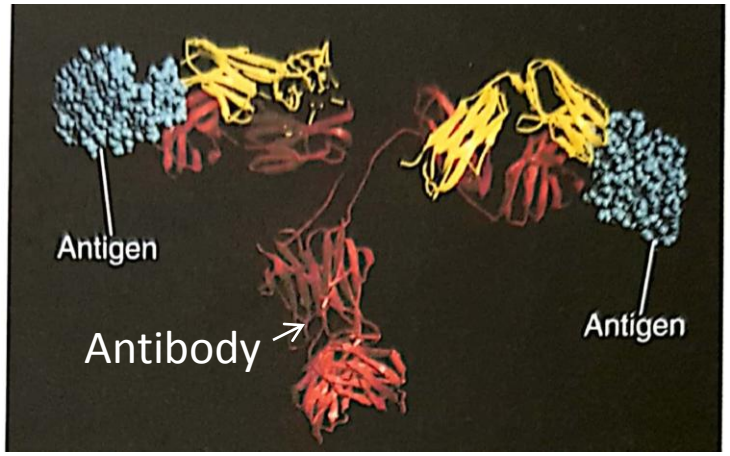
Antigen and antibody interaction

1. The antigen-antibody interaction is a bimolecular association.

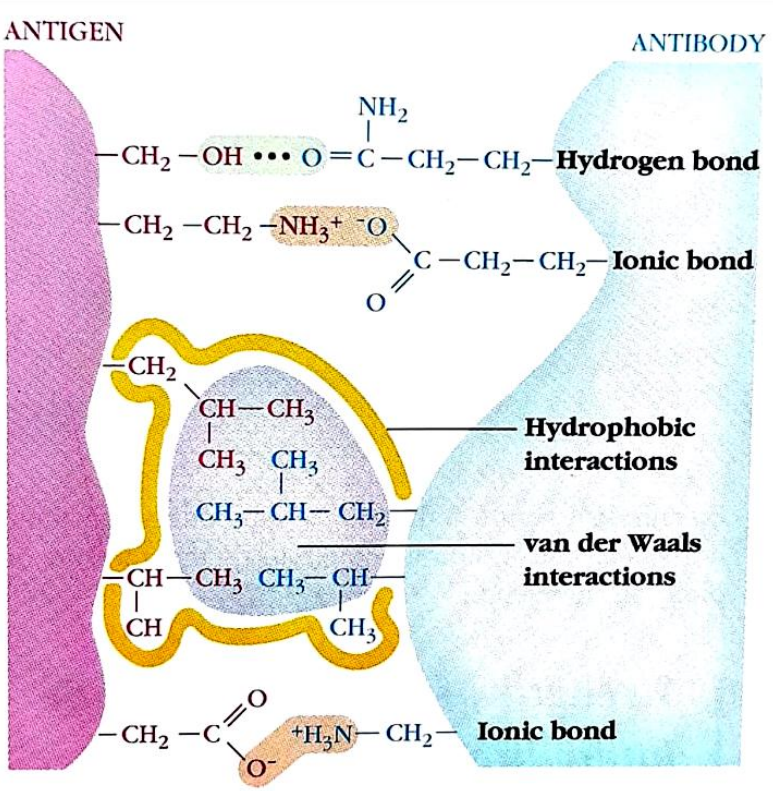
2. No chemical reaction takes place between these molecules.

3. Interaction involves various noncovalent interactions between the epitope of antigen and variable region of antibody ie., hyperveriable region or complementarity-determining region (CDR).

4. Each interaction operates over a very small distance (1 angstrom or less)

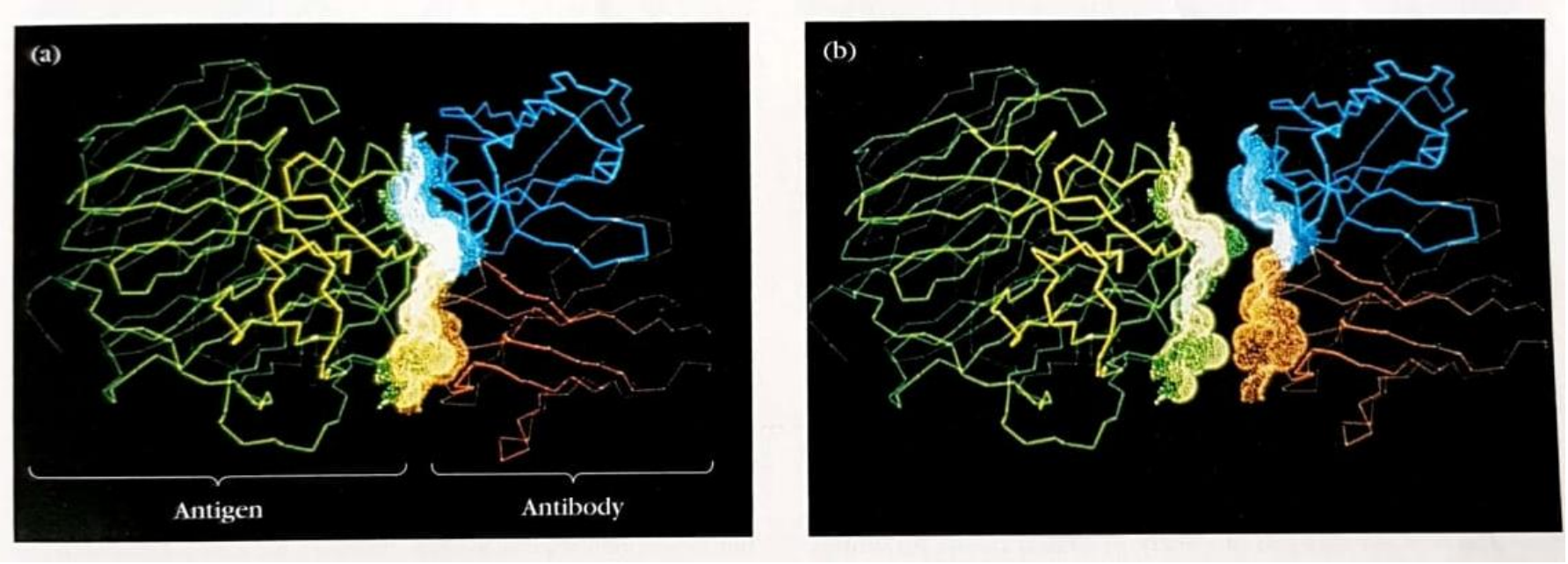


antigen + antibody = antigen-antibody complex



The interaction between an antibody and an antigen depends on four types of noncovalent forces:
(1) hydrogen bonds, in which a hydrogen atom is shared between two electronegative atoms;
(2) **ionic bonds** between oppositely charged residues;
(3) **hydrophobic interactions**, in which water forces hydrophobic groups together; and
(4) **van der Waals interactions** between the outer electron clouds of two or more atoms.

In an aqueous environment, noncovalent interactions are extremely weak and depend upon close complementarity of the shapes of antibody and antigen.



Computer simulation of an interaction between antibody and influenza virus antigen, a globular protein.

a. The antigen (yellow) in shown interacting with antibody molecule; the variable region of the heavy chain is red, and the variable region of the light chain is blue
b. The complementarity of the two molecules is revealed by separating the antigen from the antibody by 8A°. (Based on X-ray crystallography data)

Antibody Affinity

The strength of the total noncovalent interactions between a *single* antigen-binding site on an antibody and a *single* epitope is the **affinity** of the antibody for that epitope. Low-affinity antibodies bind antigen weakly and tend to dissociate readily, whereas high-affinity antibodies bind antigen more tightly and remain bound longer. The association between a binding site on an antibody (Ab) with a monovalent antigen (Ag) can be described by the equation

$$\mathrm{Ag} + \mathrm{Ab} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{Ag\text{-}Ab}$$

K1forward association rate constant, K-1 reverse dissociation rate constant

where k_1 is the forward (association) rate constant and k_{-1} is the reverse (dissociation) rate constant. The ratio of k_1/k_{-1} is the association constant K_a (i.e., $k_1/k_{-1} = K_a$), a measure of affinity. Because K_a is the equilibrium constant for the above reaction, it can be calculated from the ratio of the molar concentration of bound Ag-Ab complex to the molar concentrations of unbound antigen and antibody at equilibrium as follows:

$$K_a = \frac{[\text{Ag-Ab}]}{[\text{Ab}][\text{Ag}]}$$

Ka association constant

$$\text{Ag-Ab} \rightleftharpoons \text{Ab} + \text{Ag}$$

The equilibrium constant for this dissociation reaction, K_d, the **dissociation constant,** is equal to the reciprocal of K_a:

$$K_d = \frac{[\text{Ab}][\text{Ag}]}{[\text{Ab-Ag}]} = 1/K_a$$

The value of K_a varies for different Ag-Ab complexes and depends upon both k_1, which is expressed in units of liters/mole/second (L/mol/s), and k_{-1}, which is expressed in units of 1/second. For small haptens, the forward rate constant can be extremely high; in some cases, k_1 can be as high as 4×10^8 L/mol/s, approaching the theoretical upper limit of diffusion-limited reactions (10^9 L/mol/s). For larger protein antigens, however, k_1 is smaller, with values in the range of 10^5 L/mol/s.

Antibody Avidity

The affinity at one binding site does not always reflect the true strength of the antibody-antigen interaction. When complex antigens containing multiple, repeating antigenic determinants are mixed with antibodies containing multiple binding sites, the interaction of an antibody molecule with an antigen molecule at one site will increase the probability of reaction between those two molecules at a second site.

The strength of such multiple interactions between a multivalent antibody and antigen is called the **avidity.** The avidity of an antibody is a better measure of its binding capacity within biological systems (e.g., the reaction of an antibody with antigenic determinants on a virus or bacterial cell) than the affinity of its individual binding sites. High avidity can compensate for low affinity. For example, secreted pentameric IgM often has a lower affinity than IgG, but the high avidity of IgM, resulting from its higher valence, enables it to bind antigen effectively.

$$\text{Avidity} = K_a \times K_a = (K_a)^2 = K_{eq} = \frac{[\text{Ab-Ag}]}{[\text{Ab}][\text{Ag}]}$$

Eq=equlibrium constant

Haptens:

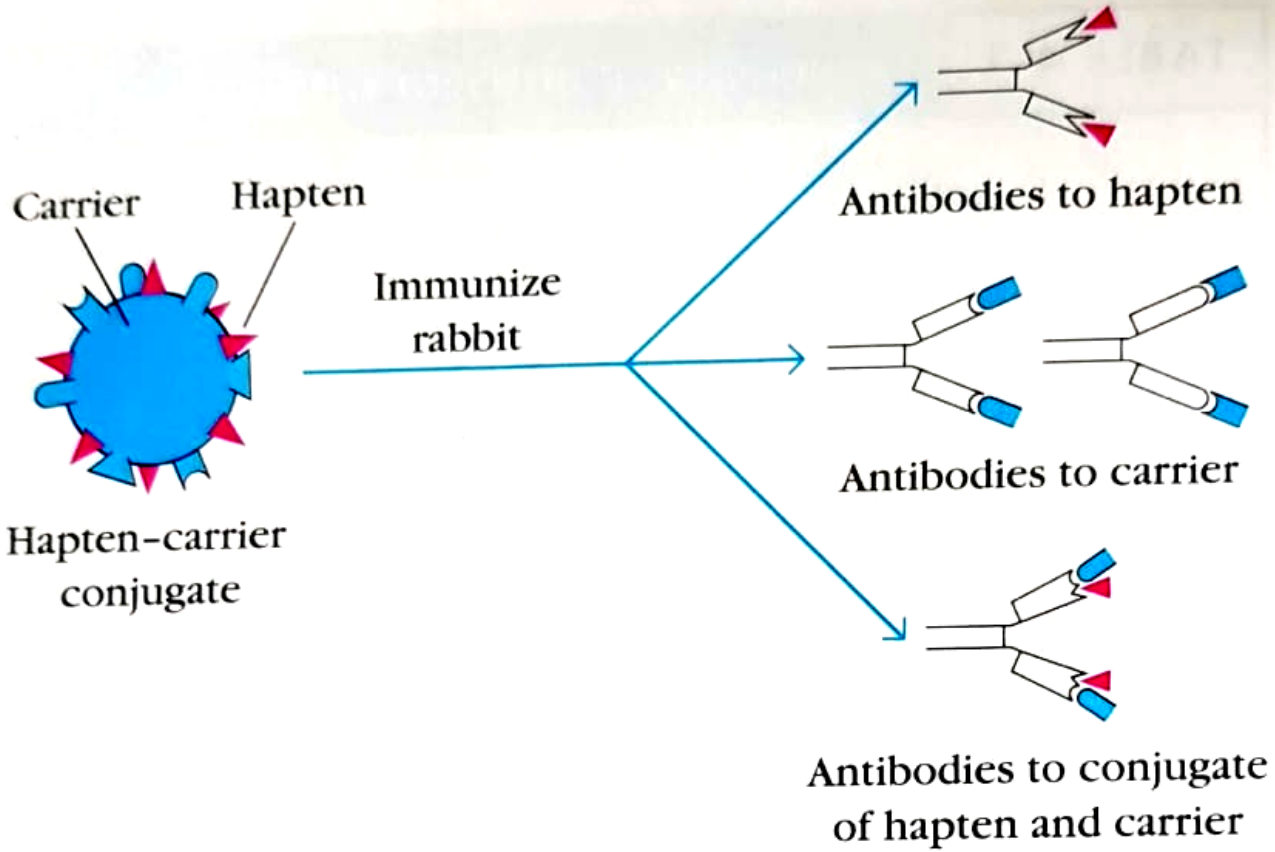
Haptens are small organic molecules that are antigenic but not immunogenic. Chemical coupling of a hapten to a large protein, called carrier, yields an immunogenic hapten-carrier conjugate.

Aniamls immunized with such a conjugate produce antibodies specific for

1. Hapten determinants
2. Unaltered epitopes on carrier protein
3. New epitope formed by combined parts both the hapten and carrier

Example; dinitrophenol chemically linked to large protein carrier such as bovine serum albumin (BSA) acts as immunogenic molecule.

1. Drugs, peptide hormones, steroid hormones.
2. Drug-protein conjugation can produce drug allergies that may be life threatening.



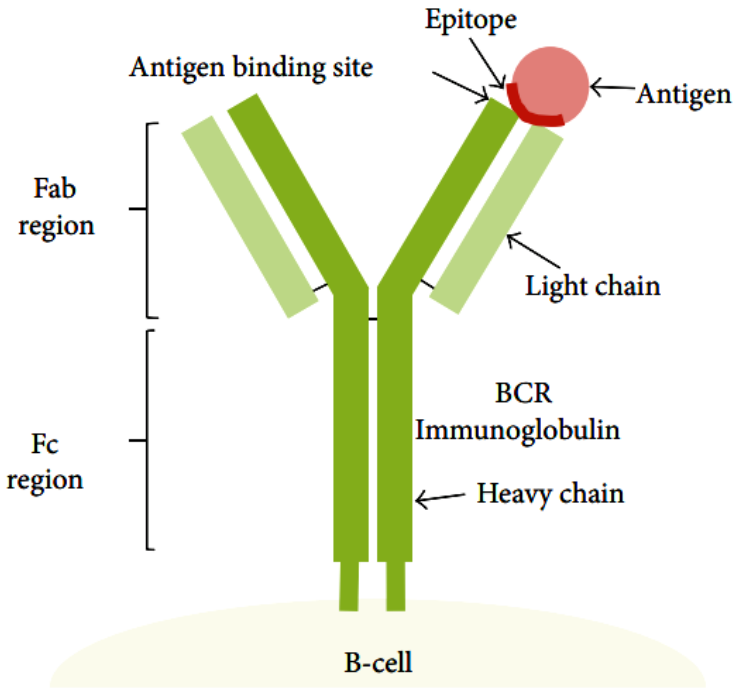
Injection with:	Antibodies formed:
Hapten (DNP)	None
Protein carrier (BSA)	Anti–BSA
Hapten–carrier conjugate (DNP-BSA)	Anti–DNP (major) Anti–BSA (minor) Anti–DNP/BSA (minor)

Cross-Reactivity

Although Ag-Ab reactions are highly specific, in some cases antibody elicited by one antigen can cross-react with an unrelated antigen.

Such cross-reactivity occurs if two different antigens **share an identical or very similar epitope.** In the latter case, the antibody's affinity for the cross-reacting epitope is usually less than that for the original epitope.

An epitope is the part of an antigen that interacts with the antigen specific receptor or antibody.



Cross-reactivity is often observed among polysaccharide antigens that contain similar oligosaccharide residues. The ABO blood-group antigens, for example, are glycoproteins expressed on red blood cells. Subtle differences in the terminal residues of the sugars attached to these surface proteins distinguish the A and B blood-group antigens.

An individual lacking one or both of these antigens will have serum antibodies to the missing antigen(s). The antibodies are induced not by exposure to red blood cell antigens but by exposure to cross-reacting microbial antigens present on common intestinal bacteria.

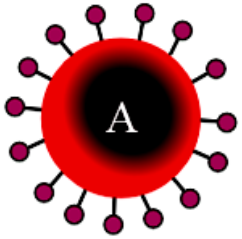
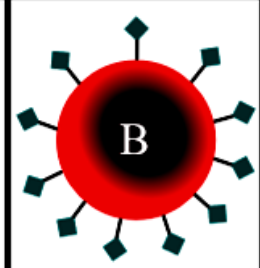
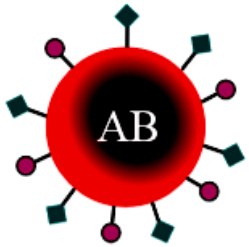
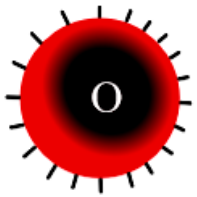
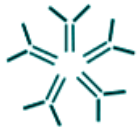
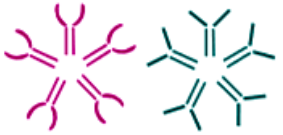
These microbial antigens induce the formation of antibodies in individuals lacking the similar blood-group antigens on their red blood cells. (In individuals possessing these antigens, complementary antibodies would be eliminated during the developmental stage in which antibodies that recognize self epitopes are weeded out.)

The blood-group antibodies, although elicited by microbial antigens, will cross-react with similar oligosaccharides on foreign red blood cells, providing the basis for blood typing tests and accounting for the necessity of compatible blood types during blood transfusions.

A type A individual has anti-B antibodies; a type B individual has anti-A; and a type O individual thus has anti-A and anti-B

ABO blood types

Blood type	Antigens on RBCs	Serum antibodies
A	A	Anti-B
B	B	Anti-A
AB	A and B	Neither
O	Neither	Anti-A and anti-B

	Group A	Group B	Group AB	Group O
Red blood cell type				
Antibodies in plasma	 Anti-B	 Anti-A	None	 Anti-A and Anti-B

Antigens in red blood cell	A antigen	B antigen	 A and B antigens	None

A number of viruses and bacteria have antigenic determinants identical or similar to normal host-cell components.

In some cases, these microbial antigens have been shown to elicit antibody that cross-reacts with the host-cell components, resulting in a tissue-damaging autoimmune reaction.

The bacterium *Streptococcus pyogenes*, for example, expresses cell-wall proteins called M antigens. Antibodies produced to streptococcal M antigens have been shown to cross-react with several myocardial and skeletal muscle proteins and have been implicated in heart and kidney damage following streptococcal infections.

Some vaccines also exhibit cross-reactivity. For instance, *vaccinia* virus, which causes cowpox, expresses cross-reacting epitopes with *variola* virus, the causative agent of smallpox.
This cross-reactivity was the basis of Jenner's method of using *vaccinia* virus to induce immunity to smallpox.

Precipitation Reactions

Antibody and soluble antigen interacting in aqueous solution form a lattice that eventually develops into a visible precipitate.

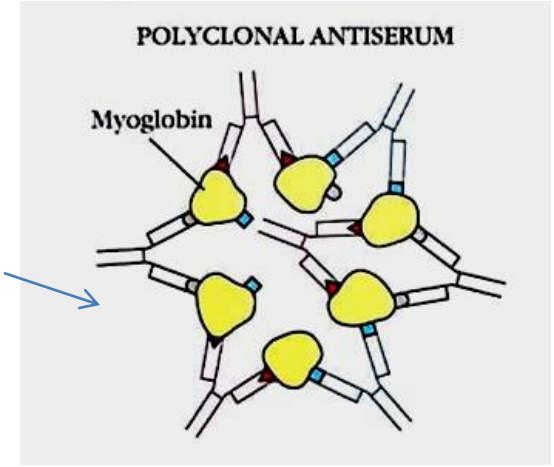
Antibodies that aggregate soluble antigens are called precipitins. Although formation of the soluble Ag-Ab complex occurs within minutes, formation of the visible precipitate occurs more slowly and often takes a day or two to reach completion.

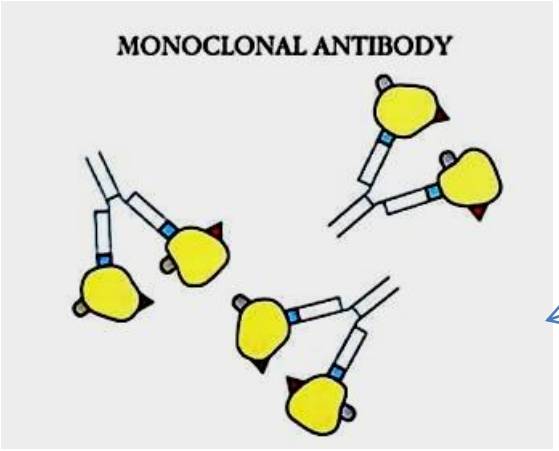
Formation of an Ag-Ab lattice depends on the valency of both the antibody and antigen:

The antibody must be bivalent; a precipitate will not form with monovalent Fab fragments.

The antigen must be either bivalent or polyvalent; that is, it must have at least two copies of the same epitope, or have different epitopes that react with different antibodies present in polyclonal antisera.

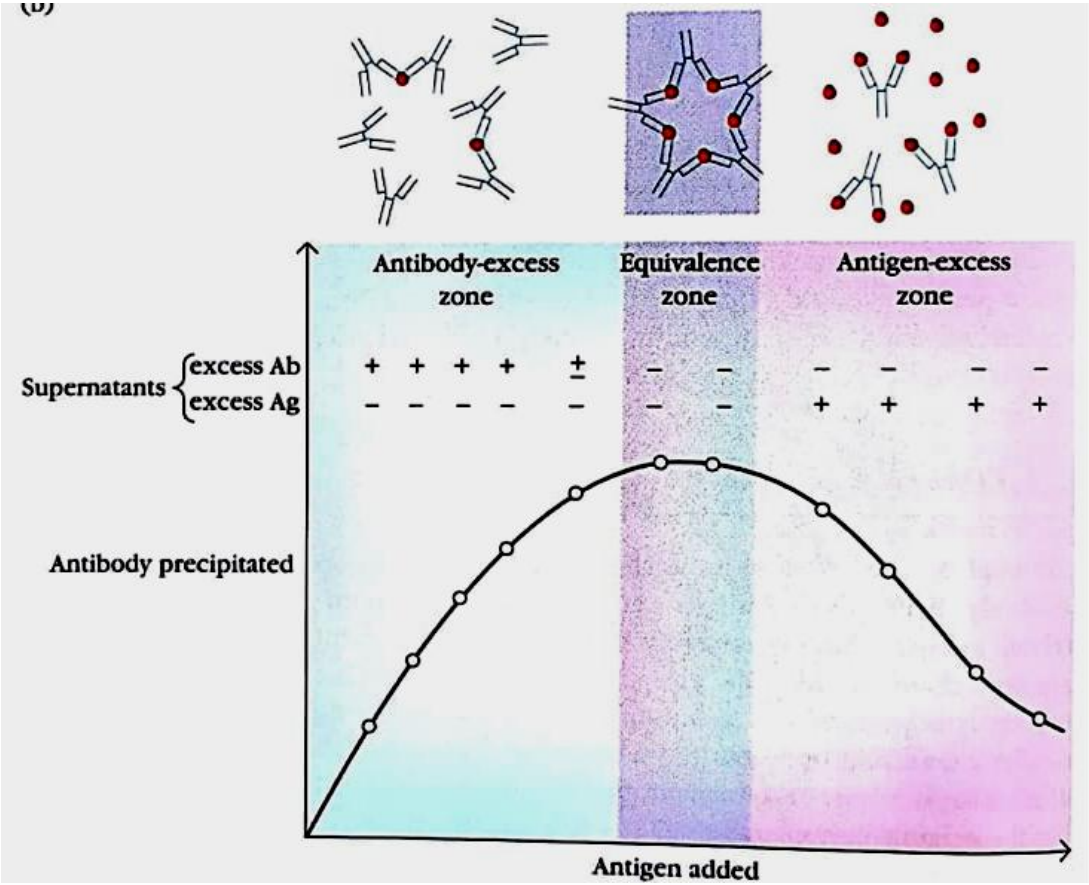
Precipitation





No precipitation

Polyclonal antibodies can form lattices, or large aggregates, that precipitate out of solution. However, if each antigen molecule contains only a single epitope recognized by a given monoclonal antibody, the antibody can link only two molecules of antigen and no precipitate is formed.



A precipitation curve for a system of one antigen and its antibodies.

This plot of the amount of antibody precipitated versus increasing antigen concentrations (at constant total antibody) reveals three zones:

1. A zone of antibody excess, in which precipitation is inhibited and antibody not bound to antigen can be detected in the supernatant;
2. An equivalence zone of maximal precipitation in which antibody and antigen form large insoluble complexes and neither antibody nor antigen can be detected in the supernatant;
3. A zone of antigen excess in which precipitation is inhibited and antigen not bound to antibody can be detected in the supernatant.

Precipitation Reactions in Gels Yield Visible Precipitin Lines

Immune precipitates can form not only in solution but also in an agar matrix.
When antigen and antibody diffuse toward one another in agar, or when antibody is incorporated into the agar and antigen diffuses into the antibody-containing matrix, a visible line of precipitation will form. As in a precipitation reaction in fluid, visible precipitation occurs in the region of equivalence, whereas no visible precipitate forms in regions of antibody or antigen excess.

Two types of immunodiffusion reactions can be used to determine relative concentrations of antibodies or antigens, to compare antigens, or to determine the relative purity of an antigen preparation.

They are **radial immunodiffusion** (the Mancini method) and

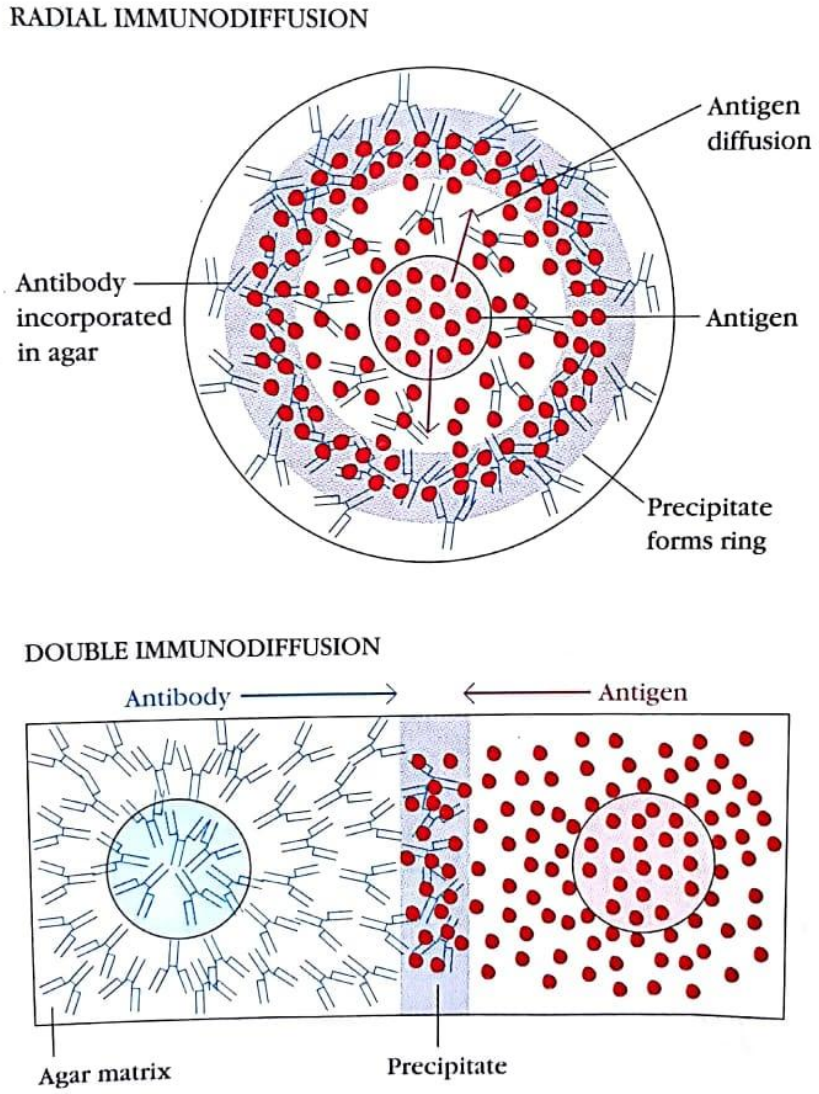
double **immunodiffusion** (the Ouchterlony method);

both are carried out in a semisolid medium such as agar.
In radial immunodiffusion, an antigen sample is placed in a well and allowed to diffuse intoagar containing a suitable dilution of an antiserum. As the antigen diffuses into the agar, the region of equivalence is established and a ring of precipitation, a precipitin ring, forms around the well.

The area of the precipitin ring is proportional to the concentration of antigen. By comparing the area of the precipitin ring with a standard curve (obtained by measuring the precipitin areas of known concentrations of the antigen), the concentration of the antigen sample can be determined.

In the Ouchterlony method, both antigen and antibody diffuse radially from wells toward each other, thereby establishing a concentration gradient. As equivalence is reached, a visible line of precipitation, a precipitin line, forms.

Precipitation reaction

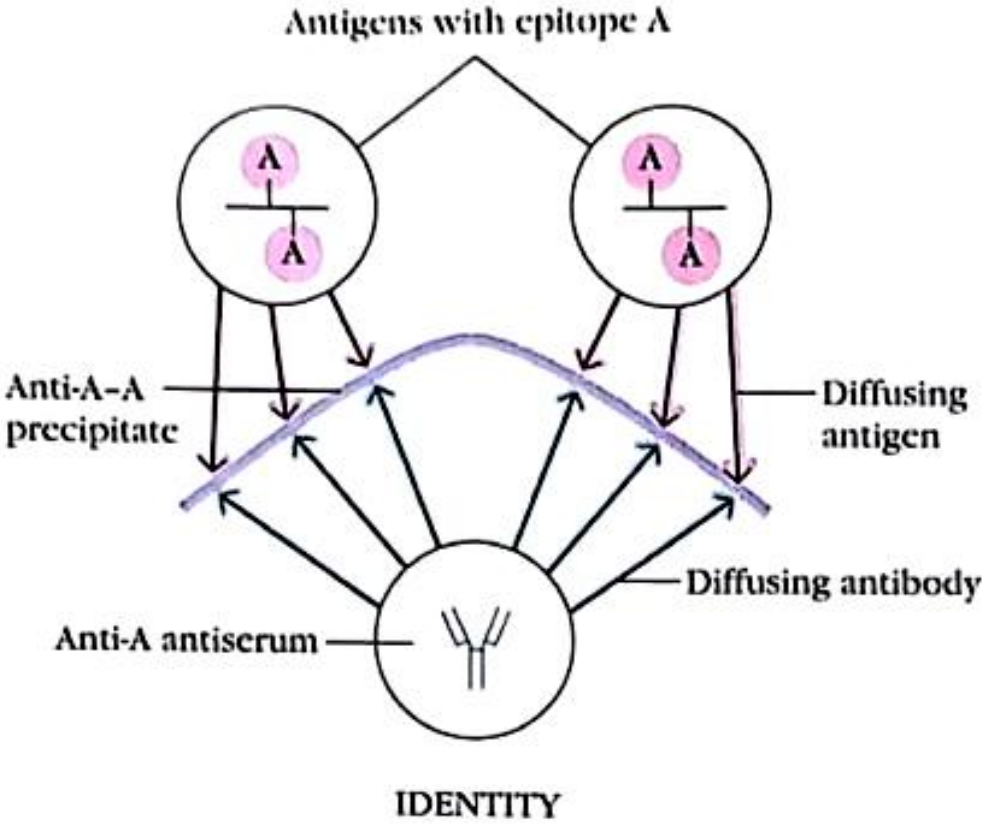


Diagrammatic representation of **radial immunodiffusion (Mancini method)** and **double immunodiffusion (Ouchterlony method)** in a gel.

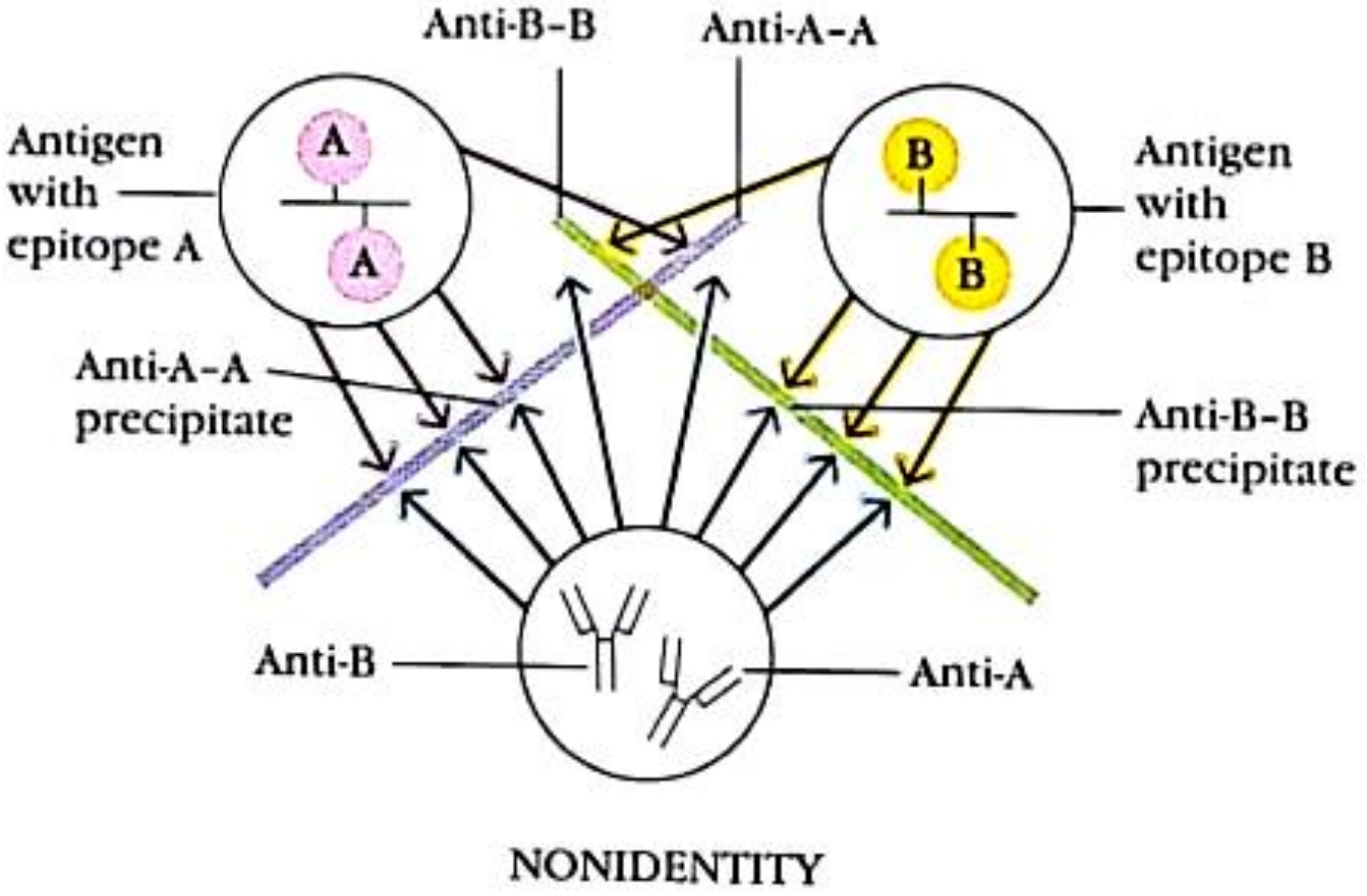
In both cases, large insoluble complexes form in the agar in the zone of equivalence, visible as lines of precipitation (purple regions).

Only the antigen (red) diffuses in radial immunodiffusion, whereas both the antibody (blue) and antigen (red) diffuse in double immunodiffusion.

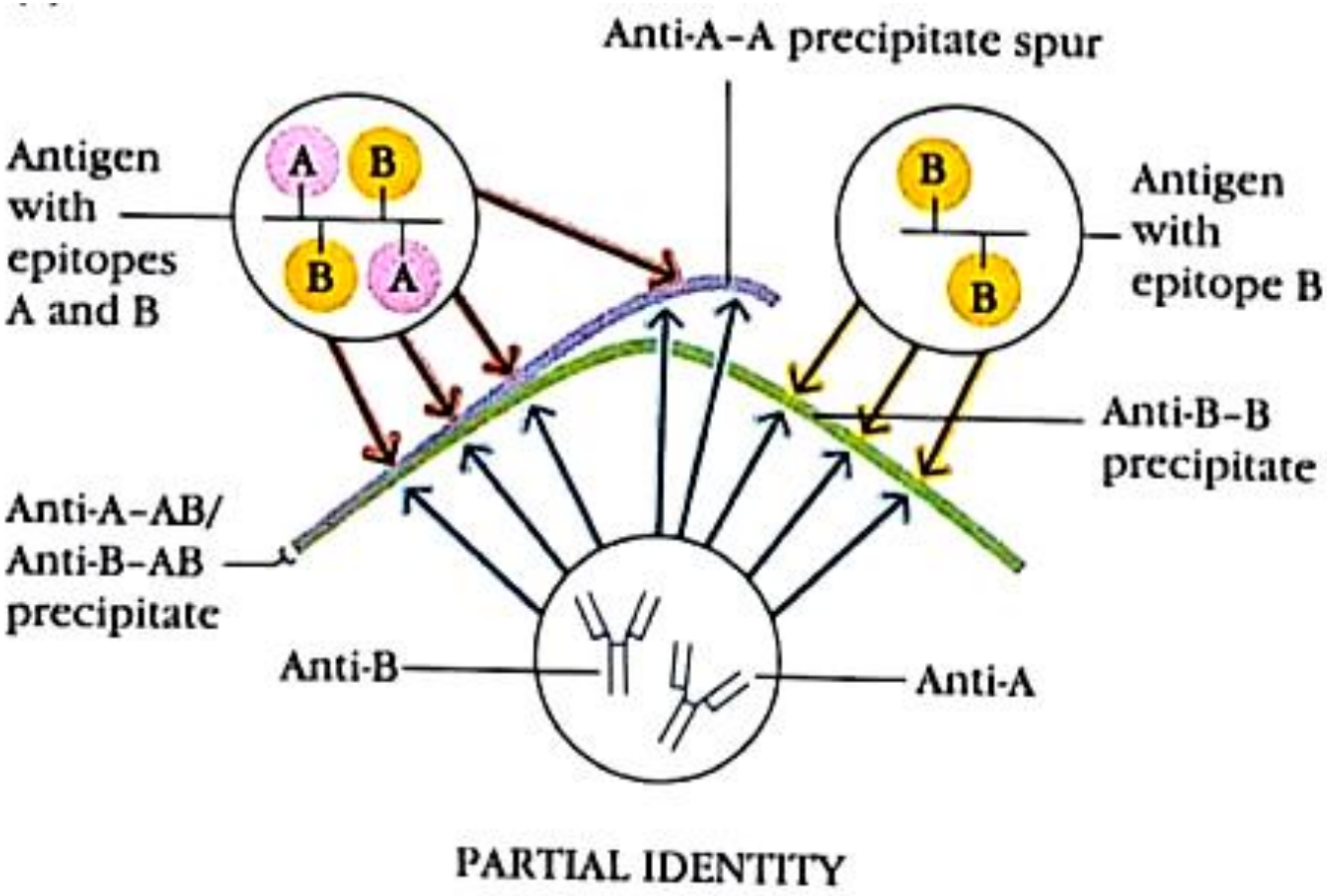
Different patterns of lines obtained on Ouchterlony double diffusion



The antibodies in the antiserum react with both the antigens resulting in a smooth continuous line of precipitation. The antibodies cannot distinguish between the two antigens. i.e., the two antigens are immunologically identical.



non-identity (i.e. the two lines cross completely).

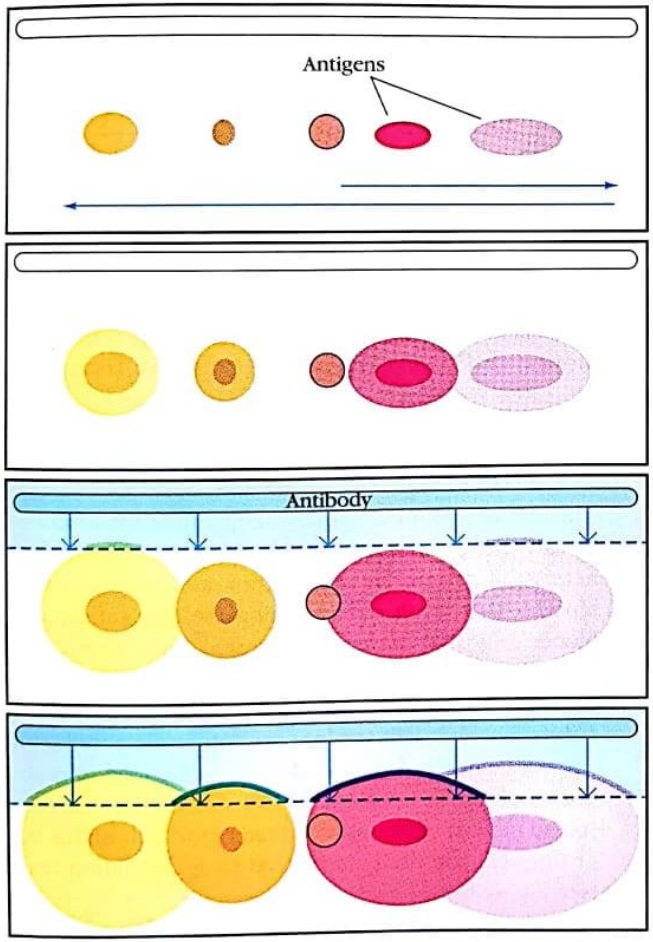


partial identity (i.e. a continuous line with a spur at one end)

Immunoelectrophoresis

Combines Electrophoresis and Double Immunodiffusion
In this method, the antigen mixture is first electrophoresed to separate its components by charge.
Troughs are then cut into the agar gel parallel to the direction of the electric field, and antiserum is added to the troughs.

Antibody and antigen then diffuse toward each other and produce lines of precipitation where they meet in appropriate proportions.



1. Antigens electrophoresed on the basis of charge.

2. Antiserum is added to the troughs and allowed to diffuse.

3. Lines of precipitation form where specific antibody and antigen react.

An antigen preparation (orange) is first electrophoresed, which separates the component antigens on the basis of charge. Antiserum (blue) is then added to troughs on one or both sides of the separated antigens and allowed to diffuse; in time, lines of precipitation (colored arcs) form where specific antibody and antigen interact.

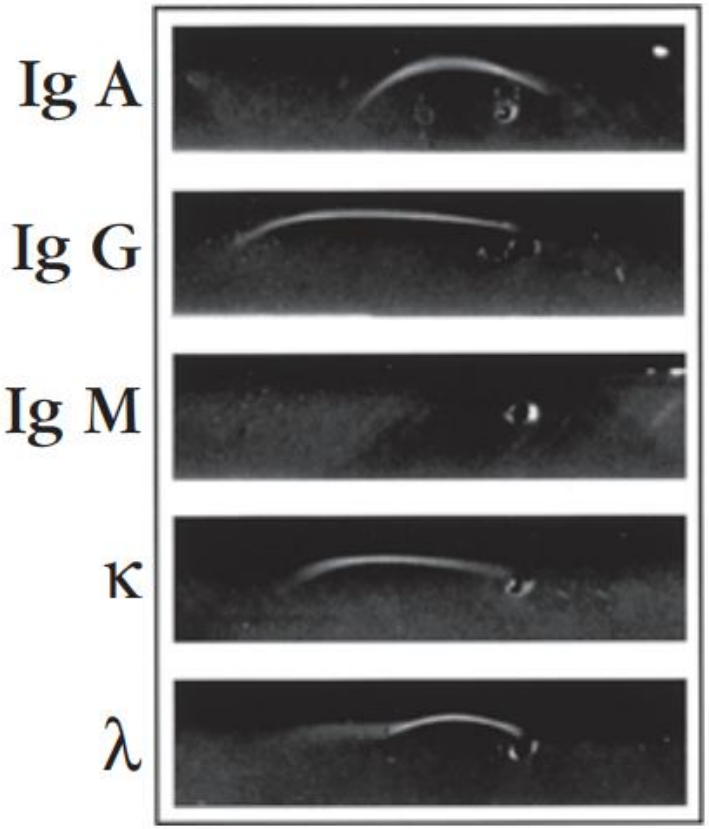
Immunoelectrophoresis is used in clinical laboratories to detect the presence or absence of proteins in the serum. A sample of serum is electrophoresed, and the individual serum components are identified with antisera specific for a given protein or immunoglobulin class .

This technique is useful in determining whether a patient produces abnormally low amounts of one or more isotypes, characteristic of certain immunodeficiency diseases.

It can also show whether a patient overproduces some serum protein, such as albumin, immunoglobulin, or transferrin.

The immunoelectrophoretic pattern of serum from patients with multiple myeloma, for example, shows a heavy distorted arc caused by the large amount of myeloma protein, which is monoclonal Ig and therefore uniformly charged .

Because immunoelectrophoresis is a strictly qualitative technique that only detects relatively high antibody concentrations (greater than several hundred g/ml), it utility is limited to the detection of quantitative abnormalities only when the departure from normal is striking, as in immunodeficiency states and immunoproliferative disorders.



Immunoelectrophoretic patterns of human serum from a patient with myeloma.

The patient produces a large amount of a monoclonal IgG (λ-light-chain-bearing) antibody.
A sample of serum from the patient was placed in the well of the slide and electrophoresed.

Then antiserum specific for the indicated antibody class or light chain type was placed in the top trough of each slide.
At the concentrations of patient's serum used, only anti-IgG and anti λ- antibodies produced lines of precipitation.

Rocket electrophoresis

A related quantitative technique, rocket electrophoresis, does permit measurement of antigen levels.

In rocket electrophoresis, a negatively charged antigen is electrophoresed in a gel containing antibody.

The precipitate formed between antigen and antibody has the shape of a rocket, the height of which is proportional to the concentration of antigen in the well.

One limitation of rocket electrophoresis is the need for the antigen to be negatively charged for electrophoretic movement within the agar matrix.

Some proteins, immunoglobulins for example, are not sufficiently charged to be quantitatively analyzed by rocket electrophoresis; nor is it possible to measure the amounts of several antigens in a mixture at the same time.

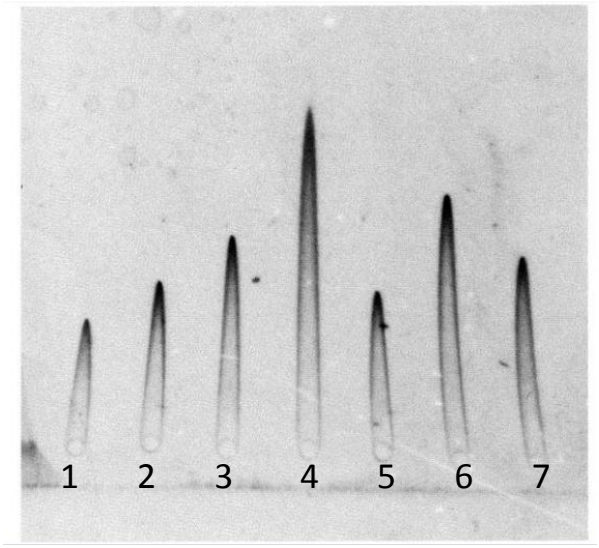


Fig. A rocket immunoelectrophoresis gel (5 x 5 cm) run at 20 mA for 3h, and stained for protein with Coomassie Brilliant Blue. (Walker, 1984 Methods Mol Biol.1:317-23. doi: 10.1385/0-89603-062-8:317.)

Rocket immunoelectrophoresis

The gel contained anti-bovine serum albumin (50 µL, anti BSA) and the sample loading (2 µL/well) were (left to right):

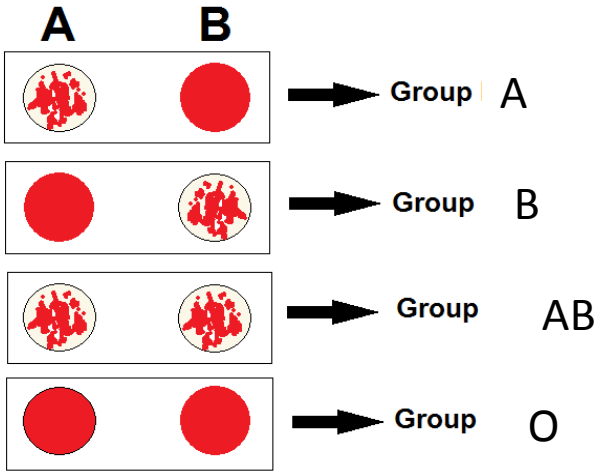
1. 40 ng BSA;
2. 67 ng BSA;
3. 100 ng BSA;
4. 200 ng BSA;
5. a 1: 1200 dilution of bovine serum;
6. 130 ng BSA;
7. 100 ng BSA

Agglutination Reactions

The interaction between antibody and a particulate antigen results in visible clumping called agglutination.
Antibodies that produce such reactions are called **agglutinins**. Agglutination reactions are similar in principle to precipitation reactions; they depend on the crosslinking of polyvalent antigens.
Just as an excess of antibody inhibits precipitation reactions, such excess can also inhibit agglutination reactions; this inhibition is called the **prozone effect**.

Hemagglutination is used in blood typing

In typing for the ABO antigens, RBCs are mixed on a slide with antisera to the A or B blood-group antigens. If the antigen is present on the cells, they agglutinate, forming a visible clump on the slide. Determination of which antigens are present on donor and recipient RBCs is the basis for matching blood types for transfusions.



Bacterial Agglutination Is Used To Diagnose Infection

A bacterial infection often elicits the production of serum antibodies specific for surface antigens on the bacterial cells. The presence of such antibodies can be detected by bacterial agglutination reactions.

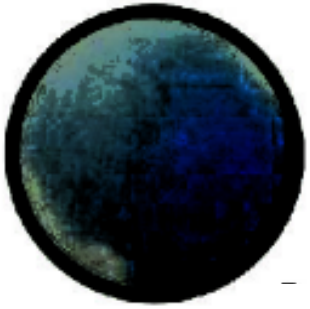
Serum from a patient thought to be infected with a given bacterium is serially diluted in an array of tubes to which the bacteria is added. The last tube showing visible agglutination will reflect the serum antibody titer of the patient.

The agglutinin titer is defined as the reciprocal of the greatest serum dilution that elicits a positive agglutination reaction.

For example, if serial twofold dilutions of serum are prepared and if the dilution of 1/640 shows agglutination but the dilution of 1/1280 does not, then the agglutination titer of the patient's serum is 640.

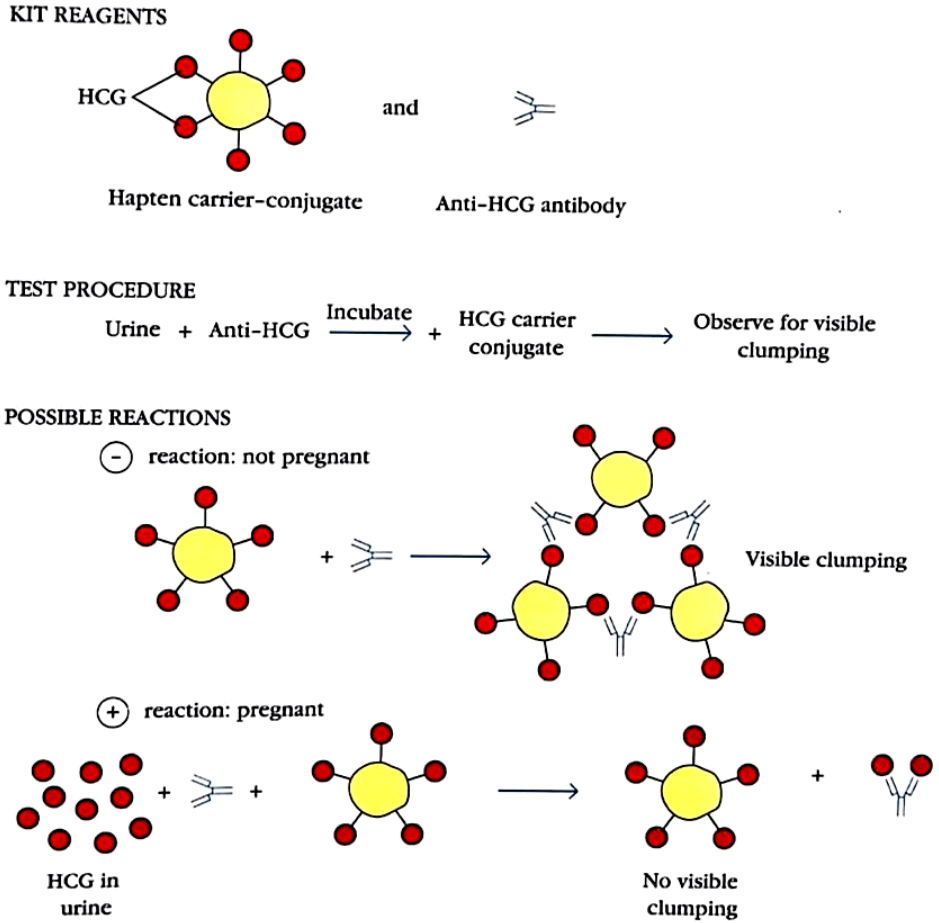
In some cases serum can be diluted up to 1/50,000 and still show agglutination of bacteria. The agglutinin titer of an antiserum can be used to diagnose a bacterial infection. Patients with typhoid fever, for example, show a significant rise in the agglutination titer to *Salmonella typhi.*

Agglutination



No Agglutination

In Agglutination Inhibition, Absence of Agglutination Is Diagnostic of Antigen



A modification of the agglutination reaction, called agglutination inhibition, provides a highly sensitive assay for small quantities of an antigen.

For example, one of the early types of home pregnancy test kits included latex particles coated with human chorionic gonadotropin (HCG) and antibody to HCG.

The addition of urine from a pregnant woman, which contained HCG, inhibited agglutination of the latex particles when the anti-HCG antibody was added; thus the absence of agglutination indicated pregnancy.

Detection methods for antibodies or antigens

Radioimmunoassay (RIA)

Enzyme-Linked Immunosorbent Assay

Chemiluminescence

Elispot Assay

Western Blotting

Immunoprecipitation

immunofluorescence

Flow Cytometry

Radioimmunoassay (RIA)

One of the most sensitive techniques for detecting antigen or antibody is radioimmunoassay (RIA). The technique was first developed in 1960 by two endocrinologists, S. A. Berson and Rosalyn Yalow, to determine levels of insulin–anti-insulin complexes in diabetics. In 1977, some years after Berson's death, the significance of the technique was acknowledged by the award of a Nobel Prize to Yalow

The principle of RIA involves competitive binding of radiolabeled antigen and unlabeled antigen to a high-affinity antibody.

The labeled antigen is mixed with antibody at a concentration that saturates the antigen-binding sites of the antibody.

Then test samples of unlabeled antigen of unknown concentration are added in progressively larger amounts.

The antibody does not distinguish labeled from unlabeled antigen, so the two kinds of antigen compete for available binding sites on the antibody.

As the concentration of unlabeled antigen increases, more labeled antigen will be displaced from the binding sites.

The decrease in the amount of radiolabeled antigen bound to specific antibody in the presence of the test sample is measured in order to determine the amount of antigen present in the test sample.

The antigen is generally labeled with a gamma-emitting isotope such as ^{125}I, but beta-emitting isotopes such as tritium (^{3}H) are also routinely used as labels.

The radiolabeled antigen is part of the assay mixture; the test sample may be a complex mixture, such as serum or other body fluids, that contains the unlabeled antigen.

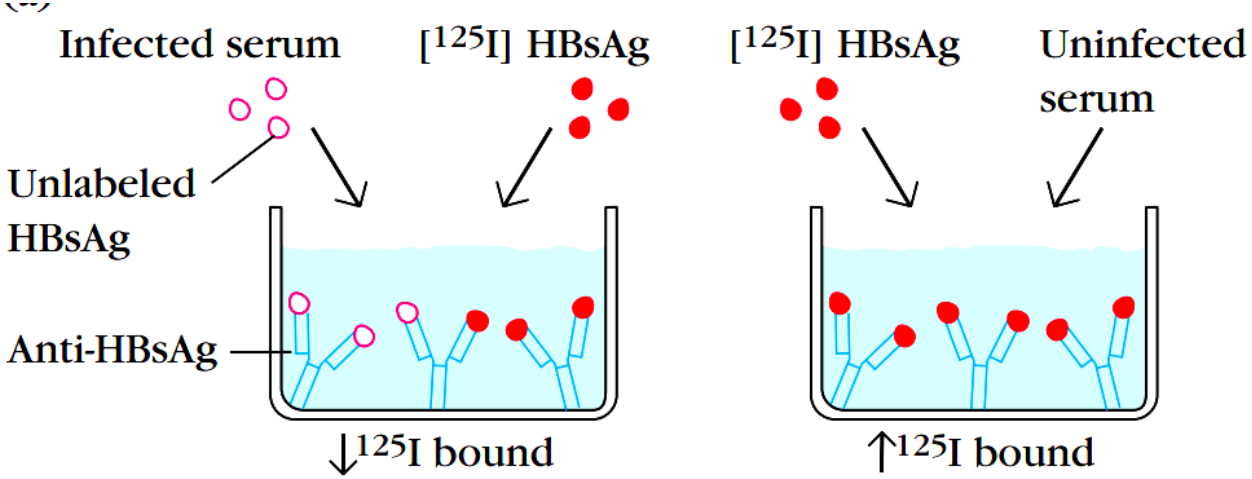
The first step in setting up an RIA is to determine the amount of antibody needed to bind 50%–70% of a fixed quantity of radioactive antigen (Ag^*) in the assay mixture. This ratio of antibody to Ag^* is chosen to ensure that the number of epitopes presented by the labeled antigen always exceeds the total number of antibody binding sites.

Consequently, unlabeled antigen added to the sample mixture will compete with radiolabeled antigen for the limited supply of antibody.

Even a small amount of unlabeled antigen added to the assay mixture of labeled antigen and antibody will cause a decrease in the amount of radioactive antigen bound, and this decrease will be proportional to the amount of unlabeled antigen added.

To determine the amount of labeled antigen bound, the Ag-Ab complex is precipitated to separate it from free antigen (antigen not bound to Ab), and the radioactivity in the precipitate is measured.

A standard curve can be generated using unlabeled antigen samples of known concentration (in place of the test sample), and from this plot the amount of antigen in the test mixture may be precisely determined.



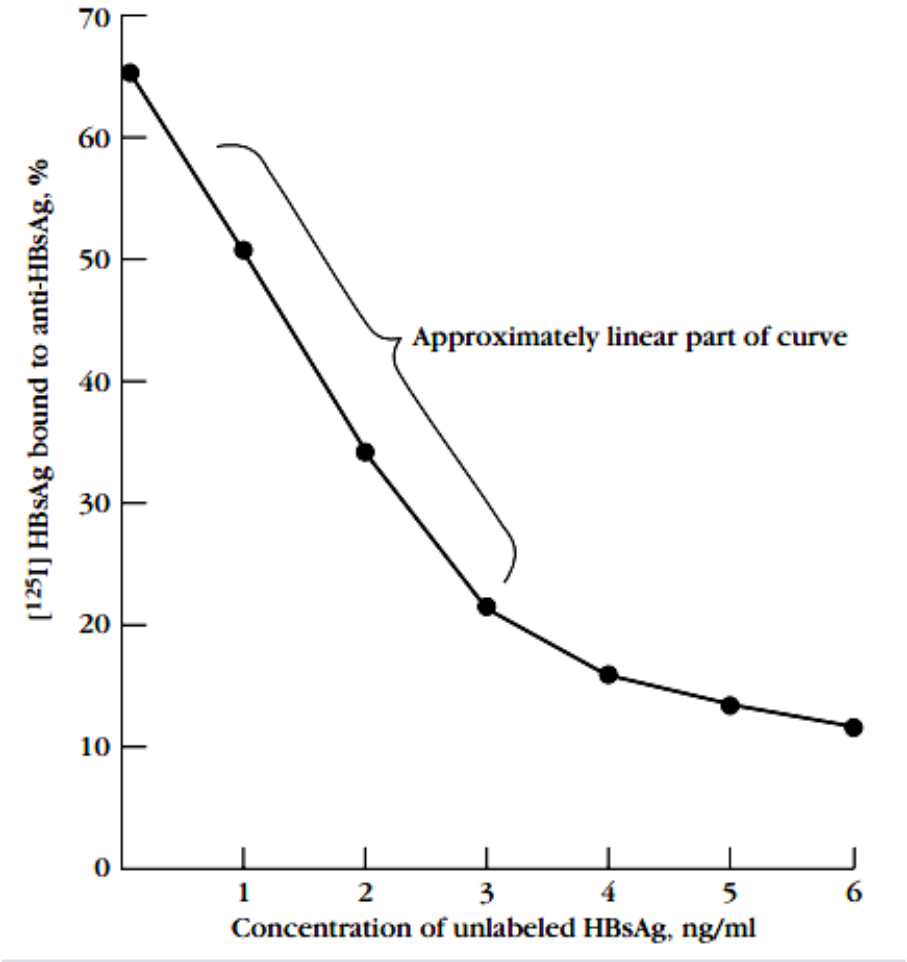
A solid-phase radioimmunoassay (RIA) to detect hepatitis B virus in blood samples.

Microtiter wells are coated with a constant amount of antibody specific for HBsAg, the surface antigen on hepatitis B virions.

A serum sample and [^{125}I]HBsAg are then added.

(Unlabeled antigen will compete with radiolabeled antigen and cause decrease in amount of radioactivity.)

After incubation, the supernatant is removed and the radioactivity of the antigen-antibody complexes is measured. If the sample is infected, the amount of label bound will be less than in controls with uninfected serum.



A standard curve is obtained by adding increasing concentrations of unlabeled HBsAg to a fixed quantity of [^{125}I]HBsAg and specific antibody. From the plot of the percentage of labeled antigen bound versus the concentration of unlabeled antigen, the concentration of HBsAg in unknown serum samples can be determined by using the linear part of the curve.

Enzyme-Linked Immunosorbent Assay

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

An enzyme conjugated with an antibody reacts with a colorless substrate to generate a colored reaction product. Such a substrate is called a chromogenic substrate.

A number of enzymes have been employed for ELISA, including alkaline phosphatase, horseradish peroxidase, and β-galactosidase.

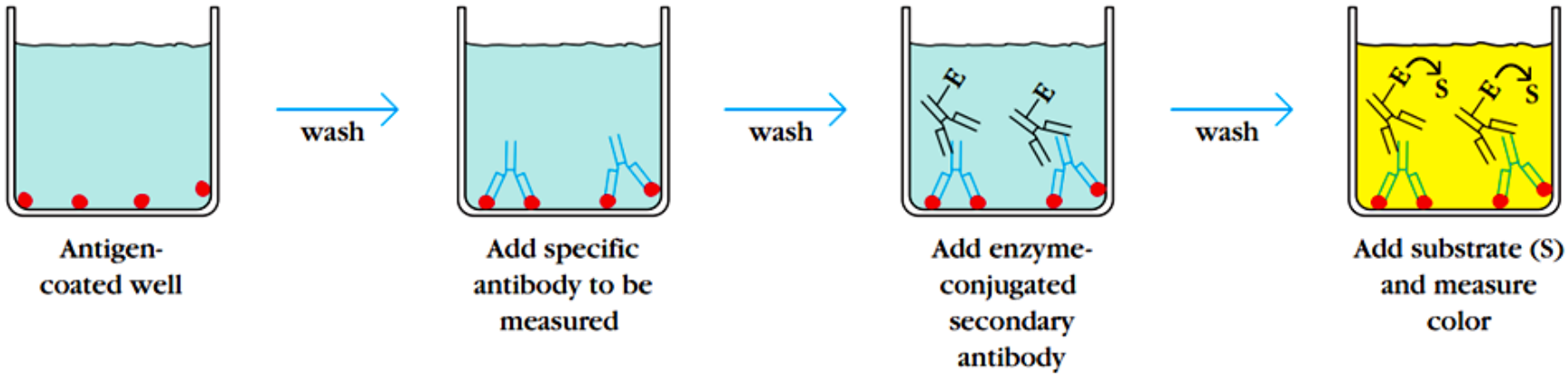
These assays approach the sensitivity of RIAs and have the advantage of being safer and less costly.

Variations in the enzyme-linked immunosorbent assay (ELISA) technique allow determination of antibody or antigen. Each assay can be used qualitatively, or quantitatively by comparison with standard curves prepared with known concentrations of antibody or antigen.

Antibody can be determined with an indirect ELISA

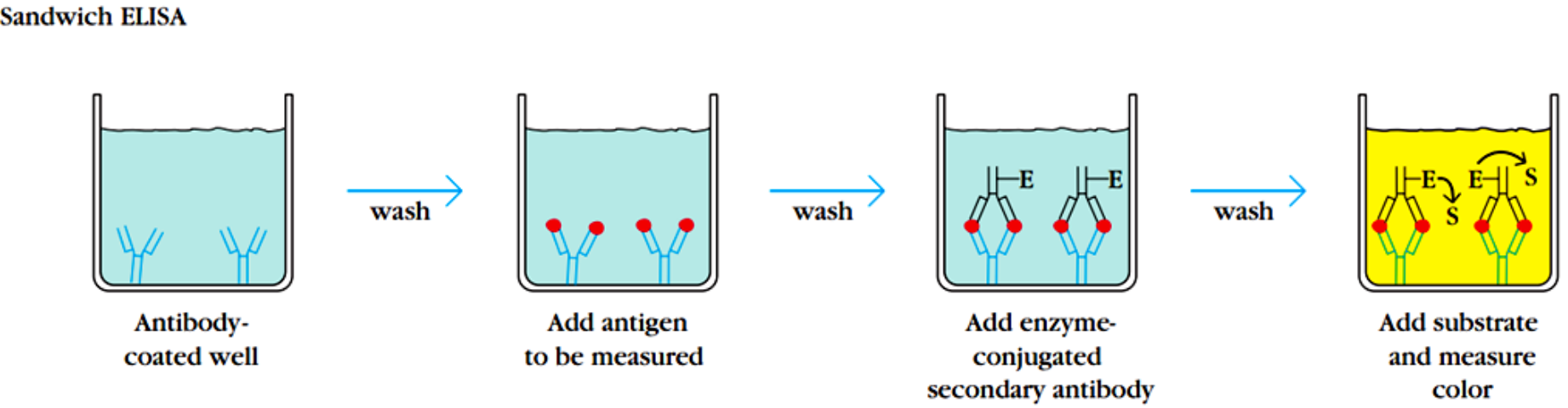
Antibody can be detected or quantitatively determined with an indirect ELISA . Serum or some other sample containing primary antibody (Ab_1) is added to an antigen-coated microtiter well and allowed to react with the antigen attached to the well. After any free Ab_1 is washed away, the presence of antibody bound to the antigen is detected by adding an enzyme-conjugated secondary anti-isotype antibody (Ab_2), which binds to the primary antibody. Any free Ab_2 then is washed away, and a substrate for the enzyme is added. The amount of colored reaction product that forms is measured by specialized spectrophotometric plate readers, which can measure the absorbance of all of the wells of a 96-well plate in seconds

Indirect ELISA



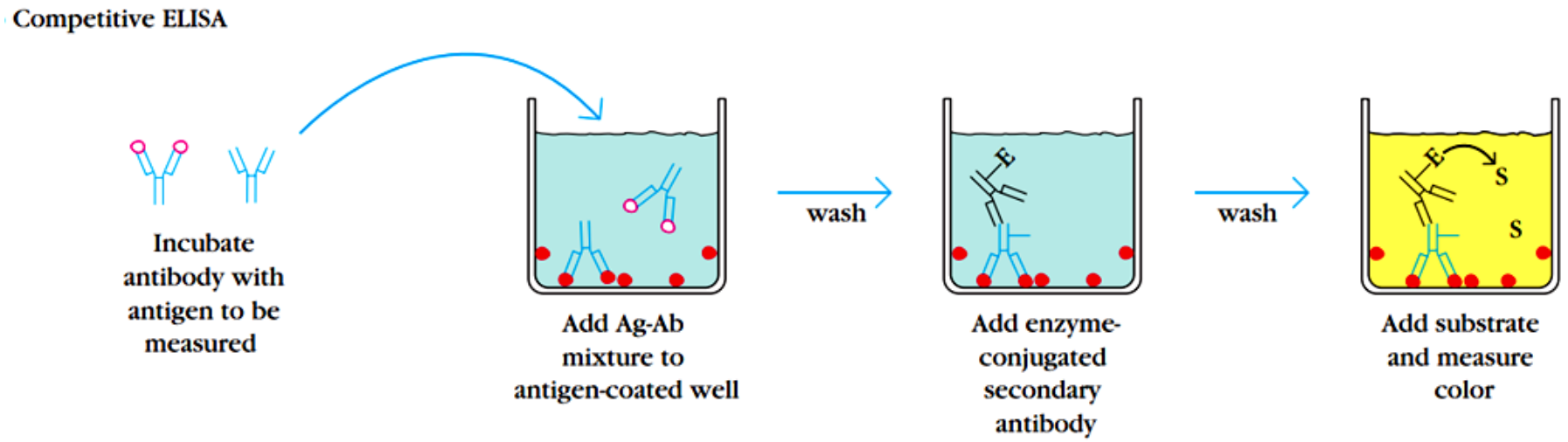
Antigen can be detected or measured by a sandwich ELISA .

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



Another variation for measuring amounts of antigen is competitive ELISA

In this technique, antibody is first incubated in solution with a sample containing antigen. The antigen-antibody mixture is then added to an antigenc oated microtiter well. The more antigen present in the sample, the less free antibody will be available to bind to the antigen-coated well. Addition of an enzyme-conjugated secondary antibody (Ab2) specific for the isotype of the primary antibody can be used to determine the amount of primary antibody bound to the well as in an indirect ELISA. In the competitive assay, however, the higher the concentration of antigen in the original sample, the lower the absorbance.



CHEMILUMINESCENCE

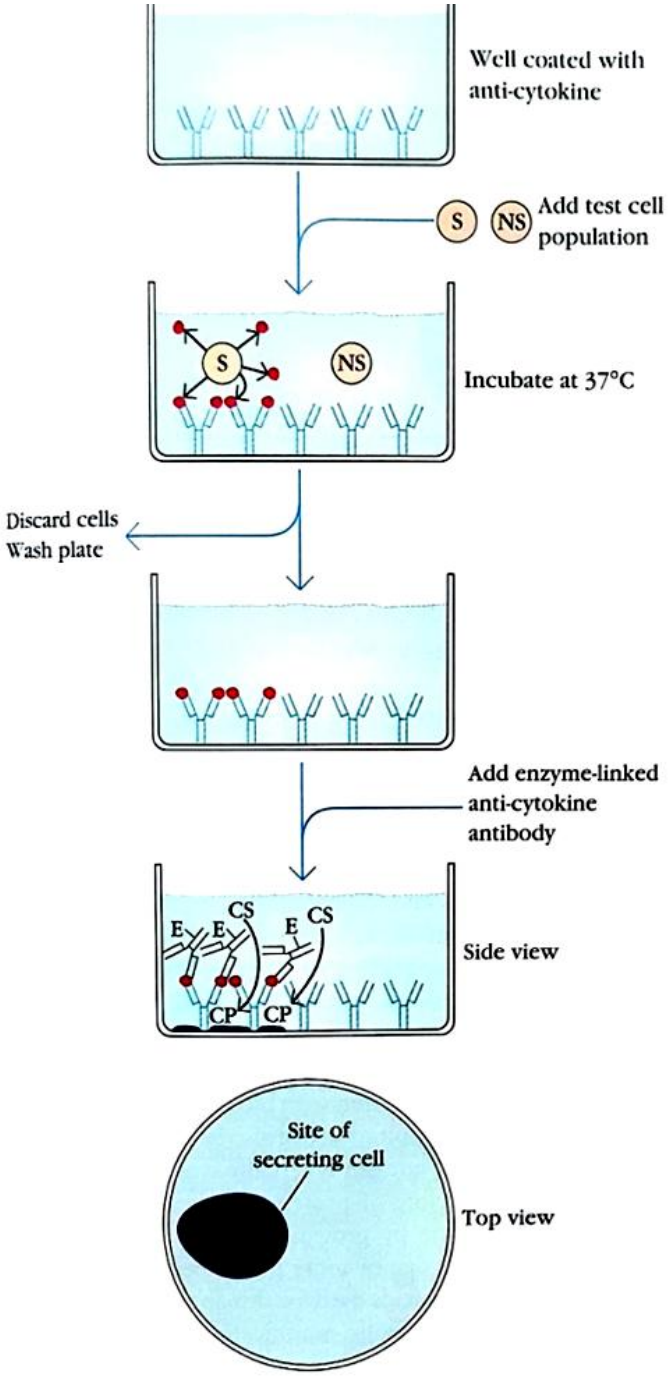
Measurement of light produced by chemiluminescence during certain chemical reactions provides a convenient and highly sensitive alternative to absorbance measurements in ELISA assays.

In versions of the ELISA using chemiluminescence, a luxogenic (light-generating) substrate takes the place of the chromogenic substrate in conventional ELISA reactions.

For example, oxidation of the compound luminol by H_2O_2 and the enzyme horseradish peroxidase (HRP) produces light:

$$\text{Ab-HRP} + \text{Ag} \longrightarrow \text{Ab-HRP-Ag} \xrightarrow{\text{luminol} + H_2O_2} \text{light}$$

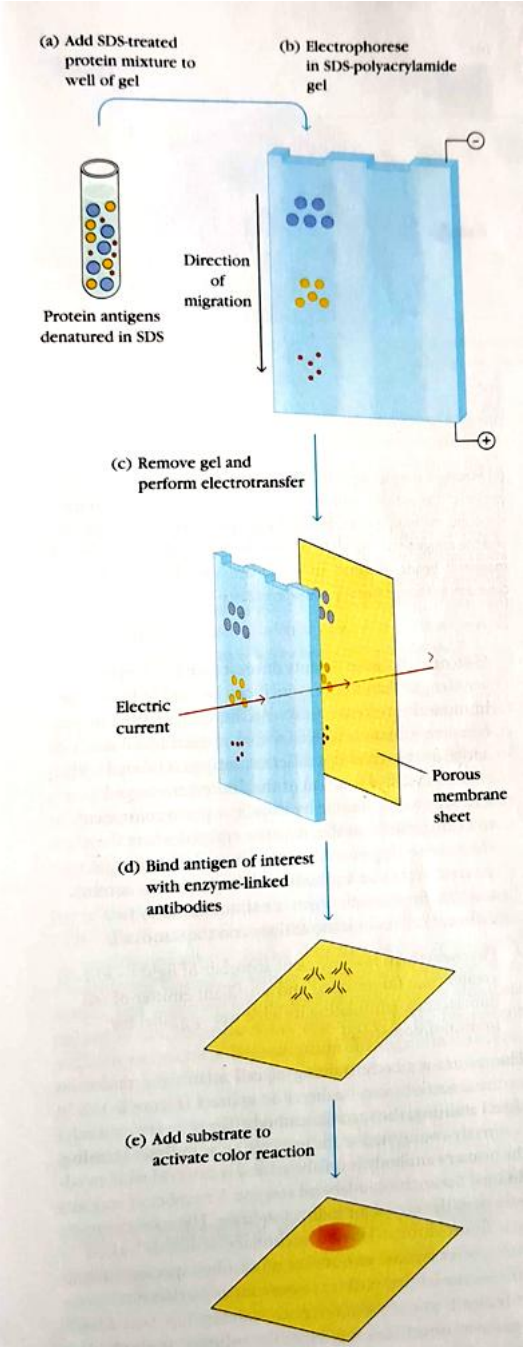
The advantage of chemiluminescence assays over chromogenic ones is enhanced sensitivity. In general, the detection limit can be increased at least ten-fold by switching from a chromogenic to a luxogenic substrate, and with the addition of enhancing agents, more than 200-fold. In fact, under ideal conditions, as little as 5×10^{-18} moles (5 attomoles) of target antigen have been detected.



ELISPOT ASSAY

A modification of the ELISA assay called the ELISPOT assay allows the quantitative determination of the number of cells in a population that are producing antibodies specific for a given antigen or an antigen for which one has a specific antibody .

In the ELISPOT assay, a well is coated with antibody against the antigen of interest, a cytokine in this example, and then a suspension of a cell population thought to contain some members synthesizing and secreting the cytokine are layered onto the bottom of the well and incubated. Most of the cytokine molecules secreted by a particular cell react with nearby well-bound antibodies. After the incubation period, the well is washed and an enzyme-labeled anti-cytokine antibody is added. After washing away unbound antibody, a chromogenic substrate that forms an insoluble colored product is added. The colored product (purple) precipitates and forms a spot only on the areas of the well where cytokine-secreting cells had been deposited. By counting the number of colored spots, it is possible to determine how many cytokine-secreting cells were present in the added cell suspension



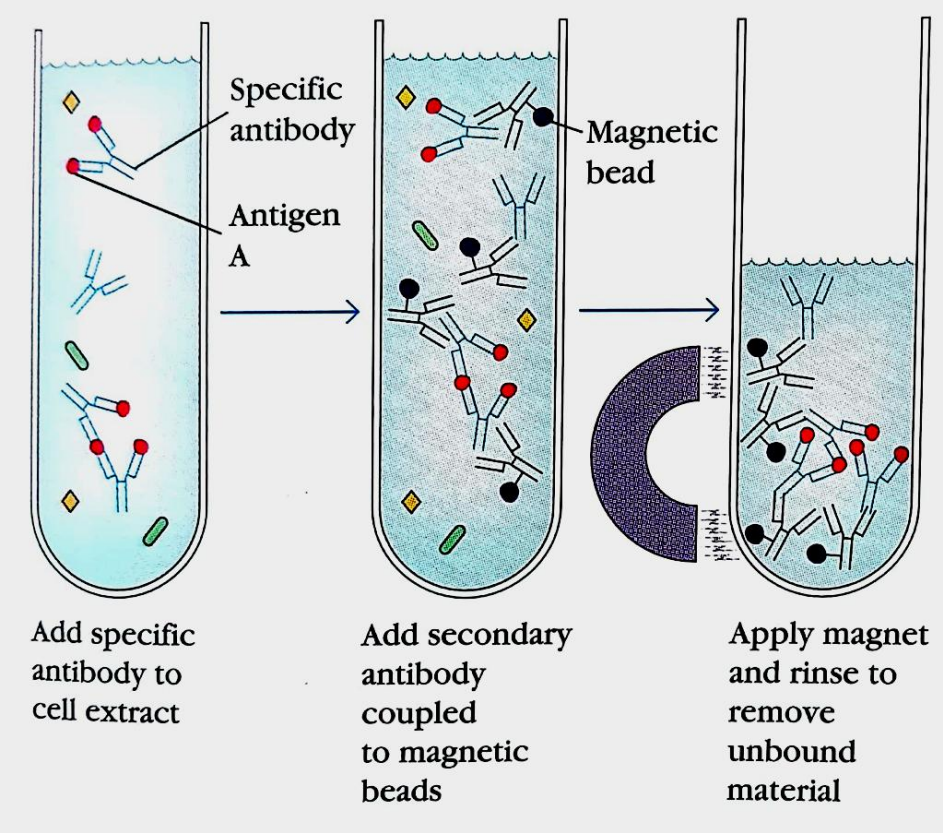
Western Blotting

Identification of a specific protein in a complex mixture of proteins can be accomplished by a technique known as Western blotting, named for its similarity to Southern blotting, which detects DNA fragments, and Northern blotting, which detects mRNAs.

In Western blotting, a protein mixture is electrophoretically separated on an SDS-polyacrylamide gel (SDS-PAGE), a slab gel infused with sodium dodecyl sulfate (SDS), a dissociating agent.

The protein bands are transferred to a nylon membrane by electrophoresis and the individual protein bands are identified by flooding the nitrocellulose membrane with radiolabeled or enzymelinked polyclonal or monoclonal antibody specific for the protein of interest.

The Ag-Ab complexes that form on the band containing the protein recognized by the antibody can be visualized in a variety of ways. If the protein of interest was bound by a radioactive antibody, its position on the blot can be determined by exposing the membrane to a sheet of x-ray film, a procedure called autoradiography. However, the most generally used detection procedures employ enzyme-linked antibodies against the protein. After binding of the enzymeantibody conjugate, addition of a chromogenic substrate that produces a highly colored and insoluble product causes the appearance of a colored band at the site of the target antigen.



Immunoprecipitation

The immunoprecipitation technique has the advantage of allowing the isolation of the antigen of interest for further analysis. It also provides a sensitive assay for the presence of a particular antigen in a given cell or tissue type. An extract produced by disruption of cells or tissues is mixed with an antibody against the antigen of interest in order to form an antigen-antibody complex that will precipitate.

Immuno precipitates can be collected using magnetic beads coupled to a secondary antibody.

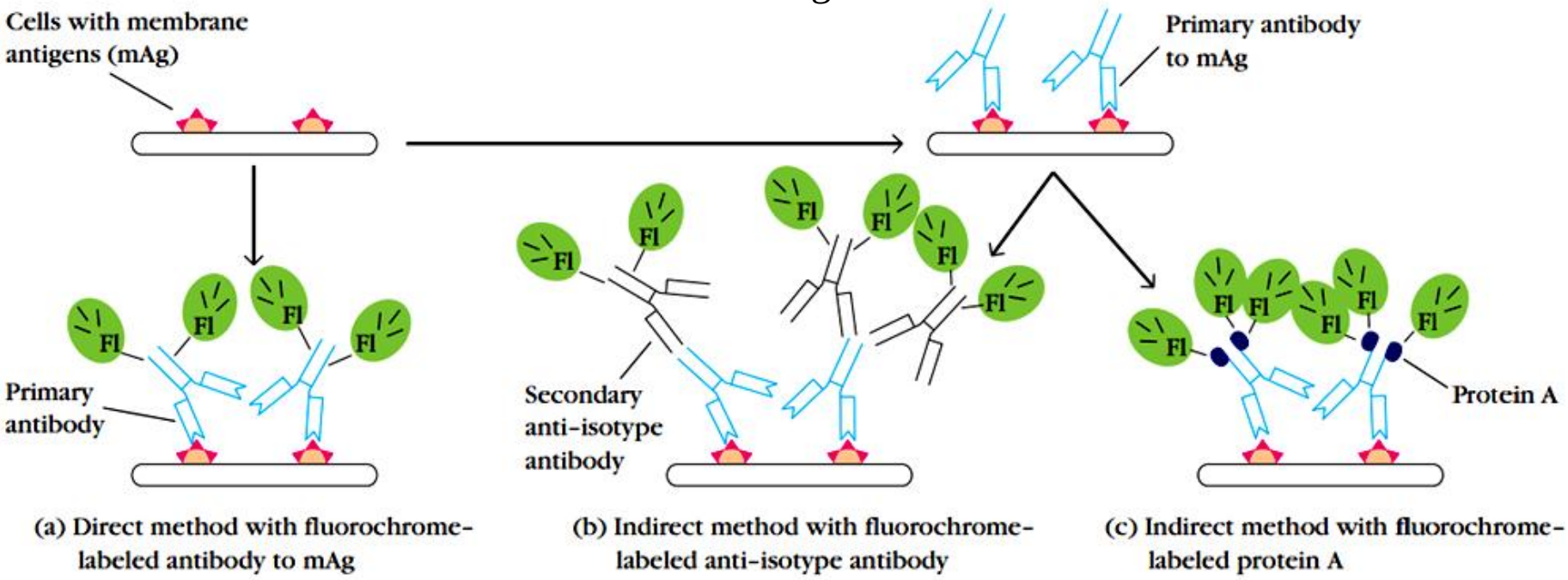
(a) Treatment of a cell extract containing antigen A (red) with a mouse anti-A antibody (blue) results in the formation of antigen-antibody complexes.

(b) Addition of magnetic beads to which a rabbit anti-mouse antibody is linked binds the antigen-antibody complexes (and any unreacted mouse Ig).

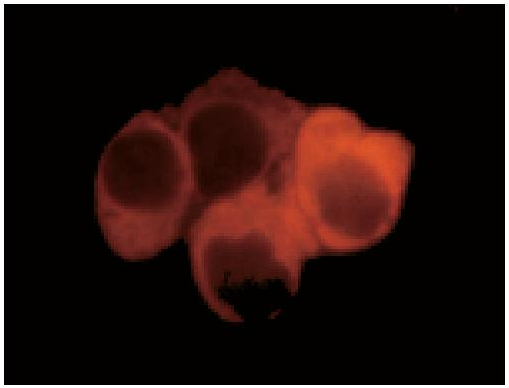
(c) Placing a magnet against the side of the tube allows the rapid collection of the antigen-antibody complexes.

After rinsing to remove any unbound material, the antigen-antibody complexes can be dissociated and the antigen studied.

immunofluorescence staining



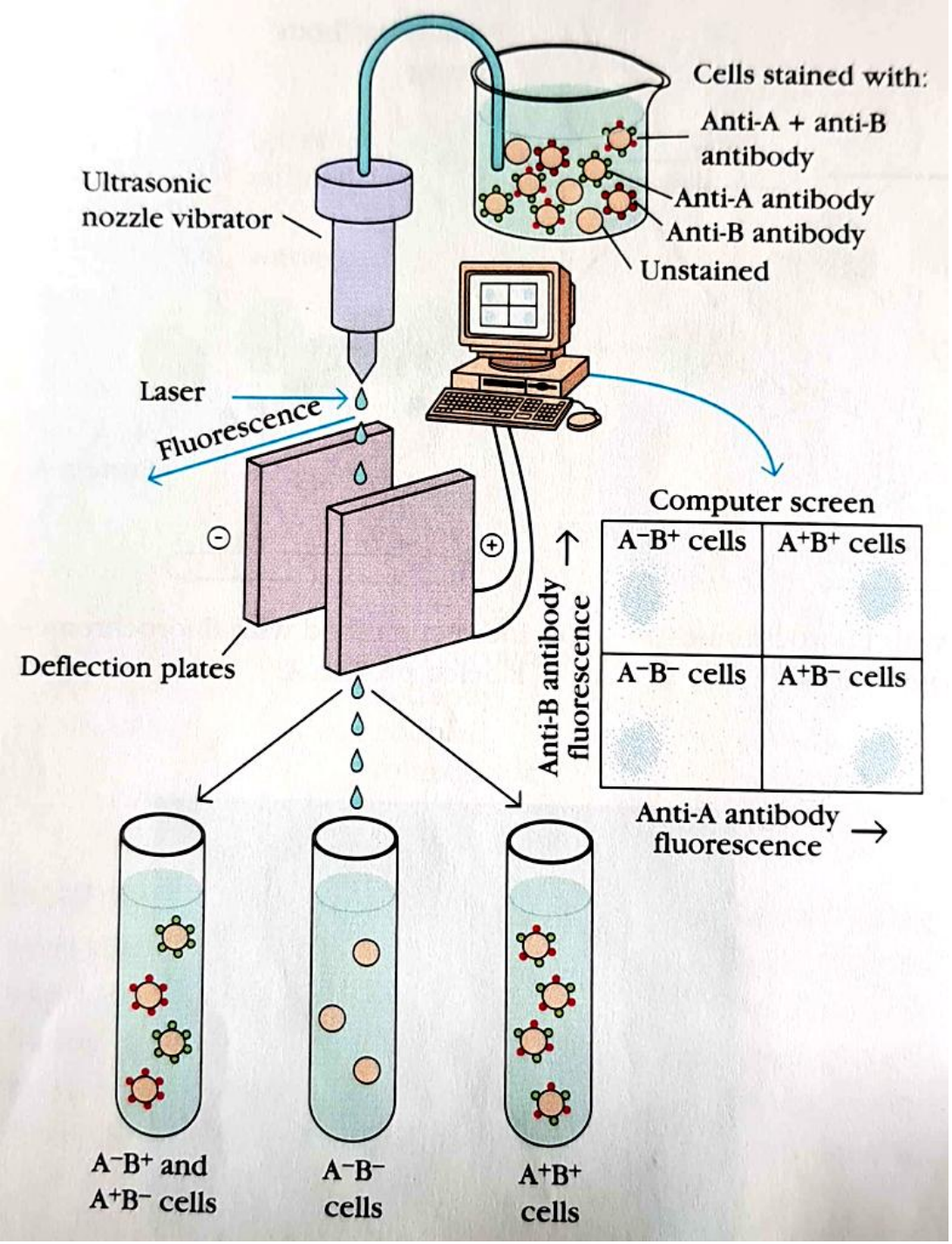
Direct and indirect immunofluorescence staining of membrane antigen (mAg). Cells are affixed to a microscope slide.
In the direct method (a), cells are stained with anti-mAg antibody that is labeled with a fluorochrome (Fl).
In the indirect methods (b and c), cells are first incubated with unlabeled anti-mAg antibody and then stained with a fluorochrome-labeled secondary reagent that binds to the primary antibody. Cells are viewed under a fluorescence microscope to see if they have been stained.
(d) In this micrograph, antibody molecules bearing μ heavy chains are detected by indirect staining of cells with rhodamine-conjugated second antibody.



Flow Cytometry and Fluorescence

Flow cytometer, is designed to automate the analysis and separation of cells stained with fluorescent antibody.
The flow cytometer uses a laser beam and light detector to count single intact cells in suspension.
Every time a cell passes the laser beam, light is deflected from the detector, and this interruption of the laser signal is recorded.
Those cells having a fluorescently tagged antibody bound to their cell surface antigens are excited by the laser and emit light that is recorded by a second detector system located at a right angle to the laser beam.
Sorting populations of cells into different containers according to their fluorescence profile is also possible by this method.

Use of the instrument to determine which and how many members of a cell population bind fluorescently labeled antibodies is called **analysis**;
Use of the instrument to place cells having different patterns of reactivity into different containers is called **cell sorting**.



Separation of fluorochrome-labeled cells with the fluorescence-activated cell sorter(FACS)

Fig. Separation of fluorochrome-labeled cells with the flow cytometer.
In the example shown, a mixed cell population is stained with two antibodies, one specific for surface antigen A and the other specific for surface antigen B. The anti-A antibodies are labeled with fluorescein (green) and the anti-B antibodies with rhodamine (red). The stained cells are loaded into the sample chamber of the cytometer.

The cells are expelled, one at a time, from a small vibrating nozzle that generates microdroplets, each containing no more than a single cell. As it leaves the nozzle, each droplet receives a small electrical charge, and the computer that controls the flow cytometer can detect exactly when a drop generated by the nozzle passes through the beam of laser light that excites the fluorochrome.

The intensity of the fluorescence emitted by each droplet that contains a cell is monitored by a detector and displayed on a computer screen. Because the computer tracks the position of each droplet, it is possible to determine when a particular droplet will arrive between the deflection plates.

By applying a momentary charge to the deflection plates when a droplet is passing between them, it is possible to deflect the path of a particular droplet into one or another collecting vessel.

This allows the sorting of a population of cells into subpopulations having different profiles of surface markers.

In the computer display, each dot represents a cell. Cells that fall into the lower left-hand panel have background levels of fluorescence and are judged not to have reacted with either antibody anti-A or anti-B.
Those that appear in the upper left panel reacted with anti-B but not anti-A, and those in the lower right panel reacted with anti-A but not anti-B. The upper right panel contains cells that react with both anti-A and anti-B. In the example shown here, the AB^{--}—and the AB^{++}— subpopulations have each been sorted into a separate tube. Staining with anti-A and anti-B fluorescent antibodies allows four subpopulations to be distinguished: A^-B^-, A^+B^+, A^-B^+, and A^+B^-.