Chapter 9. Antigen–Antibody Reaction

Table of Contents

CHAPTER PREVIEW

- General Properties
- Conventional Immunoassays
  - Precipitation Reaction
  - Agglutination Reaction
- Newer Techniques
  - ELISA and ELFA
  - Immunofluorescence Assay
  - Chemiluminescence Immunoassay
  - Rapid Tests

GENERAL PROPERTIES

The antigen (Ag)–antibody (Ab) reaction refers to the binding of antigen and antibody with each other specifically and in an observable manner.

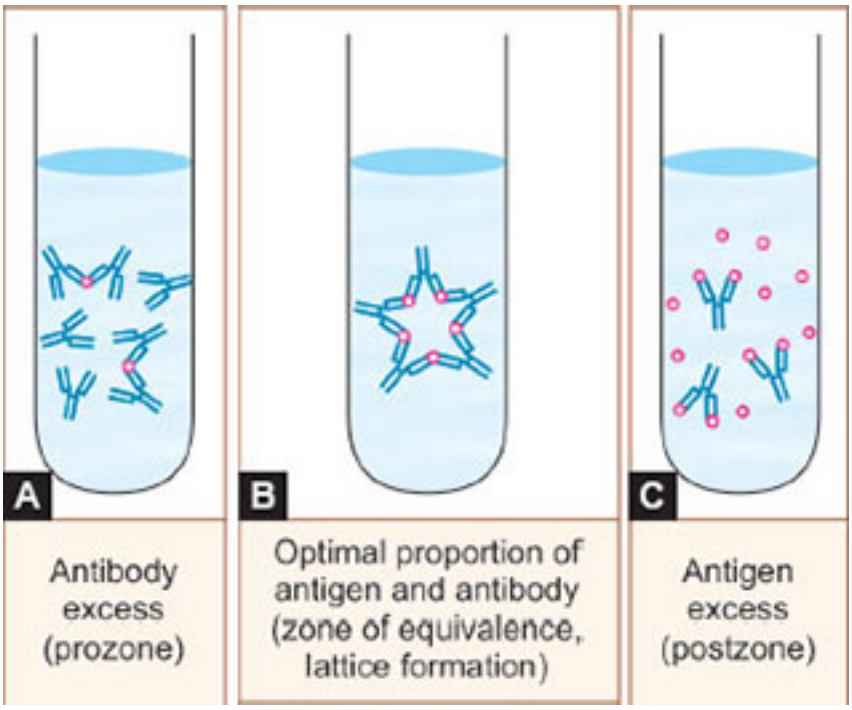
- **Specific:** Ag-Ab reaction involves specific interaction between the epitope of an antigen with the corresponding paratope of its homologous antibody
- **Noncovalent interactions:** The union of antigen and antibody requires the formation of a large number of non-covalent interactions between them
- **Immunoassays:** Because Ag-Ab reactions are specific and observable, they are extensively used in laboratories for the diagnosis of infectious diseases. Such assays are called immunoassays
  - *Antigen detection assays:* Detect antigens in the patient's sample by employing a specific antibody
  - *Antibody detection assays:* Detect antibodies in a patient's sample by employing a specific antigen.
- **Serological tests:** Immunoassays can be developed for the detection of antigens or antibodies in various clinical specimens, the most common being serum specimen. The immunoassays that are designed specifically for testing on serum specimens are called *serological tests*
- **Titer:** The exact amount of antibody in serum can be estimated by serial dilution of the patient's serum and mixing each dilution of the serum with a known quantity of antigen. The measurement of antibodies is expressed in terms of titer
  - The antibody titer of serum is the highest dilution that shows an observable reaction with the antigen
  - Antigen titer can also be similarly measured in the sera by testing the series of diluted sera against a known quantity of antibodies.

Lattice Hypothesis

When the sera-containing antibody is serially diluted (in normal saline), the antibody level gradually decreases.

- When a fixed quantity of antigen is added to such a set of test tubes containing serially diluted sera, then it is observed that the Ag-Ab reaction occurs at its best only in the middle test tubes where the amount of antigen and antibody are equivalent to each other *(zone of equivalence)*

Figs. 9.1A to C. *A.* Prozone; *B.* Zone of equivalence; *C.* Postzone.



- The Ag-Ab reaction is weak or fails to occur when the number of antigens and antibodies are not proportionate to each other *(Figs. 9.1A to C)*
  - In the earlier test tubes, *antibodies are excess*, hence the Ag-Ab reaction does not occur: This is called as *prozone phenomenon*
  - In the later test tubes, *antigen is excess*, hence the Ag- Ab reaction fails to occur: This is called as *postzone phenomenon*.

TYPES OF ANTIGEN-ANTIBODY REACTIONS

The Ag–Ab reactions used in diagnostic laboratories are based on various techniques which are broadly classified as conventional techniques and newer techniques.

- **Conventional techniques:** Examples include precipitation and agglutination reactions
- **Newer techniques:** Examples include—
  - Enzyme-linked immunosorbent assay
  - Enzyme-linked fluorescent assay
  - Immunofluorescence assay
  - Chemiluminescence immunoassay
  - Rapid tests
  - Western blot.

CONVENTIONAL IMMUNOASSAYS

PRECIPITATION REACTION

When a *soluble antigen* reacts with its antibody in the presence of optimal temperature, pH, and electrolytes (NaCl), it leads to the formation of the Ag–Ab complex in the form of:

- **Insoluble precipitation band** when gel or agar containing medium is used (called immunodiffusion), or
- **Insoluble floccules** when the liquid medium is used (called flocculation test).

Earlier, precipitation reactions were one of the widely used serological tests. However, with the advent of simple and rapid newer techniques, their application is greatly reduced. There are only limited situations where precipitation reactions are still in use, such as:

- **Slide flocculation test** (for syphilis): For example, VDRL (Venereal Disease Research Laboratory) and RPR (Rapid Plasma Reagin) tests
- **Elek's gel precipitation test:** Used for detecting diphtheria toxin.

AGGLUTINATION REACTION

When a *particulate* or *insoluble* antigen is mixed with its antibody in the presence of electrolytes at a suitable temperature and pH, the particles are clumped or agglutinated.

- **Advantages:** Agglutination is more sensitive than the precipitation test and the clumps are better visualized and interpreted as compared to bands or floccules
- **Types:** Agglutination reactions are classified as direct, indirect (passive) tests. All these agglutination tests are performed either on a slide or in a tube or on a card or sometimes in microtiter plates.

Direct Agglutination Test

Here, the antigen directly agglutinates with the antibody.

Slide Agglutination

It is usually performed to confirm the identification and serotyping of bacterial colonies grown in culture. It is also the method used for blood grouping and cross-matching.

A bacterial colony is mixed with a drop of saline on a slide to form a uniform smooth milky white suspension

↓

To this, a drop of the antiserum (serumcontaining appropriate antibody) is added and the slide is shaken thoroughly (manually or by rotator) for a few seconds

↓

A positive result is indicated by visible clumping with the clearing of the suspension **(Fig. 9.2)**

or

If the milky white suspension remains unchanged, indicates a negative result **(Fig. 9.2)**

Tube Agglutination

This is a quantitative test done for estimating antibody in serum. The *antibody titer* can be estimated as the highest dilution of the serum which produces a visible agglutination.

A fixed volume of a particulate antigen suspension is added to an equal volume of serial dilutions of a serum sample (containing appropriate antibody) in test tubes

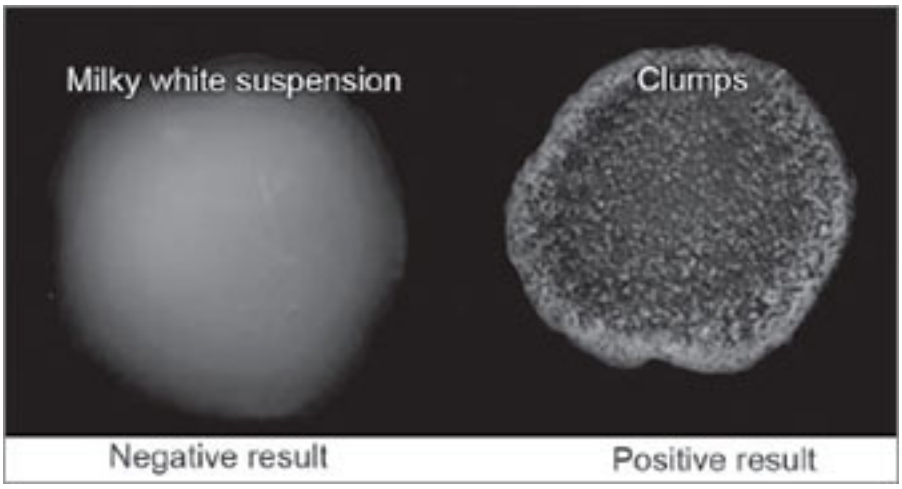
↓

A positive test indicates agglutination (clump formation at the bottom of the tube with the clearing of the supernatant)

or

A negative test indicates agglutination has not occurred (Ag suspension forms button at the bottom of the tube)

Fig. 9.2. Slide agglutination test.



Source: Department of Microbiology, JIPMER, Puducherry *(with permission).*

Tube agglutination is routinely used for the serological diagnosis of various diseases, such as:

- **Typhoid fever (Widal test):** It detects antibodies against both H (flagellar) and O (somatic) antigens of *Salmonella* Typhi
- Acute brucellosis (standard agglutination test)
- Coombs antiglobulin test
- Heterophile agglutination tests:

- Typhus fever (Weil Felix reaction)
- Infectious mononucleosis (Paul Bunnell test)
- *Mycoplasma* pneumonia (Cold agglutination test).

Microscopic Agglutination

Here, the agglutination test is performed on a microtiter plate and the result is read under a microscope. The classical example is microscopic agglutination test (MAT) done for leptospirosis.

Indirect or Passive Agglutination Test (for Antibody Detection)

As the agglutination test is more sensitive and better interpreted than the precipitation test, an attempt has been made to convert a precipitation reaction into an agglutination reaction. This is possible by coating the soluble antigen on the surface of a carrier molecule (e.g. RBC or latex) so that the antibody binds to the coated antigen and agglutination takes place on the surface of the carrier molecule.

Latex Agglutination Test (LAT) for Antibody Detection

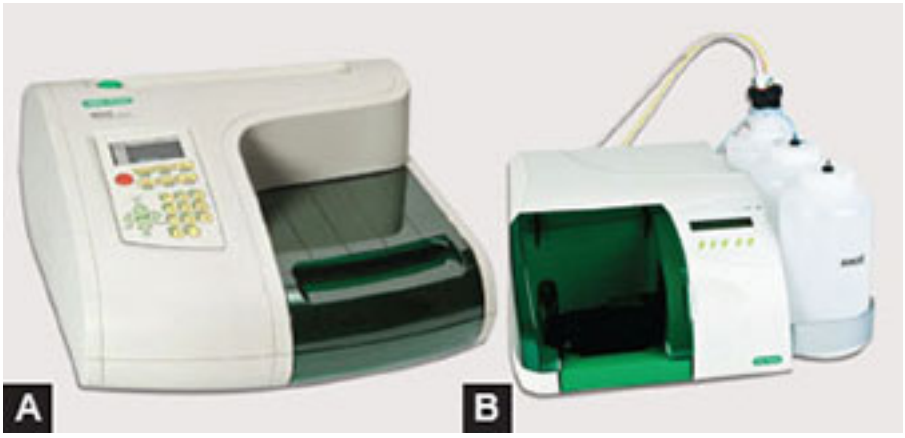
Here, latex particles are used as carrier molecules that are capable of adsorbing several types of antigens. For a better interpretation of the result, the test is performed on a black color card.

- A drop of patient's serum (containing antibody) is added to a drop of latex solution coated with the antigen and the card is rotated for uniform mixing
- A positive result is indicated by the formation of visible clumps *(Fig. 9.3)*

Fig. 9.3. Passive (latex) agglutination test.

Source: Department of Microbiology, JIPMER, Puducherry *(with permission).*

Figs. 9.4A and B. *A.* ELISA reader (Biorad); *B.* ELISA washer.



Source: Biorad Pvt. ltd *(with permission)*.

- LAT is one of the most widely used tests at present as it is very simple and rapid
- It is used for the detection of ASO (antistreptolysin O antibody).

Reverse Passive Agglutination Test (for Antigen Detection)

In this test, the antibody is coated on a carrier molecule that detects antigen in the patient's serum. The classical example is *the latex agglutination test for antigen detection*: It is used widely for the detection of CRP (C reactive protein), RA (rheumatoid arthritis factor), capsular antigen detection in CSF (for pneumococcus, meningococcus, and *Cryptococcus)* and streptococcal grouping.

NEWER IMMUNOASSAYS

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA is an immunoassay that detects either antigen or antibodies in the specimen, by using an enzyme-substrate–chromogen system for detection.

Principle of ELISA

ELISA is so named because of its two components:

- **Immunosorbent:** Here, a microtiter plate of absorbing material is used (e.g. polystyrene, polyvinyl) that specifically absorbs the antigen or antibody present in the serum
- **Enzyme** is used to label one of the components of immunoassay (i.e. antigen or antibody).

Substrate-chromogen system: It is added at the final step of ELISA. The enzyme reacts with the substrate, which in turn activates the chromogen to produce a color. The color change is detected by spectrophotometry in an ELISA reader *(Fig. 9.4A)*.

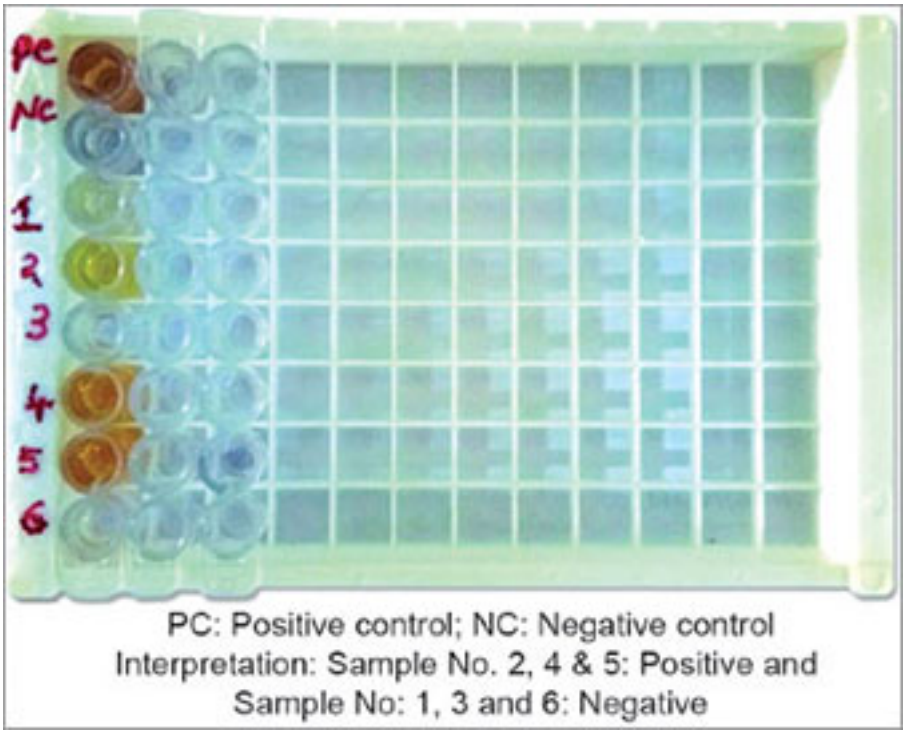
(Ag-Ab complex)-enzyme + substrate → activates the chromogen → color change → detected by spectrophotometry (ELISA reader, **Fig. 9.4A)**

Procedure of ELISA

ELISA is performed on a microtiter plate containing 96 wells *(Fig. 9.5)*, made up of polystyrene, or polyvinyl material.

- ELISA kits are commercially available; contain all necessary reagents (such as enzyme conjugate, dilution buffer, substrate/chromogen, etc.)

Fig. 9.5. ELISA for HBsAg.



Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry *(with permission)*.

- The procedure involves a series of steps done sequentially
- At each step, a reagent is added and then incubated, followed by washing of the wells by an ELISA washer *(Fig. 9.4B)*.

Types of ELISA

There are several types of ELISA, which differ from each other in their principles.

Direct ELISA

It is used for the detection of antigen in test serum. Here, the primary antibody (targeted against the serum antigen) is labeled with the enzyme *(Fig. 9.6A)*.

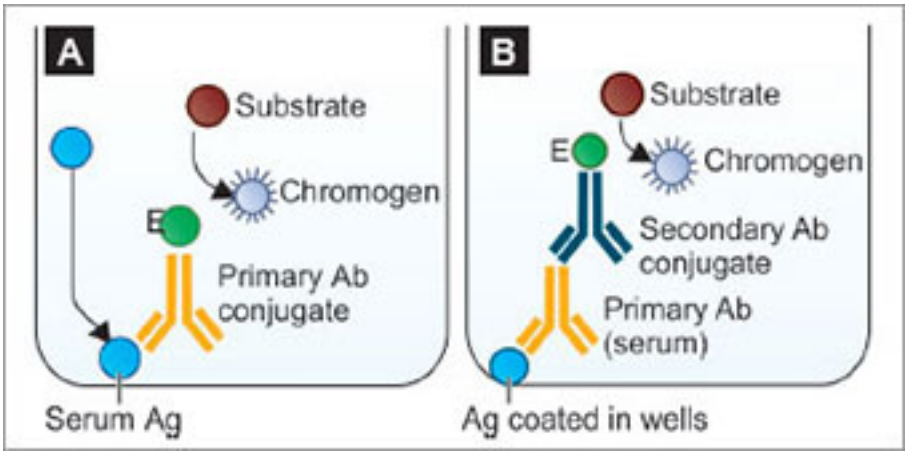
- **Step 1:** Wells of the microtiter plate are empty, not precoated with Ag or Ab
- **Step 2:** Test serum (containing antigen) is added into the wells. Antigen becomes attached to the solid phase by passive adsorption
- **Step 3:** After washing, the enzyme-labeled primary antibodies (raised in rabbits) are added
- **Step 4:** After washing, a substrate–chromogen system is added and the color is measured.

Well + Ag (test serum) + primary Ab-enzyme + substrate-chromogen → color change **(Fig. 9.6A).**

Indirect ELISA

It is used for the detection of antibody or less commonly antigen in serum. It differs from the direct ELISA in that the secondary antibody is labeled with an enzyme instead of the primary antibody. The secondary antibody is an anti-species antibody, e.g. anti-human Ig (an antibody targeted to the Fc region of any human Ig). Indirect ELISA for antibody detection is described below *(Fig. 9.6B)*.

Figs. 9.6A and B. *A.* Direct ELISA (for antigen detection); *B.* Indirect ELISA (for antibody detection).



- **Step 1:** The solid phase of the wells of microtiter plates are precoated with the Ag
- **Step 2:** Test serum (containing primary Ab specific to the Ag) is added to the wells. Ab gets attached to the Ag coated on the well
- **Step 3:** After washing, enzyme-labeled secondary Ab (anti-human immunoglobulin) is added
- **Step 4:** After washing, a substrate-chromogen system is added and the color is developed.

Wells are coated with Ag + primary Ab (test serum) + secondary Ab-enzyme + substratechromogen → development of color **(Fig. 9.6B).**

Other types of ELISA include:

- **Sandwich ELISA:** It detects the antigen in test serum. It is so named because the antigen gets sandwiched between a capture antibody (coated on the well) and a detector antibody

- **IgM antibody capture (MAC) ELISA:** This is an enzymatically amplified sandwich-type immunoassay. This format of ELISA is widely used for dengue, Japanese B encephalitis and West Nile virus, scrub typhus, leptospirosis, toxoplasmosis, etc.

- **Competitive ELISA:** It is so named because the antigen in the test serum competes with another antigen of the same type coated on the well to bind to the primary antibody.

Advantages of ELISA

ELISA is the method of choice for detection of antigens/antibodies in serum in modern days, especially in big laboratories as a large number of samples can be tested together using the 96 well microtiter plate.

- It is economical, takes 2–3 hours for performing the assay

- ELISA has a high sensitivity; that is why it is commonly used for performing screening tests at blood banks and tertiary care sites

- Its specificity used to be low. But now, with the use of more purified recombinant and synthetic antigens, and monoclonal antibodies, ELISA has become more specific.

Disadvantages of ELISA

- In small laboratories having less sample load, ELISA is less preferred than rapid tests as the latter can be performed on individual samples

- It takes more time (2–3 hours) compared to rapid tests which take 10–20 minutes

- It needs expensive equipment such as an ELISA washer and reader.

Applications of ELISA

ELISA can be used both for antigen and antibody detection.

- ELISA used for antigen detection: Hepatitis B [hepatitis B surface antigen (HBsAg) and precore antigen (HBeAg)], NS1 antigen for dengue, etc.

- ELISA can also be used for antibody detection against hepatitis B, hepatitis C, HIV, dengue, herpes simplex virus, Epstein Barr virus, toxoplasmosis, leishmaniasis, etc.

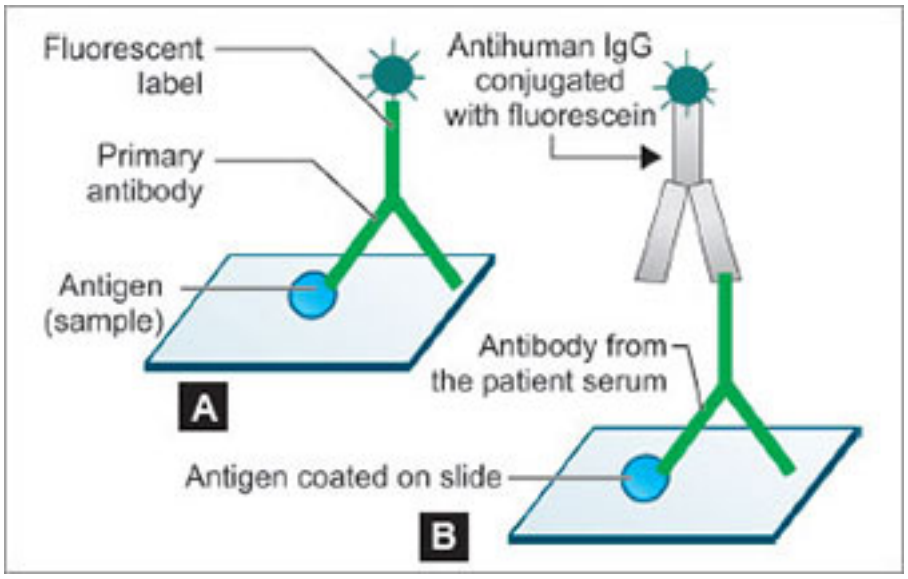
ENZYME-LINKED FLUORESCENT ASSAY (ELFA)

It is a modification of ELISA, which differs from ELISA in two ways: (i) automated system, all steps are performed by the instrument itself, and (ii) Ag-Ab-enzyme complex is detected by the fluorometric method. VIDAS and miniVIDAS (bioMérieux) are commercially available systems based on ELFA technology.

IMMUNOFLUORESCENCE ASSAY (IFA)

It is a technique similar to ELISA, but differs by the use of fluorescent dye instead of enzyme for labeling the antibody. It is of two types: direct and indirect.

Figs. 9.7A and B. Immunofluorescence assay: *A.* Direct; *B.* Indirect.



Direct Immunofluorescence Assay

- **Step 1:** Sample containing cells carrying surface antigens is smeared on a slide
- **Step 2:** Primary antibody specific to the antigen, tagged with a fluorescent dye is added
- **Step 3:** The slide is washed to remove the unbound antibodies and then viewed under a fluorescence microscope *(Fig. 9.7A)*.

Indirect Immunofluorescence Assay

This detects antibodies in the sample. Slides smeared with cells carrying known antigens are commercially available.

- **Step 1:** Test serum-containing primary antibody is added to the slide
- **Step 2:** The slide is washed to remove the unbound antibodies. A secondary antibody (antihuman antibody conjugated with fluorescent dye) is added
- **Step 3:** The slide is washed and then viewed under a fluorescence microscope *(Fig. 9.7B).*

Applications: Immunofluorescence assay has various applications, such as:

- Detection of autoantibodies (e.g. antinuclear antibodies) in autoimmune diseases
- Detecting microbial antigens, e.g. rabies antigen in corneal smear
- Detection of viral antigens in cell lines inoculated with the specimens.

CHEMILUMINESCENCE IMMUNOASSAY

The principle of chemiluminescence immunoassay (CLIA) is similar to that of ELISA; however, the chromogenic substance is replaced by chemiluminescent compounds (e.g. luminol and acridinium ester) that generate light during

a chemical reaction (luxogenic). The light (photons) can be detected by a photomultiplier, also called as luminometer *(Fig. 9.8)*.

(Ag-Ab complex)-enzyme + chemiluminescent substrate $\rightarrow$ product + light (photons) $\rightarrow$ detected by luminometer or photomultiplier.

Advantages of CLIA

- CLIA claims to be 10 times more sensitive than ELISA
- Individual specimens can be tested in CLIA in contrast to ELISA which is preferred for testing multiple samples at a time.

Applications

CLIA has limited applications in diagnostic microbiology compared to ELISA. Currently, it is available for the detection of antigens or antibodies against various infections such as hepatitis viruses, HIV, and biomarkers such as procalcitonin.

WESTERN BLOT

Western blot detects specific proteins (antibodies) in a sample containing mixture of antibodies each targeted against different antigens of same microbe.

- **Procedure:** It has three steps: (i) separation of antigen mixture into individual fragments by *gel electrophoresis*, (ii) transfer of antigen fragments onto a *nitrocellulose membrane*, (iii) detection of individual antibodies in serum against each antigenic fragments by *enzyme immunoassay*
- **Advantages:** It has an excellent specificity. Hence, it is often used as a supplementary test to confirm the result of ELISA or other immunoassays having higher sensitivity
- **Applications:** Western blot formats are available to detect antibody in various diseases such as HIV, cysticercosis, hydatid disease, etc.

RAPID TESTS

Rapid tests are revolutionary in the diagnosis of infectious diseases. They are very simple to perform (one-step method), rapid (takes 10–20 minutes), require minimal training, and do not need any sophisticated instruments.

- These tests are also called *Point-of-care* (POC) tests because unlike ELISA and other immunoassays, the POC tests can be performed independent of laboratory equipment and deliver instant results

Fig. 9.8. Chemiluminescence system (CLIA) and its principle.

Source: Department of Microbiology, JIPMER, Puducherry *(with permission)*.

- Two principles of rapid tests are available—lateral flow assay and flow-through assay
- Both formats are available for the diagnosis of various diseases such as malaria, hepatitis B, hepatitis C, HIV, leptospirosis, etc.

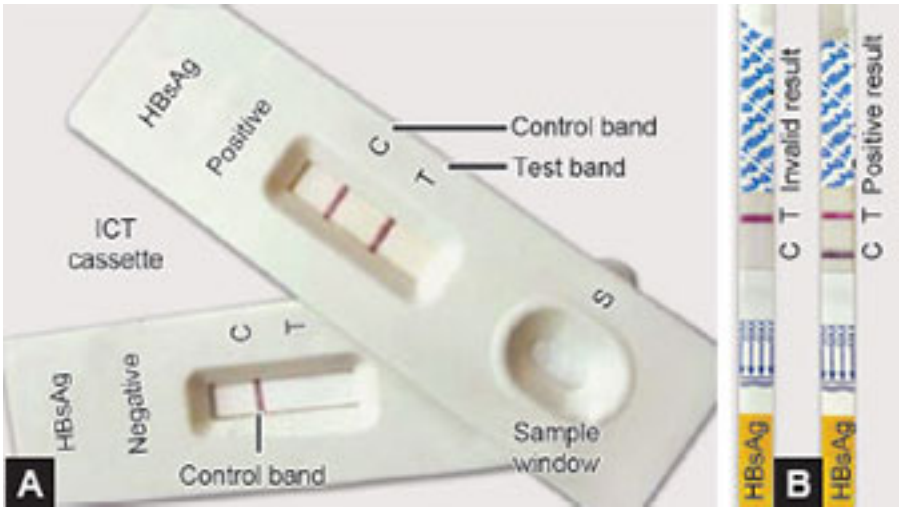
Immunochromatographic Test (Lateral Flow Assay)

The immunochromatographic test (ICT) is based on the lateral flow technique. It is widely used in diagnostic laboratories because of its simplicity, low cost, and rapidity. It can be used for both antigen and antibody detection in the sample. The principle of the antigen detection method is described below.

Principle of ICT (Antigen Detection)

The test system consists of a nitrocellulose membrane (NCM) and an absorbent pad. Two formats are available: cassette or strip *(Figs. 9.9A and B)*. The NCM is coated at two places in the form of lines—a test line, coated with monoclonal antibody targeted against the test antigen, and a control line, coated with anti-species immunoglobulin. Specific Ab against the target Ag labeled with a chromogenic marker (specific Ab tagged with *colloidal gold* or *silver*, a visually detectable marker) is infiltrated in the absorbent pad lining the sample window.

Figs. 9.9A and B. ICT for HBsAg detection: *A.* Cassette format; *B.* Strip format.



Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry *(with permission)*.

- The sample (serum) containing the test antigen is added to the sample well; it reacts with antibody labeled with a chromogenic marker *(colloidal gold* or *silver*, a visually detectable marker)
- Both 'Ag-specific Ab-colloidal gold complex' as well as the 'free colloidal gold-labeled Ab' move laterally along the nitrocellulose membrane
- **Test band:** At the test line, the Ag-labeled Ab complex is immobilized by binding to the monoclonal Ab in the test line to form a colored band *(Figs. 9.9A and B)*
- **Control band:** The free colloidal gold-labeled Ab can move further and binds to the anti-human Ig to form a color control band. If the control band is not formed, then the test is considered invalid irrespective of whether the test band is formed or not **(Figs. 9.9A and B)**.

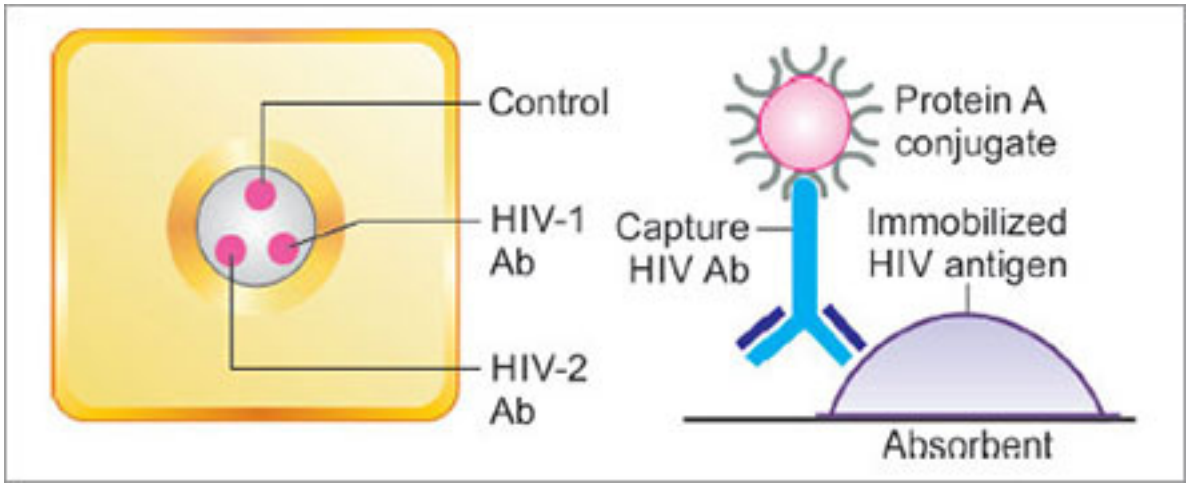
Flow-through Assay

Flow-through tests are another type of rapid diagnostic assays that differ from ICT in two aspects: (1) protein A is used for labeling antibody instead of gold conjugate, and (2) the sample flows vertically through the nitrocellulose membrane (NCM) as compared to lateral flow in ICT.

Flow-through tests can be used for both antigen and antibody detection. HIV TRI-DOT test is a classical example (described below, *Fig. 9.10A)*. It detects antibodies to HIV-1 and 2 separately in patient's serum.

- The test system is in a cassette format, consisting of a NCM and an absorbent pad. The NCM is coated at three regions- two test regions coated with HIV-1 and 2 antigens and a third control region coated with antihuman Ig
- Sample and buffer reagents are added sequentially from the top following which they pass through the membrane and excess fluid is absorbed into the underlying absorbent pad
- As the patient's sample passes through the membrane, HIV antibodies, if present bind to the immobilized antigens *(Fig. 9.10B)*

Figs. 9.10A and B. Flow-through assays: *A.* HIV TRI-DOT assay for HIV-1 and 2 antibodies detection; *B.* Principle of HIV TRI-DOT.



- **Test dots:** Protein-A conjugate (present in buffer) binds to the Fc portion of the HIV antibodies to give distinct pinkish-purple DOT(s), separately for HIV-1 and 2 antibodies
- **Control dot:** Irrespective of whether the HIV antibodies are present or not, protein-A can bind to any IgG present in serum and the IgG-protein A complex can further bind to the antihuman Ig at the control line to give a pinkish purple DOT.

EXPECTED QUESTIONS

1. I. Write an essay on:

1. Enumerate the properties and types of antigen-antibody reactions. Describe in detail the principle, types, and applications of ELISA?

2. II. Write short notes on:

1. Precipitation reaction.
2. Agglutination reactions.
3. Indirect immunofluorescence assay.
4. Immunochromatographic test.
5. Chemiluminescence immunoassay (CLIA).

3. III. Multiple Choice Questions (MCQs):

1. **The Prozone phenomenon is due to:**
   a. Excess antigen
   b. Excess antibody
   c. Hyperimmune reaction
   d. Both antigen and antibody excess

2. **All are agglutination reactions,** *except:*
    a. VDRL test
    b. Standard agglutination test
    c. Widal test
    d. Paul Bunnell test
3. **The following methods of diagnosis utilize labeled antibodies,** *except:*
    a. ELISA
    b. CLIA
    c. Precipitation test
    d. Immunofluorescence
4. **All of the following are true about immunochromatographic tests,** *except:*
    a. Require experienced personnel
    b. Low cost
    c. Simplicity of testing
    d. Rapid results

Answers

1. b	**2.** a	**3.** c	**4.** a