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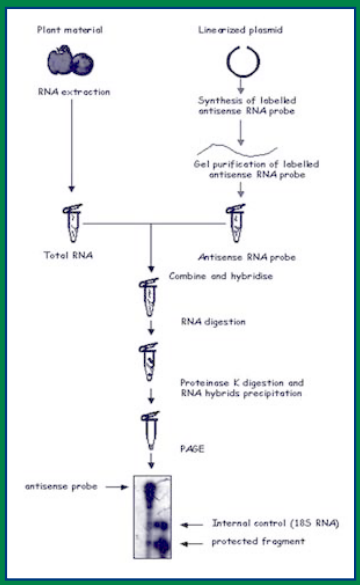
Gene Probes

Principles and Protocols

Edited by

Marilena Aquino de Muro
Ralph Rapley



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Target Format and Hybridization Conditions

Alex Reid

1. Southern Blotting

1.1. Introduction

The isolation of specific regions within the genome of an organism is now normally accomplished by polymerase chain reaction (PCR) amplification using primers specific for the region in question. However, there are occasions where this is not possible (loss of primer sites resulting in no amplification or if there are no primers available). In these cases the detection of the sequence of interest can be achieved by hybridization of a labeled probe to restricted genomic DNA immobilized on a membrane by Southern blotting ***(1)***. Genomic DNA is first digested by one or more restriction enzymes and the fragments generated separated by gel electrophoresis. The amount of DNA to be applied to the gel varies from application to application. In general 10 µg of human genomic DNA is needed for the detection of a single copy gene when using radioactively labeled probes and an overnight exposure to X-ray film. This figure can be reduced if the target is either a repetitive element (e.g., ribosomal DNA) or, if plasmid DNA or PCR products are run on the gel. Once the fragments are separated on the gel the DNA is then denatured *in situ* and transferred by capillary transfer to either a nitrocellulose or nylon membrane. The DNA fragments are then bound to the membrane, which can then be used in a hybridization reaction.

1.2. Materials

1.2.1. Specific Materials

1. 3MM filter paper (Whatman), paper towels, glass or plastic tray and support (a gel casting tray turned upside down), cling film.

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2. Hybond N membrane (Amersham Pharmacia Biotech).
3. Denaturing solution: 1.5 *M* NaCl, 0.5 *M* NaOH.
4. Neutralizing solution: 1.5 *M* NaCl, 0.5 *M* Tris-HCl, pH 7.2, 0.001 *M* EDTA.
5. 20× Saline sodium citrate (SSC): 3 *M* NaCl, 0.3 *M* Na_3 citrate.
6. UV transilluminator.
7. 100× Denhardt's solution: 10 g of bovine serum albumin (BSA) fraction V, 10 g of Ficoll 400, 10 g of polyvinylpyrrolidone (PVP) in 500 mL of distilled water. Store at –20°C in 10 mL aliquots.
8. 10% (w/v) Sodium dodecyl sulfate (SDS).
9. 10 mg/mL sheared herring testis DNA. Store at –20°C.

1.2.2. Optional Materials

1. 0.25 *M* HCl.
2. 0.4 *M* NaOH and a solution of 0.1× SSC, 0.1% (w/v) SDS, and 0.2 *M* Tris-HCl, pH 7.5.
3. Oven set at 80°C.
4. Vacuum blotting system (VacuGene system from Amersham Pharmacia Biotech).

1.3. Method

1.3.1. Preparation of the Gel for Transfer

1. Electrophorese samples in an agarose gel (*see* **Note 1**) and transfer the gel to a glass or plastic tray slightly larger than the gel.
2. If the fragments for analysis are large (>10 kb) the efficiency of transfer can be increased by depurinating the DNA. Add 0.25 *M* HCl to the tray containing the gel until the gel is just covered (*see* **Note 2**). Place on a rocking platform or orbital shaker and agitate gently for 15–25 min at room temperature.
3. Remove the 0.25 *M* HCl, rinse the gel with distilled water and cover with denaturing solution. Return the tray to the rocker and shake for 30 min at room temperature (or 15 min after the dye has returned to blue).
4. Remove the denaturing solution, rinse the gel with distilled water and cover with neutralizing solution. Shake for 15 min at room temperature.
5. Repeat with fresh neutralizing solution.
6. Set up the capillary blot.

1.3.2. Setting Up the Capillary Blot

1. Half fill a glass or plastic tray of a suitable size with 20× SSC (*see* **Note 3**). Place a support in the tray (the upturned casting tray in which the gel was cast). Cover the support with a wick made from three sheets of 3MM paper. Allow the 20× SSC to wet the wick and ensure there are no air bubbles trapped between the sheets and the support (*see* **Note 4**).
2. Carefully place the treated gel on the wick ensuring there are no air bubbles

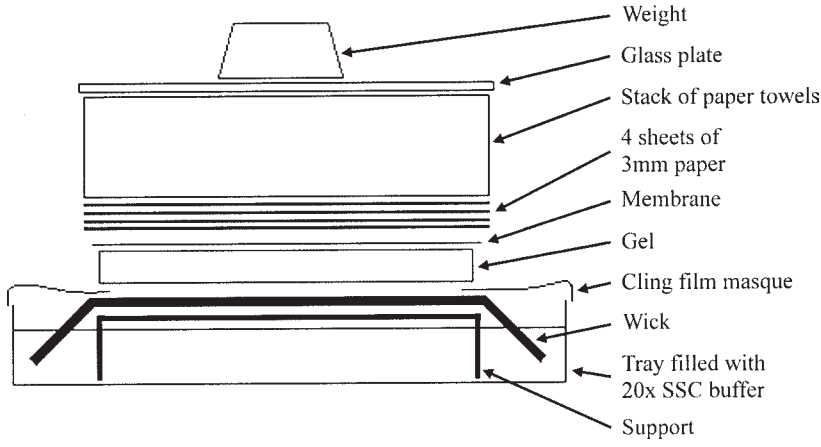

Figure 1. Schematic of a capillary Southern blot.

between the wick and the gel. Surround the gel with cling film (**Fig. 1**) to ensure that transfer occurs through the gel and not around the sides.

3. Place the membrane onto the gel (*see* **Note 5**).
4. Wet two sheets of 3MM paper cut slightly larger than the membrane with 2× SSC and place these (one at a time) on top of the membrane ensuring there are no bubbles.
5. Place a dry sheet of 3MM paper on top of the wet ones. Repeat with another sheet of 3MM paper.
6. Place a stack of paper towels 5–10 cm high on top of the 3MM paper and cover with a glass plate. Put a 500-g weight on top of the glass plate.
7. Allow transfer to proceed for several hours (preferably overnight).
8. After blotting carefully dismantle the stack of paper towels, 3MM sheets, etc. to expose the membrane. Before removing the membrane mark the edges of the gel with a pencil (if desired the wells can also be marked).
9. Remove the membrane and rinse carefully in 2× SSC to remove any adhering pieces of agarose.
10. Air-dry the membrane on a sheet of 3MM paper.
11. Fix the DNA to the membrane either by baking at 80°C for 2 h or by wrapping the membrane in cling film and placing DNA side down on a UV transilluminator for 2–5 min (*see* **Note 6**).

1.4. Optional Methods for DNA Transfer

1.4.1. Bidirectional Transfer to Two Membranes

If required the DNA in a gel can simultaneously be transferred to two membranes using the method of Smith and Summer ***(2)***. This method is of benefit if many probes need to be hybridized to the DNA in a short space of time.

1. Prepare the gel for transfer as outlined in **Subheading 1.3**.
2. After the final neutralization step cover the gel in 10× SSC and shake for 30 min.
3. Wet two sheets of 3MM paper in 2× SSC. Place one on a flat clean surface.
4. Place a nylon membrane on top of the sheet of 3MM paper, ensuring there are no air bubbles between the 3MM and the membrane.
5. Carefully place the gel on top of the membrane. Do not move the gel once it is in contact with the membrane as transfer will start immediately.
6. Place the second membrane on top of the gel followed by the second wet sheet of 3MM paper, again making sure there are no air bubbles.
7. Pick up the "gel sandwich" and place onto a stack of paper towels. Cover the top with a similar stack of towels. Place a sheet of glass on the top and weigh down as before.
8. Allow transfer to proceed as before.

1.4.2. Vacuum Blotting

There are a number of alternative methods for DNA transfer from agarose gels to membranes. One of the best of these in terms of simplicity and speed is vacuum blotting. Here the DNA is literally sucked out of the gel onto the membrane and the entire process can be carried out between 20 and 60 min. Using a vacuum blotting system several gels can be blotted in a single day. The system used in our laboratory is the VacuGene XL available from Amersham Pharmacia Biotech.

1. Set up the vacuum blot apparatus ensuring there is a liquid trap between the pump and the blotter.
2. Prewet the support screen with distilled water and place shiny side up in the blotter.
3. Place a plastic mask on the support screen with a precut hole slightly smaller than the gel to be blotted.
4. Position the membrane over the hole in the mask, ensuring there are no air bubbles between the membrane and the support screen.
5. Carefully place the gel on top of the membrane, ensuring that there are no air bubbles between the gel and the membrane and that the edges of the gel protrude over the hole in the plastic mask.
6. Clamp the top of the blotting apparatus to the lower part containing the gel.
7. Switch on the vacuum pump and pour 0.25 *M* HCl into the apparatus so that it covers the gel. Stabilize the vacuum at 50 mbar and leave for 4 min.
8. Remove the 0.25 *M* HCl by tilting the apparatus and sucking off the solution. This can be achieved by having a "T" connector between the liquid trap and the blotter, which can be opened and closed by means of a clip. Residual solutions can be removed from the gel surface by wiping with a gloved finger or a disposable pipet.
9. Pour in the denaturing solution until it covers the gel and leave for 3 min. Remove as before.

10. Cover the gel with neutralizing solution (1.0 *M* Tris-HCl, pH 5.0, 1.5 *M* NaCl, 0.001 *M* EDTA), leave for 3 min, and remove.
11. Cover the gel with 20× SSC and leave for 20–60 min (*see* **Note 7**). Make sure the gel remains immersed during transfer.
12. Remove the 20× SSC and with the vacuum still applied peel the gel off the membrane. Switch off the vacuum and remove the filter. Treat as before.

1.5. Hybridization Conditions

There are many different hybridization solutions in the literature. The one detailed here is simple to make and gives low background with the Hybond N nylon membranes. The hybridization can be carried out in either heat sealed plastic bags that can withstand the necessary temperatures or in plastic boxes with sealable lids.

1. Make up a prehybidization solution that contains final concentrations of 5× SSC, 5× Denhardt's solution, and 0.5% SDS. Allow 125 µL of solution per cm^2 of membrane. Place the prehybridization solution in a 50-mL tube and place in a 65°C water bath.
2. Boil enough herring testis DNA to give a final concentration of 100 µg/mL for 5 min and snap cool on ice. Add to the prehybridization solution.
3. Prewet the membrane to be hybridized in 5× SSC and place in an opened out plastic bag (*see* **Note 8**). Close the bag over the filter and heat seal around the edges as close to the gel as possible leaving the top open.
4. Pour in the prehybridization solution, squeeze out as much air as possible, and seal the top of the bag with a heat sealer.
5. Place the bag between two sheets of glass and place in a shaking 65°C water bath. Incubate for at least 30 min.
6. Denature the labeled probe by boiling for 5 min and snap cooling on ice. Cut one corner off the hybridization bag and pipet the probe into the prehybridization solution. Reseal the bag and incubate at 65°C in a shaking water bath overnight.
7. Prepare wash solutions (1–5 mL/cm^2 membrane) and preheat to 65°C.
8. At the end of the hybridization carefully cut one corner off the bag and pour the hybridization solution into suitable container for disposal. Open the bag and remove the membrane and place in a sealable plastic box.
9. Wash the membrane by incubating at 65°C in a shaking water bath in the following solutions: 2× SSC, 0.1% SDS for 5 min (repeat), 1× SSC, 0.1% SDS for 15 min, 0.1× SSC, 0.1% SDS for 10 min (repeat) (*see* **Note 9**).
10. Remove the membrane from the last wash solution and drain the excess liquid off. Wrap in cling film and expose to X-ray film (*see* **Note 10**). If the membrane is to be reprobed it must be kept moist.

1.6. Probe Removal from Nylon Membranes

If the membrane needs to be hybridized with more than one probe the old probe can be removed from nylon membranes (providing they have not dried out) using the following procedure.

1. Place the membrane in a sealable plastic box. Cover the membrane with 0.4 *M* NaOH and incubate at 45°C for 30 mins in a shaking water bath.
2. Pour off the NaOH solution and cover the membrane with 0.1× SSC, 0.1% SDS, 0.2 *M* Tris-HCl, pH 7.5. Incubate at 45°C for 15 min in a shaking water bath.
3. Wrap the membrane in cling film and expose to X-ray film to ensure the old probe has been removed.
4. Filters can be stored wrapped in cling film at –20°C indefinitely.

1.7. Notes

1. For genomic DNA transfer use agarose (type I; low EEO from Sigma) as this allows good transfer of the DNA out of the gel and is fairly cheap.
2. When the samples are loaded on the gel use a loading buffer containing 0.25% (w/v) bromophenol blue. The depurination step can be monitored by the change in color of the dye from blue to yellow. Once the dye has changed color leave the gel for an additional 10 min.
3. An alternative transfer buffer is 20× SSPE: 3.6 *M* NaCl, 0.2 *M* sodium phosphate, 0.02 *M* EDTA, pH 7.7. However, 20× SSC is cheaper.
4. Air bubbles are easily removed by rolling a disposable pipet gently over the surface. This method can be used at all stages of set up.
5. Small DNA fragments will start to transfer to the membrane immediately on contact. Therefore, do not move the membrane once it establishes contact with the gel. Nylon membranes do not require pre wetting before application to the gel. If a nitrocellulose membrane is to be used float the membrane on the surface of a tray filled with distilled water until it is completely wet. Carefully immerse the membrane and leave for 5 min.
6. The optimum exposure time varies between transilluminators and can also change with the age of the UV bulbs. To calibrate the transilluminator run a gel containing six lanes with 50 pg of λ DNA digested with *Hin*dIII in each lane. Blot the DNA onto a membrane and cut the filter into six strips. Expose each strip for varying lengths of time ranging from 30 s to 10 min. Hybridize these blots to λ DNA and expose to X-ray film. The optimum exposure time can be determined by the strip which gives the strongest signal.
7. Transfer times vary depending on the thickness and concentration of the gel, the size of the fragments to be transferred, and the level of vacuum applied.
8. Several membranes can be hybridized in the same bag with little loss of signal.
9. If a radioactive probe is used the progress of the washes can be monitored using a hand held counter. The membrane is ready for autoradiography when the counts fall to near background on areas of the membrane containing no DNA. If in doubt stop the washes early and expose to X-ray film. It is always possible to wash the membrane further if the signal is too strong.
10. Exposure time vary depending on the amount of DNA run on the gel, the specific activity and nature of the probe. A probe hybridizing to a single copy sequence will require longer exposure time than one for a repetitive element. The optimum exposure time will need to be determined for each experiment. In general, an

overnight exposure should suffice for most applications. Exposure times can be shortened by preflashing the X-ray film. To do this mount a flash gun on a support about 50 cm above the bench in the darkroom. Cover the lens of the flash gun with several layers of paper to reduce the amount of light emitted. Take a piece of X-ray film and place below the flash gun. Cover four fifths of the film with a sheet of card and fire the flash gun. Move the sheet of card so that three fifths of the film is exposed and fire the flash gun again. Repeat until the entire film is exposed. Develop the film to determine the optimum flash time for the film/flash gun combination. The required exposure does not alter the background of the film whereas the next exposure does. Flashing the X-ray film in this way presensitizes the film, thus reducing exposure times.

2. Slot/Dot Blots

2.1. Introduction

If large numbers of samples need to by hybridized to a probe that yields a positive/negative result (such as species specific clones), this can be achieved by dot or slot blotting. Using this technique DNA is applied directly to a membrane and therefore no gel electrophoresis is required. Commercial manifolds are available which can be attached to a vacuum source where the DNA is applied to wells from which it is sucked onto the membrane in an ordered array. Alternatively, the DNA can be pipetted directly onto the membrane using a micropipet.

2.2. Materials

2.2.1. Specific Materials

1. 3MM filter paper (Whatman).
2. Hybond N membrane (Amersham Pharmacia Biotech).
3. Denaturing solution: 1.5 *M* NaCl, 0.5 *M* NaOH.
4. Neutralizing solution: 1.5 *M* NaCl, 0.5 *M* Tris-HCl, pH 7.2, 0.001 *M* EDTA.
5. 20× SSC: 3 *M* NaCl, 0.3 *M* Na_3 citrate.
6. UV transilluminator.

2.2.2. Optional Materials

1. Commercial dot/slot blot apparatus and vacuum source (e.g., Minifold Slotblotter or Dotblotter by Schleicher & Schuell UK Ltd).

2.3. Method

1. Heat DNA samples to 95°C and snap-chill on ice. Add an equal volume of 20× SSC.
2. Place a membrane on top of a sheet of 3MM paper.
3. Spot the samples onto the membrane prewetted in 10× SSC in 2-µL aliquots

allowing to dry between applications. Take care not to allow sample spots to merge into each other. If a dot blot apparatus is used turn on the vacuum source and pipet the samples into the wells of the apparatus.
4. Immerse the membrane in denaturing solution for 5 min.
5. Transfer to neutralizing solution for 1 min.
6. Dry and fix the DNA to the membrane as for Southern blots.
7. Hybridize to a labeled probe as for Southern blots.

3. Colony Blots

3.1. Introduction

Isolation of specific sequences from DNA libraries cloned in either bacteriophage or plasmids can be achieved by plating the library out on agar plates and taking colony lifts from the agar plates. The membranes can then by used in a hybridization reaction using a suitable probe.

3.2. Materials

3.2.1. Specific Materials

1. Hybond N nylon membranes (Amersham Pharmacia Biotech) of the desired diameter (slightly less the diameter of the agar plates the colonies are grown on).
2. 3MM filter paper (Whatman).
3. Sterile needle and blunt-ended forceps.
4. Denaturing solution: 1.5 *M* NaCl, 0.5 *M* NaOH.
5. Neutralizing solution: 1.5 *M* NaCl, 0.5 *M* Tris-HCl, pH 7.2, 0.001 *M* EDTA.
6. 20× SSC: 3 *M* NaCl, 0.3 *M* Na_3 citrate.
7. UV transilluminator.

3.2.2. Optional Materials

1. 10% (w/v) SDS.
2. Oven set at 80°C.

3.3. Method

1. Plate out the bacterial cells or bacteriophage on agar plates and incubate overnight (*see* **Note 1**). Cool to 4°C for at least 30 min.
2. Bend the membrane into a U shape. Place the bottom of the U in contact with the surface of the agar plate and gently fold down so the entire membrane is in contact with the agar plate. Do not move the filter once it is in contact with the surface of the plate as this will result in smearing.
3. Mark the orientation of the membrane with respect to the plate by making three asymmetric holes with a sterile needle. These can be used for orientation of the filter after hybridization.
4. Remove the membrane after 30–60 s with a pair of blunt-ended forceps.

5. Place the membrane face up on a sheet of 3MM paper.
6. A repeat lift can be made from the plate if desired.
7. Once all of the plates have been blotted the DNA is liberated from the colonies by placing the membranes colony side uppermost up on a stack of 3MM paper saturated with denaturing solution (*see* **Note 2**). Leave for 2–5 min.
8. Transfer the membranes (colony side up) to a stack of 3MM paper soaked in neutralizing solution for 3 min.
9. Wash the membranes in 2x SSC with agitation for 2 min to remove cell debris.
10. Place the membranes DNA side up on a pad of dry 3MM paper and allow to dry.
11. Crosslink the DNA to the membrane and hybridize as for Southern blots.

3.4. Notes

1. The colonies should not be allowed to grow too large as they may merge into one another. Aim for a colony density of approx 200 per 83-mm diameter plate. Precooling the plates to 4°C prevents the colonies from smearing when blotted and lowers the amount of agar that adheres to the membrane.
2. The stack of 3MM paper should be moist, but not soaking as this will cause the colonies to diffuse. An optional lysis step can be included before denaturing the DNA by placing the filters on a stack of 3MM paper soaked in 10% SDS for 1–3 min.

4. Northern Blots

4.1. Introduction

RNA must be run on agarose gels under denaturing conditions. Two common methods can be used to achieve this. The glyoxal–dimethyl sulfoxide (DMSO) method and the formaldehyde/formamide method. The latter method is slightly easier and is described here. When working with RNA all glassware should be baked at 180°C overnight and all solutions made up containing 0.2% (v/v) diethylpyrocarbonate (DEPC) and then autoclaved to remove contaminating RNAases. It should be noted that Tris solutions cannot be DEPC-treated and that Tris stock solutions should be made up with DEPC treated water. The Tris stock for RNA work should be taken from a separate container from normal laboratory stocks and should be weighed out only by shaking the Tris out of the container (never use a spatula).

4.2. Specific Materials

1. DEPC for treating all solutions to be used. **Caution:** DEPC is a very dangerous substance and care must be exercised when handling it. Once autoclaved there is no further hazard.
2. 10× MOPS buffer: 0.2 *M* 3-[*N*-Morpholino] propanesulfonic acid, 0.5 *M* Na acetate, pH 7.0; 0.01 *M* EDTA.
3. Formaldehyde: 37% Solution, 12.3 *M*, pH >4.0.

4. Formamide (deionized).
5. 3MM filter paper (Whatman), paper towels, glass or plastic tray and support (a gel casting tray turned upside down), cling film.
6. Hybond N membrane (Amersham Pharmacia Biotech).
7. Denaturing solution: 1.5 *M* NaCl, 0.5 *M* NaOH.
8. Neutralizing solution: 1.5 *M* NaCl, 0.5 *M* Tris-HCl, pH 7.2, 0.001 *M* EDTA.
9. 20× SSC: 3 *M* NaCl, 0.3 *M* Na_3 citrate.
10. UV transilluminator.
11. 100× Denhardt's solution: 10 g of BSA fraction V, 10 g of Ficoll 400, 10 g of PVP in 500 mL of distilled water. Store at –20°C in 10 mL aliquots.
12. 10% (w/v) SDS.
13. 10 mg/mL sheared herring testis DNA. Store at –20°C.

4.3. Method

1. Prepare the Northern gel by dissolving 3 g of agarose in 250 mL of DEPC treated water. Cool to 55°C and add 17.5 mL formaldehyde and 30 mL 10× MOPS buffer (both preheated to 55°C. Cast the gel in an appropriately sized tray in a fume hood.
2. Before loading the samples prerun the gel at 5 V/cm for 5 min in 1× MOPS buffer.
3. Prepare the RNA samples (*see* **Note 1**) for electrophoresis by adding the following to the RNA sample: 5.5 µL of formaldehyde, 15 µL of formamide, 1.5 µL of 10× MOPS buffer and distilled water to a final volume of 30 µL.
4. Denature the sample at 55°C for 15 min and add 3 µL of loading buffer (50% glycerol, 0.25% bromophenol blue in DEPC-treated water). Load onto gel and run (*see* **Note 2**).
5. Place the gel in a tray and cover with DEPC-treated distilled water. Incubate with gentle agitation for 15 min.
6. Remove the water and replace with 10× SSC and shake for 15 min; repeat once.
7. Set up the capillary blot as described for Southern blotting.
8. After transfer is complete dismantle the Northern blot, remove the filter and air-dry. Do not rinse the filter as for Southern blots. Fix the RNA to the membrane by UV crosslinking or baking at 80°C as for Southern blots.
9. Hybridize membrane using the same conditions as for Southern blots.

4.4. Notes

1. The success of any experiments involving RNA depends on the quality of the RNA! The most reliable method to extract RNA from tissue samples is to use a commercially available kit.
2. If possible, recirculate the running buffer.

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2

End Labeling Procedures

An Overview

Elena Hilario

1. Introduction

The purpose of this chapter is to give an overview of the different end labeling procedures. These protocols have been standardized and optimized by several biotechnology companies and are available in kits. Unless your laboratory is involved in producing large quantities of many different probes (e.g., micrograms of each probe), it is unpractical, and perhaps more expensive, to set up your own protocol. However, a good understanding of the type of probe, the location of the label (3'-, 5'-end or distributed all along the molecule), and the number of labeled nucleotides incorporated should be considered when planning an experiment.

2. Label Location

2.1. 3'-End Labeling

The efficiency of labeling the 3'-end of a DNA molecule depends on the type of enzyme used, the type of DNA molecule (single- or double-stranded), and the length of the 3'-end (recessed, blunt end, or protruding).

DNA polymerase I Klenow fragment (exo^-) fills in the 3'-recessed ends of restriction fragments in the presence of radiolabeled nucleotides ***(1)***. The number of labeled molecules will depend on the type of labeled nucleotide added and the sequence of the complementary strand. Blunt end fragments can also be labeled by replacing the unlabeled 3'-end nucleotide by a labeled molecule.

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Klenow fragment does not label 3'-protruding ends efficiently. Labeling with Klenow fragment is an appropriate method for producing DNA size markers.

Bacteriophage T4 DNA polymerase synthesizes DNA on a 5'–3' direction. The enzyme also has exonuclease activity 3'–5' but not on the 5'–3' direction. Two steps are involved in labeling probes with bacteriophage T4 DNA polymerase ***(2)***: a replacement reaction using the 3'–5' exonuclease activity of the enzyme in the absence of dNTPs to generate 3'-protruding ends, and then filling in the ends with a mixture of unlabeled and labeled dNTPs. The resulting labeled fragment can be further digested with endonucleases and generate a mixture of probes of different sizes. Alternatively, the 3'-protruding tails of double-stranded DNA, previously digested with endonucleases, are regenerated by the bacteriophage T4 DNA polymerase in the presence of all four dNTPs (including the desired labeled dNTP). In the presence of dNTPs, T4 DNA polymerase 3'–5' exonuclease activity is inhibited and the polymerase activity predominates. However, caution should be taken to avoid long incubations, as the dNTPs could be exhausted, and the 3'–5' exonuclease activity of T4 DNA polymerase will resume and degrade double-stranded DNA as well as single-stranded DNA. Keep in mind that T4 DNA polymerase has a higher rate of 3'–5' exonuclease activity on single-stranded DNA than on double-stranded DNA. The replacement method can be difficult to control; therefore, filling in previously endonuclease digested DNA with 3'- or 5'-protruding ends in the presence of dNTPs is the best alternative when using bacteriophage T4 DNA polymerase for labeling DNA.

Terminal deoxynucleotidyl transferase (TdT) is a template independent DNA polymerase that incorporates dNTPs to the 3'-OH end of single- or double-stranded DNA, and RNA in an irreversible manner ***(3)***. This enzyme is used for the production of synthetic homo- or heteropolymers ***(4)***, for incorporating a homopolymeric tail to any type of DNA 3'-end ***(3,5–8)***, and for incorporating a single nucleotide analog such as [α-^{32}P]cordycepin-5'- triphosphate (Promega) ***(6,9)*** or digoxigenin-11-ddUTP (Roche Molecular Biochemicals) ***(10)***. Terminal dideoxynucletidyl transferase labels 3'-protruding ends more efficiently than blunt ends or 3'-recessed ends. The incorporation of dA or dT residues is more favorable than incorporating dC or dG. The type of method chosen for incorporating labeled nucleotides to the 3'-end of a DNA molecule depends on the required probe sensitivity and specificity. The 3'-end tailing reaction synthesizes highly sensitive probes owing to the addition of several labeled molecules, but the specificity decreases owing to unspecific binding of the added nucleotide tail. This inconvenience can be solved by changing the stringency conditions. If probe specificity is the priority, 3'-end labeling of the DNA molecule should be performed.

2.2. 5'-End Labeling

There are three ways of labeling DNA molecules at the 5'-end: enzymatic, chemical, or combined methods. A brief description of each method is given.

2.2.1. Enzymatic Methods

The bacteriophage T4 polynucleotide kinase catalyzes two reactions: forward and exchange. In the forward reaction, the enzyme transfers the γ phosphate of [γ-^{32}P]ATP to the 5'-hydroxy group of a DNA molecule (oligonucleotides or nucleoside 3'-monophosphates) or RNA, previously dephosphorylated with alkaline phosphatase. In the exchange reaction, T4 polynucleotide kinase transfers the 5'-terminal phosphate group of the DNA molecule to ADP. Then, the enzyme transfers the γ phosphate of [γ-^{32}P]ATP to the 5'-hydroxy group of a DNA molecule. The forward and the exchange reactions depend on the amount of ATP available ***(13,14)***. The wild-type bacteriophage T4 polynucleotide kinase has 3'-phosphatase activity ***(11)***; however, this unwanted property has been engineered and a mutant T4 polynucleotide kinase 3'-phosphatase minus enzyme is now available (MBI Fermentas, Roche Molecular Biochemicals) ***(12)***. T4 polynucleotide kinase preferentially labels protruding 5'-ends over blunt ends or recessed 5'-ends, but in the presence of polyethylenglycol 8000 the reaction conditions for labeling blunt ends or recessed 5'-ends can improve ***(13)***. Precaution should be taken in avoiding ammonium and phosphate ions during any purification procedure, as T4 polynucleotide kinase is strongly inhibited by these ions ***(13,14)***.

2.2.2. Chemical Methods

This approach is suitable for synthetic oligonucleotides with a modified 5'-end. Terminal amino function is incorporated to the 5'-end after synthesizing the oligonucleotide by adding a phosphoroamidite group. After cleavage from the synthesis support and activation, a digoxigenin molecule is covalently linked (Roche Molecular Biochemicals) ***(15)***. Large quantities of oligonucleotide can be labeled per reaction. The 3'-end remains undisturbed and available for primer extension.

2.2.3. Combined Method

Two methods have been developed by Promega to incorporate nonradioactive labels to the 5'- or/and 3'-end of unmodified or modified oligonucleotides. The T4 polynucleotide kinase incorporates a thiophosphate from adenosine-5'-*O*-(3-thiotriphosphate) to an unmodified oligonucleotide. Then, the activated thiol group of the oligonucleotide reacts with a maleimide modified hapten (fluorescein or biotin), leaving the 3'-end of the molecule unaltered

(FluoroAmpJ T4 Kinase System, Promega). The alternative method generates alkaline phosphatase conjugates at the 5'- or 3'-end of unmodified or amino modified oligonucleotides (LIGHTSMITH® II System, Promega Corp.). If the starting oligonucleotide is not modified, terminal dideoxynucleotidyl transferase incorporates an amino modified ATP to the 3'-end before the activation and conjugation of the enzyme hapten.

3. Purification of Labeled Probes

Radiolabeled probes are usually not purified after synthesis; however, if the incorporation yield is low, removing the unincorporated label might help to avoid a high background noise.

There are four methods to purify labeled probes: spin column chromatography, membrane filtration, adsorption to silica gel membranes, and ethanol precipitation. Gel size exclusion properties in spin column chromatography are not the same as in flow-dependent fractionation. In spin column chromatography, *g* force applied to the column and the centrifugation time are important factors during the exclusion process. Using prepacked and equilibrated columns prevents dilution of the applied sample. Reproducibility during purification is achieved with commercially prepacked columns (e.g., Amersham Pharmacia, Roche Molecular Biochemicals). Specific resins are used for particular purification procedures such as buffer exchange and desalting, and removal of excess primers or free nucleotides.

Membrane filtration is a fast and reliable way of removing excess label, exchanging buffers, and concentrating a sample. Microcentrifuge devices are commercially available with different cut off ranges (e.g., Microcon®, Millipore). This method is appropriate to remove primers, linkers, labeled nucleotides and desalting samples; however, modifying enzymes are usually retained together with the labeled DNA molecule. Therefore, membrane filtration is recommended mainly for chemical labeling methods.

Adsorption of DNA molecules of certain size ranges to silica-gel membrane occurs at high ionic strength and is eluted at lower ionic strength (Qiagen, Germany). No ethanol precipitation is required. Silica-gel membranes assembled on spin columns overcome the problems associated with silica-gel slurries (low yields, slurry carried over with eluted DNA, etc.). Free labeled nucleotides, modifying enzymes, reaction buffers, and other components of the labeling reaction mixtures are easily removed by this method. Recovery of oligonucleotides (17–40 bases long) and double- or single-stranded DNA fragments up to 10 kb long is feasible. This is the most efficient way to clean up any modification reaction.

Ethanol precipitation with ammonium or sodium acetate can be performed for most labeling procedures ***(13,14)***; however, for Digoxigenin-labeled probes,

lithium chloride (final concentration 0.4 *M*) should be used instead of sodium acetate. Oligonucleotides and low concentration of labeled probes are easily precipitated with carrier molecules such as glycogen (final concentration 0.4 mg/μL). Alkaline conjugates and fluorescein- or biotin-labeled probes require a combination of spin column chromatography and ethanol precipitation. The storage temperature for most probes is –20°C, or temporarily 4°C. Specific storing buffers are recommended for each method, and special care should be taken regarding pH conditions, light exposure, stabilizers (e.g., glycerol), and half-life of the probe. Storing the synthesized probe in small aliquots prevents degradation by repeated freeze–thaw cycles, and the possibility of accidental cross contamination with other probes.

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3

Photobiotin Labeling

Elena Hilario

1. Introduction

Biotin is a small vitamin found in tissue and blood and is synthesized by intestinal bacteria. Biotin functions as a prosthetic group for several carboxylases and as a CO_2 carrier. The molecule consists of a ring system covalently linked to the enzyme by a valerate side chain acting as a flexible arm. Avidin and streptavidin are two proteins that strongly bind biotin. Avidins are tetrameric proteins; each subunit has a molecular mass of 13,000–16,000 Da. Avidin is found in chicken egg whites, and a lower affinity variant is found in the yolk. Streptavidin is found in the fungus *Streptomyces avidinii*. Although the dissociation constant of avidin for biotin is higher than that of streptavidin for biotin (10-^{15} *M* and 10-^{14} *M*, respectively), the nonspecific adsorption of streptavidin to nucleic acids and negatively charged cell membranes is preferred over avidin. This is due to the low isoelectric point of streptavidin (pI 5–6), compared to pI 10 for avidin. The binding characteristics and stability of avidin–biotin and streptavidin–biotin complexes have been extensively explored and applied to develop numerous methods in immunology and molecular biology.

There are three methods for labeling DNA molecules with biotin: chemical, enzymatic, and photolabeling reactions. The type of method to be used depends on the amount of DNA available and the number of biotin molecules to be incorporated. Several commercial kits are available for DNA biotinylation. The *cis*-platinum Chem-Link reagent (Kreatech Biotechnology, B.V., The Netherlands) binds to the N7 position of guanosine and adenosine bases at 85°C, forming a stable biotin–Pt complex. This chemical reaction might denature and fragment the target DNA; however, this method is suitable for blot hybrid-

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ization. Biotin hydrazide (five carbon atoms in space arm) and Biocytin-hydrazide (10 carbon atoms in space arm) (Pierce, Perstorp Biotec) label cytosine residues in DNA and RNA via a bisulfite-catalyzed transamination reaction ***(1)***. Biotin analogs of dTTP with long spacer arms (16 carbon atoms) can be used for incorporating biotin enzymatically: by 3' end-labeling with terminal transferase and biotin-16-ddUTP, or by replacing dTTP with biotin-16-dUTP in nick translation, random priming, or polymerase chain reaction (PCR) amplifications. All the methods described (except PCR biotin labeling) require considerable amounts of starting DNA substrate, which must be free of any oligonucleotides, buffers, enzymes, additives, etc. Forster and co-workers first described the method to synthesize photobiotin acetate and bind it to nucleic acids. A molecule of photobiotin is formed by a biotin molecule bound to a linker arm of nine carbons with a positively charged tertiary amino group at the center and a photoreactive arylazide group. The reaction takes place under strong visible light or under ultraviolet light. The arylazide molecule is converted to an extremely reactive arylnitrene group that binds with the nucleic acid; however, the nature of the linkages is unknown. The spacer arm is long enough to allow the interaction of biotin and streptavidin without interference ***(2)***.

What is the advantage of using photobiotin over the chemical or enzymatic biotin labeling methods? Photobiotinylation is a simple method for labeling DNA because the reaction occurs in water and is terminated by mild alkalinization. Although ultraviolet light is used to photoactivate the molecule, the DNA damage is minimum. It does not interfere in hybridizing the biotinylated probe to the target DNA molecules in any further protocol, including delicate procedures such as subtraction hybridization ***(3,4)***.

Photobiotin acetate can be synthesized in the laboratory ***(5)***; however, it is also available from Pierce Chemical (Rockford, IL) and Sigma-Aldrich (St. Louis, MO).

2. Materials

1. 1 mg of photobiotin acetate.
2. Insect vials with flat bottom, without caps.
3. Scintillation vials without caps and a styrofoam floating boat.
4. Tray with ice water.
5. Darkroom with red safety light (e.g., KODAK safety light filter GBX2).
6. UV transilluminator lamp (MacroVue UV-25 transilluminator 302 nm Hoefer, set in HIGH 254 nm, 9000 μW/cm^2).
7. 1 *M* Tris, pH 9.0.
8. Tris–EDTA-saturated *n*-butanol:10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
9. 5 *M* Potassium acetate, pH 7.0.