

Southern Blot Analysis (from Baker lab, university of Florida)

DNA Prep

Prepare DNA via your favorite method. You may find a protocol under Mini Yeast Genomic Prep.

Restriction Digest

1. Digest DNA with appropriate restriction enzyme. Digest 10 μ g of Yeast chromosomal DNA per lane to be run. Digest 0.05 to 0.01 μ g of plasmid DNA (a massive overkill).
2. Allow restriction digest to proceed at 37°C overnight.

Electrophoresis

1. Seal the gel casting platform at both ends with tape. Prepare and pour a 100mL 0.8% agarose in 1X TBE. Insert the comb making sure there are no air bubbles trapped underneath comb and that the all bubbles on the surface of the agarose are removed before the gel sets. A 16-well comb will allow the loading of approximately 30-35 μ L of sample.
2. Prepare ³²P labeled 8/HindIII molecular weight standard. Desire 10-20K cpm to be loaded with 1Fg of cold standard for Ethidium Bromide visualization.
3. Load gel under fresh 1X TBE and run slowly (100V) to avoid smearing of samples until bromophenol blue is near the bottom. It is important to load the gel in an asymmetric pattern, (i.e have the 8/HindIII standard on the right side only.) this will prevent confusion about loading order on the final autoradiograph.
4. Stain gel in new ethidium bromide at a maximum concentration of 0.5 μ g/mL for 15 minutes.
5. Photograph preblot gel for record.

Southern Blot

1. Wash gel in 300mL of 0.25M HCl. Shake gently at room temperature on platform shaker for 30 minutes. Depurinates DNA to facilitate transfer of large DNA.
2. Wash gel in 500mL of 0.5M NaOH, 1.0 M NaCl. Shake gently at room temperature on platform shaker for 20 minutes. Denatures DNA for probe access.
3. Prepare 2 liters of 0.025M NaPO₄ pH6.5 transfer buffer (25mL 0.5M Na₂HPO₄, 75mL 0.5M NaH₂PO₄/ 2 liter).

4. Cut Hybond N+ to the size of the gel and wet with transfer buffer (Gloves a must).

5. Cut 4 pieces of Whatman 3MM paper to the same size as the gel and one twice as long to be used as a wick.

6. Set up capillary blot as per Maniatis. Transfer for at least 6 hours, normally we allow the transfer to proceed overnight.

7. Place gel support in Pyrex dish, add transfer buffer. Place long Whatman 3MM (wick) over support so that the edges are submerged in the transfer buffer. Roll out any air bubbles by gently rolling a glass tube over the surface.

8. Place 2 pieces of Whatman 3MM, same size of gel, wet with transfer buffer. Roll out any bubbles.

9. Place gel gently on top of Whatman 3MM. Very gently with wet gloves roll out bubbles.

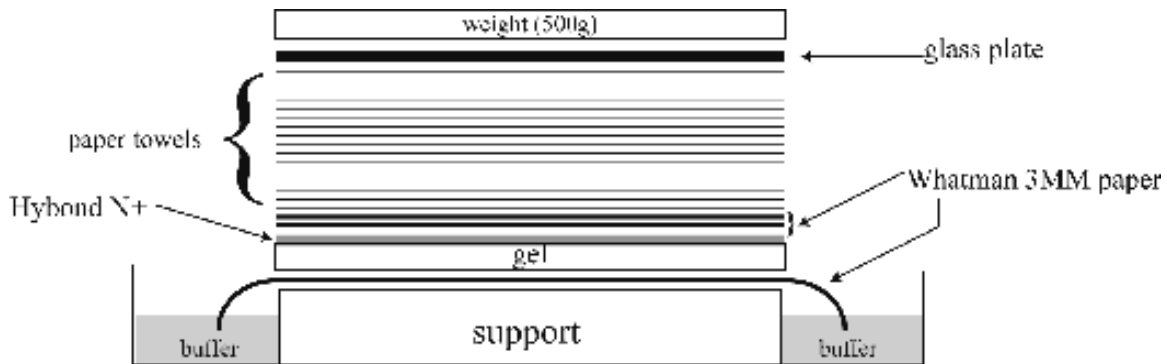
10. Cut four strips of saran wrap and place over the edges of the gel. This is to prevent the buffer from "short-circuiting". So that the buffer flows through rather than around the gel.

11. Place Hybond N+ membrane exactly over the gel. Very gently squeeze air bubbles by gently rolling a glass tube over the surface.

12. Place 2 pieces of Whatman 3MM, same size of gel, wet with transfer buffer over the Hybond N+. Try to avoid getting air bubbles under the membrane; remove any by carefully rolling a glass tube over the surface.

13. Cut paper towels to the same size as the membrane and stack these on top of Whatman 3MM papers to a height of about 10cm.

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



Disassemble the transfer pyramid

1. Once the blot is complete, disassemble the transfer pyramid and recover your membrane. Remove left bottom corner of blot, to mark DNA side.

2. Cross-link membrane by placing blot on plastic wrap, DNA-side down, on transilluminator and illuminating at full power for 5 minutes.

3. Stain transferred gel in EtBr and photograph to ensure complete blotting has been done. (Labeled Lambda should now be on filter and you may test it with the Geiger counter.)

4. Wash cross linked filter in 250mL of 0.1X SSC, 0.5% SDS at 65°C for 45 minutes to remove residual agarose. This step is critical, do not omit or the background will be very high after hybridizing.

I. Hybridization

(Church and Gilbert, 1984)

A. Prehybridization

1. Once membrane has been cross linked and washed, roll and place into glass cylinder so that it sticks completely to the walls.
2. Add 5mL of Hybridization buffer to small cylinder (8mL for large cylinder)
3. Prehybridize at 65°C for at least 15 minutes and up to 2 hrs.
4. Write in your notes the temperature and actual time of prehybridization.

B. Hybridization

1. Decant prehybridizing buffer and pour probe into cylinder prepared as indicated under probe.
2. Hybridize at 65°C overnight.
3. Make a note of temperature and total time of hybridization.

C. Washing

1. Washing is done at 65°C with prewarmed washing solution.
2. A single membrane or a maximum of two is washed in a close able box with a volume of about 200mL for about 30 minutes.
3. Change buffer 4 or 5 times.
4. Once membrane has been washed, wrap in Saran wrap, make sure there are no wrinkles and expose to film.

II Hybridization (Old method, not recommended)

Prehybridize filter in 2mL 5X "P" buffer

5mL Formamide deionized (optional)
1mL Salmon Sperm DNA 1mg/mL
0.58g NaCl (do not vortex, mix gently)

Dilute to 10mL with ddH₂O

1. Seal filter in Nylon bag without bubbles. Incubate for at least 30 minutes @ 65°C. (42°C with Formamide).
2. Add probe to bag, reseal and double bag. Incubate at least 6hrs at 65°C with slow shaking. NOTE: Probe concentration should not exceed 5-10ng/mL to avoid background.

Washing

1. Once hybridization is complete wash filter as follows.
2. Wash filter 2X with 250mL of wash A for 5 minutes at room temperature with gentle shaking.
3. Wash filter 2X with 250mL of Wash B for 30 minutes at 60°C.
4. Wash 2X with 250mL of 1:10 Wash A for 30 minutes at room temperature with gentle shaking.
5. Double wrap damp filter in plastic Wrap and expose to film.

End labeling of 8/HindIII

Digest 3µg DNA with 8/HindIII

Sample digest reaction

5µL stock DNA 1µL 10X Salt
1µL HindIII (10U/µL)
3µL ddH₂O
Digest in the 37°C for 60 minutes

1. Add 20 µL TE pH 8.0
2. 10µL A- Mix
3. 1µL Klenow (5 units)
4. 1µL 32P-dATP >3000
5. Spin and incubate @ RT for 30 minutes.
6. Add 110µL TE (150µL Final Volume)
7. Phenol extract
8. Run aqueous layer over spin column
9. Count 1µL
10. Store at -20°C properly shielded.

Probe Yip 56 with Random Primers

Random 6 Mer @ 1µg/µL concentration. We use Yip 56 @ 0.1µg/µL

1. 2µL Yip 56
2. 2.5µL 10X Klenow Salt
3. 2.5µL Random 6 Mer

4. 10 μ L ddH₂O
5. Heat to 95°C for 5 minutes, plunge on ice.
6. Add 8 μ L A- Mix
7. Add 5 U Klenow
8. Add 2 μ L dATP 32P >3000
9. Incubate @ RT x 2 hrs.
10. Add 100 μ L TE
11. Pass over spin column, equilibrated with TE
12. Heat to 95°C for 5 minutes, plunge on ice.
13. Count 1 μ L .

Solutions

1. 0.5M Na₂HPO₄ (Sodium Phosphate Monobasic)
2. 0.5M NaH₂PO₄ (Sodium Phosphate Dibasic)
3. [1M Na]HPO₄ This solution is 1M with respect to Sodium. pH 7.2

For 1L of this solution weigh 71g Na₂HPO₄, add to 800mL ddH₂O and stir, add 4mL 85% H₃PO₄ (Phosphoric Acid). Bring volume to 1L with ddH₂O. pH is critical, check and adjust if necessary.

4. Hybridization Solution (Make this solution in a 50mL conical plastic tube.)

Desired Concentration	Volume Needed
1% BSA	0.5g BSA Frac.V
1mM EDTA, pH 8.0	0.1mL 0.5M EDTA, pH 8.0
[0.5M Na]HPO ₄ , pH 7.2	25mL [1M Na]HPO ₄ , pH 7.2
7% SDS	17.5mL 20% SDS
	7.4mL ddH ₂ O

5. Wash Solution (For 2 liters)

Desired Concentration

	Volume needed
1mM EDTA, pH 8.0	4mL 0.5M EDTA, pH 8.0
[40mM Na]HPO ₄ , pH 7.2	80mL [1M Na]HPO ₄ , pH 7.2
1% SDS	20g SDS
	1916mL ddH ₂ O

6. 20% SDS

Dissolve 20g SDS in 80mL ddH₂O. Bring volume to 100mL with ddH₂O. Store at room temperature.

The following solutions are not needed if you are following the Church and Gilbert protocol for hybridization.

7. Wash A

Needed for 500mL

1.0.3M NaCl 8.8g NaCl
 2.0.06M Tris 30mL 1M Tris pH 8.0
 3.0.002M EDTA 2mL 0.5M EDTA pH 8.0

8. Wash B

Needed for 500mL

1.0.3M NaCl 8.8g NaCl
 2.0.06M Tris 30mL 1M Tris pH 8.0
 3.0.002M EDTA 2mL 0.5M EDTA pH 8.0
 4.1% SDS 5g SDS

9. 5X "P" Buffer

Needed for 40mL

1.1% BSA 0.4g
 2.1% polyvinyl-pyrrolidone 0.4g
 3.1% Ficoll 0.4g
 4.0.5% Sodium Pyrophosphate 0.2g
 5.5% SDS 2g
 6.250mM Tris pH 7.5 10mL 1M Tris pH 7.5

7.30mL ddH₂O

Aliquot 2mL into plastic tubes. Store @ -20°C

REFERENCES

Church, G. M., and W. Gilbert 1984. Genomic Sequencing. Proc. Natl. Acad. Sci. USA 81: 1991-1995.