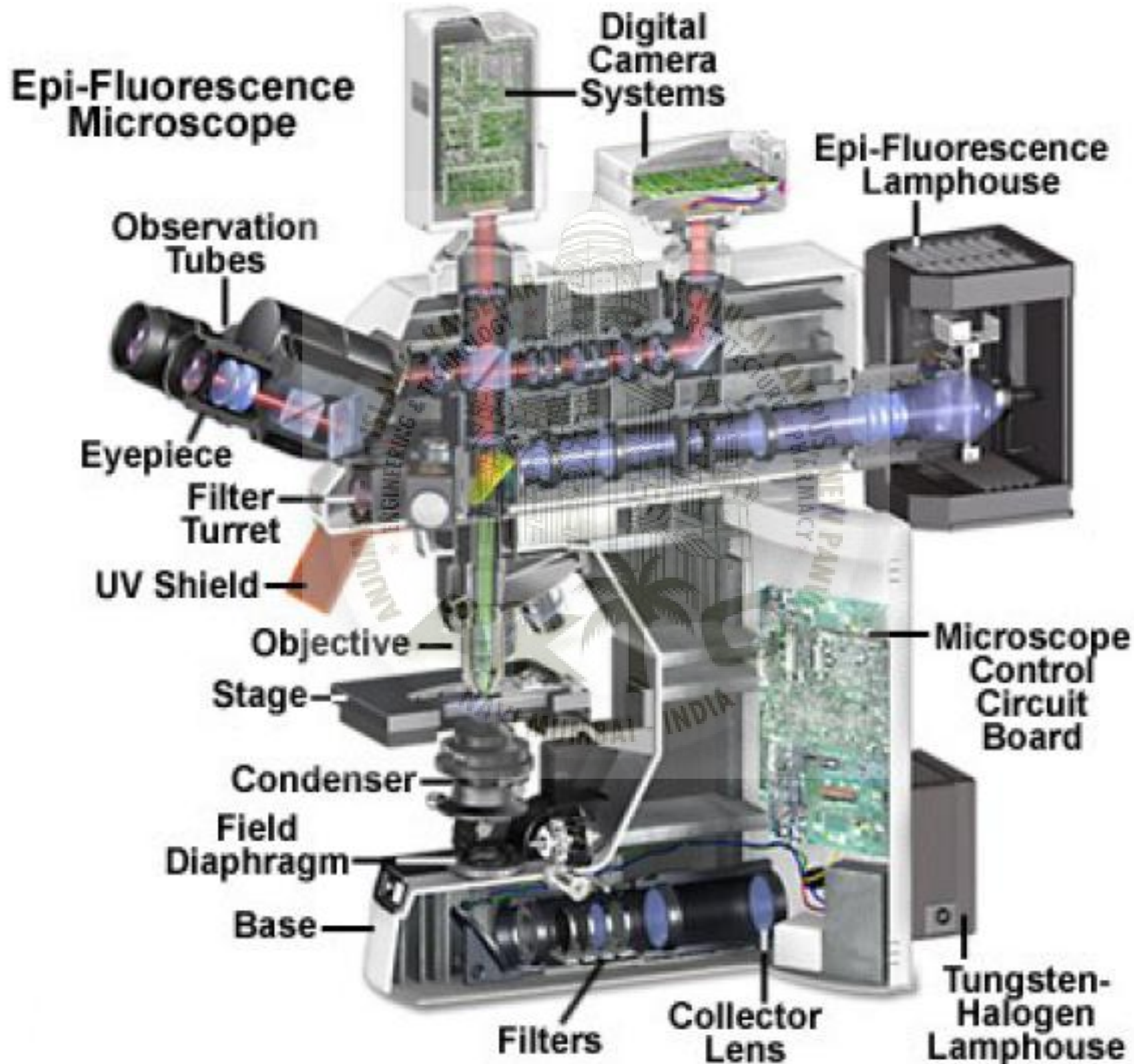


IMMUNOFLUORESCENCE

* **Immunofluorescence :** Immunofluorescence is a powerful technique that utilizes fluorescent-labelled antibodies to detect specific target antigens.
* **Fluorescein** is a dye which emits greenish fluorescence under UV light. It can be tagged to immunoglobulin molecules.
* This technique is sometimes used to make viral plaques more readily visible to the human eye.
* Immunofluorescent labelled tissue sections are studied using a fluorescence microscope.
* e.g. Dye Fluorescein, Rhodamine



FLUORESCENCE MICROSCOPY



* There are two ways of doing IF staining

1. Direct immunofluorescence
2. Indirect immunofluorescence

1. **Direct immunofluorescence**

* It's just a simple & a very common procedure in this regard.
* Ag is fixed on the slide
* Fluorescein labeled Ab's are layered over it.
* Slide is washed to remove unattached Ab's.
* Examined under UV light in an fluorescent microscope.
* The site where the Ab attaches to its specific Ag will show apple green fluorescence
* Use: Direct detection of Pathogens or their Ag's in tissues or in pathological samples.

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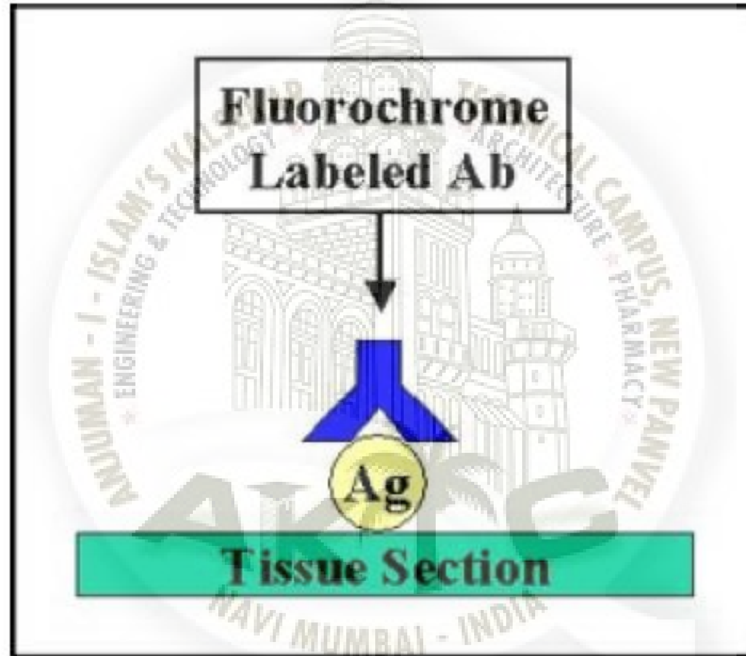
Direct Immunofluorescence-



2. Indirect immunofluorescence:

* Indirect test is a double-layer technique
* The unlabelled antibody is applied directly to the tissue substrate.
* Treated with a fluorochrome-conjugated anti-immunoglobulin serum.
* **Advantage over direct IF**
* Since several fluorescent anti-immunoglobulins can bind to each antibody present in the first layer, the fluorescence is brighter than the direct test.
* It is also more time-efficient since it is only one signal labelled reagent, the antiimmunoglobulin, is prepared during the lengthy conjugation process.

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Antibody

Antigen

Primary reaction

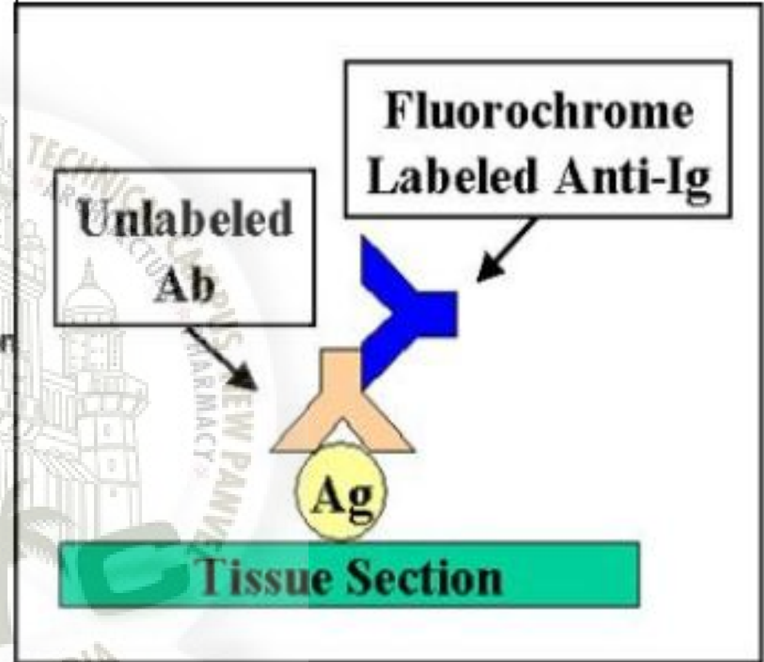
Wash

FITC-conjugated antidoby

Secondary reaction

Wash

Microscopic examination



* **Applications-**
* Immunoflourescence is a Microscopic-based technique,
* used clinically to diagnose certain cutaneous diseases
* (e.g; Lyme Disease) by the detection of AG:AB Complexes.
* Techniques including **DIF, IDIF & Salt-split Skin** are utilized depending on clinical scenario.
* **DIF** is performed on patient's skin using flourophorelabeled antibodies that directly bind to pathogenic autoantibody-antigen complexes in the skin.
* **IDIF** techniques are used in **Dermatology** primarily to detect circulating pathogenic autoantibodies.
* **Limitations:**
* **Fluorescence** signals depend on the quality & Concentration of antibodies, proper handling of specimen & detection with appropriate secondary antibodies.

References:

-B. D. Singh, A textbook of Biotechnology
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-K. Rajeshwar Reddy, Medical Microbiology

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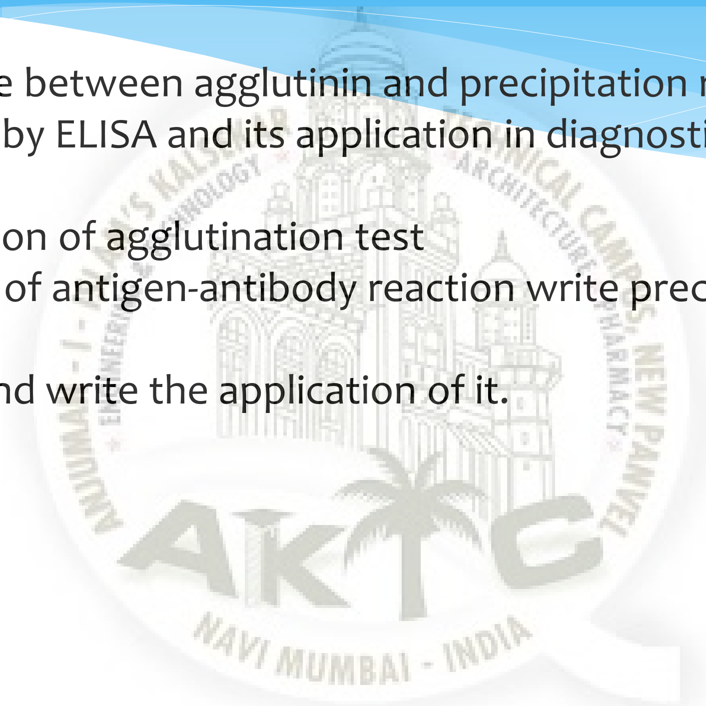
Review questions to ensure attainment of TLOs/ Cos



1. Write the difference between agglutinin and precipitation reaction .
2. What do you mean by ELISA and its application in diagnostic industry.
3. Define ELISA
4. Name two application of agglutination test
5. What are the types of antigen-antibody reaction write precipitation technique and its application.
6. Comment on RIA and write the application of it.