

### **Applications of Blotting and Hybridization Techniques**

1. Southern blotting technique is widely used to find specific nucleic acid sequence present in different plant species.
2. Northern blotting technique is widely used to find gene expression and regulation of specific genes.
3. By using blotting technique we can identify infectious agents present in the sample.
4. We can identify inherited disease.
5. It can be applied to mapping restriction sites in single copy gene.

### **Disadvantages of Blotting and Hybridization Techniques**

1. The process is a complex, cumbersome and time consuming one.
2. It requires electrophoretic separation.
3. Only one gene or RNA can be analysed at a time.
4. Gives information about presence of DNA, RNA or proteins but does not give information about regulation and gene interaction.

### **Dot Blotting Techniques**

The drawbacks of blotting techniques have lead to the development of dot blotting technique which is more advanced, less time consuming, accurate and applicable to a wide variety of gene/source simultaneously. The dot or slot blotting technique is the most widely used of all techniques for analysing. None of the blot methods require electrophoresis prior to blotting and hybridization. Hybridization of cloned DNA without electrophoretic separation is called as dot blotting.

A dot blot (or slot blot) is a technique in molecular biology used to detect biomolecules, and for detecting, analyzing, and identifying proteins. It represents a simplification of the northern blot, Southern blot, or western blot methods. In a dot blot the biomolecules to be detected are not first separated by electrophoresis. Instead, a mixture containing the molecule to be detected is applied directly on a membrane as a dot, and then is spotted through circular templates directly onto the membrane or paper substrate. This differs from the western blot because protein samples are not separated electrophoretically. This is then followed by detection by either nucleotide probes (for a northern blot and southern blot) or antibodies (for a western blot).

The technique offers significant savings in time, as chromatography or gel electrophoresis, and the complex blotting procedures for the gel are not required. However, it offers no information on the size of the target biomolecule. Furthermore, if two molecules of different sizes are detected, they will still appear as a single dot. Dot blots therefore can only confirm the presence or absence of a biomolecule or biomolecules which can be detected by the DNA probes or the antibody.

Because dot blot does not require complicated instrument to operate, lots of dot blot assays are developed using antibodies with high specificity to detect different protein targets. Dot blot is also used to evaluate or screen the effectiveness of the antibodies.

### **Protocol**

Below is just a general guide line for a antigen-antibody-antibody dot blot assay protocol. Specific concentration need to be determined for each assay. Other type of dot blot assay, such as antibody-antigen-antibody can be performed in a similar fashion.

1. spot 1-2 microliter of antigen on to a piece of membrane, let air dry for 30 min or longer;
2. incubate with blocking buffer for 30 min - 2 hr;
3. rinse with rinsing buffer, 3x5 min;
4. incubate with primary antibody, 30 min - 2 hr;
5. rinse with rinsing buffer, 3x5 min;
6. incubate with enzyme-labeled secondary antibody, 30 min - 2 hr;
7. rinse with rinsing buffer, 3x5 min;
8. add enzyme substrate, wait 5-10 min;
9. detect by eye or with colorimetric or chemiluminescent imaging system.

Dot blot is originally performed on a piece of nitrocellulose membrane or PVDF membrane. After the protein samples are spotted onto the membrane, the membrane is placed in a plastic

container and sequentially incubated in blocking buffer, antibody solutions, or rinsing buffer on shaker. Finally, for chemiluminescence imaging, the piece of membrane need to be wrapped in a transparent plastic film filled with enzyme substrate.

Vacuum-based dot blot apparatus (Bio-dot or Bio-slot from BioRad) has been used to facilitate the rinsing and incubating process by using vacuum to extract the solution from underneath the membrane, which is assembled in between several layers of plates to ensure good seal between sample wells, hold waste solution, and deliver suction force. For chemiluminescence signal detection, apparatus need to be dissembled and the membrane need to be taken out and wrapped in a transparent plastic film.

Vitrozm's 96 well Zoom Blot method (Vitrozm.com) uses an absorption plug to wick away the solution from the membranes, which are individually assembled in each well. The Zoom Plate is a self-standing device ready to use and disposable, without using any external vacuum or set up apparatus, making dot blot much easier and faster. The user never need to handle a piece of membranes as in older methods. Zoom Blot's 96 well plate format allows convenient signal detection and quantification using 96 well plate reader or imaging system.

The sensitive dot blot test can be used to detect the Chlamydia trachomatis infection and other sexually transmitted diseases. Dot blot is used to detect Antidiacyltrehalose Antibodies in Tuberculous patients and Typhoid Fever. This test could increase the number of lives saved that are affected with these diseases. Using the dot blot test can be useful for under-developed countries especially. The dot blot is a good positive predictor of these diseases for countries and regions lacking in medical facilities and laboratories. By using this test many lives could be saved and a cure could be found for the types of diseases this test can detect

**Plaque or Colony Blotting Techniques** - This method was first developed by Granstiens and Hogness (1975). This method is used to identify which colony of bacteria contains the DNA of interest among thousands. In this procedure, the bacterial colonies to be screened are transferred onto nitrocellulose or nylon membrane by using replica plating.

Due to the negative charge of the cell surface, some cells bind to the nitrocellulose membrane. Then the membrane is placed in a solution of 0.5 N NaOH to break the cell surface, convert dsDNA to ssDNA and to bind DNA to the membrane. Later, the membrane is transferred to a solution containing protease solution after neutralizing with neutralization solution.

The DNA is fixed tightly to membrane by either W cross linking or oven baking. This membrane is used for hybridization with a probe and analysed by using autoradiography or biotin method for positive hybridization. A colony whose DNA print (as replica plating provides a replica print master plate colony on the membrane) gives a positive hybridization can be picked from the master plate.

Plaque blotting is similar to colony blotting; the only difference is that instead of bacterial colony, a plaque is transferred onto the membrane. Benton and Davis developed this method

in 1977. The greatest advantage of this method is that several identical DNA prints can be easily made from a single master plate containing bacteria/plaques which are to be made.

**Dot Plot Assay Techniques** - This method is widely used to hybridize DNA from a single cell type against a wide variety of probes, for example, for a viral infection which cannot be identified by normal conventional methods or if we want to know what all genes are expressed in a single cell type (e.g. brain cell).

Cell type or cells that are to be screened are placed on the membrane as 'dot' in the order of rows and columns. Then the cells are denatured by using enzymes or detergents (SDS) and DNA is fixed by using W - cross link or oven baking. This membrane is then used for hybridization by using probes (which are specific to a gene).

## Immunological Techniques

Immunological techniques are the wide varieties of methods and specialized experimental protocols devised by immunologists for inducing, measuring, and characterizing immune responses. They allow the immunologists to alter the **immune system** through cellular, molecular and genetic manipulation. These techniques are not restricted to the field of **immunology**, but are widely applied by basic scientists in many other biological disciplines and by clinicians in human and veterinary medicine.

Most immunological techniques available are focused on the study of the adaptive immune system. They classically involve the experimental induction of an immune response using methods based on **vaccination** protocols. During a typical experiment called **immunization**, immunologists inject a test **antigen** to an animal or human subject and monitor for the appearance of immune responses in the form of specific antibodies and effector **T cells**. Monitoring the **antibody** response usually involves the analysis of crude preparations of serum from the immunized subject. The analysis of the immune responses mediated by T cells are usually performed only on experimental animals and involves the preparation of these cells from blood or from the lymphoid organs, such as the spleen and the lymph nodes. Typically, any substance that has a distinctive structure or conformation that may be recognized by the immune system can serve as an antigen. A wide range of substances from simple chemicals like sugars, and small peptides to complex macromolecules and **viruses** can induce the immune system. Although the antigenic determinant of a test substance is usually a minor part of that substance called the epitope, a small antigen referred to as a hapten can rarely elicit an immune response on its own. It is not an immunogen and would therefore need to be covalently linked to a carrier in order to elicit an immune response. The induction of such a response to even large immunogenic antigen is not easy to achieve and the dose, the form and route of administration of that antigen can profoundly affect whether a response can occur. Especially the use of certain substances called adjuvants is necessary to alert the immune system and produce a strong immune response.

According to the clonal **selection** theory, antibodies produced in a typical immunization experiment are products of different clones of B-lymphocytes that are already committed to making antibodies to the corresponding antigen. These polyclonal antibodies are multi-subunit proteins that belong to the **immunoglobulins** family. They have a basic Y-shaped structure with two identical Fab domains, which form the arms and interact with the antigen, and one Fc domain that forms the stem and determines the isotype subclass of each antibody.

There are five different isotype subclasses, IgM, Ig G, IgA, IgE, and IgD, which show different tissue distribution and half-life *in vivo*. They determine the biological function of the antibodies and appear during different stages of the immunization process. Knowledge about the biosynthesis and structure of these antibodies is important for their detection and use both as diagnostic and therapeutic tools.

Antibodies are highly specific for their corresponding antigen, and are able to detect one molecule of a protein antigen out of around a billion similar molecules. The amount and specificity of an antibody in a test serum can be measured by its direct binding to the antigen in assays usually referred to as primary interaction immunoassays. Commonly used direct assays are radioimmunoassay (RIA), enzyme-linked immunosorbent assay (**ELISA**), and immunoblotting techniques. In both ELISA and RIA, an enzyme or a radioisotope is covalently linked to the pure antigen or antibody. The unlabeled component, which most often is the antigen, is attached to the surface of a plastic well. The labeled antibody is allowed to bind to the unlabeled antigen. The plastic well is subsequently washed with plenty of **buffer** that will remove any excess non-bound antibody and prevent non-specific binding. Antibody binding is measured as the amount of radioactivity retained by the coated wells in radioimmunoassay or as fluorescence emitted by the product of an enzymatic reaction in the case of ELISA. Modifications of these assays known as competitive inhibition assays can be used that will allow quantifying the antigen (or antibody) in a mixture and determining the affinity of the antibody-antigen interaction by using mathematical models. Immunoblotting is usually performed in the form of Western blotting, which is reserved to the detection of proteins and involves an **electrophoresis** separation step followed by electroblotting of the separated proteins from the gel to a membrane and then probing with an antibody. Detection of the antigen protein antibody interaction is made in a similar way as in RIA or ELISA depending on whether a radiolabeled or enzyme-coupled antibody is used.

Antibodies can also be monitored through immunoassays that are based on the ability of antibodies to alter the physical state of their corresponding antigens and typically involve the creation of a precipitate in a solid or liquid medium. The hemmagglutination assay used to determine the ABO type of blood groups and match compatible donors and recipients for blood transfusion is based on this assay. Currently, the most common application of this immunoassay is in a procedure known as immunoprecipitation. This method allows antibodies to form complexes with their antigen in a complex mixture like the cytosol, the **nucleus** or membrane complexes of the cell. The antigen-antibody complex is precipitated either by inducing the formation of even larger complexes through the addition of excess amounts of anti-immunoglobulin antibodies or by the addition of agarose beads

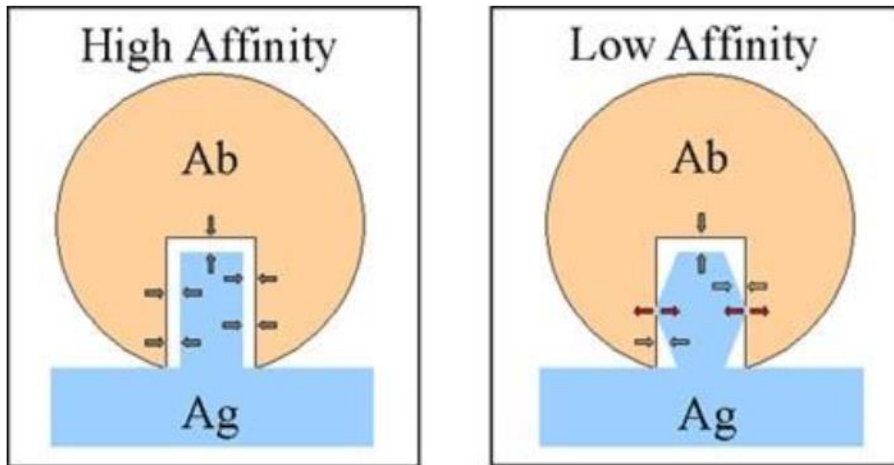
coupled to a special class of bacterial proteins that bind the Fc region of the antibody. The complex can also be precipitated by covalently linking the antibody to agarose beads forming a special affinity matrix. This procedure will also allow the purification of the antigen by immunoaffinity, a special form of affinity chromatography. Immunoprecipitation is a valuable technique that led to major discoveries in immunology and all disciplines of molecular and cellular biology. It allows the precipitation of the antigen in complex with other interacting proteins and reagents and therefore gives an idea on the function of the antigen.

The T cell immune response is detected by using monoclonal antibodies, a specific family of antibodies that recognize surface markers that are expressed by lymphocytes upon their activation. These monoclonal antibodies are highly specific, and are produced by special techniques from single clones of **B cells** and are therefore, homogenous groups of immunoglobulins with the same isotype and antigen binding affinity. These antibodies are used to identify and characterize cells by flow cytometry (FACS), immunocytochemistry, **immunofluorescence** techniques. The difficulty to isolate antigen specific T cells is due to the fact that these T cells recognize the antigen in the context of a tri-molecular complex involving the T cell receptor and the **MHC** molecules on the surface of specialized cells called antigen-presenting cells. These interactions are subtle, have low affinity and are extremely complex to study. Novel and powerful techniques using tetramers of MHC molecules were developed in 1997 that are now used to identify and isolate antigen specific T cell clones. These tetramer-based assays are proving useful in separating very rare cells, and could be used in clinical medicine. In fact, virus and tumor specific T cells usually give a stronger response and are usually more effective in killing virus infected and tumor cells. Testing for the function of activated, antigen specific T cells known as effector T cells is routinely done *in vitro* by testing for cytokine production, cytotoxicity to other cells and proliferation in response to antigen stimulation. Local reactions in the skin of animals and humans provide information about T cell responses to an antigen, a procedure that is very used in testing for allergic reactions and the efficacy of vaccination procedures. Experimental manipulations of the immune system *in vivo* are performed to reveal the functions of each component of the immune system *in vivo*. **Mutations** through irradiation, or mutations produced by **gene** targeting (e.g., knock-out and knock-in techniques), as well as animal models produced by transgenic breeding, are proving helpful to researchers in evaluating this highly complex system.

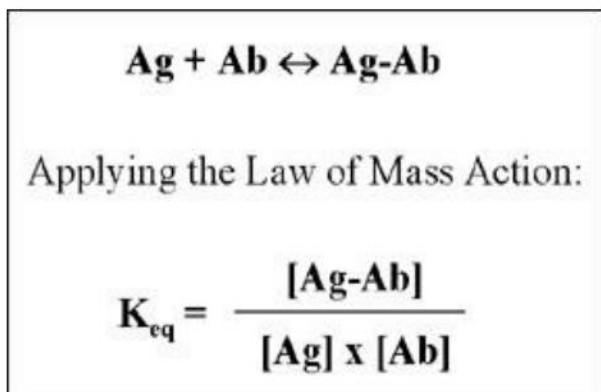
## AFFINITY AND AVIDITY

### Affinity

Antibody affinity is the strength of the reaction between a single antigenic determinant and a single combining site on the antibody. It is the sum of the attractive and repulsive forces operating between the antigenic determinant and the combining site of the antibody as illustrated in Figure 2.

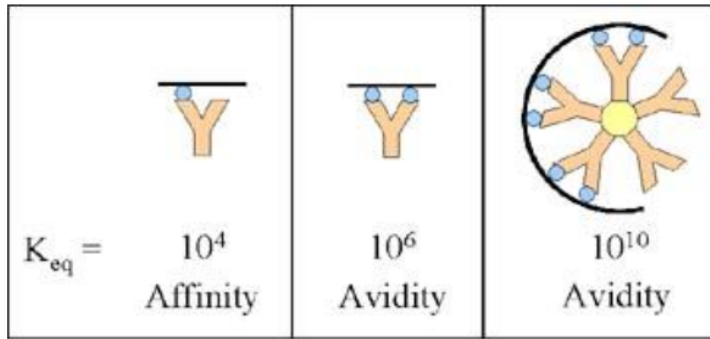


Affinity is the equilibrium constant that describes the antigen-antibody reaction as illustrated in Figure 3. Most antibodies have a high affinity for their antigens.



### Avidity

Avidity is a measure of the overall strength of binding of an antigen with many antigenic determinants and multivalent antibodies. Avidity is influenced by both the valence of the antibody and the valence of the antigen. Avidity is more than the sum of the individual affinities. This is illustrated in Figure 4.



To repeat, affinity refers to the strength of binding between a single antigenic determinant and an individual antibody combining site whereas avidity refers to the overall strength of binding between multivalent antigens and antibodies.

## SPECIFICITY AND CROSS REACTIVITY

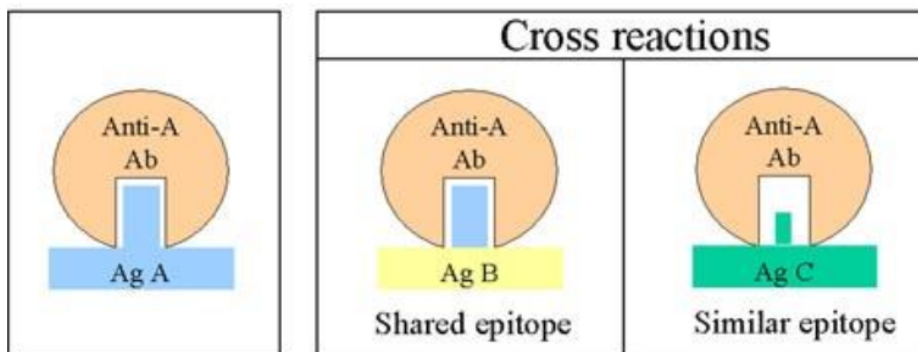
### Specificity

Specificity refers to the ability of an individual antibody combining site to react with only one antigenic determinant or the ability of a population of antibody molecules to react with only one antigen. In general, there is a high degree of specificity in antigen-antibody reactions. Antibodies can distinguish differences in:

- The primary structure of an antigen
- Isomeric forms of an antigen
- Secondary and tertiary structure of an antigen

### Cross reactivity

Cross reactivity refers to the ability of an individual antibody combining site to react with more than one antigenic determinant or the ability of a population of antibody molecules to react with more than one antigen. Figure 5 illustrates how cross reactions can arise. Cross reactions arise because the cross reacting antigen shares an [epitope](#) in common with the immunizing antigen or because it has an epitope which is structurally similar to one on the immunizing antigen (multispecificity).



## TESTS FOR ANTIGEN-ANTIBODY REACTIONS

## Factors affecting measurement of antigen-antibody reactions

The only way that one knows that an antigen-antibody reaction has occurred is to have some means of directly or indirectly detecting the complexes formed between the antigen and antibody. The ease with which one can detect antigen-antibody reactions will depend on a number of factors.

### Affinity

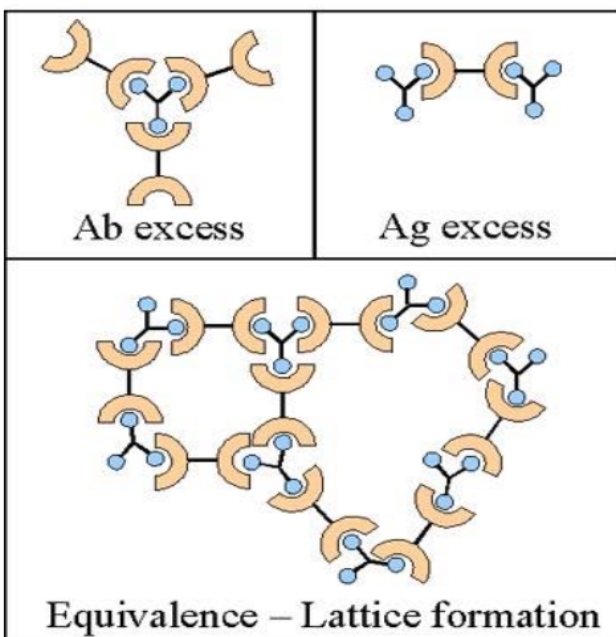
The higher the affinity of the antibody for the antigen, the more stable will be the interaction. Thus, the ease with which one can detect the interaction is enhanced.

### Avidity

Reactions between multivalent antigens and multivalent antibodies are more stable and thus easier to detect.

### Antigen to antibody ratio

The ratio between the antigen and antibody influences the detection of antigen-antibody complexes because the size of the complexes formed is related to the concentration of the antigen and antibody. This is depicted in Figure 6.



### Physical form of the antigen

The physical form of the antigen influences how one detects its reaction with an antibody. If the antigen is a particulate, one generally looks for agglutination of the antigen by the antibody. If the antigen is soluble one generally looks for the precipitation of the antigen after the production of large insoluble antigen-antibody complexes.

## Agglutination Tests

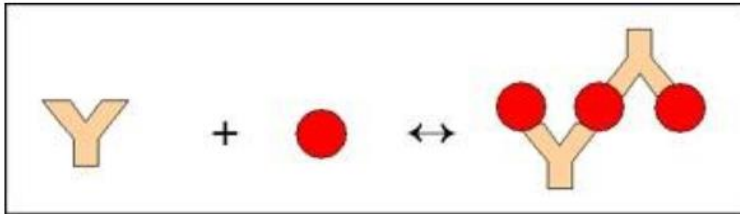
### Agglutination/Hemagglutination

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.

### 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. (Figure 7).



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.

### 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. Figure 8 illustrates a quantitative hemagglutination test.

| 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 (See Patient 6 in Figure 8). 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.

### Applications of agglutination tests

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

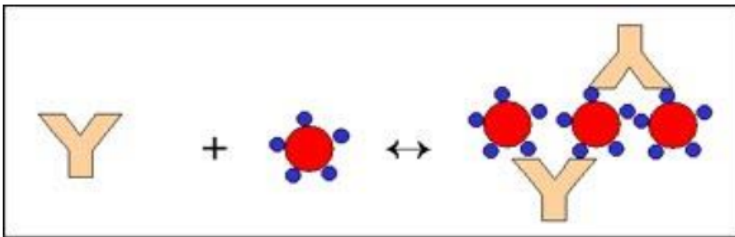
e.g. A rise in titer of an antibody to a particular bacterium indicates an infection with that bacterial type.  
N.B. a fourfold rise in titer is generally taken as a significant rise in antibody titer.

### Practical considerations

Although the test is easy to perform, it is only semi-quantitative.

### Passive hemagglutination

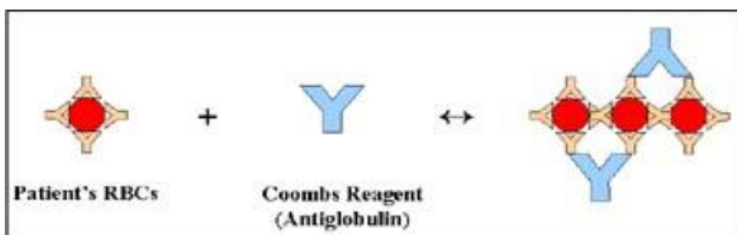
The agglutination test only works with particulate antigens. However, it is possible to coat erythrocytes with a soluble antigen (e.g. viral antigen, a polysaccharide or a hapten) and use the coated red blood cells in an agglutination test for antibody to the soluble antigen (Figure 9). This is called 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.



### Coomb's Test (Antiglobulin Test)

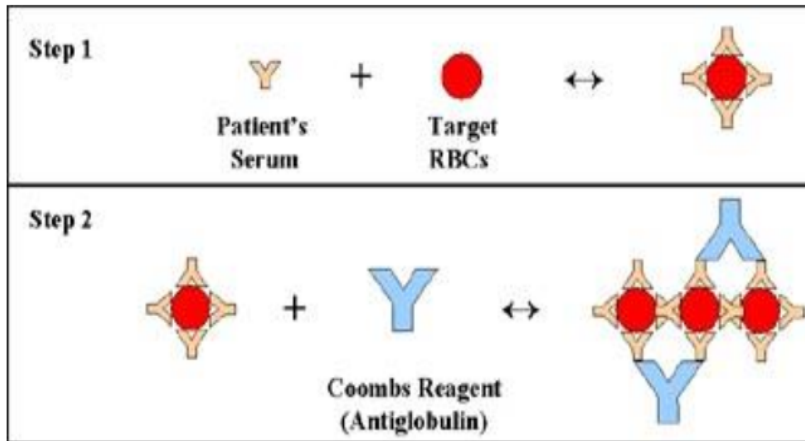
#### Direct Coomb's Test

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. This test is illustrated in Figure 10 and is known as the [Direct Coomb's test](#).



## 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 (Figure 11). 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.

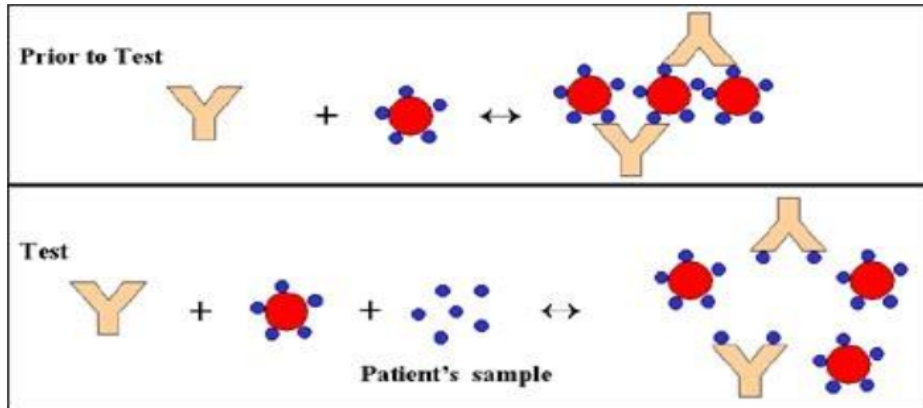


## Applications

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<sup>+</sup> children born to Rh<sup>-</sup> 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.

## 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 (see passive hemagglutination above). 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. as illustrated in Figure 12.

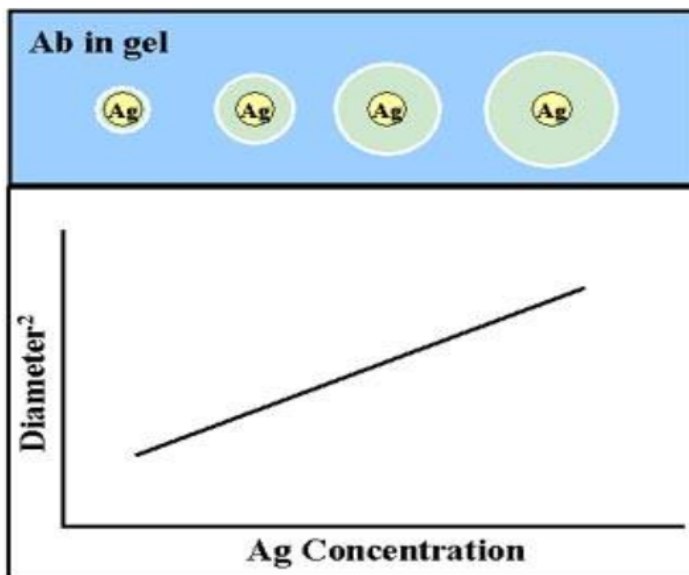


By serially diluting the sample, you can quantitate the amount of antigen in your unknown sample by its titer. This test is generally used to quantitate soluble antigens and is subject to the same practical considerations as the agglutination test.

## Precipitation tests

### Radial Immunodiffusion (Mancini)

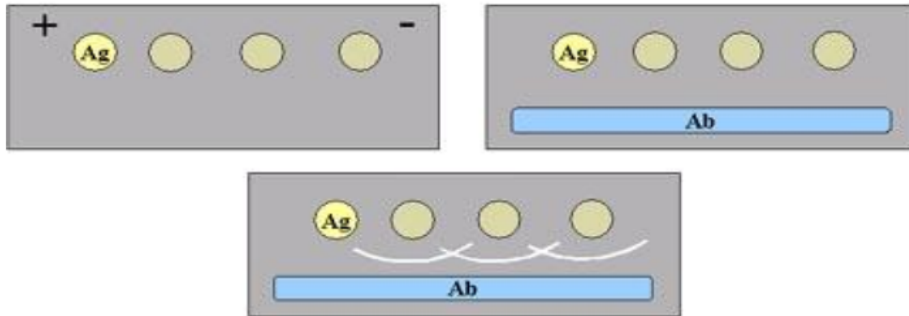
In radial immunodiffusion antibody is incorporated into the agar gel as it is poured and different dilutions of the antigen are placed in holes punched into the agar. As the antigen diffuses into the gel, it reacts with the antibody and when the equivalence point is reached a ring of precipitation is formed as illustrated in Figure 13.



The diameter of the ring is proportional to the log of the concentration of antigen since the amount of antibody is constant. Thus, by running different concentrations of a standard antigen one can generate a standard curve from which one can quantitate the amount of an antigen in an unknown sample. Thus, this is a quantitative test. If more than one ring appears in the test, more than one antigen/antibody reaction has occurred. This could be due to a mixture of antigens or antibodies. This test is commonly used in the clinical laboratory for the determination of immunoglobulin levels in patient samples.

### 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 antigen 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 as illustrated in Figure 14.



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.

### 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 as illustrated in Figure 15. 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.



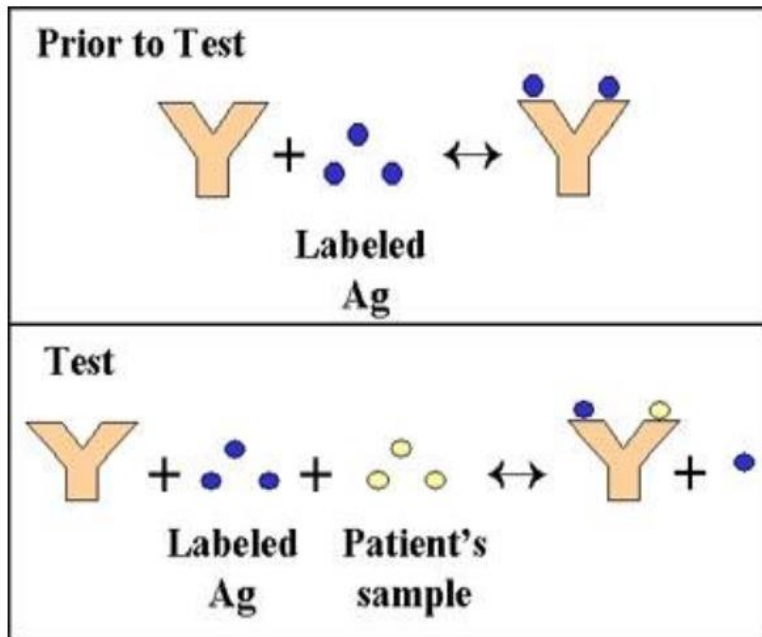
### Radioimmunoassay (RIA)/Enzyme Linked Immunosorbent Assay (ELISA)

Radioimmunoassays (RIA) are assays that are based on the measurement of radioactivity associated with immune complexes. In any particular test, the label may be on either the antigen or the antibody. Enzyme Linked Immunosorbent Assays (ELISA) are those that are based on the measurement of an enzymatic reaction associated with immune complexes. In any particular assay, the enzyme may be linked to either the antigen or the antibody.

#### Competitive RIA/ELISA for Ag Detection

The method and principle of RIA and ELISA for the measurement of antigen is shown in Figure 16. By using known amounts of a standard unlabeled antigen, one can generate a standard curve relating radioactivity (cpm) (Enzyme) bound versus amount of antigen. From this standard curve, one can

determine the amount of an antigen in an unknown sample.



The key to the assay is the separation of the immune complexes from the remainder of the components. This has been accomplished in many different ways and serves as the basis for the names given to the assay:

#### **Precipitation with ammonium sulphate**

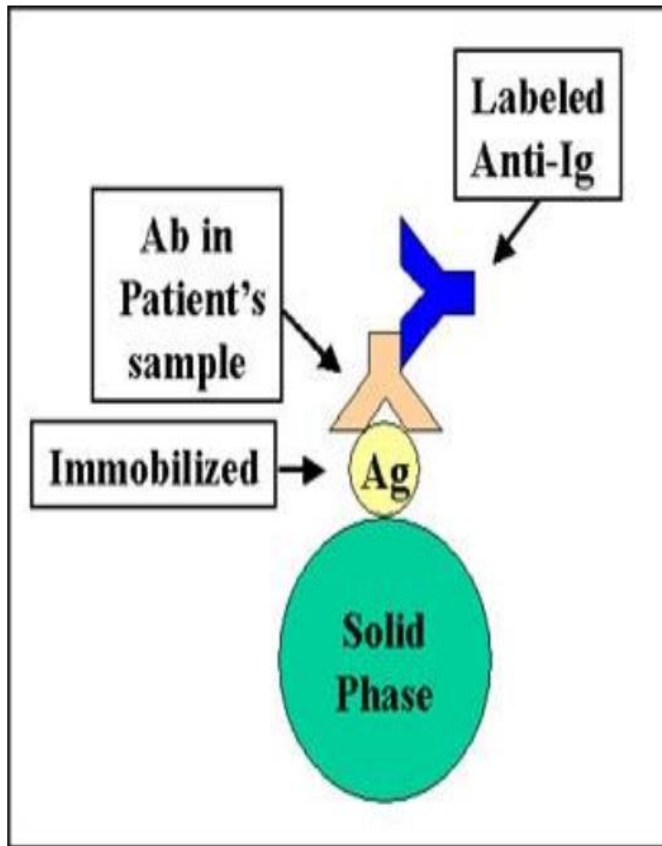
Ammonium sulphate (33 - 50% final concentration) will precipitate immunoglobulins but not many antigens. Thus, this can be used to separate the immune complexes from free antigen. This has been called the Farr Technique

#### **Anti-immunoglobulin antibody**

The addition of a second antibody directed against the first antibody can result in the precipitation of the immune complexes and thus the separation of the complexes from free antigen.

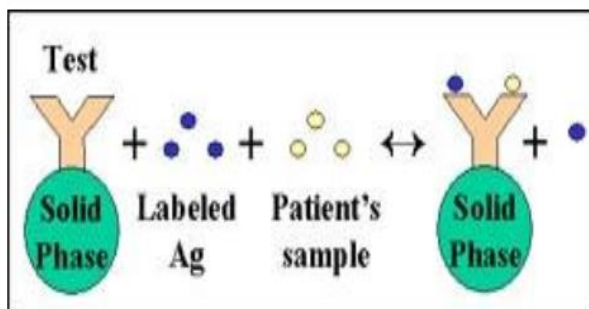
#### **Immobilization of the Antibody**

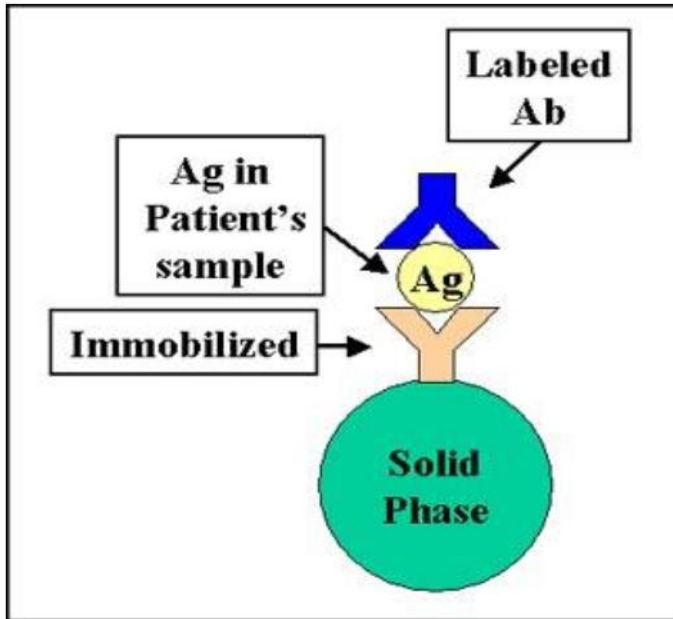
The antibody can be immobilized onto the surface of a plastic bead or coated onto the surface of a plastic plate and thus the immune complexes can easily be separated from the other components by simply washing the beads or plate (Figure 17). This is the most common method used today and is referred to as Solid phase RIA or ELISA. In the clinical laboratory, competitive RIA and ELISA are commonly used to quantitate serum proteins, hormones, drugs metabolites.



### Non-competitive RIA/ELISA for Ag or Ab

Non-competitive RIA and ELISAs are also used for the measurement of antigens and antibodies. In Figure 18, the bead is coated with the antigen and is used for the detection of antibody in the unknown sample. The amount of labeled second antibody bound is related to the amount of antibody in the unknown sample. This assay is commonly employed for the measurement of antibodies of the IgE class directed against particular allergens by using a known allergen as antigen and anti-IgE antibodies as the labeled reagent. It is called the RAST test (radioallergosorbent test). In Figure 19, the bead is coated with antibody and is used to measure an unknown antigen. The amount of labeled second antibody that binds is proportional to the amount of antigen that bound to the first antibody.





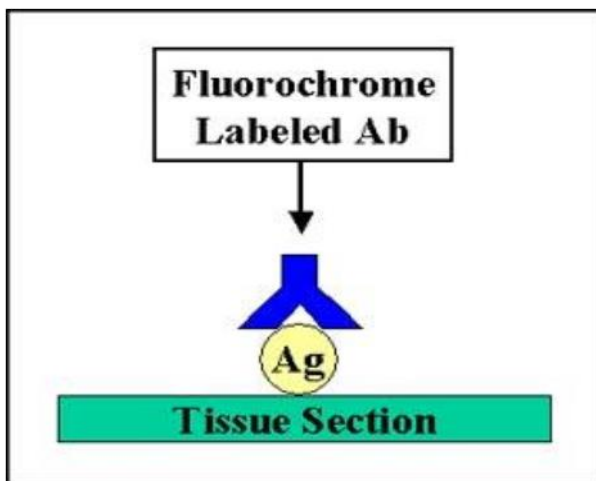
### Tests for Cell Associated Antigens

#### Immunofluorescence

Immunofluorescence is a technique whereby an antibody labeled with a fluorescent molecule (fluorescein or rhodamine or one of many other fluorescent dyes) is used to detect the presence of an antigen in or on a cell or tissue by the fluorescence emitted by the bound antibody.

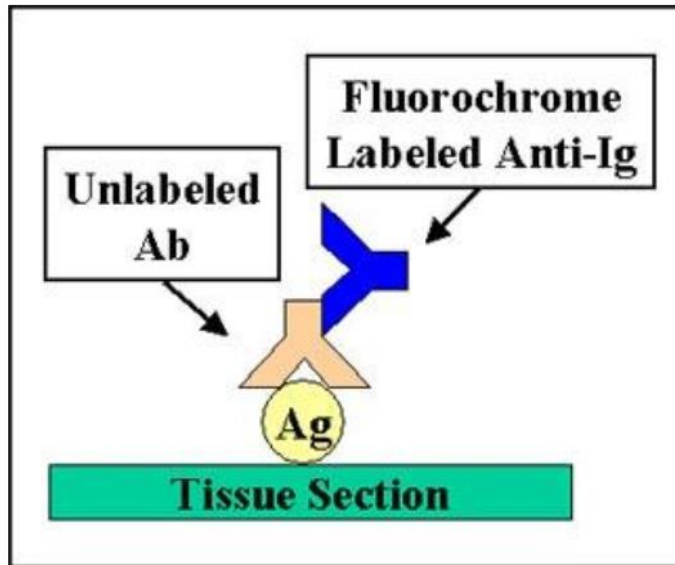
#### Direct Immunofluorescence

In direct immunofluorescence, the antibody specific to the antigen is directly tagged with the [fluorochrome](#) (Figure 20).



#### Indirect Immunofluorescence

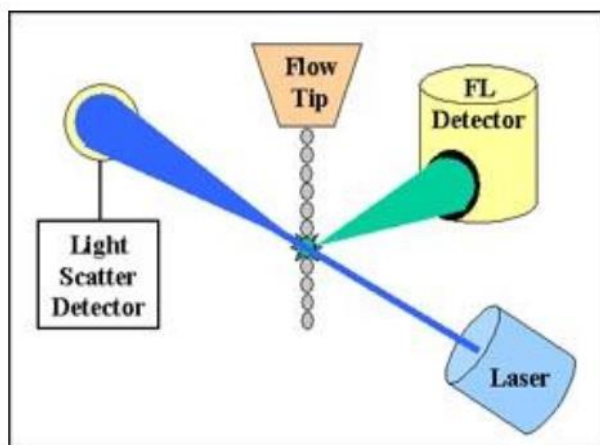
In indirect immunofluorescence, the antibody specific for the antigen is unlabeled and a second anti-immunoglobulin antibody directed toward the first antibody is tagged with the [fluorochrome](#) (Figure 21). Indirect fluorescence is more sensitive than direct immunofluorescence since there is amplification of the signal.



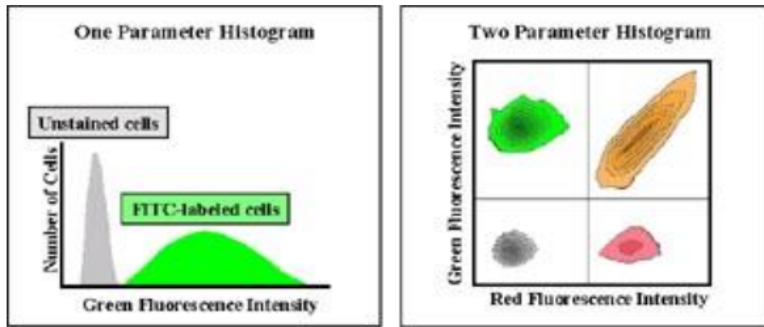
### Flow Cytometry

Flow cytometry is commonly used in the clinical laboratory to identify and enumerate cells bearing a particular antigen. Cells in suspension are labeled with a fluorescent tag by either direct or indirect immunofluorescence. The cells are then analyzed on the flow cytometer.

Figure 22 illustrates the principle of flow cytometry. In a flow cytometer, the cells exit a flow cell and are illuminated with a laser beam. The amount of laser light that is scattered off the cells as they pass through the laser can be measured, which gives information concerning the size of the cells. In addition, the laser can excite the fluorochrome on the cells and the fluorescent light emitted by the cells can be measured by one or more detectors.



The type of data that is obtained from the flow cytometer is shown in Figure 23. In a one parameter histogram, increasing amount of fluorescence (e.g. green fluorescence) is plotted on the x axis and the number of cells exhibiting that amount of fluorescence is plotted on the y axis. The fraction of cells that are fluorescent can be determined by integrating the area under the curve. In a two parameter histogram, the x axis is one parameter (e.g. red fluorescence) and the y axis is the second parameter (e.g. green fluorescence). The number of cells is indicated by the contour and the intensity of the color.



## Complement Fixation

Antigen/antibody complexes can also be measured by their ability to fix complement because an antigen/antibody complex will "consume" complement if it is present, whereas free antigens or antibodies do not. Tests for antigen/antibody complexes that rely on the consumption of complement are termed complement fixation tests and are used to quantitate antigen/antibody reactions. This test will only work with complement fixing antibodies (IgG and IgM are best).

The principle of the complement fixation test is illustrated in Figure 24. Antigen is mixed with the test serum to be assayed for antibody and antigen/antibody complexes are allowed to form. A control tube in which no antigen is added is also prepared. If no antigen/antibody complexes are present in the tube, none of the complement will be fixed. However, if antigen/antibody complexes are present, they will fix complement and thereby reduce the amount of complement in the tube. After allowing complement fixation by any antigen/antibody complexes, a standard amount of red blood cells, which have been pre-coated with anti-erythrocyte antibodies is added. The amount of antibody-coated red blood cells is predetermined to be just enough to completely use up all the complement initially added, if it were still there. If all the complement was still present (i.e. no antigen/antibody complexes formed between the antigen and antibody in question), all the red cells will be lysed. If antigen/antibody complexes are formed between the antigen and antibody in question, some of the complement will be consumed and, thus, when the antibody-coated red cells are added not all of them will lyse. By simply measuring the amount of red cell lysis by measuring the release of hemoglobin into the medium, one can indirectly quantitate antigen/antibody complexes in the tube. Complement fixation tests are most commonly used to assay for antibody in a test sample but they can be modified to measure antigen.

