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DEPARTMENT OF BIOTECHNOLOGY
FACULTY OFENGINEERING & TECHNOLOGY

LT 11. serological techniques – ELISA, RIA and western blotting.

Content Outline

1. RIA
2. Types of RIA
3. ELISA
4. Types of ELISA
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Radioimmunoassay (RIA)

•This technique was developed by Rosalyn S. Yalow and Solomon A. Berson in the 1950. This technique is a tracer procedure (using radioactive material, normally C-12 isotopes e.g C-14) used to measure trace amount of any substance having antigenic properties. The target antigen is labeled radioactively and bound to its specific antibodies (a limited and known amount of the specific antibody has to be added). A sample, for example a blood-serum, is then added in order to initiate a competitive reaction of the labeled antigens from the preparation, and the unlabeled antigens from the serum-sample, with the specific antibodies. The competition for the antibodies will release a certain amount of labeled antigen. This amount is proportional to the ratio of labeled to unlabeled antigen. A binding curve can then be generated which allows the amount of antigen in the patient's serum to be derived.

•That means that as the concentration of unlabeled antigen is increased, more of it binds to the antibody, displacing the labeled variant. The bound antigens are then separated from the unbound ones, and the radioactivity of the free antigens remaining in the supernatant is measured. A binding curve can be generated using a known standard, which allows the amount of antigens in the patient's serum to be derived.

Separation techniques:

Double antibody, charcoal, cellulose, chromatography or solid phase techniques are applied to separate bound and free radio-labeled antigen. Most frequently used is the double antibody technique combined with polyethylene. The bound or free fraction is counted in a gamma counter. Concomitantly, a calibration or standard curve is generated with samples of known concentrations of the unlabeled standards. The amount of antigen in unknown samples can be calculated from this curve.

Sensitivity:

The sensitivity can be improved by decreasing the amount of radioactively-labeled antigen and/or antibody. The sensitivity can also be improved by the so-called disequilibrium incubation. In this case radioactively labeled antigen is added after initial incubation of antigen and antibody.

Radioactive material used for labeling

125-I labels are usually applied although other isotopes such as C14 and H3 have also been used. Usually, high specific activity radio-labeled (125-I) antigen is prepared by iodination of the pure antigen on its tyrosine residue(s) by chloramine-T or peroxidase methods and then separating the radio-labeled antigen from free-isotope by gel-filtration or HPLC. Other important components of RIA are the specific antibody against the antigen and pure antigen for use as the standard or calibrator.

Advantage of RIA

RIA a very sensitive technique and can be used to measure very minute concentration of antigen. It can measure one trillionth (10^{-12}) of a gram of material per milliter of blood.

It is structurally specific as antigen: antibody reactions are highly specific.

Disadavantages

Use of radioactive substance requires extra care during handling as it risks the exposure to harmful radioactive rays to handler.

It is an indirect method of analysis.

It is a saturation analysis as active reagent added in smaller quantity than that of analyte. Therefore, such high dilution requires prolonged reaction times (in days).

Lengthy counting time.

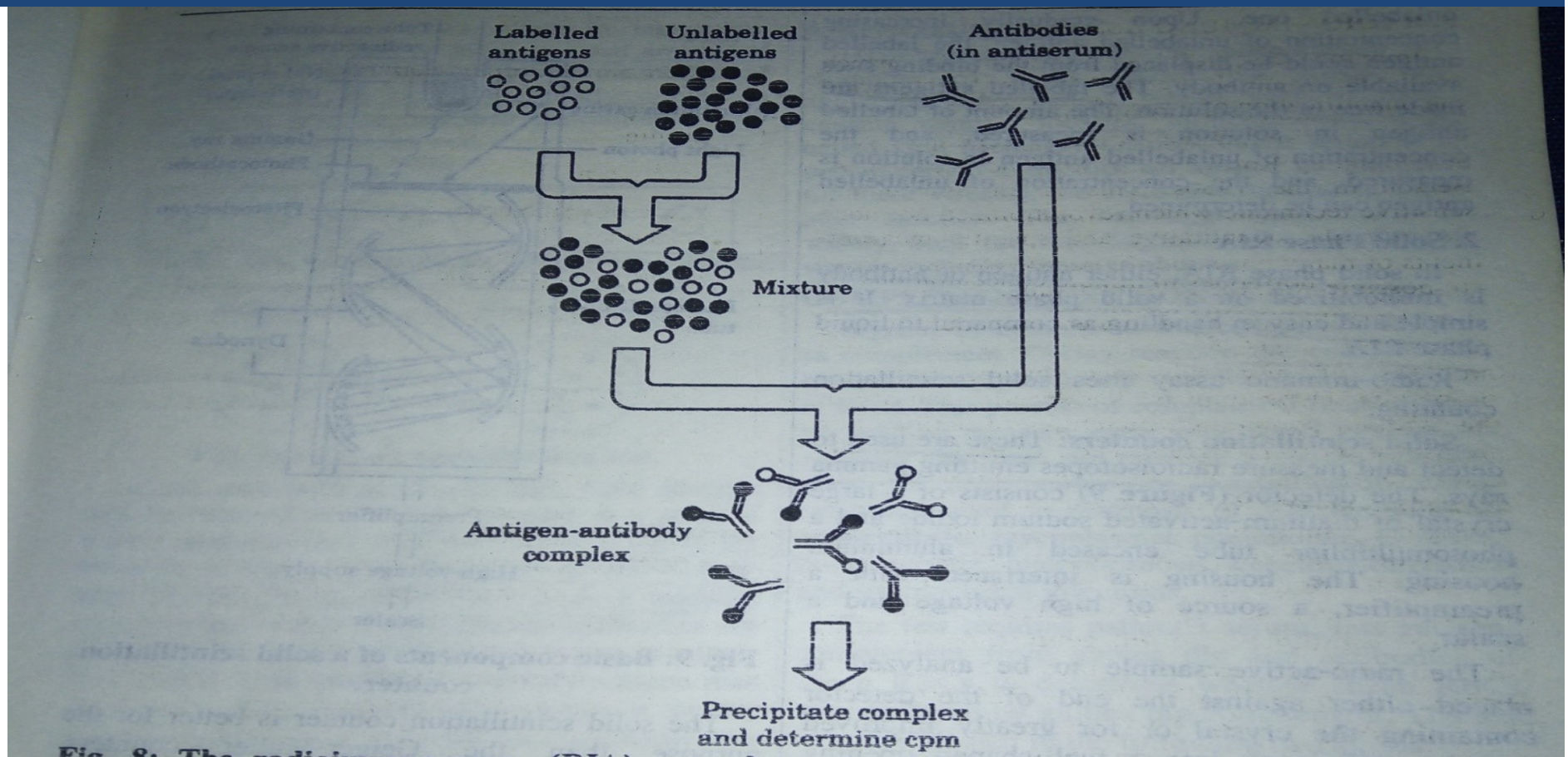


Fig. 8: The radioimmunoassay (RIA) procedure. A mixture containing a known quantity of radioactively labelled antigens and an unknown quantity of unlabelled antigens is reacted with a limiting amount of antibody and the resulting antigen-antibody complexes are then isolated. The quantity of unlabelled antigen can be determined from the radioisotope content of the isolated complexes. The technique is highly sensitive.

RIA procedure: Estimation of antigen

Variants of RIA

Liquid phase RIA

The liquid phase RIA is based on competitive binding of radiolabelled antigen and unlabelled antigen to a high affinity antibody. The antigen labeled with I^{125} is mixed with such a concentration of antibody that can just saturate the antibody. Therefore, the increasing amount of antigen (unlabelled) of unknown concentration is added. The two types of antigens now compete for available sites of the antibody. The antibody does not differentiate the labeled antigen from the unlabeled one. Upon gradually increasing concentration of unlabelled antigen, the labeled antigen could be displaced from the binding sites available on antibody. The labeled antigens are made free in the solution. The amount of labeled antigen in solution is measured, and the concentration of unlabeled antigen in solution is measured, and the concentration of unlabeled antigen can be determined.

Solid phase RIA

In solid pahse RIA, either antigen or antibody is immobilized on a solid phase matrix. It is simple and easy in handling as compared to liquid phase RIA. This technique uses solid scintillation counting.

The radioactive sample to be analyzed is placed either against the end of the detector containing the crystal of thallium –activated sodium iodide and a photomultiplier tube encased in aluminium housing. The gamma rays emitted from radioactive sample excites the electron of crystals to higher energy labels, the return of these excited energy to lower energy labels emits flashes of light or scintillation. These scintillations are proportional to the number and energy of gamma rays exciting the crystals. **Visit following link for advance reading**

- https://gpatindia.com/radioimmunoassay-riaprinciple-procedure-application-and-mcq-for-gpat-net-jrf-and-gate-exam/

ELISA

ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying peptides, proteins, antibodies and hormones. 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. The enzyme conjugated to the antibodies, typically alkaline phosphatase (AP) or horseradish peroxidase (HRP), acts as an amplifier of detection signal by converting a substrate that results in color changes in the wells.

Generalized ELISA protocol

A generalized indirect ELISA protocol has the following steps.

1. Bind the sample being tested for the presence of a specific molecule or organism to a solid support, such as a plastic microtiter plate, which usually contains 96 sample wells. Wash the support to remove unbound molecules.

2. Add a marker-specific antibody (primary antibody directed against the target antigen) to the bound material, and then wash the support to remove unbound primary antibody.

3. Add a second antibody (secondary antibody) that binds specifically to the primary antibody and not to the target molecule. Bound (conjugated) to the secondary antibody is an enzyme, such as alkaline phosphatase, peroxidase, or urease that can catalyze a reaction that converts a colorless substrate into a colored product. Wash the mixture to remove any unbound secondary antibody–enzyme conjugate.

4. Add the colorless substrate.

5. Observe or measure the amount of colored product.

There are 4 types of ELISA. Each differs by the method of antigen capture and/or signal detection

i. Direct ELISA

ii. Indirect ELISA

iii. Sandwich ELISA

iv. Competitive ELISA

1. Direct ELISA

The target antigen is first coated onto the multi-well plate, and then detected by an enzyme-linked 1' antibody.

Advantages:

Simple and quick to perform due to minimal steps required

Disadvantages:

Specificity of the primary antibody may be affected by the enzyme-linking

Linking 1' antibody for each specific ELISA experiment is expensive and time-consuming

Minimal signal amplification

2. Indirect ELISA

The target antigen, coated onto the multi-well plate, is first bound by a unconjugated 1' antibody, which in turn is detected by a enzyme-linked 2' antibody.

Advantages:

Specificity of the 1' antibody is retained

Many enzyme-linked 2' antibodies are commercially available

Better signal amplification since multiple polyclonal 2' antibodies can bind to each 1' antibody

Disadvantages:

Potential cross reactivity with 2' antibody leading to non-specific signal

3. Sandwich ELISA

Sandwich ELISA is often performed using commercially available kits since an antibody pair that targets two distinct epitopes on the target antigen is required.

The first antibody, or the capture antibody, is coated to the multi-well plate. The sample containing target antigen is added, followed by the detection of the antigen by the detection antibody that is enzyme-linked. Hence, the target antigen is "sandwiched" between the capture and detection antibodies.

Either direct or indirect detection can be used in a sandwich ELISA.

Advantages:

High specificity since the signal detection requires the binding of two 1' antibodies

Crude sample can be used: target antigen-antibody complex is immobilized to the plate, and everything else can be washed off

Disadvantages:

Commercially-prepared kits may not be available

4. Competitive ELISA

Competitive ELISA is based on the competition binding for the 1' antibody between the target antigen in a sample and the same antigen that is coated to the multi-well plate.

The 1' antibody is first added to the sample to form antigen-antibody complexes. The sample is then added to the wells that are coated with the target antigen. Only the unbound 1' antibody in the sample can bind to antigen coated in the wells. Hence, the more antigen in the sample, the less antibody is available to bind to antigen in the wells, resulting in a signal reduction.

Either direct or indirect detection can be used in a competitive ELISA.

Advantages:

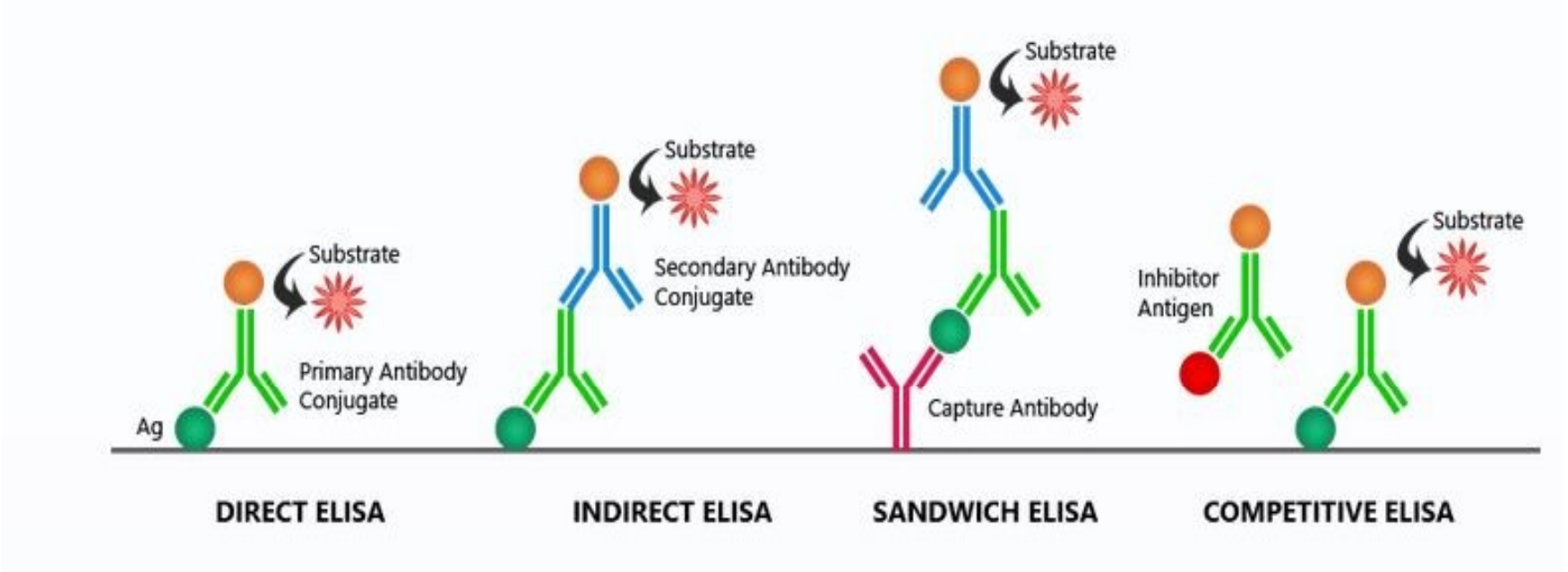
High sensitivity

Crude sample can be used

Signal can be quantified by comparing to a serial dilution standard curve

Disadvantages:

Requires 1' antibodies with high specificity to the antigen



Differences between different types of ELISAs

Western Blottinng

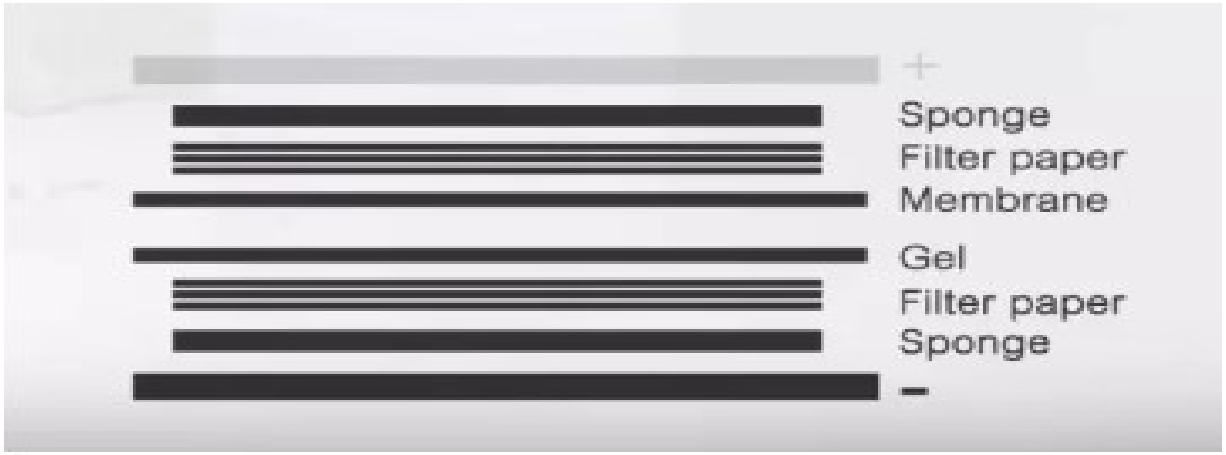
•A western blot is a laboratory method used to detect specific protein molecules from among a mixture of proteins. This mixture can include all of the proteins associated with a particular tissue or cell type. Western blots can also be used to evaluate the size of a protein of interest, and to measure the amount of protein expression. This procedure was named for its similarity to the previously invented method known as the Southern blot.

•Western blotting, also called Protein Immunoblotting because an antibody is used to specifically detect its antigen, is a widely accepted analytical technique used to detect specific proteins in the given sample

Procedure

The first step in a western blot is to prepare the protein sample by mixing it with a detergent called sodium dodecyl sulfate, which makes the proteins unfold into linear chains and coats then with a negative charge. Next, the protein molecules are separated according to their sizes using a method called gel electrophoresis. Following separation, the proteins are transferred from the gel onto a blotting membrane. Although this step is what gives the technique the name "western blotting," the term is typically used to describe the entire procedure.

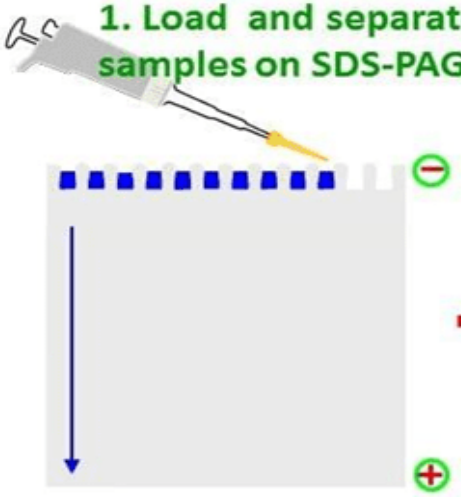
Once the transfer is complete, the membrane carries all of the protein bands originally on the gel. Next, the membrane goes through a treatment called blocking, which prevents any nonspecific reactions from occurring. The membrane is then incubated with an antibody called the primary antibody, which specifically binds to the protein of interest. Following incubation, any unbound primary antibody is washed away, and the membrane is incubated yet again, but this time with a secondary antibody that specifically recognizes and binds to the primary antibody. The secondary antibody is linked to a reporter enzyme that produces color or light, which allows it to be easily detected and imaged. These steps permit a specific protein to be detected from among a mixture of proteins.



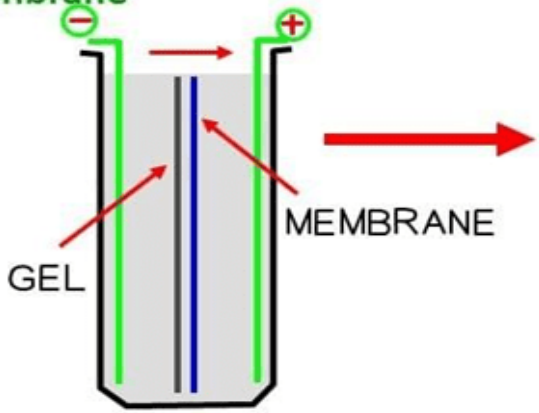
Arrangement of blot for transfer of separated bands from gel to matrix

Western Blotting Procedure

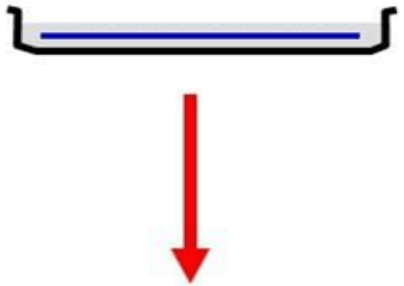
1. Load and separate protein samples on SDS-PAGE



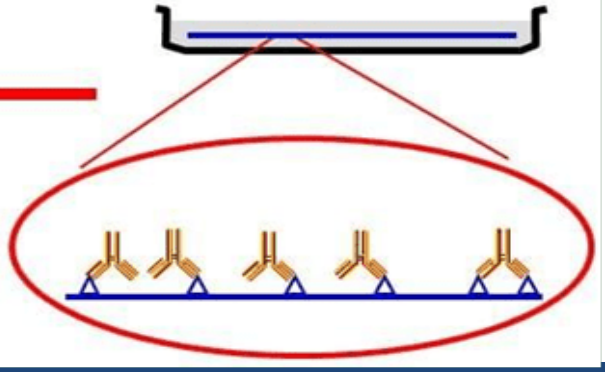
2. Electrophoretically transfer fractionated proteins onto PVDF membrane



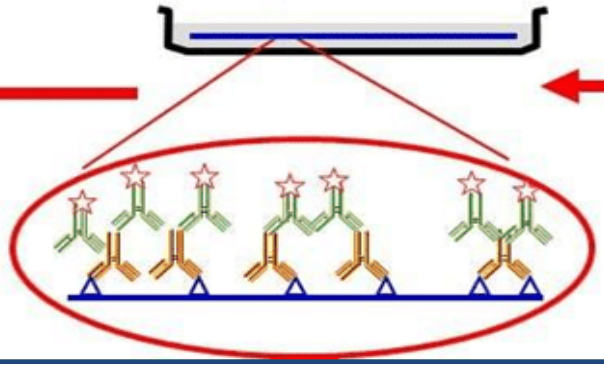
3. Block the membrane with neutral protein (BSA or milk casein)



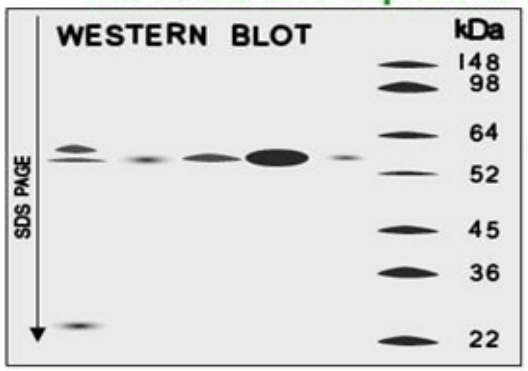
4. Incubate the membrane with primary antibody specific to target protein



5. Incubate the membrane with HRP-labeled secondary antibody specific to primary antibody



6. Incubate the blot with chemiluminescent HRP substrate and expose to film



Test your understanding

Which of the following ELISAs uses two different antibodies?
I. Direct
II. Sandwich
III. Competitive
a. I and II only
b. II and III only
c. II only
d. III only

Which technique is used to assay drug concentration in plasma ?
a. IR sepctroscopy
b. UV sepctroscopy
c. Non aqueous titration
d. RIA

Which sentence is not true about RIA?
a. The most commonly used radiolabels in RIA are tritum and iodine.
b. Centrifugation rpm is 1200-2500.
c. This techinque is very sensitivity it can detected 0.001 µg/ml
d. This techinque is very sensitivity it can detected 0.01 µg/ml

RIA standarded graph as
a. X-axis = % Radioactivity and Y.-axis = unlabled Ag(ng)
b. X-axis = unlabled Ag(ng) and Y.-axis = % Radioactivity
c. X-axis = % Radioactivity and Y.-axis = unlabled Ag(mg)
d. X-axis = Radioactivity and Y.-axis = unlabled Ag(ng)

Incubating a plate with antigen or antibody is known as _____.
a. Blocking
b. Coating
c. sandwiching
d. detection

References & Further reading

References

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Further reading

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