

# Serological tests (Antigen antibody interactions)

# Classification of antigen-antibody interactions:

1. Primary serological tests: (Marker techniques) e.g.
  - Enzyme linked immuno sorben assay (ELISA)
  - Immuno flurescent 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.

# 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. The general term agglutinin is used to describe antibodies that agglutinate particulate antigens. When the antigen is an erythrocyte the term **hemagglutination** is used. 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.


### 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.

**Serafol® ABO+D**

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

 **Anti-A**

 **Anti-B**

 **Anti-D**

Blut (Blood/Sang)

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

Geb. Dat. \_\_\_\_\_  
(Date of Birth/ Date de Naissance)

Datum \_\_\_\_\_  
(Date)

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

Kont. - Nr. \_\_\_\_\_  
(Unit No. / No. Poche)

Blutgruppe **A POS**  
(Blood Group/ Groupe Sanguin)

**SIFIN** Produced by  
SIFIN GmbH  
D-13068 Berlin

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

## b. Quantitative agglutination test

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. Then 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 | 1/1024 | Pos. | Neg. | Titer |
|---------|-----|-----|-----|------|------|------|-------|-------|-------|--------|------|------|-------|
| 1       | ●   | ●   | ●   | ●    | ●    | ●    | ○     | ○     | ○     | ○      | ●    | ○    | 64    |
| 2       | ●   | ●   | ●   | ○    | ○    | ○    | ○     | ○     | ○     | ○      | ●    | ○    | 8     |
| 3       | ●   | ●   | ●   | ●    | ●    | ●    | ●     | ●     | ●     | ○      | ●    | ○    | 512   |
| 4       | ○   | ○   | ○   | ○    | ○    | ○    | ○     | ○     | ○     | ○      | ●    | ○    | <2    |
| 5       | ●   | ●   | ●   | ●    | ●    | ○    | ○     | ○     | ○     | ○      | ●    | ○    | 32    |
| 6       | ○   | ○   | ●   | ●    | ●    | ●    | ●     | ○     | ○     | ○      | ●    | ○    | 128   |
| 7       | ●   | ●   | ●   | ●    | ●    | ○    | ○     | ○     | ○     | ○      | ●    | ○    | 32    |
| 8       | ●   | ●   | ○   | ○    | ○    | ○    | ○     | ○     | ○     | ○      | ●    | ○    | 4     |

**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.

### **c. Applications of agglutination tests**

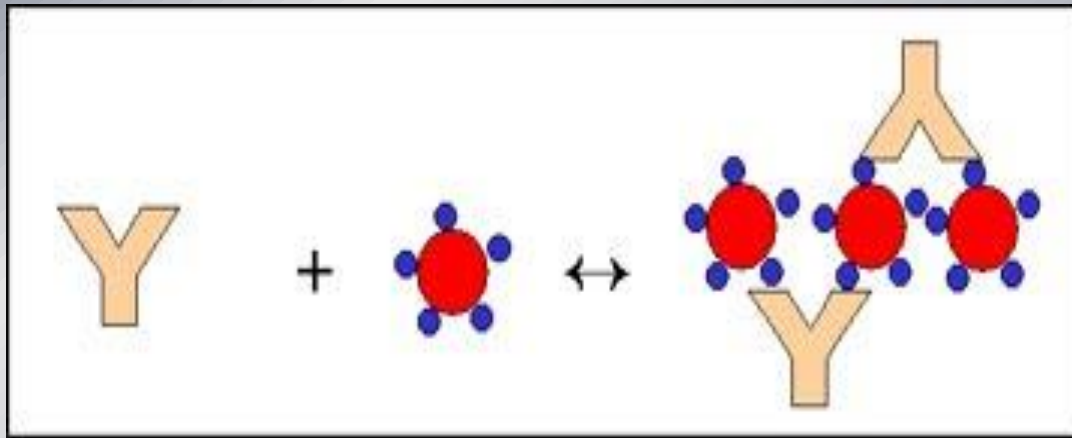
- i. Determination of blood types or antibodies to blood group antigens.
- ii. To assess bacterial infections

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

## 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.



### 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 no way is this meant to indicate that the antibodies are different in their structure, although this was once thought to be the case. Rather, it is a functional definition only. 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.

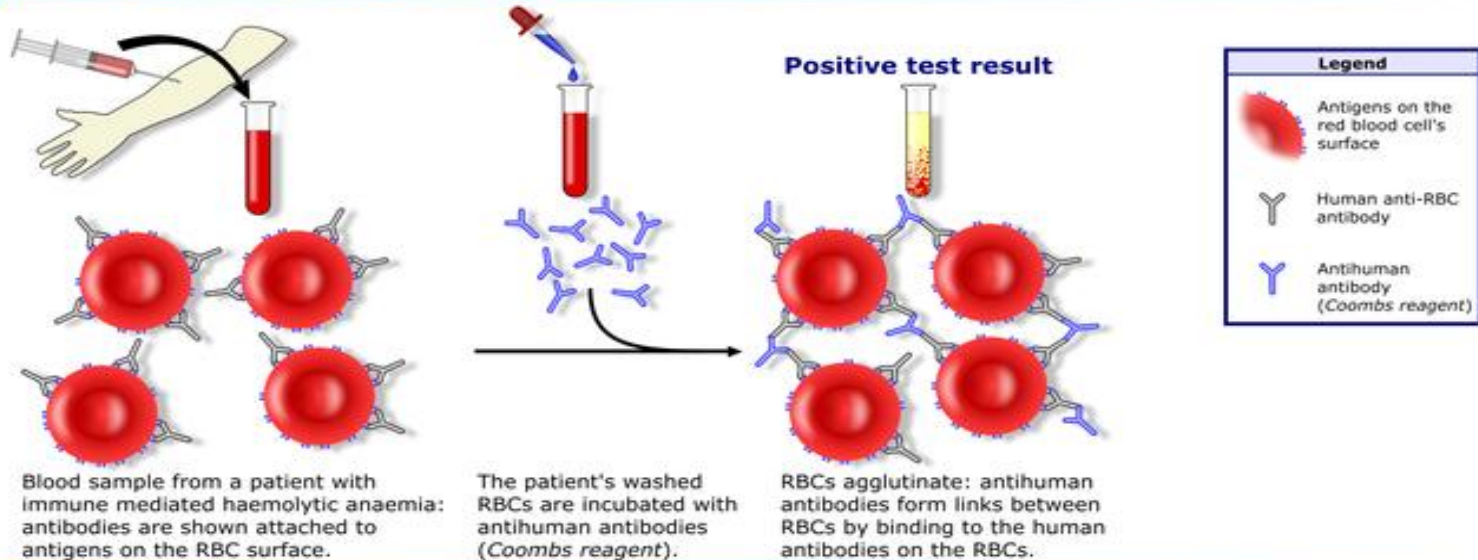
#### b. Indirect Coomb's Test

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.

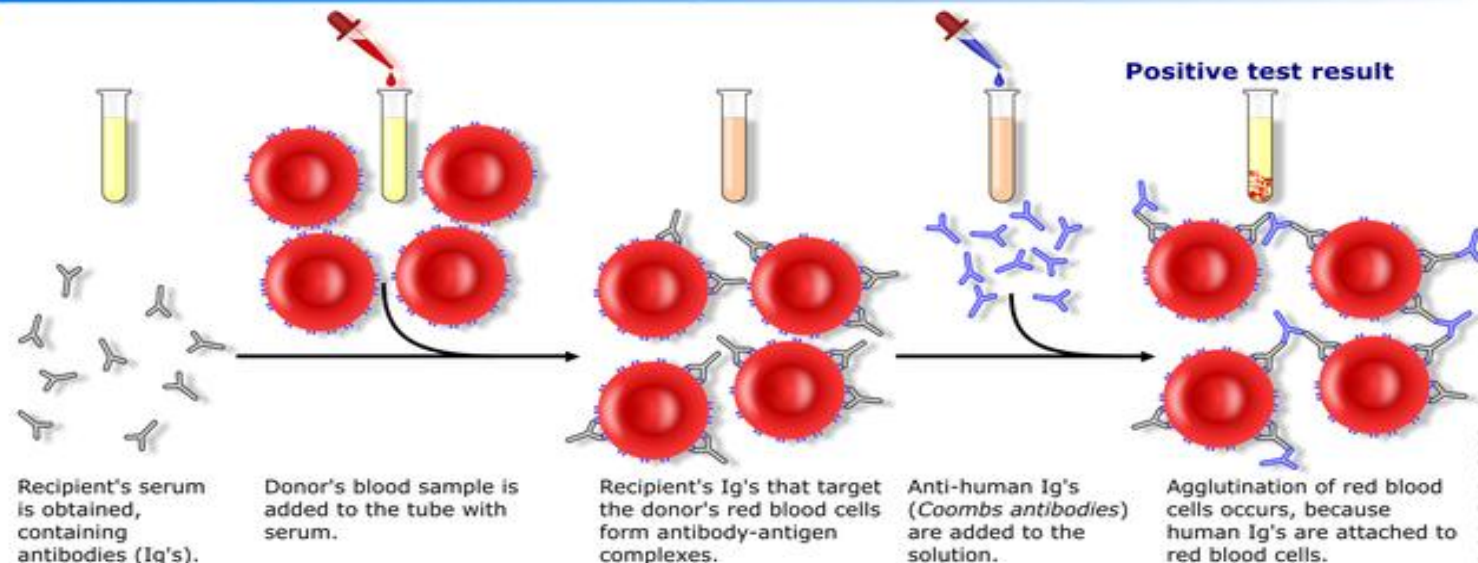
This test is done by incubating the red blood cells with the serum sample, washing out any unbound antibodies and then adding a second anti-immunoglobulin reagent to cross link the cells.



## Direct Coombs test / Direct antiglobulin test

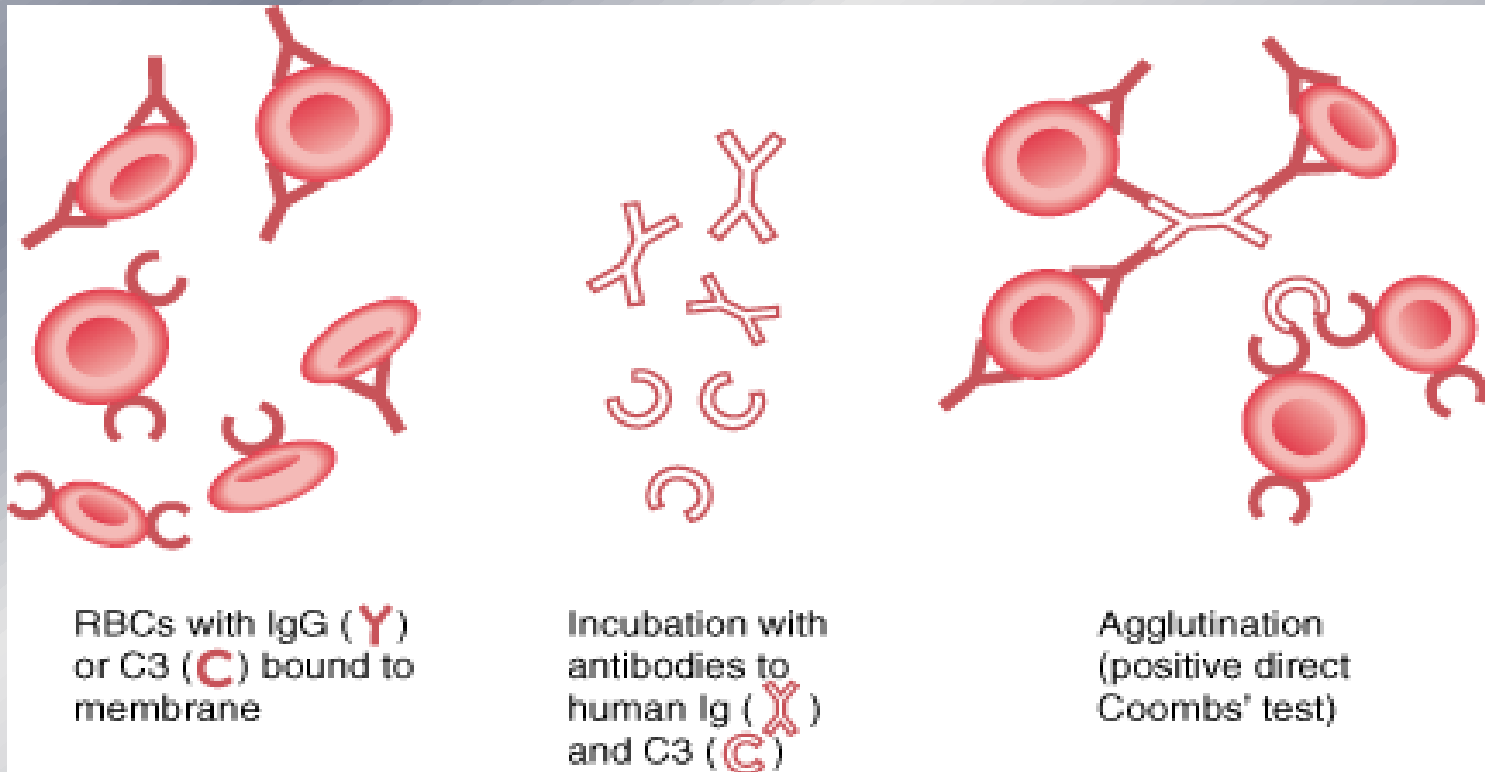


## Indirect Coombs test / Indirect antiglobulin test



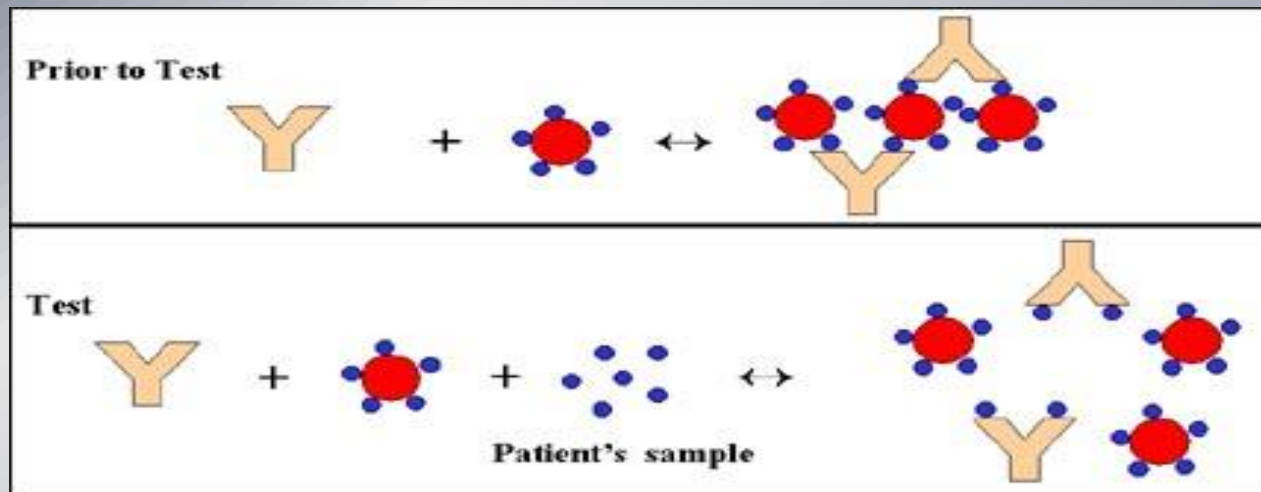
## c. Applications

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 is called hemagglutination inhibition because one 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.



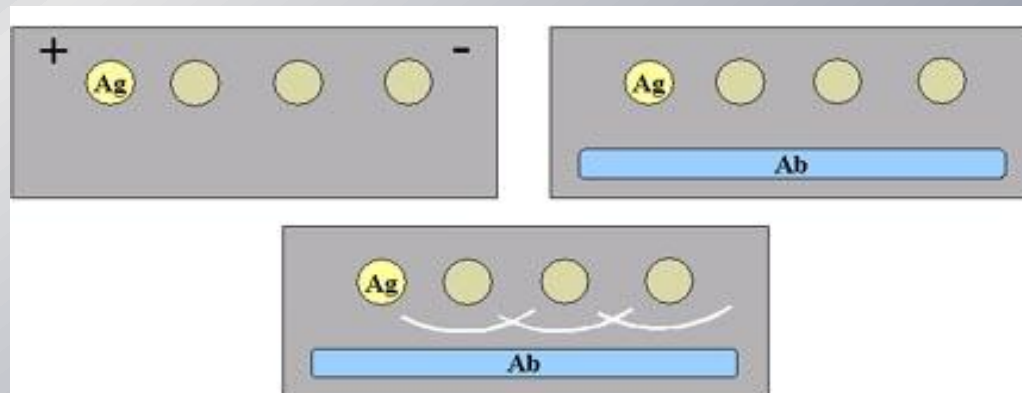
## B. Precipitation tests

### 1-Radial Immunodiffusion (Mancini)

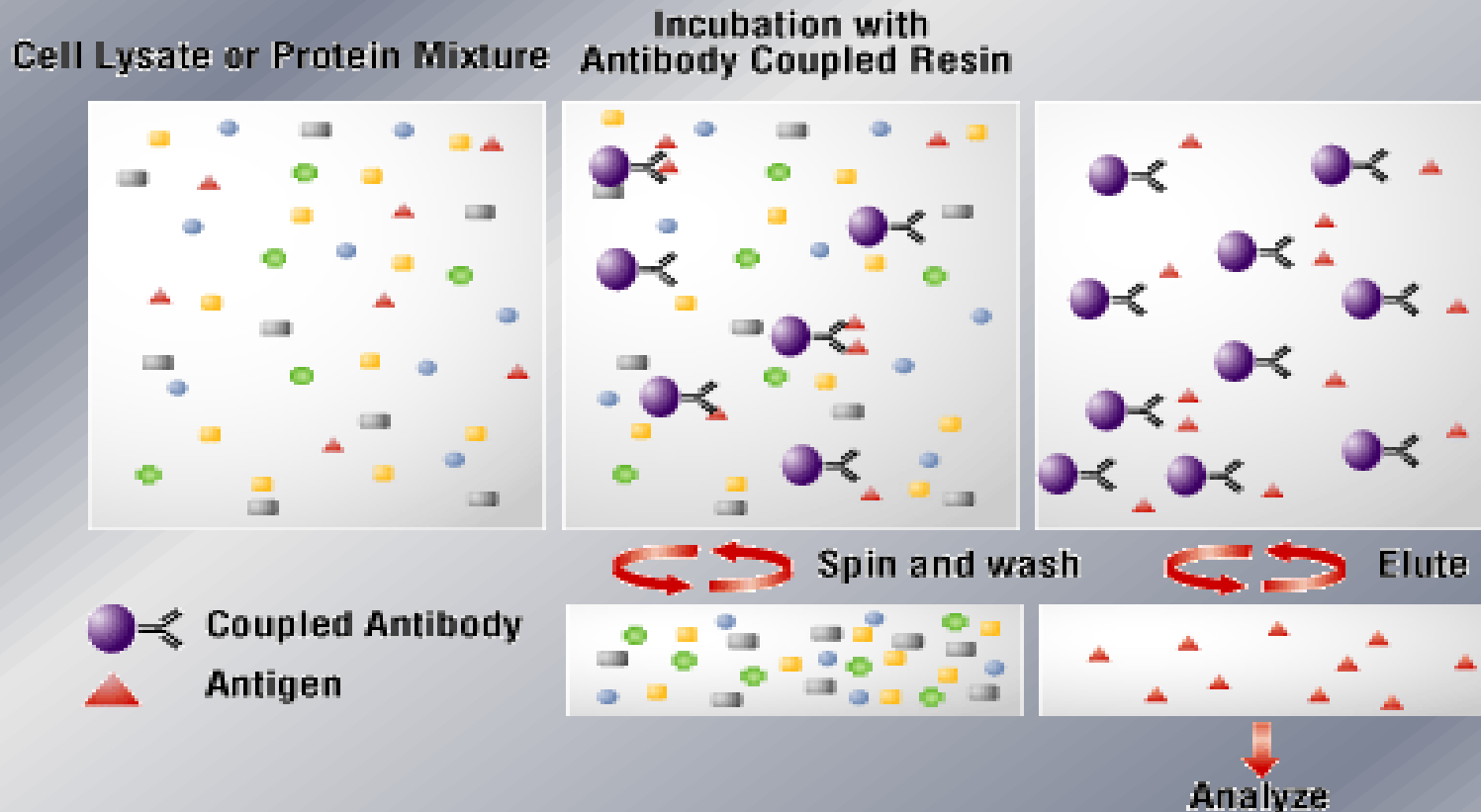
In radial immunodiffusion antibody is incorporated into the agar gel as it is poured and different dilutions of the antigen are placed in holes punched into the agar. As the antigen diffuses into the gel, it reacts with the antibody and when the equivalence point is reached a ring of precipitation is formed.

### 2-Immunelectrophoresis:

In immunelectrophoresis, a complex mixture of antigens is placed in a well punched out of an agar gel and the antigens are electrophoresed so that the antigens are separated according to their charge. After electrophoresis, a trough is cut in the gel and antibodies are added. As the antibodies diffuse into the agar, precipitin lines are produced in the equivalence zone when an antigen/antibody reaction occurs.

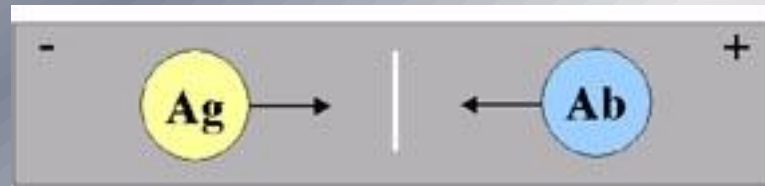


This test is used for the qualitative analysis of complex mixtures of antigens, although a crude measure of quantity (thickness of the line) can be obtained. This test is commonly used for the analysis of components in a patient's serum. Serum is placed in the well and antibody to whole serum in the trough. By comparisons to normal serum, one can determine whether there are deficiencies on one or more serum components or whether there is an overabundance of some serum component (thickness of the line). This test can also be used to evaluate purity of isolated serum proteins.



### 3-Countercurrent electrophoresis:

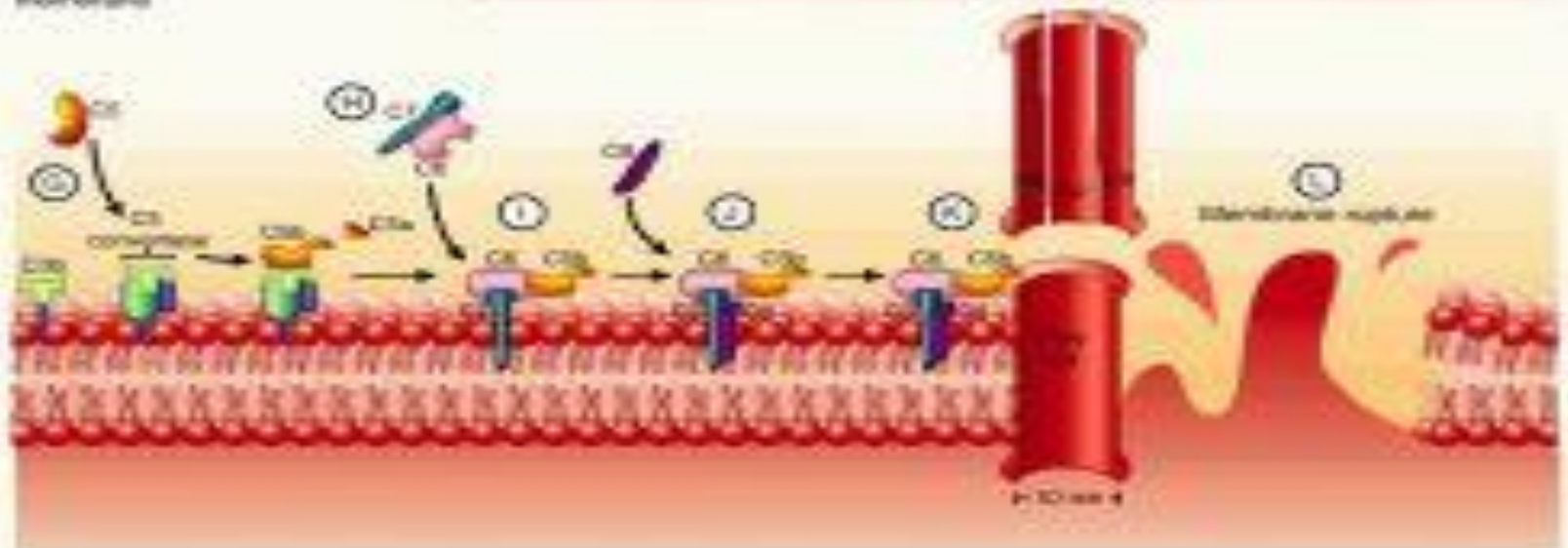
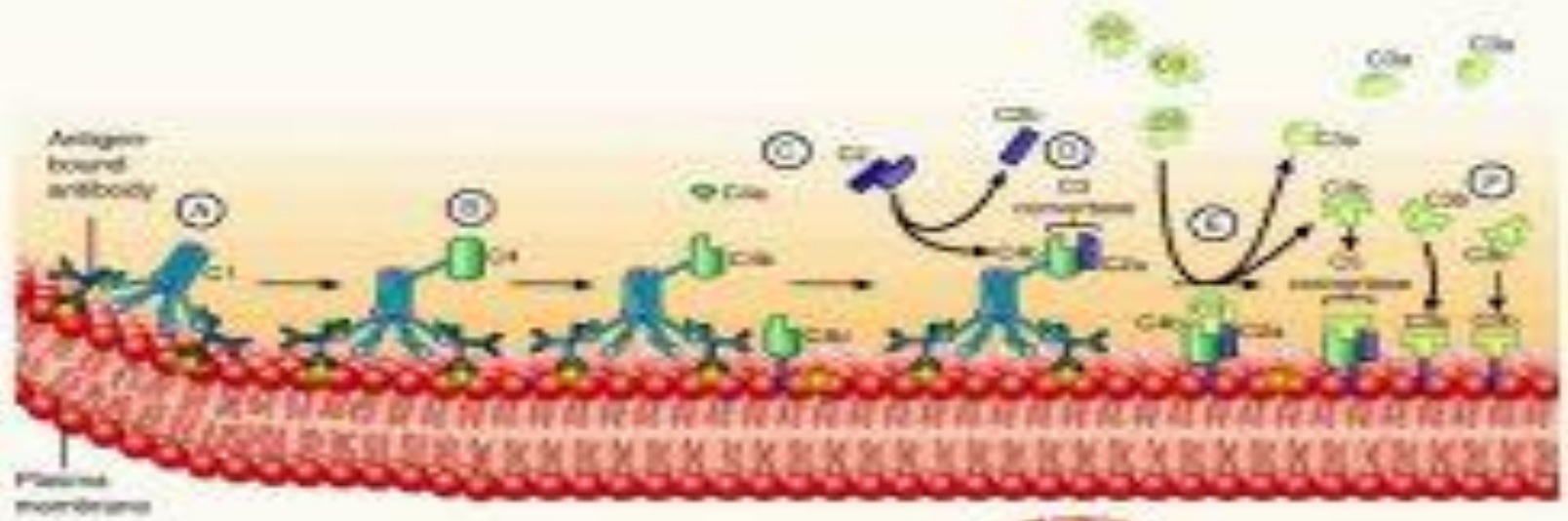
In this test the antigen and antibody are placed in wells punched out of an agar gel and the antigen and antibody are electrophoresed into each other where they form a precipitation line.



This test only works if conditions can be found where the antigen and antibody have opposite charges. This test is primarily qualitative, although from the thickness of the band you can get some measure of quantity. Its major advantage is its speed.

## C. Complement fixation test

- The **complement fixation test** is an *immunological medical* test looking for evidence of infection. It tests for the presence of either specific **antibody** or specific **antigen** in a patient's serum. It uses sheep **red blood cells** (sRBC), anti-sRBC antibody and complement, plus specific antigen (if looking for antibody in serum) or specific antibody (if looking for antigen in serum).
- If either the antibody or antigen is present in the patient's serum, then the complement is completely utilized, so the sRBCs are not lysed. But if the antibody (or antigen) is not present, then the complement is not used up, so it binds anti-sRBC antibody, and the sRBCs are lysed.
- The **Wassermann test** is one form of complement fixation test.





## D. Enzyme-Linked ImmunoSorbent Assay (ELISA)

- **Enzyme-Linked ImmunoSorbent Assay**, or **ELISA**, is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. The ELISA has been used as a diagnostic tool in medicine and plant pathology, as well as a quality control check in various industries. In simple terms, in ELISA an unknown amount of antigen is affixed to a surface, and then a specific antibody is washed over the surface so that it can bind the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal. Thus in the case of fluorescence ELISA, when light is shone upon the sample, any antigen/antibody complexes will fluoresce so that the amount of antigen in the sample can be measured.

# 1. Indirect ELISA

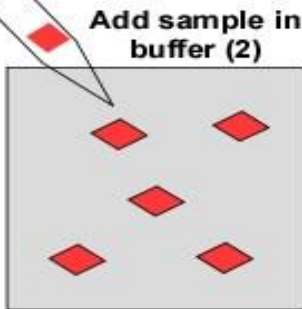
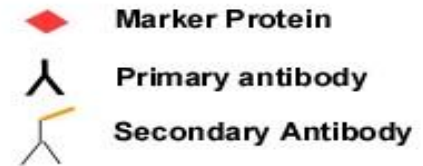
- The steps of the general, "indirect," ELISA for determining serum antibody concentrations are:
  1. Apply a sample of known antigen of known concentration to a surface, often the well of a [microtiter plate](#). The antigen is fixed to the surface to render it immobile. Simple adsorption of the protein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

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

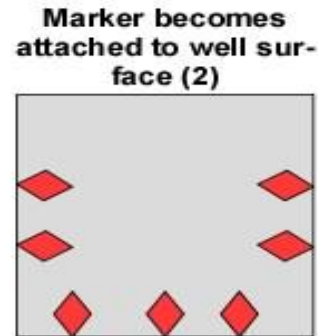
# To detect antibody (indirect ELISA):

## Indirect ELISA

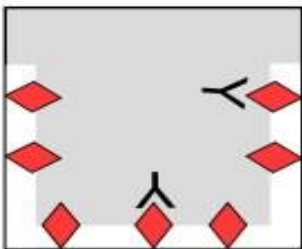
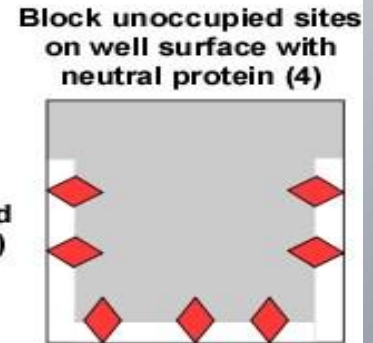
Numbers correspond to steps in text



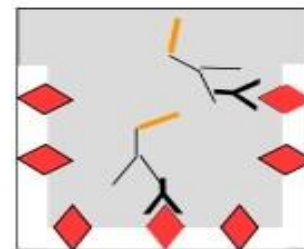
incubate (2)



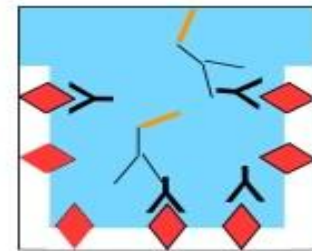
Wash to remove unattached marker (3)



Wash to remove unattached antibodies (6)



Wash to remove unattached 2° antibody (8)



## 2. Sandwich ELISA

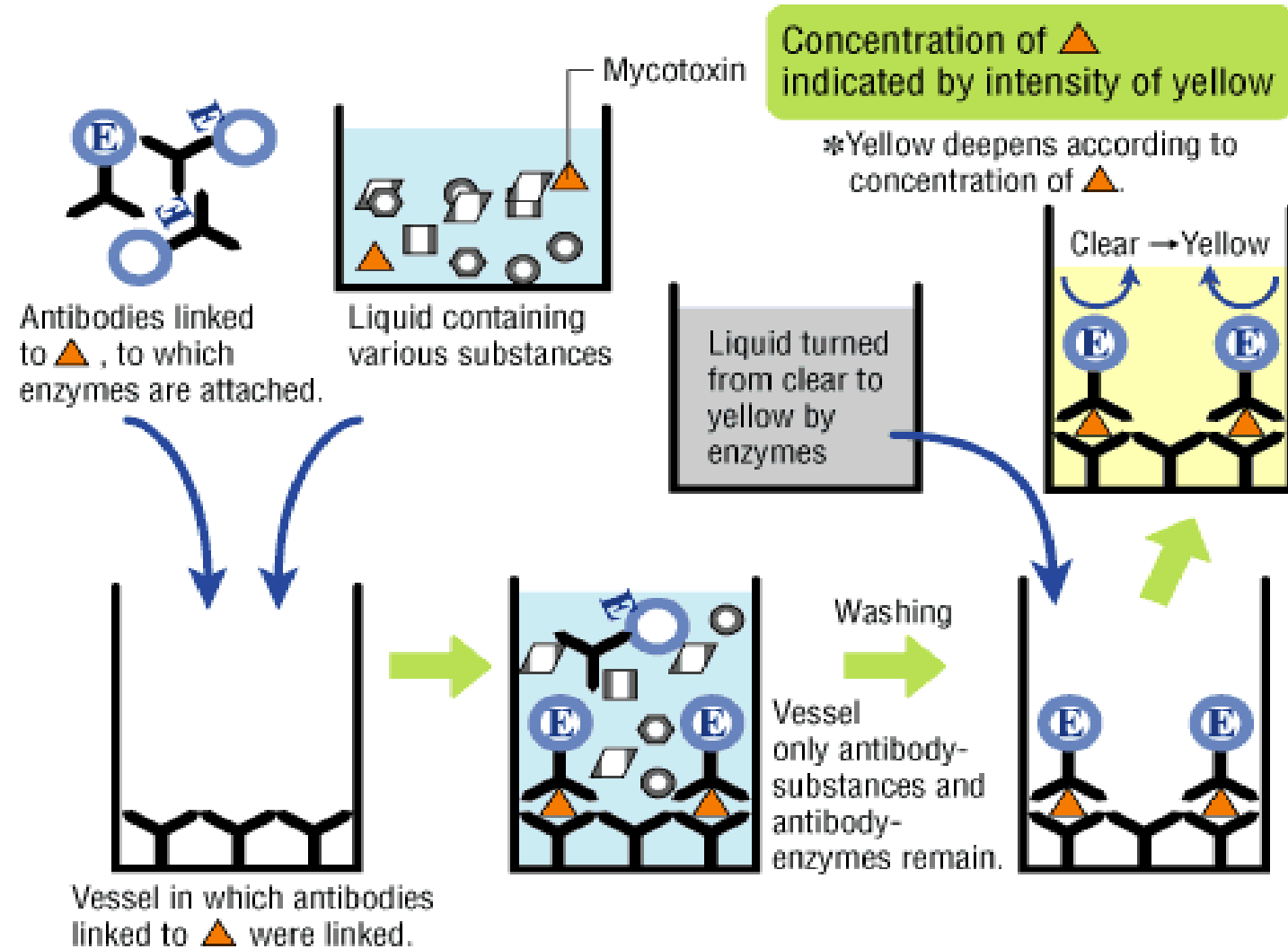
- **A sandwich *ELISA*:**
  - Plate is coated with a capture antibody
  - sample is added, and any antigen present binds to capture antibody
  - detecting antibody is added, and binds to antigen
  - enzyme-linked secondary antibody is added, and binds to detecting antibody
  - substrate is added, and is converted by enzyme to detectable form.

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

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

# To detect antigen (sandwich ELISA):

## Principle of Enzyme-Linked Immunosolvent Assay (ELISZA)

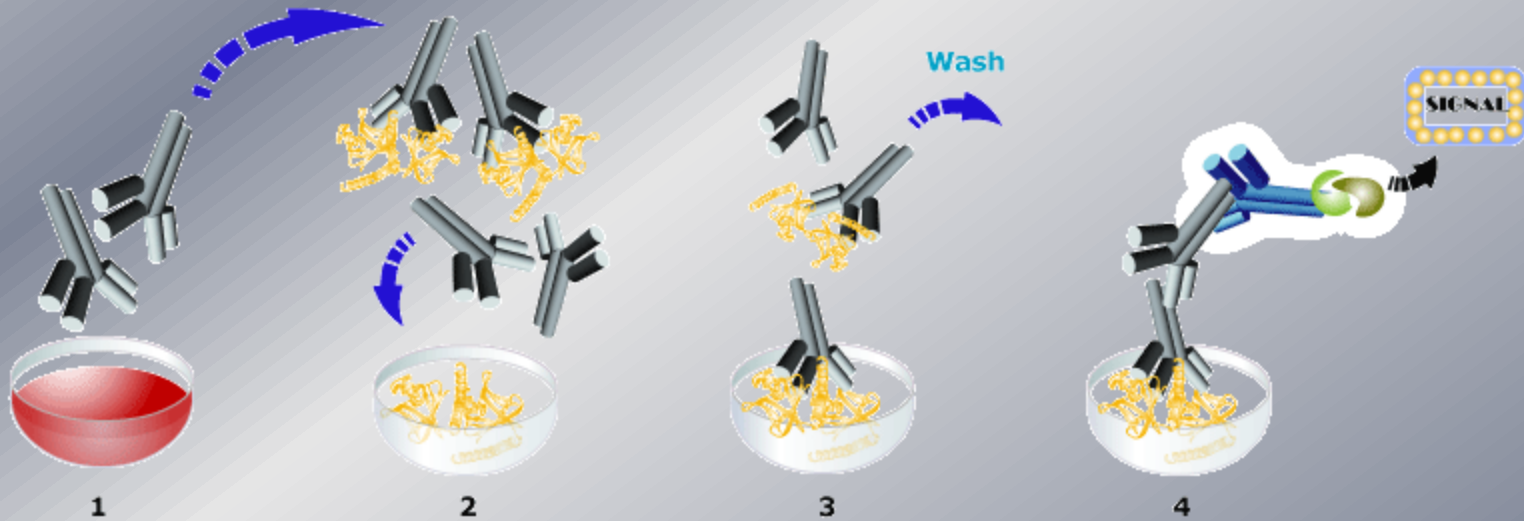


### 3. Competitive ELISA

- A third use of ELISA is through competitive binding. The steps for this ELISA are somewhat different than the first two examples:
  1. Unlabeled antibody is incubated in the presence of its antigen.
  2. These bound antibody/antigen complexes are then added to an antigen coated well.
  3. The plate is washed, so that unbound antibody is removed. (The more antigen in the sample, the less antibody will be able to bind to the antigen in the well, hence "competition.")
  4. The secondary antibody, specific to the primary antibody is added. This second antibody is coupled to the enzyme.
  5. A substrate is added, and remaining enzymes elicit a chromogenic or fluorescent signal.
- For competitive ELISA, the higher the original antigen concentration, the weaker the eventual signal.



# Competitive binding



# Applications

- Because the ELISA can be performed to evaluate either the presence of antigen or the presence of antibody in a sample, it is a useful tool both for determining serum antibody concentrations (such as with the HIV test [1] or West Nile Virus) and also for detecting the presence of antigen. It has also found applications in the food industry in detecting potential food allergens such as milk,peanuts,walnuts,almonds, and eggs [2]The ELISA test, or the enzyme immunoassay (EIA), was the first screening test commonly employed for HIV. It has a high sensitivity.In an ELISA test, a person's serum is diluted 400-fold and applied to a plate to which HIV antigens have been attached. If antibodies to HIV are present in the serum, they may bind to these HIV antigens. The plate is then washed to remove all other components of the serum. A specially prepared "secondary antibody" — an antibody that binds to human antibodies — is then applied to the plate, followed by another wash. This secondary antibody is chemically linked in advance to an enzyme. Thus the plate will contain enzyme in proportion to the amount of secondary antibody bound to the plate. A substrate for the enzyme is applied, and catalysis by the enzyme leads to a change in color or fluorescence. ELISA results are reported as a number; the most controversial aspect of this test is determining the "cut-off" point between a positive and negative result.

**THANK YOU**