

# Basic Immunologic Procedures

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Complex Serological Tests

# Classification of antigen-antibody interactions:

## 1. Primary serological tests: (Marker techniques) e.g.

- Enzyme linked immunosorbent assay (ELISA)
- Immuno fluorescent 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

## 3. Tertiary serological test: e.g.

- Determination of the protective value of an anti-serum in an animal.

# Complex Serological Tests

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- Procedures involving agglutination, and explaining the
- principle of each such as:
  - Hemagglutination inhibition.
  - Passive indirect agglutination.
  - Coombs (antiglobulin) "direct and indirect" tests.

# A. Agglutination tests:

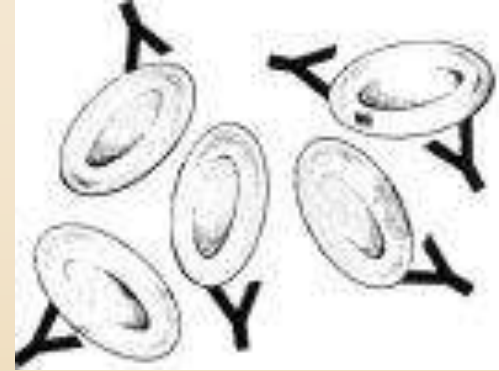
## 1. Agglutination/Hemagglutination:

When the antigen is particulate, the reaction of an antibody with the antigen can be detected by agglutination (clumping) of the antigen.

- **Definition** – the clumping together of antigen bearing cells, microorganisms or particles in the presence of specific antibodies.
- **Antigen particles** may be **erythrocyte (RBCs)** (hemagglutination), bacterial cells (coagglutination) or inert particles such as latex or charcoal coated with antigen or antibody
- **TWO step process:**
  - Sensitization
  - Lattice formation

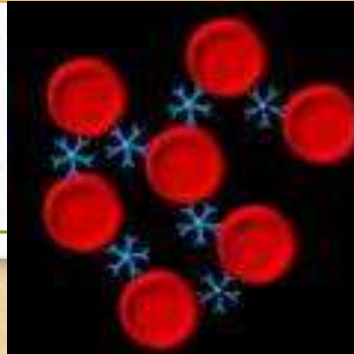
## Sensitization – not visible!

- Attachment of a single antibody to a single antigen.
- Rapid and reversible
- Affected by antibody affinity and avidity.
- IgM much more efficient than IgG.
- All antibodies can theoretically agglutinate particulate antigens but IgM, due to its high valence, is particularly good agglutinin and one sometimes infers that an antibody may be of the IgM class if it is a good agglutinating antibody.
- Antigen bearing surfaces must have sufficient quantities present, if few are present or are obscured less likely to interact with antibody.



## Lattice Formation – visible!

- Sum of the interaction between antigens and antibodies.
- Depends on environmental conditions and concentrations of antigens and antibodies.
- Physiochemical factors which affect the reaction:
  - Ionic strength
  - pH
  - Temperature
  - Electrical charges between cells if RBCs are used, especially important for IgG.



# Enhancement of Agglutination

- Additive to neutralize charge
- Viscosity
- Treatment of antigen with enzymes
- Agitation and centrifugation
- Temperature
  - IgM prefers room temperature (RT) or below
  - IgG prefers 37C = body temperature
- Optimal pH 6.7-7.2
- Timing

# Agglutination Reactions

- **Advantages**

- Easy to carry out
- No complicated equipment needed
- Can be performed as needed
- Available in pre-package kits with controls
- Reactions are **QUALITATIVE**, i.e., positive or negative
- **Titers** can be performed to give **semi-quantitative** results



# 1- Direct Agglutination

- Antigen found naturally on particle.
- **Examples of Application:**
  - Blood Grouping - antigen on cell
  - Bacterial serotyping – *Salmonella*
  - Test serum against bacteria which are difficult to grow in culture: Tularemia, Rickettsial diseases, typhoid fever.
  - Hemagglutination kits available for measles antibody detection.

## a. Qualitative agglutination test:

Agglutination tests can be used in a **qualitative** manner to assay for the presence of an antigen or an antibody. The antibody is mixed with the particulate antigen and a positive test is indicated by the agglutination of the particulate antigen.

For example, a patient's red blood cells can be mixed with antibody to a blood group antigen to determine a person's blood type.

In a second example, a patient's serum is mixed with red blood cells of a known blood type to assay for the presence of antibodies to that blood type in the patient's serum.

## Blood Grouping

**Serafol<sup>®</sup> ABO+D**

Ch. - B. Lot No. 080601    Expiry date 31.12.01

**Anti-A**      **Anti-B**      **Anti-D**

Name (Name/ Nom) \_\_\_\_\_

Geb. Dat. (Date of Birth/ Date de Naissance) \_\_\_\_\_      Kart. - Nr. (Unit No./ No. Pochet) \_\_\_\_\_

Datum (Date) 1/1      Blutgruppe (Blood Group/ Groupe Sanguin) A POS

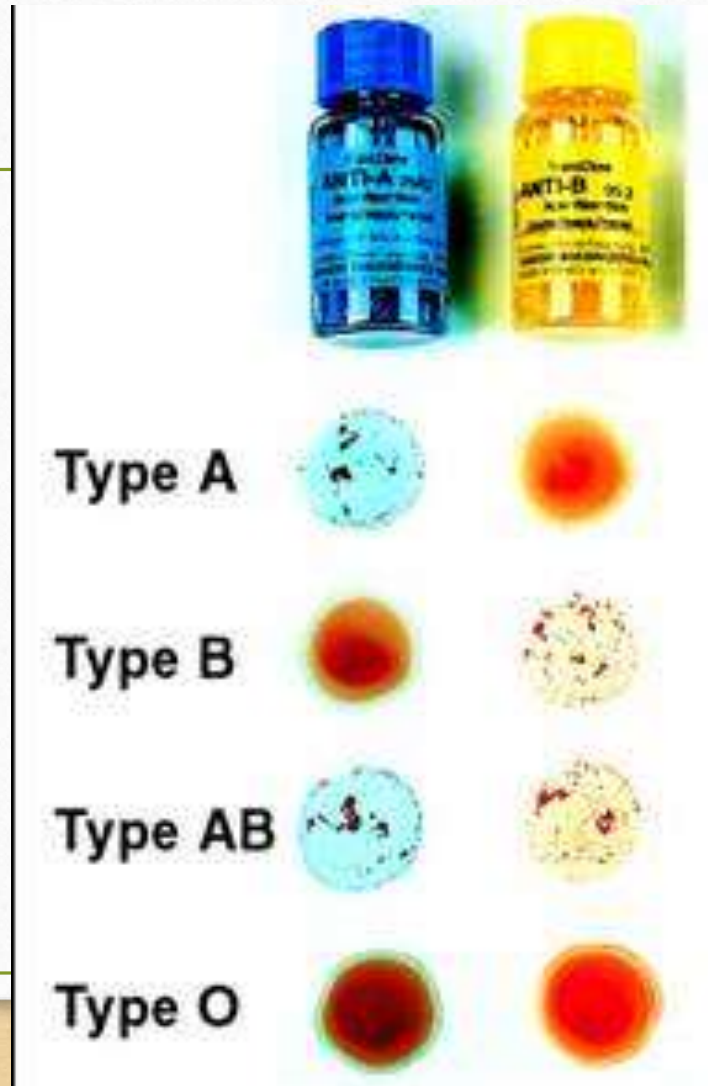
Unterschrift (Signature) \_\_\_\_\_

Blut (Blood/Sang) \_\_\_\_\_

Produced by **SIFIN** SIFIN GmbH D-13089 Berlin

Distributed by **Biotest** AG D-63303 Dreieich

# ABO Blood Grouping



## b. Quantitative agglutination:

Agglutination tests can also be used to measure the level of antibodies to particulate antigens.

In this test, **serial dilutions** are made of a sample to be tested for antibody and then a fixed number of red blood cells or bacteria or other such particulate antigen is added.

the maximum dilution that gives agglutination is determined.

The maximum dilution that gives visible agglutination is called the **titer**. The results are reported as the reciprocal of the maximal dilution that gives visible agglutination.

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

## b. Quantitative agglutination test

**Prozone effect - Occasionally**, it is observed that when the concentration of antibody is high (i.e. lower dilutions), there is no agglutination and then, as the sample is diluted, agglutination occurs.

The lack of agglutination at high concentrations of antibodies is called the *prozone* effect. Lack of agglutination in the prozone is due to antibody excess resulting in very small complexes that do not clump to form visible agglutination.

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

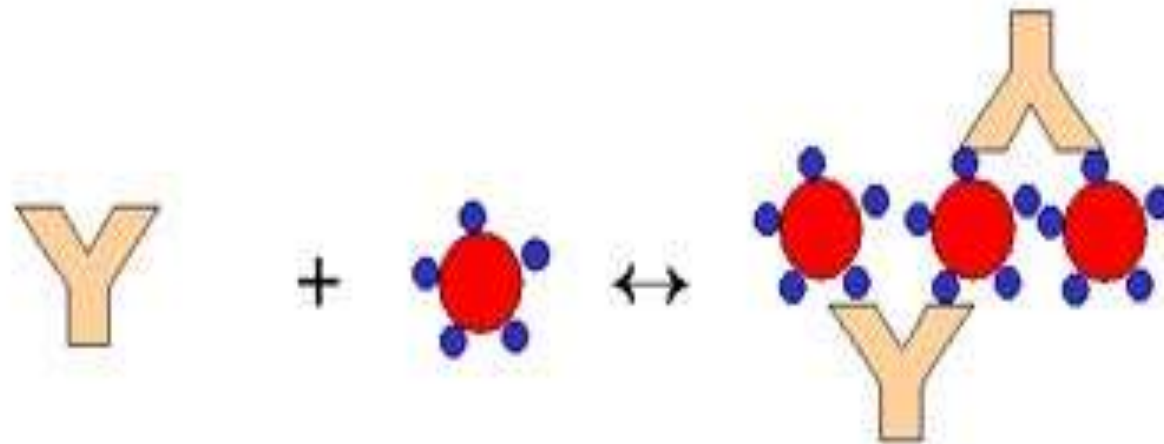
## 2-Passive Hemagglutination:

The agglutination test only works with particulate antigens.

However, it is possible to coat erythrocytes with a soluble antigen (e.g. viral antigen, a polysaccharide or a hapten)

and use the coated red blood cells in an agglutination test for antibody to the soluble antigen. This is called **passive hemagglutination**.

The test is performed just like the agglutination test. Applications include detection of antibodies to soluble antigens and detection of antibodies to viral antigens.

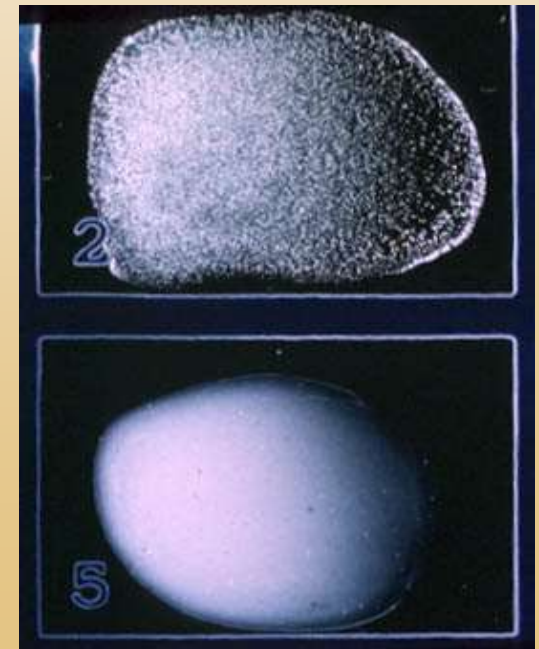


## 2-Passive Hemagglutination:

- Bind known **ANTIGENS** to inert particles to detect antibody.
- Particles used include:
  - RBCs
  - Polystyrene or latex particles
  - Bentonite particles
  - Charcoal particles
- **Artificial particles have advantages:**
  - Uniform consistency
  - Stability

## 2-Passive Hemagglutination:

- Reactions easy to read macroscopically.
- Many antigens adsorb onto RBCs spontaneously, **tanned sheep RBCs** frequently used.
- IgG naturally adsorbs onto surface of latex particles.
- The following reaction uses latex particles.
- Which reaction is positive? Negative?
- Submit answer with your question for class.





## 2-Passive Hemagglutination:

### Tests Using Passive Agglutination

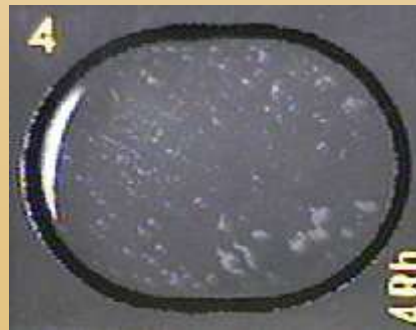
- Anti-nuclear antibodies (ANA)
- Group A strep
- Rheumatoid Factor
- Viral antibodies such as
  - cytomegalovirus
  - rubella
  - varicella-zoster

## 2-Reverse Passive Hemagglutination:

- Bind known **ANTIBODY** to carrier particle instead of antigen.
- Must orient antibody so that active site is facing outward.
- Used for detection of microbial antigens:
  - Group A and B *Streptococcus*
  - *Staphylococcus aureus*
  - *Neisseria meningitidis*
  - *Haemophilus influenzae*
  - *Cryptococcus neoformans*
  - *Mycoplasma pneumoniae*
  - *Candida albicans*

## 2-Reverse Passive Hemagglutination:

- Some organisms difficult to grow OR diagnosis needed so treatment can start.
- Widest application is in detecting soluble antigens in urine, spinal fluid and serum.
- Extraction step may be required.
- Antigens present in these fluids will attach to antibodies on particles.
- **Serologic Typing of *Shigella*: Positive Test**



# 3-Coomb's Test (Antiglobulin Test):

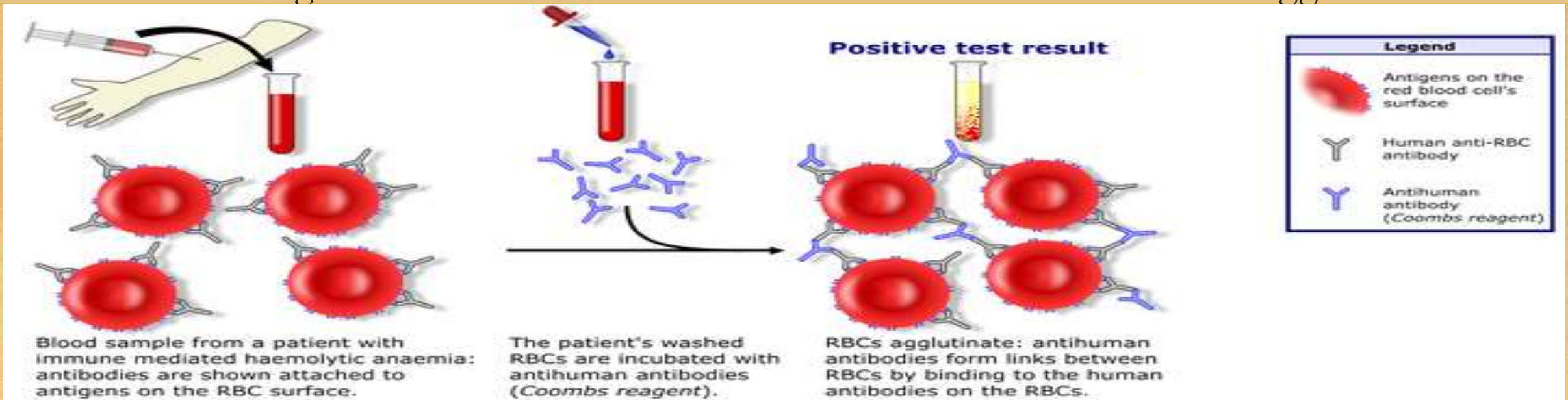
## a. Direct Coomb's Test

When antibodies bind to erythrocytes, they do not always result in agglutination.

This can result from the antigen/antibody ratio being in antigen excess or antibody excess or in some cases electrical charges on the red blood cells preventing the effective cross linking of the cells.

These antibodies that bind to but do not cause agglutination of red blood cells are sometimes referred to as **incomplete antibodies**.

In order to detect the presence of non-agglutinating antibodies on red blood cells, one simply adds **a second antibody directed against the immunoglobulin (antibody) coating the red cells**. This anti-immunoglobulin can now cross link the red blood cells and result in agglutination.

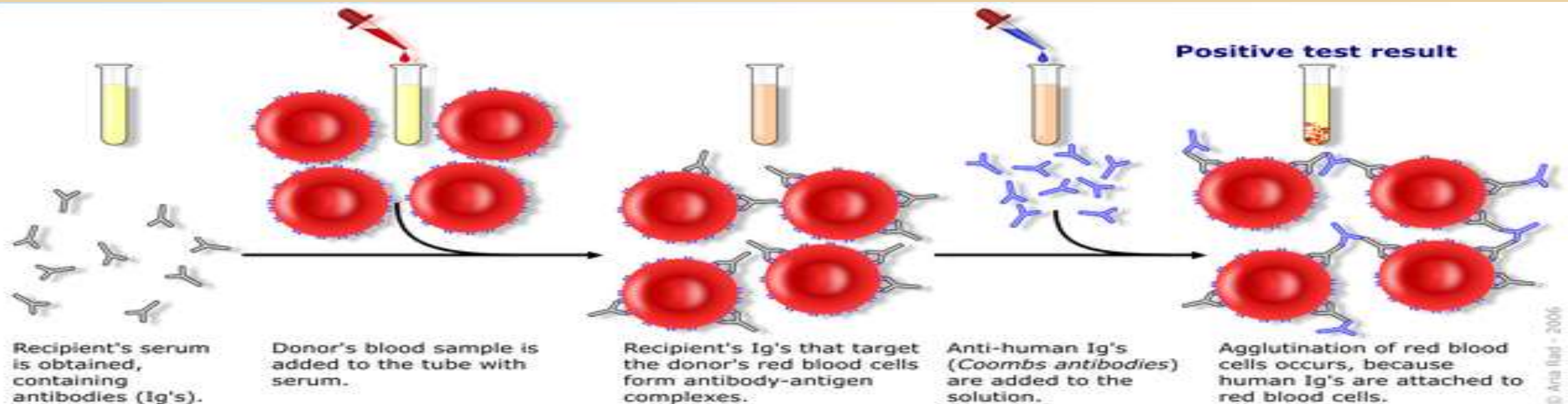


# 3-Coomb's Test (Antiglobulin Test):

## b. Indirect Coomb's Test

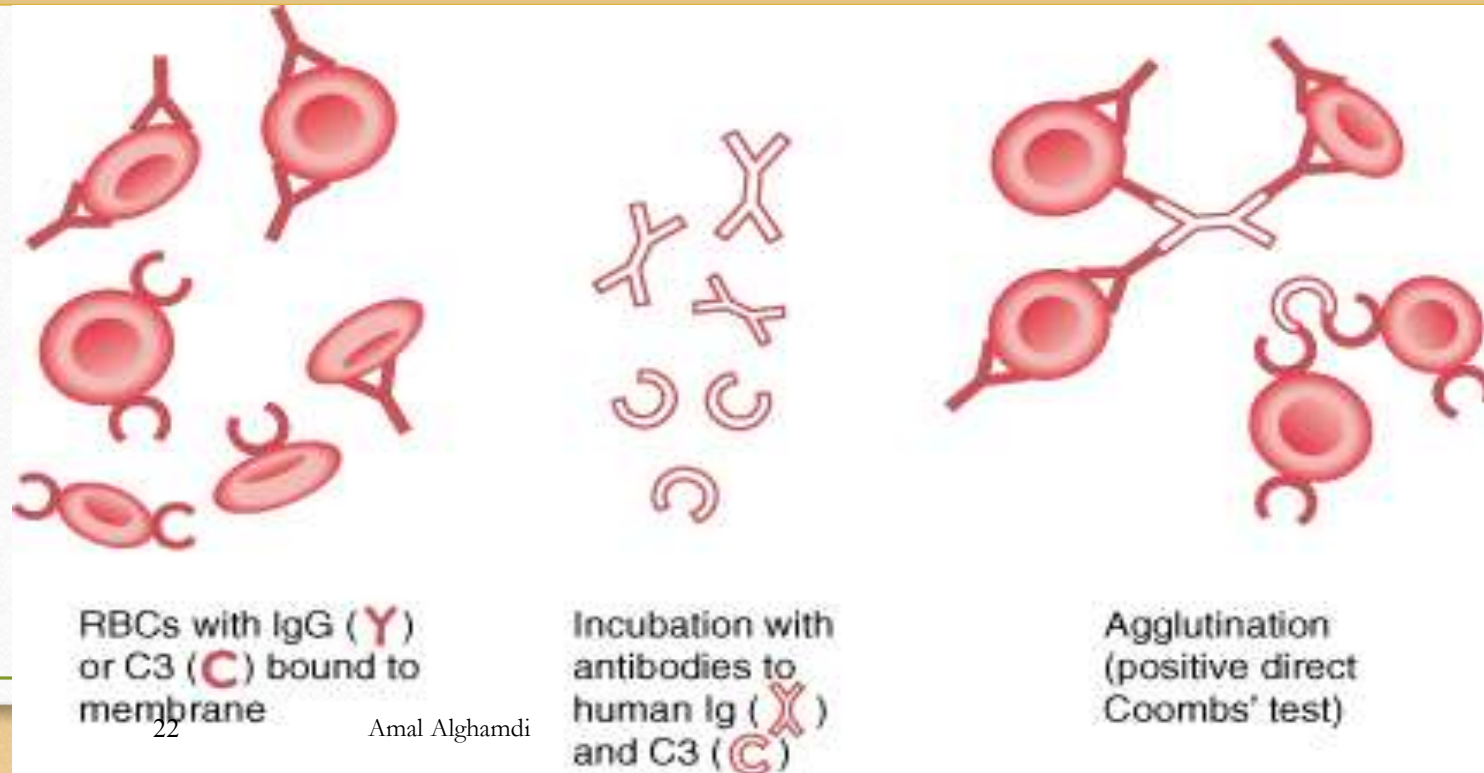
If it is necessary to know whether a serum sample has antibodies directed against a particular red blood cell and you want to be sure that you also detect potential non- agglutinating antibodies in the sample, an Indirect Coomb's test is performed.

This test is done by **incubating the red blood cells** with the **serum sample**, washing out any unbound antibodies and then adding **a second anti-immunoglobulin reagent** to cross link the cells.



## c. Applications of Coomb's Test:

These include detection of anti-rhesus factor (Rh) antibodies. Antibodies to the Rh factor generally do not agglutinate red blood cells. Thus, red cells from Rh+ children born to Rh- mothers, who have anti-Rh antibodies, may be coated with these antibodies. To check for this, a direct Coombs test is performed. To see if the mother has anti-Rh antibodies in her serum an Indirect Coombs test is performed.



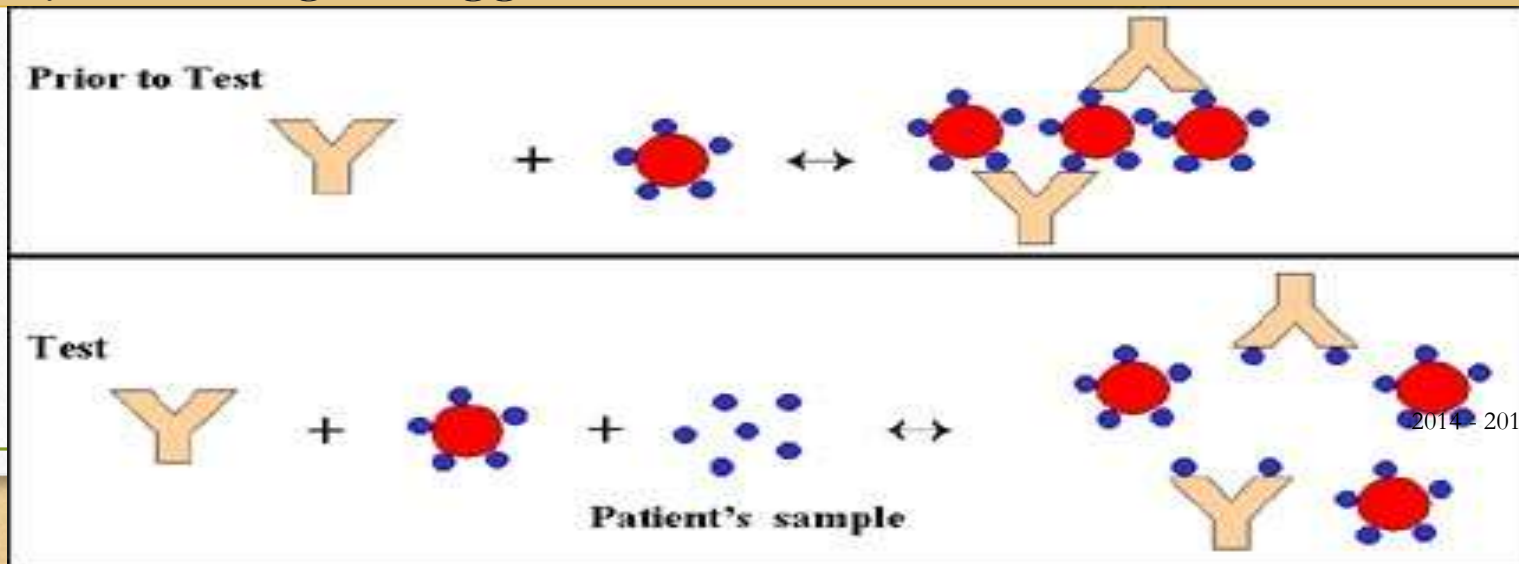
## 4-Hemagglutination Inhibition

The agglutination test can be modified to be used for the measurement of soluble antigens.

This test is called hemagglutination inhibition.

It measures the ability of soluble antigen to **inhibit** the agglutination of antigen-coated red blood cells by antibodies.

In this test, a fixed amount of antibodies to the antigen in question is mixed with a fixed amount of red blood cells coated with the antigen. Also included in the mixture are different amounts of the sample to be analyzed for the presence of the antigen. If the sample contains the antigen, the soluble antigen will compete with the antigen coated on the red blood cells for binding to the antibodies, thereby inhibiting the agglutination of the red blood cells.



# 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



Human IgG with other heavy chain isotope

+



G1 epitope specific

+



=

Patient Sample



Agglutination

THIS IS A NEGATIVE REACTION

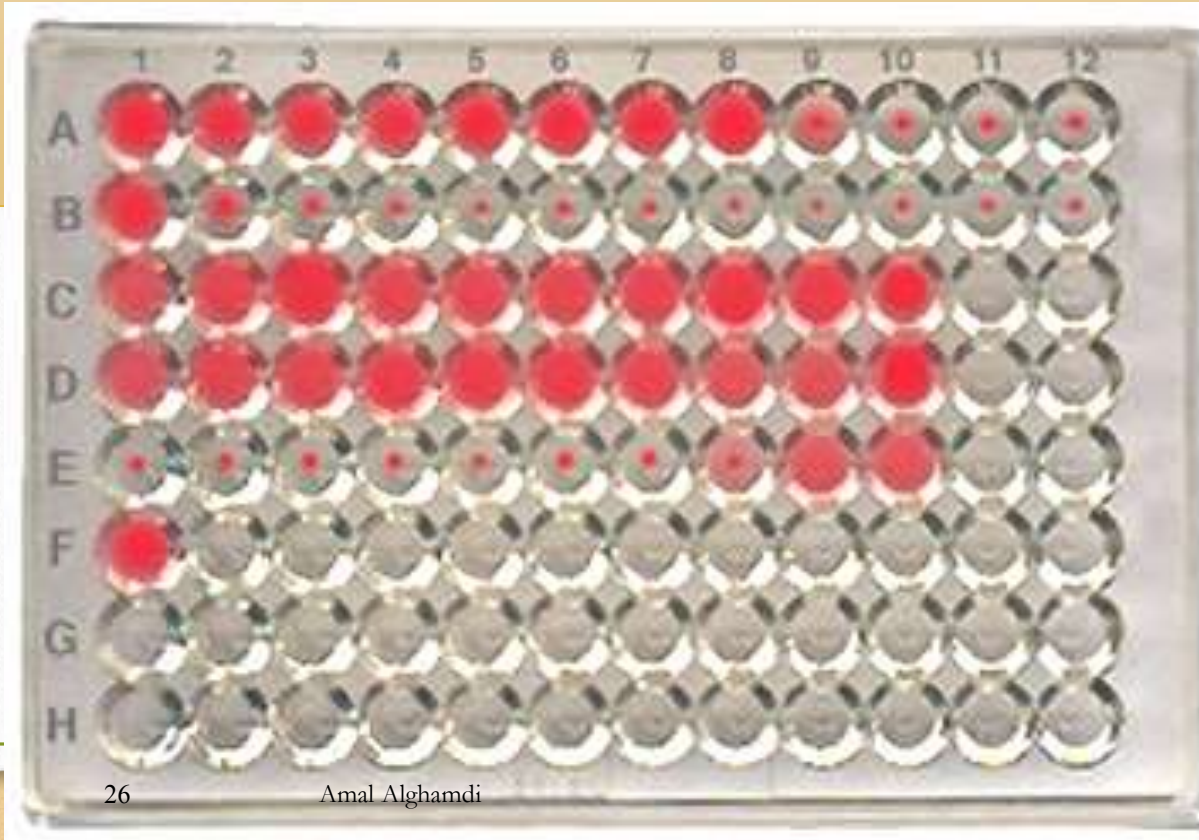


## 4-Hemagglutination Inhibition

- Based on competition between particulate and soluble antigens for limited antibody combining sites.
- Patient sample added to reagent antibody specific for antigen being tested, if antigen is present it binds to reagent antibody.
- Reagent particles (latex or RBCs) coated with the same antigen are added, if antigen was present in the sample all reagent antibody binds to it so no antibody is present to react with antigens coating the particles
- NO agglutination = POSITIVE reaction.

## 4-Hemagglutination Inhibition

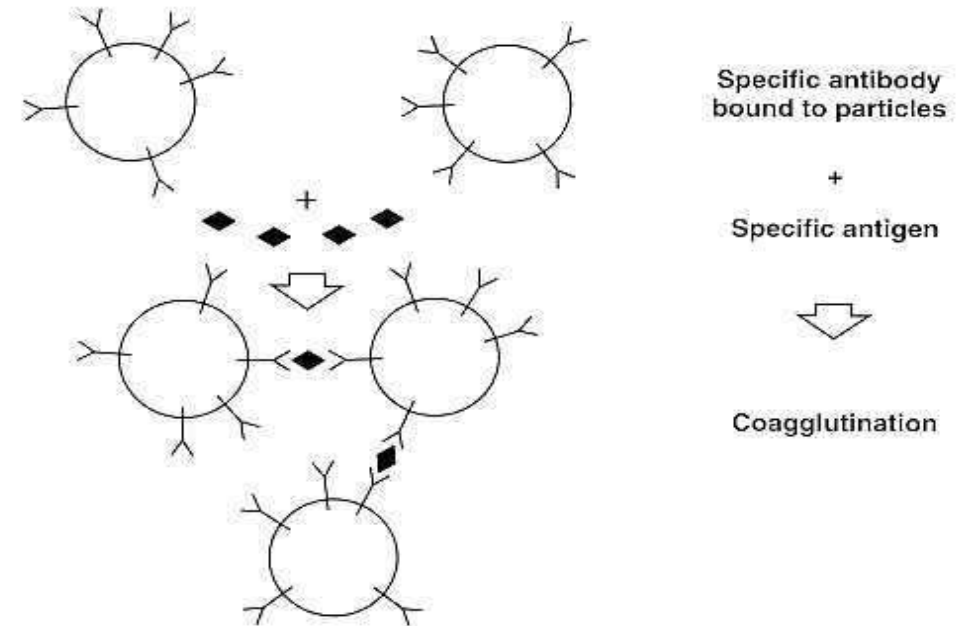
- Microtiter plate
- In wells if agglutination occurs the clumps cover the well.
- No agglutination will allow the RBCs to flow down sides and collect at the bottom.
- In row E wells 1-7 are positive – NO agglutination, 8 weakly positive, wells 9 and 10 are negative.



## 4-Coagglutination:

- Uses bacteria as the inert particle to which antibodies are attached.
- Discovered *Staphylococcus aureus* has protein A which adsorbs the Fc portion of antibody.
- Highly specific but not as sensitive as latex agglutination.
- Used for identification of streptococci, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Vibrio cholera* 0139 and *Haemophilus influenzae*.

- Name given to systems using bacteria as the inert particles to which antibody is attached.



## 5-Complement Fixation Test:

- **Antigen – antibody reactions** in which the **visible manifestation** requires the participation of accessory factors, indicator systems, and specialized equipment can be measured.

## 5-Complement Fixation Test:

### A) What is Complement ?

A protein constituent of normal blood serum, is consumed (i.e., fixed) during the interaction of antigens and antibodies.

“The Complement Fixation Test”,

is sensitive test that can be used to **detect** and **quantitate** antigens and antibodies.

The primary reacting ingredients are antigen, antibody, and complement.

## 5-Complement Fixation Test:

### Source of Complement:

**a. Normal guinea pig serum** often is used as a primary source of complement because guinea pigs have high levels of complement with efficient lytic properties.

**b. Different sources of complement** are used in different vitro test (e.g., rabbit complement is used in cytotoxicity tests performed for transplantation).

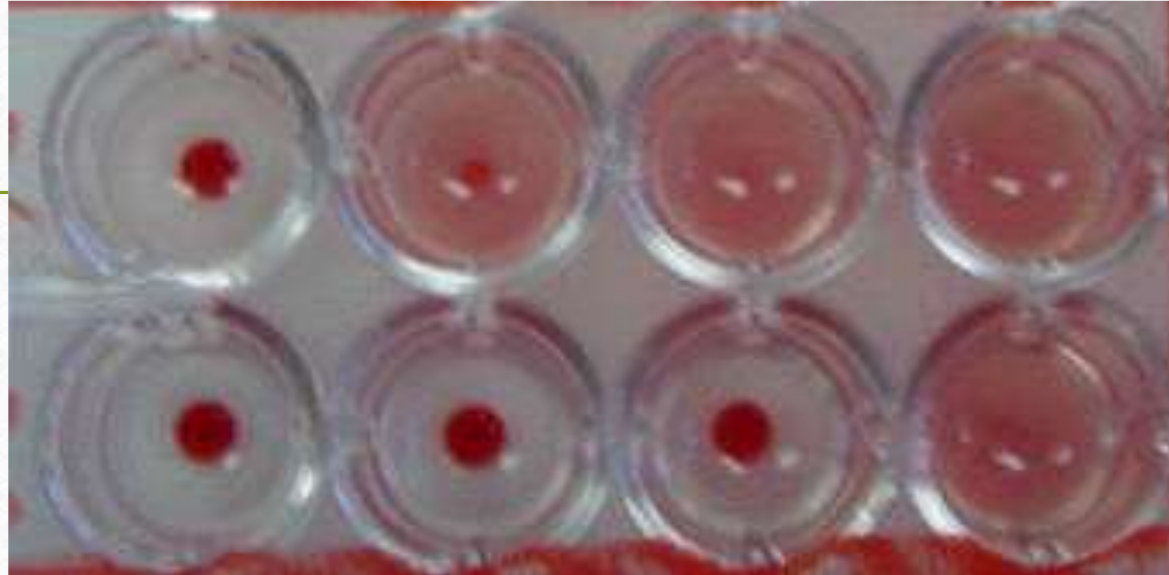
## 5-Complement Fixation Test:

### Usage of Complement Fixation Test:

To determine the presence of antibody to a known antigen in a patient's serum, a test system and an indicator system are used.

## 5-Complement Fixation Test:

### Complement Fixation Test in Microtiter Plate.



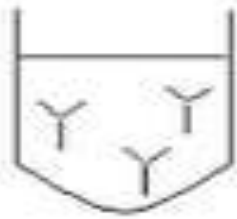
Rows 1 and 2 exhibit complement fixation obtained with acute and convalescent phase serum specimens, respectively. (2-fold serum dilutions were used) The observed 4-fold increase is significant and indicates recent infection.



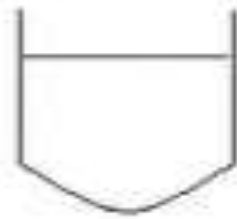
## 5-Complement Fixation Test:

- Two step process
  - Antibody (patient serum), antigen are mixed with fresh complement.
  - Sensitized sheep cells added.
  - If the patient antibody is absent the complement is free to bind to the antibody coated sheep cells causing hemolysis.
  - If the antibody is present, the antigen-antibody bind the complement and no hemolysis will occur.
  - NO hemolysis is a POSITIVE reaction

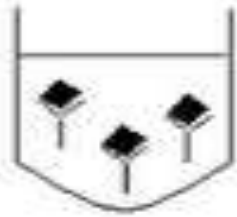
# 5-Complement Fixation Test:



Serum with antibodies



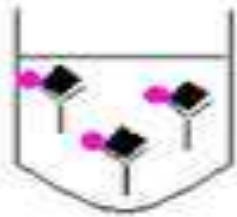
Serum without antibodies



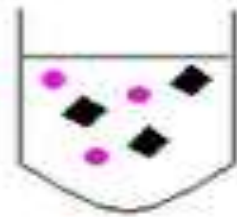
Antigen binds with antibodies



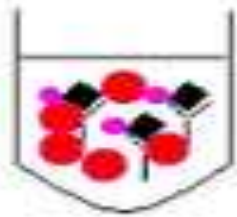
Unbound Antigen



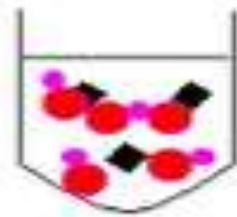
Complement binds with Ag/Ab complex



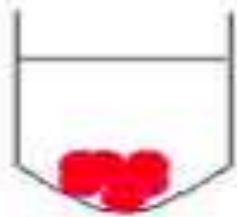
Unbound complement



Hemolysin Sensitized red blood cells serve as an indicator



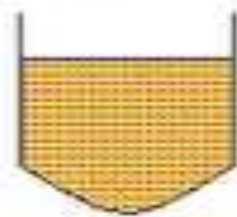
Hemolysin Sensitized RBCs serve as an indicator



RBCs settle into a pellet

no lysis

Reactive



RBCs lysed by unbound complement

lysis

Nonreactive

# References

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- <http://web.indstate.edu/thcme/PSP/labtests/precip.htm>
- <http://www.gla.ac.uk/departments/immunology/education/nursing/lectures/antibody.htm>
- <http://www.cellsalive.com/mac.htm>
- <http://jeeves.mmg.uci.edu/immunology/Assays/Assays.htm>
- <http://www.medschool.lsuhs.edu/microbiology/DMIP/dmex03.htm>
- <http://www.tulipgroup.com/Common/html/TurbidTech.pdf>
- <http://departments.oxy.edu/biology/Franck/Bio222/Lectures/Feb1lecture.htm>
- [http://www.mercodia.se/global/mainpage.asp?page\\_id=41](http://www.mercodia.se/global/mainpage.asp?page_id=41) ELISA
- <http://www.clinprointl.com/technical.htm> ELISA
- <http://www.nsbri.org/HumanPhysSpace/focus4/sf-hormonal.html>
- <http://ccm.ucdavis.edu/cpl/Tech%20updates/TechUpdates.htm> molecular diagnostics