SECTION IV

ANALYSIS OF DNA SEQUENCES BY BLOTTING AND HYBRIDIZATION

UNIT 2.9

Southern Blotting and Hybridization

DNA fragments are separated on agarose gels and denatured in situ. The fragments are then transferred from the gel to a solid support (either nitrocellulose filters or nylon membranes), where they are immobilized. After prehybridization to reduce nonspecific hybridization with the probe, the filter or membrane is hybridized to the desired radiolabeled nucleic acid probe. The filter or membrane is washed to remove unbound and weakly binding probe, and is then autoradiographed.

BASIC PROTOCOL

USING NITROCELLULOSE FILTERS

Materials

DNA molecular weight markers
10× gel loading buffer (*UNIT 2.5*)
10 mg/ml ethidium bromide
0.2 N HCl
Denaturation solution
Neutralization solution
20×, 2×, 1×, and 0.25× SSC (*APPENDIX 2*)
Prehybridization solution
Labeled probe for hybridization (*UNIT 3.5*)
Hybridization solution
0.1% sodium dodecyl sulfate (SDS)

Glass or metal decanting trays
Nitrocellulose membrane filters (Schleicher & Schuell #BA85)
46 × 57 cm Whatman 3MM filter paper
Sealable bags
Additional reagents and equipment for restriction enzyme digestion (*UNIT 3.1*), agarose gel electrophoresis (*UNIT 2.5*), and autoradiography (*APPENDIX 3*)

Preparing the samples and running the gel

1. Digest 10 pg to 10 µg of desired DNA to completion in a final volume of ~50 µl.

   The amount of DNA to be digested depends both on the complexity of the DNA and the probe to which it will be hybridized. An amount of 1 µg or less of a plasmid DNA will be sufficient to yield a signal that can be detected within hours. However, 8 to 10 µg of mammalian total genomic DNA is usually required to yield a signal that can be detected within 1 to 2 days.

2. Prepare an agarose gel as required to resolve the expected restriction fragments (see *UNIT 2.5*). Remember to include a DNA molecular weight marker lane (e.g., λ*Hin*dIII or λ*Bst*EII).

   Ideally, pour a 300-ml gel, 20 × 20 cm, with slots ~10 mm wide and 1 mm thick. This will allow 60 µl to be comfortably loaded into each slot.

3. Add gel loading buffer to sample, load samples onto gel, and electrophorese samples for 12 to 24 hr.

   Minigels can be electrophoresed at high voltage for shorter time periods, but resolution decreases as the voltage gradient increases.

4. After electrophoresis, remove gel from box. Place gel in ethidium bromide staining solution (25 μl of 10 mg/ml ethidium bromide in 500 ml water) for 30 min. Photograph the gel (minimize the time the gel is exposed to UV light).

   If a ruler is placed alongside the gel when it is photographed, a graph of the log molecular weight versus mobility of the (unlabeled) marker fragments can be drawn. In this case, it is not necessary to load radiolabeled markers onto the gel.

Southern transfer to nitrocellulose membrane filters

5. Place the gel in a tray containing 500 ml of 0.2 N HCl. Rock the tray gently for 10 min.

   For this and subsequent steps, the gel should be completely covered by solution and the tray should be rocked continuously. If the restriction fragment to be analyzed is ≤1 kb, it is not necessary to acid-treat the gel. Alternatively, if the fragment is >10 kb, the acid treatment should continue for 20 min. The acid treatment allows depurination to occur—the depurinated sites are cleaved during the alkali treatment and the smaller DNA fragments transfer more efficiently.

6. Decant the acid solution and rinse the gel several times in water. Pour off water.

   The Bromphenol Blue tracking dye should have changed from blue to yellow, indicating that the acid treatment is complete.

7. Add 500 ml denaturation solution to tray. After 15 min of rocking, decant solution and add another 500 ml to tray. Rock the tray gently for 15 min.

   The Bromphenol Blue tracking dye should once again be blue, indicating that the denaturation step is complete.

8. Decant the denaturation solution. Add 500 ml neutralization solution. Rock the tray gently for 30 min.

9. Place gel on a piece of plastic wrap and accurately measure the length and width of the gel. Put the gel back into neutralization solution.

10. Cut one piece of a nitrocellulose membrane filter such that it is 3 mm smaller in both dimensions than the gel. Wet the cut filter in a tray of water for 1 min, then place filter into a tray of 20× SSC for 5 to 10 min.

    Always use gloves when handling nitrocellulose. Oils present on ungloved hands can get on nitrocellulose and cause transfer artifacts.

11. Cut 3 to 5 sheets of Whatman 3MM paper into pieces that are 7 mm smaller in both dimensions than the piece of nitrocellulose. This should result in a 1- or 2-cm stack of cut paper.

12. Prepare a wick by cutting one piece of Whatman 3MM ~2 cm wider than the width of the gel and 30 to 40 cm long. Place several hundred milliliters of 20× SSC in a large tray. Wet the wick thoroughly in the 20× SSC; put a glass plate over the tray and place wick on plate with both ends of wick hanging over plate into the 20× SSC (see Fig. 2.9.1). Remove air bubbles trapped between the wick and glass plate by rolling a 10-ml pipet back and forth over wick.

13. Lift the gel out of the neutralization solution, allow most of the liquid to drip off the gel, and lay gel on top of the Whatman 3MM wick. Remove air bubbles trapped between gel and wick by rolling a pipet over gel as above. Remove the nitrocellulose filter from its tray of 20× SSC and lay it on top of the gel, making sure that the nitrocellulose filter does not overhang the gel. For blots of substantial amounts of plasmid or other very low complexity DNA, it is important to lay the filter

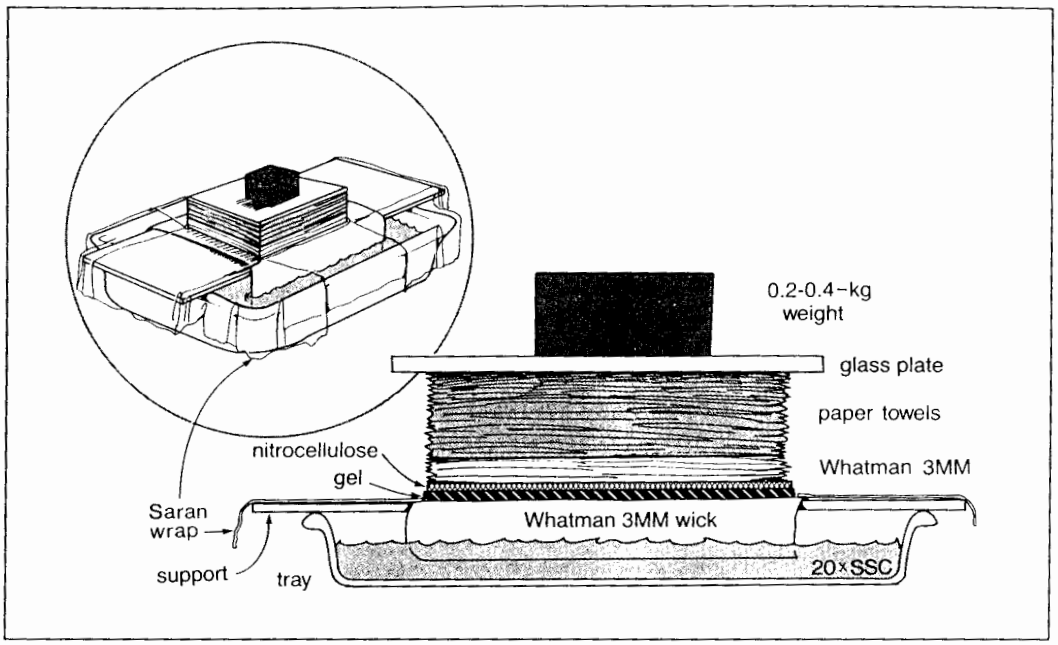

Figure 2.9.1 Transfer pyramid for Southern blotting.

down precisely the first time, as detectable transfer can take place almost immediately. Remove trapped air bubbles as above.

14. Wet one piece of the stack of cut Whatman 3MM paper in 20× SSC. Place it on a piece of dry 3MM paper to remove excess SSC and place the damp piece of 3MM paper on top of the nitrocellulose. Make sure that the piece of 3MM paper does not overhang the nitrocellulose and remove trapped air bubbles as above.

15. Place the stack of cut Whatman 3MM on top of the first piece of 3MM. Put a 2-to 3-cm-thick stack of paper towels on top of the 3MM paper. To keep the entire pyramid pressed together, place a glass plate on top and a small bottle on the glass plate.

    The final setup is shown in Figure 2.9.1. It is important to be sure that (a) no air bubbles be trapped between the gel and the nitocellulose, and (b) no part of the pyramid overhangs any part below it. This will allow an even and complete transfer.

16. Cover up the ends of the tray with plastic wrap to minimize evaporation during the transfer. Allow the transfer to proceed 2 to 12 hr.

    Thinner gels can be transferred more rapidly than thicker ones. Similarly, smaller DNA fragments transfer more rapidly than larger ones. Extent of transfer can be monitored after it is completed by noting the extent of transfer of the loading dye.

17. After the transfer is completed, take apart the pyramid so that the nitrocellulose filter is still lying on the gel. With a blue ballpoint pen, mark on the filter the location of the slot in the upper right-hand corner of the gel. It is also helpful to mark the date of the transfer on the filter. Using blunt forceps, remove the nitrocellulose filter and place it in a tray of 2× SSC for 5 min to wash away debris. Blot the filter dry on a piece of 3MM paper. Place the dried filter between two new sheets of 3MM paper and bake the nitrocellulose for 2 hr at 80°C.

    The transfer can be checked and documented by restaining and photographing the gel. If the transfer was performed properly, little or no DNA should be retained on the gel. Of course, this does not prove that the DNA is actually bound to the nitrocellulose.

Prehybridizing, hybridizing, and washing the filter

18. Place the baked filter in a sealable bag. Add 6 to 10 ml prehybridization solution (depending on the size of the filter) to the bag.

    The prehybridization solution should be added to the bottom of the bag. The filter is wetted by pulling the bag back and forth across the edge of a bench. Seal the bag.

19. Prehybridize 3 hr to overnight at the desired temperature.

    The prehybridization and hybridization temperatures will generally fall between 37° and 42°C using the prehybridization and hybridization solutions recommended here.

20. Prepare a probe by nick translation or other protocol (*UNIT 3.5*). Boil 500,000 cpm of the probe per milliliter of hybridization solution to be used (6 to 10 ml, as above) for 5 min. Add to the aliquot of hybridization solution and mix well.

21. Cut open a corner of the bag and remove the prehybridization solution. This is best accomplished by laying the bag on a flat surface and rubbing a 10-ml pipet over the bag.

22. Add the labeled probe/hybridization solution to the sealable bag. Spread the solution over the filter as above and seal the bag.

23. Hybridize 6 hr to overnight at the same temperature as prehybridization.

24. The next day, cut open the bag and remove the filter. Wash the filter in a glass or plastic tray with shaking as follows: 1× SSC, 0.1% SDS, room temperature (two times, each for 15 min), followed by 0.25× SSC, 0.1% SDS at hybridization temperature (two times, each for 15 min). Allow the filter to air dry.

    For each washing step, make sure the filter is moderately agitated. After the low-salt washes, examine the filter with a Geiger counter. If most of the filter reads at background, do not continue washing. Occasionally a more stringent wash (i.e., lower SSC concentration or higher temperature) is required.

25. Expose the filter to X-ray film.

    For blots of moderate- or high-complexity DNA, use an intensifying screen. Bands are usually visible after the film has been exposed for 1 or 2 days. This will vary, depending upon the abundance of the restriction fragment in question and the specific activity of the nick-translated probe.

SHORT PROTOCOL

SOUTHERN BLOTTING AND HYBRIDIZATION USING NITROCELLULOSE FILTERS

1. Digest samples (remember marker).
2. Prepare an agarose gel, load samples, and electrophorese.
3. Stain 30 min using 25μ l of 10 mg/ml ethidium bromide per 500 ml water. Photograph gel (with ruler).
4. Acid treat with 0.2 N HCl for 10 min.
5. Decant acid and rinse gel several times with water.
6. Add 500 ml denaturation solution for 15 min. Decant solution and repeat.
7. Decant denaturation solution and add 500 ml neutralization solution for 30 min.
8. Measure gel and set up overnight transfer:

   Wick in tray with 20× SSC
   Gel
   Nitrocellulose (soaked in H_20 and 20× SSC)
   3MM Whatman filter paper
   Paper towels
   Weight
9. Dissemble transfer pyramid and rinse nitrocellulose in 2× SSC for 5 min.
10. Bake nitrocellulose at 80°C for 2 hr.
11. Add 6 to 10 ml prehybridization solution and prehybridize overnight.
12. Prepare labeled nucleic acid probe. Add 500,000 cpm of the probe/ml hybridization solution. Remove prehybridization solution and add 6 to 10 ml hybridization solution. Hybridize overnight.
13. Wash twice, 15 min each, in 1× SSC, 0.1% SDS at room temperature. Wash twice, 15 min each, in 0.25× SSC, 0.1% SDS at hybridization temperature.
14. Expose to X-ray film.

AITERNATE PROTOCOL

USING NYLON FILTERS

The transfer protocol for nylon filters is faster and somewhat simpler than the basic protocol using nitrocellulose filters. Nylon filters are also sturdier than nitrocellulose and can be reused for several hybridizations.

Additional Materials

0.4 M NaOH
2×, 0.5×, and 0.1× SSC (*APPENDIX 2*)
SDS/prehybridization solution
SDS/hybridization solution
Nylon filters

1. Prepare samples and run the gel as in steps 1 to 4 in basic protocol.
2. Place the gel in a tray containing 500 ml of 0.2 N HCl. Rock the tray gently for 10 min.

3. Decant the acid solution and rinse the gel several times in water. Pour off water.
4. Cut a nylon filter such that it is 3 mm smaller in both dimensions than the gel. Wet the cut filter in water.

   Some manufacturers of nylon filters recommend that a particular side of the filter (usually the concave side) be in contact with the gel. Check with instructions provided by the manufacturer.

5. Set up the transfer pyramid as described in the basic protocol (steps 9 to 16) and Figure 2.9.1, *except* use 0.4 M NaOH as the transfer solution instead of 20× SSC. Transfer from 2 to 12 hr.

   In general, transfers in 0.4 M NaOH take less time than transfers in 20× SSC.

   NOTE: *If the nylon filter is to be used for repeated hybridizations (i.e., the probe will be stripped from the filter by stringent washing followed by repeated hybridizations with other probes), the transfer should be performed exactly as described in basic protocol (steps 5 to 17).*

6. Disassemble the transfer pyramid and mark the filter as described in step 17 of basic protocol. Rinse the filter in 2× SSC for 5 min.
7. Allow the filter to air dry.

   It is not necessary to bake the filter, as the alkaline transfer allows the DNA to bind covalently to the filter.

8. Place the filter in a sealable bag and add 6 to 10 ml SDS/prehybridization solution. Prehybridize several hours at 37° to 42°C.

   Some types of nylon filters used (e.g., Gene Screen Plus, Du Pont) permit the prehybridization step to be reduced to 15 min.

9. Prepare a probe by nick translation or other protocol (*UNIT 3.5*). Take 500,000 cpm of the probe per milliliter of SDS/hybridization solution to be used (6 to 10 ml, as above). Boil the probe for 5 min and add to the SDS/hybridization solution. Mix well.
10. Remove SDS/prehybridization solution from the bag and add the SDS/hybridization solution as described in steps 21 and 22 of basic protocol. Hybridize 6 hr to overnight at the prehybridization temperature.
11. Remove filter from the bag and wash with moderate agitation as follows:

    2× SSC/0.1% SDS—5 min at room temperature
    2× SSC/0.1% SDS—15 min at room temperature
    0.5× SSC/0.1% SDS—15 min at room temperature
    0.1× SSC/0.1% SDS—15 min at room temperature
    0.1× SSC/1.0% SDS—30 min at 42°C

12. Allow the filter to air dry. Expose the filter to X-ray film. Use an intensifying screen.

REAGENTS AND SOLUTIONS

Denaturation solution, 1 liter

87.75 g NaCl (1.5 M)
20.0 g NaOH (0.5 M)
Store at room temperature

Hybridization solution

Combine ingredients of prehybridization solution (below) *except* H_2O. Add 50 g dextran sulfate (for 10% final concentration) and mix overnight. Bring to 500 ml with H_2O.

Neutralization solution, 1 liter

175.5 g NaCl (3 M)
6.7 g Tris·OH, 70.2 g Tris·Cl } (0.5 M Tris)
Store at room temperature

Prehybridization solution

For 500 ml:	*Final concentration:*
12.5 ml of 1 M KPO_4, pH 7.4	25 mM KPO_4, pH 7.4
125 ml of 20× SSC (*APPENDIX 2*)	5× SSC
25 ml of 100× Denhardt's solution (*APPENDIX 2*)	5× Denhardt's solution
5 ml of 5 mg/ml salmon sperm DNA	50 μg/ml salmon sperm DNA
250 ml of 100% formamide	50% formamide
82.5 ml of H_2O	To final volume

Nonfat powdered milk can be used instead of Denhardt's solution. Store this and hybridization solution at –20°C. Shake before using if precipitate accumulates at bottom of bottle.

SDS/hybridization solution

Add 10% dextran sulfate to SDS/prehybridization solution

SDS/prehybridization solution

Add 1% SDS to prehybridization solution

COMMENTARY

Background Information

Southern blotting, the capillary transfer of DNA fragments from gels to various types of filter paper, revolutionized the study of eukaryotic genomes. The ability to detect rare sequences in a complex population of restriction fragments paved the way for the cloning of eukaryotic genes, reverse genetics, and modern molecular biology. The use of Southern transfers to analyze restriction-fragment-length polymorphisms (RFLPs) has had a dramatic impact on the diagnosis of human genetic disease, and modifications of the basic Southern concept have led to major developments in hybridization technology (e.g., northern and western blotting, *UNITS 4.9* and *10.8*).

When E.M. Southern began studying capillary transfer of DNA in the early 1970s, agarose and polyacrylamide gel electrophoresis of restriction fragments of DNA had just been described. He demonstrated that denatured DNA fragments could be transferred from agarose gels and bound efficiently to nitrocellulose filters at high-salt concentration. In addition, he found that the size of a given DNA fragment influences its ability to be transferred from a gel. Small fragments diffuse during transfer and hybridize inefficiently, which makes them difficult to detect, while large fragments are difficult to transfer (a limitation that led to the use of depurination steps prior to transfer; Wahl et al., 1979). Southern combined this methodology with existing nucleic acid hybridization technology and the Southern blot was born. Despite numerous changes in the types of gels and

filters used for transfer, the procedure in common use today is still essentially the same as the one described over a decade ago.

An alternate protocol for transfer to nylon filters is presented here, however, since they have several advantages over nitrocellulose filters. Nitrocellulose is notoriously fragile, particularly after it has been baked, and must be handled carefully, whereas nylon membranes are quite durable. In addition, nylon membranes can be exposed to basic solutions for extended periods of time (Reed and Mann, 1985), thus eliminating the need for the neutralization step present in standard Southern protocols (the transfer of DNA under these conditions prevents reannealing during the transfer, thereby increasing the sensitivity of the technique). The two transfer techniques are equally effective. Which protocol to use is largely a matter of personal preference, though recently many laboratories are choosing nylon membranes. Another major advantage of nylon membranes is that since the DNA can be covalently bound by UV irradiation, hybridized probe can be completely stripped from the membrane by denaturation and stringent washing.

For further commentary on blotting technology, see *UNIT 4.9*.

Critical Parameters

The most important variables to consider when carrying out a Southern blot are the complexity of the DNA being blotted and the homology between the probe and the sequences being detected. The total amount of the fragment(s) to be studied can vary an impressive 10^{10}-fold, from 0.1 to 1 µg for a plasmid or phage digest to 10^{-9} to 10^{-8} µg for a single-copy gene in a highly complex eukaryotic genome. This range has an obvious impact on the design of the blotting experiment: for lower complexity DNA, substantially shorter blotting, hybridization, and exposure times, a smaller amount of starting DNA, and lower specific activity probes can be used. For example, a single-copy yeast gene can be detected with an exposure time of only 1 hr starting with 1 µg of yeast DNA, blotting for 2 hr, and hybridizing for 4 to 8 hr with a probe of 10^8 cpm/µg. For a single-copy mammalian gene a conservative approach would start with 10 µg of DNA, blot overnight, and hybridize overnight in the presence of 10% dextran sulfate using a probe of greater than 10^8 cpm/µg, resulting in an exposure time of ~24 hr.

Several factors can affect the melting temperature (T_m) of the DNA-DNA hybrid between the probe and the fragment of interest, and, consequently, the appropriate conditions for hybridization and washing. In many cases the probe is not 100% homologous to the fragment. Other commonly encountered variables include the length and total G + C content of the hybridizing sequences and the ionic strength and formamide content of the hybridization buffer. The effects of all of these factors can be approximated by a single equation:

$$T_m = 81 + 16.6(\log_{10}C_i) + 0.4[\%(G + C)] - 0.6(\%\ \text{formamide}) - 600/n - 1.5(\%\ \text{mismatch})$$

where C_i is the salt concentration (monovalent ions) and n is the length of the hybrid in base pairs (slightly modified from Meinkoth and Wahl, 1984).

In designing a hybridization experiment, some factors affecting nucleic acid hybridization can be conveniently altered. The temperature of the hybridization and washes and the salt concentration during the washes are the simplest to adjust. As the temperature of the hybridization increases (i.e., hybridization stringency increases), it becomes less likely for hybridization to occur between strands that are nonhomologous, and as a result, background decreases. If the radiolabeled probe is not completely homologous with the immobilized fragment (as is frequently the case in gene family and interspecies hybridization experiments), the hybridization temperature must be reduced, and background will increase. The temperature of the washes affects the intensity of the hybridizing band and the degree of background in a similar manner. The stringency of the washes is also increased with decreasing salt concentrations.

In general, convenient hybridization temperatures in the presence of 50% formamide are: 42°C for a probe which is 95 to 100% homologous to the target fragment, 37°C for 90 to 95% homology, and 32°C for 85 to 90% homology. For lower homologies, formamide content should be lowered and temperature adjusted accordingly, using the equation above. If the homology between the probe and target DNA is not known, the simplest approach is to start with both hybridization and wash conditions which are nonstringent. If nonspecific bands or high background are observed after autoradiography, the filter can be washed

at higher stringency and reexposed. If the time required for exposure makes this approach impractical, several hybridization and/or washing stringencies should be tested in parallel.

Dextran sulfate is utilized in nucleic acid hybridization solutions because it favors the formation of networks (Wahl et al., 1979). In this context, a network is a collection of a large number of probe molecules bound to the DNA on the filter or membrane. When DNA is labeled by nick translation (*UNIT 3.5*), the resulting radiolabeled probe is, when denatured, a collection of labeled strands of varying size. The first probe strand binds to the DNA on the filter, other probe strand binds to the first strand, and a "tree" of overlapping radiolabeled fragments (the network) is formed. The practical advantage of the network is that it amplifies the amount of radiolabeled probe bound to the filter, thereby increasing the sensitivity of the technique. Since a network cannot form from a single-stranded probe, dextran sulfate is not necessary in hybridization with such probes.

Before beginning the transfer, it is essential that complete digestion of the DNA samples to be analyzed is achieved. To liberate a given restriction fragment, two cleavages are necessary—if the restriction is 70% complete, only 49% (70% × 70%) of the appropriate band will be liberated, therefore significantly reducing the attainable signal before the DNA has even been loaded onto the gel.

As for Northern transfers, setting up the transfer pyramid carefully will prevent transfer artifacts, and baking of the filter is essential. Finally, it is important to wash the nitrocellulose filters appropriately after hybridization—too little washing yields high backgrounds and too much stringent washing removes the signal of interest.

Troubleshooting

Failure to detect bands of interest is usually due to one of several simple problems. The most frequently encountered involve the input DNA, the blotting procedure, or the probe. The observation of extra unexpected bands can usually be ascribed to a problem with the input DNA. If the bands are of higher molecular weight than the expected bands and decrease in intensity with increasing molecular weight, they are probably due to partial digestion of the DNA resulting from poor quality DNA, enzyme, or both. Extra bands that are more intense than expected, and/or that vary in intensity with different DNA preparations, are frequently due to contamination of the input DNA with (usually plasmid) sequences homologous to the probe. Because of the great sensitivity of Southern blotting, an extremely low level contamination of the starting DNA, i.e., from glassware or rotors previously used for plasmid preparation, is disastrous.

Occasionally a dark smear of hybridization will be apparent on an autoradiograph, even after extensive washing. One cause of this problem is the inadvertent use of a repeated sequence as the radiolabeled probe.

The most common causes of weak signals or failure to detect any bands are problems with the blotting procedure or the probe. Simple failures in the blotting procedure can be detected by checking for the presence of DNA in the gel after transfer. It is imperative that all flow of blotting solution must be through the gel and membrane. "Short circuits" to the flow, such as a direct contact between the wick and the dry pyramid, or a crack in the gel, will prevent or decrease transfer. Problems with the probe are detectable by straightforward measurement of incorporation of radioactivity into high-molecular-weight DNA. Particularly if the enzymes used in the labeling reaction have been well characterized, low apparent specific activities can generally be attributed to poor-quality template DNA.

Anticipated Results

Using either nitrocellulose filters or nylon membranes for transfer, 10 pg of a single-copy mammalian gene present in 10 μg of DNA can be detected with a nick-translated probe of $\geq 10^8$cpm/μg with 24 hr of exposure time.

Time Considerations

As previously mentioned, the time needed to complete a Southern blotting experiment depends on the complexity of the DNA being analyzed. For detection of a single-copy gene, starting with 10 μg DNA from a highly complex genome, both the transfer and hybridization steps are frequently done overnight, and autoradiography can take up to several days. For detection of an individual fragment in a digest of a microgram of DNA from a plasmid or phage, the entire protocol—from digesting the DNA to developing the autoradiograph—can easily be done in a single day: 1 hr for restriction enzyme digestion, 1 to 2 hr for running a minigel (depending on the resolu-

tion necessary), 1 hr for denaturation and neutralization, 30 min for blotting (not a complete transfer, but sufficient), 30 min for baking (also sufficient for this, but not more stringent applications), 30 min for prehybridization, 1 hr for hybridization, 30 min for washing, and 30 min for exposure of the film (or more with a low-specific-activity probe).

It is not advisable to delay setting up the transfer once the gel has been electrophoresed—the longer the gel sits before it is transferred, the more the DNA will diffuse, resulting in fuzzy bands on the autoradiograph. It is perfectly reasonable to store the baked nitrocellulose filter or air-dried nylon membrane after the transfer. If the nitrocellulose filter is stored at room temperature for more than a few days, it should be rebaked at 80°C for 2 hr.

Literature Cited

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