Topic Introduction

Analysis of DNA by Southern Blotting

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Southern transfer and hybridization are used to study how genes are organized within genomes by mapping restriction sites in and around segments of genomic DNA for which specific probes are available. Genomic DNA is first digested with one or more restriction enzymes, and the resulting fragments are separated according to size by electrophoresis through a standard agarose gel. The DNA is then denatured in situ and transferred from the gel to a solid support (usually a nylon or nitrocellulose membrane). The DNA attached to the membrane is hybridized to a labeled DNA, RNA, or oligonucleotide probe, and bands complementary to the probe are located by an appropriate detection system (e.g., by autoradiography). By estimating the size and number of the bands generated after digestion of the genomic DNA with different restriction enzymes, singly or in combination, it is possible to place the target DNA within a context of restriction sites.

BACKGROUND

For two or three years after its introduction, the sensitivity of Southern blotting (Southern 1975) was barely sufficient to detect single-copy sequences in mammalian DNA, and the autoradiographs of the time were so speckled and streaked with background (e.g., see Botchan et al. 1976) that they certainly could not be published today. However, significant advances over the years in several areas have brought increased sensitivity and reproducibility, so that immaculate results are now the general rule rather than the rare exception. The most significant of these improvements is the use of supported nylon membranes that are far more durable and have a higher binding capacity than the original nitrocellulose membranes. In addition, DNA can now be covalently fixed to the membrane after transfer, eliminating problems caused by leaching of nucleic acids from nitrocellulose membranes during incubation at elevated temperatures (Haas et al. 1972). Other advances include:

- more efficient methods of transfer of DNA from gel to membrane, downward capillary transfer (Lichtenstein et al. 1990; Chomczynski 1992), vacuum blotting (Olszewska and Jones 1988; Trnovsky 1992), bidirectional blotting, and transfer in alkaline buffers (Reed and Mann 1985)
- facile labeling of probes in vitro to higher specific activity (Feinberg and Vogelstein 1983, 1984)
- more efficient blocking agents to prevent nonspecific attachment of radiolabeled probes to membranes (Church and Gilbert 1984)
- use of sensitive phosphorimagers to capture images with high efficiency

Many of these improvements have been incorporated into Protocol: **Southern Blotting** [Green and Sambrook 2021a], which deals with transfer of DNA from gels to membranes, and Protocol: **Southern Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Membranes** [Green and

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Sambrook 2021b], which describes hybridization of radiolabeled probes to immobilized DNAs. The techniques described are suitable for Southern analysis of restriction digests of mammalian genomic DNA but can easily be adapted to accommodate large DNA molecules separated by pulsed-field gels, as well as restriction digests of plasmids, cosmids, λ bacteriophages, bacterial artificial chromosomes (BACs), and yeast artificial chromosomes (YACs).

METHODS OF TRANSFERRING DNA FROM AGAROSE GELS TO SOLID SUPPORTS

The transfer of electrophoretically separated DNA from gels to 2D solid supports is a key step in Southern hybridization. Described below are five methods to transfer fragments of DNA from agarose gels to solid supports (nitrocellulose or nylon membranes).

Upward Capillary Transfer

In upward capillary transfer, DNA fragments are carried from the gel in an upward flow of liquid and deposited on the surface of the solid support (Southern 1975). The liquid is drawn through the gel by capillary action, which is established and maintained by a stack of dry, absorbent paper towels (see Fig. 1). The rate of transfer of the DNA depends on the size of the DNA fragments and the concentration of agarose in the gel. Small fragments of DNA (<1 kb) are transferred almost quantitatively from a 0.7% agarose gel within 1 h; larger fragments are transferred more slowly and less efficiently. For example, capillary transfer of DNAs >15 kb in length requires at least 18 h, and even then the transfer is not complete. The efficiency of transfer of large DNA fragments is determined by the fraction of molecules that escape from the gel before it becomes dehydrated. As elution proceeds, fluid is drawn not only from the reservoir, but also from the interstices of the gel itself. This flow reduces the gel to a rubbery substance through which DNA molecules cannot easily pass. The problem of dehydration due to lengthy transfer can be alleviated by partial acid/base hydrolysis of the DNA before capillary transfer (Wahl et al. 1979; Meinkoth and Wahl 1984). The DNA in the gel is exposed to weak acid (which results in partial depurination), followed by strong base (which hydrolyzes the phosphodiester backbone at the sites of depurination). The resulting fragments of DNA (~1 kb in length) can then be transferred rapidly from the gel with high efficiency. The depurination reaction must not proceed too far; otherwise, the DNA will be cleaved into small fragments that are too short to bind efficiently to the solid support. Depurination/hydrolysis can also cause the bands of the final autoradiograph to assume a "fuzzy" appearance, presumably because of increased diffusion of DNA during transfer. Therefore, depurination/hydrolysis is recommended only when it is known ahead of time that the target DNA fragments will exceed 15 kb in length.

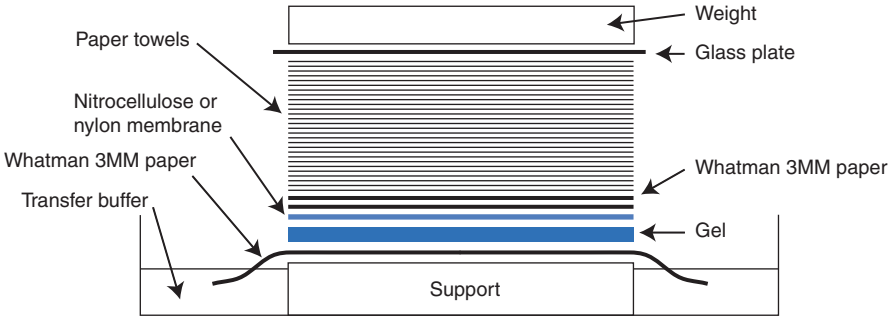

FIGURE 1. Upward capillary transfer of DNA from agarose gels. Buffer drawn from a reservoir passes through the gel onto a stack of paper towels. DNA, eluted from the gel by the moving stream of buffer, is deposited onto a nitrocellulose or nylon membrane. A weight applied to the *top* of the paper towels helps to ensure a tight connection between the layers of material used in the transfer system.

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Downward Capillary Transfer

In downward capillary transfer, DNA fragments are carried in a downward direction in a flow of alkaline buffer and are deposited onto the surface of a charged nylon membrane. Various arrangements of wicks, reservoirs, and different formulations of transfer buffers have been described to achieve downward transfer (e.g., see Lichtenstein et al. 1990; Chomczynski 1992). In our hands, the best results are achieved using 0.4 M NaOH and a setup in which the transfer buffer is drawn from reservoirs to the top of the gel through wicks and pulled through the gel by an underlying stack of paper towels. Transfer of DNA fragments is rapid, and the intensity of signal is ~30% greater than can be achieved by conventional upward transfer. This improvement probably results from a more efficient migration of DNA fragments through the interstices of the gel, which is not under pressure from weights placed on top.

Simultaneous Transfer to Two Membranes

In simultaneous transfer to two membranes, when the target DNA fragments are present in high concentration (e.g., in restriction digests of cloned DNAs), the capillary method can be used to transfer DNA simultaneously and rapidly from a single gel to two nitrocellulose or nylon membranes. The only source of transfer buffer is the liquid trapped in the gel itself, and thus the efficiency of transfer is relatively poor. This method is not recommended when high sensitivity is required (e.g., detection of single-copy sequences in mammalian DNA), but it is perfectly adequate for Southern analysis of plasmids, bacteriophages, or cosmids or the genomes of simple organisms (e.g., *Saccharomyces cerevisiae* and *Drosophila*) by Southern hybridization. Too little mammalian genomic DNA is transferred by this method to allow signal detection routinely.

Electrophoretic Transfer

The electrophoretic transfer method is not practical when nitrocellulose is used as the solid support because the high-ionic-strength buffers required to bind nucleic acids to nitrocellulose conduct electric current very efficiently. In consequence, it is necessary to use large volumes to ensure that the buffering power of the system does not become depleted by electrolysis. In addition, extensive external cooling is required to overcome the effects of ohmic heating.

Electrophoretic transfer has undergone a recent resurgence with the advent of charged nylon membranes and has become the method of choice for analysis of small fragments of DNA separated by electrophoresis through polyacrylamide gels (Stellwag and Dahlberg 1980; Church and Gilbert 1984). Nucleic acids as small as 50 bp will bind to charged nylon membranes in buffers of very low ionic strength (Reed and Mann 1985).

Although single-stranded DNA and RNA can be transferred directly, fragments of double-stranded DNA must first be denatured in situ, as described in Protocol: **Southern Blotting** [Green and Sambrook 2021a]. The gel is then neutralized and soaked in electrophoresis buffer (e.g., 1× TBE) before being mounted between porous pads aligned between parallel electrodes in a large tank of buffer. The time required for complete transfer depends on the size of the fragments of DNA, the porosity of the gel, and the strength of the applied field. However, because even high-molecular-weight nucleic acids migrate relatively rapidly from the gel, depurination/hydrolysis is unnecessary, and transfer is generally complete within 2–3 h. Because electrophoretic transfer requires comparatively large electric currents, it is often difficult to maintain the electrophoresis buffer at a temperature compatible with efficient transfer of DNA. Many commercially available electrophoretic transfer machines are equipped with cooling devices, but others are effective only when used in a cold room.

Vacuum Transfer

In vacuum transfer, DNA and RNA can be transferred rapidly and quantitatively from gels under vacuum. Several vacuum transfer devices are now commercially available in which the gel is placed in contact with a nitrocellulose or nylon membrane supported on a porous screen over a vacuum

chamber. Buffer, drawn from an upper reservoir, elutes nucleic acids from the gel and deposits them on the membrane.

Vacuum transfer is more efficient than capillary transfer and is extremely rapid. DNAs that have been partially depurinated and denatured with alkali are quantitatively transferred within 30 min from gels of normal thickness (4–5 mm) and normal agarose concentration (<1%). If performed carefully, vacuum transfer can result in a twofold to threefold enhancement of the hybridization signal obtained from Southern transfers (Olszewska and Jones 1988).

All of the commercially available apparatuses work well as long as care is taken to ensure that the vacuum is applied evenly over the entire surface of the gel. Special care should be taken with the wells of horizontal agarose gels, which tend to break during preparation of the gel for transfer. If this occurs, the wells should be trimmed from the gel before transfer. (The wells need not be trimmed from the gel as long as they are unbroken.) It is also important not to apply too much vacuum during transfer. When the vacuum exceeds 60 cm of water, the gels become compressed, and the efficiency of transfer is reduced.

MEMBRANES USED FOR SOUTHERN AND NORTHERN HYBRIDIZATION

For almost 20 years, the only available support for immobilization of DNA was nitrocellulose, which was first used in powder form (Hall and Spiegelman 1961) and subsequently as sheets (Nygaard and Hall 1960; Gillespie and Spiegelman 1965; Southern 1975). Northern hybridization was initially performed exclusively with RNA immobilized on activated cellulose papers (Alwine et al. 1977; Seed 1982). However, it was soon realized that RNA denatured by glyoxal, formaldehdye, or methylmercuric hydroxide binds tightly to nitrocellulose. For several years, nitrocellulose therefore became the support of choice for both northern and Southern hybridization. Despite its evident success, nitrocellulose is not an ideal matrix for solid-phase hybridization for the following reasons.

- Its capacity to bind nucleic acids is low (~50–100 μg/cm^2) and varies according to the size of the DNA and RNA. In particular, nucleic acids <400 bases in length are retained inefficiently by nitrocellulose.
- DNA and RNA are attached to nitrocellulose by hydrophobic interactions rather than covalent interactions, and therefore leach slowly from the matrix during hybridization and washing at high temperatures (Haas et al. 1972).
- Nitrocellulose membranes become brittle during baking under vacuum at 80°C, which is usually an integral part of the process to immobilize nucleic acids. The friable membranes cannot subsequently survive more than one or two cycles of hybridization and washing at high temperatures. This problem can be alleviated but not completely solved by using supported membranes made from mixed ester nitrocellulose, which have a higher tensile strength.
- Care is required in storing nitrocellulose membranes if they are to be used successfully for nucleic acid hybridization and western blotting. When the humidity is high, the membrane will adsorb moisture from the air and expand, resulting in curling and wrinkling. When the humidity is low, nitrocellulose membranes will dry and become charged with static electricity. In this state, the membrane is prone to cracking and splitting and becomes very difficult to wet. In most areas throughout the world, it is necessary to alter the conditions under which nitrocellulose is stored to suit seasonal changes in weather conditions. During the hot humid summers of Beijing, for example, nitrocellulose should be stored in a closed container over a dehydrating agent. In winter, the dehydrating agent can be replaced by damp pads of paper. The aim is to have wrinkle-free membranes that wet evenly and quickly (30 sec or less) and take on a bluish tinge when saturated with water.

The problems with nitrocellulose were solved by the introduction of various types of nylon membranes that bind nucleic acids irreversibly and are far more durable than nitrocellulose mem-

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TABLE 1. Properties of membranes used for Southern blotting and hybridization

Property	Type of membrane		
	Nitrocellulose	Neutral nylon	Charged nylon
Capacity (μg nucleic acid/cm^2)	80–120	~100	400–500
Size of nucleic acid required for maximal binding	>400 bp	>50 bp	>50 bp
Transfer buffer	High ionic strength at neutral pH	Low ionic strength over a wide range of pH	
Immobilization	Baking at 80°C under vacuum for 2 h	Baking at 70°C for 1 h; no vacuum required or mild alkali or UV irradiation at 254 nm; damp membranes are generally exposed to 1.6 kJ/m^2; dried membranes require 160 kJ/m^2	
Commercial products		Hybond-N GeneScreen	Hybond-N+ Zeta-Probe Nytran+ GeneScreen Plus

Polyvinylidene difluoride (PVDF) membranes are not routinely used for northern or Southern transfers. However, PVDF membranes, by virtue of their higher mechanical strength and greater capacity to bind proteins, are preferred to nitrocellulose for western blotting. Nylon membranes should not be used for western blotting because the level of nonspecific absorption of immunological probes is unacceptably high.

branes (Reed and Mann 1985). Immobilized nucleic acids can be hybridized sequentially to several different probes without damaging the membrane. Furthermore, because nucleic acids can be immobilized on nylon in buffers of low ionic strength, transfer of nucleic acids from gels to nylon can be performed electrophoretically. This method can be useful when capillary or vacuum transfer of DNA is inefficient, for example, when fragments of DNA are transferred from polyacrylamide gels.

Two types of nylon membranes are available commercially: unmodified (or neutral) nylon and charge-modified nylon, which carries amine groups and is therefore also known as positively charged or (+) nylon. Both types of nylon can bind single-stranded and double-stranded nucleic acids, and retention is quantitative in solvents as diverse as water, 0.25 N HCl, and 0.4 N NaOH. Charge-modified nylon has a greater capacity to bind nucleic acids (see Table 1), but it has a tendency to give increased levels of background, which results, at least in part, from nonspecific binding of negatively charged phosphate groups in DNA and RNA to the positively charged groups on the surface of the polymer. However, this problem can usually be controlled by using increased quantities of blocking agents in the prehybridization and hybridization steps.

Many different types of nylon membranes are available that vary in the extent and type of charge, the method used to apply it, and the density of the nylon mesh. Each manufacturer provides specific instructions for the transfer of nucleic acids to its particular product. These instructions should be followed exactly because they presumably have been shown to yield the best results.

REFERENCES

Alwine JC, Kemp DJ, Stark GR. 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc Natl Acad Sci* **74:** 5350–5354.

Botchan M, Topp W, Sambrook J. 1976. The arrangement of simian virus 40 sequences in the DNA of transformed cells. *Cell* **9:** 269–287.

Chomczynski P. 1992. One-hour downward alkaline capillary transfer for blotting of DNA and RNA. *Anal Biochem* **201:** 134–139.

Church GM, Gilbert W. 1984. Genomic sequencing. *Proc Natl Acad Sci* **81:** 1991–1995.

Feinberg AP, Vogelstein B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132:** 6–13.

Feinberg AP, Vogelstein B. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *[Addendum] Anal Biochem* **137:** 266–267.

Gillespie D, Spiegelman S. 1965. A quantitative assay for DNA–RNA hybrids with DNA immobilized on a membrane. *J Mol Biol* **12:** 829–842.

Green MR, Sambrook J. 2021a. Southern blotting. *Cold Spring Harb Protoc* doi:10.1101/pdb.prot100487.

Green MR, Sambrook J. 2021b. Southern hybridization of radiolabeled probes to nucleic acids immobilized on membranes. *Cold Spring Harb Protoc* doi:10.1101/pdb.prot100495.

Haas M, Vogt M, Dulbecco R. 1972. Loss of simian virus 40 DNA–RNA hybrids from nitrocellulose membranes; implications for the study of virus–host DNA interactions. *Proc Natl Acad Sci* **69:** 2160–2164.

Hall BD, Spiegelman S. 1961. Sequence complementarity of T2-DNA and T2-specific RNA. *Proc Natl Acad Sci* **47:** 137–163.

Lichtenstein AV, Moiseev VL, Zaboikin MM. 1990. A procedure for DNA and RNA transfer to membrane filters avoiding weight-induced gel flattening. *Anal Biochem* **191:** 187–191.

Meinkoth J, Wahl G. 1984. Hybridization of nucleic acids immobilized on solid supports. *Anal Biochem* **138:** 267–284.

Nygaard AP, Hall BD. 1960. A method for the detection of DNA–RNA complexes. *Biochim Biophys Acta* **40:** 85–92.

Olszewska E, Jones K. 1988. Vacuum blotting enhances nucleic acid transfer. *Trends Genet* **4:** 92–94.

Reed KC, Mann DA. 1985. Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Res* **13:** 7207–7221.

Seed B. 1982. Diazotizable arylamine cellulose papers for the coupling and hybridization of nucleic acids. *Nucleic Acids Res* **10:** 1799–1810.

Southern EM. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98:** 503–517.

Stellwag EJ, Dahlberg AE. 1980. Electrophoretic transfer of DNA, RNA and protein onto diazobenzyloxymethyl (DBM)–paper. *Nucleic Acids Res* **8:** 299–317.

Trnovsky J. 1992. Semi-dry electroblotting of DNA and RNA from agarose and polyacrylamide gels. *Biotechniques* **13:** 800–804.

Wahl GM, Stern M, Stark GR. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. *Proc Natl Acad Sci* **76:** 3683–3687.

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