SEROLOGICAL TECHNIQUES – I

(Secondary Binding Tests)

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Antigen antibody reactions

Ag-Ab reaction occurs in three stages:

- Primary Stage
  - Formation of Ag-Ab complex **combined by weaker intermolecular forces**
- Secondary stage
  - leads precipitation
  - agglutination
  - lysis of cells etc.
- Tertiary stage (reaction):
  - Leads to tissue damage
  - Destruction of Ag or its Neutralization

Types of Antigen antibody reactions

- **Types of antigen- antibody reactions in vivo**:
  - Agglutination
  - Complement fixation
  - Neutralization
  - Antibody dependent cell mediated cytotoxicity (ADCC)
  - Opsonization
- **Types of antigen antibody reactions used in vitro:**
  - Precipitation
  - Agglutination
  - Neutralization
  - Complement fixation
  - Fluorescent-antibody technique (FAT)
  - ELISA- Enzyme-linked immunosorbent assay
  - RIA-Radioimmunoassay
  - Immunochromatography (ICT)

Why Use Serology?

- Antibodies are produced in large quantities and circulate in the blood
- Antigens and antibodies are easier to detect than finding the organism directly.
- Soluble antigens frequently enter the circulation of the host and are sometimes excreted in the urine. They are produced in discrete infected lesions.
- Culture is relatively insensitive due to the low concentration of the agents in tissues. Multiple cultures are usually required.

TYPES

- **Antigen test** – detection of antigen

- **Antibody test -** demonstration of immune response
  - paired sera samples
  - acute and convalescent phase
  - 21 days

CLASSIFICATION

- **Primary binding test -** directly measure the binding of antigen to antibody e.g. RIA, IF, ELISA.
- **Secondary binding test -** measure the results of antigen – antibody interaction *in vitro*, e.g. precipitation, complement fixation.
- ***In vivo* test -** measures the actual protective effect of antibodies in a host, e.g. passive cutaneous anaphylaxis.

TABLE 6-3 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 (RIA)	0.0006–0.006
Enzyme-linked immunosorbent assay (ELISA)	~0.0001–0.01
ELISA using chemiluminescence	~0.00001–0.01†
Immunofluorescence	1.0
Flow cytometry	0.006–0.06

*The sensitivity depends on the affinity of the antibody used for the assay as well as the epitope density and distribution on the antigen.
†Note that the sensitivity of chemiluminescence-based ELISA assays can be made to match that of RIA.

SOURCE: Updated and adapted from N. R. Rose et al., eds., 1997, *Manual of Clinical Laboratory Immunology,* 5th ed., American Society for Microbiology, Washington, DC.

Table 6-3
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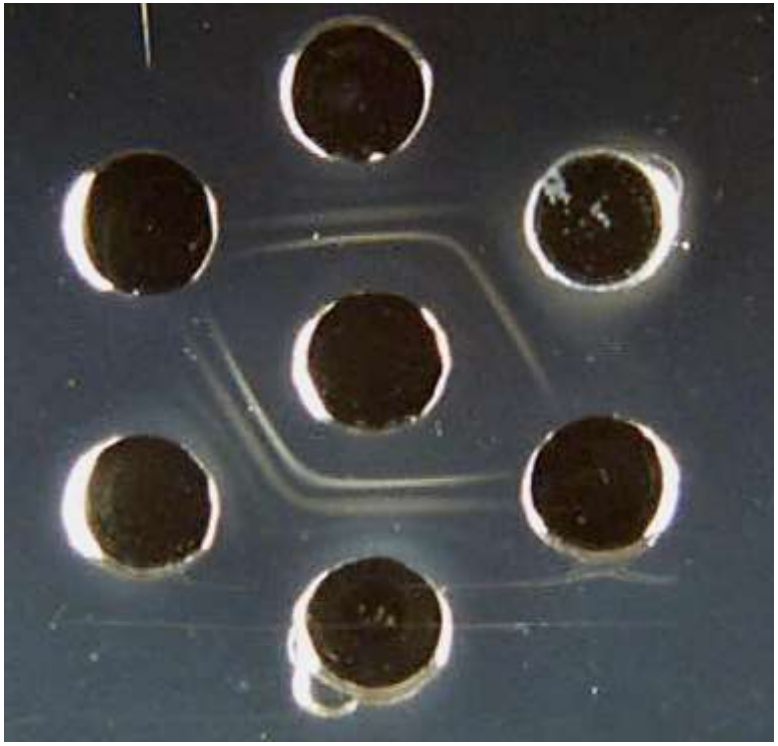
PRECIPITATION REACTIONS

PRECIPITATION REACTIONS

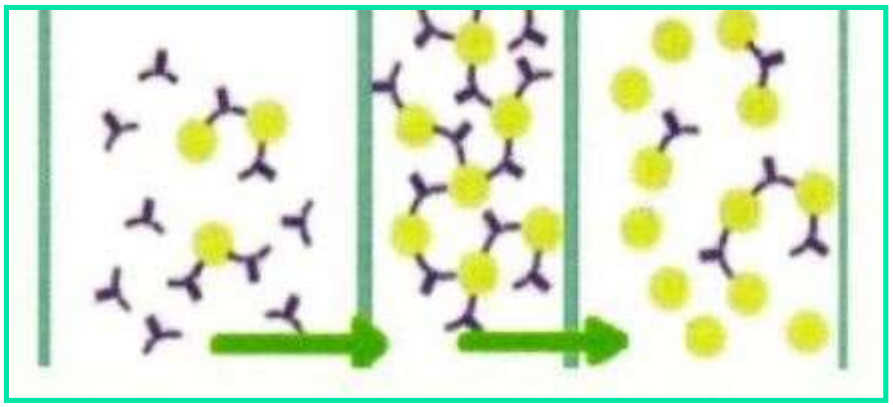
- Precipitation reactions are based on the interaction of antibodies and soluble antigens (acellular, e.g. viruses) in presence of electrolytes (NaCl) at a suitable temperature and pH
- The two soluble reactants come together to make one insoluble product, the precipitate.
- Precipitate settles to the bottom of the tube (in aqueous solution) or appears as an opaque white line (in gel).
- Precipitation is sensitive in detection of antigens
- As little as 1 g of protein can be detected by precipitation tests.
- However, precipitation is relatively less sensitive for the detection of antibodies.
- The amount of precipitate formed is greatly influenced by the:
  - Relative proportions of antigens and antibodies
  - The Ab avidity

PRECIPITATION REACTIONS

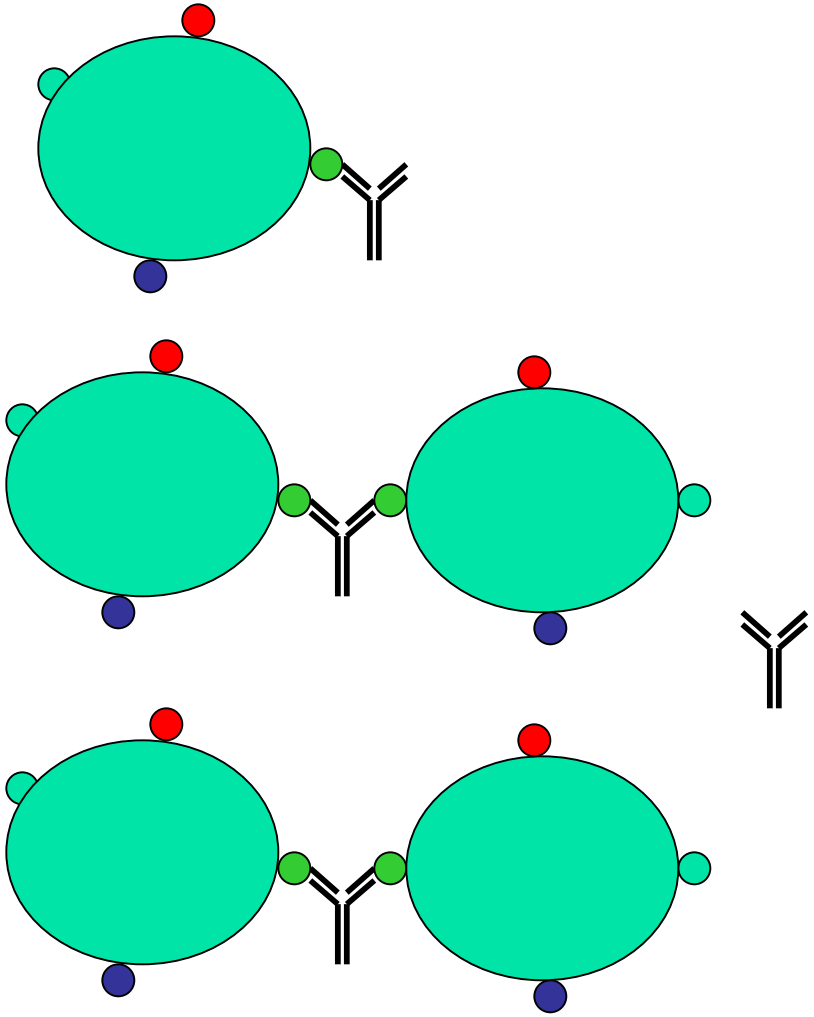


MARRACK'S HYPOTHESIS

- **Marrack (1934) proposed the lattice hypothesis**
- Based on the interaction of antibodies and antigens.
- Antibody and antigen must be multivalent**.**
- Two soluble reactants that come together in presence of an electrolyte to make one insoluble product , the precipitate .
- When antigen and antibody are in optimal proportions, the formation of lattices (cross-links) occurs.

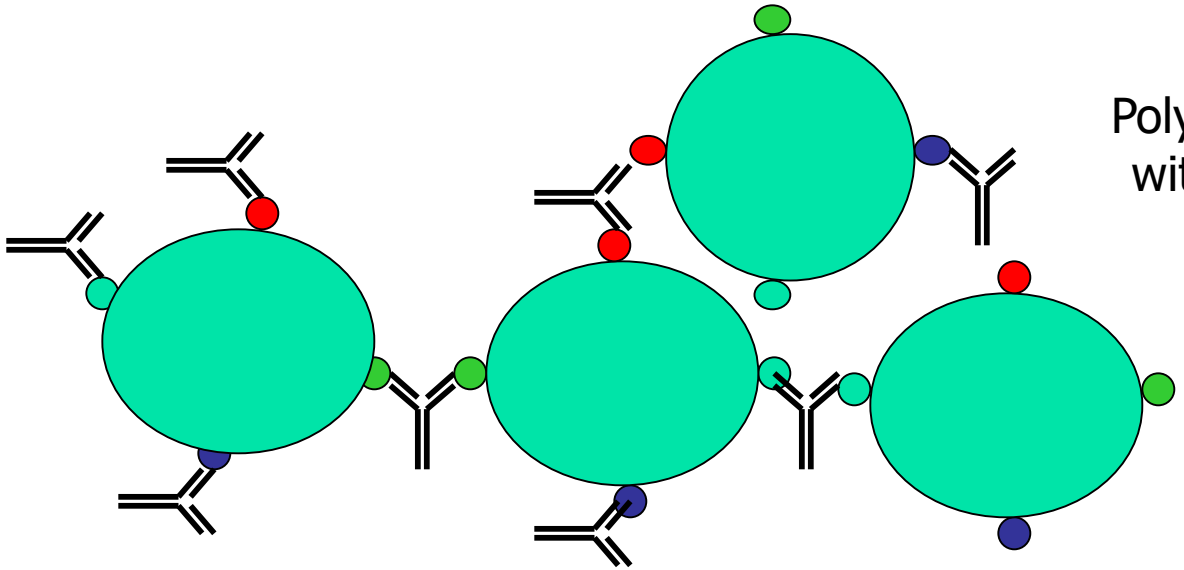


When do antibody-antigen lattices form?

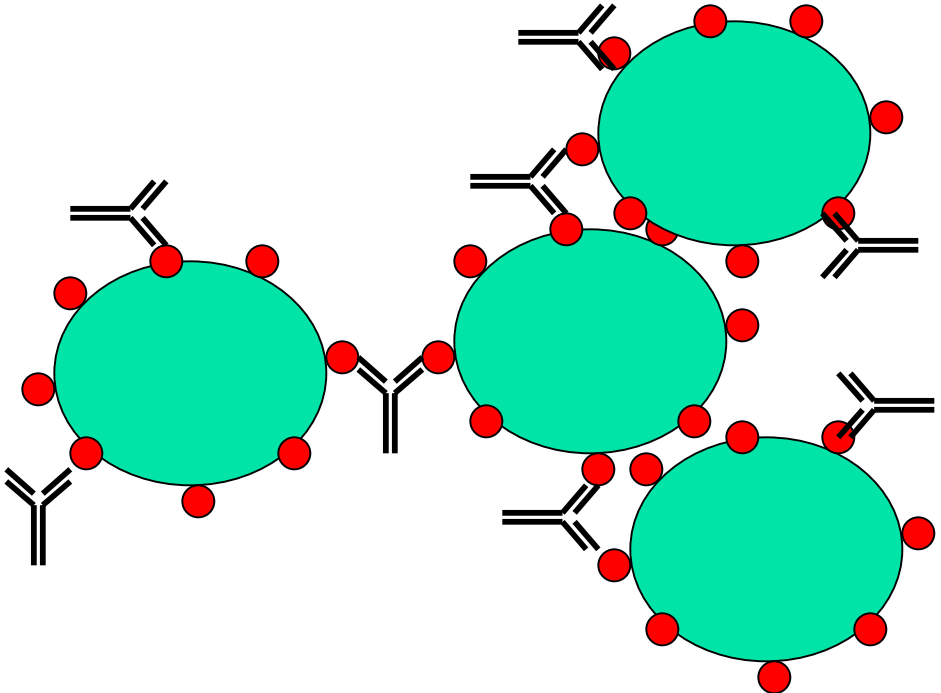


Not with monoclonal antibody and antigen with multiple distinct epitopes

When do antibody-antigen lattices form?



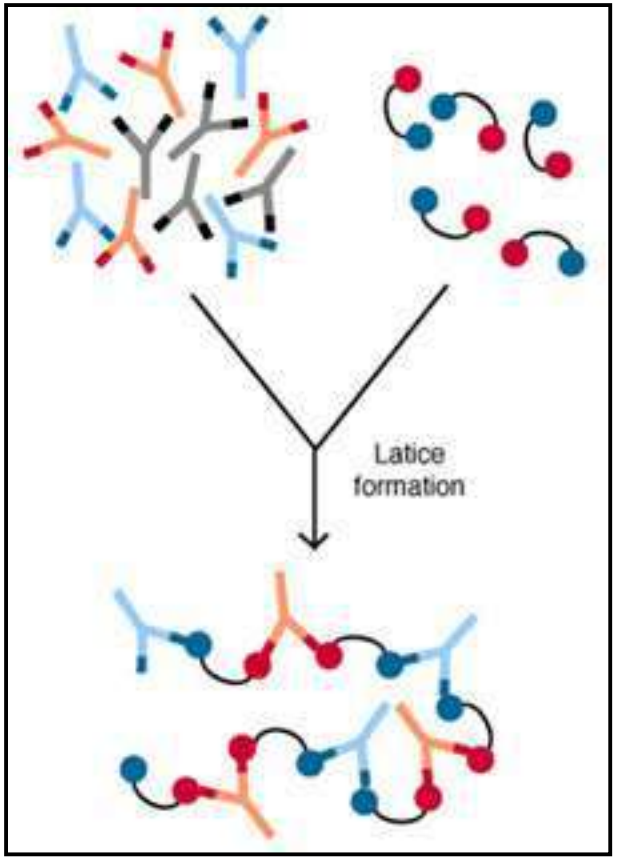
Polyclonal antibody and antigen with multiple distinct epitopes



Monoclonal antibody and antigen with repeating pattern of identical epitopes

When do antibody-antigen lattices form?

- bivalent antibody
- bivalent or polyivalent antigen
- extensive only when the ratio of multivalent antigen to bivalent antibody is optimal



The Zonal Phenomenon

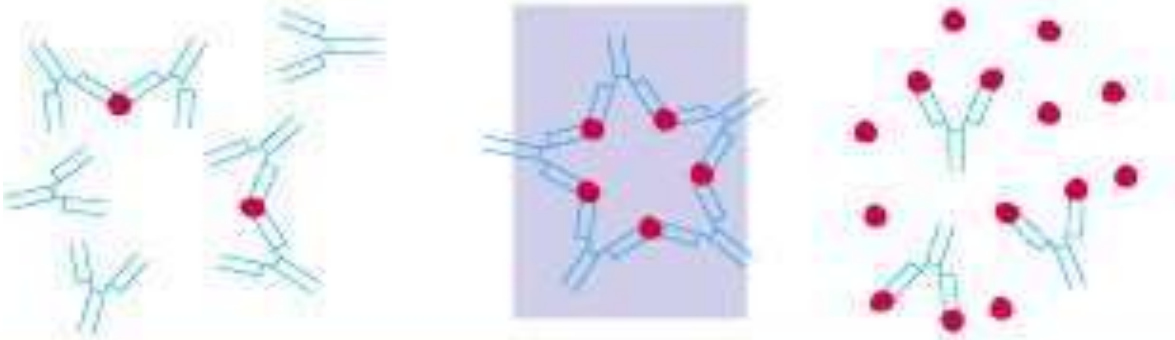
In quantitative precipitation or agglutination test normally the quantity of precipitate or agglutinate formed should be in a decreasing trend with increasing dilution of antibodies. However, at times no precipitation or agglutination is observed at higher concentration of antibodies followed by appearance at higher dilution, reaching to a peak and then again decreasing with increasing dilution. This is referred to as **"Zonal phenomenon"** in which agglutination or precipitation does not take place in zone having excess of either antigen or antibody; maximum precipitate or agglutinate when both the reactants are in optimal proportion. The initial zone with no immune complexes is called as **prezone or prozone**, the middle zone with highest concentration of immune complexes is called as **zone of equivalence** and the last zone with decreasing concentration of immune complexes is called as **post zone.**

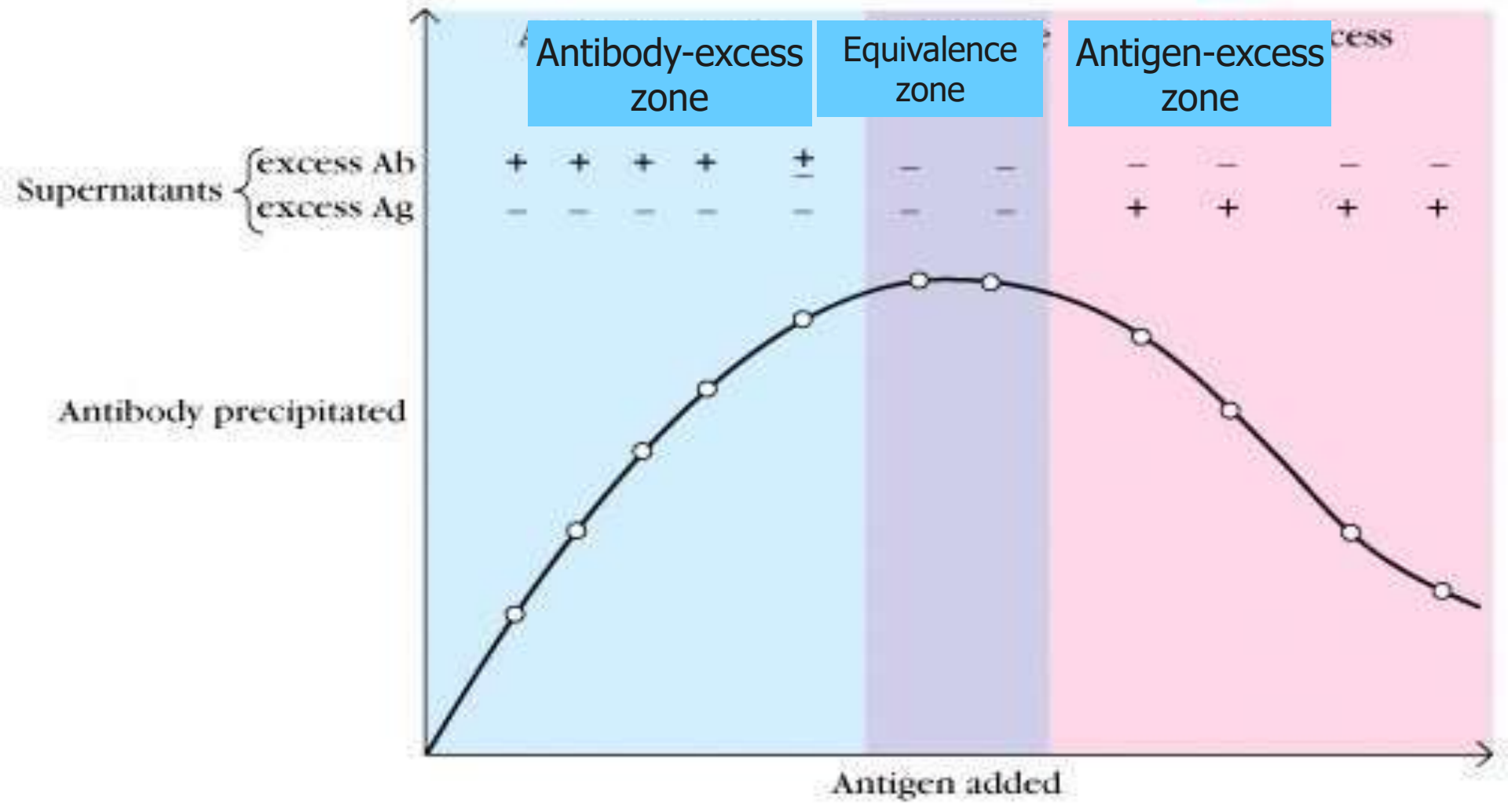
The Zonal Phenomenon can be explained by Marrack's Hypothesis according to which the formation of lattices is extensive only when the ratio of multivalent antigen to bivalent antibody is optimal

Precipitin Reaction

precipitates form most efficiently when antibody and antigen are at similar concentrations:

the Equivalence Zone





The Prozone Phenomenon

- **Prozone phenomenon** is a false negative response resulting from high antibody titer which interferes with formation of antigen-antibody lattice, necessary to visualize a positive precipitation or agglutination test.
- The Prozone phenomenon may be either due to presence of excess antibodies in serum (e.g., Brucellosis, secondary syphilis, Human immunodeficiency virus (HIV) co-infection, and pregnancy) or blocking antibodies or to non-specific inhibitors in serum.
- The monovalent antibodies (blocking antibodies) or absence of cross linking of antigen by bivalent antibodies both prevent formation of lattices.

An example of prozone phenomenon.

	Serum dilution										
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Titre
Sample #1	4+	3+	3+	2+	1+	1+	--	--	--	--	32
Sample #2	--	--	3+	4+	4+	3+	3+	2+	1+	--	256

* Sample 2 is an example of the prozone phenomenon

TYPES OF PRECIPITATION REACTIONS

Precipitation reaction can be broadly of three types:

- Precipitation in solution
- Precipitation in agar
- Precipitation in agar with an electric field

Precipitation in solution

Ring test and flocculation tests are examples of precipitation in solution:

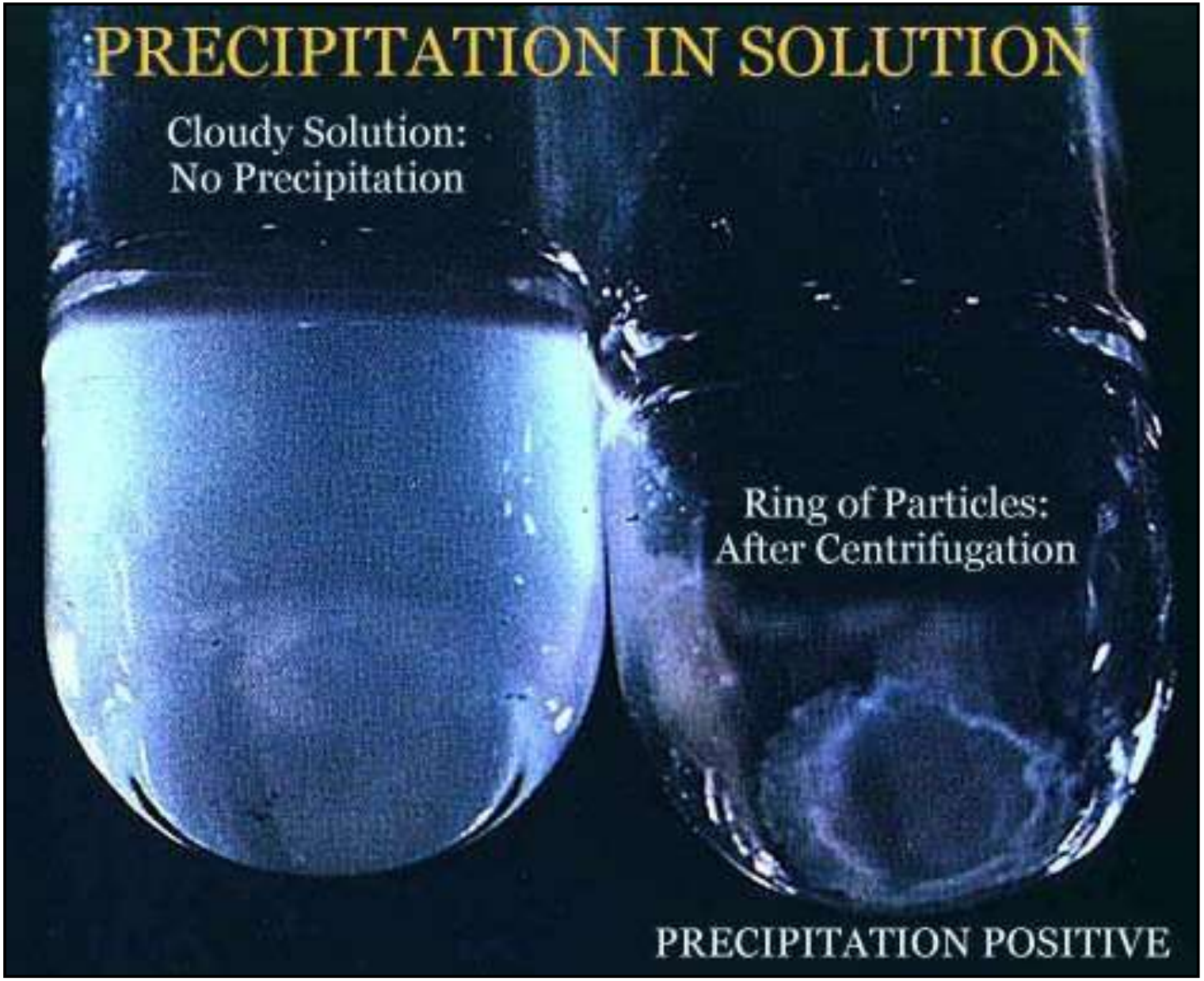
- **Ring test:**
  - In this test, the antigen solution is layered over antiserum in a test tube.
  - Precipitation between antigen and antibody in antiserum solution is marked by the appearance of a ring of precipitation at the junction of two liquid layers.
  - C-reactive protein (CRP) and Streptococcal grouping by the Lanoefield methods are the examples of ring test.

Precipitation in solution

Ring test and flocculation tests are examples of precipitation in solution:

- **Flocculation test:**
  - Flocculation test may be performed in a slide or tube.
  - In this test, a drop of antigen solution is added to a drop of patient's serum on a cavity slide and the result is recorded after shaking the slide.
  - In a positive test, the floccules appears which can be seen by naked eye or demonstrated well under the microscope.
  - Example: VDRL test for syphilis (slide)

    Khan test for syphilis (tube)


PRECIPITATION IN SOLUTION
Cloudy Solution:
No Precipitation
Ring of Particles:
After Centrifugation
PRECIPITATION POSITIVE

Precipitation in gel

- Precipitation carried out in gel is known as **'immunodiffusion'**
- In this test, reactants are added to the gel and antigen-antibody combination occurs by the means of diffusion
- Gels are prepared from 1-2% agar or agarose solution in a suitable buffer, such as barbitone buffer
- Either (single) or both (double) reactants diffuse due to electrical charge on their surface.
- The rate of diffusion is affected by the size of the particle, temperature, gel viscosity, amount of hydration and interaction between the matrix and reactants.
- Speed of movement can be increased by applying electric current
- Reactants diffuse out and form an opaque white line of precipitate where they meet in optimal proportions

Types of immunodiffusion reaction

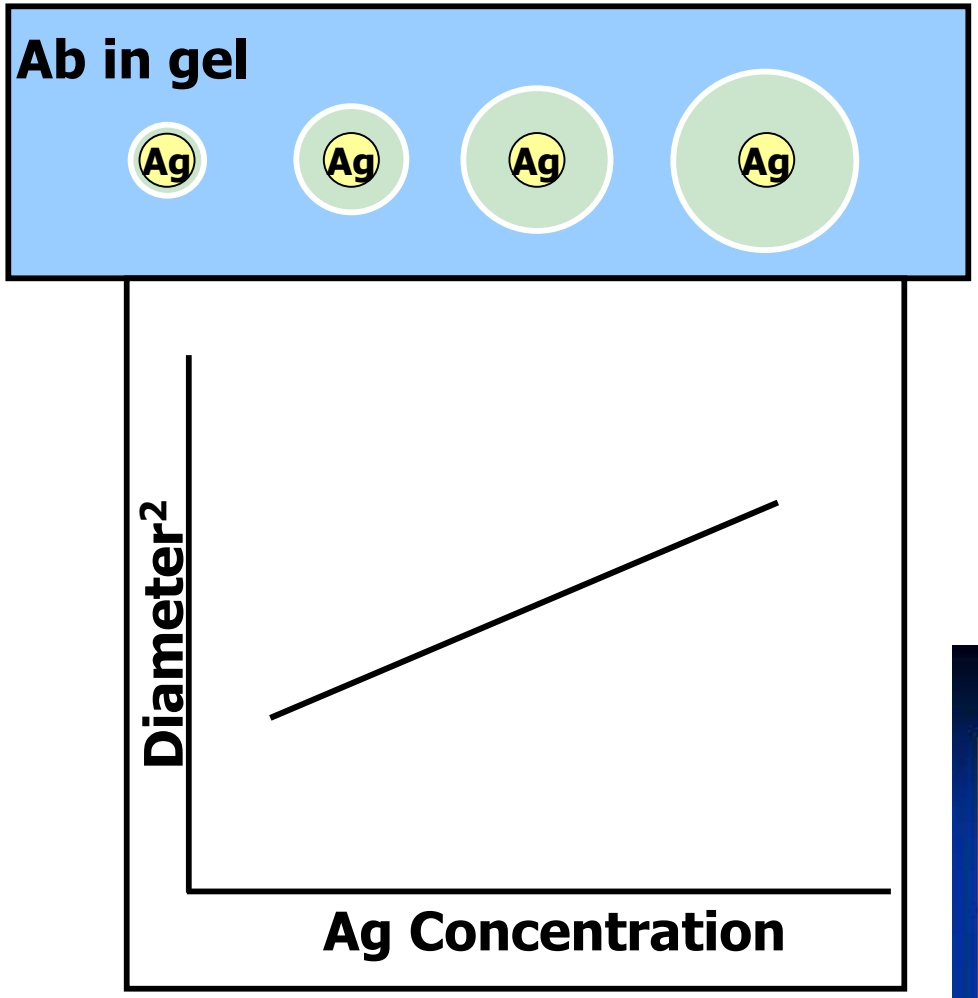
Immunodiffusion reaction are classified based on the:

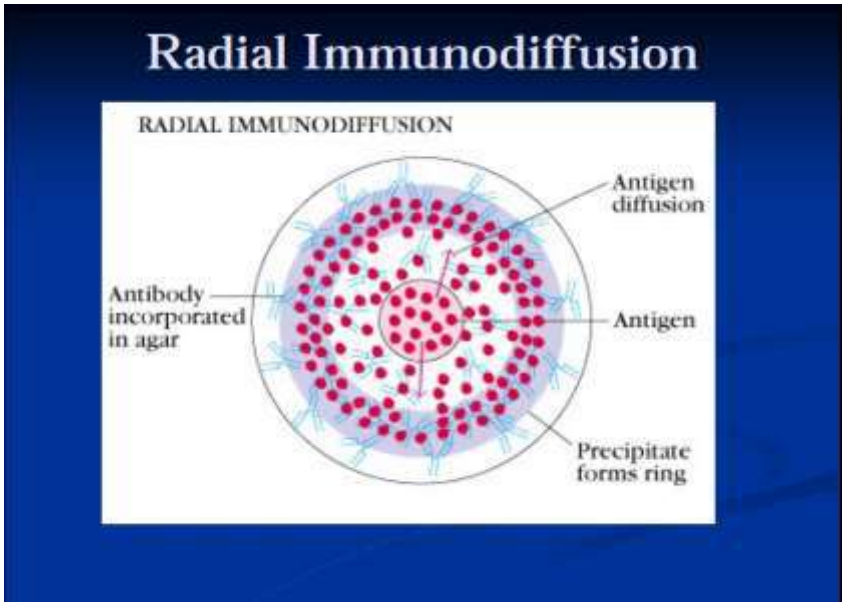
- Number of reactant diffusing and
- Direction of diffusion, as follows

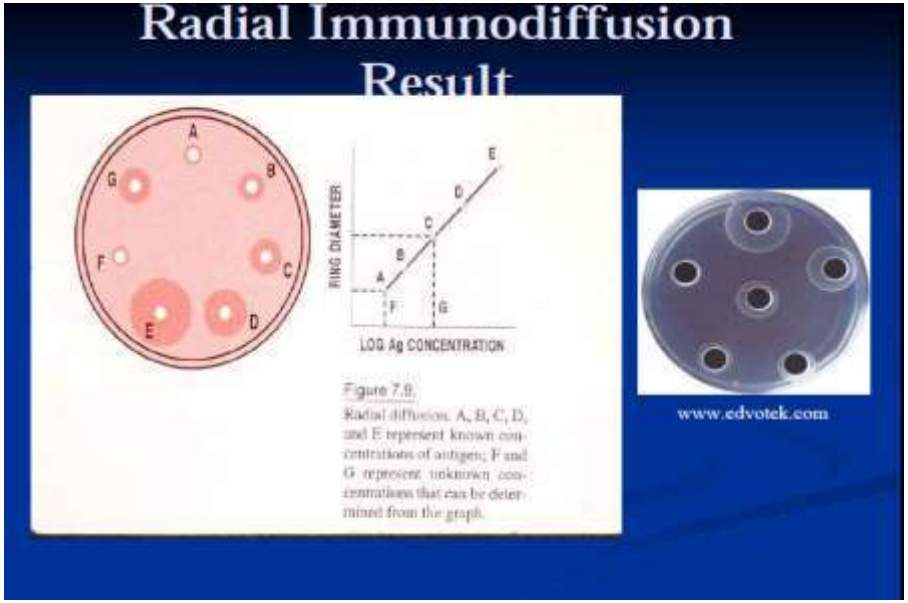
- **Single diffusion in one dimension:**
  - It is the single diffusion of antigen in a agar in one dimension.
- **Single diffusion in two dimension:**
  - It is also called radial immunodiffusion
- **Double diffusion in one dimension**
- **Double diffusion in two dimension**

Radial Immunodiffusion (Mancini)

- Quantitative measurement of Ag
- Ab in gel, Ag in well
- Antibody present in the gel reacts with the antigen, which diffuses out of the well, to form a ring of precipitation around the wells.
- Interpretation
  - Diameter of ring is proportional to the concentration of Ag
- Generate standard curve using known concentration of Ag
- Add unknown sample. Read from standard curve


Ab in gel
Ag
Ag
Ag
Ag
Diameter²
Ag Concentration


Radial Immunodiffusion
RADIAL IMMUNODIFFUSION
Antigen diffusion
Antibody incorporated in agar
Antigen
Precipitate forms ring


Radial Immunodiffusion Result
A
B
C
D
E
F
G
RING DIAMETER
LOG Ag CONCENTRATION
Figure 7.9
www.edvotek.com

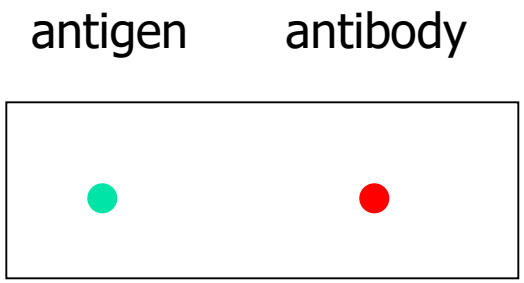
Double diffusion in one dimension

- In this method, the antibody is incorporated in agar gel in a test tube above which a layer of plain agar is placed. The antigen is then layered on the top of this plain.
- During the course of time, the antigen and antibody move toward each other through the intervening layer of plain agar.
- In this zone of plain agar, both antigen and antibody react with each other to form a band of precipitation at their optimum concentration.
- Example: Ascoli's precipitation test

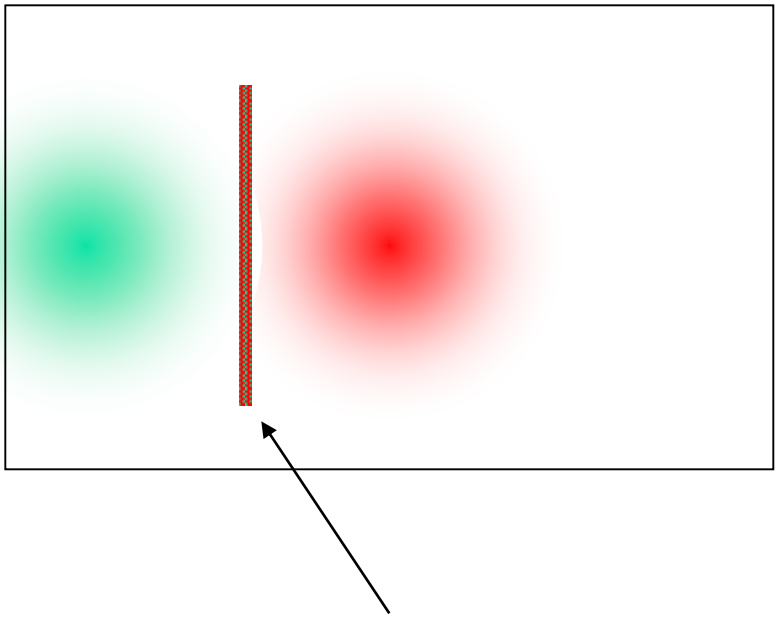
Double diffusion in two dimensions
(Ouchterlony technique)

- In this method, both the antigen and antibody diffuse independently through agar gel in two dimensions, horizontally and vertically.
- The test is performed by cutting wells in the agar gel poured on a glass slide or in a petri dish.
- The antiserum consisting of antibody is placed in the central well and different antigens are added to the wells surrounding the center well or vice versa
- After an incubation period of 12-48 hours in a moist chamber, the lines of precipitin are formed at the zone of optimal concnetration of antigen and antibody.

Double-diffusion assay (Ouchterlony method)



Antigen & antibody embedded in agar gel atop glass slide



DOUBLE IMMUNODIFFUSION

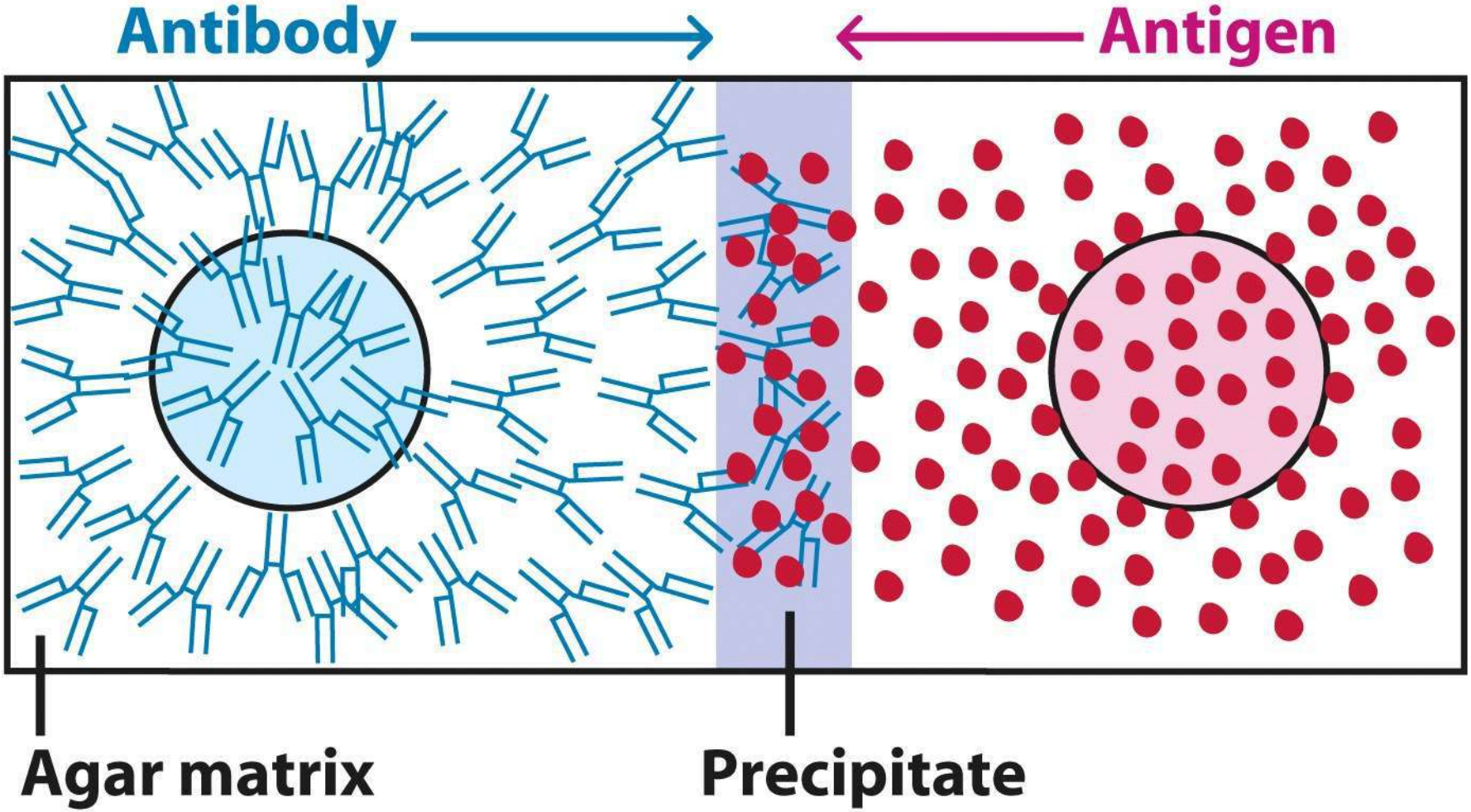


Figure 6-6 part 2
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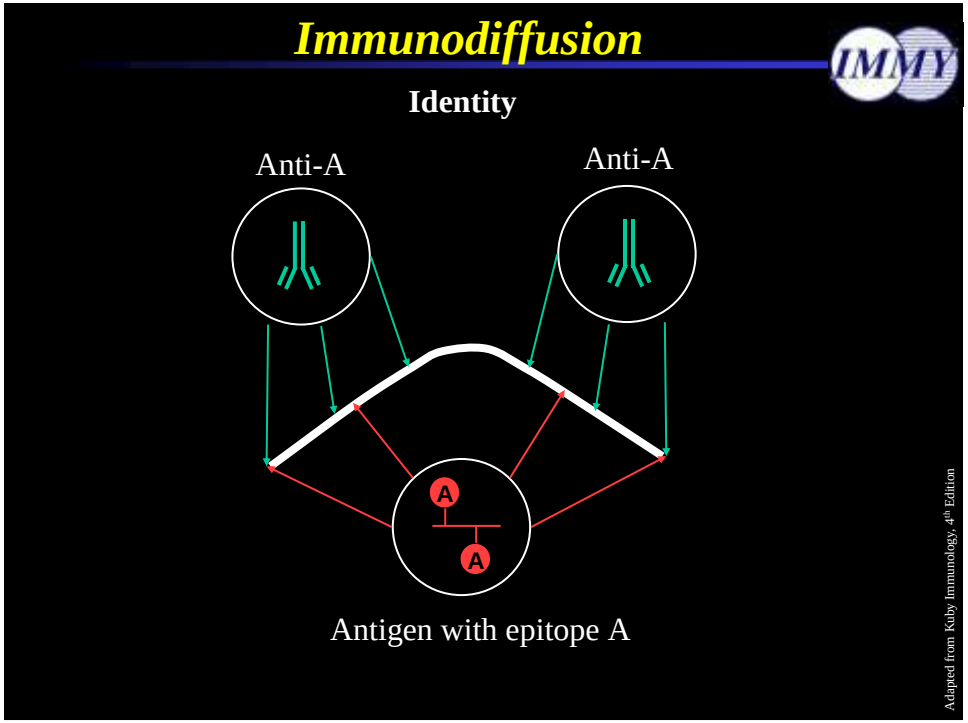
Loading gel with antibodies and antigen

TYPES OF REACTION - I

Reaction of identity

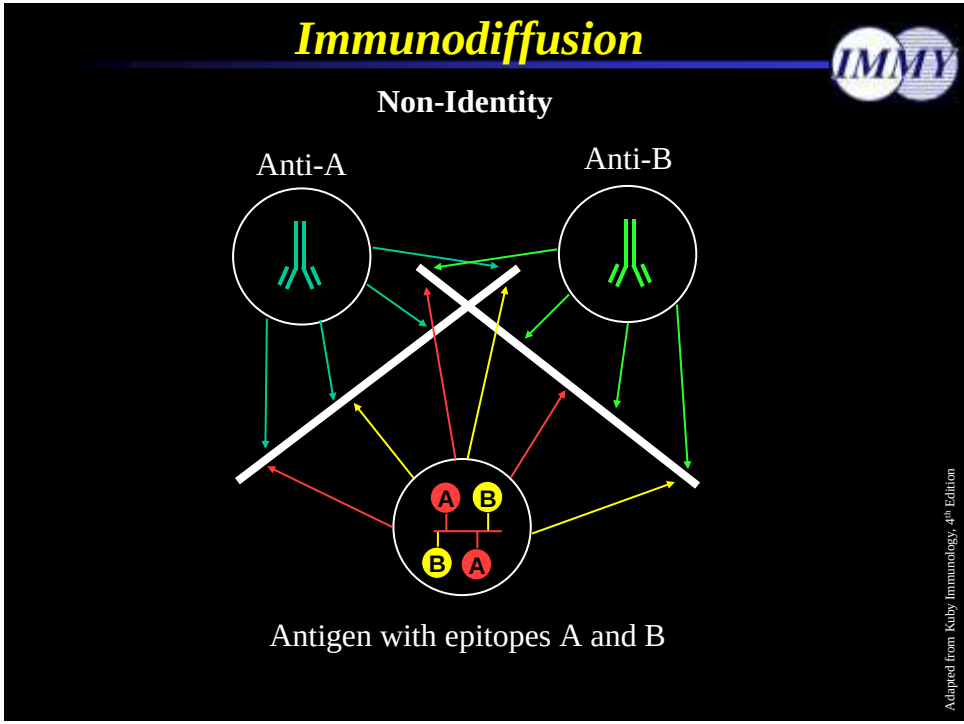
- characterized by fusion of two precipitin lines
- occurs when two antigens share identical epitopes



TYPES OF REACTION - II

Reaction of non-identity

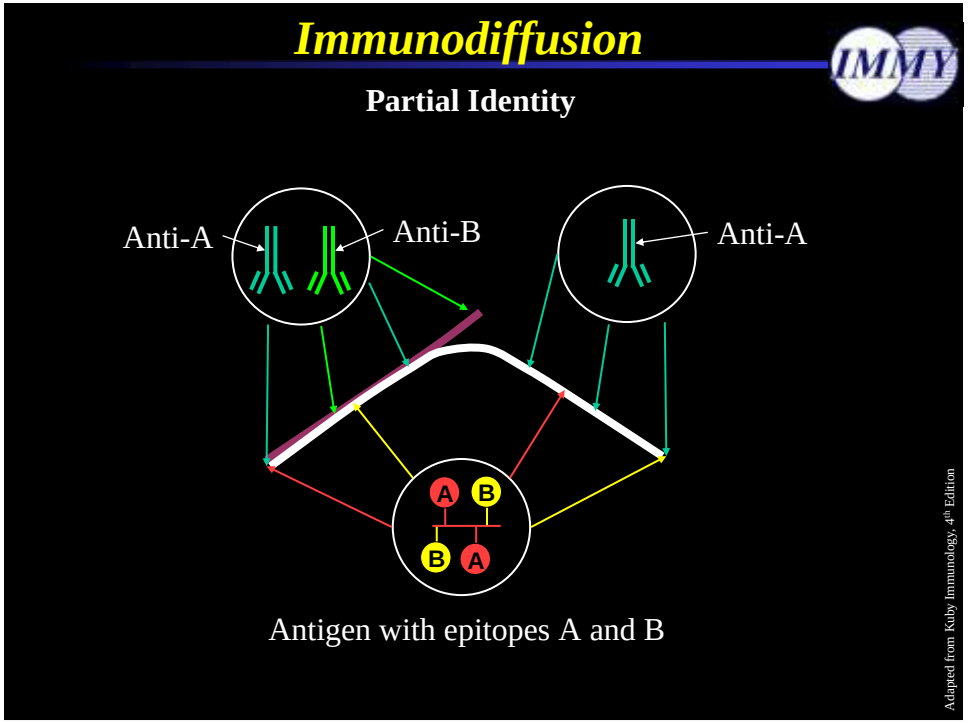
- Characterized by intersection or crossing of two precipitin lines
- occurs when two antigens do not share any epitope



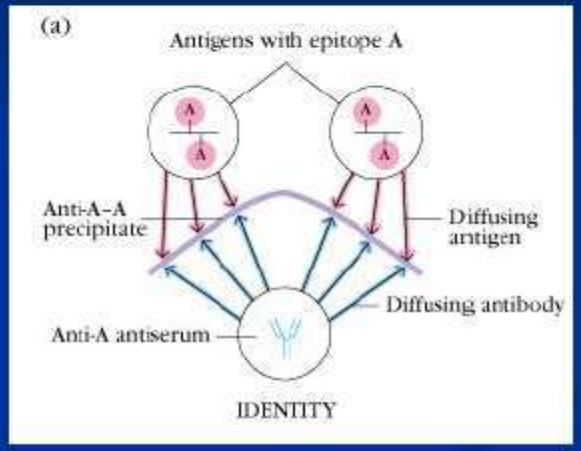
TYPES OF REACTION - III

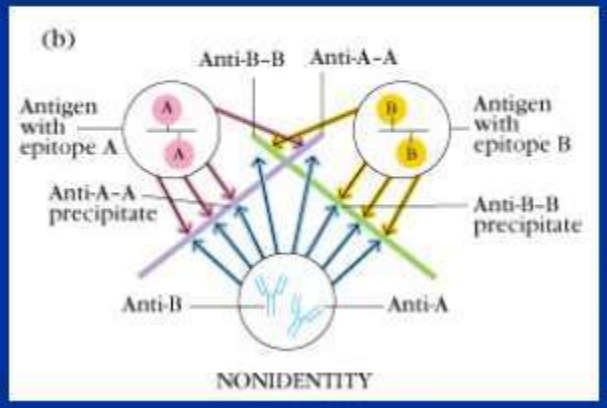
Reaction of partial identity

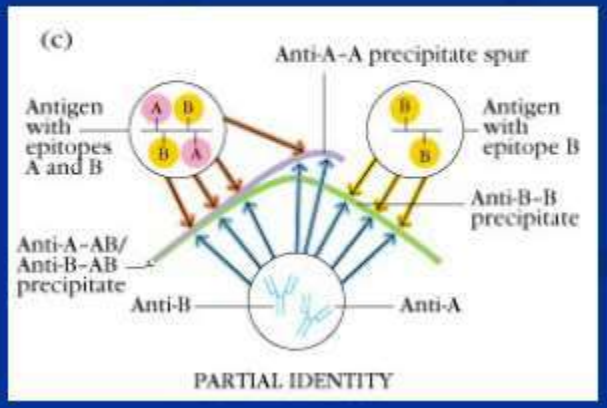
- characterized by formation of a line of identity with a curved spur
- occurs when two antigens share some epitopes but not all



Double diffusion results







Precipitation in agar with an electric field

There are three methods:

- Immunoelectrophoresis
- Countercurrent immunoelectrophoresis
- Rocket electrophoresis

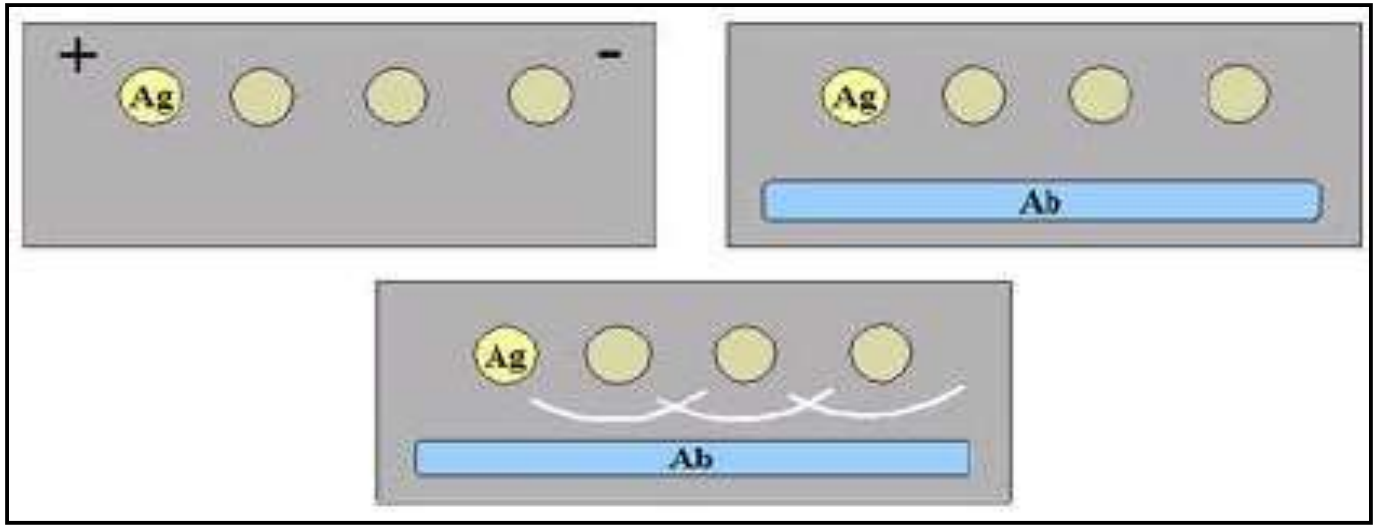
Immunoelectrophoresis

- It is a process of combination of immunodiffusion and electrophoresis.
- In this method soluble antigens are separated by electrophoresis in an agar gel according to their charge under an electric field.
- A mixture of antigen is added into a well in a agar on a glass slide.
- An electric current is then passed through the agar. During electrophoresis, antigen moves in the electric field according to their charge and size.
- A trough is then cut into the agar gel parallel to the direction of the electric field and antiserum containing a mixture of antibodies is added to the trough.
- Abs for all antigens in the mixture diffuse at a right angle to the direction of electrophoresis of Ags

Immunoelectrophoresis

- **Interpretation**

  - Precipitin arc represent individual antigens
- Estimates minimum no. of antigens in a mixture
- Estimates over- or under-production of antigens, such as myeloma proteins in human serum.



Immunoelectrophoresis Result

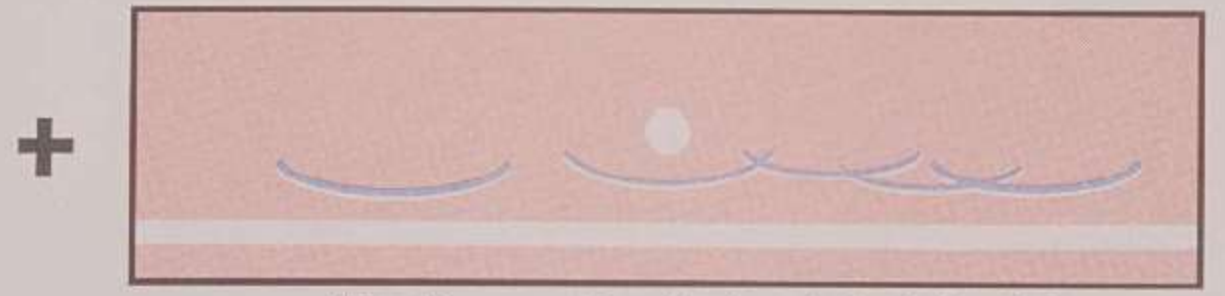
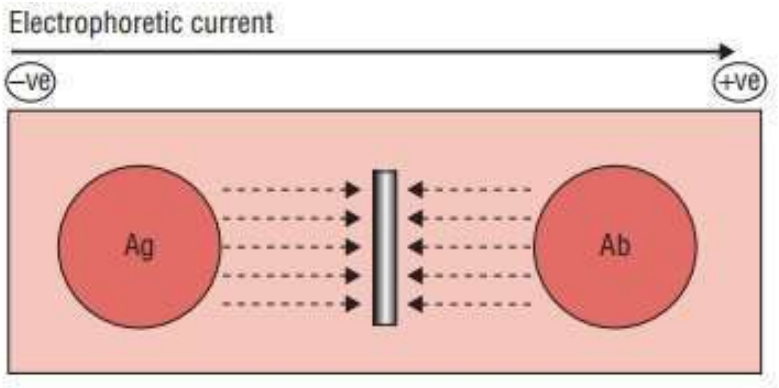


Figure 7.10.

Patterns of immunoelectrophoresis of serum proteins.

Countercurrent immunoelectrophoresis

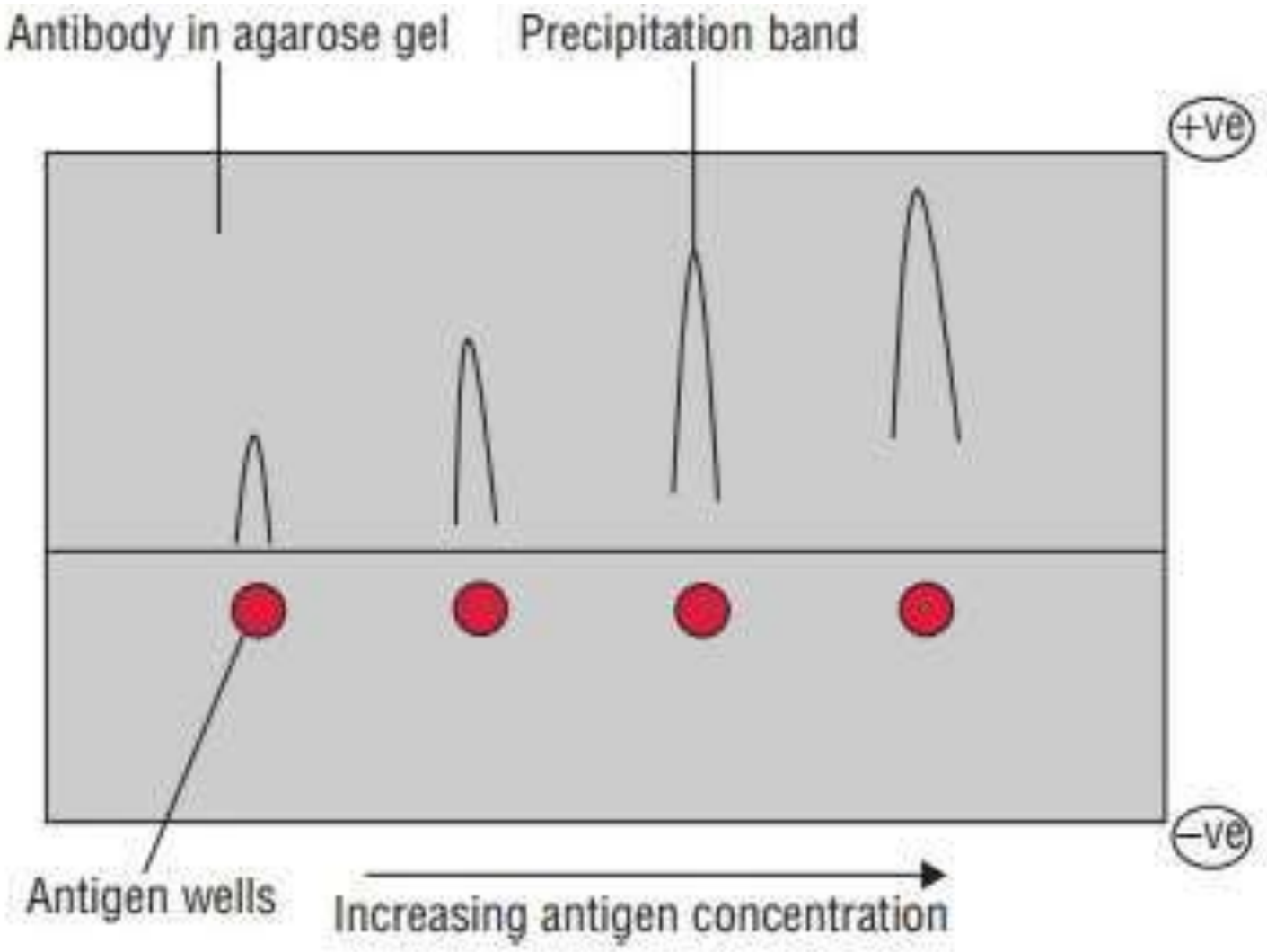
- Both Ag and Ab are placed in wells in an agar gel and made to migrate toward each other by electrophoresis
- pH of buffer is so chosen (8.6) that the Ab is positively charged and the Ag is negatively charged.
- Ag is placed in the cathodic well (negative end) and antiserum is placed in the anodic well (positive end).
- Ag will migrate towards anode while Ab migrates towards cathode and precipitation occur at equivalence within 30 to 60 minutes.



Rocket electrophoresis

- This technique is a modification of radial immunodiffusion using electrophoresis, developed by Laurell
- It is called so due to the appearance of the precipitin bands in the shape of cone like structure (rocket appearance) at the end of the reaction.
- Antibody is incorporated in the gel and antigen is placed in wells cut in the gel.
- Electric current is then passed through the gel, (a negatively charged antigen is electrophoresed in a gel containing antibody which facilitates the) which facilitates the migration of the antigen into agar.
- This results in the formation of the precipitin line which has the shape of rocket.
- The height of the rocket, measured from the well to the apex, is directly proportional to the amount of antigen in the sample.
- Rocket electrophoresis is mainly used for the quantitative estimation of the antigen in the serum.

Rocket electrophoresis



Two-dimensional Immunoelectrophoresis

- Two-dimensional immunoelectrophoresis is a variant of rocket electrophoresis.
- It is a double diffusion technique used for qualitative as well as quantitative analysis of sera for a wide range of antigens.
- This test is a two-stage procedure.
- In the first stage, antigens in solution are separated by electrophoresis.
- In the second stage, electrophoresis is carried out again, but perpendicular to that of first stage to obtain rocket-like precipitation.

AGGLUTINATION REACTIONS

AGGLUTINATION REACTIONS

- Agglutination reactions are based on the interaction of antibodies (agglutinins) and particulate antigens (e.g. bacteria) in presence of electrolytes (NaCl) at a suitable temperature and pH
- The binding of bi- or multivalent antibodies with particulate antigens form large clumps or aggregates, easily visible without magnification, when exposed to specific antibodies.
- The test could be qualitative (e.g. RBPT) or quantitative (e.g. SAT)
- Agglutination tests are easy to perform and in some cases are the most sensitive tests currently available.
- The amount of agglutinate formed is greatly influenced by the:
  - Relative proportions of antigens and antibodies
  - The Ab avidity
  - time of incubation with the antibody source
  - conditions of the test environment (e.g., pH and protein concentration)

Agglutination test

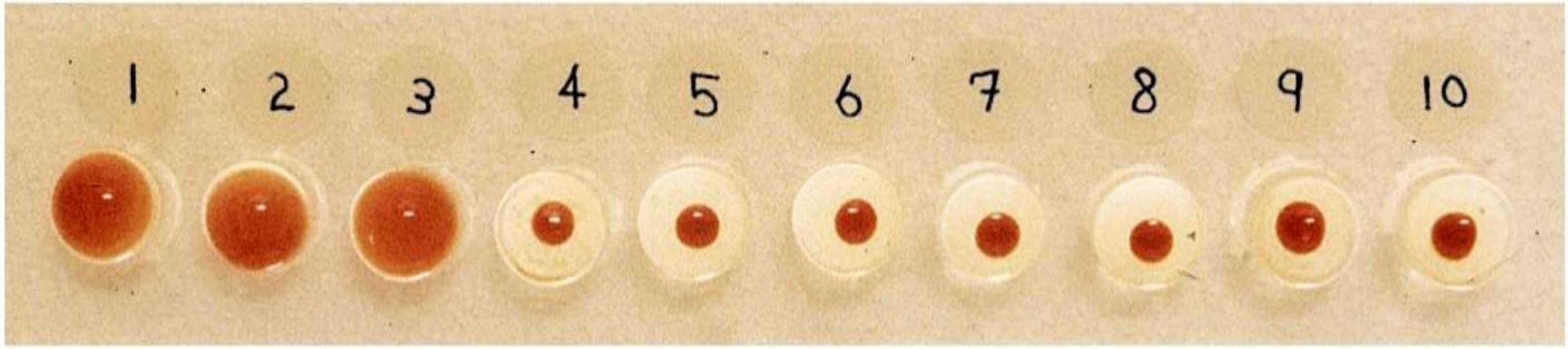


Figure 6-8
Kuby IMMUNOLOGY, Sixth Edition

- Practical considerations
  - Easy
  - Semi-quantitative
- Applications
  - Blood typing
  - Bacterial infections
    - Fourfold rise in titer

TYPES OF AGGLUTINATION REACTIONS

Precipitation reaction can be broadly of the following types:

- **Direct (active) agglutination** - reactions where the antigens are found naturally on a particle are known as direct agglutination.
  - Example between a cellular antigen and its antibody (RBPT, SAT, Blood group typing, etc)

- **Indirect (passive) agglutination –** reactions employing carrier particles that are coated with soluble antigens
  - Using biological carrier (e.g., erythrocytes)
  - Using artificial carrier (e.g., latex or charcoal particles)

- **Co-agglutination** - using Protein A

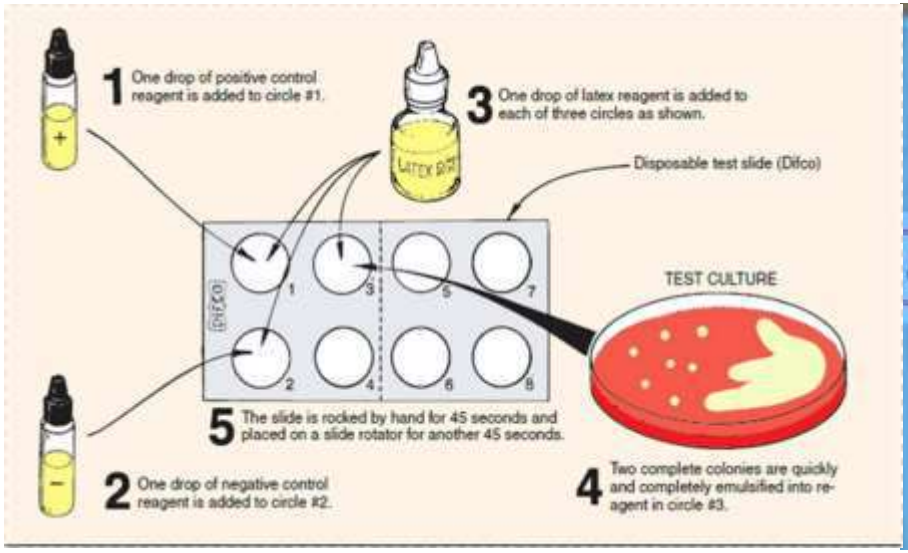
Direct (Active) Agglutination

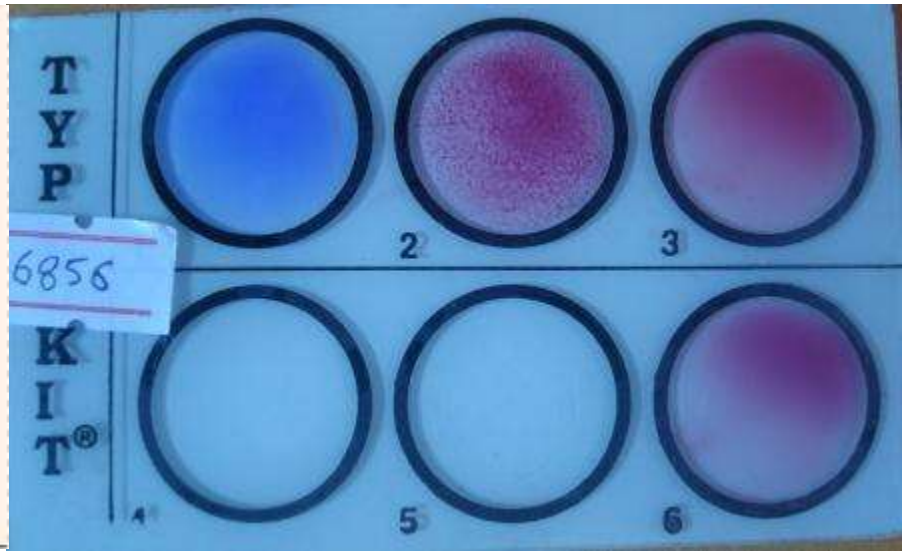
- Agglutination reactions where the antigens are found naturally on a particle are known as direct agglutination. This is different from passive agglutination, which employs particles that are coated with antigens not normally found on their surfaces.
- Direct bacterial agglutination uses whole pathogens as a source of antigen. The binding of antibodies to surface antigens on the bacteria results in visible clumps.
- Direct agglutination reactions can be of the following types:
  - (a) slide agglutination,
  - (b) tube agglutination,
  - (c) heterophile agglutination - demonstration of heterophilic antibodies in serum present in certain bacterial infections
  - (d) antiglobulin (Coombs') test.

Slide agglutination test

- It is a qualitative agglutination reaction performed on a slide.
- Examples - Blood typing, RBPT, *Salmonella Pullorum* plate test, Lancefield method for serotyping of streptococci, etc.
- Slide tests are commonly used for rapid identification of bacteria.
- These tests can be used for detection of antigen (e.g., Lancefield method) or antibodies (e.g., RBPT)
- The method consists of mixing a drop of antigen and antibody on a glass slide or a porcelain plate. One of the reagent is known and
- A positive reaction is indicated by formation of visible clumps within a minute.
- An antigen control by mixing a drop of antigen with normal saline should be included to rule out false positives due to autoagglutination of the antigen and also to validate the results

Slide agglutination test





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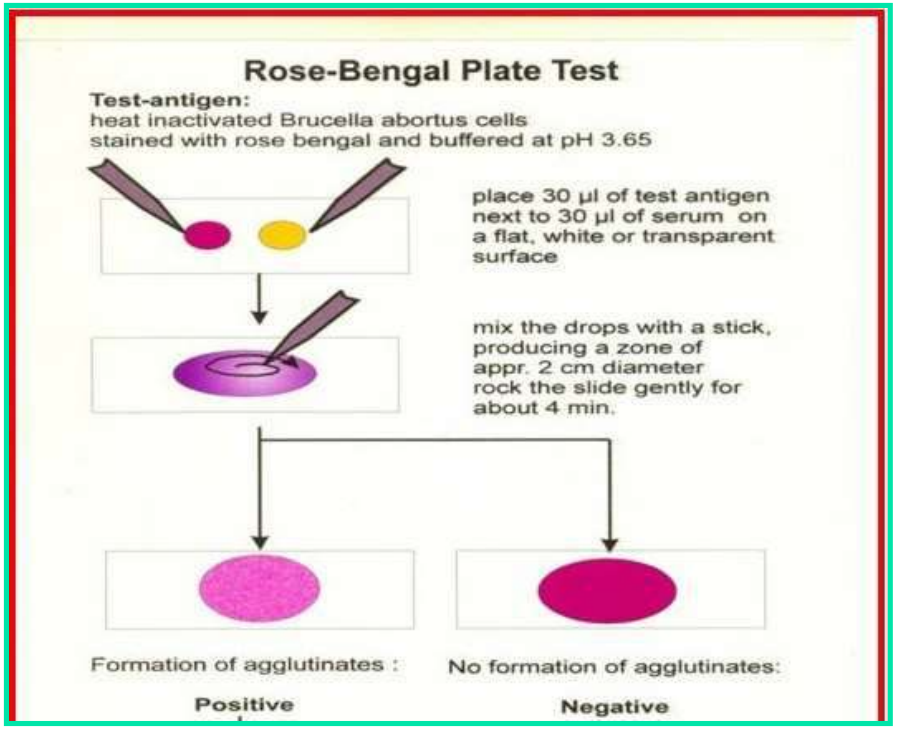
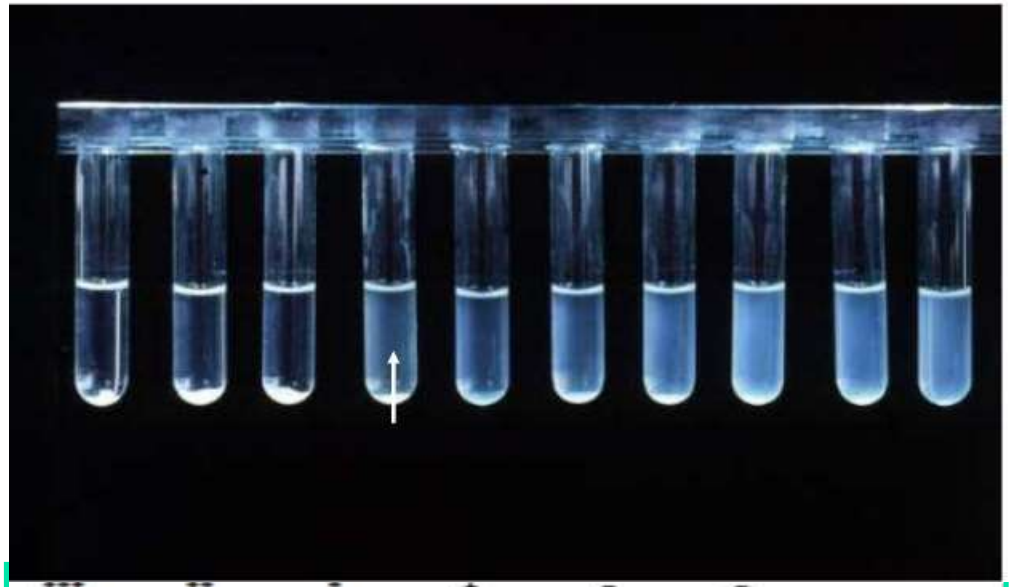
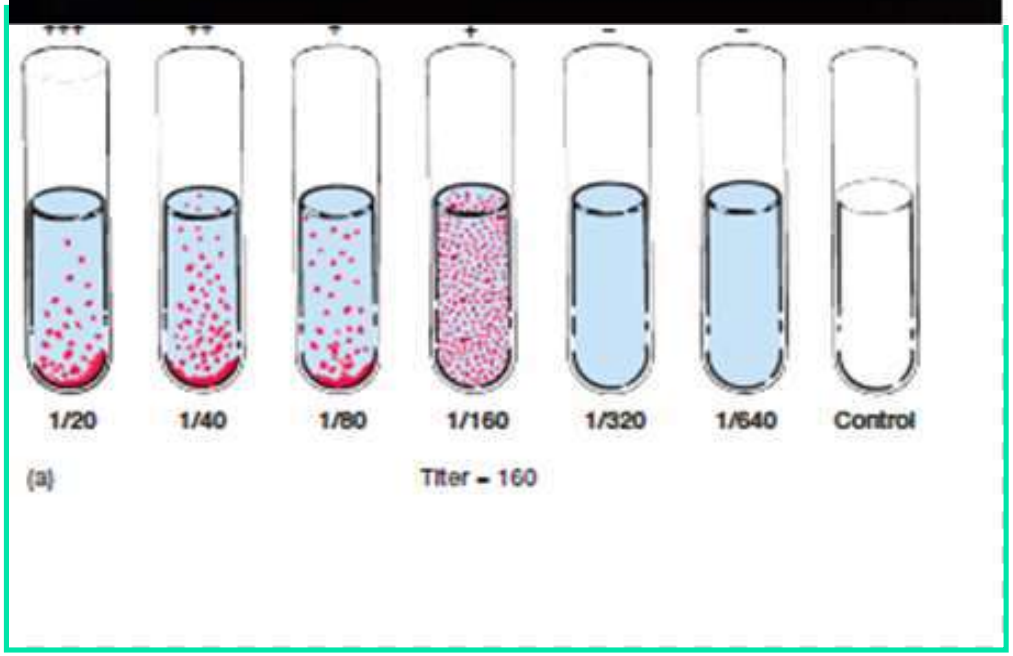


Tube agglutination test

- It is an agglutination reaction performed in test tubes.
- Tube tests are commonly used as a quantitative test to determine the titer of antibodies in a given serum sample
- Examples – STAT for Brucellosis, Widal test for typhoid, etc.
- The method consists of adding a fixed volume of antigen into test tubes containing serial dilutions of antiserum in normal saline.
- A positive reaction is indicated by formation of visible clumps after a pre-determined period of incubation.
- The reciprocal of highest dilution of anti-serum giving positive reaction is the titre of antibodies.
- Appropriate antigen and serum controls should be included to validate the results .
- Prozone phenomenon may be observed.

Tube Agglutination Test

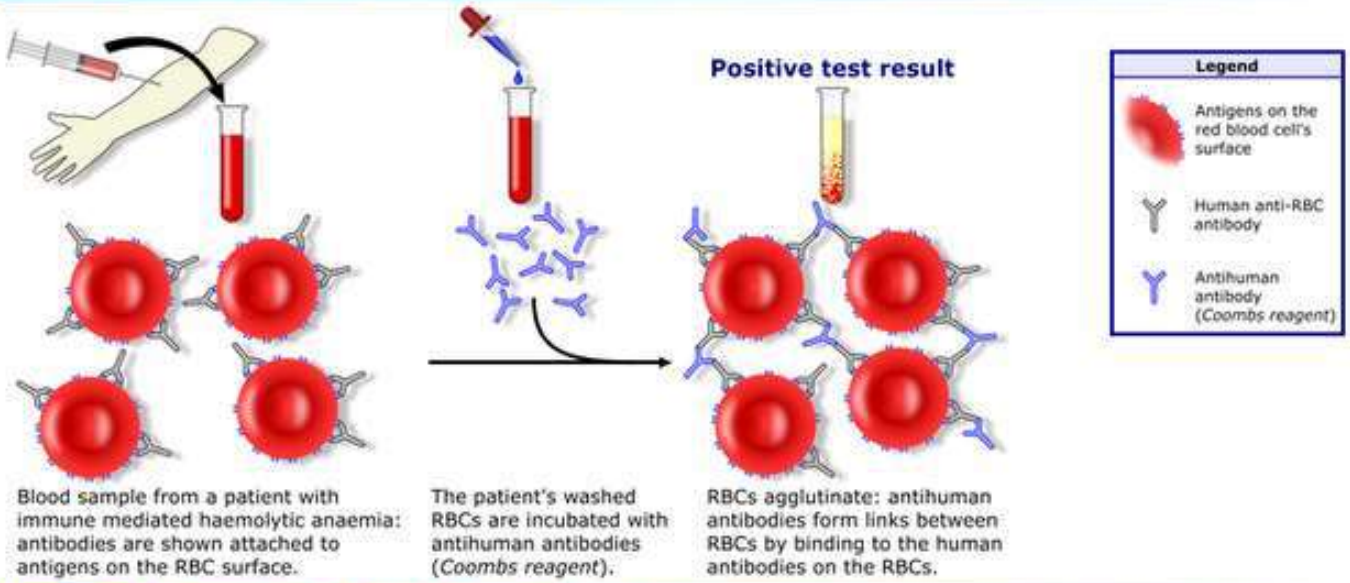




Antiglobulin (Coombs') test

- Coombs' test was devised originally by Coombs', Mourant, and Race for detection of incomplete anti-Rh antibodies
- These incomplete anti-Rh antibodies coats the surface of erythrocytes, but does not cause any agglutination when serum is mixed with Rh1 erythrocytes in saline.
- On adding antiglobulin or Coombs' serum (rabbit anti-human IgG) to such anti-Rh antibodies coated erythrocytes, however, the latter are agglutinated.
- Coombs' tests are used for detection of anti-Rh antibodies, autoimmune hemolytic anemia, and incomplete antibodies in brucellosis and other diseases.
- Coombs' test is of two types:
  - (a) direct Coombs' test - detection of incomplete antibodies on patients RBCs
  - (b) indirect Coombs' test - detection of incomplete antibodies in patients sera

Direct Coombs test / Direct antiglobulin test

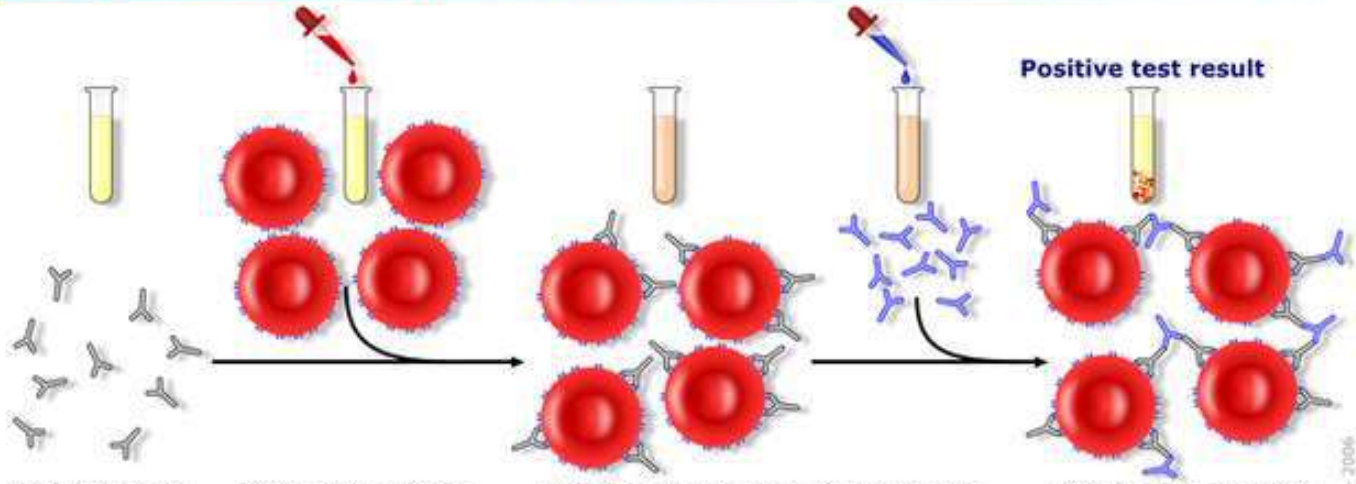


Blood sample from a patient with immune mediated haemolytic anaemia: antibodies are shown attached to antigens on the RBC surface.

The patient's washed RBCs are incubated with antihuman antibodies (*Coombs reagent*).

RBCs agglutinate: antihuman antibodies form links between RBCs by binding to the human antibodies on the RBCs.

Indirect Coombs test / Indirect antiglobulin test



Recipient's serum is obtained, containing antibodies (Ig's).

Donor's blood sample is added to the tube with serum.

Recipient's Ig's that target the donor's red blood cells form antibody-antigen complexes.

Anti-human Ig's (*Coombs antibodies*) are added to the solution.

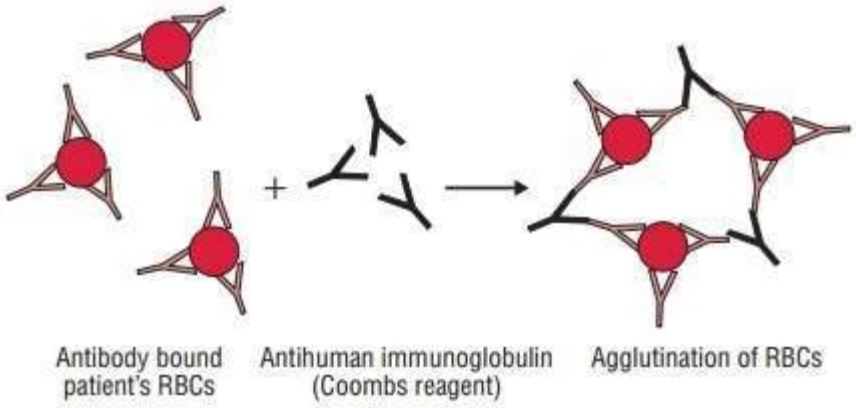
Agglutination of red blood cells occurs, because human Ig's are attached to red blood cells.

Antiglobulin (Coombs') test - principle

- The antibodies produced against Rh antigen are of IgG type (against IgM antibodies produced against ABO antigens). As IgG antibodies are only bivalent they are unable to crosslink Rh epitopes present in the surface of RBCs and, therefore, are unable to agglutinate RBCs.
- Negative charge on the surface of RBCs prevents them from coming closer than within 20 nm of each other. Moreover, the no. of Rh epitopes are less and sparsely placed on the surface of RBCs . Hence, IgG antibodies are unable to cross-link red cells as the Fab arms, even when fully extended, are unable to span the distance between two closely placed Rh epitopes
- Therefore, the antibodies formed against Rh antigens are called incomplete antibodies (misnomer, as IgG antibodies are complete with bivalency and the problem is with Rh antigen)

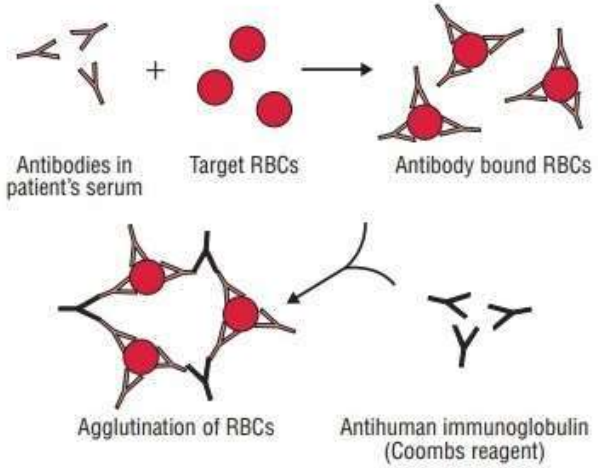
Direct Coombs' test

- In this test, the sensitization of red blood cells (RBCs) with incomplete antibodies takes place *in vivo*.
- The test procedure involves the addition of Coombs serum (AHG – anti-human IgG) directly to a patient's washed RBCs.
- The occurrence of agglutination means that the patient's RBCs have cell bound antibodies.
- Direct Coombs test is used for diagnosis of:
  - hemolytic transfusion reaction,
  - hemolytic disease of the fetus and newborn (HDFN) and
  - autoimmune hemolytic anemia (AIHA) etc.



Indirect Coomb's Test

- The indirect Coombs test, detects antibodies against human RBCs in the patient's serum.
- In this method, a patient's serum is incubated with RBCs of a known type followed by the addition of adding Coombs serum (AHG).
- If test serum contains incomplete circulating Abs they will sensitize RBCs *in vitro* and agglutination will result.
- The indirect Coombs test is used in crossmatching before blood transfusions and in prenatal testing of pregnant women.

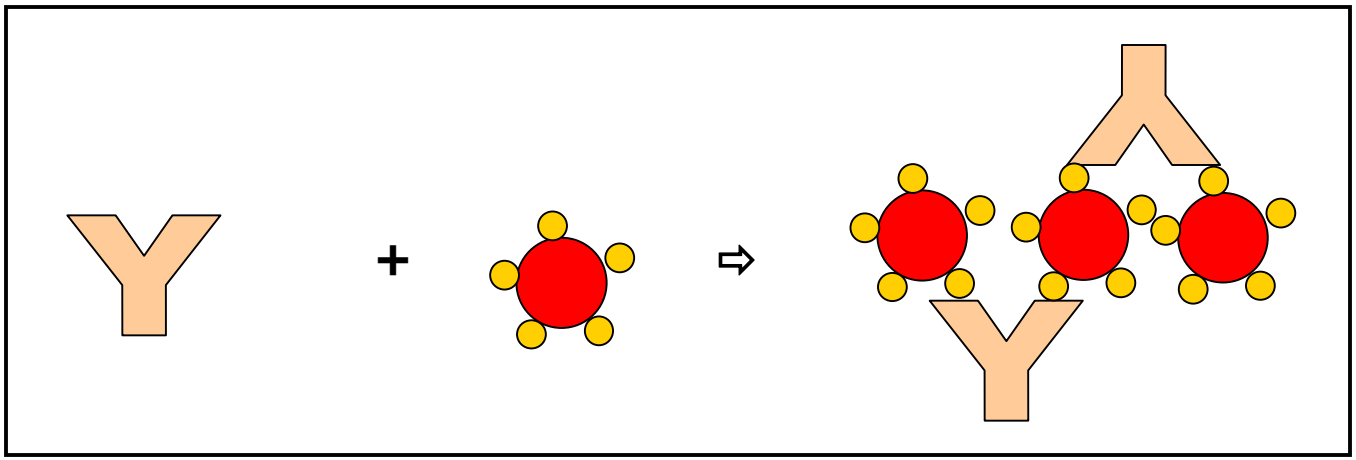


Indirect (Passive) Agglutination

- Agglutination reactions where the soluble antigens are coated or adsorbed on a cell or particle, called as carrier, are known as direct agglutination. These antigens are not normally found on the surfaces of such carriers
- Coating 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 anti-bodies.
- The binding of antibodies to homologous antigens adsorbed on the surface of carriers results in the agglutination of carrier particles forming visible clumps as in direct agglutination.
- If the antibody instead of antigens is adsorbed on the carrier particle for detection of antigens, it is called as **reverse passive agglutination.**

Passive Agglutination

Agglutination test done with a soluble antigen coated onto a particle



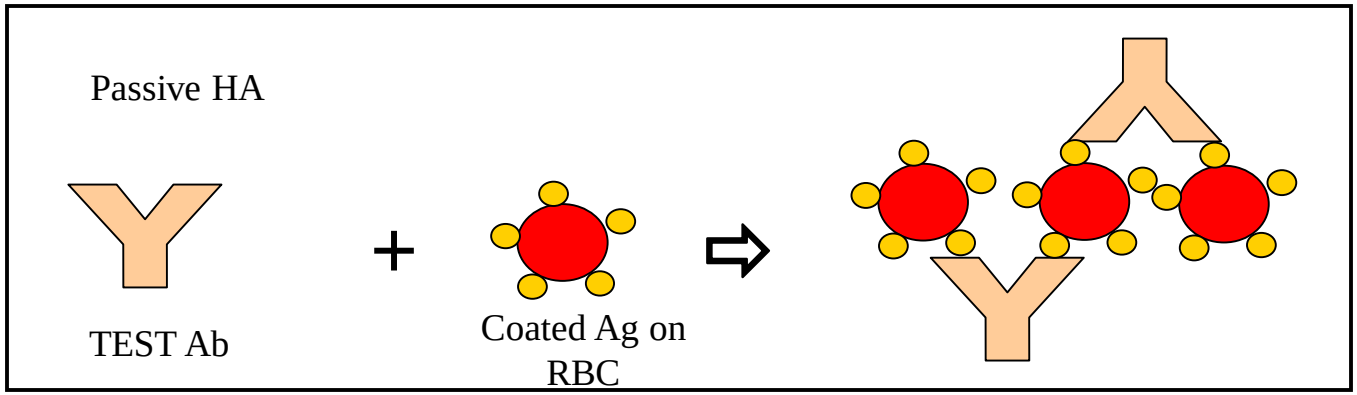
Indirect (Passive) Agglutination

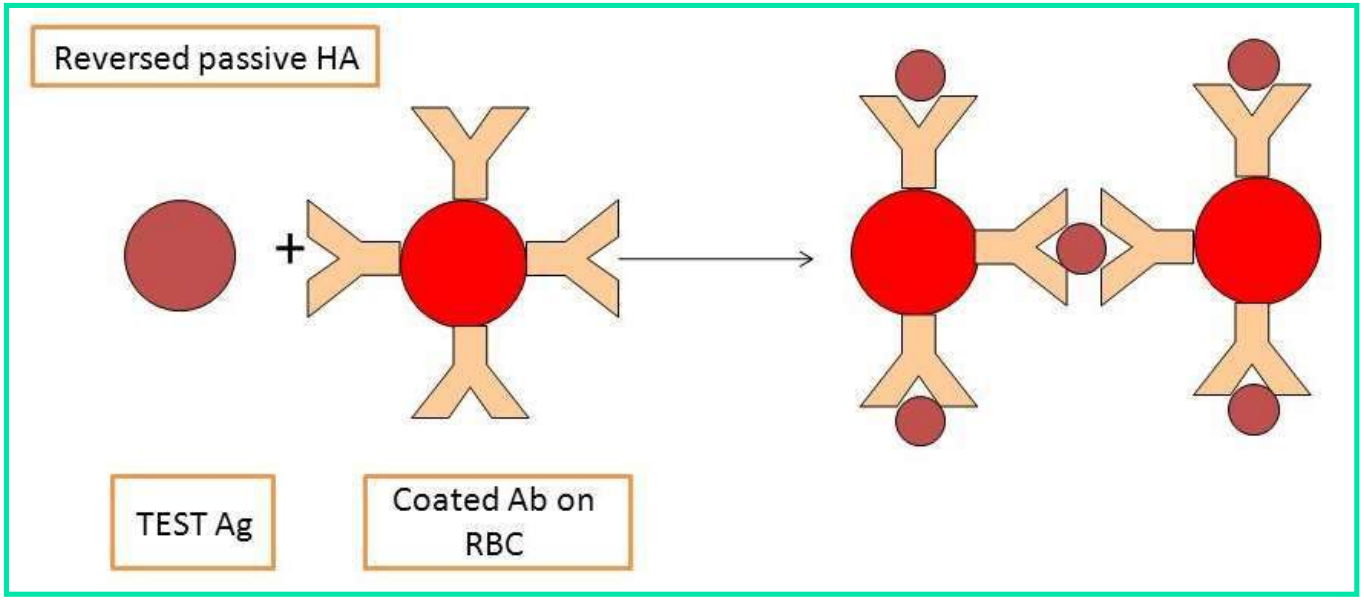
- Earlier erythrocytes were the major carrier particles used for coating of antigens, the test being called as **passive or indirect haemagglutination (PHA/IHA)**
- Nowadays inert particles, such as polystyrene latex, bentonite, and charcoal are used for this purpose.
- Particle size vary from 7 µm for RBCs to 0.05 µm for very fine latex particles.
- The use of synthetic beads or particles provides the advantage of consistency, uniformity, and stability. Reactions are also easy to read visually.
- Indirect agglutination reactions, depending on the carrier particles used, can be of the following types:
    - (i) passive hemagglutination test,
    - (ii) latex agglutination test, and
    - (iii) coagglutination test.

PASSIVE HAEMAGGLUTINATION

- Antigen–coated red blood cells (sensitized red cells) are prepared by mixing a soluble antigen with red blood cells that have been treated with tannic acid or chromium
- On adding antiserum these sensitized cells in wells of a microtitre plate are agglutinated
- RBCs of sheep, human, chicken, etc. are commonly used in the test.
- PHA is commonly used for serodiagnosis of many parasitic diseases including amoebiasis, hydatid disease, and toxoplasmosis.
- When antibodies are attached to the RBCs to detect microbial antigen, it is known as **Reverse Passive Hemagglutination(RPHA*)***. The RPHA has been used extensively to detect viral antigens, such as in HBsAg in the serum for diagnosis of hepatitis B infection and sheep pox

Passive Haemagglutination

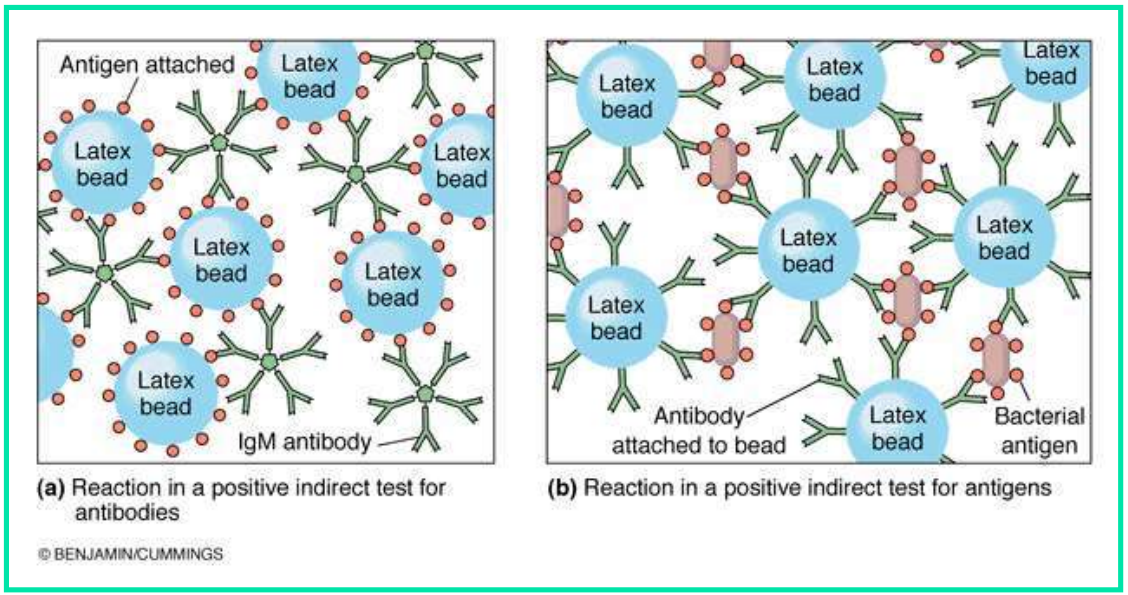




LATEX AGGLUTINATION TEST

- A group of passive agglutination tests carried out by coating either antigen or antibody on an artificial carrier particle, called latex bead, are known as **latex agglutination test (LAT)**.
- If antigen and antibodies are homologous, then clumping of beads occur.
- LAT can be used either to detect antibody or antigen; the latter is sometimes known as Reverse Passive Latex Agglutination Test.
- Latex beads are polystyrene latex particles; 0.8- 1 µm in diameter.
- The number of antibody or antigen molecules bound to each latex particle is large, resulting in a high number of exposed potential binding sites.
- Antigen or antibody present in a specimen binds to the combining sites of the corresponding antibody/antigen exposed on the surfaces of the latex beads, forming cross-linked aggregates of latex beads and antigen/antibody., which are visible as clumps.

Latex agglutination test



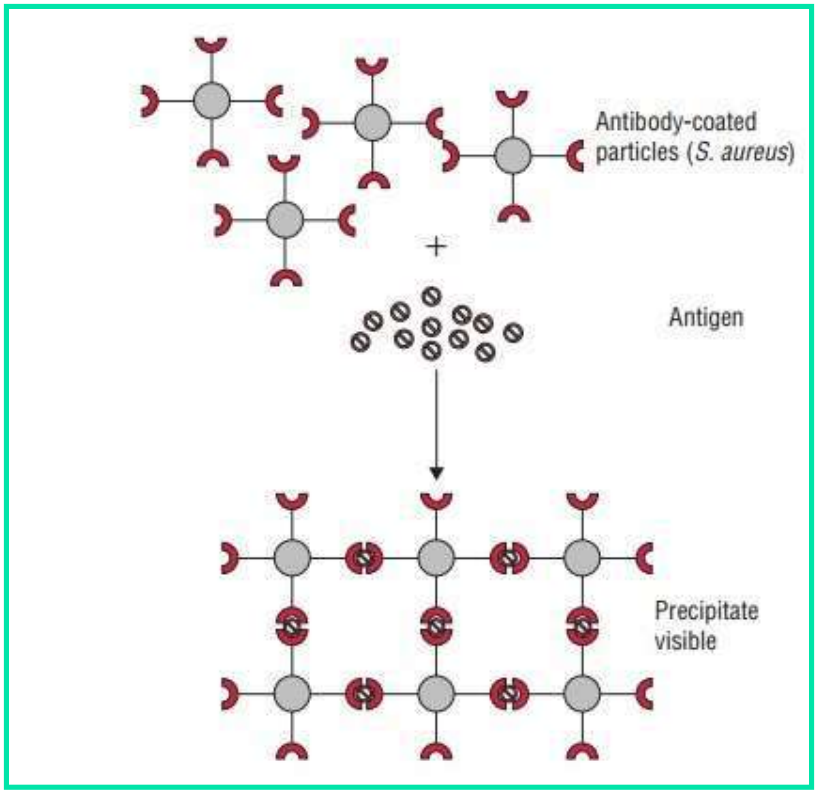
Latex tests are very popular in clinical laboratories to detect antigen to *Cryptococcus neoformans* in CSF or serum and to confirm the presence of beta-hemolytic streptococcus from the culture plates. Latex tests are also available to detect *Streptococcus agalactiae*, *Clostridium difficile* toxins A and B and rotavirus.

LATEX AGGLUTINATION TEST

- **Interpretation** - depending on the procedure, some reactions are reported as positive or negative and other reactions are graded on a 1+ to 4+ scale,
- **Adavntages**
  - large particle size of latex facilitates the visualization of the antigen-antibody reaction.
  - easy to do and rapid to perform
  - inexpensive, relatively stable and not subject to cross reactivity with other antibodies.
  - sensitivity for bacterial polysaccharides detection is 0.1 ng/mL.
- **Limitations**
  - the pH, osmolarity, and ionic concentration of the solution influence the amount of binding that occurs
  - test needs to be properly standardised
  - some constituents of body fluids, such as rheumatoid factor, have been found to cause false positive reactions in the LAT

COAGGLUTINATION TEST

- Protein 'A', produced by *Staphytococcus aureus* Cowan-I strain has high affinity for IgG antibodies of certain species.
- Such bacterial cells can be coated with specific antiviral antibodies, which bind via their Fc portion to the protein A molecules, leaving Fab portion free.
- In a positive test, protein A bearing *S. aureus* coated with antibodies will be co-agglutinated if mixed with specific antigen.
- *Staphytococcus aureus* Cowan-I strain are more stable, economical and resistant to ionic changes than latex beads
- The coagglutination reaction is highly specific but may not be as sensitive for detecting small quantities of antigen as latex agglutination.



Haemagglutination Inhibition (HAI) Test

- Some viruses can directly bind and agglutinate mammalian and avian red cells. This phenomenon is called as **Haemagglutination**.
- (Direct) Haemagglutination is not a serological test
- Haemagglutinating viruses include orthomyxoviruses, paramyxoviruses, alpha viruises, flaviviruses, and bunyaviruses as well as some adenoviruses, reoviruses, paravoviruses, and coronoaviruses.
- Inhibition of viral haemagglutination by specific antibody can be used either as a method for identifying a specific virus or to measure antibody levels in the serum; **this serological reaction is called as HAI**

Haemagglutination Inhibition (HAI) Test

- Dilutions of test sera are made, followed by the addition of an equal volume of virus suspension diluted to contain approximately 4 to 8 HA units
- The appropriate RBC suspension is then added and the plates are gently mixed and allowed to incubate for 1 – 2 hours
- If the specific antibody is present in the test serum, agglutination of RBC will be inhibited and the RBC's will settle out in a well-defined "button".
- Agglutinated cells, in contrast, will settle out in a thin layer over the entire bottom of the test well.

HA and HAI Tests

HA

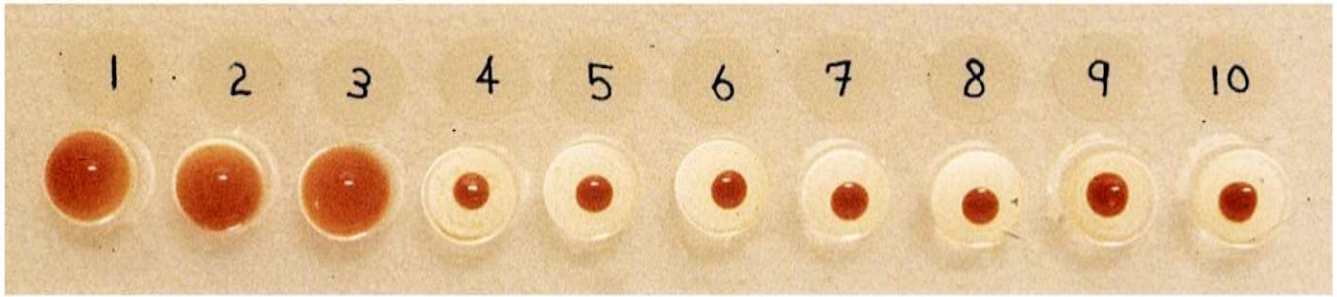


Figure 6-8
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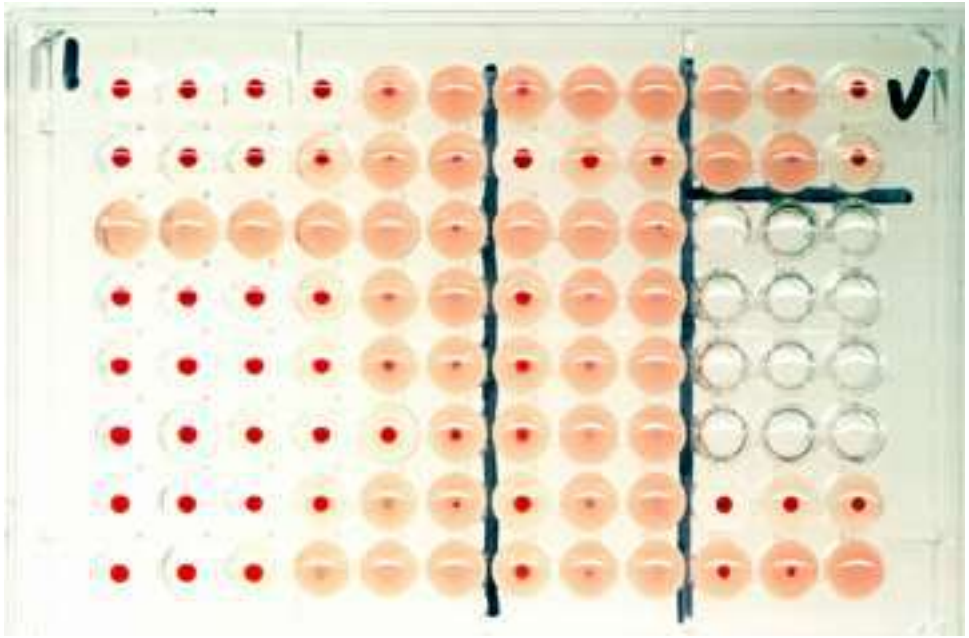
HAI



COMPLEMENT FIXATION TEST

Complement Fixation Test (CFT)

- The activation of the classical complement pathway by IgG or IgM bound to antigen results in the generation of membrane attack complexes that can disrupt the membrane where compliment is fixed. If the antibody is bound to red cells, these are ruptured and haemolysis occurs. This phenomenon can be used to measure serum antibody levels in a test called the **complement fixation test.**
- Complement fixation tests are most useful as an aid in the diagnosis of acute or recent infection, because they primarily detect IgM class of antibody.
- The complement fixation test consists of two systems – antibody complement systems: (*a*) an indicator system and (*b*) a test system.
- In classical CFT, fresh guinea pig serum is used as a source of complement.

Complement Fixation Test

- The CFT **indicator system** consists of sheep red blood cells (SRBC) and anti-SRBC antibody (haemolysin).
- The CFT **test system** consists of antigen, antibody and guinea pig complement.
- The serum is first heated to 56°C to inactivate the native complement.
- Then the inactivated serum is adsorbed with washed sheep RBC to eliminate broadly cross-reactive anti-RBC antibodies (also known as Forssman-type antibodies), which could interfere with the assay.
- The serum is then mixed with purified antigen and with a dilution of fresh guinea pig serum, and incubated for 30 minutes at 37°C .
- If the ANTIBODIES SPECIFIC FOR THE ANTIGEN IN THE ASSAY IS PRESENT in the patient's serum, then complement is completely consumed in the reaction and there is none left to bind to the SRBC/anti-SRBC complexes.
- **A Test Positive For Ab = NO HEMOLYSIS**

Complement Fixation Test

- If patient's serum does not have antigen specific antibodies, then immune complex is not formed and , therefore, complement does not get fixed.
- Complement is thus free to bind to the SRBC/anti-SRBC complexes (haemolysin) and cause lysis of SRBCs.
- **A Test Negative for Ab = HEMOLYSIS**
- CFT used to be a test of choice for diagnosis of FMDV prior to ELISA

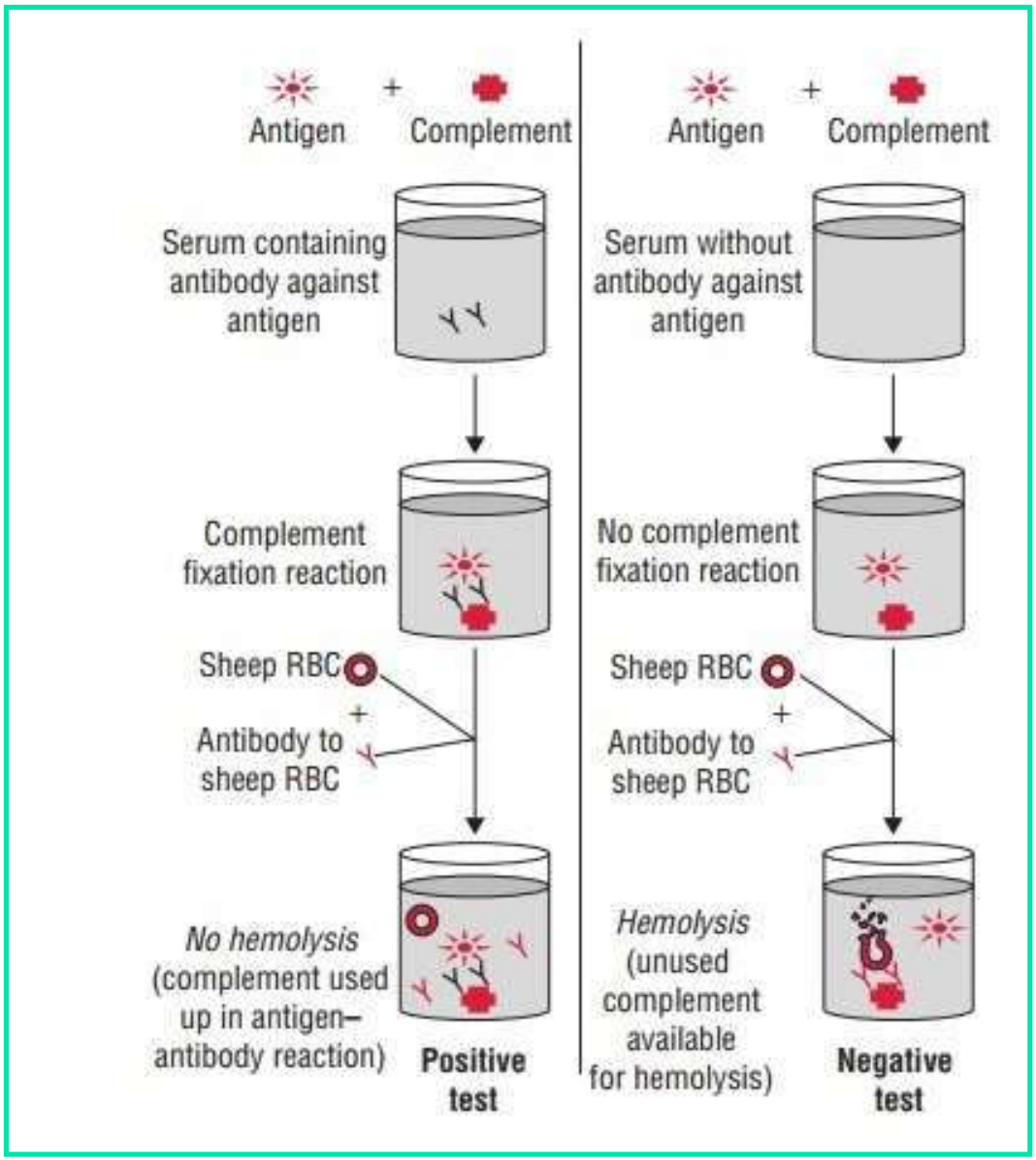
Advantages of Complement Fixation Test

- Ability to screen against a large number of viral and bacterial infections at the same time.
- Economical

Disadvantages of Complement Fixation Test

- Not sensitive – cannot be used for immunity screening
- Time consuming and labor intensive
- Often non-specific e.g. cross-reactivity between HSV and VZV

Complement Fixation Test



Other Types of Complement Fixation Test

- Indirect complement fixation test
- Congulatinating complement absorption test
- Immune adherence
- Immobilisation test
- Cytolytic tests

Indirect complement fixation test

- Indirect CFT is carried out to test the sera that cannot fix guinea pig complement, such as avian sera (e.g., parrot, duck) and mammalian sera (e.g., cat, horse).
- The test is carried out in duplicate
- After step 1, standard antiserum to antigen which is known to fix complement is added to one set.
- If antibodies were not present in the test serum then the antigen would react with the standard antiserum fixing the complement.
- On the other hand if antibodies are present in the test serum the antigen would be utilized in the first step. Hence, no reaction would occur between the standard antiserum and the antigen and therefore no fixation of complement which would then get fixed on SRBC and cause haemolysis. Thus in this case haemolysis indicates a positive result.

Congultinating complement absorption test

- It is an alternative method for systems that do not fix guinea pig complement.
- In this test, horse complement which is non-haemolytic is used.
- Sheep erythrocytes sensitized with bovine serum are used as the indicator system.
- The **bovine serum contains conglutinin**, a β-globulin that acts as antibody to the complement.
- Conglutinin causes agglutination of sensitized sheep erythrocytes if these are combined with complement, which is known as *conglutination*.
- If the horse complement had been used up by the antigen–antibody reaction in the first step, the agglutination of the sensitized cells does not occur.
- **Thus, agglutination of SRBC = negative and no agglutination is positive reaction**

Immune adherence

Certain pathogens (e.g., *Vibrio cholerae*, *Treponema pallidum*, etc.) react with specific antibodies in the presence of complement and adhere to erythrocytes or platelets. The adherence of cells to bacteria is known as **immune adherence, which facilitates phagocytosis of the bacteria**.

Immobilization test

Immobilization test is a complement-dependent test in which certain live bacteria, such as *T. pallidum,* are immobilized when mixed with patient's serum in the presence of complement. This forms the basis of *T. pallidum* immobilization test. A positive test shows serum to contain treponemal antibodies.

Cytolytic test

The incubation of a live bacterium with its specific antibody in the presence of complement leads to the lysis of the bacteria cells. This is the basis of **Vibriocidal antibody test** measure anti-cholera antibodies.

NEUTRALIZATION TEST

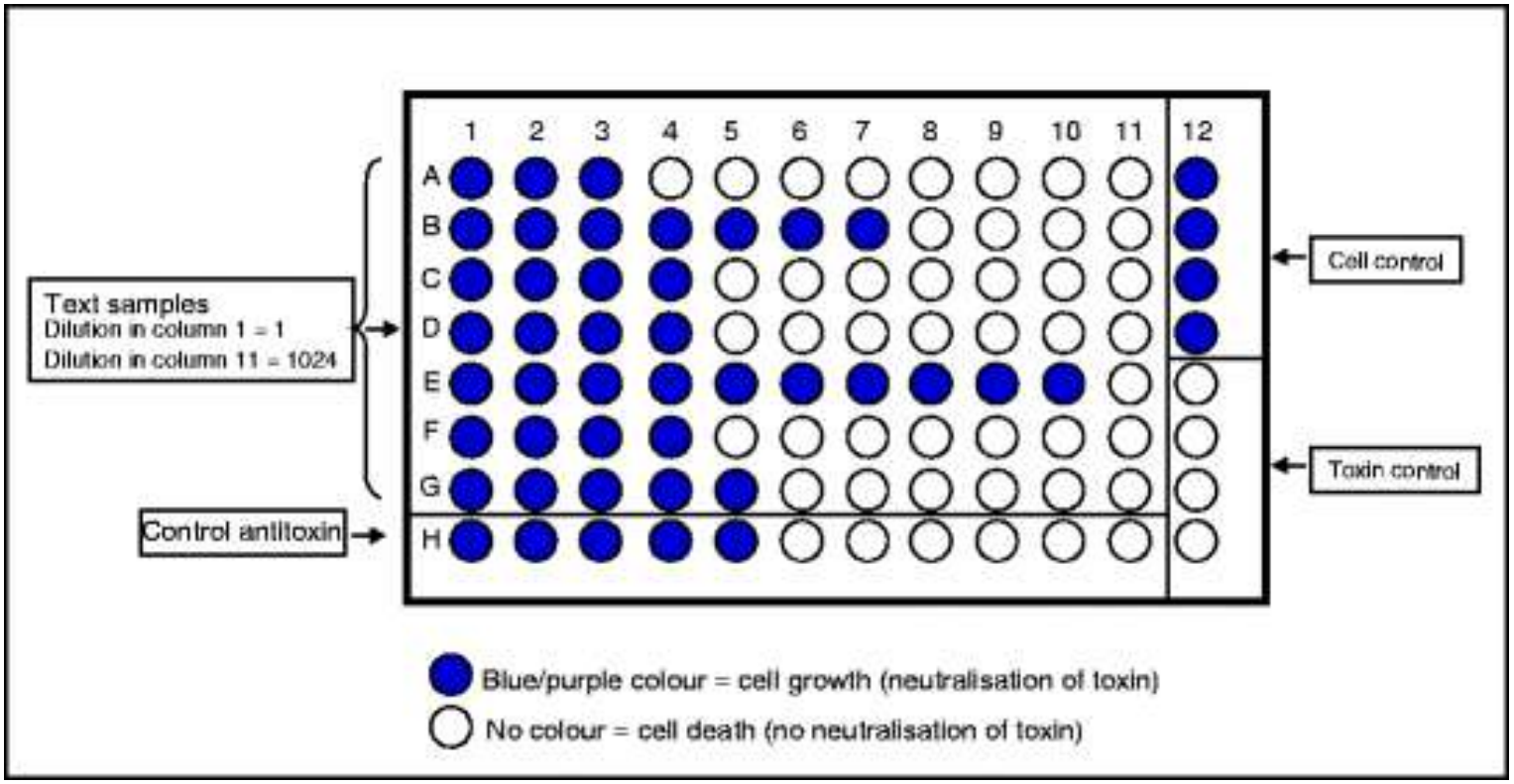
Neutralization Test

- Neutralization is an antigen–antibody reaction in which the biological effects of viruses and toxins are neutralized by homologous antibodies known as neutralizing antibodies.
- These tests are broadly of two types: (*a*) virus neutralization tests and (*b*) toxin neutralization tests.
- Neutralizing antibodies block or distort the antigen sufficiently, so that pathogen fails to exert its biological activity.
- Neutralization reactions can occur *in vitro* or i*n vivo.*
- Laboratory animals or tissue culture cells are used as "indicator systems" in neutralization tests. The toxin or virus to be assayed should have known effects on the indicator system which is neutralized by antibodies.

Toxin Neutralization Test

- Toxin neutralization tests are based on the principle that biological action of toxin is neutralized on reacting with specific neutralizing antibodies called antitoxins.
- Examples of toxin neutralization tests include:
  - In vivo:
    - (a) Schick test to demonstrate immunity against diphtheria and
    - (b) *Clostridium welchii* toxin neutralization test in guinea pig or mice.
  - In vitro:
    - (a) antistreptolysin O test and
    - (b) Nagler reactionused for rapid detection of *Cl. welchii*

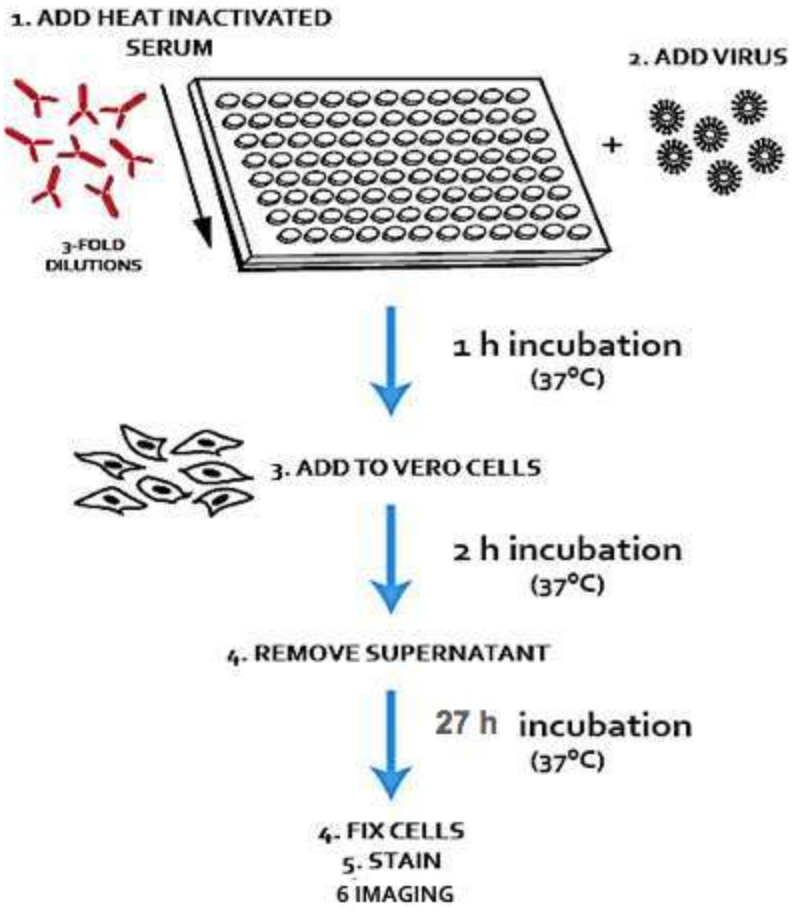
Toxin Neutralization Test



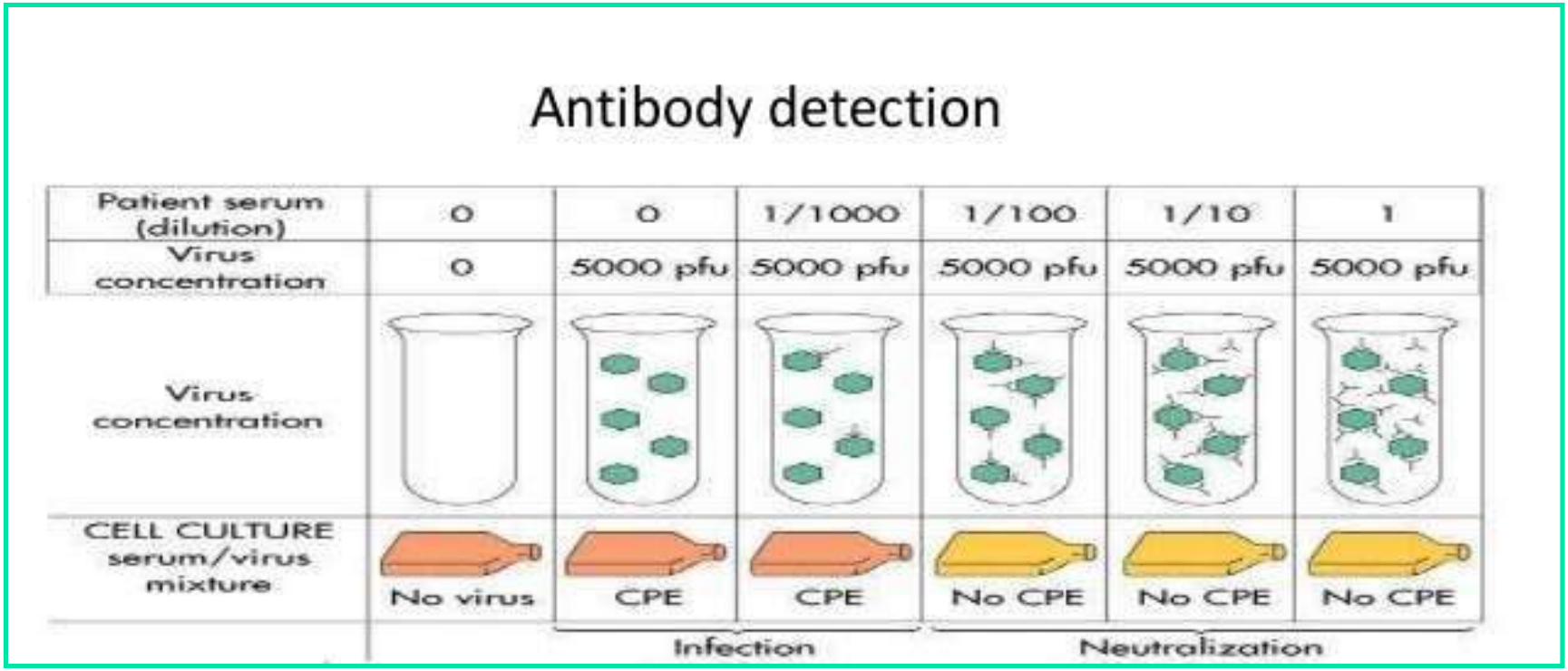
Virus Neutralization Test

- Neutralization of viruses by their specific antibodies are called **virus neutralization tests**.
- Inoculation of viruses in cell cultures, eggs, and animals results in the replication and growth of viruses. When virus-specific neutralizing antibodies are injected into these systems, replication and growth of viruses is inhibited. This forms the basis of virus neutralization test.
- **Neutralizing antibodies** may interfere with virion binding to receptors, block uptake into cells, prevent uncoating of the genomes in endosomes, or cause aggregation of virus particles.
- Neutralizing antibody is directed against the surface proteins of the virus. Antibodies formed against internal components of the virus do not neutralize the infectivity of the virus; rather such **non-neutralizing antibodies** may enhance their infectivity

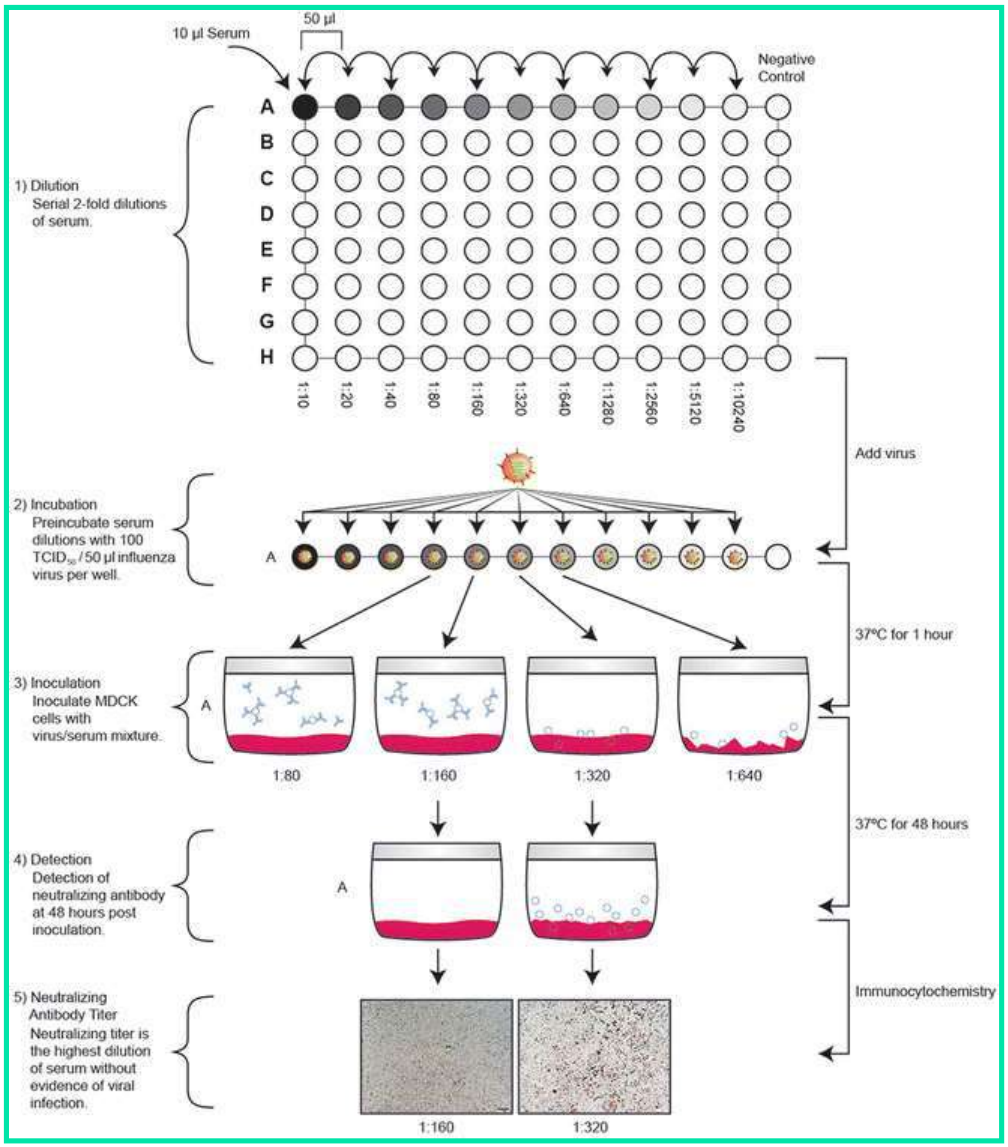
Virus Neutralization Test



Virus Neutralization Test



Virus Neutralization Test



Virus Neutralization Test (VNT)

- In a **neutralization test**, serum and virus are reacted together in equal volumes and inoculated into a susceptible animal host, embryonated eggs or cell culture. If antibodies to the virus are present then clinical disease, mortality in eggs or CPE will not be observed. This test can be used for detection of antigen (VNT) or antibody (Serum Neutralization Test – SNT)
- **Applications of VNT**
  - **Disease diagnosis:** Neutralization tests are used for the diagnosis of various infections. For example, IBR cuased by BHV-1
  - **Passive Immunization:** e.g., Anti Tetanus Serum, Rabies immunoglobulins
  - **Active Immunization:** e.g., vaccine against Rabies, NDV, FMDV, PPRV, etc
  - **Serotyping of viruses:** Virus neutralization assay is used for the serotyping of viruses. For example, Blue Tongue and FMD viruses

Haemagglutination Inhibition (HAI) Test

- Hemagglutination inhibition test is a type of virus neutralization test frequently used for the diagnosis of viruses which have the property of agglutinating the red blood cells (haemagglutination),such as influenza, NDV, etc.
- Haemagglutination inihibition antibodies, if present in serum, will occupy receptors on surface of RBCs and prevent virus from combining with these. Thus the agglutination of the red blood cells by virus will be inhibited.
- HAI test is used for serodiagnosis of viruses which cause HA. The test is also used to assess antibodies titre post vaccination for diseases such as Newcastle in poultry.
- Serial dilutions of patient's sera are allowed to react with a fixed dose of viral haemagglutinin, followed by the addition of agglutinable erythrocytes. In the presence of antibody, the ability of the virus to agglutinate the erythrocytes is inhibited.

Haemagglutination Inhibition (HAI) Test

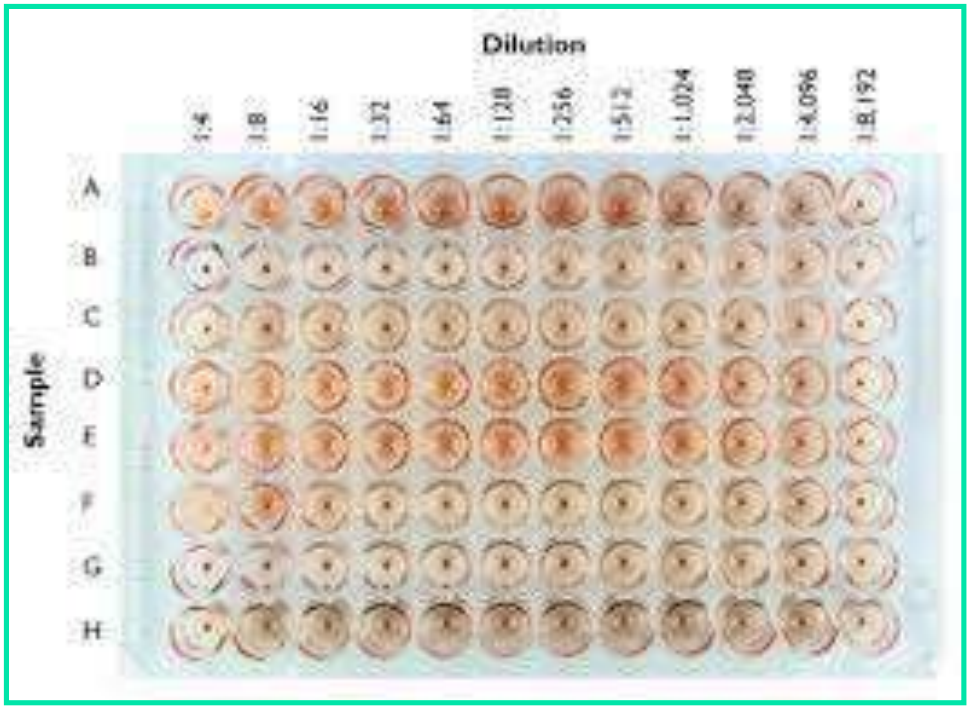
- Prior to test, HA titer of virus is determined. For HAI test 4 or 8 HA units of virus are used.
- The HAI test may be complicated by the presence of non-specific inhibitors of viral haemagglutination and naturally occurring agglutinins of the erthrocytes.
- Non-specific inhibitors of viral haemagglutination may be removed by the treatment of sera before testing by kaolin, RDE, potassium periodate (KIO) or by heat inactivation.
- Non-specific agglutinins for erythrocytes may be removed by the addition of erythrocytes to the sera prior to testing to allow the erythrocytes to absorb the non-specific agglutinins.
- A positive test is indicated by the formation of button of erythrocytes in the bottom, whereas in negative samples there is a lattice formation due to HA caused by the virus.

Haemagglutination Inhibition (HAI) Test

- Serum and erythrocytes control should be included in each test.
- The erythrocytes control should show a button at the bottom of the well. The serum controls for each serum should show the absence of agglutination.
- The haemagglutinin back titration should show agglutination at 4, 2 and 1 HA units.
- The HAI test is simple to perform and requires inexpensive equipment and reagents
- The disadvantages are low sensitivity, the actual reading of results is subjective and the reagents should be fresh or else abnormal agglutination patterns may arise

Haemagglutination Inhibition Tests

	Components	Interaction	Microtiter Results
A	RBCs		No Reaction
B	Virus + RBCs	→	Hemagglutination
C	Virus + Antibody + RBCs	→	Hemagglutination Inhibition

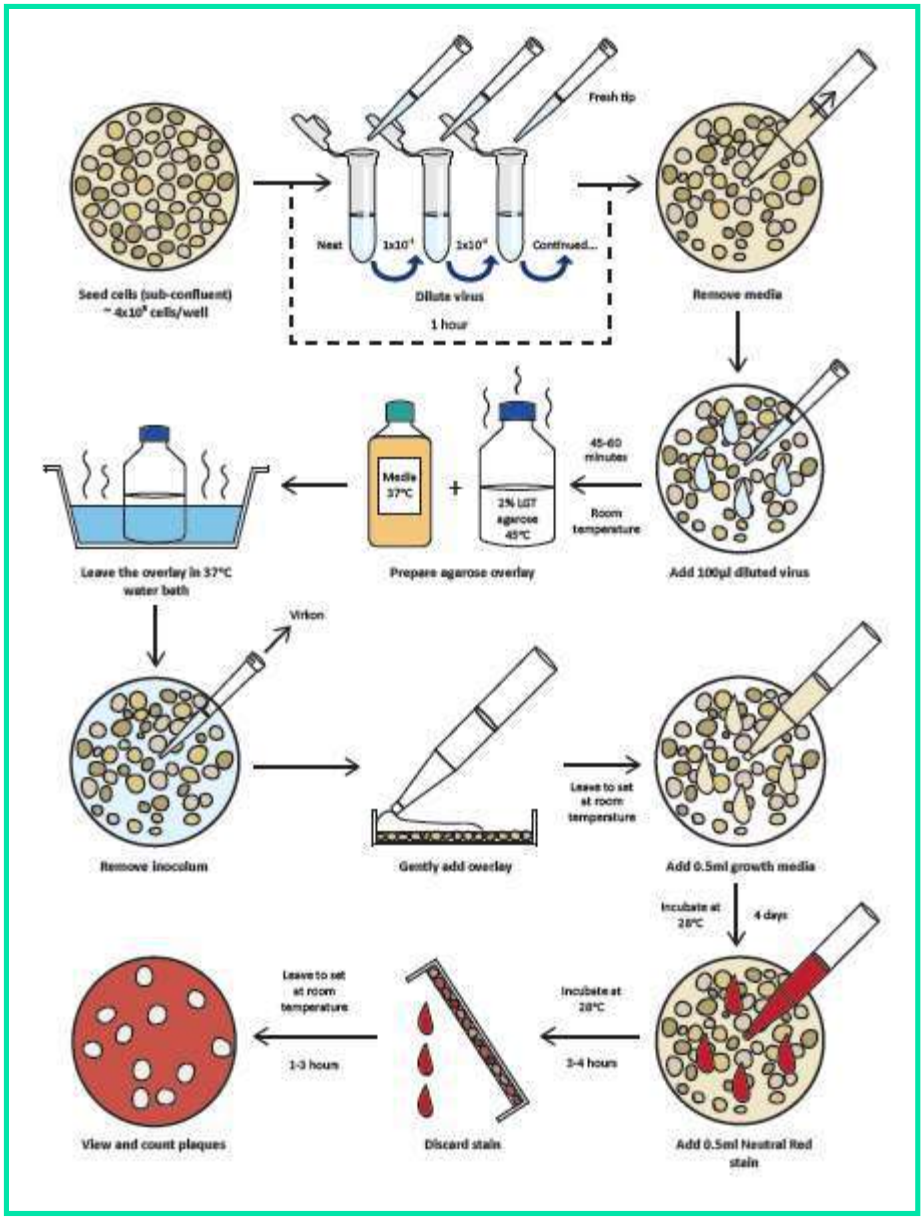


Plaque Reduction Neutralization Test (PRNT)

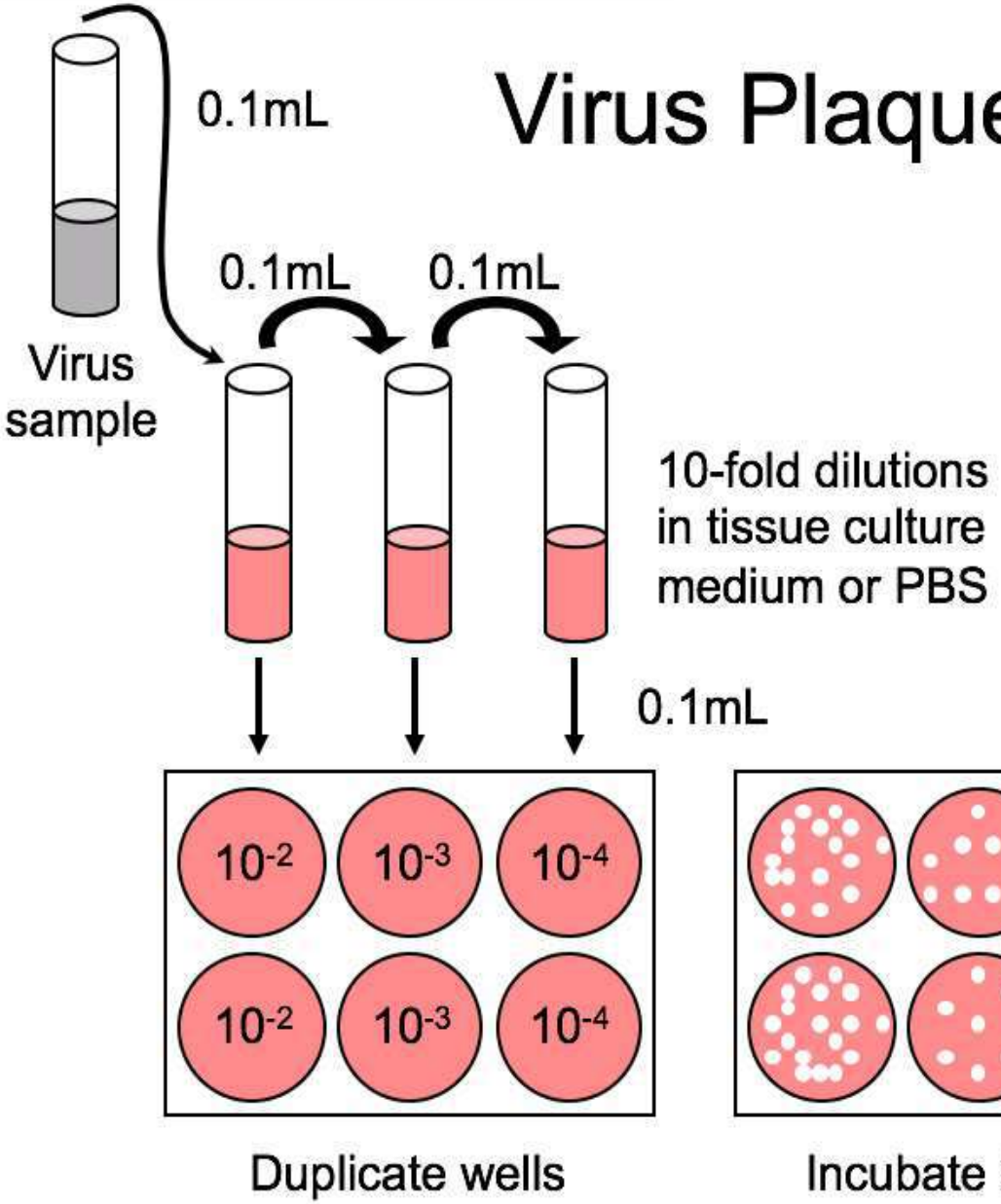
- A plaque is a local area of cell death caused by a virus in a cell monolayer.
- On an agar overlay after staining an infected cell sheet with vital dyes, such as neutral red, the plaque appear as unstained area.
- Each plaque represents one infectious unit in the original virus sample and is called as plaque forming unit (pfu).
- The plaques assay is carried out by inoculating a constant volume of 10-fold serial dilutions of a virus inoculum in a cell monolayer, each monolayer with one dilution.
- The procedure is depicted in figures.
- The no. of plaques are counted and no of pfu/ml of original viral inoculum is calculated by the following formula:

Pfu/ml=no of pfu*1/dilution factor

Viral Plaque Assay

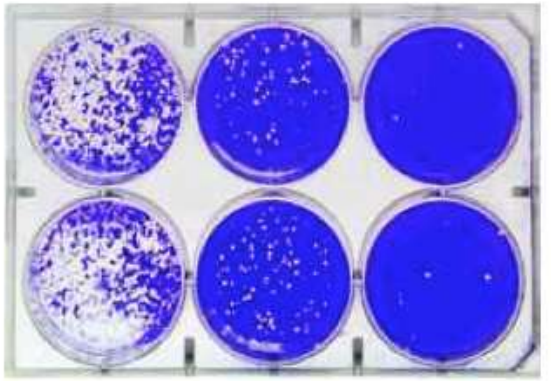


Virus Plaque Assay



1. Stain cells with dye,
2. plaques become visible, count 3. calculate original concentration "virus titer":

Example: 8 pfu x 1/dilution = 8×10^3 pfu/mL



Each plaque represents one infectious unit (virion or infected cell) in the original virus sample.

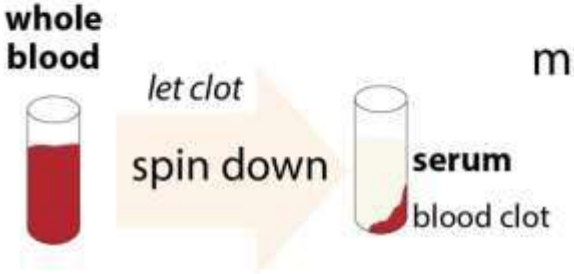
Plaque Reduction Neutralization Test (PRNT)

- The PRNT is a serological test which utilizes the ability of a specific antibody to neutralize a virus, in turn, preventing the virus from causing the formation of plaques in a cell monolayer.
- The assay involves mixing a constant amount of virus of known pfu/ml with dilutions of the serum specimen being tested, followed by plating of the mixture onto cells of an appropriate cell line for the individual virus.
- The concentration of plaque forming units can be determined by the number of plaques formed after a few days. A vital dye (e.g. neutral red) is then added for visualization of the plaques and the number of plaques in an individual plate is divided by the original number of virions to calculate the percentage neutralization.
- Interpretation is typically based on 70% neutralization, which is the last dilution of serum capable of inhibiting 70% of the total plaques (virions).

Plaque Reduction Neutralization Test

this is the standard method of testing for neutralizing antibodies
(the type of antibodies that prevent a virus from infecting cells)

take blood **serum**

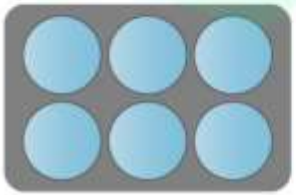


heat it up to kill any live virus

make a **dilution series** (e.g. dilute in half then in half then in half)

mix that with **live virus**

add it to plated **cells**

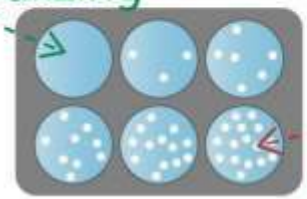


cover it with an **agarose** gel layer to prevent it from just randomly spreading all over...

...**wait** to see how many cells get infected

& **compare** to the no-serum-added control

the *more/stronger* neutralizing antibodies are present, the *fewer* the # of infected cell clusters (plaques)



the dilution that gives you a 50% decrease in plaques is the **$PRNT_{50}$**

Plaque Reduction Neutralization Test (PRNT)

- Currently, the PRNT test is considered the "gold standard" for detecting and measuring antibodies that can neutralize the viruses that cause many diseases.
- The results are dependent on type of cell monolyer used and multiplicity of infection
- Advantages
  - higher sensitivity
  - Specificity
- Disadvantages
  - Time consuming
  - Cumbersome
  - DI particles may interfere with results
- PRNT is used for diagnosis of EEE, WEE, VEE, Dengue, JE, Zika viruses, etc.