Swayam Course - Analytical Techniques

Week 11, Tutorial 29 - Blotting techniques for Nucleic acids

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Introduction

Blotting is a method in which a macromolecule is immobilized on a solid matrix and subsequently probed with a detectable ligand to determine whether the macromolecule binds specifically to its ligand. Depending on whether the immobilized macromolecule is DNA, RNA or protein, one generates DNA blots (Southern blots), RNA blots (Northern blots) (1), or protein blots (Western blots). The macromolecule can be applied to the blotting matrix directly (dot blot), or it can be derived and eluted from an electrophoretic gel (gel blot).

Blotting techniques are used to separate DN, RNA and protein types of molecules. In cells, they exist as a mixture. Blotting allows researchers to find one protein among many, like a needle in a haystack. Blotting is generally done by letting a mixture of DNA, RNA or protein flow through a slab of gel. This gel allows small molecules to move faster than bigger ones. The separated molecules are then pressed against a membrane, which helps move the molecules from the gel onto the membrane. The molecules stick to the membrane, but stay in the same location, apart from each other, as if they were still in the gel.

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Introduction :

Blotting is a common laboratory procedure in which biological molecules in a gel matrix are transferred onto nitrocellulose or nylon membrane for further scientific analysis. The biological molecules transferred in this process are DNA, RNA or proteins. The blotting procedure is named differently depending on the type of the molecules being transferred. When DNA fragments are transferred the procedure is called a Southern bl, named after Edward Southern that first developed it. The Northern blotting procedure, which transfers RNA molecules, was developed shortly thereafter and humorous named Northern blotting.

Western blotting involves the transfer of proteins. All blotting procedures begin with a standard process called gel electrophoresis when DNA, RNA, or proteins are loaded on to an agarose or acrylamide gel and separated on the gel through an electric field. Two types of gels are commonly used: agarose gels and acrylamide gels. Transfer is initiated when nitrocellulose or nylon membrane is laid on top of the gel and biological molecules are transfer from the gel to the membrane. Hybridization / blotting is a technique in which biological molecular (DNA, RNA or protein) are immobilized onto a nylon or nitrocellulose membrane. A probe (a piece of nucleic acid with identical and specific sequence to the organism or gene of interest) can then hybridize (join) to the biological molecules (DNA, RNA or protein) with identical sequence on the membrane.

The hybridization between the blotted DNA and probe is visualized by labeling the probe in some way.Short fragments of DNA that have a nucleotide sequence complementary to the molecule being analyzed are normally used as probes in Southern and Northern blots.

Blotting technique is an extremely powerful tool for analyzing gene structure and used to study gene expression, once cloned cDNA is isolated..

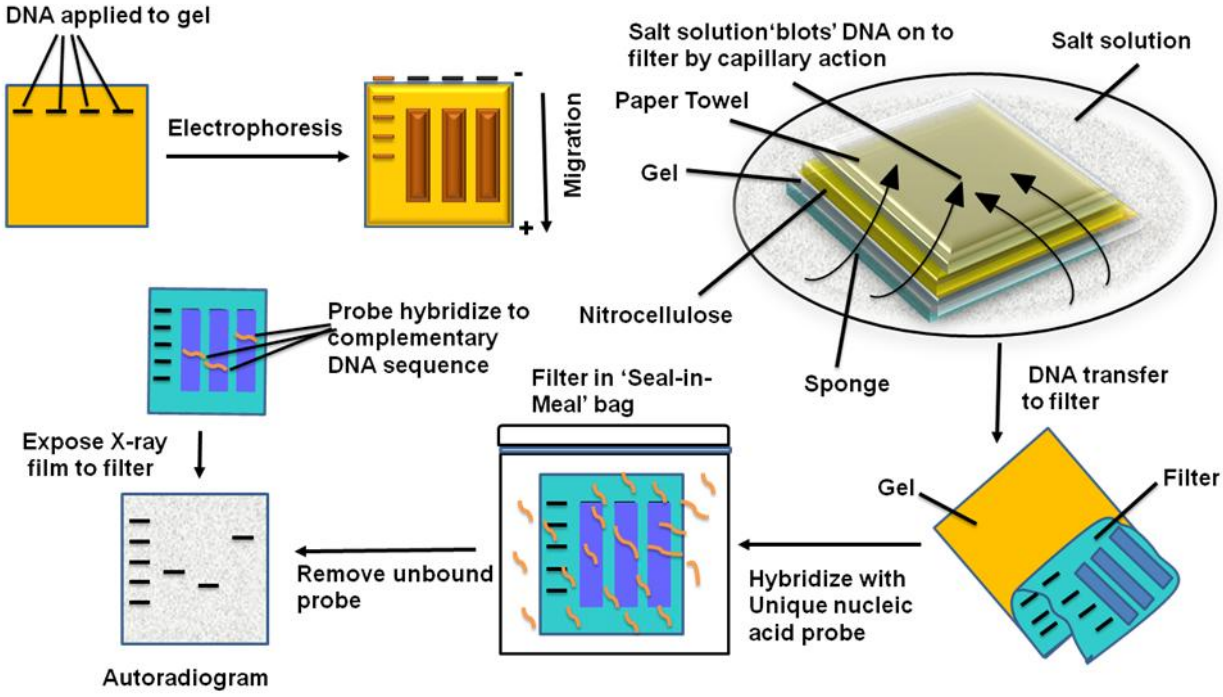
Technique # 1. Southern Blotting:

Developed by E.M. Southern, the technique of Southern blotting is one of the most important methods used in molecular biology. In Southern blotting, DNA is transferred from a gel to a

membrane for hybridization analysis. In this technique, the DNA is cut with suitable restriction enzymes and run on a gel. Treatment with NaOH denatures the DNA to form single strand.

- Southern blotting is an example of RFLP (restriction fragment length polymorphism).. Southern blotting is a hybridization technique for identification of particular size of **DNA** from the mixture of other similar molecules**.** This technique is based on the principle of separation of DNA fragments by gel electrophoresis and identified by labelled probe hybridization.
- Basically, the DNA fragments are separated on the basis of size and charge during electrophoresis. Separated DNA fragments after transferring on nylon membrane, the desired DNA is detected using specific DNA probe that is complementary to the desired DNA.
- A hybridization probe is a short (100-500bp), single stranded DNA. The probes are labeled with a marker so that they can be detected after hybridization.

The transfer of DNA from agarose gel to the membrane is performed by capillary action. The gel is placed above the buffer saturated filter paper. The nitrocellulose membrane is placed above the gel and covered by 2-3 layers of dry filter paper towel. A flow of buffer occurs through the gel and membrane to the top papers.



<u>Work flow for Southern Blot</u>

Step 1: DNA digestion

Obtaining complete fragmentation of your DNA at the intended restriction enzyme sites is a critical step in Southern blot analysis.

Step 2: Gel electrophoresis

Fragmented DNA is typically electrophoresed on an agarose gel to separate the fragments according to their molecular weights. Acrylamide gels can alternatively be used for good resolution of smaller DNA fragments (<800 bp).

Agarose gel electrophoresis has proven to be an efficient and effective way of separating nucleic acids.

Agarose's high gel strength allows for the handling of low percentage gels for the separation of large DNA fragments.

Molecular sieving is determined by the size of pores generated by the bundles of agarose[7] in the gel matrix. In general, the higher the concentration of agarose, the smaller the pore size.

Traditional agarose gels are most effective at the separation of DNA fragments between 100 bp and 25 kb. To separate DNA fragments larger than 25 kb, one will need to use pulse field gel electrophoresis, which involves the application of alternating current from two different directions.

In this way larger sized DNA fragments are separated by the speed at which they reorient themselves with the changes in current direction. DNA fragments smaller than 100 bp are more effectively separated using polyacrylamide gel electrophoresis.

Unlike agarose gels, the polyacrylamide gel matrix is formed through a free radical driven chemical reaction. These thinner gels are of higher concentration, are run vertically and have better resolution.

In modern DNA sequencing capillary electrophoresis is used, whereby capillary tubes are filled with a gel matrix. The use of capillary tubes allows for the application of high voltages, thereby enabling the separation of DNA fragments (and the determination of DNA sequence) quickly.

Step 3: Blotting

After electrophoresis, DNA is transferred to a positively charged nylon membrane. Traditional transfer of DNA is done overnight using an upward-transfer method. For reliable and consistent transfer with minimal background, Nylon Membranes are highly recommended. The membranes are ideal for use with radiolabeled and nonisotopic probes to achieve maximum hybridization signal.

A range of membranes is now available for hybridization experiments.

Nitrocellulose, Originally was the most widely used membrane for its sensitivity and low background signal.

Single Stranded DNA(ssDNA) is immobilized on Nitrocellulose membrane under conditions of High Salt(> 1M Nacl) and must be heated at 800C in a vacuum to irreversibly attach nucleic acid.

Nylon based membranes are physically much stronger and can bind all nucleic acids under a wide range of salt concentration

Irreversible or covalent attachment can be achieved by UV irradiation for 5min or less by treatment with 0.4M NaOH.

Step 4: Probe labeling

A nucleic acid probe with sequence homologous to the target sequence under study is labeled with radioactivity, fluorescent dye, or an enzyme that can generate a chemiluminescent signal when incubated with the appropriate substrate. The choice of the label depends on several factors such as the nature of your probe or probe template, sensitivity needed, quantification requirements, ease of use, and experimental time.

VARIOUS LABELLING METHODS:

- **Radioactive Detection**, Originally 32P was routinely used because of its high energy and ease of incorporation into the phosphate groups of dNTPs. Radioactively labeled probes are detected using X-ray film.

Disadvantages of 32P are its short half-life(about 2 weeks), contamination problems (all materials and equipment must be dedicated to radioactive work only), and expense of disposal of radioactive waste.

Many different non-radioactivedetection systems are available, depending on the label used.

- **Non-radioactive detection** are colourimetric, fluorescent, and chemiluminescent.

- **Colourimetric detection** generally involves the production of a coloured precipitate which can be seen with the naked eye. In a typical system, the DNA probe itself is labeled with an antigen such as digoxigenin; following hybridization to its target it would be exposed to an anti-digoxigenin antibody conjugated to an enzyme capable of catalyzing a colourimetric reaction
- **Fluorescent detection** involves probes which are directly labeled with fluorophores, or more likely, probes which are coupled to fluorescent molecules indirectly.

  Fluorophores emit light when excited by light of an appropriate wavelength; the emitted light can then be detected.

- **Chemiluminescence** is the result of a chemical or enzymatic reaction that triggers the release of ordinary visible light which can then be detected.

  Examples include firefly luciferase and enhanced chemiluminescence (ECL) using horseradish peroxidase enzyme (HRP).In a luciferase reaction, light is emitted when luciferase acts on the appropriate luciferin substrate.

  HRP catalyzes the conversion of enhanced chemiluminescent substrate into a sensitized reagent, which, on further oxidation by hydrogen peroxide, produces a triplet carbonyl group which emits light when it decays to the singlet carbonyl.

HOW ARE DNA PROBES LABELLED(Southern Blotting)?

The label (radioactive or non-radioactive) is attached to one of the four nucleotides (dNTPs) and then incorporated into the probe using one of the following methods:

PCR labeling is as traight forward and now very popular method of incorporating labeled nucleotides into a probe. Primers are designed to amplify the desired probe sequence, and a labeled dNTP is simply included during the PCR.

Nick translation involves the action of two enzymes (DNaseIand DNA polymerase I) on the double-stranded DNA (dsDNA) to be labeled in the presence of a labeled nucleotide. Under conditions designed to limit its activity, DNaseI will randomly introduce a few nicks (i.e. single strand breaks) in the dsDNA backbone. These nicks are repaired by DNA polymerase I.DNA polymerase I synthesizes DNA in a 5'-3' direction and it also has exonuclease activity. These two activities act together to remove and then replace a few nucleotides down from each nick, incorporating the labeled dNTP in the process. The newly formed bits of DNA are now labeled and can be used as a probe.

Random oligo primed synthesisis a technique in which dsDNA is denatured into single strands and annealed to random hexamer oligonucleotides. These randomprimers can then be extended using DNA polymerase (Klenow), incorporating labeled nucleotides .

End labeling involves techniques in which one end of a DNA (or RNA) molecule is specifically labeled. The 5' or 3' end can be labeled.With 5' end labeling a terminal phosphate group (usually radioactive 32P) is donated from a dNTP to the 5'–OH group, and with 3' ends a small chain of identical labeled nucleotides are added.

Step 5: Hybridization & washing

Hybridization (procedure where probe and membrane are incubated to allow annealing of probe to immobilizedDNA/RNA on membrane) and washes (the probe that did not bind or bound non-specifically to DNA/RNA on membrane must be washed from the membrane) must be done at specific temperatures.

All double-stranded nucleic acids -whether dsDNA, dsRNAor RNA:DNA hybrids -have specific "melting temperatures", which depend mainly on their specific guanine + cytosine (G + C) content, but also on whether they are DNA, RNA, or a mixture (RNA:RNA hybrids have the highest melting temperatures, followed by DNA:RNA hybrids, then dsDNA), and on the ionic strength of the solution.

Based on the composition of the DNA sequence, the melting temperature (Tm) of the probe must be determined.

During hybridization, the labeled probe is incubated with the DNA fragments that are immobilized on the blot under conditions that promote hybridization of complementary sequences. When used for both prehybridization and hybridization, can increase sensitivity up to 100 times compared to other hybridization solutions by pushing hybridization to completion without increasing background. As few as 10,000 target molecules can be detected. Because ULTRAhyb® buffer maximizes blot sensitivity, for many targets hybridization can typically be performed in just 2 hours.

After hybridization, the unhybridized probe is removed by washing in several changes of buffer. Low stringency washes (e.g., with 2X SSC or SSPE) remove the hybridization solution and unhybridized probe. High-stringency washes (e.g., with 0.1X SSC or SSPE) remove partially hybridized probe molecules. The result is that only fully hybridized labeled probe molecules, with complementary sequence to the region of interest, remain bound.

Step 6: Detection

In the detection step, the bound, labeled probe is detected using the method required for the particular label used. For example, radiolabeled probes may be detected using X-ray film or a phosphorimaging instrument, and enzymatically labeled probes are typicallly detected by incubating with a chemiluminescent substrate and exposing the blot to X-ray film.

Applications of Southern blotting:

1. Southern blotting technique is used to detect DNA in given sample.
2. DNA finger printing is an example of southern blotting.
3. Used for paternity testing, criminal identification, victim identification
4. To isolate and identify desire gene of interest.
5. Used in restriction fragment length polymorphism
6. To identify mutation or gene rearrangement in the sequence of DNA
7. Used in diagnosis of disease caused by genetic defects
8. Used to identify infectious agents

Applications in research :

A relatively new method for the diagnostic characterization of malignant lymphomas was established with the introduction of gene probes usinfg Souther Blot for the immunoglobulin and T cell receptor gene segments . Though the method is not absolutely specific for the determination of lineage and clonality of a given lymphoid neoplasm, it provides a lot of additional information for the pathologist. It is the first method that gives proof to the clonality of T cell lymphomas; reactive lymph node processes can be distinguished from true neoplasms; within lymph nodes of mixed lymphoid populations the clonally proliferated can be detected and the lineage can be determined. Therefore the Southern Blot Analysis is a method that should be applied for the diagnosis of malignant lymphomas together with histology and immunohistochemistry. Using the combination of all these methods an extensive characterization of lymphoid neoplasms can be made.

Reference : Pathol Res Pract. 1989 Apr;184(4):455-63.

Southern Blotting Applications in Medical Microbiology

Southern blotting is primarily used for DNA fingerprinting, gene sequencing and genetic engineering.

It has been used in the identification of strains in microbes such as concluding the type of human papillomavirus extracted from a condyloma.

However in this case it provided to be unreliable as it produced false-negatives, as PCR and in-situ hybridisation were deemed to be more efficient .

Another use of Southern blotting was in the detection of a strain of Listeria monocytogenes. In this study it was deemed an important technique in verifying species identification and in the characterization of epidemic strains .

This method can be used to DNA fingerprint most microbes and determine a diagnosis and treatment for a patient suffering from their pathogenicity. However it is found to be too laborious, time consuming and requires large amounts of high quality DNA for most routine laboratories yet variations of this technique are still widely performed.

The western blot, which uses antibodies as the probe to detect proteins instead of DNA, is a confirmatory test in the diagnosis of a human immune-deficiency virus(HIV) infection . Since the established use of PCR there has become a decreased need for these laborious techniques in DNA sequencing.

Technique # 2. Northern Blotting:

RNA Isolation

A northern blot is a laboratory method used to detect specific RNA molecules among a mixture of other population of RNA. Northern blotting can be used to analyze a sample of RNA from a particular tissue or cell type in order to measure the RNA expression of particular genes. This method was named for its similarity to the technique known as a Southern blot.

The first step in a northern blot is to denature, or separate, the RNA within the sample into single strands, which ensures that the strands are unfolded and that there is no bonding between strands. The RNA molecules are then separated according to their sizes using a method called gel electrophoresis. Following separation, the RNA is transferred from the gel onto a blotting membrane. (Although this step is what gives the technique the name "northern blotting," the term is typically used to describe the entire procedure.) Once the transfer is complete, the blotting membrane carries all of the RNA bands originally on the gel. Next, the membrane is treated with a small piece of DNA or RNA called a probe, which has been designed to have a sequence that is complementary to a particular RNA sequence in the sample; this allows the probe to hybridize, or bind, to a specific RNA fragment on the membrane. In addition, the probe has a label, which is typically a radioactive atom or a fluorescent dye. Thus, following hybridization, the probe permits the RNA molecule of interest to be detected from among the many different RNA molecules on the membrane.

Obtaining high-quality, intact RNA is a critical step in performing northern blot analysis. All protocols, techniques, and commercially available kits used to isolate RNA share these common attributes:

- Cellular lysis and membrane disruption
- Inhibition of ribonuclease activity
- Deproteinization
- Recovery of intact RNA

The detail steps involved are :

The steps involved in Northern analysis include:

- RNA isolation (total or poly(A) RNA)
- Probe generation
- Denaturing agarose gel electrophoresis
- Transfer to solid support and immobilization
- Prehybridization and hybridization with probe
- Washing
- Detection
- Stripping and reprobing (optional)

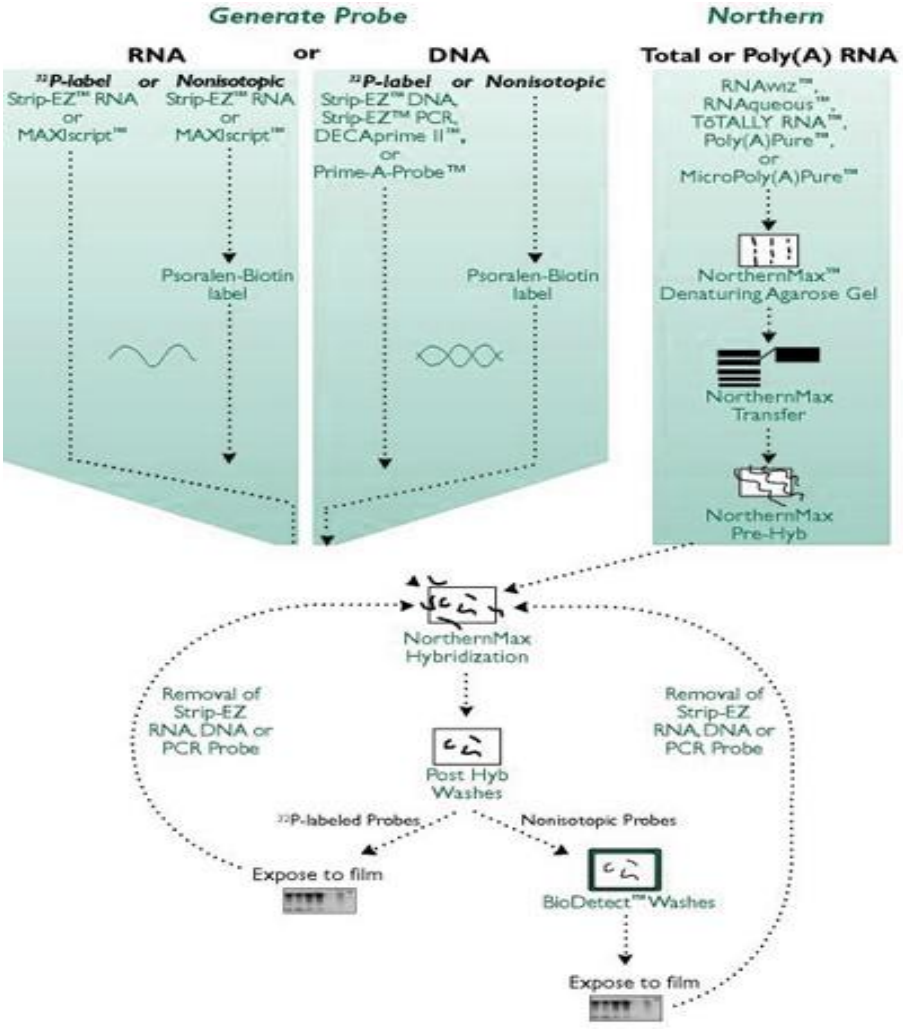
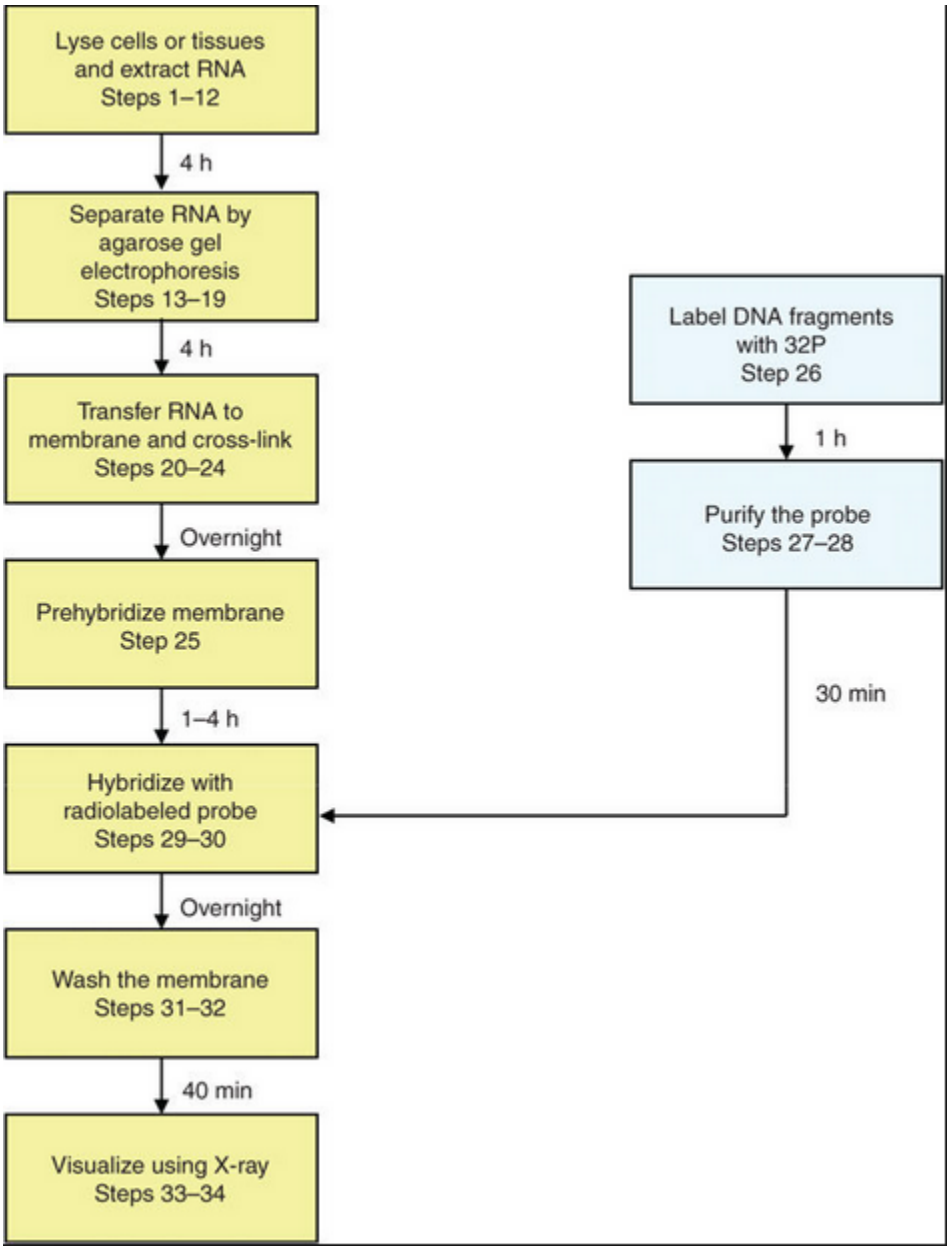


Image adapted from : https://www.thermofisher.com

Northern blots can be probed with radioactively or nonisotopically labeled RNA, DNA or oligodeoxynucleotide probes. Research at Ambion has revealed startling differences in the signal sensitivities on Northern blots achieved by three methods of probe synthesis when using standard formamide or aqueous hybridization buffers — random-priming of DNA, asymmetric PCR-generated DNA and in vitro transcription of RNA. While probes for Northerns and Southerns have been historically synthesized by random-primed labeling, our results indicate that probes synthesized by asymmetric PCR are 3-5 fold more sensitive than random-primed probes, and that RNA probes provide an additional 10-fold increase in sensitivity. RNA probes have the added advantage that they can be hybridized and washed under more stringent conditions, which results in lower background and fewer problems with cross-hybridization.



Applications for Northern Blot :

- Southern blots are used in gene discovery , mapping, evolution and development studies, diagnostics and forensics (It is used for DNA fingerprinting, preparation of RFLP maps)
- identification of the transferred genes in transgenic individuals, etc.
- It is an invaluable method in gene analysis.
- Important for the conformation of DNA cloning results.
- Highly useful for the determination of restriction fragment length polymorphism (RFLP) associated with pathological conditions.

Difference between Norther and Southern Blots :

Southern blotting	Northern blotting
Southern name of inventor	Northern a misnomer
Separation of DNA	Separation of RNA
Denaturation needed	Denaturation not needed
Nitrocellulose filter membrane	Amino benzyloxymethyl filter paper Membrane
DNA-DNA Hybridization	RNA-DNA Hybridization

Technique #3 Reverse North Blotting:

Reverse Northern blot is a variant of the regular Northern blot

- Isolated DNA fragments is the substrate (that is affixed to the membrane)
- the probe is RNA extracted from a tissue and radioactively labelled.

RNA is converted to cDNA before hybridization.

- The use of DNA microarrays that have come into widespread use in the late 1990s and early 2000s is more akin to the reverse procedure, in that they involve the use of isolated DNA fragments affixed to a substrate, and hybridization with a probe made from cellular RNA.
- Thus the reverse procedure, though originally uncommon, enabled northern analysis to evolve into gene expression profiling, in which many (possibly all) of the genes in an organism may have their expression monitored.

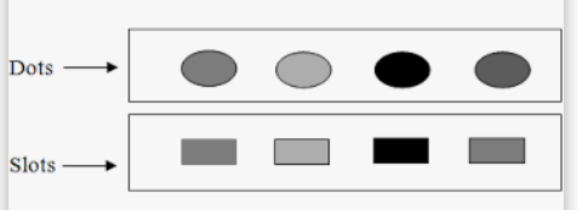
Technique No. 4 DOT/SLOT BLOT

Dot and slot blotting are simple techniques for immobilizing bulk unfractionated DNA on a nitrocellulose or nylon membrane.

Does not involve any electrophoresis step.

Hybridization analysis can then be carried out to determine the relative abundance of target sequences in the blotted DNA preparations. The protocol is based on a sample denaturation by temperature (i.e., PCR products or crude extracts) and the direct transfer to the membrane by the use of a dot-blot or slot-blot apparatus, without any previous electrophoretic separation.

Schematic representation of Dot and Slot Blot



Applications of Dot and Slot Blot:

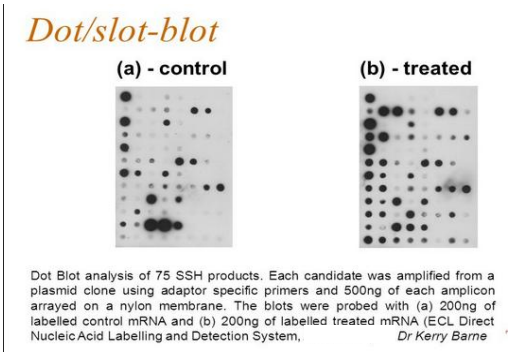
Dot Blot:

- Amplification analysis
- Expression analysis(RNA)
- Mutation analysis

Slot Blots:

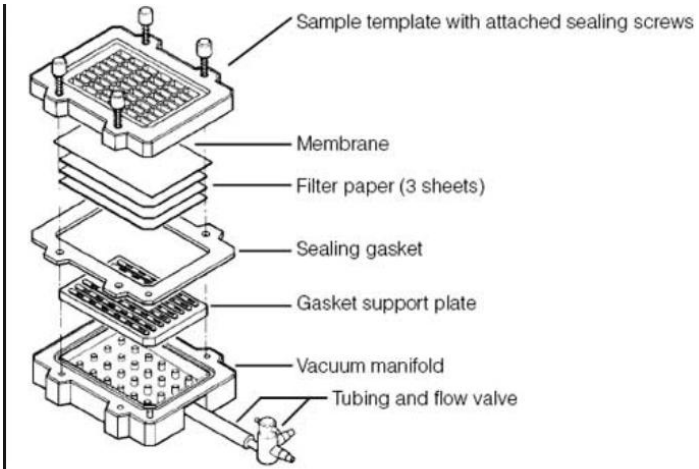
- Amplification analysis
- Expression analysis

Representation of the data output from Dot/slot blot



Dot Blot analysis of 75 SSH products. Each candidate was amplified from a plasmid clone using adaptor specific primers and 500ng of each amplicon arrayed on a nylon membrane. The blots were probed with (a) 200ng of labelled control mRNA and (b) 200ng of labelled treated mRNA (ECL Direct Nucleic Acid Labelling and Detection System, *Dr Kerry Barne*

Figure showing the arrangement of a Dot/slot Blot experiment



DOT BLOT APPARATUS

SLOT BLOT APPARATUS

Technique No. 5 Reverse DotBlot Hybridization

Amplification and labeling of DNA sequence of Interest

Immobilzation to Nylon/PVDF membrane

Hybridization to oligonucleotides(the probe)

Technique No. 6 Southwestern and NorthWestern Blotting

Southwestern blotting, based along the lines of Southern blotting (which was created by Edwin Southern) and first described by B. Bowen, J. Steinberg and colleagues in 1980, is a lab technique which involves identifying and characterizing DNA-binding proteins (proteins that bind to DNA) by their ability to bind to specific oligonucleotide probes.

The proteins are separated by gel electrophoresis and are subsequently transferred to nitrocellulose membranes, similar to other types of blotting.

The name *southwestern blotting* is based on the fact that this technique detects DNA-binding proteins, since DNA detection is by Southern blotting and protein detection is by western blotting.

The genomic DNA region of interest is digested by restriction enzymes selected to produce fragments of appropriate but different sizes, which are subsequently end-labeled and allowed to bind to the separated proteins.

The specifically-bound DNA is eluted from each individual protein-DNA complex and analyzed by polyacrylamide gel electrophoresis.

The **Northwestern blot**, also known as the Northwestern assay, is a hybrid analytical technique of the Western blot and the Northern blot, and is used in molecular biology to detect interactions between RNA and proteins.

RNA binding proteins are separated by gel electrophoresis, transferred to a membrane and probed with a labeled RNA of interest.

SUMMARY:

1. Blotting is a method in which a macromolecule is immobilized on a solid matrix

and **s**ubsequently probed with a detectable ligand to determine whether the

macromolecule binds specifically to its ligand.

2. Depending on whether the immobilized macromolecule is DNA, RNA or protein,

Blotting techniques can be divided into three main categories

- DNA blots (Southern blots),
- RNA blots (Northern blots)
- protein blots (Western blots).

3. Southern blotting is an example of RFLP (restriction fragment length

polymorphism). It was developed by Edward M. Southern (1975).

4. Southern blotting is a hybridization technique for identification of particular size of **DNA** from the mixture of other similar molecules**.**

5. This technique is based on the principle of separation of DNA fragments by gel electrophoresis and identified by labelled probe hybridization.

6. Basically, the DNA fragments are separated on the basis of size and charge during electrophoresis. Separated DNA fragments after transferring on nylon membrane, the desired DNA is detected using specific DNA probe that is complementary to the desired DNA.

7. A hybridization probe is a short (100-500bp), single stranded DNA. The probes are labeled with a marker so that they can be detected after hybridization.

8. Some (but certainly not all) applications of southern blot that you cant easy do with PCR,

Detecting multiple homologous genes in a genome - PCR tends to be very specific and when two bands are seen, it is more likely non-specific or contamination

9. Detecting orthologous or paralogous genes in similar or distant species where you might not know anything about the sequence divergence and hence primer sites (you can increase or decrease specificity depending on hybridization conditions) - if you dont know the primer sites, you cant do this

10. . Detecting insertions of a plasmid/viral vector when making stable transgenics and determining copy number (similar to #8 really). Cant with normal PCR, qPCR you could

a. Easier to multiplex / detect multiple products

b. you can analyse methylation patterns that affect restriction sites (as you digest with a restriction enzyme before probing)

11. Northern blot analysis is useful for revealing the size of the mRNA encoded by a gene.

12. In some cases, a number of different mRNA species encoded by the same gene have been identified in this way, suggesting that different promoter sites or different terminator sites are used or that alternative mRNA processing can take place.

13. . Northern blot analysis can be used to investigate whether an mRNA is present in a cell type or tissue and in how much quantity it is present.

. It is useful for determining the levels of gene activity.

Example: During development, in different cell types of organisms, or in cells before and after they are subjected to various physiological stimuli.

14. Reverse Northern Blotting uses isolated DNA fragments as the substrate (that is affixed to the membrane) and the probe is RNA extracted from a tissue and radioactively labelled.

RNA is converted to cDNA before hybridization.

15. Southern Blotting is based on the fact that this technique detects DNA-binding proteins, since DNA detection is by Southern blotting and protein detection is by western blotting.

16. Dot and slot blotting are simple techniques for immobilizing bulk unfractionated DNA on a nitrocellulose or nylon membrane. Does not involve any electrophoresis step.

17. Hybridization analysis can then be carried out to determine the relative abundance of target sequences in the blotted DNA preparations.

18. The **Northwestern blot**, also known as the Northwestern assay, is a hybrid analytical technique of the Western blot and the Northern blot, and is used in molecular biology to detect interactions between RNA and proteins.

END OF MODULE