

## **EXPERIMENT NINE: SOUTHERN BLOT**

**The objectives of this experiment are to:**

- (1) restrict genomic DNA for Southern blot electrophoresis
- (2) electrophorese restricted gDNA for Southern blotting
- (3) perform a Southern transfer
- (4) hybridize and wash a Southern transfer
- (5) detect the labeled probe on the Southern membrane
- (6) determine the gene fragment sizes in samples on the Southern blot.

**Exeprimental expectations are:**

# Southern Blotting

## Introduction

The Southern blot is an example of a basic nucleic acid hybridization technology. Hybridizations are useful for understanding gene organization and copy number, cloning genes from libraries, detecting polymorphisms (such as RFLPs used in forensics) and detecting expressed transcripts.

In the Southern Blot procedure, DNA fragments from an agarose electrophoresis gel are first transferred onto a membrane and then detected by a labeled "probe." The transfer step starts the procedure and is problematic for at least two reasons. First, electrophoretic transfer cannot be used because excessive heat would result from the high ionic strength required for DNA to bind to most membranes (especially small fragments, < 500 bp.) Thus, a slower "capillary" transfer is required which allows high ionic strength. Second, as the transfer proceeds, the gel shrinks and retards the migration of the larger pieces of DNA, which are often the size containing a gene or a substantial portion of a gene. Thus, cleaving DNA into smaller fragments (HCl depurination followed by NaOH), along with long periods of time (18-48 hours) are usually needed to effectively transfer large DNA fragments. Cellulose nitrate and Nylon are the most common membranes used and the procedure below may be used for both.

After transfer to a membrane, the DNA fragments are detected by hybridization with a "probe." The probe is labeled with an enzyme, a radionucleotide, or a small organic molecule (which may be detected with another "signal" system). In this experiment, the probe is a "family" of ACT1 fragments directly labeled with alkaline phosphatase. This probe is specific for the gene of interest and will be used to detect the gene sequence in three different DNA sources: genomic DNA fragments, an amplicon-containing plasmid, and PCR products.

The following are other examples of procedures that utilize hybridization technology:

Northern blots were developed for the analysis of RNA. Northern blots are used for characterizing one or a few specific mRNA transcripts within multiple RNA samples. Usually total RNA samples are fractionated by gel electrophoresis, transferred to a blot membrane, and probed with specific RNA or DNA probes. Northern blots may be used to compare the amount of specific mRNAs in two or more samples, to reveal the mRNA transcript size, or to identify splice variants of a gene.

Dot/Slot blots use the same technology as Southern or Northern blots, except the RNA or DNA is not fractionated by gel electrophoresis before blotting. Instead, the nucleic acids are applied to the membrane as a dot or through a slotted manifold. Dot/slot blots are generally used for quantitative comparisons of target nucleic acids among many samples.

DNA microarrays or Gene Chips consist of hundreds or thousands of gene specific nucleic acids (e.g. cDNA clones or oligos) spotted geometrically onto a single membrane, glass slide or other matrix. These arrays may then be used to compare the gene expression patterns of two or more samples. For instance, mRNA from two samples (e.g. treated or untreated cells) are first converted to separate cDNA populations with labeled nucleotides. These separate "probe" populations are then hybridized to separate identical arrays, or if they are labeled with different fluorescent dyes, they are mixed and hybridized to the same array. The amount of hybridized probe signal from any detected spot is proportional to the amount of mRNA in the original cell sample, reflecting the gene expression in the different cell samples.

Genomic DNA to be size fractionated on agarose gels should be digested with a restriction enzyme. Restriction enzymes which are "6-cutters" are typically used because the fragments they generate are "gene-sized", while "4-cutters" would probably result in too many small pieces of DNA due to their sites occurring more frequently.

## Part A: Digestion of Genomic DNA and Southern blot

### Materials and Equipment Required

#### 1. Restriction digest of genomic DNA

37°C water bath

70°C heat block

#### 2. Gel electrophoresis & blot

Electrophoresis unit/casting tray/comb(s)

power supply

GelDoc imaging station

*pipet tip container with lid (no insert)*

*7 mm of gel-sized blotting paper*

*4-5 cm of gel-sized brown paper towels*

*gel-sized charged nylon membrane*

*50 ml sterile conical centrifuge tube*

*UV Cross-linker*

### Reagents Required

#### 1. Restriction digest of genomic DNA

Sterile water

Genomic DNA prep

2  $\mu$ L 10 restriction enzyme buffer

10-20 U ? restriction enzyme

#### 2. Gel electrophoresis & blot

gel loading dye

10  $\mu$ L kb DNA ladder

Ethidium bromide

pCR2.1/GPDH plasmid

0.1 ng/ $\mu$ L GPDH amplicon

*100 ml 0.25 N HCl solution (per gel)*

*400 mL 0.5 M M NaOH/1.5 M NaCl(per gel)*

*Southern transfer – per 4 students:*

### Genomic DNA digest and electrophoresis procedure

1. Make a master mix for all of the digests that your table will perform by combining the following reagents (in order) in a 1.5 mL microfuge tube:

10  $\mu$ L genomic DNA

\_\_\_  $\mu$ L 1X restriction enzyme buffer from 10X stock

\_\_\_  $\mu$ L BSA (0.1 mg/mL from a 10 mg/mL stock)

\_\_\_  $\mu$ L water (or adjust to have total volume of 20  $\mu$ L)

1  $\mu$ L \_\_\_\_\_ restriction enzyme, ~10 U/ $\mu$ L final

total vol = 20  $\mu$ L

2. Incubate at 37°C for 1-3 hours to ensure complete digestion.

***Done by instructor:***

3. *Pulse microfuge to collect liquids and then store at 4°C or on ice until ready to electrophorese. This will be your "restricted genomic DNA".*

**Next lab period:**

4. Prepare a 1% mini agarose gel in **50mL** of 1X TAE as previously done; use the 12-well comb and prepare rows of combs in each gel (see gel diagram below).
5. Prepare the following samples for running on the gel (*italicized* is prepared for you):
  - 5  $\mu$ L 1:250 *ACT1* amplicon (~0.5 ng total) + water and gel loading dye
  - 5  $\mu$ L 1:100 dilution of *pCR2.1/ACT1(-intron)* plasmid (~1 ng total) + water and dye
  - 5  $\mu$ L genomic DNA + water and gel loading dye
  - 20  $\mu$ L restricted genomic DNA + gel loading dye
6. Each student will load the following samples into 5 wells of the gel with 2 lanes separating students in each row (so 4 students will share one gel; 2 students using the top row; 2 students using the bottom row):

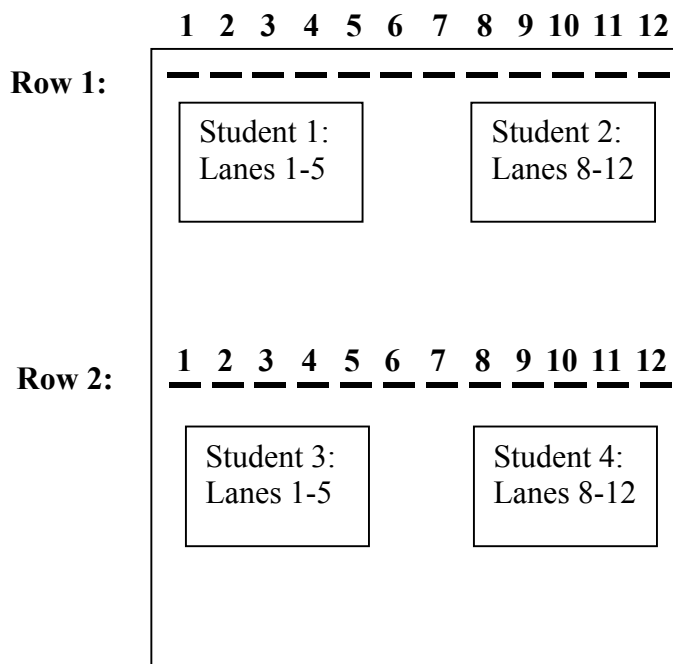
**Sample 1:** 10  $\mu$ L 1 kb ladder

**Sample 2:** ~15-20  $\mu$ L restricted genomic DNA (as much as will fit into well without overflowing)

**Sample 3:** uncut genomic DNA (from above)

**Sample 4:** pCR2.1/ACT1 plasmid (from above)

**Sample 5:** ACT1 amplicon (from above)

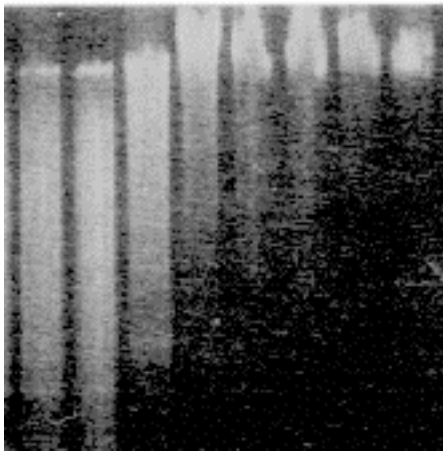


7. Electrophorese in 1X TAE for 60 minutes at 75 V.  
The unrestricted DNA should run as one band at an apparent size of 40-50 kb, while the restricted DNA should run as a smear from the top of the 1 kb ladder standard to ~1000 bp. See figures 9.1 and 9.2 (following page) as examples of genomic DNA restrictions.
8. Capture the image of your gel using a fluorescent ruler (why would you need to do this?) and begin preparing for Southern transfer of DNA onto the nylon membrane.

**Figure 9.1.**

Sample pattern of partially  
restricted genomic DNA

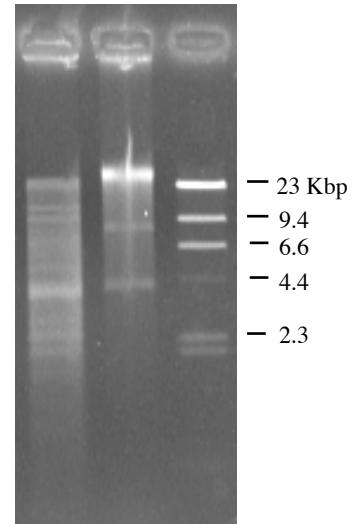
Decreasing amounts of restriction enzyme -->



**Figure 9.2.**

Restricted and Native  
yeast DNA

restricted yeast DNA, EcoRI    native yeast DNA    □ phage HindIII digest std.



**Figure 9.1 and 9.2. Example gels of genomic DNA digests.**

The gel shown in Figure 9.1 shows genomic DNA samples digested with decreasing amounts of enzyme. Notice the more complete digests in the left lanes compared to those on the right. The left lanes demonstrate the desired results. Figure 9.2 shows the differences between restricted (left-most lane) and unrestricted yeast genomic DNA (middle lane). The restricted sample shows a greater degree of DNA fragment size variation. The distinct bands seen in the unrestricted lane may be yeast plasmids present in the cell. Sizes of DNA standards are shown in kb on the right.

## Alkaline Transfer Procedure

1. Rinse gel in distilled water (DI water) in the bottom of a pipet tip container (yellow) for a few minutes. *Cut off a small corner of the upper right of the gel to help with orientation later.*
2. Replace water with 0.25 N HCl solution (Depurination solution), so gel is just covered with the solution.
3. Agitate for 10 minutes. The bromophenol blue dye should turn yellow-green: indicating diffusion of HCl throughout the gel.  
\*The HCL partially depurinates the DNA.
4. Decant HCl solution and rinse gel in DI water.
5. Add Denaturation and Transfer solution to wash container so it is 2/3 full and agitate for 15-30 minutes. The bromophenol blue should return to its normal color.  
\*During this step, the NaOH cleaves the DNA at the sites of depurination producing short pieces of DNA which will transfer more effectively than long pieces of DNA. The NaOH/NaCl also denatures DNA into single strands (important for binding the target DNA to the membrane and the probe).
6. Set up the transfer of the entire gel; build transfer apparatus from bottom to top as follows and illustrated in Figure 9.3 on following page:
  - Place 1 cm blotting paper on bottom of pipet tip container.
  - Use 50-80 mL Denaturation and Transfer solution to saturate lower layer of blotting paper.
  - Place gel **face down** on top of wet blotting paper (be sure that there are no bubbles between the gel and paper).
  - Cut same corner of membrane as the gel and mark membrane with name of your group using pencil (this will be on the DNA side). Place membrane **face down** on top of gel (note that the opposite corner is cut when the gel/membrane are facing down - obviously). Mark both rows of wells by making a hole in the membrane over the first and last well in each row. You will be cutting the membrane after transfer into 4 separate sections for each of you to hybridize separately.
  - Place 1 sheet of soaked blotting paper on top of membrane followed by 2 dry.
  - Place 4-5 cm blotting paper or brown paper towels on top of dry blotting paper.
  - Place lid to pipet tip box on top with weight.
7. Allow DNA to transfer for two hours to overnight.

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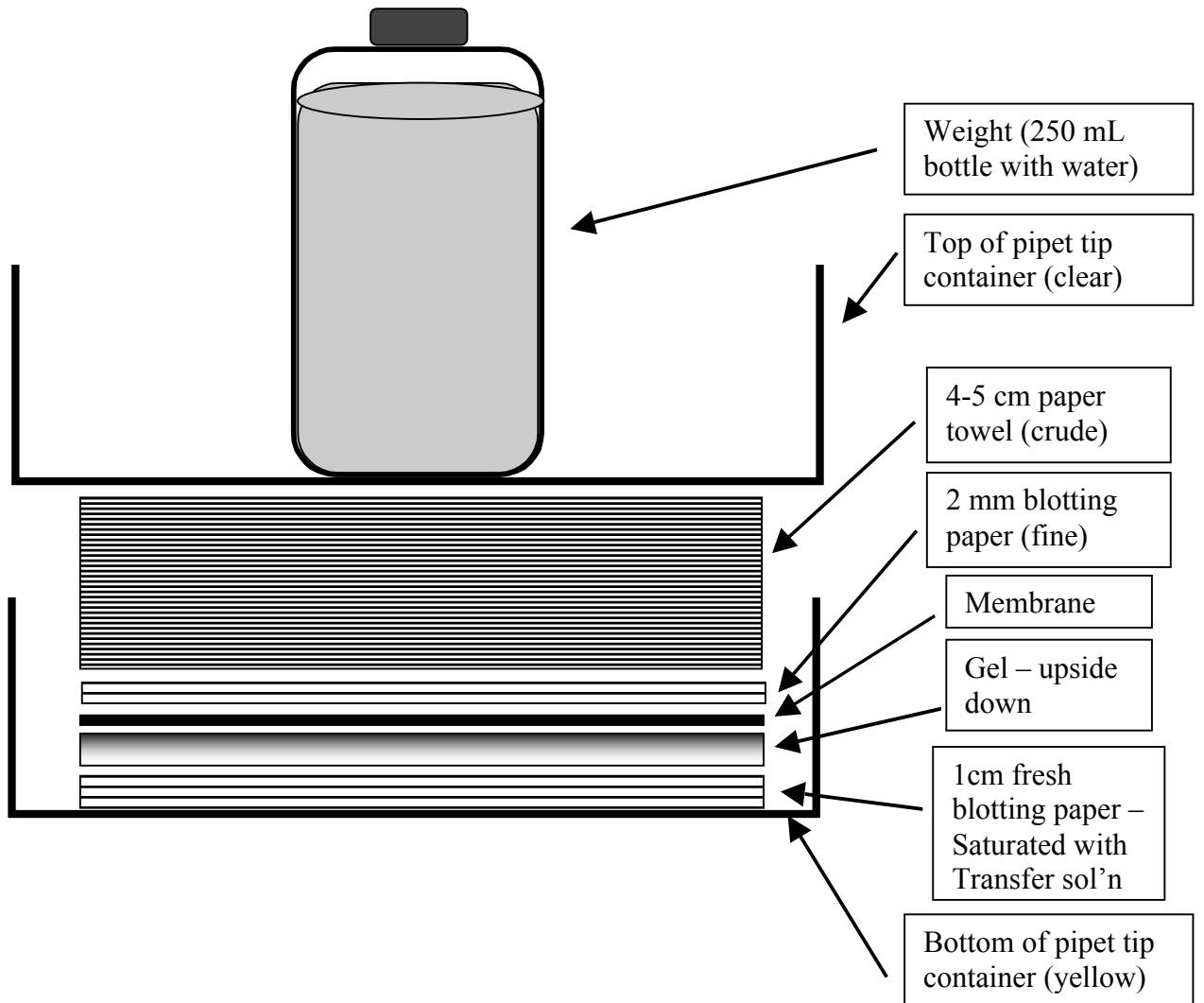
### ***Done by instructor:***

8. *After transfer is complete, the instructor will remove the top layers of filter paper, mark the wells on your nylon, peel membrane from gel and, keeping DNA sample side up and rinse in 20 mL 2X SSC inside pipet tip container lid. **Be sure you marked the DNA side of membrane!** Membrane will be dried at room temperature between filter papers.*

### **During the next lab period:**

9. UV cross-link the DNA to the membrane. Be sure to place membrane face-up inside the UV cross-linker and expose to appropriate amount of UV light (Program C2, 50 mJ). Cut membrane into 4 squares according to marks and place each membrane into a separate sterile 50 mL tube for hybridization.

Figure 9.3 Southern Transfer Set-up



## Part B: Southern hybridization

### Materials and Equipment Required

Hybridization oven set at 55°C

### Reagents Required

4.5 mL Hybridization buffer (0.25 mL/cm<sup>2</sup> membrane)

\*Prepare Hybridization solution with added 0.5M NaCl and 4% (w/v) blocking agent from

5-10 ng/mL labeled DNA probe (total of 23 ng in 6.4 µL)

\* Made fresh (within 2-3 hours of class)

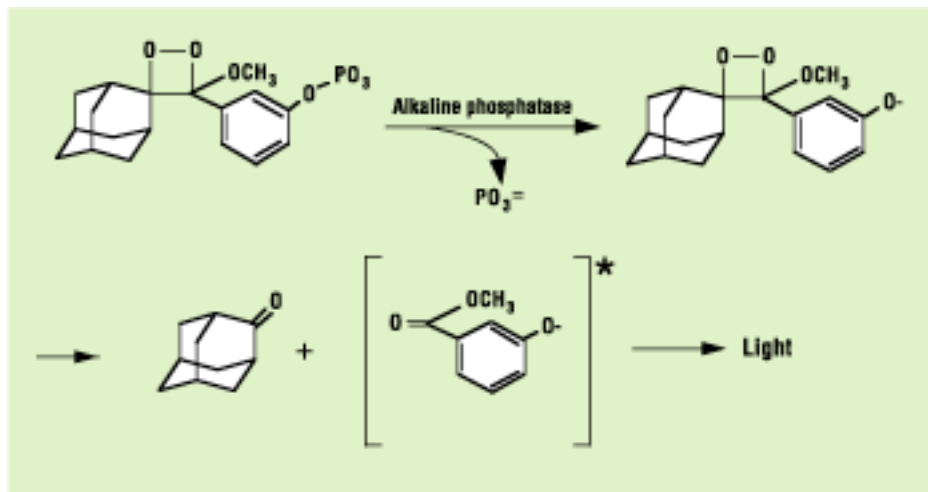
### Procedure

1. For each blot (~15 cm<sup>2</sup>), pipet 4.5 mL (~ 0.25 mL/cm<sup>2</sup> membrane) of pre-warmed hybridization solution (55°C) into the 50 mL tube containing your membrane (be sure membrane is oriented with DNA side facing interior of tube). Check for leaks after capping and holding the tube on its side.
2. Prehybridize the membrane at 55°C for 15 minutes.
3. After prehybridization, add 14 µL of stock probe containing glycerol (~24 ng; 5-10 ng/mL hybridization solution) directly to liquid. Mix probe into hybridization solution at bottom of tube, then swirl over blot.
4. Return tube to hybridization oven. Allow it to incubate for 18-24 hours at 55°C.

### Signal Generation and Probe Detection

The CDP-Star chemiluminescent detection reagent supplied by Amersham Pharmacia Biotech utilizes the probe-bound alkaline phosphatase enzyme to catalyze the decomposition of a stabilized dioxetane substrate (Figure 9.4). Dephosphorylation results in the formation of a metastable anion; charge transfer from the phenolate to the dioxetane ring causes the cleavage of the cyclic peroxide. The result is an excited-state anion that emits light at 477nm. This reaction has a rapid light output with a very short lag phase; the light reaches its maximum at 4 hours and will last for several days. The light is detected with an imaging machine.

**Figure 9.4 The light emitting mechanism of 1,2 dioxetane AP substrates.**



## Signal Generation and Detection

### Materials and Equipment Required

Small pipet rack lid (for washing)  
Blunt-end forceps for manipulating membrane  
Saran wrap

### Reagents Required

20 mL primary wash solution  
100 mL secondary wash solution  
525  $\mu$ L CDP-Star<sup>†</sup> detection reagent (30-40  $\mu$ L/cm<sup>2</sup>)

### Procedure

1. Discard the hybridization solution, but keep the membranes in the tube. Wash the membranes with 10 mL of preheated (water bath) primary wash buffer at 55°C for 10 minutes.
2. Pour off wash and repeat with fresh, preheated primary wash buffer.
3. Remove blots from tube and place in small pipette rack lid (**DNA side up!**) and add 50 mL secondary wash buffer. Mix with gentle agitation for 5 minutes at room temperature.
4. Pour off wash and repeat.
5. Drain off excess wash solution and add 525  $\mu$ L CDP-Star detection reagent directly onto blot surface. Let sit for 2-5 minutes.
6. Drain off excess detection reagent and wrap blot in saran wrap and proceed to detection of chemiluminescent signal using the Bio-Rad Fluorimager.

<sup>†</sup> CDP-Star detection reagent contains an aqueous solution of <1.5% (w/v) disodium 2-chloro-5-(4-methoxyspiro [1,2 dioxetane-3,2'-(5'-chloro)-tricycl [3,3,1,1<sup>3,7</sup>]decan]-4yl) phenyl phosphate. The toxicity of this substance is not known, so handle with care.

### Results and Analysis

After obtaining the results from the Fluorimager, you will need to answer the following and place them in your notebook:

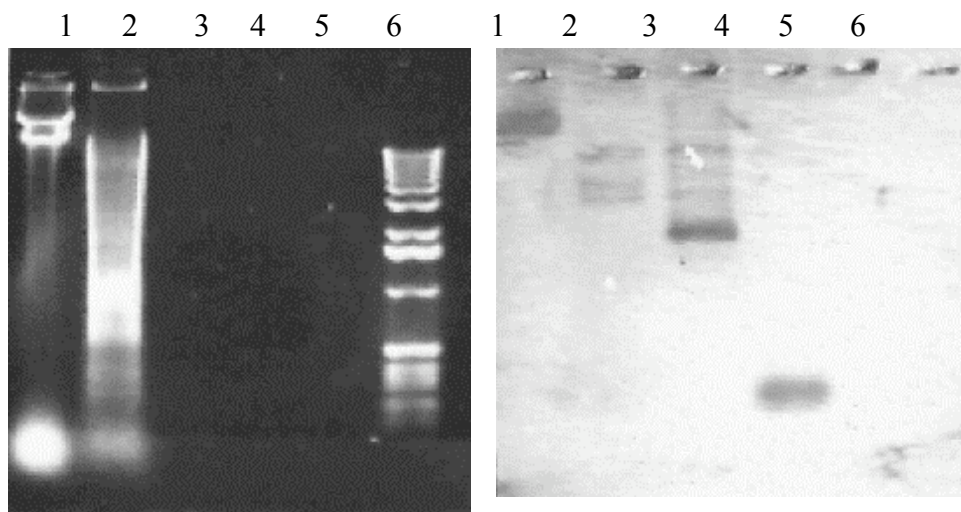
1. What is the size of each band of DNA in each lane that hybridized to the probe? You will need to state the lane (*e.g.*, digested genomic DNA), the number of bands present in each lane and the size of each band, as determined by the EtBr gel photograph taken prior to the blot.

*Note: you will need to use a conversion factor to relate your gel image ruler to the ruler you are using to measure the fluorescent bands.*

2. Trip to the Bio Computer Lab:
  - a. What sized fragments from the digested DNA (be sure to state which enzyme you used) would be expected based upon analysis of the sequence from the database?
  - b. Discuss the accuracy of your attempt to determine the proper size of the bands. How could this have been improved?

**Figure 9.3 Example Southern Blot.**

Left image is an agarose gel electrophoresis pattern stained with ethidium bromide and the right image is the resultant membrane probed with a biotinylated GPDH probe.



Lane Legend:

- |    |   |    |                                       |
|----|---|----|---------------------------------------|
| 1. | Yeast genomic DNA, ~5 $\mu$ g                     | 4. | GPDH amplicon, ~0.5 ng                |
| 2. | Yeast genomic DNA, restrict. Hind III, ~5 $\mu$ g | 5. | pUC19/GPDH <sub>mouse</sub> , ~0.5 ng |
| 3. | pCR2.1-TOPO/GPDH <sub>yeast</sub> , ~0.5 ng       | 6. | 1 kb linear dsDNA                     |

Lanes 3, 4, and 5 in the ethidium bromide-stained blot (left image) do not show up because of the low amount of DNA present. However, in the Southern blot (right image) the higher sensitivity of the hybridization technique allows visualization of the bands in lanes 3 and 4. Lanes 5 and 6 do not show up in the Southern Blot because no GPDH-specific DNA are in these lanes.

### **Reagents for Southern Blotting**

*100X Denhardt's solution (not used in Expt 9)*

2.0 g bovine serum albumin (BSA)

2.0 g Ficoll 400

2.0 g Polyvinylpyrrolidone

*Add approximately 50 ml di water and mix to dissolve. Make up to 100 ml final volume. Store at -20°C for up to 3 months.*

*Hybridization buffer (not used in Expt 9)*

5X SSC

5X Denhardt's solution

0.5% (w/v) SDS

*Add the following to the manufacturer's supplied buffer the day of hybridization:*

0.5 M NaCl

4% (w/v) blocking agent

*Stir at RT for 1-2 hours to dissolve. Preheat to 55°C for prehybridization.*

*20X SSC (not used in Expt 9)*

88.23 g Na<sub>3</sub>citrate•2H<sub>2</sub>O (fw = 297)

175.32 g NaCl

1000 mL final volume with water

*Check pH is between 7 and 8.*

### **Depurination Solution (0.25 M HCl)**

11 ml HCl

989 ml di water

Mix, store at RT for up to 1 month.

### **Denaturation and Transfer solution**

87.66 g NaCl (1.5 M)

20 g NaOH (0.5 M)

Mix in 800 ml di water and mix to dissolve. Make up to final of 1000 ml. Store at RT for up to 3 months.

### **Amersham Pharmacia Biotech formulas for Southern hybridization:**

Primary Wash Solution: amount/L (may be kept for up to 1 week at 4°C)

2 M Urea	120 g
0.1% SDS	1 g
50 mM NaPO <sub>4</sub> (pH 7.0)	100 mL of 0.5 M stock
150 mM NaCl	8.7 g
1 mM MgCl <sub>2</sub>	1 mL of 1.0 M stock
0.2% Blocking reagent	2 g

Secondary Wash Solution: amount/L (may not be stored)

2mM MgCl <sub>2</sub>	2 mL of 1.0 M stock
1X Tris/NaCl solution	50 mL of 20X stock

20X Tris/NaCl solution: amount/L (may be kept for up to 4 mo at 4°C)

1 M Tris base	121 g
2M NaCl	112 g
pH to 10.0	