Southern blotting

Southern blotting is one of the central techniques in molecular biology. First devised by E. M. Southern (1975). Southern blotting is the transfer of DNA fragments (usually restriction fragments) from an electrophoresis gel to a membrane support, resulting in immobilization of the DNA fragments, so the membrane carries a semipermanent reproduction of the banding pattern of the gel. Southern blotting makes upward capillary transfer of DNA from an agarose gel onto a nylon or nitrocellulose membrane, and subsequent immobilization by UV irradiation (for nylon) or baking (for nitrocellulose). After immobilization, the DNA can be subjected to hybridization analysis, enabling bands with sequence similarity to a labeled probe to be identified. Southern blotting is therefore a method for 'detection of a specific restriction fragment against a background of many other restriction fragments.'

Procedure

1. DNA isolation and Digestion of the DNA

Genomic DNA is isolated from cells or tissue with appropriate protocol and become extensively fragmented by random shearing during the extraction process, so that specific restriction fragments of 20 kb and more can be obtained. DNA has been treated with one or more restriction endonucleases.

2. Run the digest on an agarose gel

Restriction fragments are fractionated by agarose gel electrophoresis and the gel then pretreated prior to setting up the Southern blot.

3. Pretreatment of DNA

The pretreatment (usually while it is still on the gel) has two objectives.

First, it is desirable to break the DNA molecules in individual bands within the gel into smaller fragments, because smaller fragments transfer more quickly than larger ones. This is achieved by soaking the gel in 0.25 mol L^{-1} HCl for 30 mins, which results in a small amount of depurination – cleavage of the β-N-glycosidic bond between purine bases (adenine or guanine) and the sugar component of their nucleotides – which is followed by decomposition of the sugar structure and breakage of the polynucleotide chain.

The second pretreatment is with an alkaline solution that denatures the double-stranded DNA molecules by breakage of their hydrogen bonds, so the molecules become single-stranded. This aids their transfer and subsequent binding to the membrane, and also ensures that after binding the base-pairing components of the polynucleotides are available for hybridization with the probe.

Note: If a nitrocellulose membrane is being used then the alkali pretreatment is followed by neutralization of the gel by soaking in a Tris-salt buffer, this step being essential because DNA does not bind to nitrocellulose at a pH of greater than 9.0.

4. Transfer the denatured DNA to the membrane

Traditionally, a nitrocellulose membrane is used, although nylon or a positively charged nylon membrane may be used. Nitrocellulose typically has a binding capacity of about 100µg/cm, while nylon has a binding capacity of about 500 µg/cm. Many scientists feel nylon is better since it binds more and is less fragile. Transfer is usually done by capillary action, which takes several hours. Capillary action transfer draws the buffer up by capillary action through the gel an into the membrane, which will bind ssDNA.

Vacuum blot apparatus may be used instead of capillary action. In this procedure, a vacuum sucks SSC through the membrane. This works similarly to capillary action, except more SSC goes through the gel and membrane, so it is faster (about an hour).

Note: SSC means the high salt transfer buffer needed to transfer DNA. It comprises 3.0 mol L^{-1} NaCl and 0.3 mol L^{-1} sodium citrate. The same buffer can be used for transfer to a nylon membrane. For a positively charged nylon membrane an alkaline transfer buffer (0.4 mol L^{-1} NaOH) is used because, as described earlier, this results in immediate covalent attachment of the transferred DNA to the membrane. With this type of transfer the alkali pretreatment is unnecessary. The blot is then left for at least 18 h for a high-salt transfer or 2 h for an alkaline blot.

5. Immobilization of DNA

After blotting, the transfer setup is dismantled and the membrane rinsed in 2 X SSC and left to dry. If the blot has been made onto a nitrocellulose or uncharged nylon membrane, then the DNA is only loosely bound to the membrane at this stage. After transfer of DNA to the membrane (nylon), it is treated with UV light. This help to cross links (via covalent bonds) the DNA to the membrane. Membrane can be baked (nitrocellulose) at about 80°C for a couple of hours, but be aware that it is very combustible (noncovalent but semi-permanent attachment of DNA to a nitrocellulose membrane).

6. Prehybridization

To block the unused DNA binding sites on the membrane, the prehybridization step is necessary. If this step is omitted, then the probe will bind nonspecifically to the surface of the membrane and the signal resulting from hybridization to the specific restriction fragment will be difficult if not impossible to identify. To prehybridize, non-specific ssDNA is added to the membrane. Sonicated salmon sperm DNA is commonly used (unrelated to the one whose DNA has been blotted).

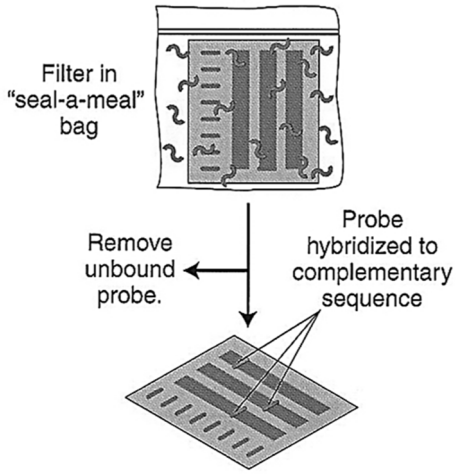
Denhardt's solution containing Ficoll (biological polymers, a carbohydrate-based compound) and Polyvinylpyrrolidone (nonbiological polymeric compounds) and BSA (bovine serum albumen, a

non-specific protein), SDS (sodium dodecyl sulfate), and formamide (destabilizing agent) are added during prehybridization process.

Prehybridization takes between 15 min and 3 h at 68°C, depending on the type of membrane.

7. Probe the membrane with labeled ssDNA (hybridization) and posthybridization washing

A hybridization probe is a short, single stranded DNA. The probes are labeled with a marker so that they can be detected after hybridization. This process relies on the ssDNA hybridizing (annealing) to the DNA on the membrane due to the binding of complementary strands. Hybridization analysis is carried out by soaking the Southern blot in a buffer containing the hybridization probe, usually in a tube that is constantly rotated so all parts of the membrane are exposed to the probe, or alternatively in a sealed plastic bag that is placed on a shaker.



Prehybridization buffer is used during hybridization step. Altering the concentrations of formamide, SSC, temperature and SDS affects "stringency," or specificity. Actual hybridization, which is carried out in a high-salt buffer containing a detergent, usually 2 X SSC plus 1% SDS.

Two issues are critical at this stage. First, enough probe DNA must hybridize to the target restriction fragment to produce a clear signal that can be discerned by the detection system appropriate for the label carried by the probe. The second critical factor that must be considered during the hybridization step is the specificity of the reaction. If the probe DNA has been carefully chosen, then it will contain a region that is completely complementary to all or a part of the blotted restriction fragment that is being sought. If this hybridizing region in the probe is not completely complementary to the target, then it will at least have a region of strong similarity so that a stable hybrid can form. The problem is that the probe also has the potential to hybridize to any other blotted DNA fragments with which it has partial complementarity.

If the probe is >100 bp in length, for example a cloned restriction fragment, then the initial hybridization is usually carried out at 68°C in a high-salt buffer, this representing highly stringent conditions under which only stable hybrids are expected to form, with very little if any nonspecific hybridization. If a short oligonucleotide probe is used (15–25 nucleotides in length), then the hybridization step is usually carried out under conditions of low stringency (typically at a temperature several degrees below the calculated Tm for the desired hybrid), so that all potential hybrids, including nonspecific ones, are able to form. Specificity is then achieved by a series of washes (posthybridization washes) at increasing temperatures so that, hopefully, only the desired hybrid remains at the end of the procedure.

Probing is often done with ^{32}P labeled ATP, biotin/streptavidin or a bioluminescent probe.

Note:

A. Radioactive method

i. Probe with ^{32}P labeled ATP (Nick Traslation)

Treat the dsDNA fragment that you are using as a probe with a limiting amount of DNAse, which causes double-stranded nicks in DNA. Add ^{32}P, dATP, and other dNTPs to DNA polymerase I, which has 5' to 3' polymerase activity and 5' to 3' exonuclease activity.

Nick translation occurs and as the nick is translated down the DNA strand, the polymerase activity continues to nick while the exonuclease activity continues to fill in the nick. As this happens, ^{32}P becomes incorporated into, and thus labels, the DNA. Heat the DNA to make it single stranded, then immediately place it on ice to keep the two strands from reannealing to each other.

ii. **Random oligo primed synthesis**

DNA is denatured into single strands and annealed to random hexamer oligonucleotides. These random primers can then be extended using DNA polymerase (Klenow), incorporating labeled nucleotides.

iii. **End labeling**

The end of a DNA (or RNA) molecule is specifically labeled. The 5’ end can be labeled with the enzyme polynucleotide kinase, which donates the terminal (gamma) phosphate group (usually radioactive ^{32}P) from a dNTP to the 5’ OH. Note that only one marked residue per probe molecule is incorporated by this method, so the specific activity of the label (radioactive counts per minute per microgram of DNA) is lower than in the other methods.

Disadvantages of ^{32}P

i. Short half-life (about 2 weeks) means probes must be used immediately, and the labeling reagent cannot be stored for long.
ii. Contamination problems: all materials and equipment must be dedicated to radioactive work only. Regular lab-wide testing for contamination is required. Expense of disposal of radioactive waste.
iii. Must have access to a dark room to set up and develop films.

B. Nonradioactive method

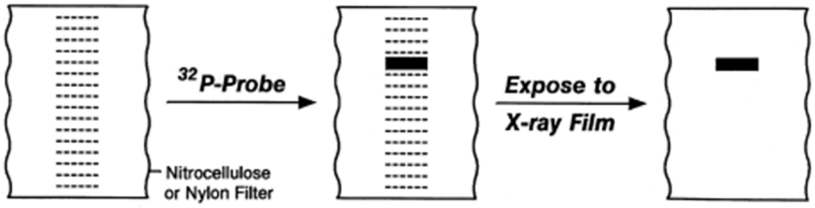
i. **Colorimetric detection** generally involves the production of a colored precipitate which can be seen with the naked eye. In a typical system, the DNA probe itself is labeled with an antigen such as digoxigenin; following hybridization to its target it would be exposed to an anti-digoxigenin antibody conjugated to an enzyme capable of catalyzing a colorimetric reaction (one commonly used example is alkaline phosphatase which will act on substrates NBT & BCIP to produce a dark purple product).

ii. **Fluorescent detection** involves probes which are directly labeled with fluorophores, or more likely, probes which are coupled to fluorescent molecules indirectly. For example, if probe is labeled with biotin, it would be exposed to avidin conjugated to a fluorescent tag. (Biotin and avidin strongly and specifically bind together, like an antibody and its antigen.) Fluorophores emit light when excited by light of an appropriate wavelength.

iii. **Chemiluminescence** is sort of a combination of these two: an enzymatic reaction that triggers the release of ordinary visible light (firefly luciferase).

8. Visualization

If radiolabeled ^{32}P probe is used, then it is visualized by autoradiograph. Biotin/streptavidin detection is done by colorimetric methods, and bioluminescent visualization uses luminesence.



Application of Southern blotting

- Southern blotting technique is used to detect specific DNA in given sample.
- Used during DNA finger printing.
- To identify clone.
- Used for paternity testing, criminal identification, victim identification.
- To isolate and identify desire gene of interest.
- Used in restriction fragment length polymorphism.
- To identify mutation or gene rearrangement in the sequence of DNA.
- Used in diagnosis of disease caused by genetic defects.
- Used to identify infectious agents.