ANALYSIS OF DNA SEQUENCES BY BLOTTING AND HYBRIDIZATION

Southern Blotting

Southern blotting is the transfer of DNA fragments from an electrophoresis gel to a membrane support. The transfer or a subsequent treatment results in immobilization of the DNA fragments, so the membrane carries a semipermanent reproduction of the banding pattern of the gel. After immobilization, the DNA can be subjected to hybridization analysis (*UNIT 2.10*), enabling bands with sequence similarity to a labeled probe to be identified.

When setting up a Southern transfer, choices must be made between different types of membrane, transfer buffer, and transfer method. The most popular membranes are made of nitrocellulose, uncharged nylon, or positively charged nylon. Although these materials have different properties, the three types of membrane are virtually interchangeable for many applications. The main advantage of nylon membranes is that they are relatively robust and so can be reprobed ten or more times before falling apart. Nitrocellulose membranes are fragile and can rarely be reprobed more than three times; however, these are still extensively used, as they give lower backgrounds with some types of hybridization probe. The properties and advantages of the different membranes are discussed more fully in the commentary.

The basic protocol describes Southern blotting via upward capillary transfer of DNA from an agarose gel onto a nylon or nitrocellulose membrane, using a high-salt transfer buffer to promote binding of DNA to the membrane. With the high-salt buffer, the DNA becomes bound to the membrane during transfer but not permanently immobilized. Immobilization is achieved by UV irradiation (for nylon) or baking (for nitrocellulose). A support protocol describes how to calibrate a UV transilluminator for optimal UV irradiation of a nylon membrane.

The first alternate protocol details transfer using nylon membranes and an alkaline buffer, and is primarily used with positively charged nylon membranes. The advantage of this combination is that no post-transfer immobilization step is required, as the positively charged membrane binds DNA irreversibly under alkaline transfer conditions. The method can also be used with neutral nylon membranes but less DNA will be retained.

The second alternate protocol describes a transfer method based on a different transfer-stack setup. The traditional method of upward capillary transfer of DNA from gel to membrane described in the first basic and alternate protocols has certain disadvantages, notably the fact that the gel can become crushed by the weighted filter papers and paper towels that are laid on top of it. This slows down the blotting process and may reduce the amount of DNA that can be transferred. The downward capillary method described in the second alternate protocol is therefore more rapid than the basic protocol and can result in more complete transfer.

Although the ease and reliability of capillary transfer methods makes this far and away the most popular system for Southern blotting with agarose gels, it unfortunately does not work with polyacrylamide gels, whose smaller pore size impedes the transverse movement of the DNA molecules. The third alternate protocol describes an electroblotting procedure that is currently the most reliable method for transfer of DNA from a polyacrylamide gel.

SOUTHERN BLOTTING ONTO A NYLON OR NITROCELLULOSE MEMBRANE WITH HIGH-SALT BUFFER

The procedure is specifically designed for blotting an agarose gel onto an uncharged or positively charged nylon membrane. With the minor modifications detailed in the annotations, the same protocol can also be used with nitrocellulose membranes.

The protocol is divided into three stages. First, the agarose gel is pretreated by soaking in a series of solutions that depurinate, denature, and neutralize the DNA and gel matrix. The second stage is the transfer itself, which occurs by upward capillary action. Finally, the DNA is immobilized on the membrane by UV irradiation (for nylon) or baking (for nitrocellulose).

Materials

DNA samples to be analyzed
0.25 M HCl
Denaturation solution: 1.5 M NaCl/0.5 M NaOH (store at room temperature)
Neutralization solution: 1.5 M NaCl/0.5 M Tris·Cl, pH 7.0 (store at room temperature)
20× and 2× SSC (*APPENDIX 2*)

Oblong sponge slightly larger than the gel being blotted
Whatman 3MM filter paper sheets
Nylon *or* nitrocellulose membrane (see Table 2.9.1 for suppliers)
UV-transparent plastic wrap (e.g., Saran Wrap) for nylon membranes
UV transilluminator (*UNIT 2.5*) or UV light box (e.g., Stratagene Stratalinker) for nylon membranes

Additional reagents and equipment for restriction endonuclease digestion (*UNIT 3.1*) and agarose gel electrophoresis (*UNIT 2.5*)

Table 2.9.1 Properties of Materials used for Immobilization of Nucleic Acids[a]

	Nitrocellulose	Supported nitrocellulose	Uncharged nylon	Positively charged nylon	Activated papers
Application	ssDNA, RNA, protein	ssDNA, RNA, protein	ssDNA, dsDNA, DNA, protein	ssDNA, dsDNA, RNA, protein	ssDNA, RNA
Binding capacity (μg nucleic acid/cm^2)	80-100	80-100	400-600	400-600	2-40
Tensile strength	Poor	Good	Good	Good	Good
Mode of nucleic acid attachment[b]	Noncovalent	Noncovalent	Covalent	Covalent	Covalent
Lower size limit for efficient nucleic acid retention	500 nt	500 nt	50 nt or bp	50 nt or bp	5 nt
Suitability for reprobing	Poor (fragile)	Poor (loss of signal)	Good	Good	Good
Commercial examples	Schleicher & Schuell BA83, BA85; Amersham Hybond-C; PALL Biodyne A	Schleicher & Schuell BA-S; Amersham Hybond-C extra	Amersham Hybond-N; Stratagene Duralon-UV; Du Pont NEN GeneScreen	Schleicher & Schuell Nytran; Amersham Hybond-N$^+$; Bio-Rad ZetaProbe; PALL Biodyne B; Du Pont NEN GeneScreen Plus	Schleicher & Schuell APT papers

[a]This table is based on Brown (1991), with permission from BIOS Scientific Publishers Ltd.
[b]After suitable immobilization procedure (see text).

Preparation and Analysis of DNA

2.9.2

CAUTION: Wear gloves from step 2 of the protocol onward to protect your hands from the acid and alkali solutions and to protect the membrane from contamination.

Prepare the gel

1. Digest the DNA samples with appropriate restriction enzyme(s), run in an agarose gel with appropriate DNA size markers, stain with ethidium bromide, and photograph with a ruler laid alongside the gel so that band positions can later be identified on the membrane.

   The gel should contain the minimum agarose concentration needed to resolve bands in the area of interest (see commentary to UNIT 2.5A) and should be ≤7 mm thick, preferably less. The amount of DNA that must be loaded depends on the relative abundance of the target sequence that will subsequently be sought by hybridization probing (see recommendations in commentary to this unit and UNIT 2.10).

2. Rinse the gel in distilled water and place in a clean glass dish containing ~10 gel volumes of 0.25 M HCl. Shake slowly on a platform shaker for 30 min at room temperature.

   This step results in partial depurination of the DNA fragments, which in turn leads to strand cleavage. The length reduction improves the transfer of longer molecules. The step is not necessary with PCR products <4 kb in length, or if efficient transfer of molecules >5 kb is not required. The time needed for the acid treatment depends on the concentration and thickness of the gel. To check that the treatment has been sufficient, watch the xylene cyanol and bromophenol blue dyes. When these change color to green and yellow respectively, the gel has equilibrated with the acid. Adequate depurination takes a further 10 min.

3. Pour off the HCl and rinse the gel with distilled water. Add ~10 vol denaturation solution and shake as before for 20 min. Replace with fresh denaturation solution and shake for a further 20 min.

   Denaturation unzips the DNA to give single-stranded molecules that have unpaired bases and are suitable for subsequent hybridization analysis.

4. Pour off the denaturation solution and rinse the gel with distilled water. Add ~10 vol neutralization solution, shake as before for 20 min, then replace with fresh neutralization solution and shake for a further 20 min.

   The aim of the neutralization step is to bring the gel pH down to <9.0. At higher pH, the transferred DNA will not bind to nitrocellulose. After neutralization, the gel pH can be checked using a pH meter. If the pH is >9.0, carry out a third washing in neutralization solution. Neutralization is less critical with a nylon membrane but should still be carried out.

Set up the transfer

5. Using Figure 2.9.1 as a guide, place an oblong sponge, slightly larger than the gel, in a glass or plastic dish (if necessary, use two or more sponges placed side by side). Fill the dish with enough 20× SSC to leave the soaked sponge about half-submerged in buffer.

   The sponge forms the support for the gel. Any commercial sponge will do; before a sponge is used for the first time, it should be washed thoroughly with distilled water to remove any detergents that may be present. As an alternative, use a solid support with wicks made out of Whatman 3MM filter paper (see Fig. 2.9.1). An electrophoresis tank should not be used, as the high-salt transfer buffer will corrode the electrodes.

6. Cut three pieces of Whatman 3MM paper to the same size as the sponge. Place these on the sponge and wet them with 20× SSC.

7. Place the gel on the filter paper and squeeze out air bubbles by rolling a glass pipet over the surface.

8. Cut four strips of plastic wrap and place over the edges of the gel.

   This is to prevent the buffer from "short-circuiting"—i.e., so that it flows through rather than around the gel.

9. Cut a piece of nylon membrane just large enough to cover the exposed surface of the gel. Pour distilled water ~0.5 cm deep in a glass dish and wet the membrane by placing it on the surface of the water. Allow the membrane to submerge, then leave for 5 min.

   If a nitrocellulose membrane is being used, submerge in distilled water; replace the water with 20× SSC and leave for 10 min. Avoid handling nylon and nitrocellulose membranes even with gloved hands—use clean blunt-ended forceps instead.

10. Place the wetted membrane on the surface of the gel. Try to avoid getting air bubbles under the membrane; remove any that appear by carefully rolling a glass pipet over the surface.
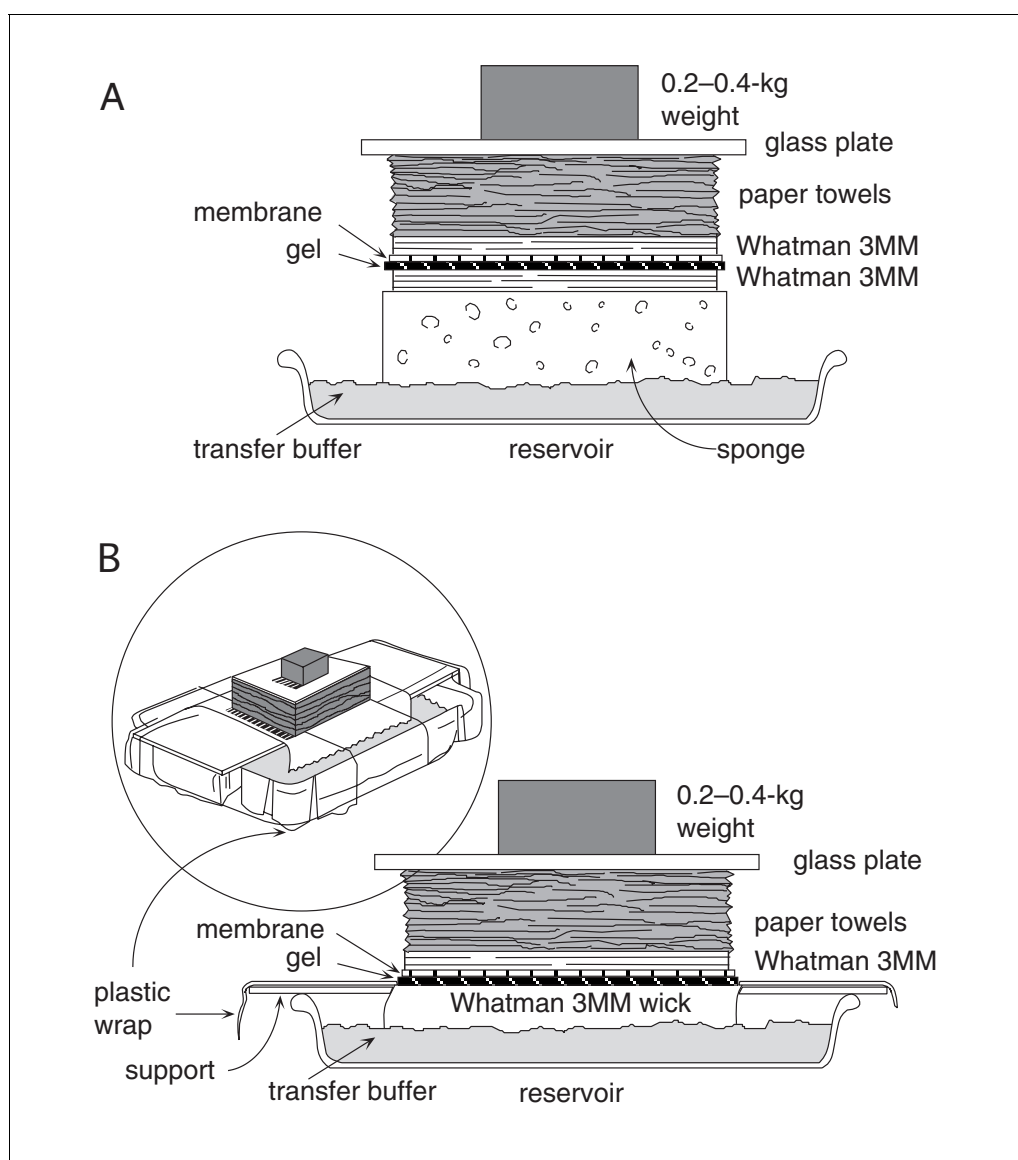

Figure 2.9.1 Two alternative transfer pyramid setups for Southern blotting via upward capillary transfer. (**A**) Sponge method. (**B**) Whatman 3MM filter paper wick method.

For blots of substantial amounts of plasmid or other very-low-complexity DNA, it is important to lay the membrane down precisely the first time, as detectable transfer can take place almost immediately, and moving the membrane may cause "mobile" bands as streaks on the autoradiograph.

11. Flood the surface of the membrane with 20× SSC. Cut five sheets of Whatman 3MM paper to the same size as the membrane and place these on top of the membrane.

12. Cut paper towels to the same size as the membrane and stack these on top of the Whatman 3MM papers to a height of ~4 cm.

13. Lay a glass plate on top of the structure and place a weight on top to hold everything in place. Leave overnight.

    The weight should be sufficient to compress the paper towels to ensure good contact throughout the stack. Excessive weight, however, will crush the gel and retard transfer.

    An overnight transfer is sufficient for most purposes. Extend the transfer time if the gel concentration is >1%, or transfer of fragments >20 kb is desired. Make sure that the reservoir of 20× SSC does not run dry during the transfer. If the gel contains large amounts of DNA (see annotation to step 10), high transfer efficiencies may not be required and shorter transfer times (~1 hr) may be used.

Disassemble the transfer pyramid

14. Remove the paper towels and filter papers and recover the membrane. Mark in pencil the position of the wells on the membrane and ensure that the up-down and back-front orientations are recognizable.

    Pencil is preferable to pen, as ink marks may wash off the membrane during hybridization. An alternative for a nylon membrane only is to cut slits with a razor blade to mark the positions of the wells (do not do this before transfer or the buffer will short-circuit). The best way to record the orientation of the membrane is by making an asymmetric cut at one corner.

15. Rinse the membrane in 2× SSC, then place it on a sheet of Whatman 3MM paper and allow to dry.

    The rinse has two purposes: to remove agarose fragments that may adhere to the membrane and to leach out excess salt. The gel can be restained with ethidium bromide to assess the efficiency of transfer.

Immobilize the DNA

16. Wrap the membrane UV-transparent plastic wrap, place DNA-side-down on a UV transilluminator (254-nm wavelength) and irradiate for the time determined from the support protocol.

    CAUTION: *Exposure to UV irradation is harmful to the eyes and skin. Wear suitable eye protection and avoid exposure of bare skin.*

    Nitrocellulose membranes should not be UV irradiated. Instead, place between two sheets of Whatman 3MM paper and bake under vacuum for 2 hr at 80°C. This results in noncovalent attachment of the DNA to the membrane; the vacuum is needed to prevent the membrane from igniting.

    UV crosslinking is recommended for a nylon membrane as this leads to covalent attachment and enables the membrane to be reprobed several times. The membrane must be completely dry before UV crosslinking; check the manufacturer's recommendations. A common procedure is to bake for 30 min at 80°C prior to irradiation. Plastic wrap is used to protect the membrane during irradiation, but it must be UV-transparent. A UV light box can be used instead of a transilluminator (follow manufacturer's instructions).

Southern Blotting

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17. Store membranes dry between sheets of Whatman 3MM paper for several months at room temperature. For long-term storage, place membranes in a desiccator at room temperature or 4°C.

CALIBRATION OF A UV TRANSILLUMINATOR

When immobilizing DNA on a nylon membrane by UV crosslinking as described in the basic protocol, the intensity of irradiation is critical. Too little results in submaximal immobilization, and too much causes DNA degradation. UV transilluminators must therefore be calibrated to determine their output. The calibration should be repeated from time to time as the output of the lights changes with age. If a UV exposure meter (e.g., Baxter Scientific Blak-Ray) is available, it can be used to calculate the period of irradiation needed to produce the optimal exposure described in the manufacturer's support literature for the membrane (see also *UNIT 3.19*). Alternatively, the optimal exposure time can be determined experimentally as described in this protocol.

UV light boxes designed specifically for crosslinking are now commercially available. Most have automatic devices that vary output according to the age of the lights, but it is still a good idea to calibrate them regularly.

Additional Materials

DNA probe labeled to a specific activity of 10^8 dpm/μg

Additional reagents and equipment for DNA dot blotting (*UNIT 2.9B*) and hybridization analysis (*UNIT 2.10*)

CAUTION: Exposure to UV irradiation is harmful to the eyes and skin. Wear suitable eye protection and avoid exposure of bare skin.

1. Prepare five identical series of DNA dot blots (*UNIT 2.9B*) on nylon membrane strips, each with a range of DNA quantities from 1 to 100 pg.

    The dot blots should be prepared on nylon membrane from the batch that is being used for Southern transfers.

2. Dry the nylon strips, wrap each one in UV-transparent plastic wrap, and place on the UV transilluminator, DNA-side-down.

3. Put on eye protection and cover exposed skin, then switch on the transilluminator. Remove individual strips after 30 sec, 45 sec, 1 min, 2 min, and 5 min.

4. Hybridize the strips with a suitable DNA probe labeled to a specific activity of 10^8 dpm/μg and prepare autoradiographs (*UNIT 2.10*).

5. Determine which nylon strip gives the most intense hybridization signals; the exposure time used for that strip is the optimal exposure time.

    Note that the optimal exposure time depends on the dryness of the membrane. The test membrane strips should therefore be dried in exactly the same way as the Southern blots.

    At no or low UV dose, the signal will be weak; with increasing dose, it will reach a plateau. At long exposures the signal will again decline, as the radiation will damage the DNA so that it hybridizes poorly.

SOUTHERN BLOTTING ONTO A NYLON MEMBRANE WITH AN ALKALINE BUFFER

With a positively charged nylon membrane, the transferred DNA becomes covalently linked to the membrane if an alkaline transfer buffer is used. The protocol can also be carried out with uncharged nylon membranes using the minor modifications detailed in the annotations to the protocol steps below, but the transfer will be less complete. However, this transfer technique is not suitable for nitrocellulose membranes, as these do not retain DNA at pH >9.0 and fall apart after long exposure to alkali. The resistance of the nylon membrane to alkali should be checked before use, as some types of nylon membrane are less resistant than others.

Additional Materials

0.4 M (for charged membrane) *or* 0.25 M (for uncharged membrane) NaOH
0.25 M NaOH/1.5 M NaCl for uncharged membrane
Positively charged *or* uncharged nylon membrane (see Table 2.9.1 for suppliers)

CAUTION: Wear gloves to protect your hands from the acid and alkali solutions and to protect the membrane from contamination.

1. Prepare a gel and treat with 0.25 M HCl as described in steps 1 and 2 of the basic protocol.

2. Rinse the gel with distilled water. Pour 10 gel volumes of 0.4 M NaOH into the dish and shake slowly on a platform shaker for 20 min.

   This is the denaturation step. If an uncharged nylon membrane is being used, use 0.25 M NaOH in place of 0.4 M NaOH.

3. Follow steps 5 to 14 of the basic protocol to carry out the transfer, using 0.4 M NaOH as the transfer solution in place of 20× SSC. Leave to transfer ≥2 hr.

   Alkaline transfer is quicker than high-salt transfer, so the blot can be taken apart any time after 2 hr.

   A positively charged nylon membrane does not have to be prewetted, but can be placed directly onto the gel. If uncharged nylon is being used, use 0.25 M NaOH/1.5 M NaCl as the transfer solution. Check that the paper towels are resistant to the alkali solution; some types go brown and should not be used.

4. Remove the paper towels and filter paper and recover the membrane. Rinse the membrane in 2× SSC, place on a sheet of Whatman 3MM filter paper, and allow to air dry. Store as described in step 17 of the basic protocol.

   The rinse has two purposes: to remove agarose fragments that may adhere to the membrane and to neutralize the membrane. Baking or UV crosslinking is not needed with a positively charge membrane; in fact, UV crosslinking is detrimental. With an uncharged membrane, immobilize as described in step 16, basic protocol.

SOUTHERN BLOTTING BY DOWNWARD CAPILLARY TRANSFER

One disadvantage with the upward capillary method (basic protocol) is that the gel can become crushed by the weighted filter papers and paper towels that are laid on top of it, reducing capillary action. The problem worsens as the transfer proceeds, as the paper towels become soaked in transfer buffer, increasing the pressure on the gel. This retards the blotting process and as a result the transfer must be carried out for 16 to 24 hr.

This alternate protocol describes a simple downward capillary transfer system that does not cause excessive pressure to be placed on the gel. Transfer from a 4-mm-thick 1% agarose gel is complete in just 1 hr. The protocol is suitable for all types of membrane and
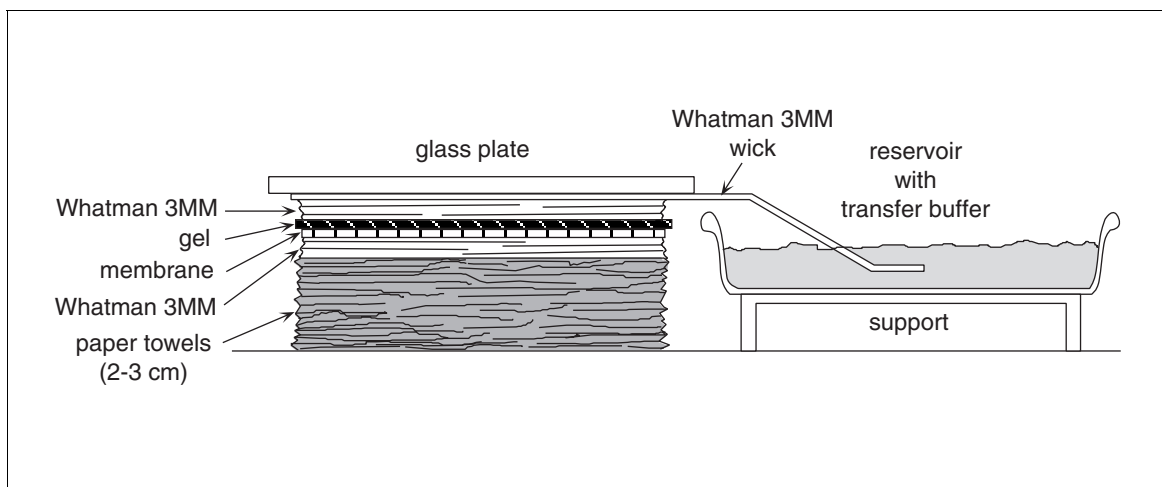
Figure 2.9.2 Transfer pyramid setup for Southern blotting via downward capillary transfer. Adapted with permission from Academic Press.

can be used with either high-salt or alkaline transfer buffers.

CAUTION: Wear gloves to protect your hands from the acid and alkali solutions used in gel preparation and transfer and to protect the membrane from contamination.

1. Prepare a gel as described in steps 1 to 4 of the basic protocol (high-salt transfer) or steps 1 to 2 of the first alternate protocol (alkaline transfer).

2. Make a stack of paper towels 2 to 3 cm high in a glass dish. The towels should be slightly wider than the gel.

3. Using Figure 2.9.2 as a guide, place four pieces of Whatman 3MM filter paper on top of the paper towels. Wet a fifth filter paper with transfer buffer and place on top.

4. Wet the membrane as described in step 9 of the basic protocol.

    The membrane can be slightly larger than the gel.

5. Place the membrane on the top filter paper. Remove bubbles by rolling a glass pipet over the surface of the membrane.

    Plastic wrap should be placed around the gel to prevent short-circuiting, as in step 8 of the basic protocol.

6. Place the gel on the membrane and remove bubbles.

    No part of the gel should extend over the edge of the membrane.

7. Soak three pieces of Whatman 3MM paper cut to the same size as the gel with transfer buffer and place on top of the gel.

8. Place two larger pieces of Whatman 3MM paper together and soak in transfer buffer. As shown in Figure 2.9.2, place the glass dish containing transfer buffer on a support and use the two pieces of soaked Whatman 3MM paper to form a bridge between the gel and the reservoir.

9. Place a light plastic cover (e.g., a gel plate) over the top of the stack to reduce evaporation. Leave for 1 hr.

10. Remove the paper towels and filter papers and recover the membrane. Treat the membrane as described in steps 16 and 17 of the basic protocol (high-salt transfer) or step 4 of the first alternate protocol (alkaline transfer).

Preparation and Analysis of DNA

2.9.8

ELECTROBLOTTING FROM A POLYACRYLAMIDE GEL TO A NYLON MEMBRANE

Capillary transfer, although reliable and needing no special equipment, has the disadvantage that it does not work with polyacrylamide gels, whose pore sizes are too small for effective transverse diffusion of DNA. Polyacrylamide gels must therefore be blotted by electrophoretic transfer. This requires a transfer buffer of low ionic strength, so nylon membranes are generally used, with uncharged nylon being slightly preferable to positively charged.

In conjunction with UV crosslinking (support protocol), this method transfers small DNA fragments and retains them quantitatively on the membrane; complete transfer and retention is crucial to the success of many procedures, including genomic sequencing and polymerase chain reaction (PCR) methods for quantitation of rare DNAs (Chapter 15). The polyacrylamide gel containing the samples of interest is placed in contact with an appropriately prepared membrane. These are then sandwiched into an electroblotting apparatus and the DNA is transferred out of the gel onto the membrane using electric current. After a buffer rinse, the membrane is ready to be UV-crosslinked.

There are several types of electroblotting apparatus on the market and the precise details of the procedure depend on the equipment being used. This protocol refers specifically to the Trans-Blot electroblotter (Bio-Rad). With a different piece of equipment, modify the procedure in accordance with the manufacturer's instructions.

Additional Materials

> 0.5× TBE electrophoresis buffer (*APPENDIX 2*)
> Scotch-Brite pads (supplied with Trans-Blot apparatus)
> Trans-Blot electroblotting cell (Bio-Rad) with cooling coil, or other electroblotting apparatus (*UNIT 10.8*)
>
> Additional reagents and equipment for nondenaturing (*UNIT 2.7*) or denaturing (*UNIT 2.12*) polyacrylamide gel electrophoresis and for electroblotting (*UNIT 10.8*)

CAUTION: Wear gloves to protect the membrane from contamination.

1. Run DNA samples in a nondenaturing or denaturing polyacrylamide gel.

2. When electrophoresis is almost complete, cut a piece of nylon membrane sufficient in size to cover the relevant parts of the gel. Pour distilled water ~0.5 cm deep in a glass dish. Wet the membrane by floating it on the surface of the water, then submerge it. Leave for 5 min.

   The membrane need not be exactly the same size as the gel. Ensure that the membrane is large enough to cover all the DNA bands, but do not worry if not all parts of the gel are in contact with the membrane. Avoid handling the membrane even with gloved hands—use clean blunt-ended forceps instead.

3. When electrophoresis is complete, remove one glass plate from the electrophoresis apparatus and stain and photograph the gel (if nondenaturing). Cut a piece of Whatman 3MM filter paper slightly larger than the gel. Lay the filter paper on the surface of the gel and remove trapped air bubbles by rolling a glass pipet over the surface. The gel should adhere to the filter paper. Lift the gel off the glass plate by peeling the filter paper away.

   This manipulation is designed to prevent breakage of a thin polyacrylamide gel. If the gel is sufficiently sturdy, it can instead be lifted directly onto a piece of Whatman 3MM paper that has been soaked in 0.5× TBE electrophoresis buffer.

4. Soak two Scotch-Brite pads in 0.5× TBE and remove air pockets by repeated

squeezing and agitation. Cut seven pieces of Whatman 3MM paper to the same size as the gel and soak these for 15 to 30 min in 0.5× TBE.

5. Place the opened gel holder of the Trans-Blot cell in a shallow tray, with the grey panel resting flat on the bottom, and place one of the saturated Scotch-Brite pads on the inner surface of the grey panel. Place three soaked filter papers on the pad. To ensure that there are no trapped air bubbles, build up the stack of filter papers one by one, carefully searching for trapped bubbles and removing them by rolling a glass pipet over the surface of the top paper.

> *Removing air bubbles is crucial to the success of electroblotting. It is also important that the filter papers be totally saturated with buffer. If heat build-up and bubble formation during transfer are a problem, the filters were probably not completely saturated.*

6. Flood the filter paper carrying the gel with 0.5× TBE and place on top of the filter-paper stack. Flood the surface of the gel with 0.5× TBE and place the prewetted membrane onto the gel.

> *Again, it is important to remove air bubbles. Placing the stack on a light box makes trapped bubbles stand out more clearly.*

7. Flood the surface of the membrane with 0.5× TBE and place the remaining four sheets of saturated Whatman 3MM paper on top, followed by the second saturated Scotch-Brite pad. Close the gel holder.

8. Half-fill the Trans-Blot cell with 0.5× TBE and place the gel holder in the cell with the grey panel facing towards the cathode. Fill the cell with 0.5× TBE and electroblot at 30 V (~125 mA) for 4 hr under the conditions recommended by the manufacturer.

> *Higher voltages can be used to reduce the transfer time (e.g., 40 V for 2 hr).*

> *Some protocols suggest using precooled 0.5× TBE when filling the cell as well as performing the electroblotting in a cold room. This may not provide sufficient cooling to prevent heat build up and bubble formation, especially if a voltage >30 V is used. For more efficient transfer, the temperature must be kept at 4°C using the cooling coil. With other makes of equipment, it may be possible to control the temperature of the blot by circulating water from a cooling water bath.*

9. Switch off the power and remove the gel holder. Take the assembly apart, marking the orientation of the membrane in pencil or by cutting a corner as in step 14 of the basic protocol.

10. If the gel was nondenaturing, denature the membrane by placing it for 10 min, DNA-side-up, on three pieces of Whatman 3MM paper soaked in 0.4 M NaOH.

> *This denaturation step is required for subsequent rehybridization. If the gel was run under denaturing conditions, this step is not required.*

11. Rinse the membrane in 2× SSC, place on a sheet of Whatman 3MM paper, and allow to dry. Immobilize the DNA and store membranes as in steps 16 and 17 of the basic protocol.

COMMENTARY

Background Information

Southern blotting has been one of the cornerstones of DNA analysis since its first description by E.M. Southern (1975). Immobilization of DNA by binding to nitrocellulose, either powdered or in sheet form, had been utilized in biochemistry and molecular biology for several years (Hall and Spiegelman, 1961; Nygaard and Hall, 1963), but Southern was the first to show how immobilization of size-fractionated DNA fragments could be carried out in a reliable and efficient manner. The advent of Southern transfer and the associated hybridization techniques (*UNIT 2.10*) made it possible for the first time to obtain information about the physical organization of single and multicopy sequences in complex genomes. This expedited the first successful cloning experiments with eukaryotic genes and was directly responsible for breakthroughs such as the discovery of introns (Doel et al., 1977). The later application of Southern transfer and hybridization to the study of restriction fragment length polymorphisms (RFLPs) opened up new possibilities such as genetic fingerprinting (Jeffreys et al., 1985) and prenatal diagnosis of genetic disease (Davies, 1986).

The term Southern blotting, now used to describe any type of DNA transfer from gel to membrane, originally referred solely to capillary transfer onto nitrocellulose. The fragility of nitrocellulose membranes prompted the search for alternative types of support matrix, resulting in the introduction of nylon membranes in the early 1980s. In comparison with nitrocellulose, nylon membranes have less stringent requirements regarding the composition of the transfer buffer, and in recent years several novel buffer formulations have been devised. The most important innovation has been the use of alkaline transfer buffers with positively charged nylon membranes, resulting in immediate covalent attachment of the DNA to the membrane and thereby eliminating the need for the post-transfer immobilization steps required with nitrocellulose and uncharged nylon. Attempts have also been made to reduce the time needed for transfer by either changing the architecture of the transfer assembly (Lichtenstein et al., 1990; Chomczynski, 1992) or using noncapillary methods such as electroblotting, first described for protein transfers (Towbin et al., 1979; *UNIT 10.8*).

Critical Parameters

The amount of DNA that must be loaded depends on the relative abundance of the target sequence to which hybridization will take place (*UNIT 2.10*). The detection limit for a radioactive probe with a specific activity of 10^8 to 10^9 dpm/μg is about 0.5 pg DNA. Thus, for human genomic DNA, 10 μg—equivalent to 1.5 pg of a single-copy gene 500 bp in length—is a reasonable minimum quantity to load. For more information about appropriate DNA quantities, see the commentary to *UNIT 2.10*.

Optimization of the parameters that influence Southern blotting must be carried out in conjunction with hybridization analysis, as the efficiency of transfer can be assessed only from the appearance of the autoradiograph obtained after the membrane is probed. It can therefore take a considerable amount of time to identify the precise conditions needed for optimal transfer, and there is often a temptation to make do with a suboptimal setup, so long as it works sufficiently well. This is understandable, but quality control of Southern transfers should not be neglected, as quite dramatic improvements can sometimes be achieved simply by using a more appropriate membrane or transfer system. The following paragraphs describe the most important factors that should be considered.

Choice of membrane

Nylon and nitrocellulose membranes have very different properties (Table 2.9.1). It is generally accepted that the main advantage of a nylon membrane is its greater tensile strength and the fact that the DNA can be bound covalently by UV crosslinking (Li et al., 1987) or, in the case of positively charged nylon, by transfer with an alkaline buffer (Reed and Mann, 1985). Nylon membranes can therefore be reprobed up to about 12 times without becoming broken or losing their bound DNA. In contrast, nitrocellulose membranes are fragile and do not bind DNA covalently: baking to immobilize the DNA leads to a relatively weak hydrophobic attachment through exclusion of water. After about three rounds of hybridization, a nitrocellulose membrane is usually in pieces, with most of the DNA leached off (Haas et al., 1972). If reprobing is important, then a nylon membrane (charged or uncharged) should be used.

The most clearly recognized disadvantage of nylon membranes (both charged and uncharged) is the amount of background signal

seen after hybridization. This can be a problem with any type of hybridization probe but is especially bad with some nonradioactive DNA probes (*UNITS 3.18 & 3.19*). There are several ways of reducing background hybridization signals (see *UNIT 2.10*, troubleshooting), but these may not be fully effective with a nylon membrane; if background is a problem, the only answer may be to change to nitrocellulose.

Nylon membranes are able to bind about five times more DNA per cm^2 than nitrocellulose (Table 2.9.1), but this is not a factor in Southern blotting, as the maximum binding capacity of the membrane is never approached. Size limits for efficient DNA retention are more important. Nylon retains DNA fragments down to 50 nucleotides in length, but nitrocellulose is inefficient with molecules <500 nucleotides. The only reason that PCR products shorter than 500 bp give reasonable hybridization signals after transfer to nitrocellulose is because there is normally a lot of DNA in the band to start with. Nitrocellulose is not a good choice for restriction-digested genomic DNA if the target band for hybridization probing is likely to be <500 bp.

In addition to nitrocellulose, uncharged nylon, and positively charged nylon, there are several other transfer matrices that are used less frequently (Table 2.9.1). Supported nitrocellulose membranes, an interesting innovation of the late 1980s, attempt to improve the tensile strength of nitrocellulose by supporting the matrix on a more rigid platform. In practice these membranes tend to combine the worst feature of nitrocellulose (loss of bound DNA) with elevated background caused by the support material. A second alternative to the "traditional" types of membrane are the activated cellulose papers, ABM- (Noyes and Stark, 1975) and APT-papers (Seed, 1982). The cellulose matrix of these papers carries aromatic groups that, after chemical activation, bind DNA covalently. These papers have relatively low binding capacities and for routine work offer no advantages over nylon (which they predated). They can, however, bind oligonucleotides down to just 2 nucleotides in length, considerably below the lower limit for nylon or nitrocellulose.

Many commercial suppliers market transfer membranes of one type or another. Quality is variable, especially with charged nylon membranes. The products listed in Table 2.9.1 reflect the author's experiences and do not necessarily include all the reliable brands.

Transfer buffer

With nitrocellulose, the transfer buffer must provide a high ionic strength to promote binding of the DNA to the membrane (Southern, 1975; Nagamine et al., 1980). Several formulations exist, but 20× SSC is recommended because it is easy to make up and can be stored for several months at room temperature. The solution does not need to be filtered prior to use as a transfer buffer, but will need filtering if the same stock is to be used to prepare hybridization solutions (*UNIT 2.10*). Lower SSC concentrations (e.g., 10×) should not be used with nitrocellulose membranes, as the lower ionic strength may result in loss of smaller DNA fragments during transfer. In addition, alkaline transfer is not suitable for nitrocellulose membranes, as they do not retain DNA at pH 9.0 and fall apart after long exposure to alkali (Dyson, 1991).

Nylon membranes are able to bind DNA under a variety of conditions (acid, neutral, alkaline, high or low ionic strength), but a high-salt buffer such as 20× or 10× SSC appears to be beneficial (Khandjian, 1987). The addition of 2 mM *N*-laurylsarcosine (Sarkosyl) to the buffer may aid transfer to certain brands of nylon membrane (Chomczynski, 1992). Positively charged nylon membranes can be used with SSC buffers as described in the basic protocol, but this does not exploit their full potential. The ability of these membranes to bind DNA covalently after transfer in an alkaline buffer (0.4 M NaOH) is a major advantage, and the technique is well worth assessing if low efficiency is suspected with high-salt transfers. The only real problem with alkaline blotting is that high backgrounds may result if a chemiluminescent detection system (*UNIT 3.19*) is used. Alkaline transfer can also be carried out with uncharged nylon, but less DNA is retained than with charged nylon (Dyson, 1991).

Duration of transfer

The most difficult parameter to evaluate is duration of transfer. In a capillary system, rate of transfer depends on the size of the DNA, thickness of the gel, and agarose concentration. Upward capillary transfer is slow, as the architecture of the blot crushes the gel and retards diffusion of the DNA. With a high-salt buffer, it takes ~18 hr to obtain acceptable transfer of a 15-kb molecule from a 5-mm-thick 0.7% agarose gel; with the same gel 90% of the 1-kb molecules will be transferred in 2 hr. This problem is partially alleviated by the depurination step in the basic protocol, which breaks

larger molecules into fragments 1 to 2 kb in length, thereby reducing the time needed for their transfer. Even so, the blot should be left for at least 12 hr. If the gel is thicker than 5 mm or has an agarose concentration >1.0%, it cannot be assumed that the larger fragments will have transferred to a sufficient extent after 12 hr and a longer blotting period (up to 24 hr) may be necessary. The required blotting period can be assessed only by trial and error: if it is possible that the target band for the hybridization probe is >10 kb and a clear signal is not seen, the blot should be repeated using a longer transfer period.

Alkaline blots are more rapid, with most of the DNA being transferred during the first 2 hr. Therefore, if transfer of large bands is essential, alkaline transfer onto positively charged nylon should be considered as an alternative to a high-salt blot. Prolonging the alkaline transfer beyond 2 hr apparently does no harm (Chomczynski, 1992), and often these blots are left overnight.

More rapid transfers can be achieved with the downward capillary blot procedure developed by Chomczynski, described in the second alternate protocol. Variables in this system have been comprehensively evaluated (Chomczynski, 1992) and a downward blot is recommended if maximal transfer efficiency is required. If speed is more important than efficiency (e.g., if the gel being blotted contains a restriction-digested plasmid with few bands, each containing a relatively large amount of DNA) then any one of several quick-blot techniques can be used (e.g., Smith and Summers, 1980). These are not described in the protocols because in practice an overnight blot is usually just as convenient—everyone has to sleep sometime.

Transfer method

Capillary transfer is still the most popular method of Southern blotting. Its advantages are simplicity, reliability, and the lack of special equipment requirements. Alternative transfer methods have not yet achieved widespread use but are gaining prominence for certain applications.

Electroblotting, originally developed for protein transfers (see UNIT 10.8), does not work well with high-salt buffers and so is less appropriate for nitrocellulose membranes; it is worth attempting with nitrocellulose only if there is a really good reason for using this type of membrane (Smith et al., 1984). The technique has been applied to both nylon membranes and activated papers (Bittner et al., 1980; Stellwag and Dahlberg, 1980), but problems often arise with overheating, which leads to bubble formation between the layers of the sandwich enclosing the gel, in turn leading to uneven transfer. Electroblotting is therefore only recommended for polyacrylamide gels, which cannot be blotted by capillary transfer.

The second alternative to capillary transfer is vacuum blotting, which was likewise first used with protein gels (Peferoen et al., 1982). The transfer buffer is drawn through the gel by vacuum pressure, enabling a 5-mm-thick 1% agarose gel to be blotted onto nitrocellulose (in 20× SSC) or nylon (in water) in as little as 30 min. The vacuum pressure must be controlled carefully to avoid compressing the gel and retarding transfer, but once mastered, the technique is very efficient. In a side-by-side comparison, a vacuum-blotted gel usually gives a significantly higher hybridization signal than a capillary blot. Reliable vacuum blotters are the StrataVac (Stratagene), VacuGene (Pharmacia Biotech), Model 785 (Bio-Rad), and TransVac (Hoefer). A protocol for their use is not provided here, as each device has its own special features; the individual manufacturer's instructions should be followed. Vacuum blotting should be seriously considered in any laboratory where Southern transfers are the rate-limiting step in the research program.

Immobilization technique

The aim of immobilization is to attach as much of the transferred DNA as possible to the membrane as tightly as possible. The standard procedures for the three common types of membranes, in increasing order of desirability, are as follows: for nitrocellulose, baking in a vacuum (leading to relatively weak noncovalent interactions), for uncharged nylon, UV irradiation (leading to covalent crosslinking), and for positively charged nylon, immobilization during alkaline transfer (giving covalent linkages).

Baking and alkaline transfer are straightforward procedures that cannot be improved on if carried out according to the instructions. UV crosslinking is more variable. Calibration of the UV source (see support protocol) is essential even if a light box rather than a transilluminator is used. Calibration should be repeated from time to time to check that the performance of the lights has not declined, and should also be carried out with each new batch of membrane, especially if the supplier has been

changed. Before irradiation, a nylon membrane must be thoroughly dried. Some manufacturers recommend air drying; others suggest a 30-min bake at 80°C. The manufacturer's recommendations should also be consulted if UV crosslinking is to be carried out after a nonalkaline transfer to a positively charged nylon membrane; with some brands, UV treatment can result in a loss of DNA.

Troubleshooting

Troubleshooting Southern blotting can be difficult and laborious, as transfer efficiency cannot be assessed accurately until the membrane has been subjected to hybridization analysis. The gel can be restained with ethidium bromide immediately after blotting in order to determine if a large quantity of DNA remains in the gel, which indicates a major problem with the transfer; however, the absence of DNA on the gel does not mean that all of it has become bound to the membrane. Bound DNA can sometimes be visualized by staining the membrane with methylene blue, but this is unreliable. Often a problem is only recognized when a less-than-perfect autoradiograph is obtained after probing, and even then it may be difficult to decide whether the transfer or hybridization technique is to blame. Discussion of how to troubleshoot Southern transfer is therefore provided in more detail in the commentary of UNIT 2.10.

Early warning signs of problems may be provided by the appearance of the membrane before and during transfer. Membranes should be pure white when removed from their wrapping and should remain white during the transfer and immobilization procedures. Membranes that are discolored, dusty, or dirty should not be used, and a membrane that is difficult to wet should be treated with suspicion. Yellow patches on a nitrocellulose membrane after transfer indicate inadequate neutralization. The only permissible color changes are a slight pinkness after transfer, from ethidium bromide staining of the DNA, or blue marks from transfer of the gel dye markers.

Anticipated Results

The Southern transfer procedures described in this unit should all yield a clear white membrane, possibly with pink and blue patches as described above. No data is generated until the membrane is subjected to autoradiography; anticipated results of autoradiography are discussed in the commentary to UNIT 2.10.

Time Considerations

The time required for Southern blotting is variable, as discussed in critical parameters. The basic protocol can be carried out in 24 hr as follows: restricting the DNA and running the agarose gel, 4 hr; preparing the gel, 2 hr; setting up the transfer, 30 min; transfer, overnight; taking the blot apart and drying the membrane, 30 min; immobilization, 2 hr for nitrocellulose, 5 min for nylon. More rapid transfer systems allow the procedure to be completed in a working day, mainly by reducing transfer time.

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2.9.14

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Key Reference

Southern, 1975. See above.

First description of capillary transfer from gel to membrane.

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Dot and Slot Blotting of DNA

Dot and slot blotting are simple techniques for immobilizing bulk unfractionated DNA on a nitrocellulose or nylon membrane. Hybridization analysis (*UNIT 2.10*) can then be carried out to determine the relative abundance of target sequences in the blotted DNA preparations. Dot and slot blots differ only in the geometry of the blot, a series of spots giving a hybridization pattern that is amenable to analysis by densitometric scanning.

Samples are usually applied to the membrane using a manifold attached to a suction device. The basic protocol describes such a procedure for dot or slot blotting on an uncharged nylon membrane; annotations to the steps detail the minor modifications that are needed if blotting onto nitrocellulose. The first alternate protocol describes the more major changes required for blotting with a positively charged nylon membrane. A second alternate protocol describes preparation of dot blots by spotting the samples onto the membrane by hand.

CAUTION: In all of the protocols, wear gloves to protect your hands from the alkali solution and to protect the membrane from contamination. Avoid handling nitrocellulose and nylon membranes even with gloved hands—use clean blunt-ended forceps instead.