Immunochemical techniques III

Objectives

Our main objective is to understand the underlying principles of complement fixation test and the western blotting techniques, which are used commonly in the various field of molecular biology, clinical applications etc.

Introduction

All vertebrates have advanced immune system of their body. The more complex the organism the more advanced the immune system. The immune system of mammals has evolved over a million challenges that arise in the body. Immunity in our body is monitored or controlled by specific cells from stem cells in bone marrow. The most important cell types are B and T lymphocytes, which have the ability to act against bacterial and other viruses. B-cells release antibodies against stimulation of a foreign substance into the body. An antigen is a foreign substance capable of an immune response leading to the production of antibodies. They are the targets to which antibodies bind. So antibodies are antigen specific that is it binds to the antigen that initiated its production. This chapter focuses on the detection and analysis of the specific antibody or antigen present in the sample using complement fixation test and western blotting technique.

1. Complement Fixation Test (CFT)

Principle

Complement is a kind of protein present in normal body serum that can be activated directly by pathogens or indirectly by pathogen-bound antibody, leading to a cascade of reactions that occurs on the surface of pathogens and generates active with components with various effective function. This whole cascade pathway is term as complement system and it is made up of nine protein components, C1 to C9. Complement binds to Antigen-Antibody (complex. When antigen forms a complex with antibody on surface of a cell, complement causes lysis of cell.

CFT is a technique that has been used over many years to detect and quantify antibody that serology does not agglutinate or precipitate when reacted with its antigen, but can be demonstrated by its use, or fixation, of complement. Antigen-Antibody reactions lead to immune complex formation that produces complement fixation via the classical pathway. That is when complement takes part in antigen-antibody reaction; it is bound or fixed to the antigen-antibody complex. When this complex is on bacteria, red cells or other cells, the complement brings about the lysis of cells involved. This can be exploited to determine the amount of antigen-antibody present in the patient sample. CFT can detect antibody at a level of less than one microgram per millilitre.

Complement fixation test require two steps

Step 1

A known antigen and inactivated patient's serum are incubated with a standardized, limited amount of complement. If the serum contains specific, complement activating antibody the complement will be activated or fixed by the antigen-antibody complex. However, if there is no antibody in the patient's serum, there will be no formation of antigen-antibody complex, and therefore complement will not be fixed but will remain free.

Step 2

The second step detects whether complement has been utilized in the first step or not. This is done by adding the indicator system. If the complement is fixed in the first step owing to the presence of antibody there will be no complement left to fix to the indicator system. However, if there is antibody in the patient's serum, there will be no antigen-antibody complex, and therefore, complement will be present free or unfixed in the mixture. This unfixed complement will now react with the antibody- coated sheep red blood cells to bring about their lysis. Thus, if no lysis of sheep red blood cells takes place then it is said to be positive CFT which indicates the presence of antibody in the test serum, while lysis of sheep red blood cells will give Negative CFT which indicates the absence of antibody in the serum.

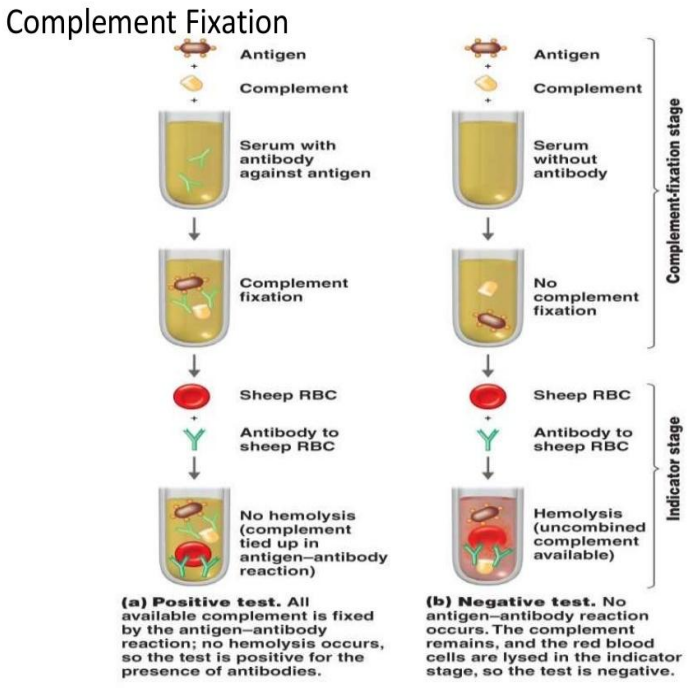

Figure: Complement fixation Test

Advantages of CFT

1. Ability to screen against a large number of viral and bacterial infections at the same time.
2. It is not very expensive.

Disadvantages of CFT

1. Not sensitive - cannot be used for immunity screening
2. Time consuming and labor intensive
3. Often non-specific e.g. cross-reactivity between Herpes simplex virus and Varicella zoster virus

Modifications of complement fixation test

(a) **Indirect complement fixation test:**

This modification is used when serums which don't fix guinea pig complement is to be tested. Here, the test is set up in duplicate. After step 1, standard antiserum to antigen which is known to fix complement is

added to one set. If antibodies were not present in the test serum then the antigen would react with the standard antiserum fixing the complement. On the other hand if antibodies are present in the test serum the antigen would be utilized in the first step. So, no reaction would occur between the standard antiserum and the antigen and therefore no fixation of complement would cause lysis of sheep red blood cells. Thus, in this case haemolysis indicates a positive result.

(b) **Conglutinating complement absorption test**:

Here horse complement which is non-haemolytic is used. The indicator system used is sensitized sheep red blood cells mixed with bovine serum. Bovine serum contains a beta globulin called conglutinin would also combine with this complement causing agglutination of the sheep red blood cells, indicating a negative result.

(c) **Immune adherence**:

When some bacteria such as Vibrio cholera or Treponema pallidum combine with their specific antibody in the presence of complement and some particles such as erythrocytes or platelets, they adhere to the erythrocytes or platelets. This is called immune adherence.

(d) **Immobilisation test:**

Here antigen is incubated with patient's serum in presence of complement. If specific antibody is present it would immobilize the antigen. Eg. Treponema palladium immobilization test, considered gold standard for the serodiagnosis of syphilis.

(e) **Cytolytic tests:**

The incubation of a live bacterium with its specific antibody in the presence of complement leads to the lysis of the bacteria cells. This is the basis of vibriocidal antibody test used to measure anticholera antibodies.

1. Western blotting

Western blotting is also known as immunoblotting, it 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.

Working principle

In Western blotting, a protein mixture is electrophoretically separated on an SDS-polyacrylamide gel, a slab gel infused with sodiumdodecyl sulphate. Then protein bands are transferred to a nylon membrane by electrophoresis and the individual protein bands are identified by flooding the nitrocellulose membrane with radiolabel or enzyme-linked polyclonal or monoclonal antibody specific for the protein of interest.

Steps involved in the Western blotting

Although the details of Western blotting protocols may vary from application to application, with adaptations to suit specific protein characteristics and the level of information required, they all follow some common basic steps.

1. **Sample preparation:**

All sources of protein, from single cells to whole tissues as well as extracellular matrices , biological fluids and proteins secreted in vitro, are open to analysis by Western blotting. One should follow a popular and particular extraction method which is suitable with the source, at the same time, obtaining a sufficient yield of material at an acceptable level of purity is very important.

Table 2.1. Overview of extraction options for different cells and tissues

Sample	Typical lysis options
Tissue culture	Detergent lysis
Cell suspensions	Ultrasonication
Most plant and animal tissues	Mechanical homogenization (e.g. Waring** blender or Polytron**)
Soft animal tissues and cells	Dounce (manual) and/or Potter-Elvehjem (mechanical) homogenization
Bacterial and mammalian cells	Freeze/thaw lysis
Bacteria, erythrocytes, cultured cells	Osmotic shock lysis
Solid tissues and plant cells	Manual grinding with mortar and pestle
Cell suspensions, yeast cells	Grinding with abrasive component (e.g. sand, glass beads, alumina)
Bacteria, yeast, plant tissues, fungal cells	Enzymatic digestion
Bacteria, yeast, plant cells	Explosive decompression (nitrogen cavitation)
Microorganisms with cell walls	French press
Plant tissues, fungal cells	Glass bead milling

Figure: overview of extraction options for different cells and tissues

2. Immunoelectrophoresis

Electrophoresis is a commonly used method for separating proteins on the basis of size, shape and/or charge. Polyacrylamide is gels are the most commonly used matrices in research laboratories for separation of proteins and nucleic acids, respectively.

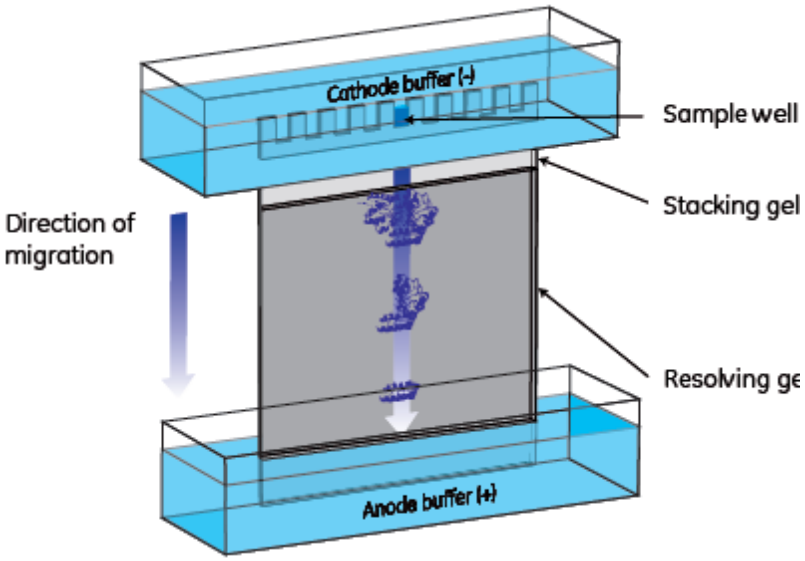

Figure: SDS-polyacrylamide gel electrophoresis

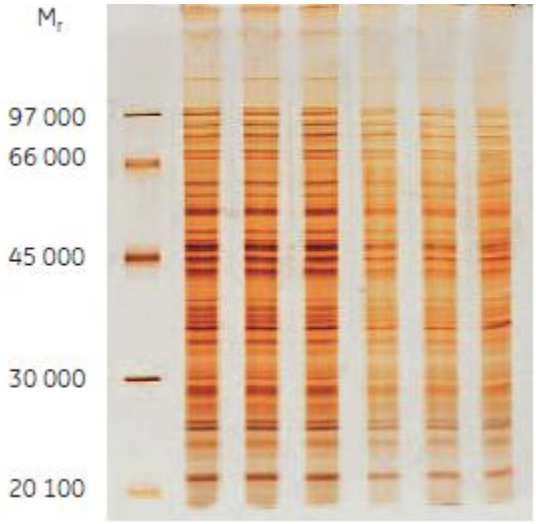

Figure: Protein bands seen after SDS-polyacrylamide gel electrophoresis

3. Transfer of protein bands

On completion of the separation of proteins, the next step is to transfer the proteins from the gel to a solid support membrane, usually made of a chemically inert substance, such as nitrocellulose or polyvinylidene difluoride (PVDF). Blotting makes it possible to detect the proteins on the membrane using specific antibodies. The proteins transferred from the gels are immobilized at their respective relative migration positions at the time point when the electric current of the gel run was stopped. It is important that transfer is as uniform as possible across the entire area of the gel:membrane sandwich in order to ensure that large and small proteins are transferred with similar efficiency.

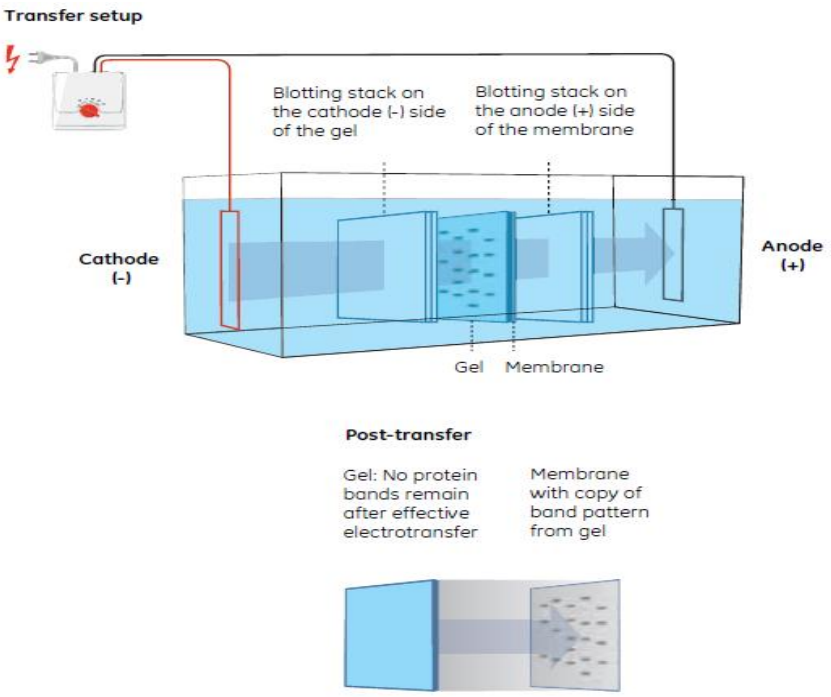

Figure: Transfer of protein bands from SDS-PAGE to immobile nitrocellulose membrane

Blocking:

Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein – typically bovine serum albumin (BSA) or non-fat dry milk in tris-buffered saline with a minute percentage of detergent such as Tween 20 or Triton X-100. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces background in the final product of the western blot, leading to clearer results, and eliminates false positives.

Primary Antibody

After blocking, a dilute solution of primary antibody which is generated when a host species or immune cell culture is exposed to the protein of interest is incubated with the membrane under gentle agitation. Typically, the solution is composed of buffered saline solution with a small percentage of detergent. The antibody solution and the membrane can be sealed and incubated together for anywhere from 30 minutes to overnight.

Secondary Antibody

The unbound primary antibodies are removed by rinsing and the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. Antibodies come from animal sources (or animal sourced hybridoma cultures. The secondary antibody is usually linked to radioactive labelled probe, fluorescent labelled or a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. Enzyme linked secondary is used to cleave a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein.

Detection:

A variety of detection systems, based on chemiluminescence, fluorescence, chromogenic or radioisotopic detection are available.

- Chemiluminescence detection

  Chemiluminiscence detection methods depend on incubation of the western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by CCD cameras which capture a digital image of the western blot or photographic film. The use of film for western blot detection is slowly disappearing because of non linearity of the image (non accurate quantification). The image is analysed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density. Newer software allows further data analysis such as molecular weight analysis if appropriate standards are used.

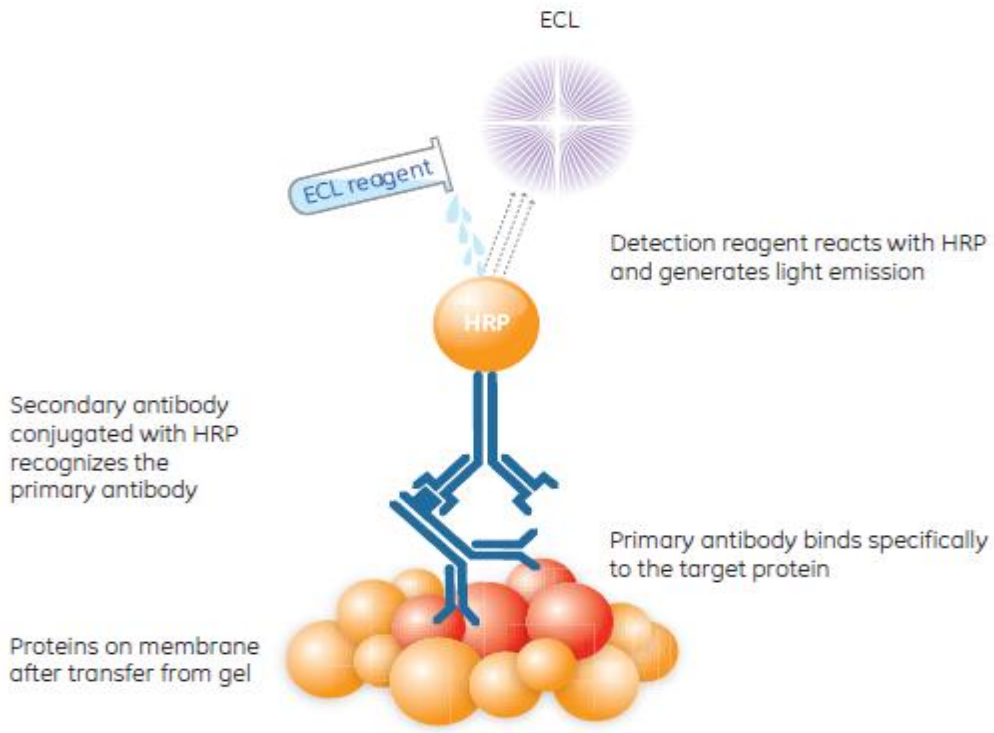

Figure: The principle of chemiluminescence detection in Western blotting

- Radioactive detection

Radioactive labels allow the placement of medical X-ray film directly against the western blot, which develops as it is exposed to the label and creates dark regions which correspond to the protein bands of interest. The importance of radioactive detections methods is declining due to its hazardous radiation because it is very expensive, health and safety risks are high, and ECL (enhanced chemiluminescence) provides a useful alternative.

- Fluorescent detection

The fluorescently labeled probe is excited by light and the emission of the excitation is then detected by a photosensor such as a CCD camera equipped with appropriate emission filters which captures a digital image of the western blot and allows further data analysis such as molecular weight analysis and a quantitative western blot analysis. Fluorescence is considered to be one of the best methods for quantification but is less sensitive than chemiluminescence.

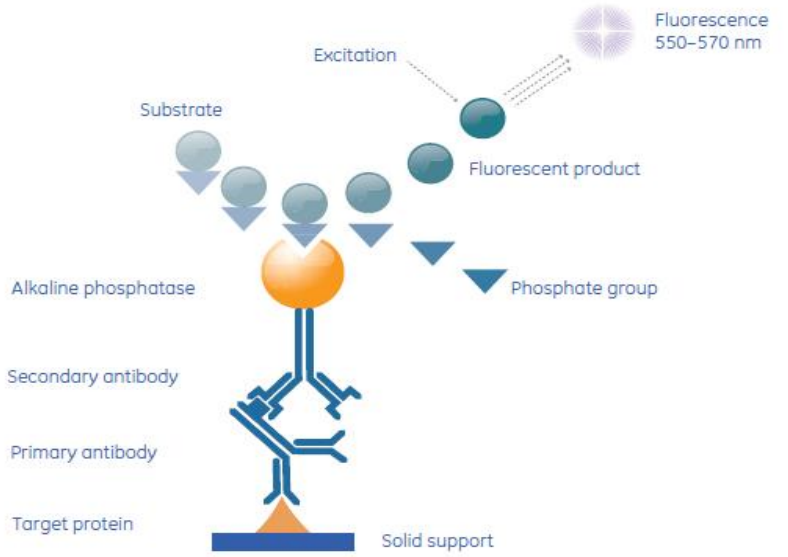

Figure: Proteins are detected by fluorescence detection method

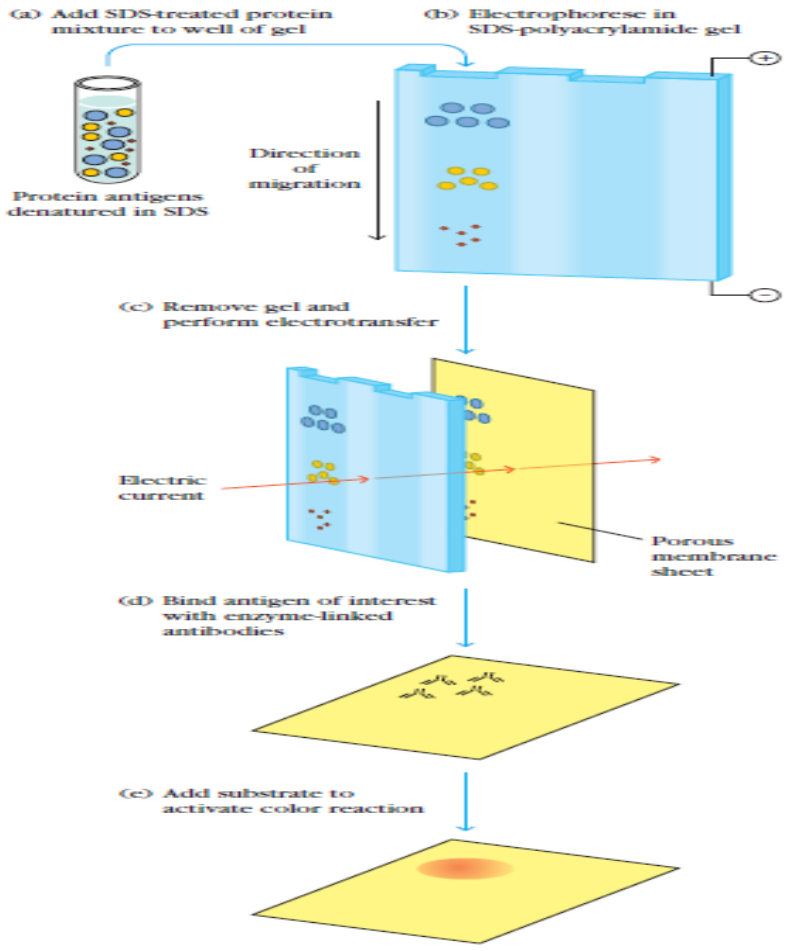

Figure: Overall process of western blotting

Conclusion

In this topic, we have learnt the princples of complement fixation test and western blotting. These two methods are widely used in the fields of molecular biology, biochemistry, immunogenetics and other biology disciplines. They played a central role, from confirmation of the presence of target proteins. Western blotting is the most widely used procedure in confirmatory testing for HIV, where it is used to determine whether the patient has antibodies that react with one or more viral proteins.

Summary

The complement fixation test is an immunological medical test that can be used to detect the presence of either specific antibody or specific antigen in a patient's serum, based on whether complement fixation occurs or not. Certain modification of complement fixation test is also being done like Indirect complement fixation test, Immune adherence, Conglutinating complement absorption test, Immobilisation test etc. On other, western blotting used to identify proteins from samples after electrophoresis. The sample may be tissue homogenate in origin or an extract of cells or other biological source. It relies upon three key elements to accomplish this task: the separation of protein mixtures by size using gel electrophoresis; the efficient transfer of separated proteins to a solid support; and the specific detection of a target protein by appropriately matched antibodies. Once detected, the target protein will be visualized as a band on a blotting membrane, X-ray film, or an imaging system. Since Western blotting is accomplished rapidly, using simple equipment and inexpensive reagents, it is one of the most common laboratory techniques.

Glossary

1. **Antibody:** It is an immunoglobulin protein molecules synthesized on exposure to antigen and form a complex with it.
2. **Antigen:** A foreign substance that can induce an immune response.
3. **Complement proteins:** Complement is a kind of protein present in normal body serum that can be activated directly by pathogens or indirectly by pathogen-bound antibody.

4. **Complement fixation test:** A test for infection with a microorganism which involves measuring the amount of complement available in serum to bind with an antibody–antigen complex.
5. **Chemiluminesence**: Chemiluminescence is the production of light from a chemical reaction. Two chemicals react to form an excited intermediate, which breaks down releasing some of its energy as photons of light to reach its ground state.
6. **Immunoelectrophoresis:** A procedure involving sequential electrophoresis, used to identify the proteins in a sample.
7. **Nitrocellulose membrane**: It is a matrix used in protein blotting because of their high protein-binding affinity, compatibility with a variety of detection methods (chemiluminescence, chromogenic, and fluorescence), and the ability to immobilize proteins, glycoproteins, or nucleic acids.
8. **Primary Antibody:** A primary antibody is an immunoglobulin that specifically binds to a particular protein or other biomolecule of research interest for the purpose of purifying or detecting and measuring it.
9. **Secondary Antibody**: Antibody which generally conjugated with enzyme and binds to the primary antibody to assist in detection, sorting and purification of target antigens in western blotting techniques.
10. **Western blotting**: It is an analytical technique used in molecular biology, immunogenetics and other molecular biology disciplines to detect specific proteins in a sample of tissue homogenate or extract.

FAQS:

Q1. What do you understand by the term complement?

Answer: A group of serum proteins which can be activated (fixed) by antigen,antibody complexes or other substances, which may result in lysis of a microbial target, or a variety of other biological effects important in both innate and adaptive immunity.

Q2. Give the advantages and the limitations of CFT.

Answer: The advantages of CFT are having ability to screen against a large number of viral and bacterial infections at the same time and it is not very expensive. And the disadvantages of CFT are less sensitivity means it cannot be used for immunity screening, it is also time consuming and labor intensive. It is

often non-specific e.g. cross-reactivity between *Herpes simplex* virus and *Varicella zoster* virus.

Q3. Mention certain modifications of complement fixation test?

Answer: Indirect complement fixation test, Conglutinating complement absorption test, Immune adherence, Cytolytic tests, Immobilisation test are some of the modifications being done.

Q4.What is western blotting?

Answer: The western blot (sometimes called the protein immunoblot) is a widely used analytical technique used in molecular biology, immunogenetics and other molecular biology disciplines to detect specific proteins in a sample of tissue homogenate or extract.

Q5. Describe the steps involve in Complementary Fixation Test?

Answer: It required two main stages, the complement fixation stage and the indicator stage. A known antigen and inactivated patient's serum are incubated with a standardized, limited amount of complement in the first stage followed by addition of an indicator, antibody- coated sheep red blood cells. If no lysis of sheep red blood cells takes place then it is said to be positive while lysis of sheep red blood cells will give negative CFT.

Q6. What are the basic steps in immunoblotting?

Answer: Sample preparation, separation of protein by electrophoresis, Transfer of proteins band on immobile substance, blocking specific binding, introduction of primary antibody, secondary antibody binding step and detection using various detecting method are the basic steps in immunoblotting.

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4. Lesley-Jane Eles , immunology for Life Scientists(2nd edition), John Wiley & Sons Ltd, UK,2003

Links

1. https://en.wikipedia.org/wiki/Complement_fixation_test
2. https://en.wikipedia.org/wiki/Western_blot
3. http://www.spacesrl.com/wpcontent/uploads/2011/03/WesternBlottingBrochure.pdf
4. http://jeeves.mmg.uci.edu/immunology/CoreNotes/Chap05.pdf