



AGGLUTINATION REACTIONS

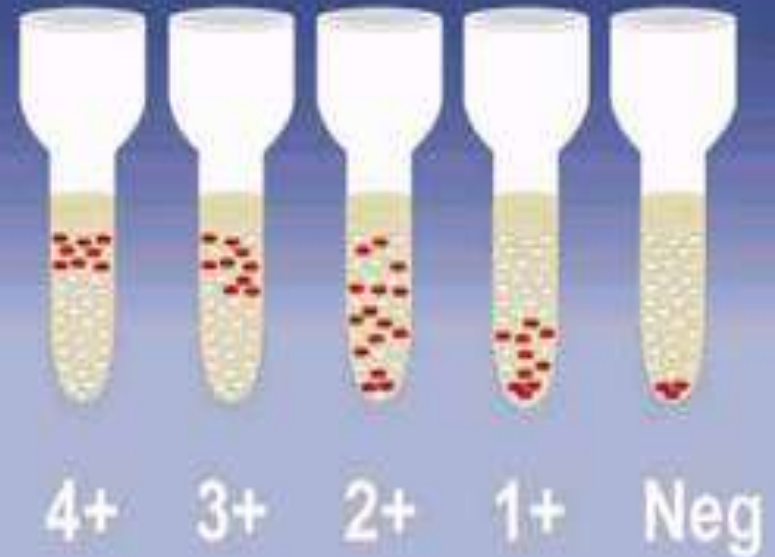
Agglutination

- Is the aggregation of particulate matter caused by the combination with specific antibody
- 1896: First observed by Gruber and Durham when serum antibody was found to react with bacterial cells

Agglutination

- Agglutinins
 - Antibodies that produce such reactions
- Involves two-step process:
 - Sensitization or initial binding
 - Lattice formation or formation of large aggregates

Column Agglutination Reactions



Agglutination


- Types of particles that participate in such reactions:
 - Erythrocytes
 - Bacterial cells
 - Inert carriers such as latex particles
- Each particle must have multiple antigenic or determinant sites, which are cross-linked to sites on other particles through the formation of antibody bridges

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 *Antibodies* 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**.



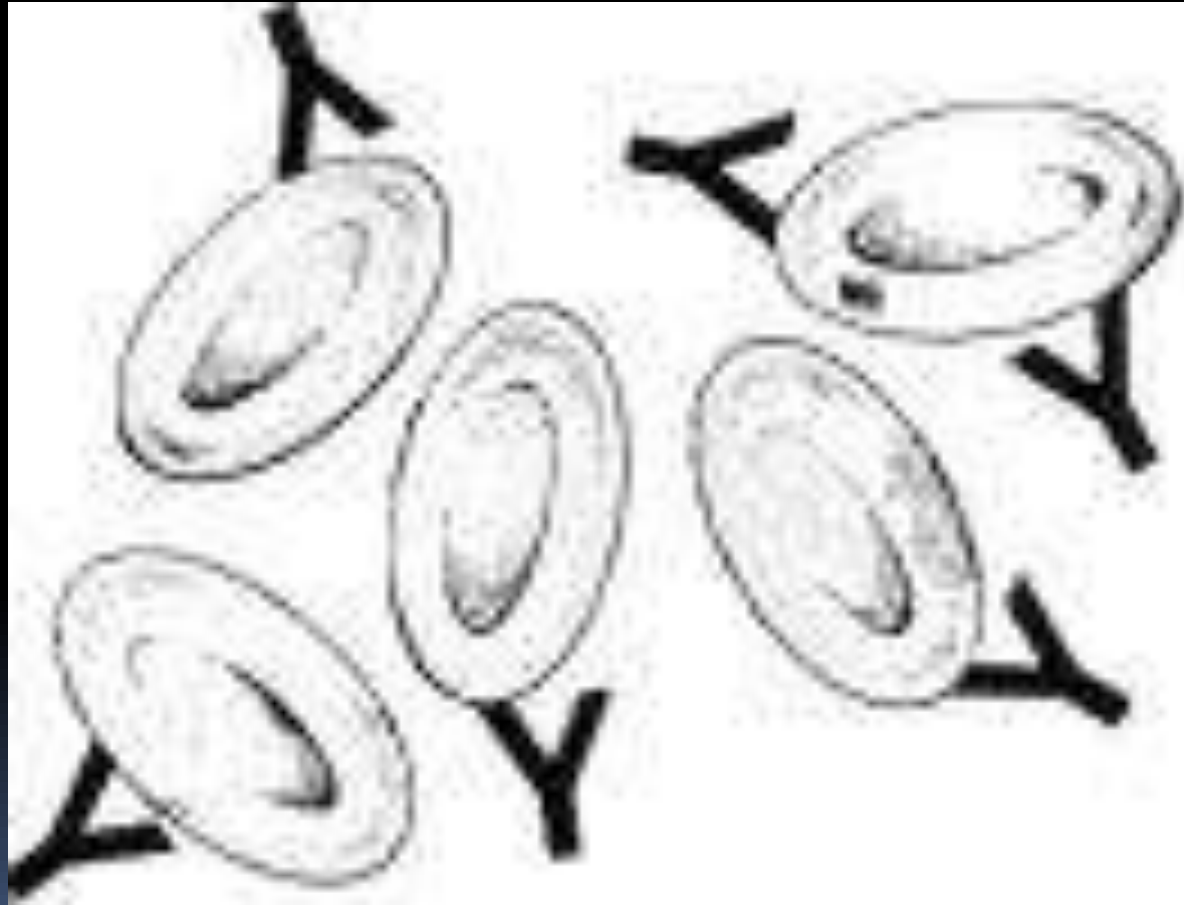
Agglutination reactions can be classified into several distinct categories:


- Direct
 - Passive
 - Reverse passive
 - Agglutination inhibition
 - Coagglutination
- 

Steps in Agglutination

- Primary phenomenon (SENSITIZATION)
 - First reaction involving Ag-Ab combination
 - Single antigenic determinant on the surface particle
- Initial reaction: rapid and reversible
- Cross link formation → visible aggregates (stabilization)

SENSITIZATION

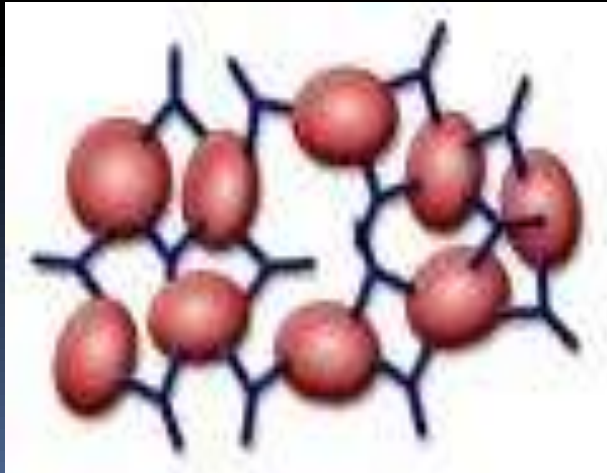


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- Secondary phenomenon:(
 - LATTICE FORMATION
 - Ab + multivalent Ag \rightarrow stable network (visible reaction)
 - conc. of Ag and Ab

 - Governed by physiochemical factors:
 - Ionic strength of milieu
 - pH
 - temperature

Secondary Phenomenon

- Lattice Formation
- The Fab portion of the Ig molecule attaches to antigens on 2 adjacent cells-visible results in agglutination
- If both antigen and antibody are SOLUBLE reaction will become visible over time, ie, precipitation



Other techniques that enhance agglutination, especially that of red blood cells, include:

- **Viscosity**

- can be increased by adding agents such as dextran or polyethylene glycol (PEG).
- These agents reduce the water of hydration around cells and allow them to come into closer proximity for antibody to join together.

- **Enzymes**

- Bromelin, papain, trypsin, and ficin
- work by reducing the surface charge on red blood cells through cleaving of chemical groups and decreasing hydration.



- Ficin

- cleaves sialoglycoproteins from the red blood cell surface; in addition to reducing the charge, this may change the external configuration of the membrane to reveal more antigenic determinant sites.



- Agitation and centrifugation

- provide a physical means to increase cell–cell contact and thus heighten agglutination.⁴




■ Temperature

- has an influence on the secondary, or aggregation, phase.
- IgG class antibodies agglutinate best at 30°C to 37°C
 - Ex. Antibodies to other human blood groups
- IgM antibodies react best at temperatures between 4°C and 27°C.
 - ex. Ab against the ABO blood groups



- pH

- Most reactions produce optimal antigen–antibody combination when the pH is between 6.5 and 7.5
 - Exceptions:
 - human anti-M and antiP₁, which react best at a lower pH.
- 



TYPES OF AGGLUTINATION REACTIONS

DIRECT AGGLUTINATION

- occurs when antigens are found naturally on a particle.
- Detection of antibodies is primarily used in diagnosis of diseases for which the bacterial agents are extremely difficult to cultivate.

DIRECT AGGLUTINATION

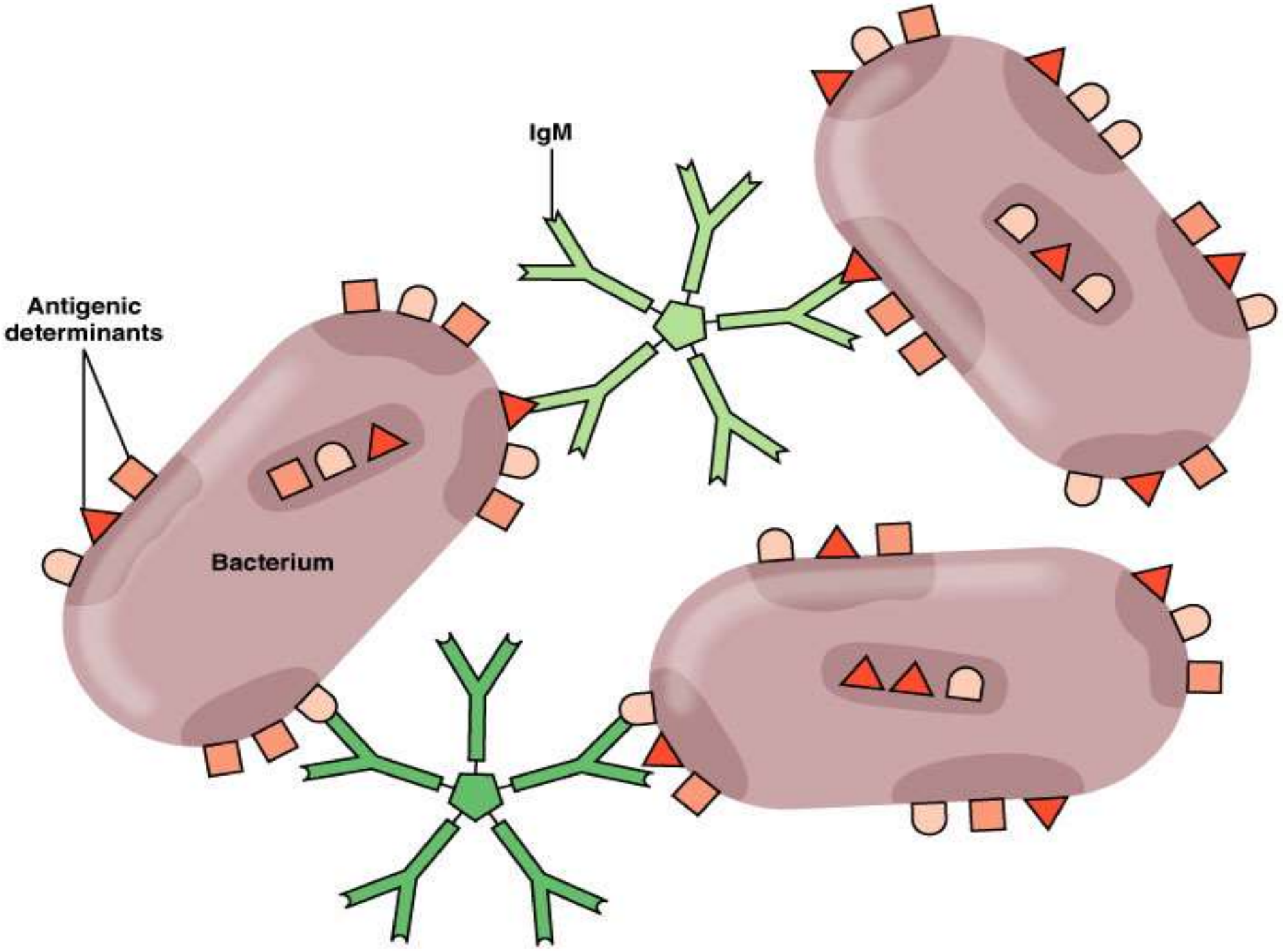
- Test patient serum against large, cellular antigens to screen for the presence of antibodies.
- Antigen is naturally present on the surface of the cells.
- In this case, the Ag-Ab reaction forms an agglutination, which is directly visible.

DIRECT AGGLUTINATION

- The particle antigen may be a bacterium.
 - e.g.: Serotyping of *E. coli*, *Salmonella* using a specific antiserum
- The particle antigen may be a parasite.
 - e.g.: Serodiagnosis of Toxoplasmosis
- The particle antigen may be a red blood cell.
 - e.g.: Determination of blood groups

DIRECT AGGLUTINATION

- Ex.
- Widal test
 - a rapid screening test to help determine presence of typhoid fever
- The antigens used in this procedure include:
 - Salmonella O (somatic)
 - H (flagellar) antigens.
- A significant finding is a fourfold increase in antibody titer over time when paired dilutions of serum samples are tested with any of these antigens.



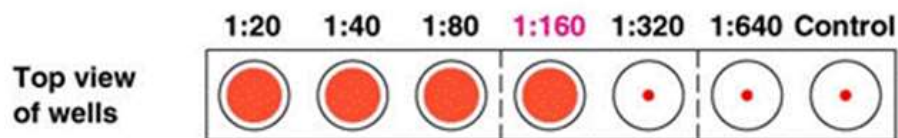
DIRECT AGGLUTINATION

- These reactions can be performed on slides (rapid tests) or on microtiter plates or tubes for Antibody titration if required.



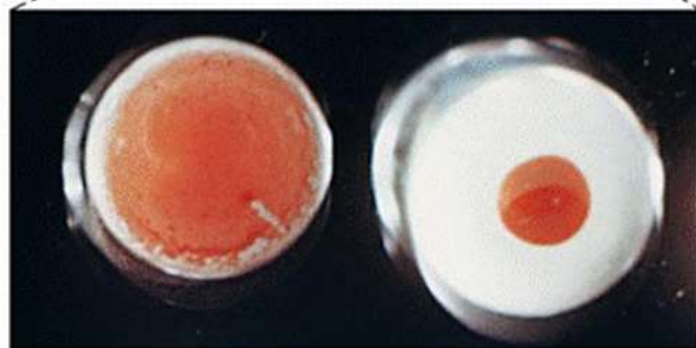
Slide Agglutination Test

- Used for serotyping (e.g. Salmonella)
- Antigen: isolated Salmonella in suspension
- Antibody: specific antisera against Salmonella
- Place test Salmonella in a drop of saline on a slide
- Add a drop of antiserum, mix and rock slide for approx. 1 minute
- Examine for agglutination

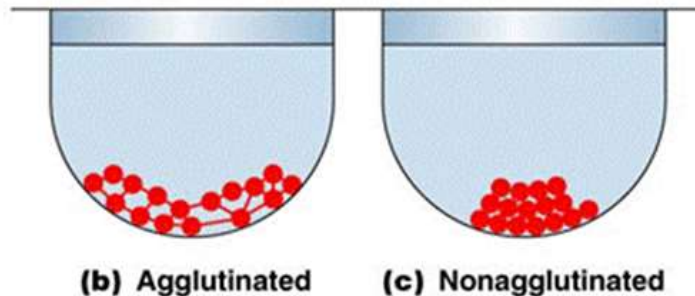


(a)

Enlarged photo of wells



Side view of wells



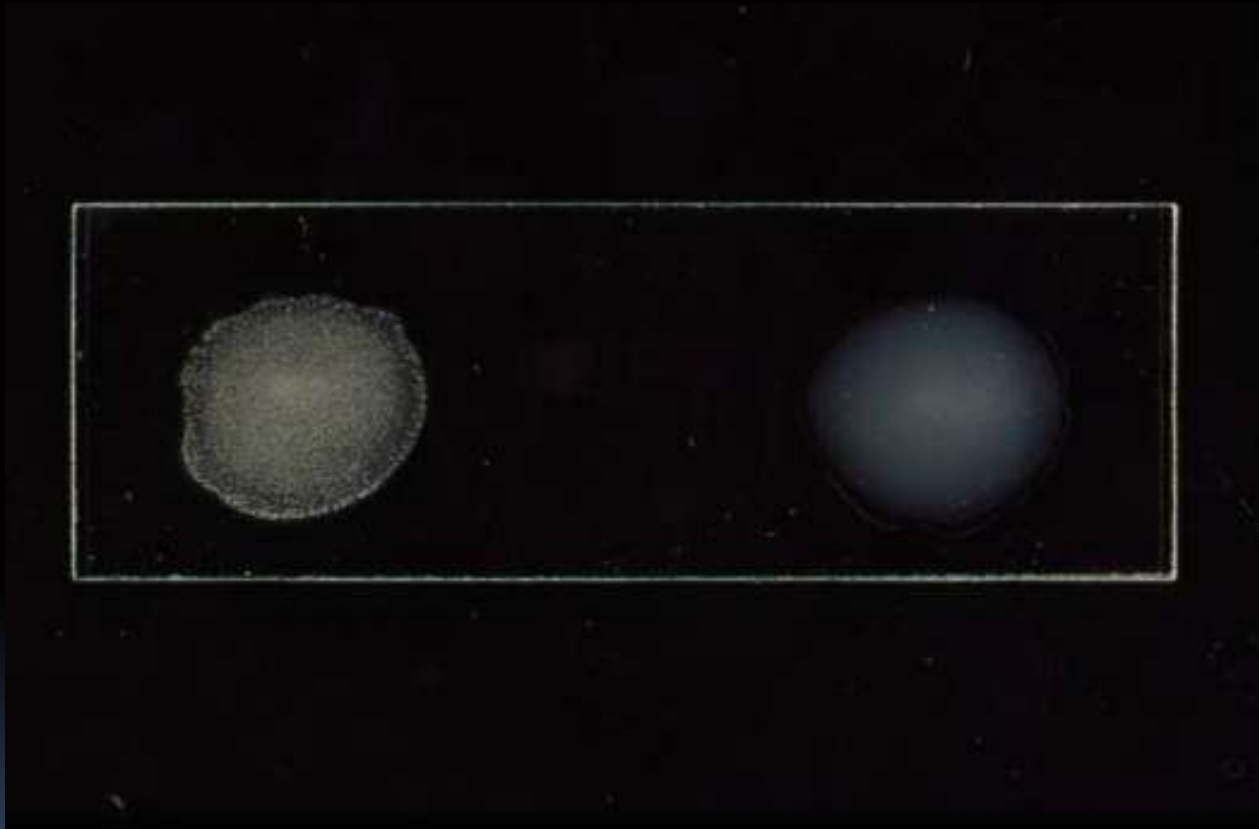
(a) Each well in this microtiter plate contains, from left to right, only half the concentration of serum that is contained in the preceding well. Each well contains the same concentration of particulate antigens, in this instance red blood cells.

(b) In a positive (agglutinated) reaction, sufficient antibodies are present in the serum to link the antigens together, forming a mat of antigen-antibody complexes on the bottom of the well.

(c) In a negative (nonagglutinated) reaction, not enough antibodies are present to cause the linking of antigens. The particulate antigens roll down the sloping sides of the well, forming a pellet at the bottom. In this example, the antibody titer is 160 because the well with a 1:160 concentration is the most dilute concentration that produces a positive reaction.

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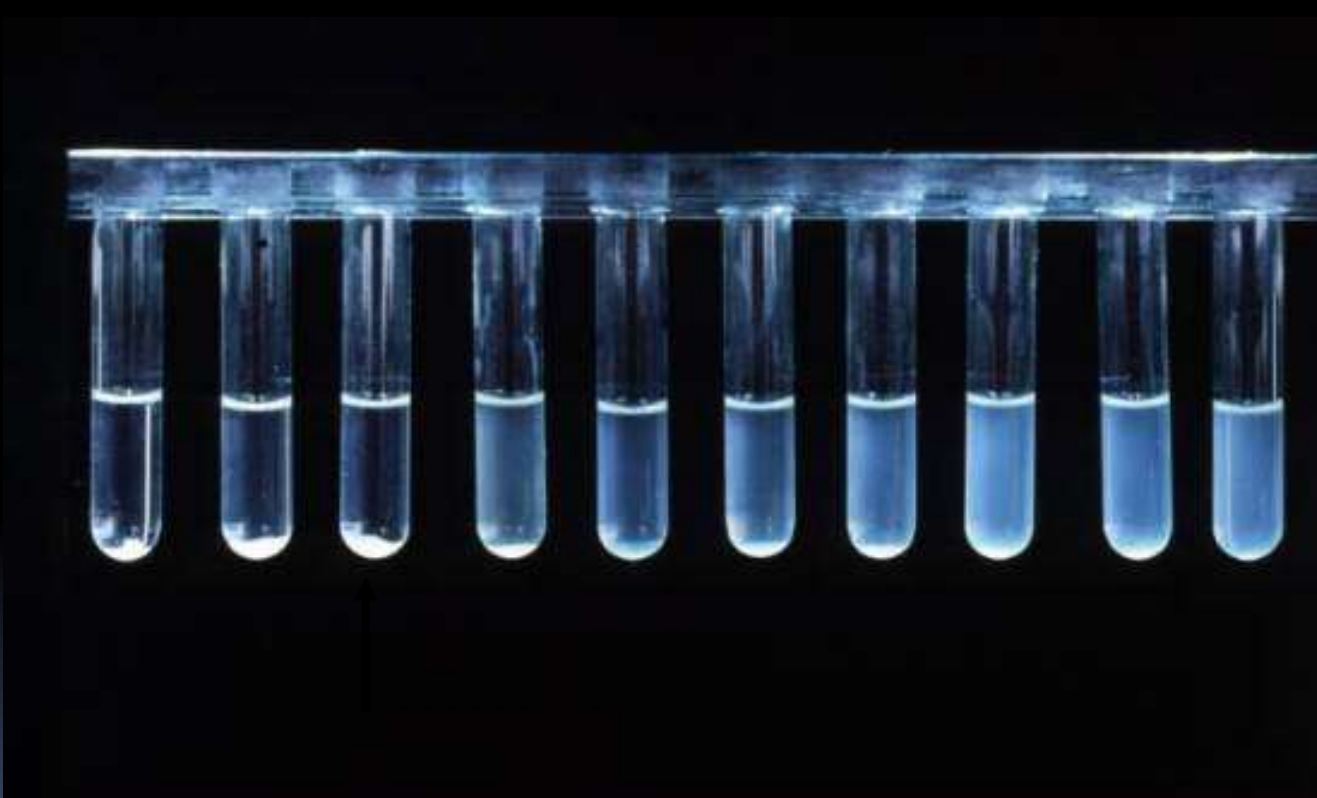
Slide Agglutination Test



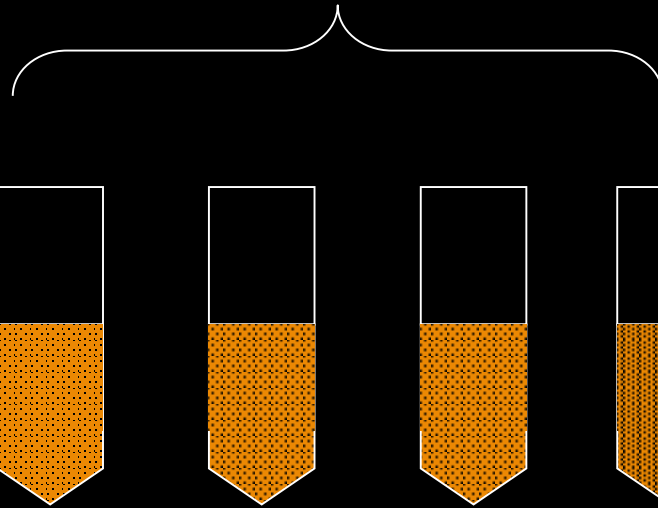
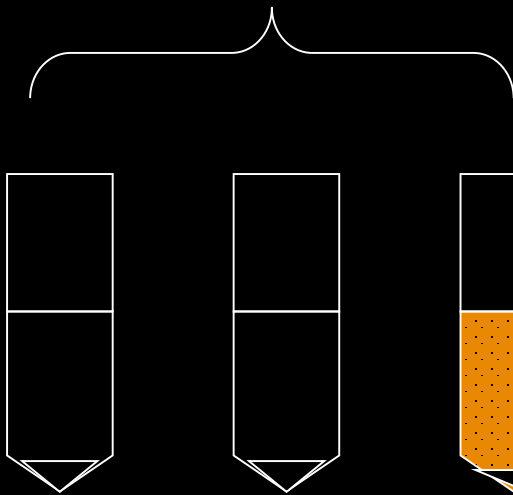
Tube Agglutination Test

- Also known as the standard agglutination test or serum agglutination test (SAT)
- Test serum is diluted in a series of tubes (doubling dilutions)
- Constant defined amount of antigen is then added to each tube and tubes incubated for ~20h @37°C
- Particular antigen clumps at the bottom of the test tube
- Test is read at 50% agglutination
- Quantitative
- Confirmatory test for ELISA reactors
- Example: Brucellosis screening , Widal Testing

Tube Agglutination Test



TUBE AGGLUTINATION TEST



1/10

1/20

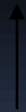
1/40

1/80

1/160

1/320


Neg. ctrl





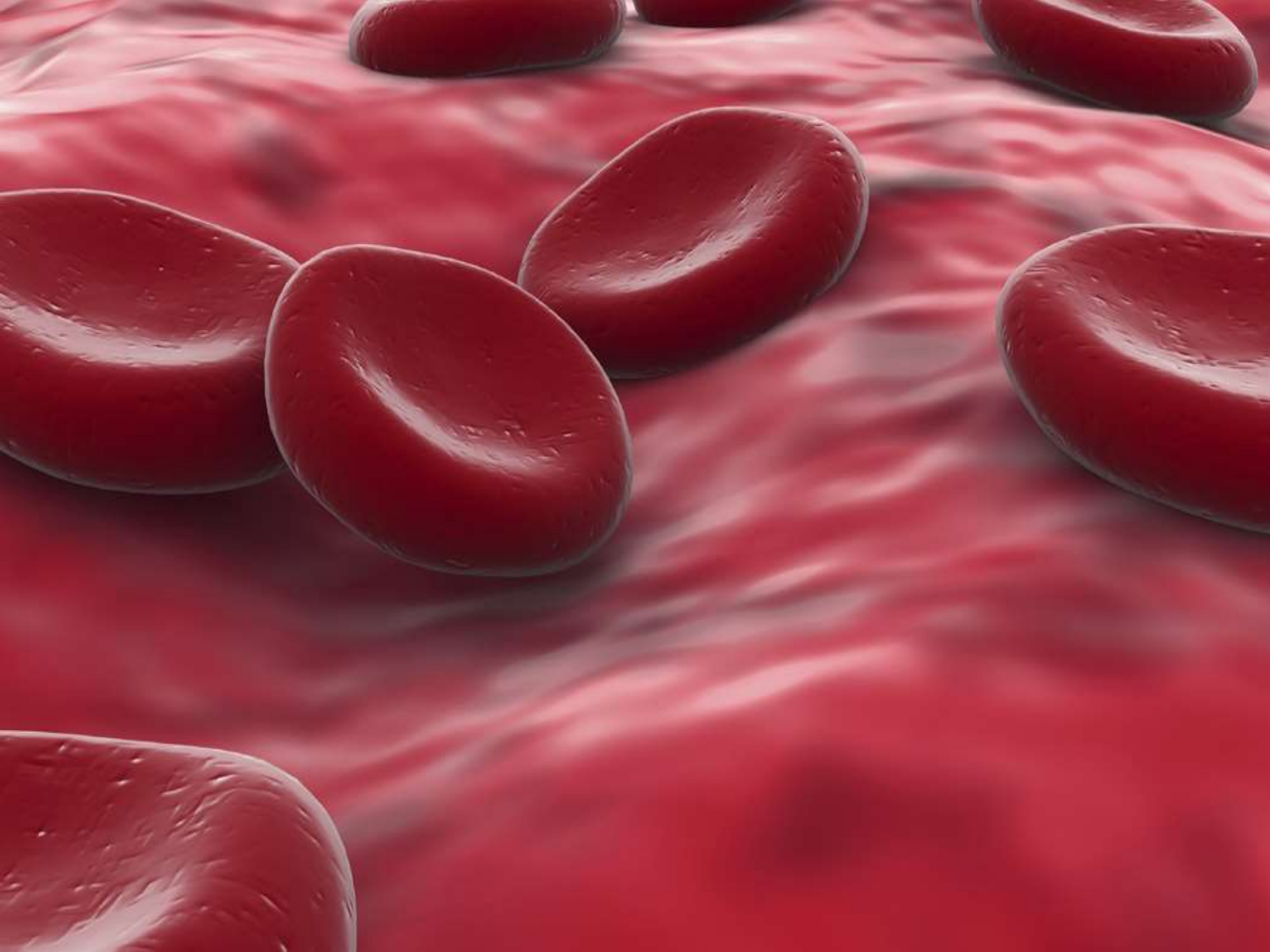
In this case, the titre is 1/40

Hemagglutination

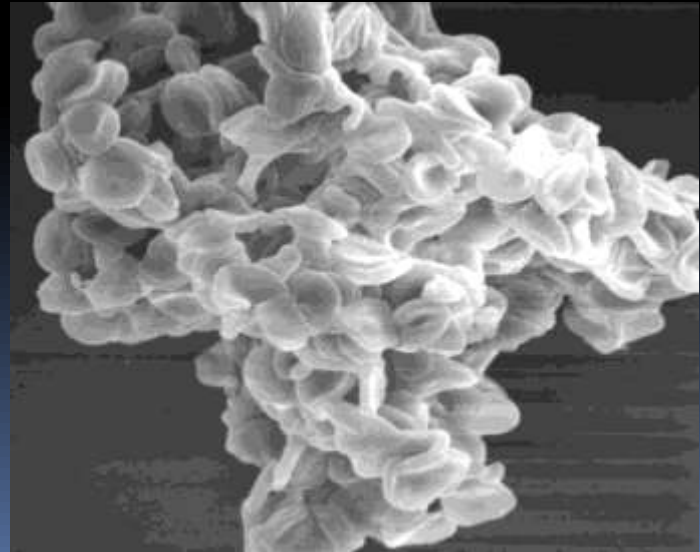
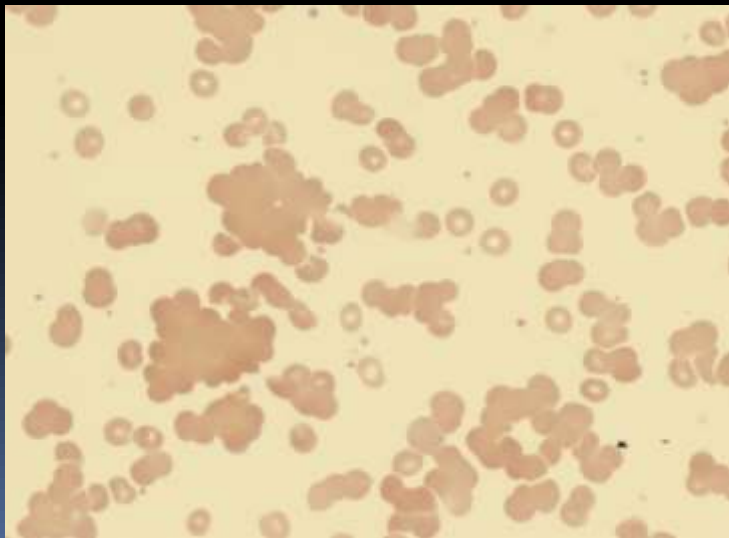
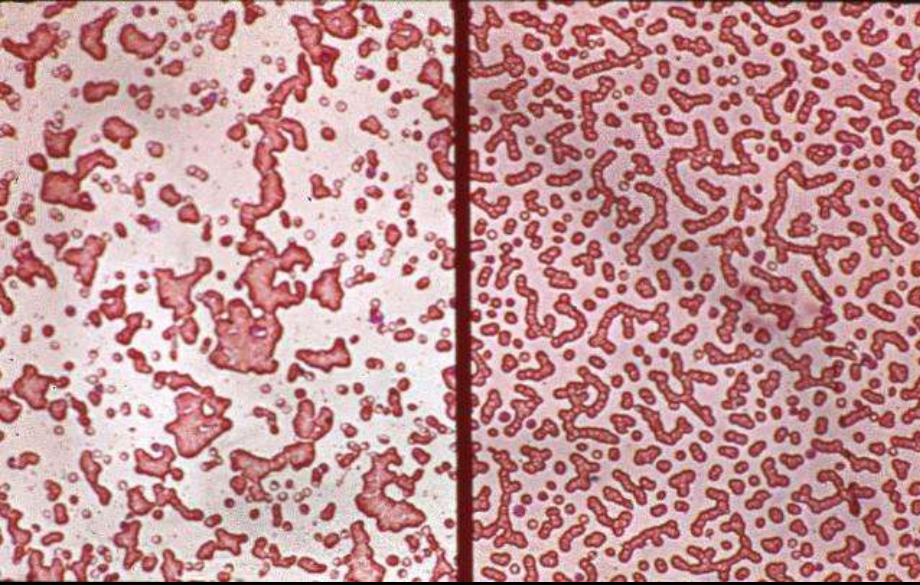
- If an agglutination reaction involves red blood cells
- Ex:
- ABO typing
 - one of the world's most frequently used immunoassays.
 - IgM type antisera
 - usually performed at room temperature without the need for any enhancement techniques.
 - Very simple to perform, is relatively sensitive, and is easy to read.

- 
- Can be interpreted using cell sedimentation pattern.
Microtiter well
 - Negative: dark red, smooth button at the bottom
 - Positive: have cells that are spread across the well's bottom, usually in a jagged pattern with an irregular edge.
-
- Test tubes
 - Negative: if the cell button can be evenly resuspended when shaken with no visible clumping
 - Positive: presence of cell button, can be graded to indicate the strength of the reaction

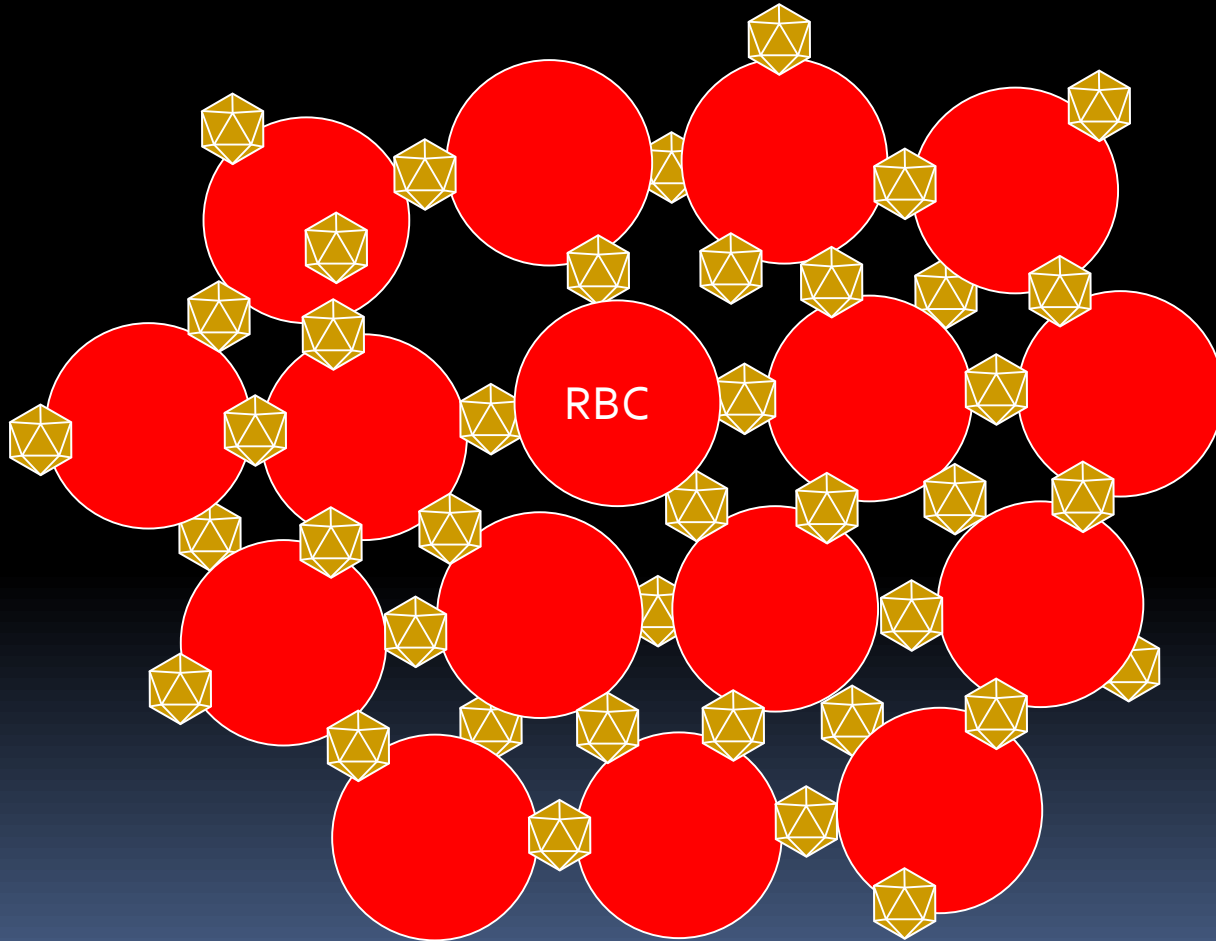
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- 
- Hemagglutination kits are now available for detection of antibodies to hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) I and II, to cite just a few examples.



Haemagglutination



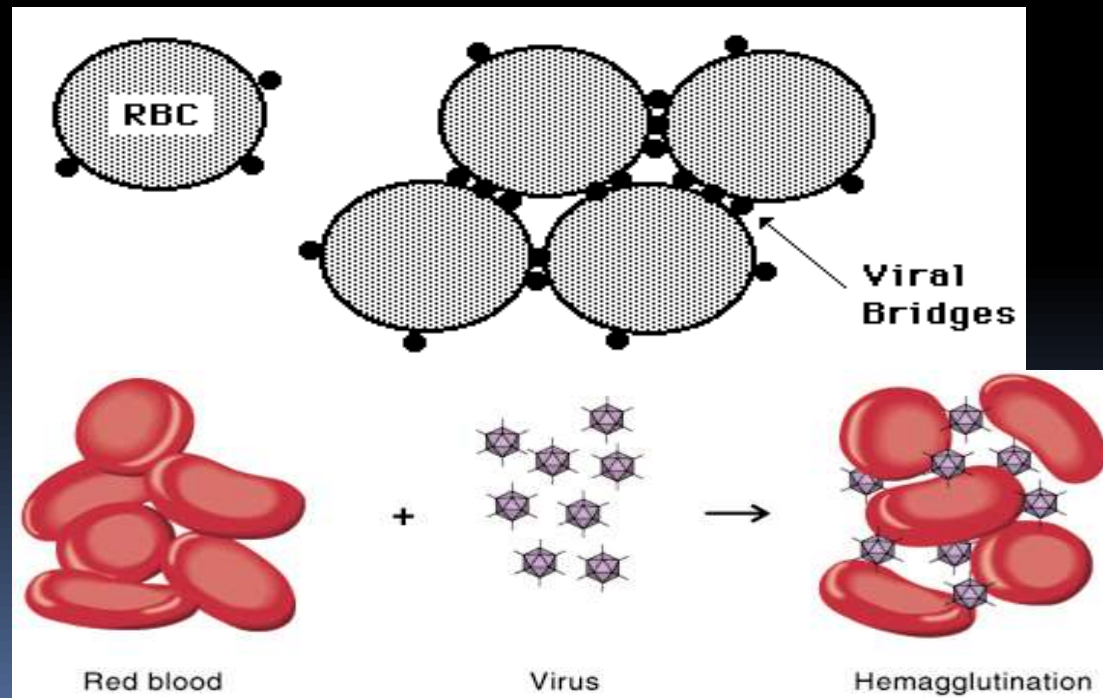
Haemagglutination



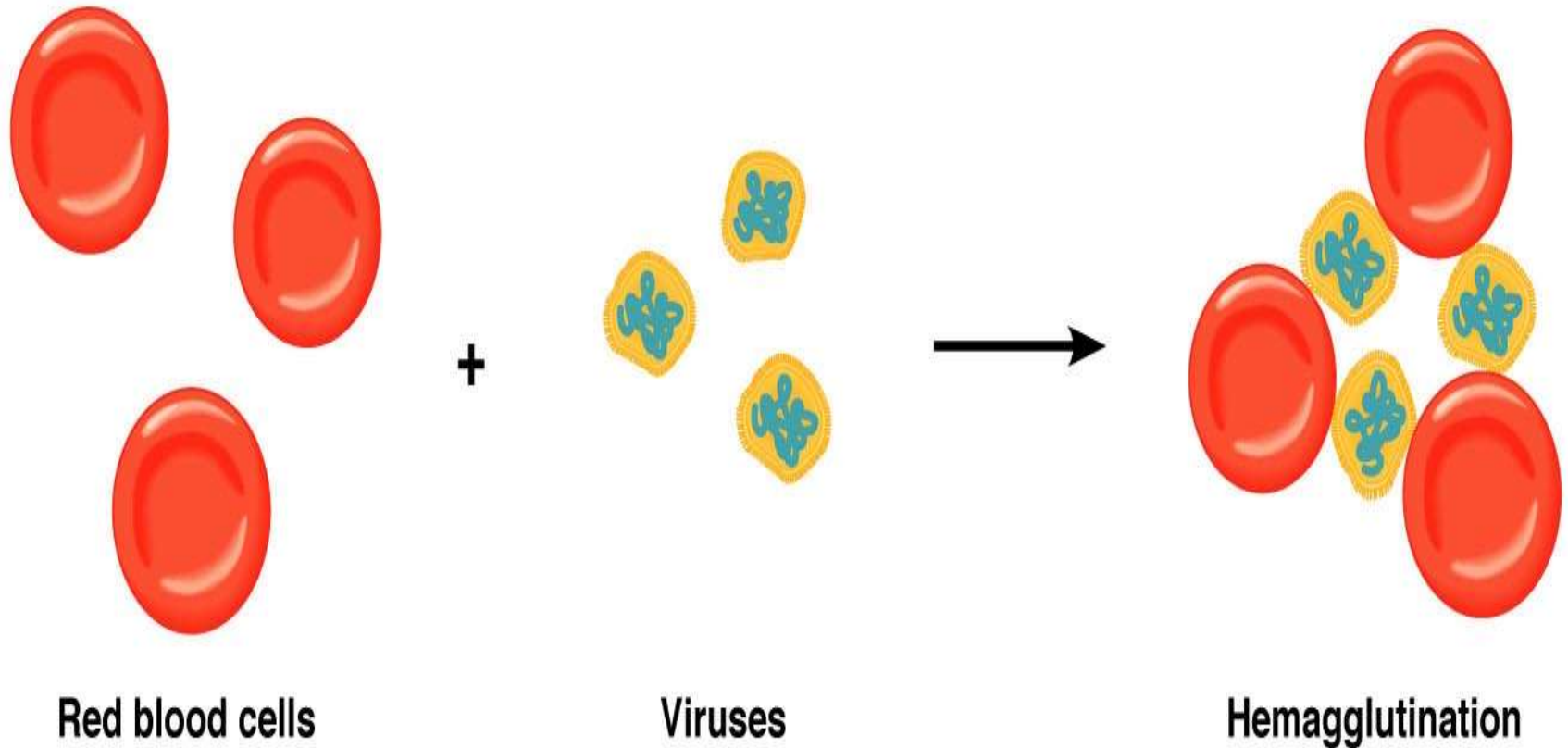
Viral Haemagglutination

- Some viruses and microbes contain proteins which bind to erythrocytes (red blood cells) causing them to clump together

- NDV
- Adenovirus III
- AIV
- IBV
- Mycoplasma



Viral Hemagglutination

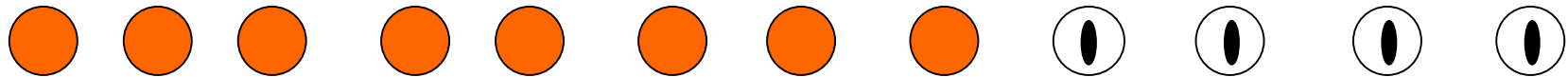


- the attachment of viral particles by their receptor sites to more than 1 cell.
- As more and more cells become attached in this manner agglutination becomes visible

Readings The results

- **Titer:** The maximum dilution that gives visible agglutination.
- **The end point:** is the well with the lowest concentration of the virus where there is haemagglutination

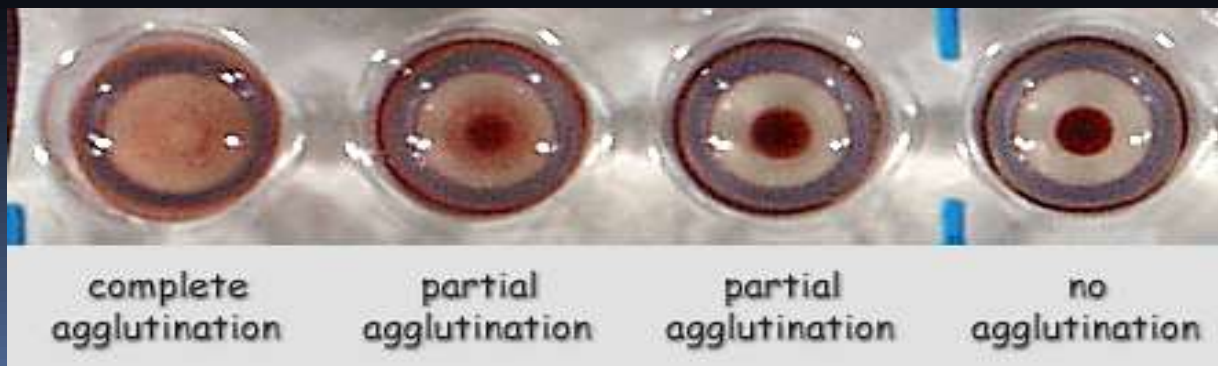
2 4 8 16 32 64 128 256 512 1024 2048 4096



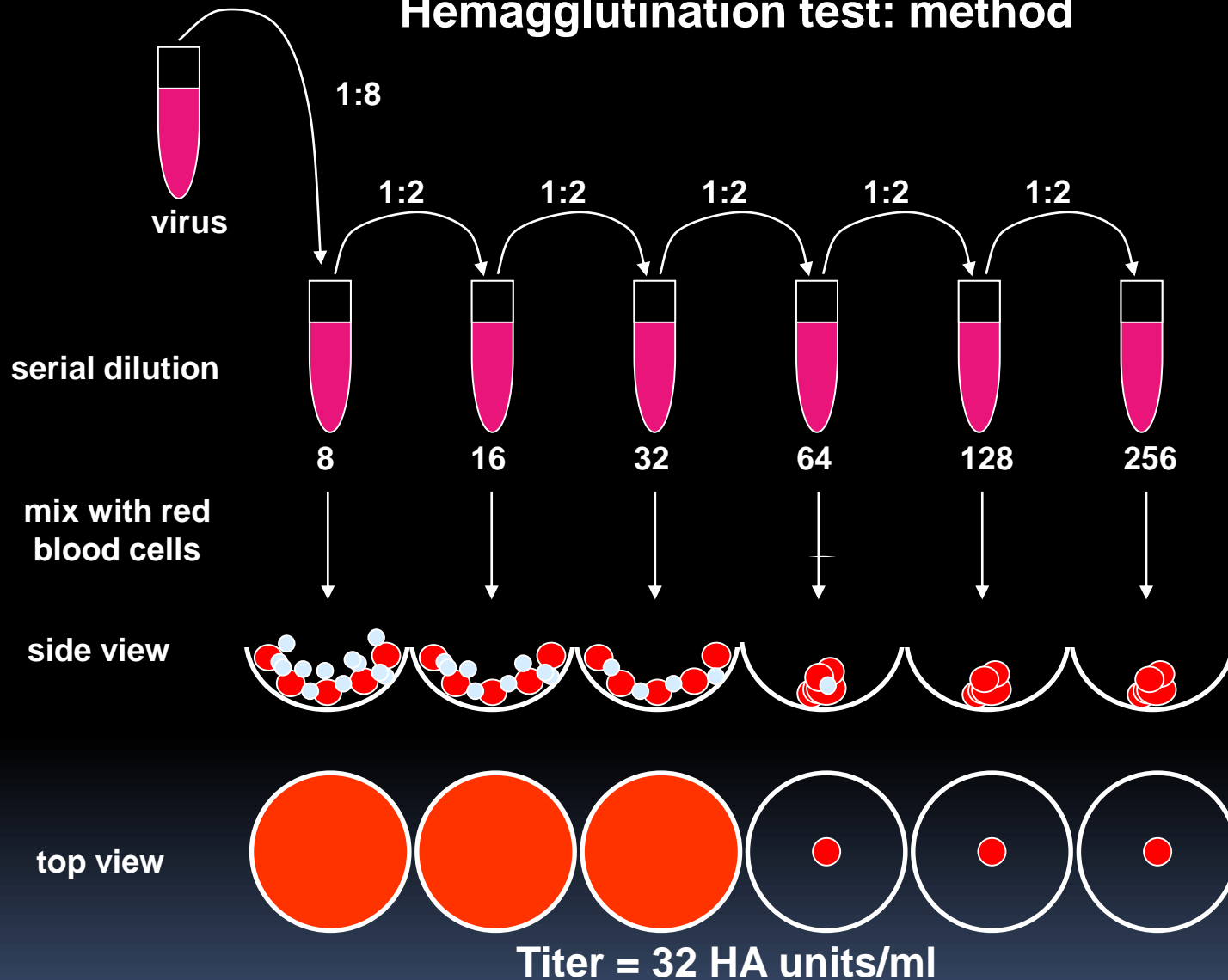
The HA titer of this virus in this row is 256 or 2^8
(1:256 dilution contains **(1 HA unit)** (one haemagglutinating unit))

Example of readings

Patient	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	Pos.	Neg.	Titer
1	●	●	●	●	●	●	○	○	○	○	●	○	64
2	●	●	●	○	○	○	○	○	○	○	●	○	8
3	●	●	●	●	●	●	●	●	●	○	●	○	512
4	○	○	○	○	○	○	○	○	○	○	●	○	<2
5	●	●	●	●	●	○	○	○	○	○	●	○	32
6	○	○	●	●	●	●	●	○	○	○	●	○	128
7	●	●	●	●	●	○	○	○	○	○	●	○	32
8	●	●	○	○	○	○	○	○	○	○	●	○	4

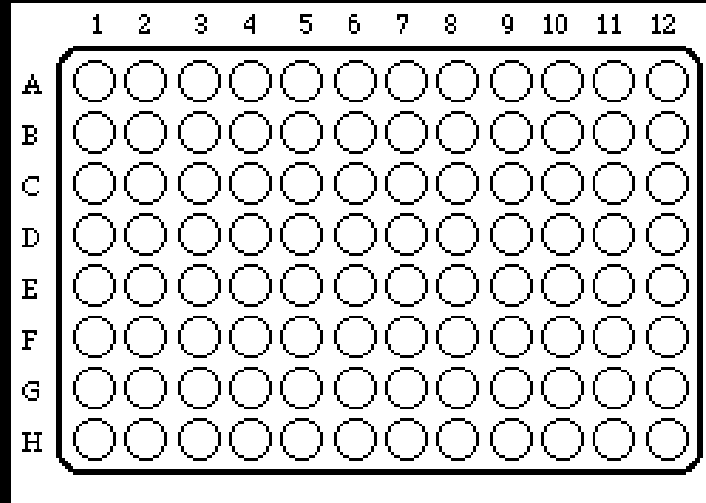


Hemagglutination test: method



One HA unit : minimum amount of virus that causes complete agglutination of RBCs

WHAT DO WE NEED?



PROCEDURE

(CONTROLS)

Always run four control rows:

_ **Positive:** Contains antibodies against the specific virus

_ **Negative:** Contains no antibodies against the specific virus

_ **Antigen**

_ **RBCs**

WASHING RBCs



Why do we have to wash RBCs?

- To obtain pure RBCs and to get rid from any other blood components such as WBCs, immune complexes, and Abs

Washing process

- Take place 4-5 time .
- Until get clear solution above the RBCs after centrifugation .
- Using PBS or normal saline .

Note :(avoid using water to wash RBCs because it will definitely lead to RBCs lyses)

Red Blood Cells

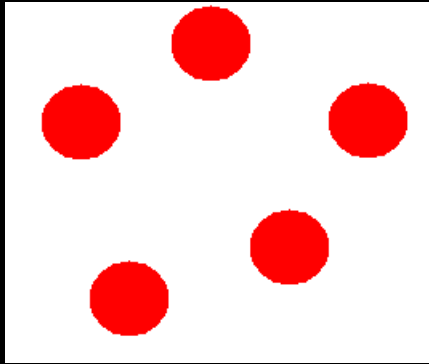


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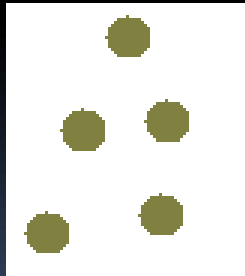
Procedure

- Obtain blood samples in tubes, spin at 1500 RPM for 5 minutes.
- Draw off the supernatant using Pasteur pipette.
- Add 2ml PBS to each tube and move to a clean test tube.
- Centrifuge again. Each time draw off the washing solution and add 10 ml PBS until the solution above the RBCs layer becomes clear.

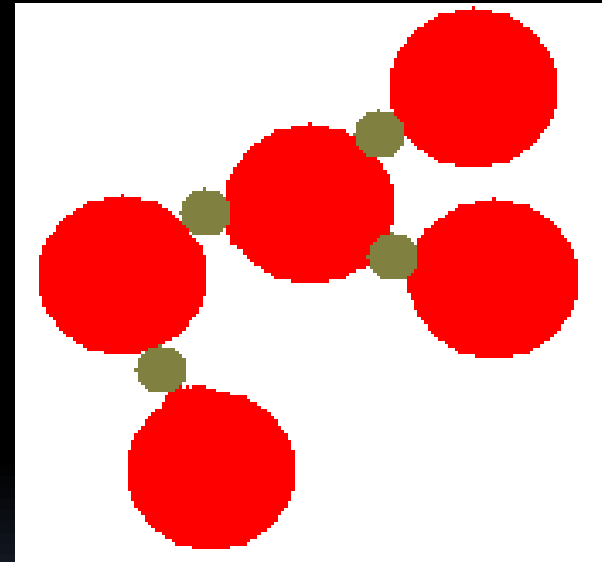
In the absence of anti-virus antibodies



Erythrocytes

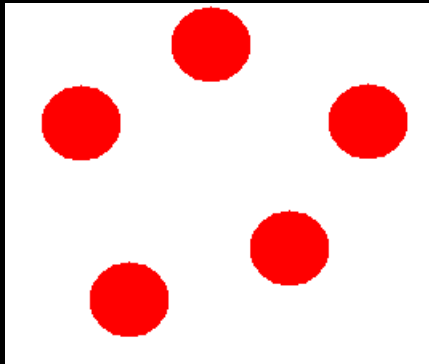


Virus

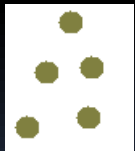


Virus agglutination of erythrocytes

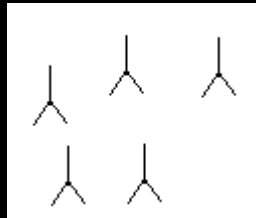
In the presence of anti-virus antibodies



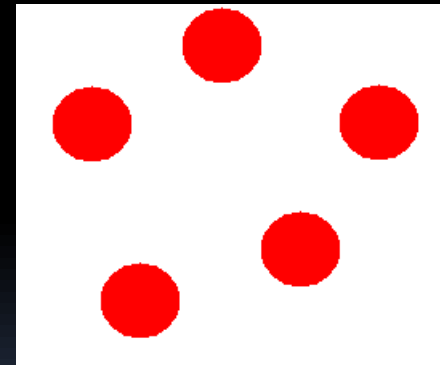
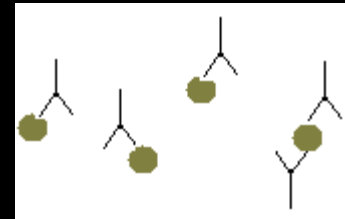
Erythrocytes



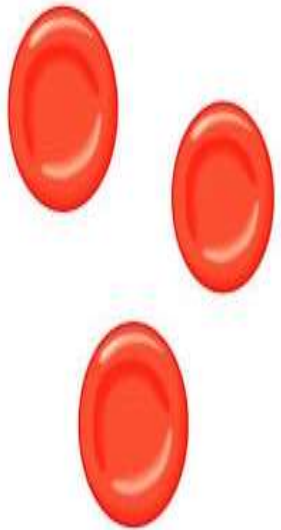
Virus



Anti-virus antibodies

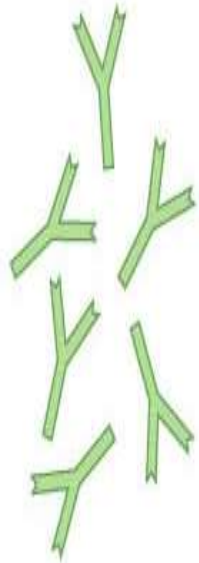


Viruses unable to bind to the erythrocytes



Red blood cells

+

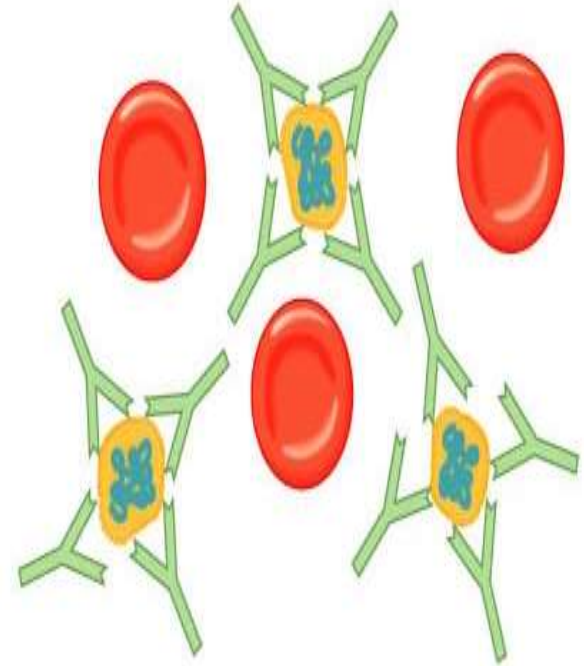


Antiviral antibodies
from serum

+




Viruses




Viruses neutralized and
hemagglutination inhibited

Passive Agglutination

- **Also known as Indirect agglutination**
- employs particles that are coated with antigens not normally found on their surfaces.
- A variety of particles, including erythrocytes, latex, gelatin, and silicates, are used for this purpose.
- The use of synthetic beads or particles provides the advantage of consistency, uniformity, and stability.¹
- Reactions are easy to read visually and give quick results.
- Particle sizes vary from 7 μm for red blood cells down to 0.8 μm or less for fine latex particles.¹¹

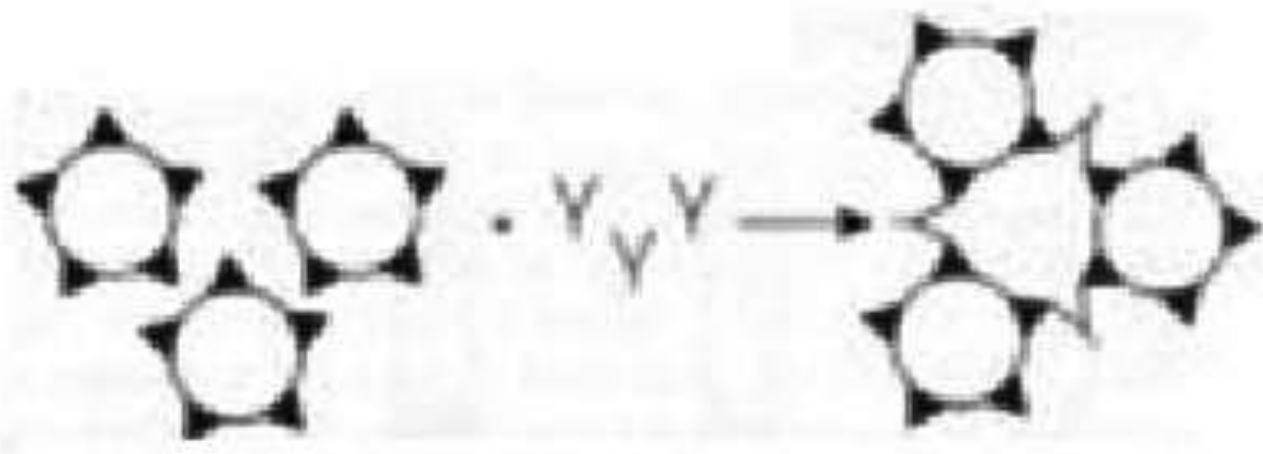
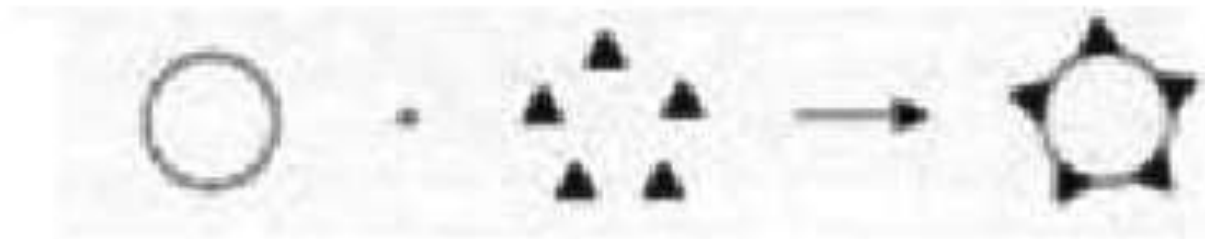
- 
- Many antigens, especially polysaccharides, adsorb to red blood cells spontaneously, so they are relatively easy to manipulate.
 - Problems encountered with the use of erythrocytes as carrier particles include the possibility of crossreactivity, especially with heterophile antibody if the cells used are nonhuman.

- 
- Ex.
 - Rheumatoid factor;
 - Antinuclear antibody occurring in the disease lupus erythematosus;
 - Antibodies to group A streptococcus antigens;
 - Antibodies to *trichinella spiralis*;
 - Antibodies to *treponema pallidum*;
 - Antibodies to viruses such as cytomegalovirus, rubella, varicella-zoster, and HIV-1/ HIV-2.

➤ **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

Passive Agglutination

- An agglutination reaction that employs particles that are coated with antigens not normally found in the cell surfaces
- Particle carriers include:
 - Red blood cells
 - Polystyrene latex
 - Bentonite
 - charcoal

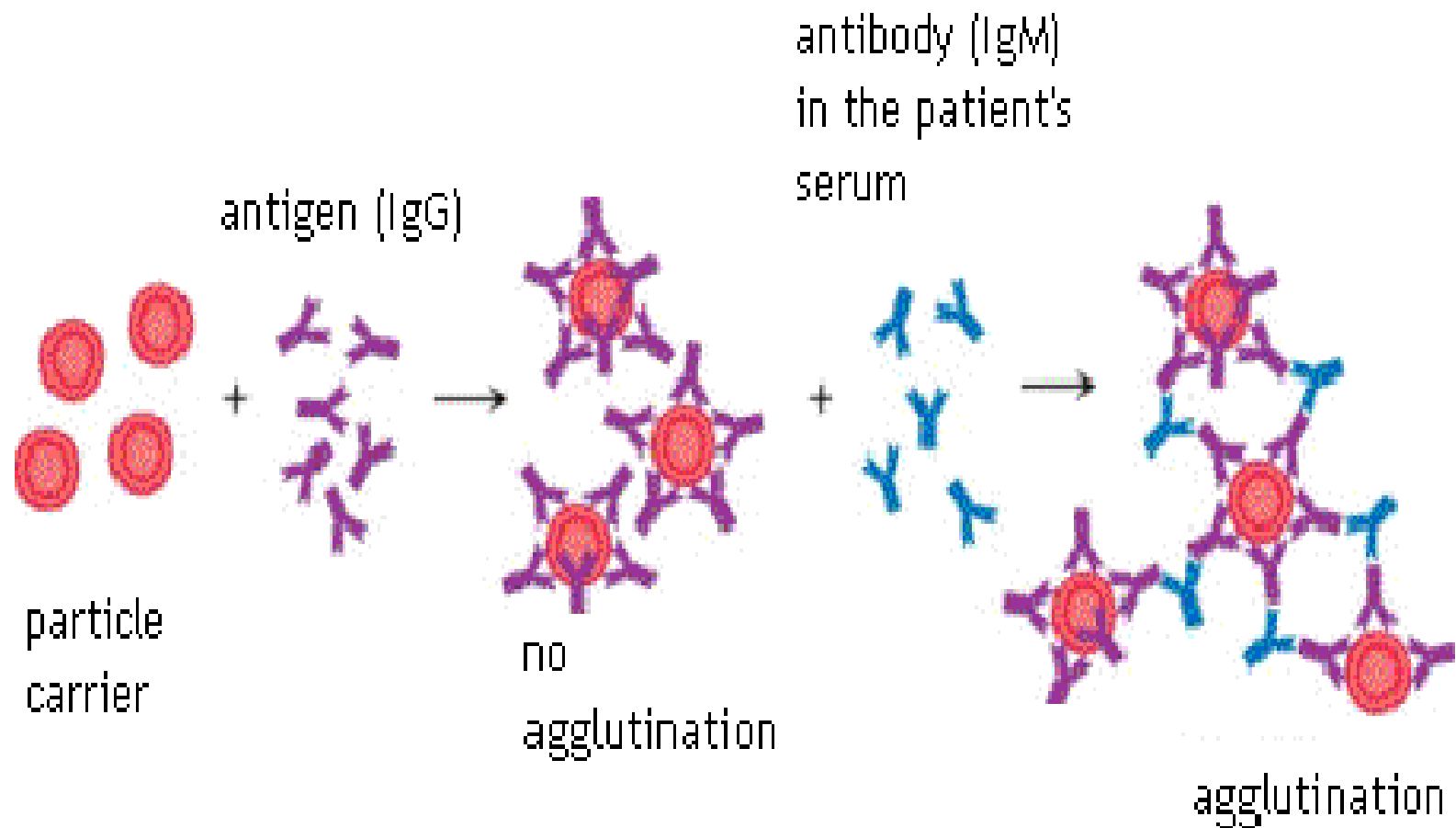
Passive Agglutination

- Passive agglutination has been used in the detection of :
 - Rheumatoid factor
 - Antinuclear antibody in LE
 - Ab to group A streptococcus antigens
 - Ab to *Trichinella spiralis*

Passive Agglutination Test


- Converting a precipitating test to an agglutinating test
- Chemically link soluble antigen to inert particles such as LATEX or RBC
- Addition of specific antibody will cause the particles to agglutinate
- Reverse PAT: antibody linked to LATEX
e.g. Lancefield grouping in Streptococci.

The latex particles are coated with IgG and mixed with the patient's serum



Reverse Passive Agglutination

- In **reverse passive agglutination**, antibody rather than antigen is attached to a carrier particle.
- The antibody must still be reactive and is joined in such a manner that the active sites are facing outward.
- Adsorption may be spontaneous, or it may require some of the same manipulation as is used for antigen attachment.
- Principle:
 - Latex particles coated with antibody are reacted with a patient sample containing the suspected antigen.

- 
- Numerous kits are available today for the rapid identification of antigens from such infectious agents as group B streptococcus
 - *Staphylococcus aureus*
 - *Neisseria meningitidis*
 - streptococcal groups A and B
 - *Haemophilus influenzae*
 - Rotavirus
 - *Cryptococcus neoformans*
 - *Vibrio cholera* 01
 - *Leptospira*

 - Rapid agglutination tests have found the widest application in detecting soluble antigens in urine, spinal fluid, and serum.


REVERSE PASSIVE Agglutination Tests


- **PRINCIPLE:** latex particles coated with antibody are reacted with a patient sample containing suspected antigen





Agglutination Inhibition

- based on competition between particulate and soluble antigens for limited antibody-combining sites, and a lack of agglutination is an indicator of a positive reaction.
- Typically, this type of reaction involves haptens that are complexed to proteins; the hapten–protein conjugate is then attached to a carrier particle.
- The patient sample is first reacted with a limited amount of reagent antibody that is specific for the hapten being tested.

- 
- Indicator particles that contain the same hapten one wishes to measure in the patient are then added.
 - If the patient sample has no free hapten, the reagent antibody is able to combine with the carrier particles and produce a visible agglutination.
 - In this case, however, agglutination is a negative reaction, indicating that the patient did not have sufficient hapten to inhibit the secondary reaction

- 
- Either antigen or antibody can be attached to the particles.
 - The sensitivity of the reaction is governed by the avidity of the antibody itself. It can be a highly sensitive assay capable of detecting small quantities of antigen.
 - Ex.
 - Detection of illicit drugs such as cocaine or heroin

- 
- **Hemagglutination inhibition** reactions use the same principle, except red blood cells are the indicator particles.
 - Ex:
 - used to detect antibodies to certain viruses, such as rubella, mumps, measles, influenza, parainfluenza, HBV, herpes virus, respiratory syncytial virus, and adenovirus.

- 
- Red blood cells have naturally occurring viral receptors. When virus is present, spontaneous agglutination occurs, because the virus particles link the red blood cells together. Presence of patient antibody inhibits the agglutination reaction.

Agglutination

Inhibition

- Based on the competition between particulate and soluble antigens for limited antibody combining site
- Lack of agglutination is indicator of a positive reaction
- Usually involves haptens complexed with proteins

Agglutination Inhibition Tests

- **Pregnancy Testing**
 - classic example of agglutination inhibition
- ▣ **Human chorionic gonadotropin (hCG)**
 - Appears in serum and urine early in pregnancy



Agglutination Inhibition

Urine



Antiserum

No hCG in urine:
Anti-hCG free

hCG in urine:
Anti-hCG neutralized

Carriers coated with hCG added

Carriers coated with hCG added

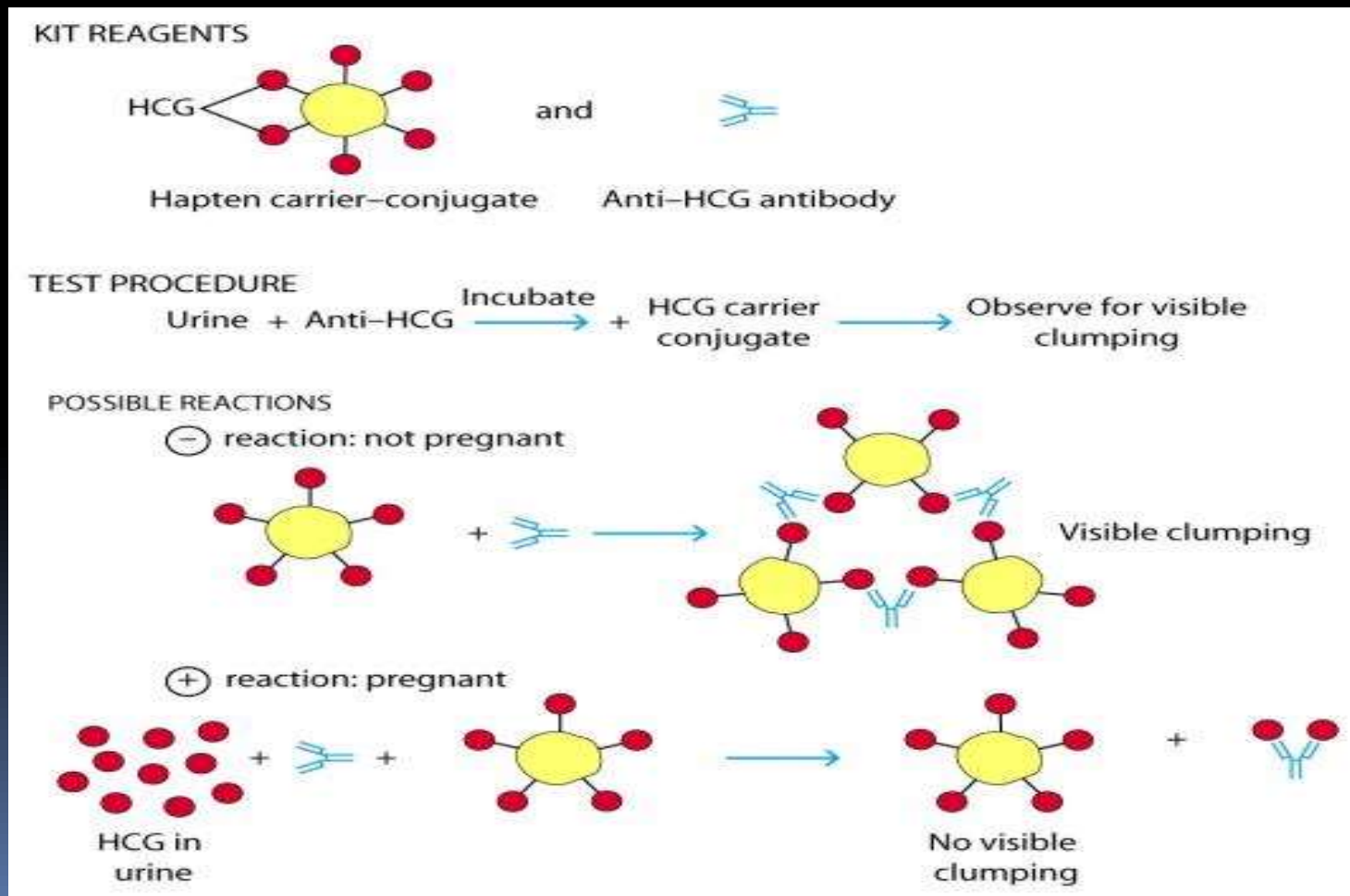
AGGLUTINATION of carriers:
Negative test for hCG
NOT PREGNANT

NO AGGLUTINATION of carriers:
Positive test for hCG
PREGNANT

Agglutination Inhibition:

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

Example: First home pregnancy test



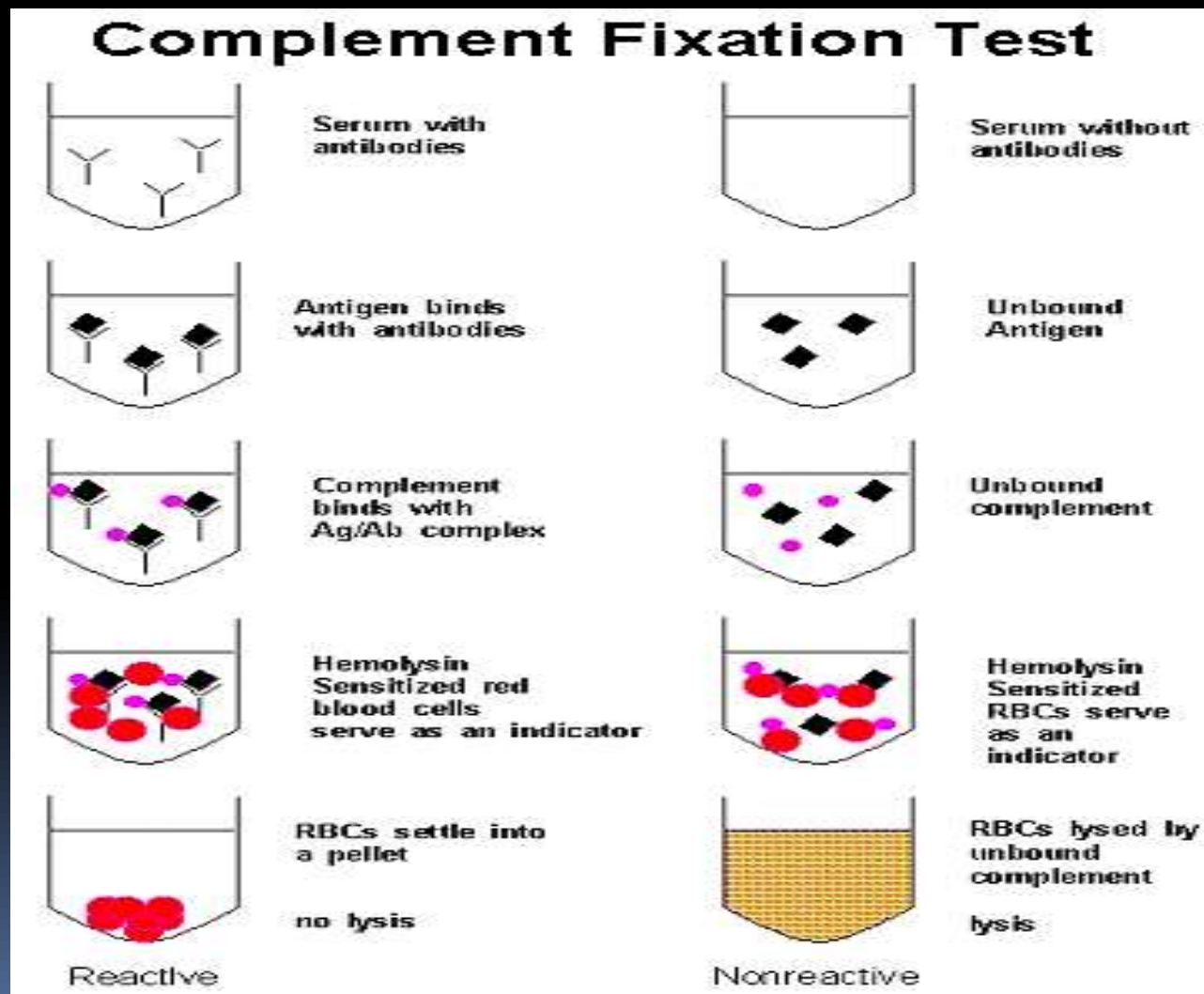
Complement Fixation:

- *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 every 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.




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



HEMAGGLUTINATION INHIBITION

Patient Sample } Human IgG expressing the G1 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

Patient Sample } Human IgG with other heavy chain isotope +  G1 epitope specific +  = **Agglutination**

THIS IS A NEGATIVE REACTION

Coagglutination

- uses bacteria as the inert particles to which antibody is attached.
- *Staphylococcus aureus*
 - is most frequently used
 - contains protein on its outer surface, called *protein A*, which naturally adsorbs the fragment crystallizable (FC) portion of antibody molecules.
 - The active sites face outward and are capable of reacting with specific antigen

- These particles exhibit greater stability than latex particles and are more refractory to changes in ionic strength.
- However, because bacteria are not colored, reactions are often difficult to read.
- Such testing is highly specific, but it may not be as sensitive for detecting small quantities of antigen, as is latex agglutination.¹¹
- Ex:
 - identification of streptococci
 - *Neisseria meningitidis*,
 - *Neisseria gonorrhoeae*
 - *Vibrio cholera* 0139
 - *Haemophilus influenzae*

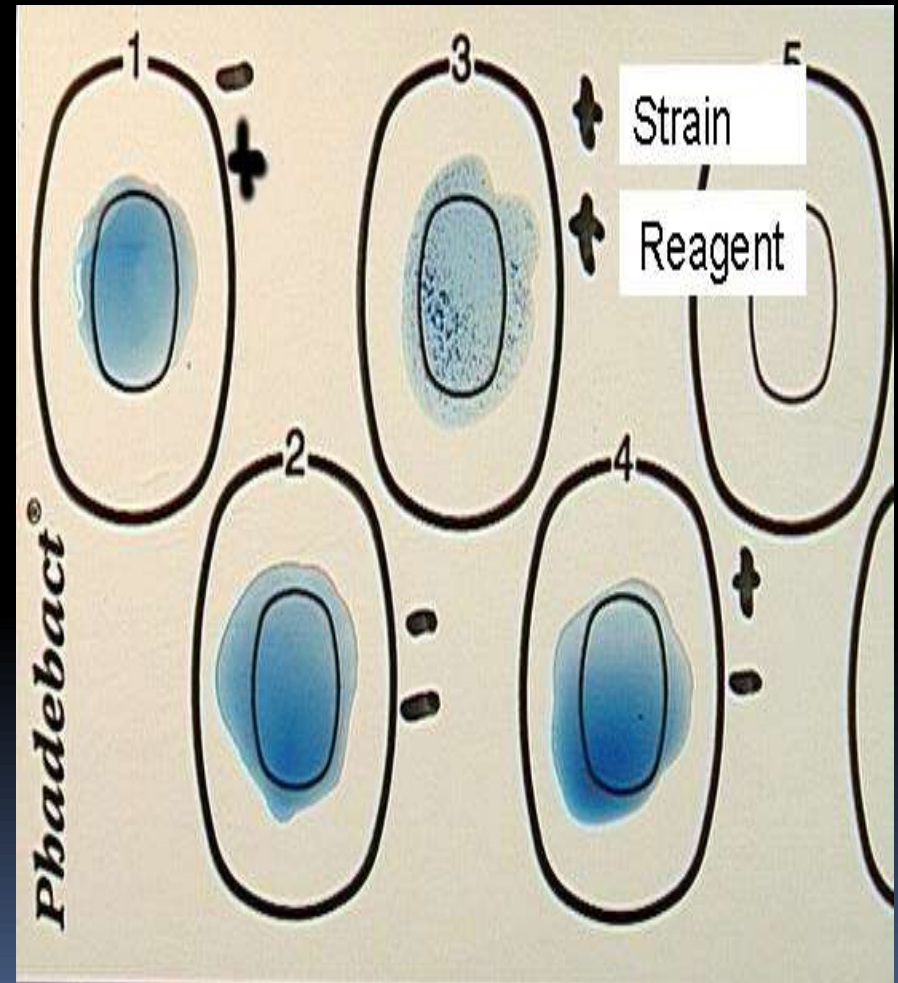
Co-agglutination

- Co agglutination is similar to the latex agglutination technique for detecting antigen (described above). Protein A, a uniformly distributed cell wall component of *Staphylococcus aureus*, is able to bind to the Fc region of most IgG isotype antibodies leaving the Fab region free to interact with antigens present in the applied specimens. The visible agglutination of the *S. Aureus* particles indicates the antigen-antibody reactions

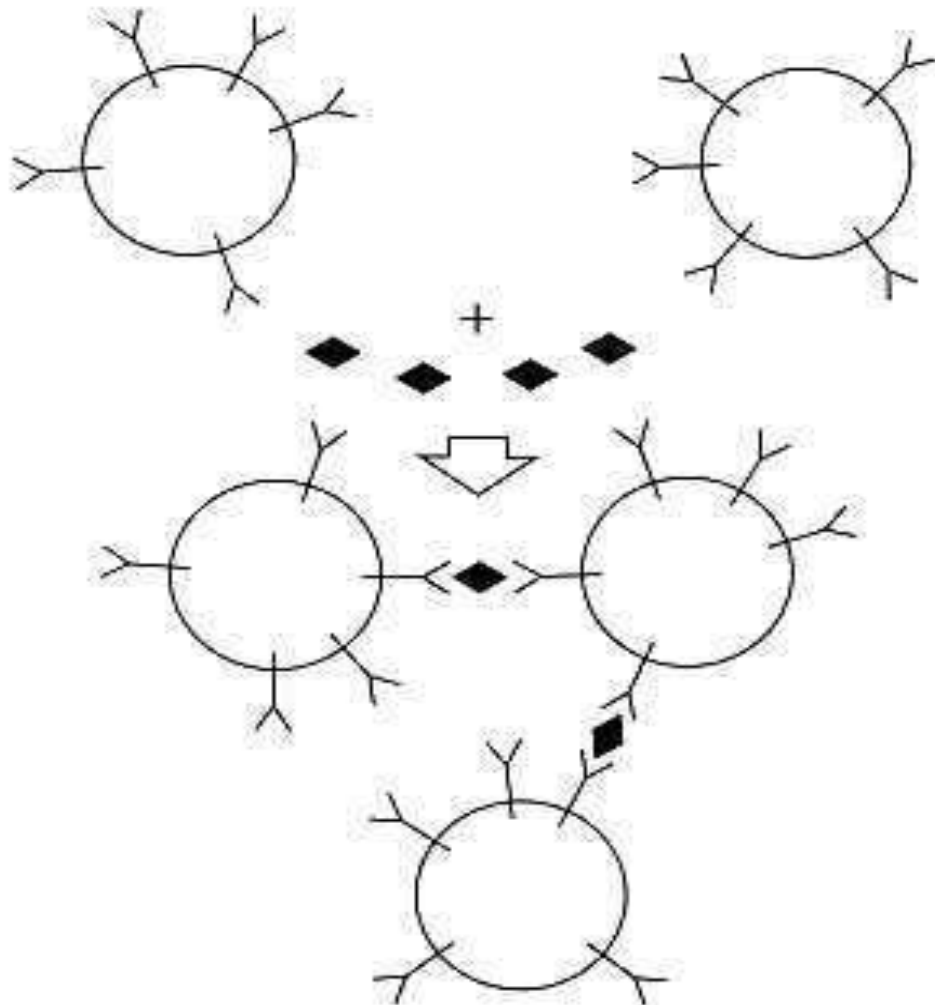


Coagglutination

- Name given to systems using inert bacteria as the inert particles to which the antibody is attached
- *S.aureus*: most frequently used because it has protein A in its outer surface that naturally adsorbs the Fc portion of the antibody



Highly specific but not very sensitive
in detecting small quantities of



Specific antibody
bound to particles

+

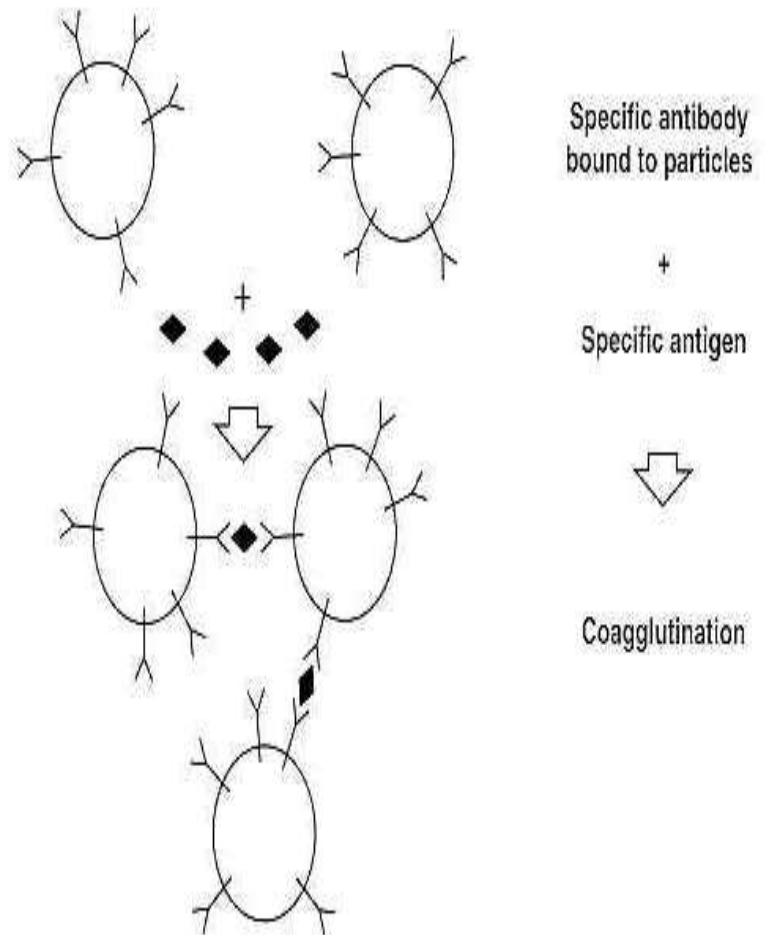
Specific antigen



Coagglutination


Co agglutination Test


Agglutination test in which inert particles (latex beads or heat-killed *S aureus* Cowan 1 strain with protein A) are coated with antibody to any of a variety of antigens and then used to detect the antigen in specimens or in isolated bacteria.



ANTIGLOBULIN-MEDIATED AGGLUTINATION

- antihuman globulin test(AHG)
- also known as the *Coombs' test*
- is a technique that detects nonagglutinating antibody by means of coupling with a second antibody.
- It remains one of the most widely used procedures in blood banking.

- 
- The key component of the test is antibody to human globulin that is made in animals or by means of hybridoma techniques.
 - Such antibody will react with the FC portion of the human antibody attached to red blood cells.
 - Agglutination takes place because the antihuman globulin is able to bridge the distance between cells that IgG alone cannot do.


- 
- The strength of the reaction is proportional to the amount of antibody coating the red blood cells.
 - The Coombs' test can be divided into two different types:
 - Direct
 - Indirect


Direct Antiglobulin Test

- used to demonstrate in vivo attachment of antibody or complement to an individual's red blood cells.
- This test serves as an indicator of autoimmune hemolytic anemia, hemolytic disease of the newborn, sensitization of red blood cells caused by the presence of drugs, or a transfusion reaction.
- The test is called *direct*, because red blood cells are tested directly as they come from the body.

Indirect Antiglobulin Test

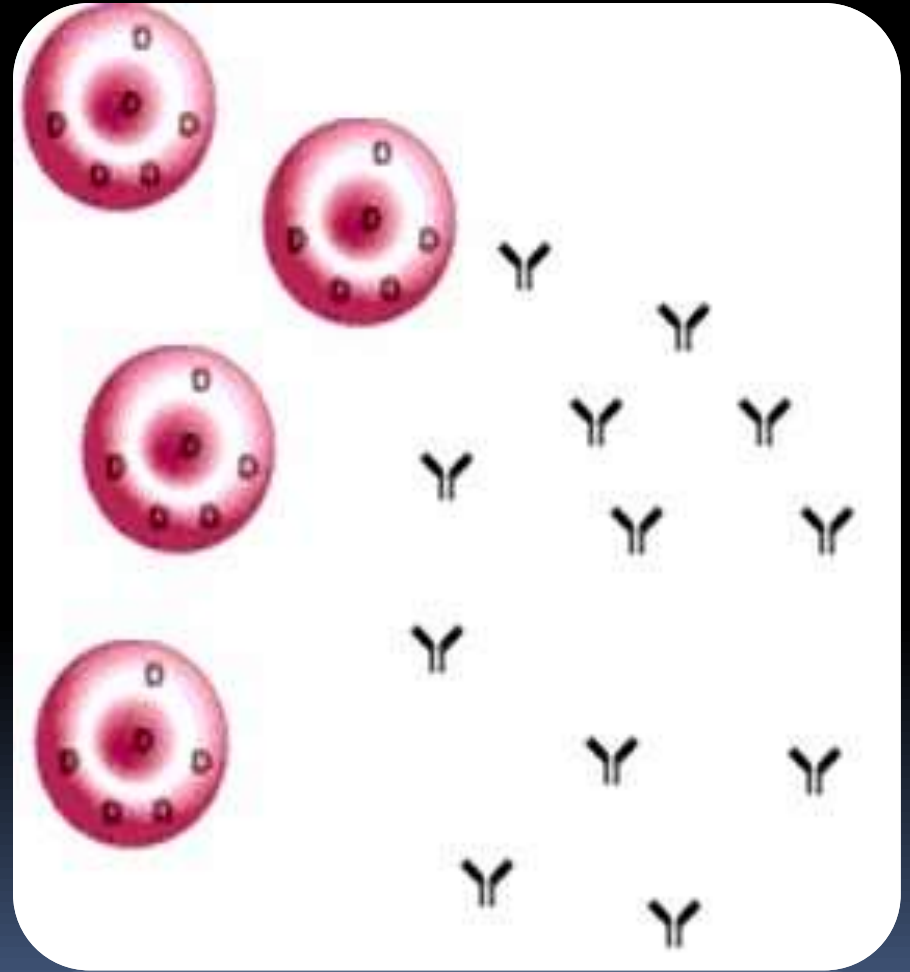
- The **indirect antiglobulin test**, or indirect Coombs' test
- is used to determine the presence of a particular antibody in a patient, or it can be used to type patient red blood cells for specific blood group antigens.
- This is a two-step process, in which washed red blood cells and antibody are allowed to combine at 37°C , and the cells are then carefully washed again to remove any unbound antibody.
- When antihuman globulin is added, a visible reaction occurs where antibody has been specifically bound.

- 
- This test is most often used to check for the presence of clinically significant alloantibody in patient serum when performing compatibility testing for a blood transfusion
 - In this case, patient serum is used to combine with reagent red blood cells of known antigenicity. All reactions are run at 37°C to detect clinically significant antibodies.
 - Cells are then washed, and antihuman globulin is added. Tubes are centrifuged and read for agglutination.

- 
- Possible sources of error in performing the Coombs' test include failure to wash cells, improper centrifugation, failure to add test serum or antihuman globulin, and use of expired reagents or those that have not been properly stored.
 - In addition, an improper concentration of red cells may alter the results. Too heavy a red cell concentration may mask agglutination, while too light a concentration will make the reaction hard to read.
 - Thus, it is important to use quality controls and to interpret results carefully.

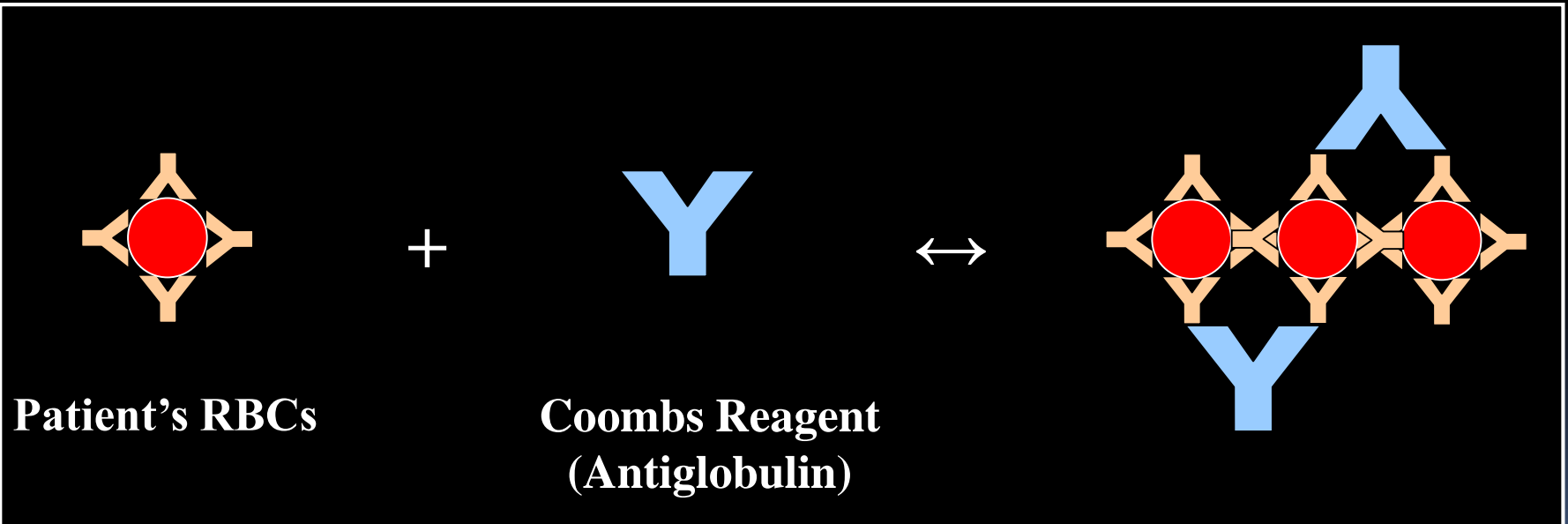
Coombs Test an Agglutination Test

- The Coombs test is actually two separate tests: the "direct" and "indirect" Coombs tests. Both aim to identify autoimmune haemolysis of red blood cells (erythrocytes).



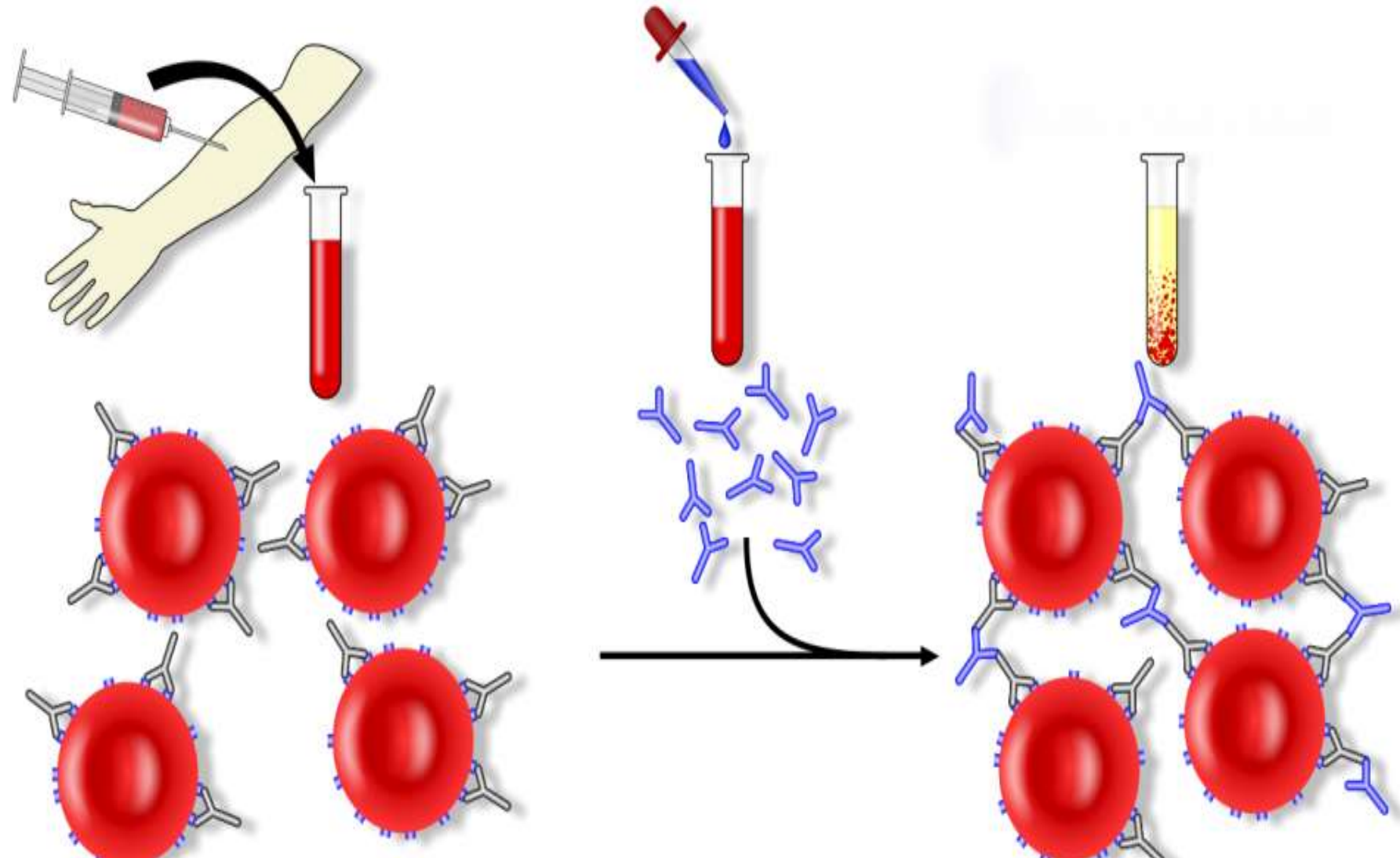
Coombs (Antiglobulin) Tests

- Incomplete Ab
- Direct Coombs Test
 - Detects antibodies on erythrocytes



Coombs Test

Direct ant globulin test (also



Coombs (Antiglobulin) Tests

- Indirect Coombs Test

- Detects anti-erythrocyte antibodies in serum

Step 1



Step 2



Application of Coombs (Antiglobulin) Tests

- Applications
 - Detection of anti-Rh Ab
 - Autoimmune hemolytic anemia

