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Review

Southern Blot Hybridization: Principles and Applications of Course: Advanced Microbial Genetics and Molecular Biology

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Abstract: Southern blot hybridization is a classical molecular technique that enables the detection, characterization, and quantification of specific DNA sequences within complex genomic samples. Developed by Edwin Southern in 1975, the method remains foundational in microbial genetics and molecular biology despite the emergence of high-throughput sequencing and PCR-based technologies. The technique integrates restriction enzyme digestion, agarose gel electrophoresis, alkaline denaturation, membrane transfer via capillary or electroblotting, and sequence-specific hybridization using labeled nucleic acid probes. Southern blotting provides high specificity, sensitivity, and reliability for analyzing gene structure, copy number variation, genetic polymorphisms, and large DNA fragments that are difficult to amplify. This article reviews the principles, historical development, reagents, materials, and procedural steps of Southern blotting, including advances such as non-radioactive labeling systems, nylon membrane adaptation, and improvements in hybridization chemistry. Furthermore, it highlights key applications in microbial genetics, pathogen detection, clinical diagnostics, genetic engineering, and forensic analysis. Despite modern innovations, Southern blotting continues to be indispensable for studying genomic organization, validating recombinant constructs, detecting long genomic repeats, and confirming results obtained from PCR or sequencing platforms. Its robustness, precision, and ability to analyze high-molecular-weight DNA justify its continued relevance in advanced molecular biology research and medical microbiology.

Keywords: Southern blotting, DNA hybridization, Molecular diagnostics, Genomic analysis, Restriction enzymes, Nucleic Acid probes, Electrophoresis, Membrane transfer, Gene detection, Molecular biology techniques.

INTRODUCTION

Blotting is used in molecular biology for the identification of proteins and nucleic acids and is widely used for diagnostic purposes. This technique immobilises the molecule

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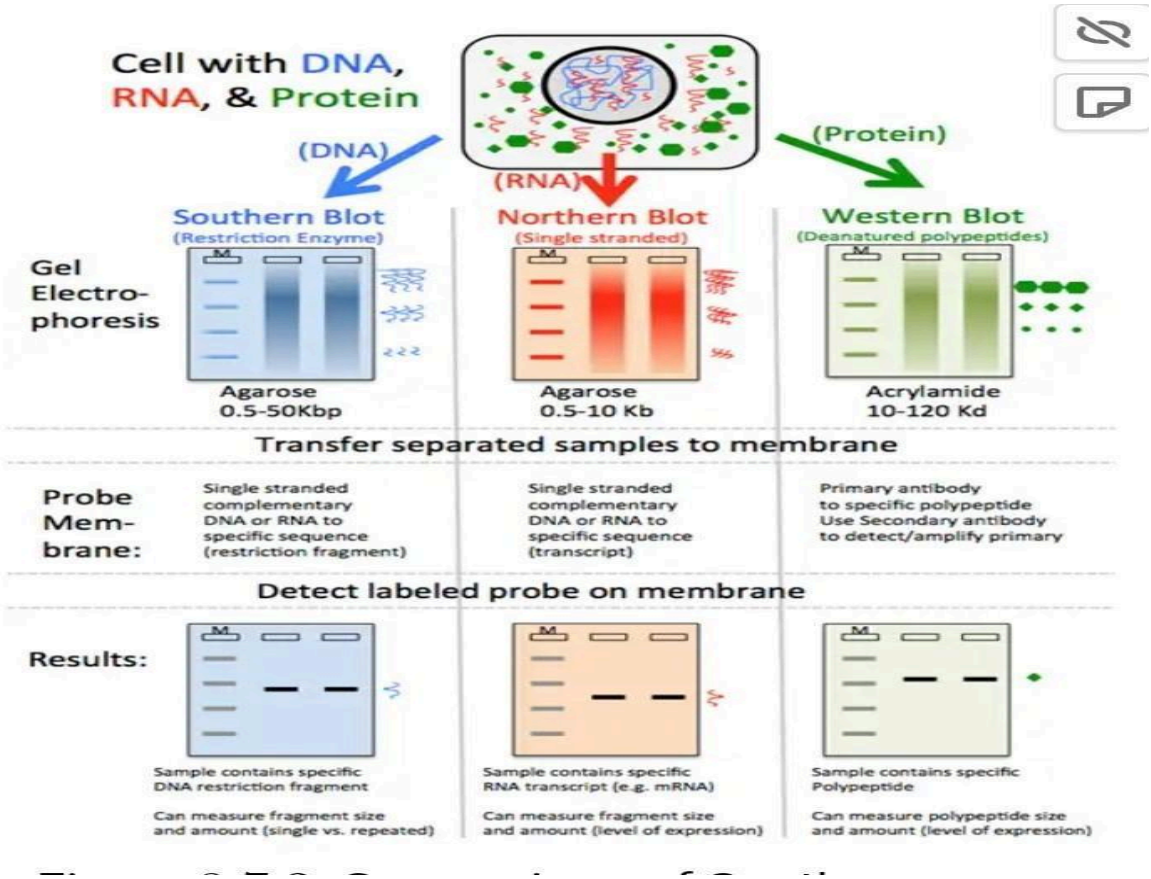
of interest on a support, which is a nitrocellulose membrane or nylon. It uses hybridisation techniques for the identification of specific nucleic acids and genes. The blotting technique is a tool used in the identification of biomolecules such as DNA, mRNA, and protein during different stages of gene expression. Protein synthesis involves the expression of a DNA segment, which gets converted to mRNA to produce the respective protein. Molecules such as DNA, RNA, and proteins are subjected to biochemical analysis, which is separated using blotting techniques. In the case of a cell, these molecules are present altogether, and hence, with the help of blotting, scientists are able to recognise a specific molecule out of all others. Blotting is performed by allowing a mixture of molecules of interest to pass through a block of gel, which separates the molecules based on their molecular sizes. The processed molecules are required to be hard-pressed against a suitable membrane, which will, in turn, transfer the molecules from the gel onto a suitable membrane (nylon, nitrocellulose, or PVDF) via capillary action. After the molecules are transferred to the membrane, their position does not change.

Southern blotting was introduced by Edwin Southern in 1975 as a method to detect specific sequences of DNA in DNA samples. The other blotting techniques that emerged from this method have been termed Northern (for RNA), Western (for proteins), Eastern (for post-translational protein modifications), and Southwestern (for DNA-protein interactions) blotting.

Blotting approaches are viewed as an aid to gel electrophoresis, which is generally applied for the separation of DNA, RNA, and protein, yielding reproducible results attributed to their excellent resolving power. Specific molecules can thereby be detected amid the combination of molecules that are subjected to the separation. Most methods have a general step wherein the molecules of interest are transferred, once separated, from the gel to a solid membrane phase, which is accomplished by drenching in a solution across the gel and the membrane via penetrable paper. Many types of multifaceted apparatus are also provided by numerous suppliers for electroblotting, which is more specifically useful for transfer initiated from less porous polyacrylamide gels compared to commonly used porous agarose gels. In the case of DNA and RNA, the detection of specific sequences in the membrane is carried out via hybridisation with nucleic acid labelled probes, which, in the case of proteins, is replaced by the use of labelled antibody probes. The initially developed protocols applied radioactive probes labelled with radioactive isotopes for detection purposes via the implementation of autoradiography procedures. In this process, the pattern

of decay emissions radiated from a radioactive material is applied to produce an image on X-ray film, which can also be made available as a digital image by the application of scintillation-based gas detectors or systems based on phosphor imaging. Keeping in mind the harmful effects of exposure to radioactivity, other kinds of labeling systems have been developed, which include fluorescent and chemiluminescent reagents. Additionally, several other modifications have been implemented in contrast to the original method, such as DNA probes being more commonly applied rather than RNA, nylon membranes replacing traditional nitrocellulose, and to avoid the renaturation of DNA sequences during transfer, the transfer is carried out in alkaline conditions, which was supposed to be in neutral solution as per the original protocol. The DNA is subjected to acid treatment to reduce its size in order to increase the transfer rate of larger fragments. The gel strips and tube gels applied in the original protocol are no longer used; instead, gel formats are applied. Though there have been many changes in the original protocol, modern-day protocols still retain most of the fundamental features of the original protocol.

Southern blotting was applied in many important studies, such as the genetic mapping of the human genome, which was based on blotting-based detection of restriction fragment length polymorphisms. Additionally, DNA fingerprinting was first developed via hybridisation of the human DNA restriction digestion products with minisatellite probes. However, most of the primary applications of this method have now been substituted by DNA sequencing and polymerase chain reaction (PCR), as they provide more extensive data and are also easier to implement. Nevertheless, blotting is still applied in many areas, such as in the measurement of copy number, analysis of long stretches of DNA that are difficult to amplify or sequence using PCR or DNA sequencing, and in the structural analysis of DNA, wherein the physical forms of DNA are separated using two-dimensional gel electrophoresis and subsequently detected using blotting of specific components.



General Procedure for Blotting

Homogenisation of the sample, which involves the purification of DNA, RNA, and proteins, is performed after extraction from a variety of sources, such as cells or tissue.

Digest the DNA with restriction enzymes into fragments, which are not required for RNA (northern blot).

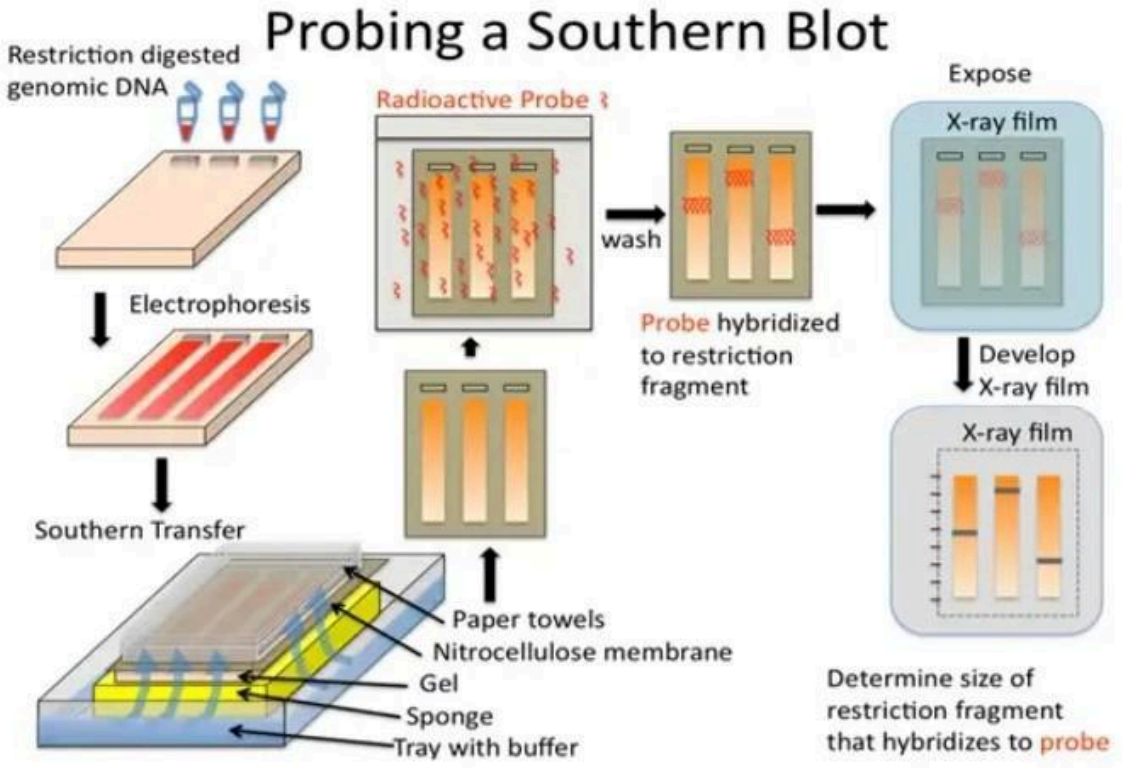
Separation of the molecule of interest by an electrophoresis membrane is generally performed on an agarose gel for DNA fragments. In the case of RNA samples, they can be separated on an agarose gel in the presence of formaldehyde as the denaturing agent. This is necessary as formaldehyde confines the secondary structures of RNA molecules.

Transferring the molecules (DNA/RNA fragments) to a nitrocellulose membrane or nylon membrane from the gel.

Prehybridisation (Blocking): Washing of the nylon membrane with a prehybridisation or blocking solution comprising salmon sperm DNA is required in order to block non-specific DNA interactions; this also helps in the reduction of background noise. As an alternative, there are some commercially available blocking buffers, like Perfect

Hyb™ Plus buffer, in which there is no requirement for salmon sperm DNA for blocking purposes.

For the preparation of the probe, fresh probe DNA labelled with 32P alpha-labelled dCTP is prepared. Hybridisation or identification of the molecule is achieved by incubating the blot with the specific labelled probe. For the detection of the probe and the sequence of interest, DNA/RNA, the film is exposed to -80°C.



SOUTHERN BLOTTING

The first of these techniques developed was the Southern blot, named after Dr. Edwin Southern, who developed it to identify specific DNA sequences. Southern blotting is a detection technique used to find the target DNA sequences in the DNA sample in the field of molecular biology. The process starts with the electrophoresis of DNA molecules, which are hybridised in a blotting membrane, followed by a transfer step where DNA from the gel is transferred onto the blotting membrane.

HISTORICAL BACKGROUND

The ability of the DNA strands to hybridise comes from their complementary nature, clearly depicted in Watson-Crick's DNA structure model. In the beginning, experiments were carried out to study the renaturing ability of DNA and the formation of

DNA/RNA hybrids for understanding tissue or cell-specific expression. Thereafter, hybridisation came into the picture with the development of probes that can detect specific sequences within the target and the immobilisation of the target sequences onto a solid support, like nitrocellulose powder, which further gave rise to nitrocellulose membranes. The demonstration by Southern that DNA fragments separated by gel electrophoresis can be transferred onto a membrane allowed the characterisation of DNA by hybridisation pattern and by their molecular sizes. Later, this method was applied for the spatial identification of targets and blotting of bacterial colonies.

The electrophoresis step can be done using two different types of gels, such as polyacrylamide gel (PAGE) with urea and sodium dodecyl sulphate (SDS) with urea. These two gels have different applications. When a PAGE gel is used, the same quantity of DNA fragments is transferred to the blotting paper. If SDS is used, the resolution of the bandwidth formed remains the same. In this technique, a DNA molecule of size 100 pg can be identified. The technique can be summarised as the formation of double-stranded DNA, which has one strand from the target DNA and the other from the DNA probe. The DNA probe is produced in vitro for the sequence of interest.

PRINCIPLE

Restriction endonucleases, which are enzymes, are used to break the DNA into small fragments. These fragments are then separated using electrophoresis. The fragments obtained are then classified according to their size (kDa). Thus, DNA fragments are transferred to the blotting paper, where they are incubated with probes. Probes used in Southern blotting can be highly selective. They can selectively bind with a resolution of 1 in a million and possess the characteristics to bind to the intended target fragments.

MATERIALS REQUIRED

Reagents

The buffer used for electrophoresis is TAE or TBE.

The agarose preferred should be of electrophoresis grade.

For staining the DNA, ethidium bromide (0.5 μg ml-1, dissolved in H2O) is used. However, there are other DNA staining dyes, such as SYBR Green, which can replace ethidium bromide for safe handling.

Note: Ethidium bromide, being a mutagen, requires careful handling. It is advised to wear gloves during its usage and to follow the pertinent regulations for the disposal of pipette tips. Avoid touching any objects with ethidium bromide-contaminated gloves.

2X and 20X SSC (the composition of 20X SSC includes 3.0 M NaCl and 0.3 M sodium citrate)

6X DNA loading buffer composed of 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in water.

Suitable DNA markers of varying molecular weight, also referred to as DNA ladders, are used as standards for reference.

The prehybridisation and hybridisation mixture consists of 0.5% SDS, 6X SSC, 5X Denhardt's solution, and 100 mg ml–1 sheared, denatured salmon sperm DNA or yeast tRNA.

Denhardt's solution, widely used for hybridisation, is made up of 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin (BSA).

Paraffin oil

Cellulose nitrate or nitrocellulose membrane filter with uniform porosity (e.g., Millipore 25 HAWP; nylon membranes used for blotting protocols are available under various trade names from commercial suppliers).

RNase A (20 pg ml-1 in 2X SSC) for the specific cleavage is needed.

Restriction enzymes and an appropriate buffer are used.

Radioactively labelled RNA serves as a probe for specific detection, where autoradiography is conducted.

Note: Handling of radioactive probes must be handled carefully, which necessitates reliable safety measures and legal regulations.

For the detection of RNA labelled with tags such as 3H, 35S, 125I, or 14C, there is a requirement of 2,5-Diphenyloxazole (PPO) in toluene at a concentration of 20% wt/vol.

EQUIPMENT

The transfer from narrow strips of gel can be achieved by using three pieces of glass or Plexiglas, each measuring 5 cm × 20 cm, with a thickness similar to that of the gel.

There is a requirement for thick, dry filter paper (four to five in number) or paper towels measuring 10 cm × 18 cm.

The hybridisation vessel possesses larger dimensions (0.8 mm deep × 2 cm high × ~1 cm longer) than those of the membrane used for hybridisation, and the material used for developing the hybridisation vessel is Perspex (note: several alternative procedures are followed for hybridisation).

Four narrow pieces of Perspex possess a thickness similar to that of gel. The length of Perspex is sufficient to surround the gel at a spacing of approximately 3 mm.

A tray having a depth of 20–50 mm (approx.), and a length and width larger than the gel of 20 mm (approx.)

A glass sheet with a length sufficient to be placed on the tray and narrow enough to have a gap of 10 mm on each side.

Several thick pieces of filter paper have a large area compared to the gel. The length of the filter paper is adequate to cover the glass sheet and can be dipped in the tray.

A moistened piece of nitrocellulose membrane, having a wider area that can cover the entire gel. The nitrocellulose membrane is placed on top of four strips made of Perspex. The moistening of the nitrocellulose membrane is done using 2× SSC.

Paper towels are stacked one on top of the other.

Apparatus for casting gel.

A gel tank for carrying out electrophoresis is necessary.

Power supply for the entire device setup is required.

DNA: The entire procedure is initiated by employing enzyme-digested DNA of varying concentrations, which will quantify the optimum DNA concentration and specify the restriction enzyme to be used. Generally, an amount of 1 µg of DNA derived from clones (e.g., from plasmid or bacteriophage clones) is adequate for plasmids having a low copy number. We require larger amounts for carrying out the separation of complex DNA (e.g., genomic DNA). The advisable range to be considered would be 5-10 µg.

The electrophoresis buffer used is TAE, which has a composition of 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, with a pH range of 7.4-8.2. This buffer is normally made as a stock concentration of 20X, or TBE, made up of 89 mM Tris, 2.5 mM EDTA, and 89 mM borate, normally made as a 10X stock.

TAE is recommended to be best when we run gels for a shorter interval of time, and when the recovery of DNA fragments from the gel is to be carried out.

PROCEDURE

Step 1: DNA purification

To extract the DNA present inside the nucleus of a cell, we must first lyse the entire cell to enable the expulsion of the DNA. Incubating the cell culture with detergent lyses the entire cell. Now the lysed sample contains DNA, protein, and debris. Protein is lysed by

adding the proteinase enzyme and incubating. DNA is purified and separated by alcohol precipitation, and fibres are removed by using a buffer.

Apart from standard manual isolation procedures, there are commercially available kits, such as GenElute™, for the isolation of DNA from a variety of sources, including mammalian cells, plants, bacteria, and fungi. High purity of the isolated DNA is ensured in the case of using commercial kits.

Step 2: Fragmentation

The long nucleotide sequences should be broken into smaller fragments for the purification or identification process. This is done by the restriction endonuclease enzyme.

All the reagents necessary for the digestion process should be kept on ice before setting up a restriction digestion reaction with suitable enzymes and appropriate DNA concentrations. The components are added to the multiwall plates or microcentrifuge tubes (PCR tubes) and mixed by aspirating the contents with the help of a pipette slowly to avoid the formation of any bubbles. It is to be taken care that the enzyme has to be added at the last step, and until then it should be stored at −20 °C. To ensure complete digestion of the DNA, a surplus amount of enzyme is supposed to be added, as the fragments produced due to partial digestion can cause ambiguity in the results, leading to inaccurate analysis of the subsequent blot to be analysed. Nevertheless, the concentration of the enzyme added in total should not surpass one-tenth of the total volume of the digest, because the glycerol content generally found in stock of the enzymes has the capacity to impede the digestion process when present at high concentrations. Also, in order to minimise the probable errors due to pipetting, master mixes should be prepared accordingly when there is a requirement for the analysis of a large number of samples.

The incubation of the digests is carried out at 37 °C either in an incubator or a water bath. A water bath is generally preferred. For DNA samples obtained from cloning, 1-2 h is deemed sufficient. In the case of genomic DNA, there is a requirement for overnight digestion, wherein there is a chance that the enzyme is inactive in between. To ascertain that the overnight reaction is successful, half of the enzyme is to be added at the beginning of the digestion reaction, and the second half can be added in the morning, and the incubation can be continued for an hour.

Once the digestion is over, it may be the case that a concentration step is required to ensure that a suitable volume of DNA is present for loading into the gel, which is generally fixed at 20 µl per well. This makes the total volume 24 µl after the addition of 6X loading

buffer. A standard method for concentrating the DNA samples involves precipitation in the presence of ethanol, after which the DNA sample is resuspended in ddH2O. The traces of ethanol should be removed completely, as any remaining ethanol would lead to spilling of the samples out of the wells once the samples are loaded.

Step 3: Gel Electrophoresis

Nucleic acids are negatively charged molecules. Therefore, they move towards the anode in an electrophoresis chamber. The movement of the DNA fragments differentiates the rate of transport, thus enabling separation by size.

The percentage of the gel that is to be used and the size of the gel must be determined. The percentage will decide the size of the fragments that will be separated, and the size of the gel, on the other hand, will give the range of fragments that are feasible to be resolved. Longer gels are generally needed in the case of the separation of genomic DNA or multiple fragments that have similar sizes in order to guarantee appropriate separation. Generally, a 0.7–2% gel is considered adequate for most applications. However, in the case of some genomic DNA samples, it may be required that a low percentage of gel be run for the necessary separation of the fragments. When a gel of less than 0.8% is required, a high base gel percentage is necessary (up to 2%) to provide support, as low percentage gels are very fragile. It is necessary that the base gel is dispensed before the comb is positioned. As soon as the base gel is set, the low percentage gel is poured on top, and the comb is placed in a way that it does not touch the base gel at the bottom.

A 1X electrophoresis buffer is made after dilution of the prepared stock solution in ddH2O, and agarose is added to it in a conical flask, which is kept under constant stirring to avoid the formation of clumps. Thereafter, the agarose is melted in a microwave or on a stirrer with heating under constant stirring. In case a microwave is used, the gel should be swirled every 30 s to ensure even consistency is maintained. In most standard microwaves, the gel completely melts within 3 min.

Note: The agarose should be checked regularly during heating, as it is susceptible to boiling in a short time. To ensure there is no spillage, it is recommended that a conical flask sufficiently larger in volume be used during agarose preparation.

The flask containing the molten gel is required to be transferred to a magnetic stirrer, and the gel is allowed to cool down slowly to around 60ºC under gentle but constant stirring. The ethidium bromide is supposed to be added to the gel at this stage, at a concentration of 5 μg ml-1.

By the time the gel is cooled down, the 1X electrophoresis buffer is prepared in an appropriate amount to fill the tank reservoir up to the level of a few millimetres above the gel slab to ensure proper immersion.

The gel casting tray is prepared with a comb of suitable tooth size, depending on the sample volume to be loaded. The casting tray should be on a straight platform before pouring the gel onto it. The formation of bubbles is to be avoided as far as possible, as they cause the DNA to run in an irregular manner. If bubbles are formed, they can be burst by using a pipette tip, and this should be done as soon as the gel has been poured.

As soon as the gel is set, it can be understood by observing its opalescent appearance, and the comb should be gently removed. The gel is then transferred to the tank, and 1X running buffer is added to it in order to immerse the gel. The buffer should be added in a sufficient amount, keeping in mind the maximum capacity of the tank, because it increases the buffering effect as well as protects the gel from melting down due to the generation of heat, particularly if the gel is to be run for a longer period of time. One has to be careful while removing the combs so as not to tear the wells. In case the gel apparatus in use provides for removing the comb after the gel is placed in the tank and immersed in the buffer, the comb should be removed in the final step to avoid breakage of the wells. When a gel of lower percentage is used, before removal of the comb, brief refrigeration could help in sustaining the wells in proper shape.

The DNA samples are prepared by adding 1X loading buffer to each 5 µl of sample. The loading dye and the sample should be mixed well without the formation of any bubbles, which will eventually lead to the spilling of the samples from the wells once they are loaded.

The samples are then loaded carefully into the wells, and one or preferably two of the wells are left blank for the molecular weight marker to be loaded, which generally comes in a ready-to-load formulation. While loading, the pipette tip is supposed to be placed below the corner at the top of the well so that the pipette tip does not pressurise against the bottom of the well, which may also lead to perforation of the well. The sample is to be loaded slowly to avoid spillage out of the wells and mixing of samples with the contents of the next well.

The lid is then placed on top and connected to the power pack with the voltage set at an appropriate level. In order to ascertain if the setup is working properly, small bubbles can be seen popping up as the power supply is switched on. In the case of genomic DNA,

the voltage should be set low, preferably not exceeding 20 V, and for plasmid DNA, it can be around 100 V. The electrodes should be located in the correct position for the DNA to run in the appropriate direction. The positive socket is generally red, and the negatively charged one is black in colour.

Step 4: Denaturation

DNA thus attained is double-stranded in nature. For our purpose of probe hybridisation, we need single-stranded DNA. DNA is therefore denatured in an alkaline solution. This results in the formation of denatured DNA.

Step 5: Blotting

Blotting is the transfer of the fragmented DNA sequence to the nitrocellulose membrane or nylon membrane. The process is done by either electroblotting or capillary blotting. The DNA molecule is saturated using a NaCl solution and permanently fixed using either UV radiation or drying.

Choice of the membrane for blotting

In the original protocol, nitrocellulose membranes have been used for blotting in the case of Southern blotting, but in recent times, nylon membranes have been implemented for the blotting process due to their ability to bind a greater amount of DNA efficiently, which allows the Southern blot to be carried out with a lesser amount of target DNA. The preparation time for transfer takes approximately 2-3 h. In the case that the gel is run without ethidium bromide, it needs to be immersed in electrophoresis buffer mixed with ethidium bromide for 0.5-2 h.

Thereafter, the gel is transferred carefully to a gel documentation system for capturing the photograph of the gel under ultraviolet light of 256 nm, although when DNA is present in high amounts, it can also be detected at 310 or 365 nm. If a scale is placed along with the gel, it helps in correlating the fluorescence photograph with the final autoradiograph that will be generated. If sufficient separation of the DNA has not occurred, then the gel can be run for a longer time after returning to the tank. Distinct bands generally appear in the case of clonal DNA and genomic DNA.

DNA mostly yields a smear in which the repetitive elements of the DNA are represented by the appearance of brighter bands. Thereafter, the portion of the gel to be subjected to blotting can be cut apart using a blade. In the experiments where large fragments need to be transferred, the gel should be subjected to a dilute acid bath for approximately 10 min in order to bring about depurination.

In the next step, the gel is denatured completely by submerging it for 15-30 min in the presence of 1.5 M NaCl and 0.5 M NaOH in a tray that is placed on a rocking platform. The above-mentioned solution is then substituted with 0.5 M Tris-HCl (pH 7) and 3 M NaCl. It is further incubated for 15-30 min, which leads to the neutralisation of the gel. The time required for transfer is about 15 min.

In the case of the transfer of strips, the constituents of gel transfer are prepared such that there is a portion of thick filter paper of appropriate dimensions, preferably 20 cm × 18 cm, dipped in 20X SSC buffer, along with a nitrocellulose filter that is equal to the length of the strip of the gel, having a width of 1 cm, dipped in 2X SSC. The filter paper is required to be immersed, and the membrane should be transferred by initially floating it on the solution surface to avoid uneven transfer due to trapped air.

The large filter paper is laid on a glass or plastic platform after being soaked in 20× SSC in the case of narrow strips. Utmost care should be taken so that no air is trapped below. In the case of wider strips, the tray is first filled with 20× SSC before a glass plate is laid on it. Thereafter, a compact arrangement of filter paper soaked in 20× SSC is placed on it before being dipped into the tray.

A glass or a Perspex sheet is placed on the uppermost part and towards one of the sides of the wet paper.

The gel is then removed from the above neutralising solution and is laid parallel to the glass approximately 2-3 mm apart. Similarly, on the opposite side of the gel, the second glass is laid around 2-3 mm apart.

The nitrocellulose membrane is then laid on the top portion of the gel with the ends balanced on the glass, forming an air space between them. Air should not be trapped between the membrane and the gel; hence, the alignment has to be carried out with care. The membranes should not be shaken once they are placed. The absorbent paper is finally placed on top. It is to be noted that placing a heavy weight on the top may disorient the gel, in turn affecting the final results.

The transfer process is then started for approximately 3 h, which varies depending on the concentration of the gel as well as the size of the fragment in question. In order to avoid drying during the transfer, 20× SSC must be replenished to avoid shrinkage of the gel. During this process of the addition of buffer, it must not fill the gap between the gel and the glass or sheets.

Once the transfer is completed, the nitrocellulose membrane is removed carefully in a way that the gel remains attached. Then the nitrocellulose membrane is turned, and the boundaries of the gel are traced using a pencil. The gel is then removed from the nitrocellulose membrane, and if some amount of DNA is left out during the transfer, it can be visualised under UV light, as explained before. The portion of the nitrocellulose membrane that was touching the gel should be removed using a blade.

The cut portion is then kept immersed in 2X SSC for approximately 10-20 min before it is subjected to baking at 80 °C for 2 h in a vacuum oven. Alternatively, the DNA can also be fixed via UV crosslinking mediated by exposure to short-wavelength UV light in a commercial setup. If desired, the dry membranes can also be stored at room temperature for future reference. Once the transfer process is completed, the preparation for the hybridisation reaction must be carried out. For this, a blocking step that usually takes around an hour is mandatory for eliminating non-specific reactions. This step is also known as the prehybridisation step.

The membrane is incubated in standard Denhardt's solution for 1 h, or in certain cases, more time is required, depending on the type of reaction. Salmon sperm DNA is also widely applied as a blocking agent. Commercially available prehybridisation solutions, like Perfectly™ Plus buffer, are used for preparing blocking solutions containing Salmon sperm DNA. Briefly, after incubating the prehybridisation solution to 42°C, the heat-snap-chilled salmon sperm DNA is added to it at a concentration of 50 µg/mL. The prehybridisation solution containing the sperm DNA is allowed to interact with the blotted membrane inside a hybridisation chamber for up to 5 h.

Step 6: Hybridisation

Labeled probe is added to the membrane buffer and incubated for several hours as it takes for the probe to find the exact target sequence. The time required for hybridisation usually 1–16 h depends on factors like the complexity of the probe and

CONCENTRATION

The radioactive RNAs are present in limited amounts as they are derived from cells, making them an important factor for the original protocol. Due to this, the volume of the hybridisation solution used is supposed to be less so that the desired RNA concentration can be achieved. There can be two approaches for the hybridisation experiment, where one uses a smaller volume, and the other utilises a vessel designed to hold the membrane in a low volume of liquid. The suitable setup possesses a plastic bag that can be sealed using

heat, and it consists of an optimum amount of hybridisation mix and can be dipped in a water bath or a hybridisation tube with capped ends. In the case of the tube, it must be ensured that the membrane is wet when the tube is subjected to rotation cycles in a rotisserie oven. The probes applied can be either RNA or DNA. The cells act as a source for the RNA; alternatively, they can also be obtained from clones or PCR products via in vitro transcription. In the case of DNA, they can be either clones or PCR products. The procedure adopted for the labelling method relies on the source from which the probe is derived.

In the case of hybridisation by immersion in paraffin oil, a drop of solution containing the probe RNA of appropriate concentration, depending on the membrane size, is placed on a plastic sheet.

One of the ends of the nitrocellulose membrane is allowed to soak up the liquid from the drop by slowly moving the membrane over the drop's surface. Once it is wet, the same procedure is repeated for the other side of the membrane.

The membrane is then dipped in a hybridisation solution saturated with paraffin oil at a temperature of 40-65 °C, which depends on the type of solvent in use. It is important to consider that when the membrane is baked in the presence of 2X SSC, this leads to the introduction of salt, which in turn will be the deciding factor for the solvent in which RNA will be dissolved. This process yields good results but suffers from disadvantages such as an uneven background, which can be resolved if the hybridisation is conducted at 40 °C in the presence of 2X SSC and 40% formamide. This method also works well in cases of hybridisation involving large fragments.

In the case of hybridisation using a vessel destined to place the membrane in the presence of a small quantity of liquid, the vessel is initially filled with the solvent that is required for hybridisation to take place. The membrane is then fed into it through a thin aperture at the top.

The solvent is then drained off, and the probe solution is added. The amount of the solution needed depends on the size of the membrane. For wider membranes, this type of vessel is not suitable; instead, they can be hybridised inside a close-fitted cylindrical tube for transferring several gels that are too wide to be hybridised in this type of vessel. In case the hybridisation is carried out inside a water bath, the top is sealed as the water bath contains a lid. Moreover, another advantage is that, in this case, RNA can be reused.

The membrane is then subjected to hybridisation for a suitable period of time, depending on the concentration and purity of RNA, as well as other conditions. It is appropriate to leave it overnight in most cases. Once the hybridisation period is over, the membranes are removed and blotted against filter papers. Thereafter, they are washed for 20-30 min using a large amount of solvent used in hybridisation and at the same temperature at which hybridisation is carried out.

The excessive amount of the probe is washed using sodium chloride and buffers comprising detergents. If the salt concentration is lowered, the wash is considered to be more stringent, and a greater amount of such a nonspecific probe that is bound to the membrane is removed with a gradual increase in the number of stringent washes. The application of a high temperature while washing also increases the stringency of the wash. Once the wash is completed, only the specific hybrids remain on the blot, and the nonspecific ones are removed.

The membrane is checked for background using a radiation monitor. In case it is high, the membrane is treated with RNase for 30 min at 20 °C and then rinsed using 2X SSC. Finally, the membranes are air-dried. Detection time is approximately 1-48 h, which mostly depends on the nature of the probe in the detection of radioactivity of RNA. There are two options, namely 32P-labelled RNA detection and 3H-, 35S-, 125I-, or 14C-labelled RNA detection.

For the detection of 32P-labelled RNA, the membrane is wrapped in plastic with no air bubbles trapped in it and laid on X-ray film. With the application of slight pressure, the membranes are flattened.

For the detection of 3H-, 35S-, 125I-, or 14C-labelled RNA, fluorography is implemented. The membranes are soaked in a solution of PPO in toluene and then air-dried. Thereafter, they are laid on the X-ray film and maintained at -70 °C. The approximate timings for all the processes are crucial in obtaining good results. The restriction digestion usually takes 2-24 h, which can comprise overnight digestion. Similarly, in the case of electrophoresis, the timing can vary between 1-16 h and can be implemented overnight. The preparation for the transfer takes around 0.5-3 h. For setting up the transfer apparatus, approximately 15 min is required. Transfer time can be between 3-16 h, including overnight transfer. Blocking to reduce non-specificity can be done for up to 1 h. The hybridisation process takes 1-16 h, and depending on the probe nature and amount, it can continue

overnight. The detection takes around 1-48 h, which is affected by the yield in hybridisation and probe specificity.

TROUBLESHOOTING

For troubleshooting of the process, the following steps can be considered: the samples may leak out of the wells due to damage or puncture of the wells. One must be careful while removing the comb as well as while loading the gel.

Floating of the samples out of the well is commonly observed due to residual ethanol and also due to the loading of the gel in haste. The ethanol is to be completely removed before sample loading, and the gel should be loaded slowly.

The samples are not run straight, and bands have a frowned appearance. This may happen when the gel is run at too high a voltage, and the problem can be solved by choosing a lower voltage.

DNA, including the molecular weight markers, is not visible. This may be due to a lesser amount of ethidium bromide used, which can be resolved by using an appropriate concentration.

In cases where the molecular weight markers can be seen but the samples cannot be visualised, it may be that the DNA is degraded. The sample quality of the DNA must be analysed.

If the DNA as well as the molecular weight markers are not separated, it may be due to the fact that the gel is made up of water instead of 1× running buffer.

In the case of the appearance of a background with spots, it might be due to the powder from gloves; hence, gloves without powder are recommended. Increasing the number of washes can also eliminate the background. The signal intensity is lower. This might be due to less sample content, and hence, the DNA concentration should be increased.

REUSE OF BLOTS

In the case of the analysis of clinical samples where there is a lesser amount of target DNA, it might be advisable to re-use the blots. For this purpose, the probe can be removed by warm alkali treatment for a brief interval of time. Thereafter, the membrane undergoes a neutralisation step, and then it can be hybridised with a different probe.

Comparison of Southern Blotting with PCR and Chromatography

In most applications, PCR technology has replaced the Southern blotting technique. However, PCR technology is dependent on accurate DNA sequence information for the

design of specific primers, whereas Southern blotting is independent of such a requirement. Additionally, in PCR, if the design of the primers fails, considering the gene rearrangement that is being analysed, Southern blotting will be able to provide at least some information. In comparison to chromatography, the advantage of the Southern blot method is that it amplifies the detection limit levels that are necessary or useful for investigating particular genomic loci. However, this method faces drawbacks, such as the requirement of a high starting concentration of target DNA, and it is also dependent on the efficiency of the restriction enzyme in use, which in turn affects the results.

RESULTS

The hybridisation of the probe to the specific DNA on the membrane results in a conclusion that the probe is specific to a fragment on the membrane due to the presence of a complementary sequence in the fragment. A probe that has hybridised with a single fragment of DNA not being digested by restriction enzymes will result in only one band in the final blot. In cases where the probe binds to many similar sequences, it will result in multiple bands. The probe specificity, as well as decreased non-specific hybridisation, is generally modulated by optimising the temperature at which the hybridisation is carried out, and the salt concentration also needs to be regulated.

APPLICATION

Southern blotting is used in a number of applications. The primary usage of Southern blotting is to identify a specific DNA sequence in a DNA sample. It is mostly used in the identification of viral infections and certain bacterial infections. In rDNA technology, the Southern blotting technique is used to isolate a particular DNA fragment. It is also useful in the study of mutations and gene rearrangements; this property is used to diagnose neonatal diseases and genetic disorders. Due to the precision in DNA identification, this technique is used in phylogenetic studies, paternity and maternity analysis, forensic studies, and personal identification.

Southern blotting can be applied in studying the structure of a gene or to elucidate restriction enzyme maps. Particularly, Southern blotting can be used in the identification of the methylated sites present in some genes in particular. Applying restriction nucleases such as MspI and HpaII, which can both identify and cleave among the identical sequences, can implement this technique. The discovery of RFLPs by Southern blotting has helped in the mapping of several genomes that were crucial to be mapped. In the arena of immunology, the clonal rearrangements of the immunoglobulins, as well as the T cell receptor genes that

play an important role in eliciting an immune response, can be analysed by Southern blotting.

CONCLUSION

Southern blot hybridisation remains a foundational technique in molecular genetics, providing valuable insights into DNA structure and function. Although newer, faster methods such as PCR and next-generation sequencing have emerged, Southern blotting continues to be used in specific applications where precise DNA analysis is required. Its ability to detect and analyse specific DNA sequences has made it an indispensable tool in genetic research, forensic science, and medical diagnostics. These sources cover the principles, methodology, and applications of Southern blot hybridisation in molecular biology and genetics. If you need more specific references or access to full texts, let me know.

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Contribution to Knowledge

This article contributes to scientific knowledge by providing a comprehensive, updated synthesis of the principles and applications of Southern blot hybridisation, emphasising its continued relevance in modern molecular biology despite the rise of advanced sequencing technologies. It highlights improvements in membrane chemistry, probe labelling, and hybridisation strategies that have enhanced the specificity, sensitivity, and safety of the technique. The work also clarifies the unique strengths of Southern blotting, particularly its ability to analyse large genomic fragments, detect structural variations, and validate genetically engineered constructs, which are essential in microbial genetics, clinical diagnostics, and forensic science. By integrating classical methodology with modern advancements, this study provides researchers, students, and laboratory professionals with a clearer understanding of how Southern blotting remains a gold-standard reference technique in contemporary genomic research.

Conflicts of Interest

The author declares that there are no conflicts of interest regarding the publication of this article. No financial, personal, or institutional relationships influenced the research, writing, or interpretation of the content presented in this work.

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