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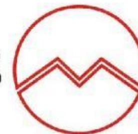

Review on Immunodiagnostics

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Abstract: Immunodiagnostic is an antibody-based method allowing the specific detection, quantification and localisation of antigens by means of antibody binding.Traditionally, the diagnosis of infectious diseases has been accomplished by the isolation of the infecting microorganism in pure culture. Classical methods of microbial isolation and identification have been invaluable in the study of bacterial, viral and fungal infections. However, cultivation systems offer disadvantages for the rapid diagnosis of infectious diseases. For example, many microorganisms, especially viruses and slow growing bacteria, require a considerable period of time in cultivation that the results from cultures are often not available at a time when the result can alter the course of therapy. Thus, more sensitive means must be applied for detecting and identifying a wide range of infectious diseases.
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LIST OF ABBREVIATIONS

CFT	Complement Fixation Test
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
FAT	Fluorescent Antibody Technique
IHA	indirect haemagglutination
I-PCR	Immuno-Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
RBC	Red Blood Cells
SDS-PAGE	Sodium Dodecylsulfate–Polyacrylamide Gel Electrophoresis

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1. INTRODUCTION

Immunodiagnostic is an antibody-based method allowing the specific detection, quantification and localisation of antigens by means of antibody binding.Traditionally, the diagnosis of infectious diseases has been accomplished by the isolation of the infecting microorganism in pure culture. Classical methods of microbial isolation and identification have been invaluable in the study of bacterial, viral and fungal infections. However, cultivation systems offer disadvantages for the rapid diagnosis of infectious diseases. For example, many microorganisms, especially viruses and slow growing bacteria, require a considerable period of time in cultivation that the results from cultures are often not available at a time when the result can alter the course of therapy. Thus, more sensitive means must be applied for detecting and identifying a wide range of infectious diseases.

The development of rapid, simple, sensitive and specific diagnostic tests for infectious diseases has been much slower than the other disciplines of medicine. This is partially attributed to the complexity and diversity of the pathogenic organisms and to the difficulty in detecting low concentrations of these organisms in samples. With the advent of technology, new methods are available to health professionals.

Immunological and molecular biology-based techniques are rapidly advancing the field of diagnostics in human and animal diseases. These new methods offer specificity and sensitivity and possessing the advantage that they are often able to produce results in a single day, thus providing the opportunity of taking more effective control measures.

Immunodiagnostic tests use an antigen-antibody reaction to detect and identify a specific antigen or antibody associated with a disease-causing organism. The antigen-antibody reaction itself is very specific. Consequently, if the correct antibodies can be obtained, immunodiagnostic techniques have the advantage of being able to identify the presence of a specific pathogen directly in the specimens and also can be used to detect the specific antibodies produced as a result of the immune response of the host to the organism. The primary component in the test is the antibody. The specificity, and to some extent the sensitivity, of the assay depends on the quality of the antibodies used in the reagents.

Immunodiagnosis has three main objectives:

a) Recognition of the acute infection, allowing an early treatment
b) Identification of a previous infection
c) Acquisition of epidemiological information.

2IMMUNODIAGNOSTIC METHODS

There are different methods for identification of unknown organisms by using immunodiagnostic methods. These are:

- ❖ Agglutination
- ❖ Precipitation
- ❖ Complement fixation
- ❖ Fluorescent antibody technique (FAT),
- ❖ Enzyme linked immunosorbent assay (ELISA)
- ❖ Western blot analysis.
- ❖ Immuno-PCR

1.1.

1.2. Agglutination

Agglutination reactions are among the most easily performed of immunological tests. Agglutination is a term come from “glu-” which means ‘adhesion’ and is broadly used in medical-clinical area to designate the formation of visible aggregates as a result of interaction among specific antibodies and insoluble particles containing antigenic determinants. The agglutination can occur with particles having natural antigenic determinants on their surface (erythrocytes, bacteria, protozoa, etc.) and with inert particles (latex, polystyrene, bentonite, etc.), or even with antigenically unrelated cells (blood cells) which adsorb or attach to soluble antigens. The principle is that if the serum contains antibodies against a surface antigen, they will agglutinate the bacterial cells.

The procedure is used both to demonstrate the presence of antibodies in serum and to identify antigens on microbial cell surfaces. A diagnostic assay for an infectious agent can be used to demonstrate the presence or absence of infection, or

to detect evidences of a previous infection (for example, the presence of antibodies). Agglutination reactions are much employed for the laboratory diagnosis of diseases caused by viruses, bacteria, protozoa, fungi, and autoimmune diseases. In Microbiology, agglutination is an important technique for diagnosis commonly used as a method of identifying bacteria and its specific antigens

In order for agglutination between antibody and antigen to occur, the antibody and antigen epitope must be combined in the proper proportions, called the zone of equivalence. When this happens, the antibody molecules bind to epitopes on two or more different antigens, forming a crosslinked network. If enough antigens and antibodies are present, the mass of agglutination becomes visible to the naked eye as clumping. If the antibody molecules are in too high a concentration, they will not form the crosslinked network, even though they recognize the antigen. This is called the prozone effect. If the antigen is in excess, this will also prohibit formation of a crosslinked network, termed a postzone effect. False negative reactions can occur when antibody or antigen concentrations are in either prozone or postzone regions.

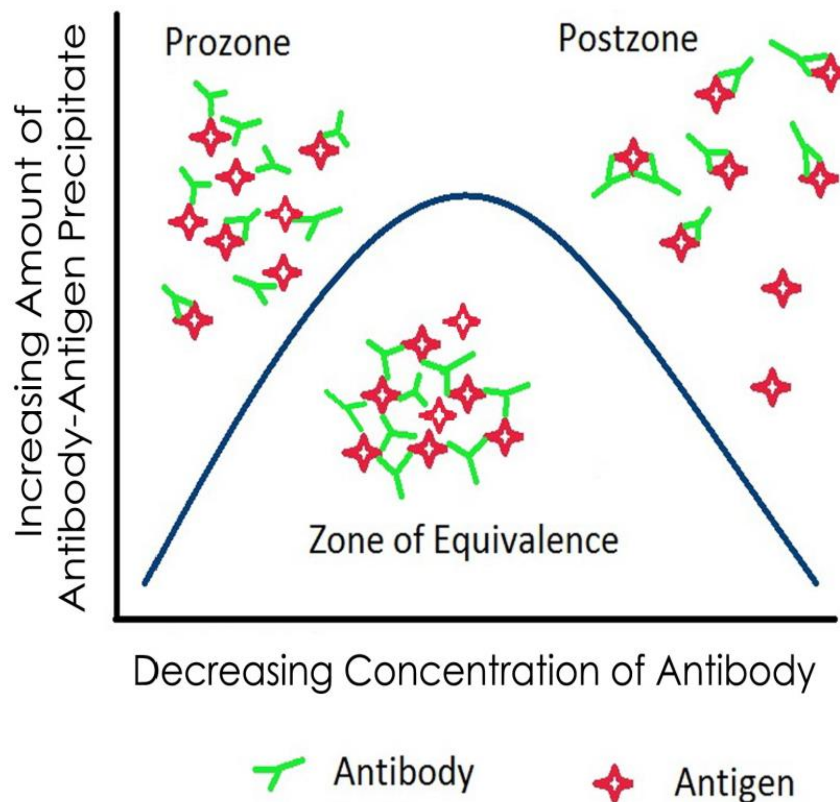

Figure 1:- Effect of antibody and antigen concentrations on agglutination.

Regions of excess antibody concentration are in the prozone region, while regions of excess antigen are in the postzone region. Antibody and antigen concentrations are optimal in the zone of equivalence.

Agglutination interactions may occur through a direct or indirect form. In both agglutination reactions, the biological fluid is tested for the presence of antibodies that will bind the antigens. The agglutination reaction involves in vitro aggregation of microscopic carrier particles (usually of polymeric nature, referred to as latex). This aggregation is mediated by the specific reaction between antibodies and antigens, one of which is immobilized on the surface of the latex particles to enhance the sensitivity and extend the point of equivalence.

In direct agglutination test the biological sample is mixed with a suspension containing antibodies against the antigen. If antigen is present in the sample it will react with the antibodies to form an aggregate. If no antigen is present in the sample the mixture will keep its appearance as a smooth suspension. This method is applicable to the detection of polyvalent antigens, e.g., proteins and micro-organisms. Insoluble antigenic particles in direct agglutination are used at their entire or fragmented forms. Bacteria,

fungi, protozoa and erythrocytes can be directly agglutinated by antibody.

An indirect agglutination test is used for the detection of an antibody in a biological sample. This works based on similar principles whereby antigens of the antibody corresponding are bound to latex particles. In the indirect agglutination the erythrocytes and inert particles (latex, bentonite, yeast, etc.) can be sensitized by passive adsorption via chemical agents such as tannic acid and chromium chloride, and by conjugation of the antigen by means of covalent chemical bonds by providing stable reagents. The agglutination assays can be performed in tubes or plates. This approach is applicable to mono- and polyvalent antigens, e.g., drugs, steroid hormones, and proteins

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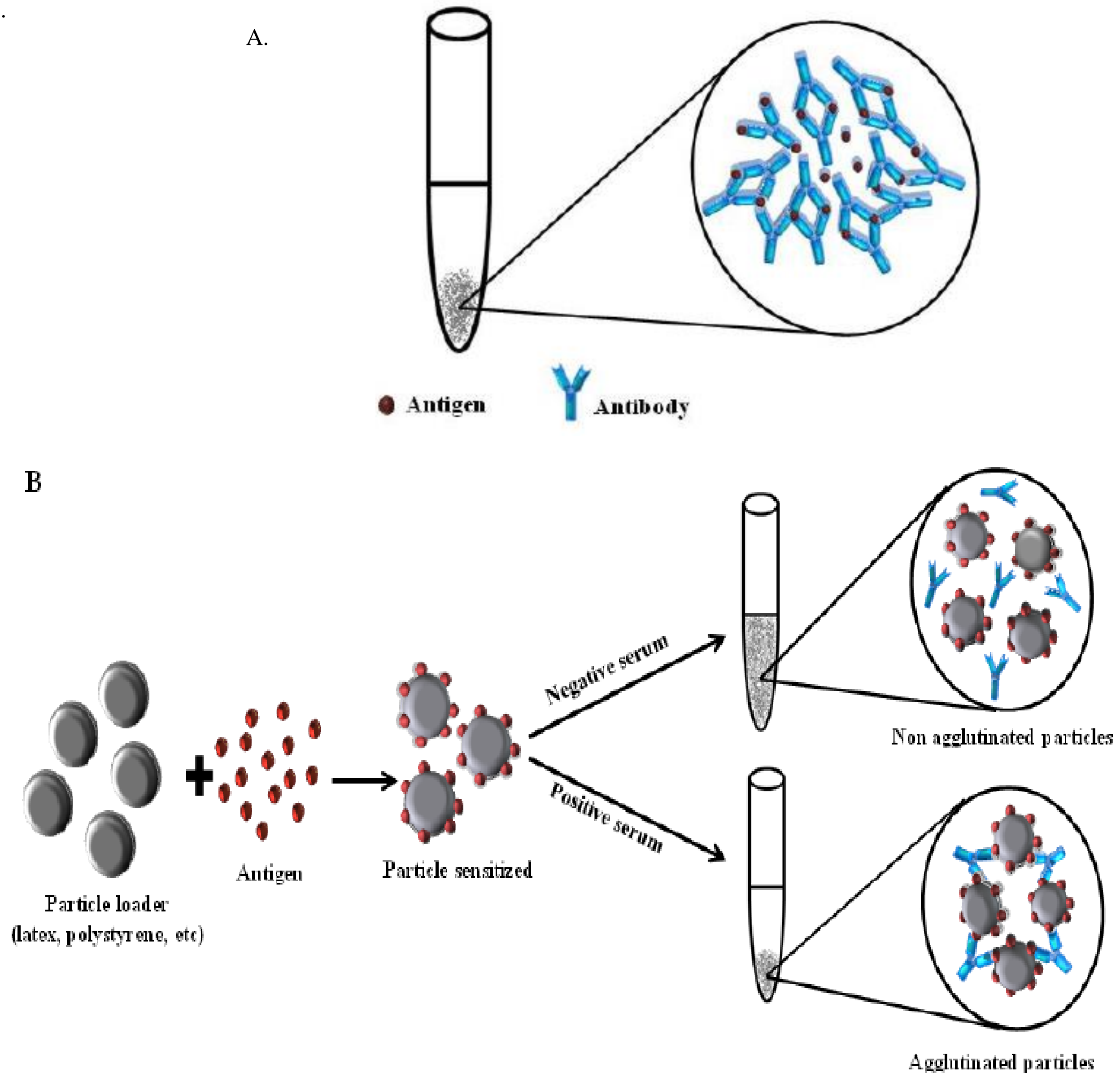


Figure 2:- Schematic representation of agglutination interactions.

A: Direct agglutination reaction where antibodies recognize and establish links with antigens forming aggregates. B: Indirect agglutination using particles. These reactions occur when antigens or parts of antigens are first bound (adsorbed) to a carrier particle to become resistant and more easily recognized by specific antibodies. Sensitized carrier particles amplify the recognition by antibodies and clump.

When agglutination interactions use erythrocytes can be called hemaglutination. Erythrocytes have been found to be convenient carriers for antigen or

antibody. Indirect agglutination with antigen coated erythrocytes is referred to as indirect (passive) haemagglutination (IHA) and erythrocytes coated with antibody are used in the reverse IHA test for assay of antigen.

The IHA test is versatile and it is possible to coat many antigens on the surface of erythrocytes. The IHA tests are simple, rapid, easy to perform, specific, sensitive and reproducible. In these assays, the erythrocyte has been described as a label, tag or marker for antibody. The erythrocyte as a carrier replaces markers used for visualization of primary antigen-antibody reaction.

Advantages

- ✓ Most widely used
- ✓ Very simple
- ✓ No instrument is required
- ✓ Cheap
- ✓ Fairly sensitive

Disadvantages

- ✓ Not highly specific
- ✓ Not highly sensitive.

Serially diluted serum samples are placed in a microtiter plate to which the viral antigen and the RBCs are added. The last serial dilution that yields total inhibition of agglutination is the serum titer for the patient. Hemagglutination inhibition assays are quite tedious and labor-intensive because they require fresh erythrocytes and antigen dilutions each time the test is performed. In recent years the IHA test for assay of antibody and reverse IHA test for assaying antigen have been widely used in bacteriology, .virology, immunology, parasitology, mycology and vaccinology

1.3.

1.4. Precipitation

The precipitin test is an example of a clinical test based on antigen-antibody reaction. Antibodies are able to precipitate antigens through multivalent binding, in which 2 Fab fragments in a single antibody can simultaneously bind to 2 antigens. A matrix of antigen:antibody complexes in a solution will then lead to a formation of a visible precipitate. In the precipitin test, a soluble antigen and antibody diffuse toward each other, and a visible precipitate forms when the 2 solutes meet at an optimal concentration.

The antibody cross-links antigen molecules in variable proportions, and aggregates (precipitates) form. In the zone of equivalence, optimal proportions of antigen and antibody combine; the maximal precipitates forms, and the supernatant contains neither an excess of antibody nor an excess of antigen. In the zone of antibody excess, there is too much antibody for efficient lattice formation, and precipitation is less than maximal. In the zone of antigen excess, all antibodies have combined but precipitation is reduced because many antigen-antibody complexes are too small to precipitate.

1.5. Complement Fixation

Complement fixation (CF) tests are used for detecting the presence of a specific antibody in a patient's serum by using the antigen, complement, and red blood cells (RBCs). If antibody is present, it will bind to the specific antigen. Addition of a complement that binds to the antigen-antibody complex forms a system, which allows RBCs to settle out of the serum as a pellet. If no antigen-specific antibodies are present, no complex is formed, and the complement will lyse the RBCs when they are added.

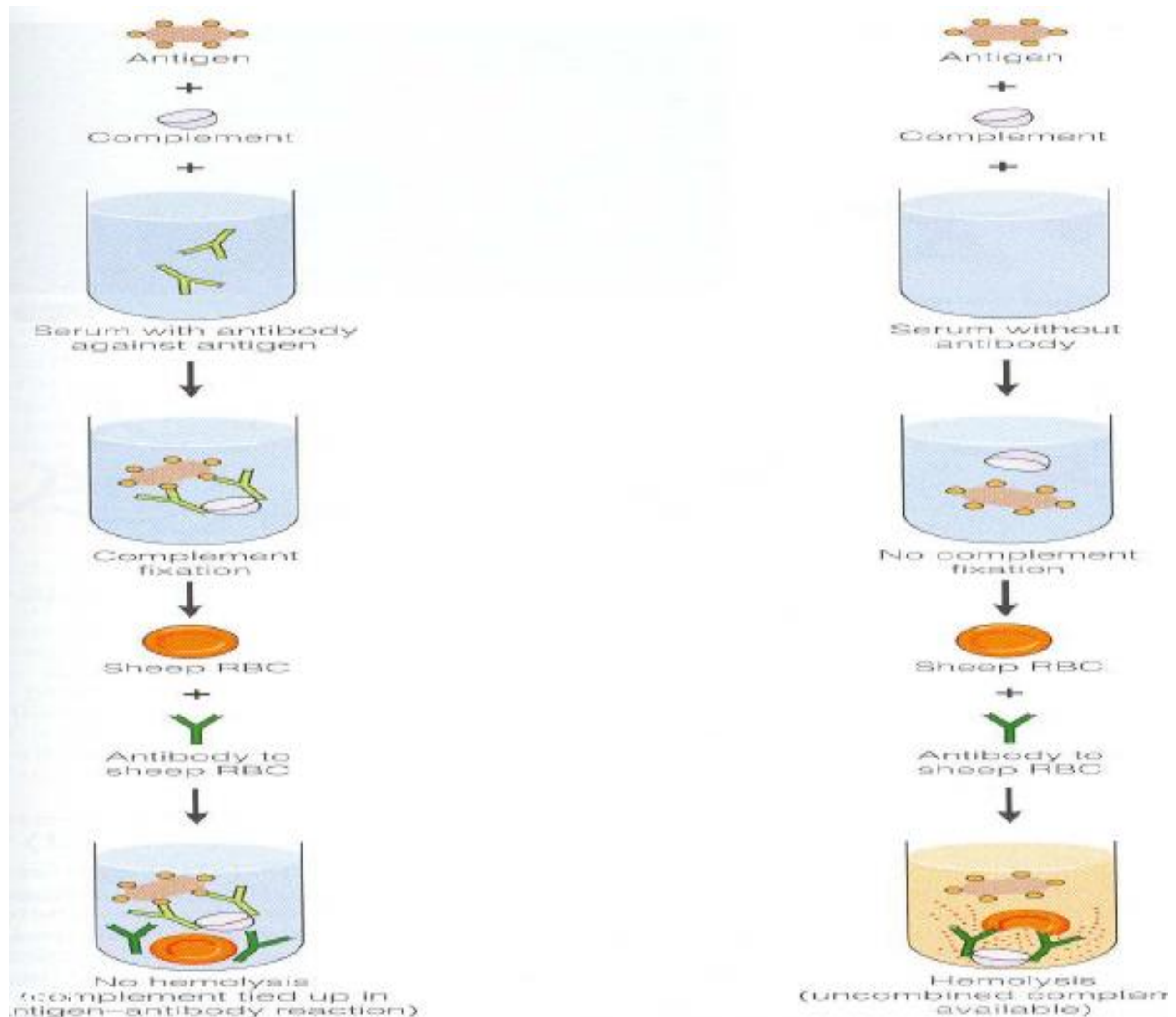


Figure 3:- Complement Fixation Reaction.

1.6.

1.7. Fluorescent antibody technique (FAT)

In this case pecific monoclonal or polyclonal antibodies are conjugated to fluorescent dyes (fluorescein isothiocyanate, rhodamine isothiocyanate, Texas red), which can be visualized using a fluorescence microscope, fluorometer, fluorescence scanner, or flow cytometer. These dyes can be used as tags, conjugated to target antibody molecules, and when the antibody complexes with the specific antigen, then the complex are "lighted".

In a direct fluorescent antibody test (DFAT), the antigen-specific labeled antibody is applied to a fixed specimen on a microscope slide, incubated, washed, and visualized under a fluorescence microscope. When a secondary, species-specific antibody is labeled with a fluorophore instead of the primary, the method is called indirect fluorescent antibody test (IFAT).

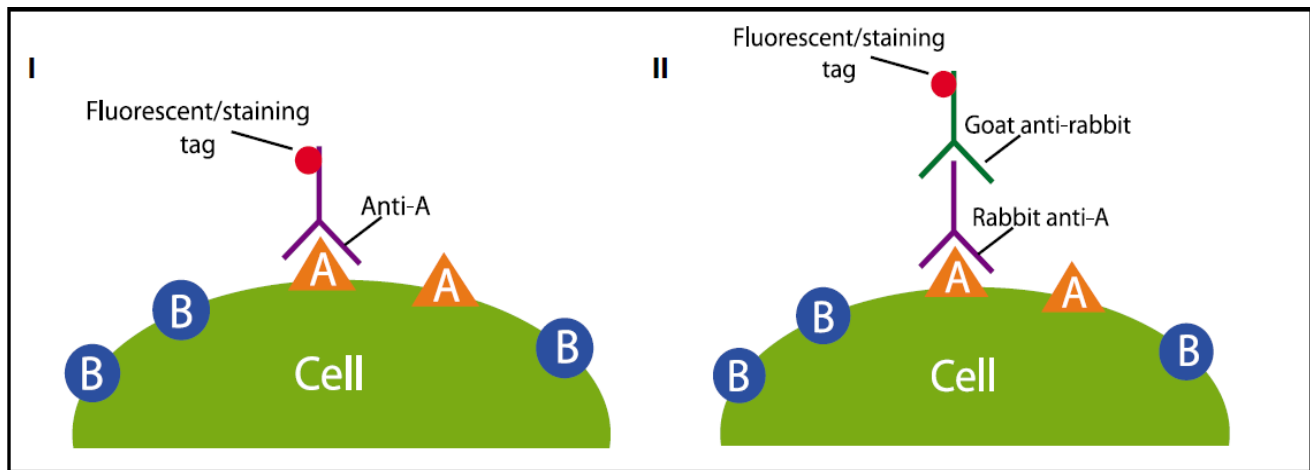


Figure 4:- Direct and indirect fluorescent antibody test format.

Direct fluorescent antibody test (**I**) uses antibodies that recognize specific antigens on the cell surface. In the indirect test format (**II**), the detection is based on a secondary (anti-species) antibody conjugated to a fluorescent label.

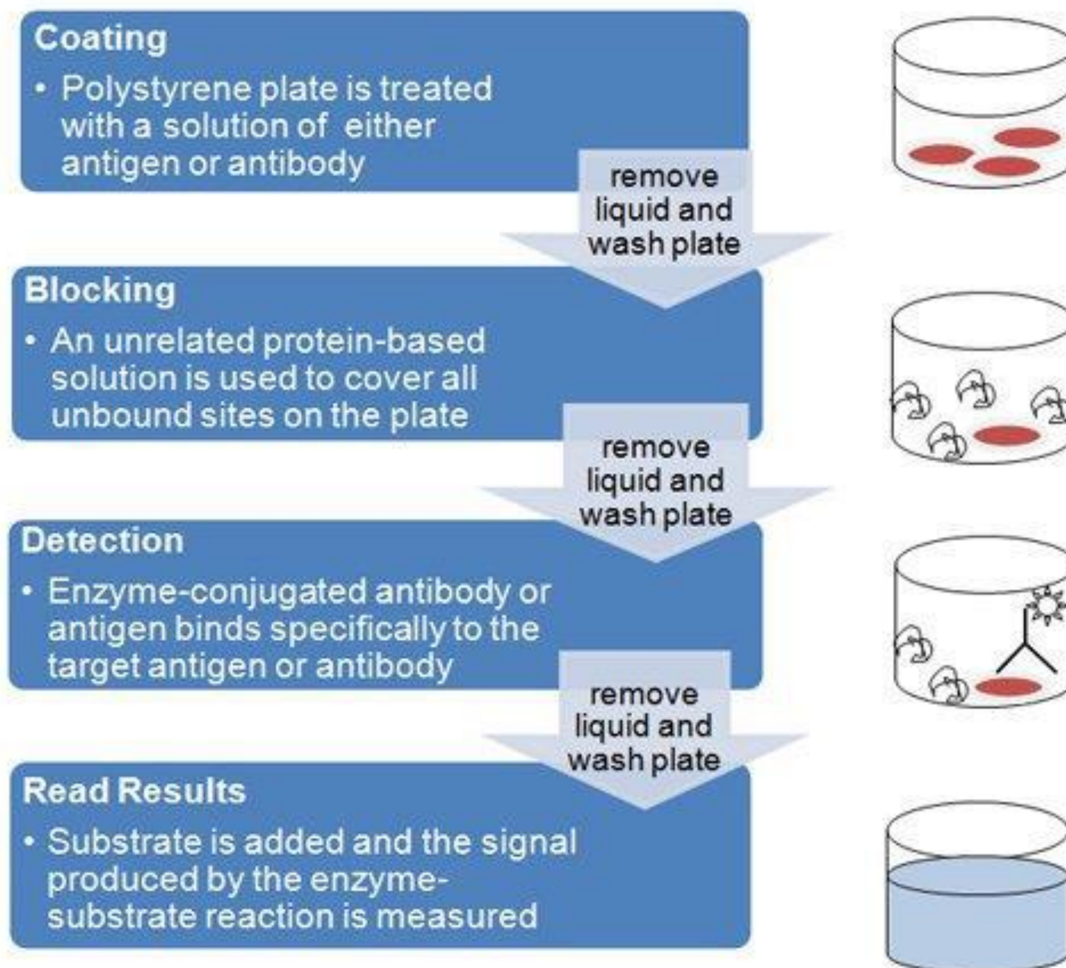
1.8. Enzyme linked immunosorbent assay (ELISA)

The ELISA is one of the most powerful of all immunochemical techniques. It employs a wide range of methods to detect and quantitate antigens or antibodies and to study the structure of antigens (proteins, peptides, hormones). It is a widely used clinical diagnostic tool used to detect a wide range of diseases from infectious diseases to cancer biomarkers. It is described as a precise, sensitive, versatile and quantifiable diagnostic method.

ELISAs are typically performed in 96-well (or 384-well) polystyrene plates, which will passively bind antibodies and proteins. It is this binding and immobilization of reagents that makes ELISAs so easy to design and perform. Having the reactants of the ELISA immobilized to the microplate surface makes it easy to separate bound from non-bound material during the assay. This ability to wash away nonspecifically bound materials makes the ELISA a powerful tool for measuring specific analytes within a crude preparation.

In an ELISA, an antigen must be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction.

General ELISA Procedures: Unless a kit with a plate that is pre-coated with antibody is used, an ELISA begins with a **coating** step, in which the first layer, consisting of a target antigen or antibody, is adsorbed onto a 96-well polystyrene plate. This is followed by a **blocking** step in which all unbound sites are coated with a blocking agent. Following a series of washes, the plate is **incubated with enzyme-conjugated antibody**. Another series of washes removes all unbound antibody. A **substrate** is then added, producing a calorimetric signal. Finally, the plate is **read**



ELISA Types

ELISAs can be performed with a number of modifications to the basic procedures and can be grouped into the four main categories: direct, indirect, sandwich, and competitive ELISAs.

1.8.1. Direct ELISA

For direct detection, an antigen coated to a multi-well plate is detected by an antibody that has been directly conjugated to an enzyme [such as; alkaline phosphatase (AP) or horseradish peroxidase (HRP)]. This detection method is a good option if there are no commercially available ELISA kits for the target protein.

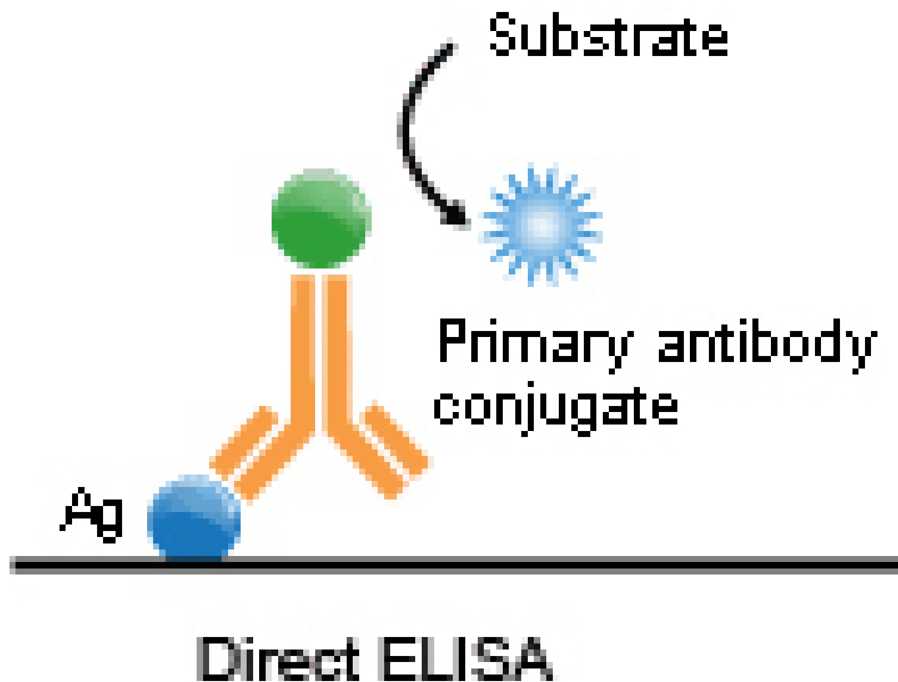


Figure 5:- Direct ELISA.

The direct ELISA technique is typically used when the immune response to an antigen needs to be analyzed.

Advantages

- ✓ Faster than other ELISA – the technique has fewer steps
- ✓ Less prone to error – as less reagents and fewer steps are required
- ✓ Cross-reactivity of secondary antibody is eliminated

Disadvantages

- ✓ Antigen immobilization is not specific - may cause higher background noise than indirect ELISA. Mainly because all proteins in the sample, including the target protein, will bind to the plate
- ✓ Less flexible - each target protein needs a specific conjugated primary antibody
- ✓ Labeling primary antibodies for each specific ELISA system is time-consuming and expensive.
- ✓ Minimal/No signal amplification - reduces assay sensitivity

1.8.2. Indirect ELISA

For indirect detection, the antigen coated to a multi-well plate is detected in two stages or layers. First an unlabeled primary antibody, which is specific for the antigen, is applied. Next, an enzyme-labeled secondary antibody is bound to the first antibody. The secondary antibody is usually an anti-species antibody and is often polyclonal.

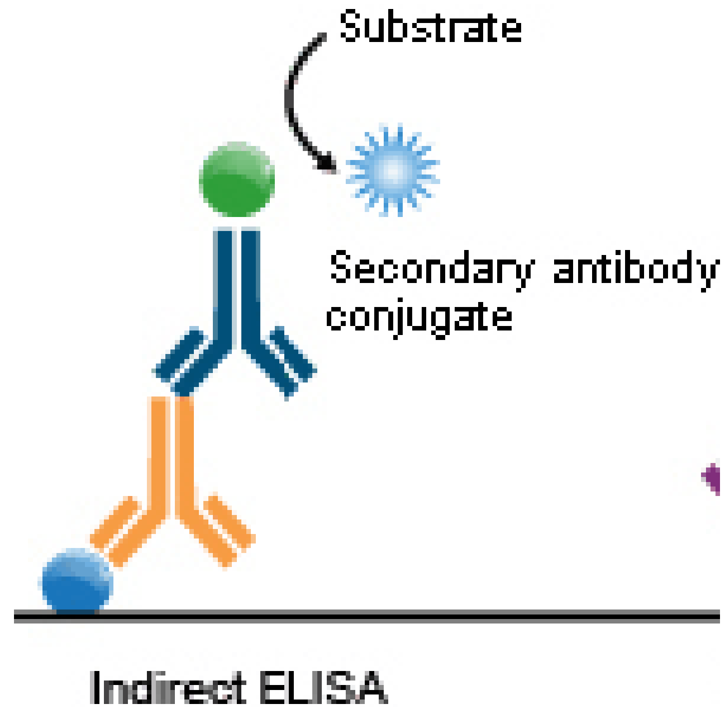


Figure 6:- Indirect ELISA.

The indirect assay is the most popular format for ELISA. The indirect ELISA is most suitable for determining total antibody concentration in samples.

Advantages

- High sensitivity - more than one labeled secondary antibody can bind the primary antibody because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody, allowing for signal amplification
- Economical - fewer labeled antibodies are needed
- Greater flexibility - different primary antibodies can be used with a single labeled secondary antibody
- A wide variety of labeled secondary antibodies are available commercially.
- Versatile because many primary antibodies can be made in one species and the same labeled secondary antibody can be used for detection.
- Maximum immunoreactivity of the primary antibody is retained because it is not labeled.

Disadvantages

- Possibility of background noise – secondary antibody may be cross-reactive
- Longer procedure than direct ELISA technique - additional incubation step for secondary antibody needed

1.8.3. Sandwich ELISA

Sandwich ELISAs typically require the use of matched antibody pairs (capture and detection antibodies), where each antibody is specific for a different, non-overlapping part (epitope) of the antigen molecule. A first antibody (known as capture antibody) is coated to the wells. The sample solution is then added to the well. A second antibody (known as detection antibody) follows this step in order to measure the concentration of the sample. The capture antibody, as its name implies, binds the antigen that can then be detected in a direct ELISA or in an indirect ELISA configuration.

The procedure for a sandwich ELISA firstly requires the well of an ELISA plate to be coated with a capture antibody. The analyte or sample is then added, followed by a detection antibody. The detection antibody can be enzyme conjugated, in which case this is referred to as a direct sandwich ELISA. If the detection antibody used is unlabeled, a secondary enzyme-conjugated detection antibody is required. This is known as an indirect sandwich ELISA.

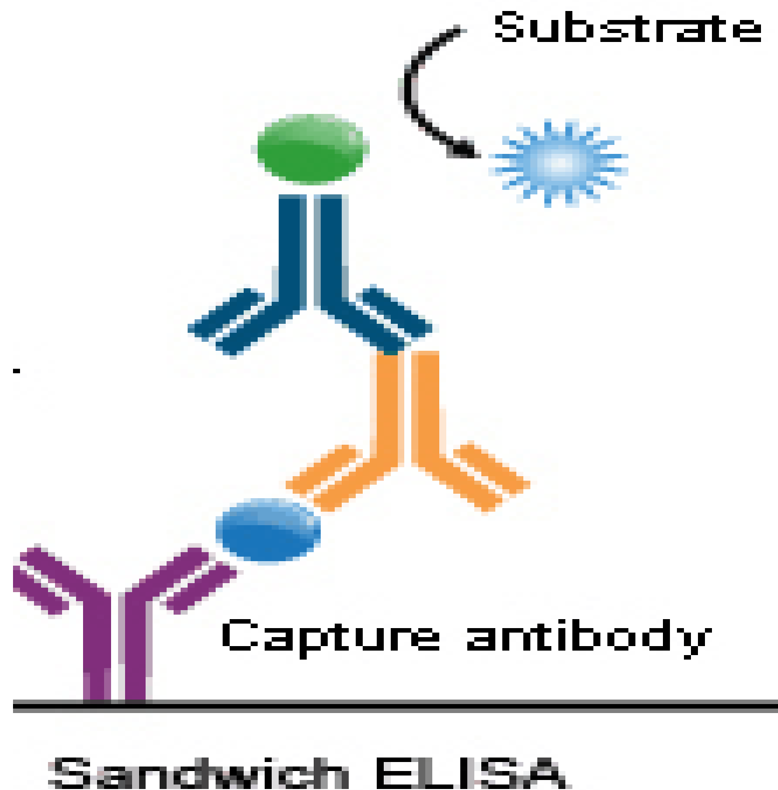


Figure 7:-Sandwich ELISA.

Sandwich ELISAs are particularly suited to the analysis of complex samples, since the antigen does not need to be purified prior to the assay yet still delivers high sensitivity and specificity (e.g. measuring cytokine levels in an immune response).

Advantages

- ✓ High sensitivity - 2-5 times more sensitive than direct or indirect ELISA
- ✓ High specificity - two antibodies are involved in capture and detection ofthe antigen/analyte
- ✓ Flexibility - both direct and indirect detection can be used
- ✓ Suitable for complex (or crude/impure) samples: the antigen does not require purification prior to measurement

Disadvantages

Antibody optimization can be difficult - cross-reactivity may occur between the capture and detection antibodies.

Needs a standardized ELISA kit or tested antibody pair.

1.8.4. Competition/Inhibition ELISA

The competition/inhibition ELISA, also known as a blocking ELISA, is perhaps the most complex of all the ELISA techniques. It is predominantly used to measure the concentration of an antigen or antibody in a sample by detecting interference in an expected signal output. The key event of competitive ELISA (also known as inhibition ELISA) is the process of competitive reaction between the sample antigen and antigen bound to the wells of a microtiter plate with the primary antibody.

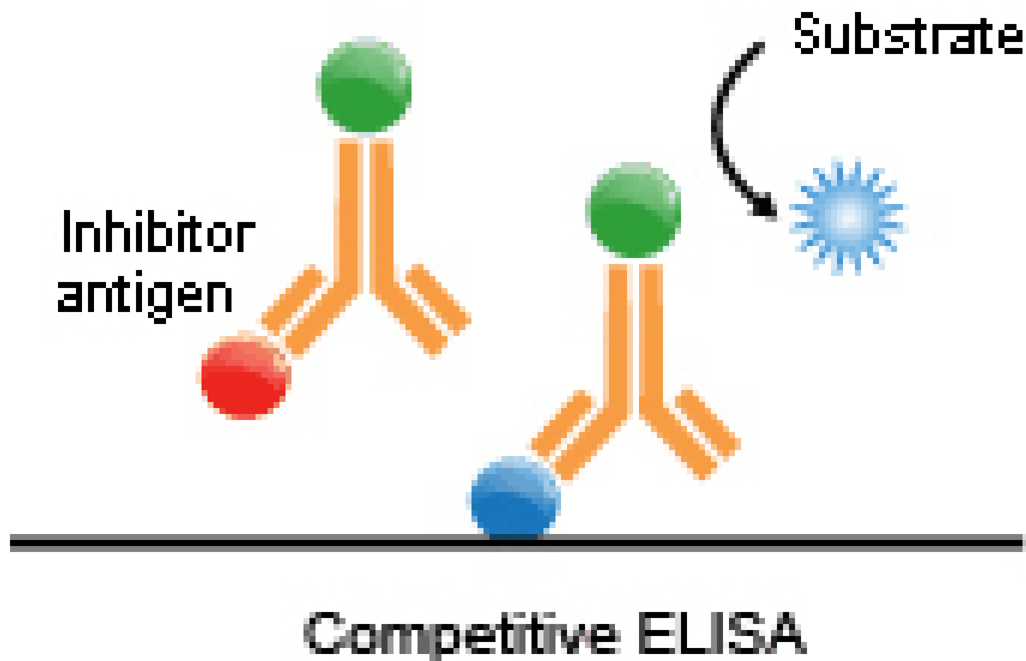


Figure 8:-Competitive ELISA.

First, the primary antibody is incubated with the sample antigen and the resulting antibody–antigen complexes are added to wells that have been coated with the same antigen. After an incubation period, any unbound antibody is washed off. The more antigens in the sample, the more primary antibody will be bound to the sample antigen. Therefore, there will be a smaller amount of primary antibody available to bind to the antigen coated on the well, resulting in a signal reduction indicating that the signal output inversely correlates with the amount of antigen in the sample.
This type of ELISA commonly used when only one antibody is available for the antigen of interest. It is also suitable for detecting small antigens that cannot be bound by two different antibodies such as in the sandwich ELISA technique.

Advantages

- Main advantage - no sample processing is required and crude or impure samples can be used
- More robust - less sensitive to sample dilution and sample matrix effects than the sandwich ELISA
- More consistent - less variability between duplicate samples and assays
- Maximum flexibility - it can be based on direct, indirect or sandwich ELISA

ELISA Data Interpretation

The ELISA assay yields three different types of data output:
1) **Quantitative**: ELISA data can be interpreted in comparison to a standard curve (a serial dilution of a known, purified antigen) in order to precisely calculate the concentrations of antigen in various samples.
2) **Qualitative**: ELISAs can also be used to achieve a yes or no answer indicating whether a particular antigen is present in a sample, as compared to a blank well containing no antigen or an unrelated control antigen.
3) **Semi-Quantitative**: ELISAs can be used to compare the relative levels of antigen in assay samples, since the intensity of signal will vary directly with antigen concentration.

1.9. Western blot analysis

The term, "Western blotting", was applied specifically to the transfer of proteins and their detection by antibodies. Western blotting, also known as immunoblotting, is a well established and widely used technique for the detection and analysis of proteins. The method is based on building an antibody : protein complex via specific binding of antibodies to proteins immobilized on a membrane and detecting the bound antibody with one of several detection methods. The Western blotting method was first described in 1979 and has since become one of

the most commonly used methods in life science research.

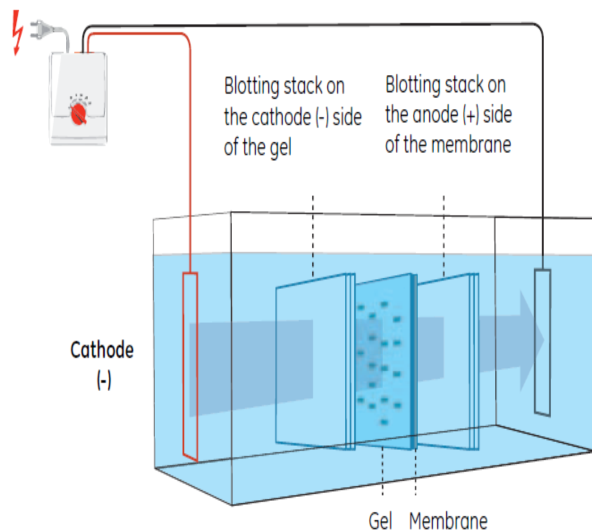
Western blotting is a rapid and sensitive assay for the detection and characterization of proteins. The technique allows one to identify particular proteins by utilizing the specificity inherent in antigen-antibody recognition. This technique is powerful, since it combines electrophoretic separation of proteins, glycoproteins and lipopolysaccharides with immunological identification.

Once such antigens have been detected, they can be further characterized by Western blotting. Initially, a sample is subjected to electrophoresis to separate antigens according to their charge and size, or size alone. A second electrophoretic step transfers the antigens from the gel to an immobilizing surface, such as nitrocellulose paper where they are bound irreversibly. After this transfer, the paper is blocked with blocking agent to prevent nonspecific binding of anti-body and probed with a specific enzyme-conjugated antibody (horseradish peroxidase-anti-immunoglobulin conjugate). A chromogenic substrate is then added to determine which electrophoretic band is bound by the antibody.

The technique is useful for a number of purposes including characterization of unknown antigens or antibody specificities, confirmation of the presence of bacterial antigens in sera or tissues and detection of seropositive individuals which have been exposed to a pathogen. The primary advantage of Western blotting, as opposed to other immunoassays, is the high degree of specificity in resolving distinct antigens. However, there are two disadvantages: first, it is mainly a qualitative assay and quantification of antibody or antigen is difficult; and second, if the antigen sample must be denatured (such as in SDS-PAGE), antigenic activity may be reduced or destroyed.

An initial consideration for successful Western blotting is the empirical determination of the optimal percentage of acrylamide for resolution of the antigens of interest. Next, it must be determined whether the antigens are capable of binding to the nitrocellulose. Finally, the antigen detection procedure must be highly specific and sensitive. The technique can be used to detect as little as 1 ng of a protein antigen that has been previously separated under denaturing conditions by SDS-PAGE, provided that an antibody that recognizes the denatured form of the protein is available.

The entire procedure can be completed in 1 to 2 days, depending on transfer time and type of gel. Western blotting has been useful for characterizing the specificities of polyclonal antisera (rabbit and salmonid) and monoclonal antibodies to extracellular and cell surface antigens.



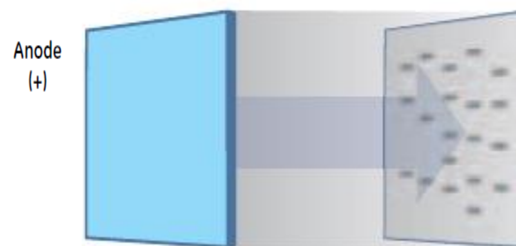


Figure 9:- Western blot protein transfer setup.

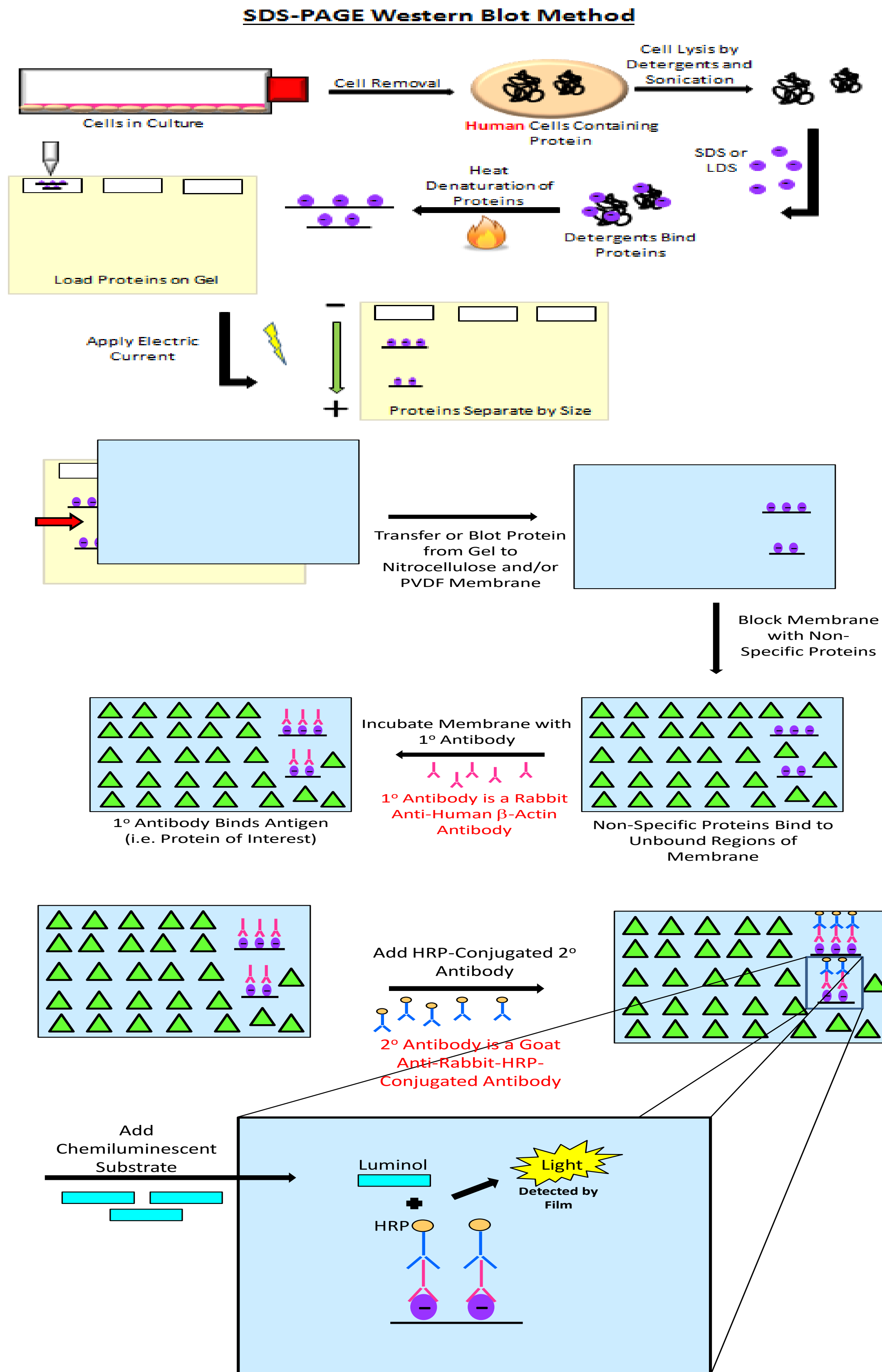


Figure 10:- SDS-PAGE western blot method.

1.10. Immuno-PCR

The development of a reliable, sensitive, specific and rapid diagnostic test is a major challenge for determining effective treatment of infectious diseases. A large variety of methods are available: for the direct detection of the infectious agents, culture and microscopy are widely used; however, these methods are limited in the case of fastidious slow-growing microorganisms.

PCR has become one of the most popular methods for the direct detection of nucleic acids from an infectious agent. The very high sensitivity of PCR, which is capable of detecting a single molecule of DNA, makes it an excellent choice for microbiological diagnostic purposes. The efficacy of PCR is based on its ability to amplify a specific DNA segment flanked by a set of primers. The enormous amplification capability of PCR allows the production of large amounts of specific DNA products, which can be detected by various methods. PCR is widely used as a routine laboratory technique for the detection of nucleic acid molecules, but it cannot detect non-nucleic acid molecules. However in some cases, the protein target is expressed at higher copy numbers than the nucleic acid.

Immunoassays allow a diagnostic method to directly detect the proteins of pathogens and also indirectly detect antibodies produced against microorganisms. The enzyme-linked immunosorbent assay (ELISA) is a commonly used technique to detect antibodies or antigens in samples using the reaction of antibodies to their antigens. ELISA combines the specificity of antibodies with the sensitivity of simple enzyme assays and antibodies are coupled to an easily assayed enzyme. Despite its effectiveness and its specificity, ELISA is unable to detect some antigens when these are present at low concentrations, which seems to use the low level of transcription of its genes as a persistence mechanism in the host. By combining the enormous amplification power of PCR with antibody based immuno- assays, Immuno PCR (I-PCR) allows the detection of proteins at a level of a few hundred molecules.

Immuno-PCR (I-PCR) was first introduced by Sano *et al*. in 1992 and combines the amplification power of PCR with the versatility of ELISA resulting in improved conventional antigen detection sensitivity. The first I-PCR was developed by using a detection antibody coupled to a biotinylated DNA molecule through a streptavidin–protein A chimaeric molecule and could detect as few as 580 BSA antigen molecules.

In immuno-PCR, a linker molecule with bispecific binding affinity for DNA and antibodies is used to attach a DNA molecule (marker) specifically to an antigen-antibody complex, resulting in the formation of a specific antigen-antibody- DNA conjugate. The attached marker DNA can be amplified by PCR with the appropriate primers. The presence of specific PCR products demonstrates that marker DNA molecules are attached specifically to antigen-antibody complexes, which indicates the presence of antigen. A streptavidin-protein A chimera was used as the linker. The chimera has two independent specific binding abilities; one is to biotin, derived from the streptavidin moiety, and the other is to the Fc portion of an immunoglobulin G (IgG) molecule, de-rived from the protein A moiety. This bifunctional specificity both for biotin and antibody allows the specific conjugation of any biotinylated DNA molecule to anti-gen-antibody complexes.

The detection procedure used in I-PCR is similar to conventional enzyme-linked immunosorbent assay (ELISA). Instead of an enzyme-conjugated secondary antibody directed against the primary antibody, as in typical ELISA, a biotinylated linear plasmid DNA (pUC19) conjugated to the streptavidin-protein A chimera was targeted to the antigen-antibody complexes. A segment of the attached marker DNA was amplified by PCR with appropriate primers, and the resulting PCR products were analyzed by agarose gel electrophoresis after staining with ethidium bromide.

Several commonly used formats of universal I-PCR include direct I-PCR, indirect I-PCR, sandwich I-PCR and indirect sandwich I-PCR.

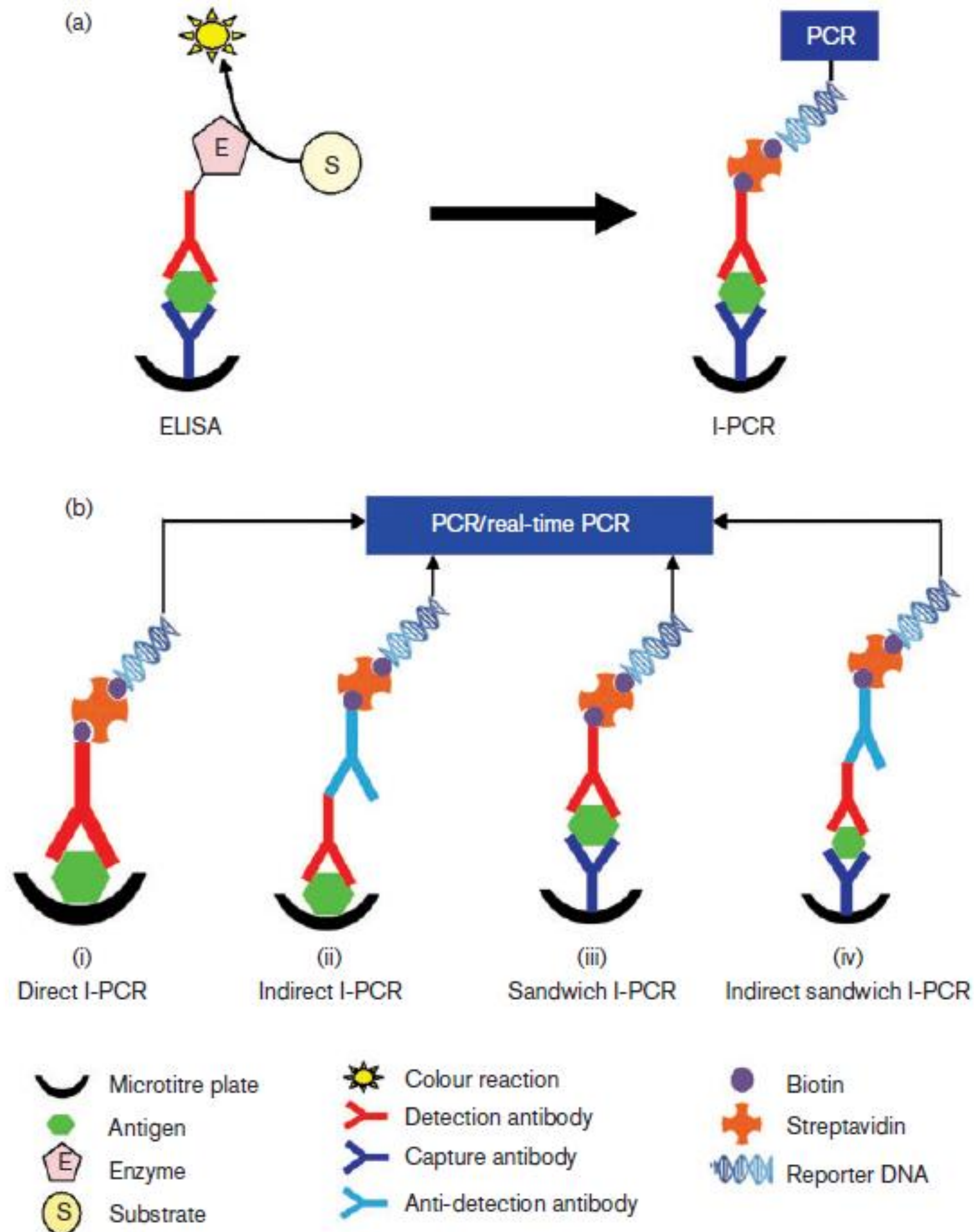


Figure 11:- Evolution of I-PCR and different formats of universal I-PCR.

(a) Evolution of I-PCR. The general set-up of I-PCR is almost similar to that of antigen detection with ELISA, but in IPCR, a conjugate comprising an antibody and a reporter DNA is amplified by PCR for signal generation. (b) Different formats of universal I-PCR. Streptavidin acts as a linker molecule between the biotinylated detection antibody and the biotinylated reporter DNA; the biotinylated reporter DNA is amplified by PCR/real-time PCR.

(i) Direct I-PCR: the antigen is captured on the wells of a microtitre plate and detected directly by biotinylated antibody, which is attached to biotinylated DNA through streptavidin.

(ii) Indirect I-PCR: a biotinylated antibody against the detection antibody (anti-detection antibody) is attached to the detection antibody, and is further attached to biotinylated DNA through streptavidin.

(iii) Sandwich I-PCR: the antigen is sandwiched between a capture antibody and the detection antibody, which is further attached to biotinylated DNA through streptavidin.

(iv) Indirect sandwich I-PCR: the antigen is sandwiched between a capture antibody and the detection antibody, and an antidetection biotinylated antibody is attached to the detection antibody, which is further attached to biotinylated DNA through streptavidin.

However, the limited availability of streptavidin–protein A fusion protein, and the widely varied affinities of protein A with antibodies of various classes and subclasses from different species restricted its immediate applications. Moreover, production of the fusion proteins was a laborious and time-consuming process. Then subsequent studies have developed the immuno-PCR methodology further by improving the reagents and assay formats to make them suitable for clinical applications.

By substituting the fusion protein with avidin-biotinylated and streptavidin-biotinylated DNA complexes, the immuno-PCR system becomes commercially available. Despite the strong binding of avidin or streptavidin with biotin the tetrameric structure of both avidin and streptavidin leads to the formation of heterogeneous DNA-antibody conjugates, which likely affects the batch-to-batch reproducibility of immuno-PCR.

With the development of crosslinking techniques, the direct conjugation of detection antibody with the reporter DNA through covalent binding with a chemical compound such as succinimidyl 4-[N-maleimidomethyl]- cyclohexane-1-carboxylate (SMCC) has become easier and reduced procedural complexity since the DNA-antibody conjugates can be prepared before beginning the assay.

Another important strategy is phage displaymediated I-PCR (PD-I-PCR). Instead of using mAb and streptavidin–biotin/chemically linked DNA in the conventional I-PCR, a recombinant phage particle is used as a ‘ready reagent’ for I-PCR. The surface displayed single chain variable fragment (scFv) and phage DNA themselves can serve directly as detection antibody and PCR template, respectively. The target antigen is captured by the immobilized capture antibody coated on a microtitre plate, whilst the recombinant phage particles are anchored through the interaction between the displayed scFv and bound target antigen. The phage DNA is released by heat lysis and serves as PCR template for amplification.

In recent years, nanobiotechnology has emerged as the most promising tool for the development of a powerful strategy for drug delivery diagnostics. Besides circumventing the background noise, the use of magnetic beads and nanoparticle-based I-PCR can further improve detection limits and reduce washing steps, as well as reducing incubation steps, thus improving the assay. In the magnetic bead-based I-PCR assay, the capture antibody is adsorbed to the magnetic beads to capture the antigen and the sandwich I-PCR format is then followed using streptavidin as a bridge between the detection antibody and the reporter DNA.

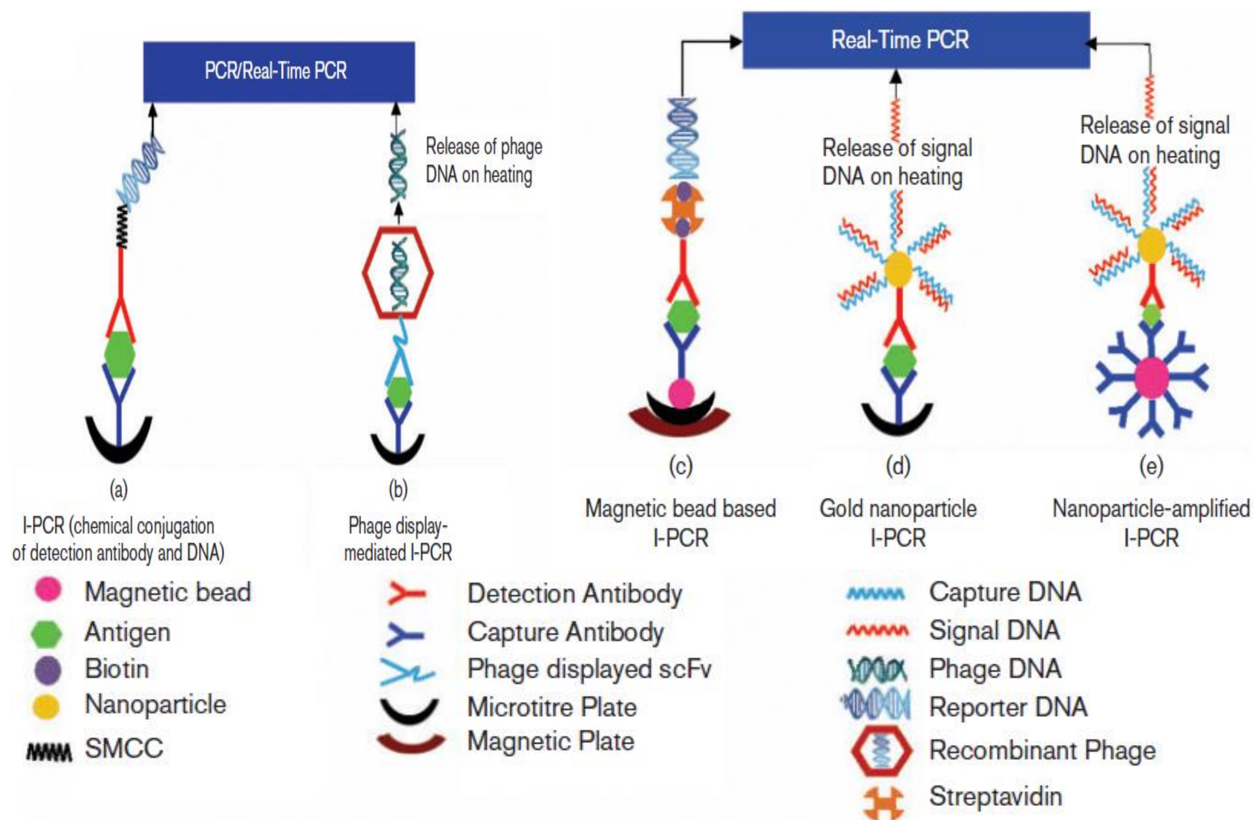


Figure 12:- Modification of the I-PCR assay.

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