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A REVIEW ON BLOTTING TECHNIQUE

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ABSRTACT

Blotting is a classical technique for detecting macro-biomolecules of interest from a mixture of molecules. The technique is generally composed of four steps: 1) gel electrophoresis-based separation of molecules, 2) blotting (transfer and immobilization) of separated molecules from the gel onto the membrane, 3) specific hybridization of probes to target molecules on the membrane, and 4) visualization of the probe/target molecule complex. The present article briefly introduces blotting techniques for readers, since the direction-named techniques are confusing for people beyond the biotechnology field. Particularly, the smartness of the blotting step in a blotting technique is emphasized. Specifically, the blotting step allows for fixation of size-separated molecules on the membrane, and permits subsequent hybridization of the target molecule to labeled probe, collectively allowing target molecules to be detected by imaging. In this article, we will discuss about the blotting techniques, their principles and fabulous procedures. Blotting techniques play a pivotal role in molecular biology, enabling scientists to analyze and visualize biomolecules with precision. These techniques have revolutionized the way we study DNA, RNA, and proteins, offering valuable insights into cellular processes. In this article, we will explore the fundamentals of blotting techniques, their applications, and advancements in the field. We will also provide references to learn and acknowledge the importance of blotting techniques.In conclusion, not only is blotting a smart strategy for enabling the detection of molecules of interest but also the concept of blotting plays an important role in biotechnology.

KEYWORDS

Blotting,southern blot,northern blot,western blot

INTRODUCTION

The word blot mean dark mark , stain or ink , thus blotting a technique of transferring the sample onto the membrane in order to analyze or detect. It has following types: Southern blotting, Western blotting and Northern blotting. The technique was given in 1970s and it proved to be very serviceable in the biological fields. Recent years have witnessed advancements in blotting techniques, with the integration of innovative technologies. For instance, quantitative PCR (qPCR) and RNA-Seq have complemented traditional Northern blotting, offering higher sensitivity and specificity in RNA analysis. Similarly, advancements in mass spectrometry have expanded the capabilities of protein analysis, providing a more comprehensive understanding of the proteome.Blotting techniques involve the separation (via electrophoresis) and transfer of DNA, RNA, or proteins onto a blotting membrane. The target DNA is then attached to a molecule in

order to aid detection. Southern blotting is used to evaluate for specific DNA sequences and may be used in identification of genetic mutations and in forensics.[13],[9].

TYPES OF BLOTTING TECHNIQUES

Southern blotting
Northern blotting
Western blotting

1.SOUTHERN BLOTTING

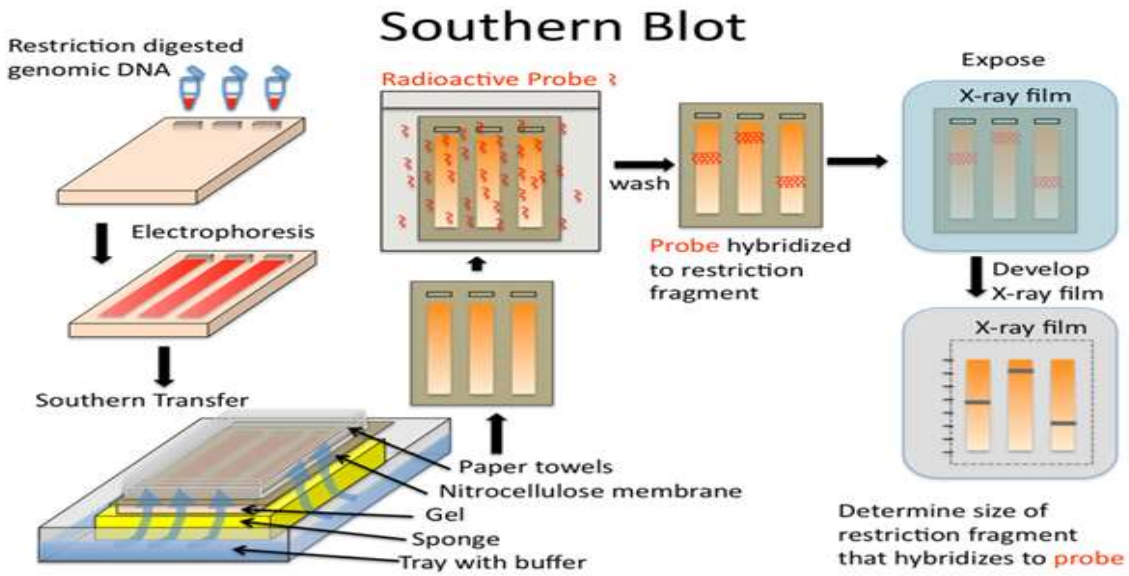
It is defined as "Southern blot is a laboratory technique used in molecular biology to detect and analyze specific DNA sequences."It named after the scientist Edwin Southern, who introduced this method in the 1970s. This technique helps to analyze and characterize DNA by separating it and then transferring it onto a solid support, which can be a nitrocellulose or nylon membrane. In most applications, the mixture of restriction DNA fragments is size-separated by agarose gel electrophoresis. The nitrocellulose or nylon paper is then exposed to labeled cDNA probes. These probes hybridize with complementary DNA molecules on the paper. The paper after thorough washing is exposed to X-ray film to develop autoradiograph. This reveals specific bands corresponding to the DNA fragments recognized by cDNA probe[13].

PRINCIPLE

Southern blotting is based on the principle of analyzing specific DNA sequences fragment in a sample. Firstly, DNA extracted from cells or tissue samples using various methods, such as enzymatic digestion or chemical lysis. Then DNA of genome is first fragmented using restriction enzymes and then separated through gel electrophoresis. The denatured DNA fragments are transferred from the gel to a membrane, where a labeled DNA probe, which is complementary to the target sequence, hybridizes with specific fragments. These probes can be radioactive or fluorescently labeled (produce specific light). Detection of the labeled probes allows to identify and analyze specific DNA fragments on the membrane.The underlying principles of all blotting techniques are almost identical. In Southern blotting, DNA fragments are first separated using gel electrophoresis and then the separated molecules are transferred to a membrane surface. In the next step, hybridization analysis is carried out on the membrane using labeled probes complimentary to the target sequence to be identified, thus detecting the presence of DNA fragment of interest.[8]

PROCEDURE\STEPS INVOVLED IN SOUTHERN BLOTTING

The first step in Southern blotting is to extract the DNA from the samples of interest. This could involve isolating genomic DNA from cells, tissues, or even viruses using different extraction techniques. The extracted DNA then cut into smaller pieces by using restriction enzymes. These enzymes recognize specific DNA sequences and cleave the DNA at those sites, generating fragments of varying lengths. The DNA fragments loaded onto an agarose gel and electrophoresis done. In this step, an electric field applied, due to which negatively charged DNA molecules start to move through the gel matrix towards positive end. Smaller fragments move faster and travel than larger fragments which move very slowly. Transferring onto Membrane, the water move from tray upto tissues via capillary action .When water has travelled half of the pile remove the membrane . The DNA has also travelled along the capillary action. The membrane has now DNA that may not be visible. The membrane with transferred DNA fixed on place to prevent loss of DNA. Mostly UV light used. The membrane exposed to UV light which form covalent bonding between the DNA and the membrane. Membrane baked at a high temperature up to 80-120°C.This process helps the DNA to adhere to the membrane. The transferred DNA fragments on the membrane is treated with labeled probes that are complementary to the target DNA sequences. These probes hybridize with the specific DNA fragments of interest and form stable double-stranded complexes with DNA fragments. After hybridization the membrane goes through washing steps to remove unbound or non-specific bound probe molecules .Then detection and result analysis take place. [8]



FIGURENO:1[SOUTHERN BLOTTING STEPS]

APPLICATIONS OF SOUTHERN BLOTTING

1.The Southern blotting methodology can be extremely sensitive.
2. It can be applied to mapping restriction sites around a single-copy gene sequence in a complex genome such as that of humans.
3. It is an invaluable method in gene analysis.
4. Important for confirmation of DNA cloning.
5. Forensically applied to detect minute quantities of DNA (to identify parenthood, thieves, rapists etc.)
6. when a "mini-satellite" probe is used it can be applied forensically to minute amounts of DNA.

2. Northern blotting

It is a blotting technique that is developed by Ames Alvin, David camp and George at Stanford University in 1979. It a technique that used to detect the RNA or it can be performed to determine the gene expression. It is very similar to the southern blotting.

PRINCIPLE

The principle of the northern blot is the same as all other blotting technique that is based on the transfer of biomolecules from one membrane to another. The RNA samples are separated on gels according to their size by gel electrophoresis. Since RNAs are single-stranded, these can form secondary structures by intermolecular base pairing. The electrophoretic separation of the RNA segments is thus performed under denaturing conditions. The separated RNA fragments are then transferred to a nylon membrane. Nitrocellulose membrane is not used as RNA doesn't bind effectively to the membrane.The transferred segments are immobilized onto the membrane by fixing agents. The RNA fragments on the membrane are detected by the addition of a labeled probe complementar to the RNA sequences present on the membrane.The hybridization forms the basis of the detection of RNA as the specificity of hybridization between the probe, and the RNA allows the accurate identification of the segments. Northern blot utilizes size-dependent separation of RNA segments and thus can be used to determine the sizes of the transcripts[13].

PROCEDURE\STEPS INVOLVED IN NORTHERN BLOTTING

A. Seperation of RNA on Denaturation of Gel

The RNA gel solution is prepared by adding formaldehyde to the agarose solution.The cast is assembled, and the prepared denaturing gel is poured into the cast. As the gel begins to set, a comb with appropriate teeth is added to form wells.Once the gel is set, the comb is removed, and the gel is equilibrated with a

running buffer for 30 minutes before running.15 µg RNA sample is mixed with an equal volume of RNA loading buffer. Three µg of RNA markers are added in the same volume of RNA loading buffer.
The samples are incubated at 65°C on a heating block for about 12-15 minutes.The samples are loaded to the equilibrated gel, and the first row of wells is filled with RNA markers.The gel is then run at 125V for about 3 hours.[1]

B. Transfer of Gel from Nylon Membrane

A nylon membrane is cut that is larger than the size of the denaturing gel, and a filter paper with the same size as the nylon membrane is also prepare .Once the electrophoresis process is complete, the RNA gel is removed from the tank and rinsed with water.An oblong sponge that is slightly larger than the gel is placed on a glass dish, and the dish is filled with SSC to a point so as to leave the soaked sponge about half-submerged in the buffer.A few pieces of Whatman 3mm papers are placed on top of the sponge and are wetted with SSC buffer.
The gel is then placed on top of the filter paper and squeezed out to remove air bubbles by rolling a glass pipette over the surface.The nylon membrane prepared is wetted with distilled water on an RNase-free dish for about 5 minutes.The wetted membrane is placed on the surface of the gel while avoiding any air bubbles formation.The surface is further flooded with SSC, and a few more filter papers are placed on top of the membrane.A glass plate is placed on top of the structure in order to hold everything in place. The structure is left overnight to obtain an effective transfer.[2]

C.Immobilisation

Once the transfer is complete, the gel is removed and rinsed with SSC, and allowed to dry.The membrane is placed between two pieces of filter paper and baked in a vacuum oven at 80°C for 2 hours.In some cases, the membrane can be wrapped in a UV transparent plastic wrap and irradiates for an appropriate time on a UV transilluminator.

D. Hybridisation

The DNA or RNA probes to be used are to be labeled to a specific activity of >108 dpm/µg, and unincorporated nucleotides are to be removed.The membrane carrying the immobilized RNA is wetted with SSC.The membrane is placed in a hybridization tube with the RNA-side-up, and 1 ml of formaldehyde solution is added.The tube is placed in the hybridization oven and incubated at 42°C for 3 hours.If the probe used is double-stranded, it is denatured by heating in a water bath or incubator for 10 minutes at 100°C.The desired volume of the probe is pipette into the hybridization tube and furthe incubated at 42°C.The solution is poured off, and the membrane is washed with a wash solution.The membrane is then observed under autoradiograph .

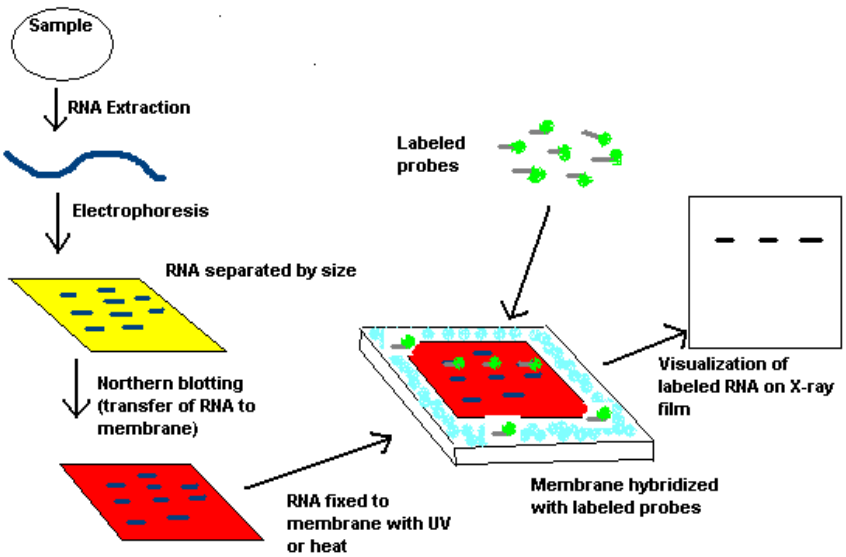


FIGURE NO;2[NORTHERN BLOTTING]

APPLICATIONS

1.The technique can be used for the identification and separation of RNA fragments collected from different biological sources.
2.Northern blotting is used as a sensitive test for the detection of transcription of DNA fragments that are to be used as a probe in Southern Blotting.
3.It also allows the detection and quantification of specific mRNAs from different tissues and different living organisms.
4.Northern blotting is used as a tool for gene expression studies related to overexpression of cancer-causing genes, and gene expression during transplant rejects.
5.Northern blotting has been used as a molecular tool for the diagnosis of diseases like Crohn's disease.
6.The process is used as a method for the detection of viral microRNAs that play important roles in viral infection.

3.WESTERN BLOTTING

The term "western" blotting was coined by Burnette 1981. It involves the transfer of electrophoresed protein bands from a polyacrylamide gel on to a membrane of nitrocellulose or nylon, to which they bind strongly (Gershoni & Palade 1982, Renart & Sandoval 1984). The bound proteins are then available for analysis by a variety of specific protein–ligand interactions. Most commonly, antibodies are used to detect specific antigens. Lectins have been used to identify glycoproteins. Western blotting, which is also known as protein immunoblotting, is a laboratory technique used to detect specific proteins in a given sample." This technique basically combines the principles of gel electrophoresis and immunoblotting to separate and identify proteins on the basis of their molecular weight and antigenic properties. By using antibodies that bind to specific proteins, it is possible to study protein expression, post-translational modifications and protein-protein interactions, and post-translational modifications.Western blotting or protein blotting depends on the specificity of interaction between the protein of interest and the probe used for the detection of the protein.Western blotting has a number of advantages over other similar techniques as the process only requires the use of a small amount of reagents, and the same protein transfer can be used for multiple analyses. [4]

PRINCIPLE

The principle of western blotting is the interaction between the proteins and the probes used for the detection of the proteins.The proteins used for western blotting are separated by gel electrophoresis to obtain them on a gel matrix.The proteins are then transferred to a nitrocellulose or polyvinylidene fluoride (PVDF) membrane, where they are immobilized. The transfer of the protein is known as blotting.The protein on the membrane can either be detected by the use of a reporter-labeled primary antibody directed against the protein or a reporter-labeled secondary antibody directed at the primary antibody.The reporter or probe present on the antibody can be an enzyme that produces a color reaction or a luminescent signal at the antigen-antibody binding site that produces a fluorescent signal in the presence of a particular substrate.The signal or color generated by the probe requires a detection system that is appropriate for the signal or intensity generated.[6]

PROCEDURE\STEPS INVOLVED IN WESTERN BLOTTING

Process is start by extracting protein from cells or tissues. Extraction done by using suitablelysis buffer to release the proteins of interest. Next step is to prepare a polyacrylamide gel and load the protein samples into wells. An electric current applied to separate the proteins based on their size. After the separation, the proteins separated from the gel onto a solid support membrane, such as a nitrocellulose or PVDF membrane. Then membrane incubated by using blocking solution (often 5% milk or BSA) to prevent binding of non-specific antibodies. Incubate the membrane with primary antibodies which is specific to the target proteins. After washing, the membrane incubated conjugated to an enzyme (e.g., HRP or alkaline phosphatase) which is complementary to primary antibody. Target proteins identified by using proper detection methods. Apply a substrate that reacts with the enzyme on the secondary antibody. Then analysis take place.[5]

A. Sample Preparation

The most commonly used samples for western blot are cell lysates which are collected by the process of extraction.The extraction can be achieved by different means like mechanical destruction, chemical extraction, or the use of enzymes.The extraction of often performed at cold temperature in the presence of protease inhibitors in order to prevent the denaturation of the proteins.

B. Gel Electrophoresis

The protein sample is diluted with the sample buffer and is heated and shaken for 10 minutes at 70°C.The sample is then centrifuged at 5000g.The gel case is removed from the pouch and is placed in the buffer tank against the rubber seal with the gel walls facing the inside of the tank reservoir.The running buffer is poured onto the upper reservoir while ensuring that no buffer leakage occurs on the lower tank.Each of the wells is then loaded with an equal volume of heat-denatured sample, and one of the lanes is reserved for the protein ladder.The lid is placed on the tank, and it is connected to the power supply.The run is allowed to run at 200 V constant for 50 minutes.[6]

C.Protein Transfer

The transfer buffer is prepared by adding 10% methanol to the buffer.The transfer case is taken and laid out. It is then covered with a transfer buffer.A foam sponge is taken and laid on the backside, over which goes the filter paper. These should be placed to ensure that both of them are wet and slightly submerged.The gel is taken out from the tank and placed on the wet filter paper. The nitrocellulose membrane is wet with the transfer buffer and is placed on top of the gel in a way that there are no bubbles between the gel and the membrane.The transfer case is placed into the transfer tank, which is further filled with transfer buffer.The tank is then connected to power at 100V for 1 hour.Once the transfer is complete, the transfer case is removed, and the nitrocellulose membrane is removed from the gel.[14]

D.Immunodetection

The membrane is washed with Tris-buffered saline for 5 minutes in a Petri dish.The 10% nonfat dry milk is mixed with the Tris buffer, and the membrane is covered with the mixture for 30 minutes at room temperature.The membrane is washed with the Tris buffer to remove any excess mixture remaining on the membrane.with the help of the forceps, the membrane is transferred to a new Petri dish onto which the primary antibody is added.The membrane with the antibody is incubated for 3 hours at room temperature. The membrane is washed after incubation with the Tris buffer.The membrane is transferred again to a new Petri dish, where a secondary HRP-conjugated antibody is added.The membrane incubated for 1 hour. The concentration of secondary antibodies often remains at 1 µg/ml, but this also depends on the dilution.The membrane is washed again with Tris buffer to remove excess antibodies from the surface.The membrane is incubated with the substrate for 5 minutes, and the observation is made.[12]

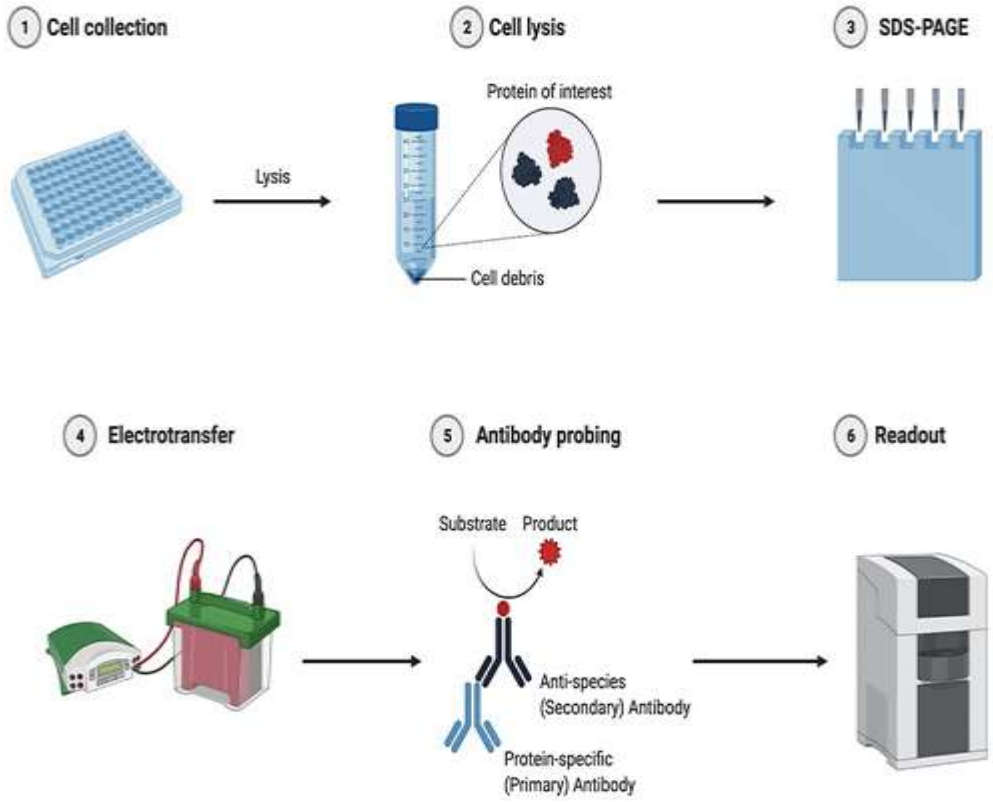


FIGURE NO:3 [WESTERN BLOTTING]

APPLICATIONS

1.Western blotting is an excellent method with high sensitivity in order to detect a particular protein even in low quantity.
2.Western blotting has been used in the clinical diagnosis of different diseases. The confirmatory test for HIV involves a western blot by detecting anti-HIV antibodies in the serum.
3.The technique has been used to quantify proteins and other gene products in gene expression studies.
4.Since western blotting detects the proteins by their size and ability to bind to the antibody, it is appropriate for evaluating the protein expressions in cells and further analysis of protein fractions during protein purification.
5.Western blotting is also used for the analysis of different biomarkers like growth factors, cytokines, and hormones.[6]

Comparison of blotting techniques:

{characteristics of southern,northern,western,blots}[15]

Technique	Southern blot	Northern blot	Western blot
Target	DNA sequences	RNA sequences	Protein
Separation	Electrophoresis	Electrophoresis	Electrophoresis
Blotting method	Capillary transfer	Capillary transfer	Electrophoretic transfer
Probe	Oligonucleotides	Oligonucleotides	Antibodies
Common detection methods	• X-ray • Colorimetry • Chemiluminescence	• X-ray • Colorimetry • Chemiluminescence	• Colorimetry • Chemiluminescence

ADVANTAGES &DISADVANTAGES

GENERAL ADVANTAGES

All kinds: good specificitc

Southern blot: can detect a wide variety of mutations

Western blot:

Good sensitivity

Can evaluate for several target proteins

GENERAL DISADVANTAGES

Requires a large amount of DNA or RNA

Slow, labor-intensive process

Expensive

Use of radioactive materials can potentially be hazardous[10].

LIMITATIONS

Southern blotting hasTime consuming and labour-intensive.
Requires radioactive or non-radioactive probes.[10]

Northern blotting has a lower sensitivity as compared to other modern techniques like RT-PCR and nuclease protection assays.The method requires a large amount of sample RNA, and these should be of high quality.The technique is time-consuming and complex, especially in cases where multiple probes are to be added.

Western blotting can only be performed for proteins if the primary antibodies for the proteins are available.Some antibodies might exhibit off-target effects by interacting with more than one protein in the sample.The technique is a costly process with the cost of antibodies and expensive detection.[9]

CONCLUSION

Blotting techniques have been instrumental in unraveling the intricacies of molecular biology, enabling researchers to decode the language of nucleic acids and proteins. From the classic Southern blotting to the cutting-edge applications in genomics and proteomics, these techniques continue to shape the landscape of biological research. As technology evolves, so too will the potential for deeper insights into the molecular mysteries that govern life.At present, many of the applications of Southern blot and northern blot have been largely replaced by polymerase chain reaction (PCR) and Sanger sequencing, however, the western blot is still a standard methodology for protein analysis. Furthermore, the basic concept of blotting, as a smart strategy for enabling the detection of molecules of interest, continues to play a vital role in molecular biotechnology.

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