

Southern Blotting

Materials

⌘ Depurination Solution

200 mM HCl

⌘ Denaturation Solution (1L)

1 M NaCl (58.4 g)
0.5 M NaOH (20 g)

⌘ Neutralization Solution (1L)

3 M NaCl (175.3 g)
0.5 M Tris (60.6 g)
pH to 7.4 with NaOH or HCl before voluming to 1L

⌘ 20x SSC (1L)

0.3 M Na(3) citrate, $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7\cdot 2\text{H}_2\text{O}$ (88.2 g)
3 M NaCl (175.3 g)

⌘ 20% SDS (1L)

200 g SDS (sodium dodecyl sulfate)
heat and stir to dissolve

⌘ 1 M NaPO₄ pH 7.0 (200 ml)

<u>Solution</u>	<u>Stock</u>	<u>Volume (ml)</u>
NaH ₄ PO ₄	2 M	39
Na ₄ HPO ₄	2 M	61
Water		100

⌘ 50x Denhardt's Solution (1L)

1% BSA, bovine serum albumin (10 g)
1% PVP, polyvinyl pyrrolidone (10 g)
1% FilcollA (10 g)

⌘ Herring Sperm DNA (10 mg/ml)

⌘ Pre-Hybridization Solution (100 ml)

<u>Solution</u>	<u>Stock</u>	<u>Volume (ml)</u>
NaPO ₄ pH 7.0	1M	4
SSC	20x	30
SDS	20%	5
Water		50

Microwave 30 sec until clear

Add 6.5 ml Denhardt's Solution and 3.5 ml water
 Add 1 ml Herring Sperm DNA boiled for 10 min

Keep at 65°C until it turns cloudy or freeze at -20°C.

⌘ **Prime-It II Random Primer Labeling Kit (Stratagene)**

⌘ **Rinse (500 ml)**

2x SSC, 0.4% SDS

<u>Solution</u>	<u>Stock</u>	<u>Volume (ml)</u>
SSC	20x	50
SDS	20%	10
Water		440

⌘ **Wash 1 (500 ml)**

1x SSC, 0.25% SDS

<u>Solution</u>	<u>Stock</u>	<u>Volume (ml)</u>
SSC	20x	25
SDS	20%	6.25
Water		468.75

⌘ **Wash 2 (500 ml)**

0.5x SSC, 0.25% SDS

<u>Solution</u>	<u>Stock</u>	<u>Volume (ml)</u>
SSC	20x	12.5
SDS	20%	6.25
Water		481.25

⌘ **Probe Removal Solution**

0.1 M NaOH
 0.1% SDS

⌘ **Probe Removal Neutralization Solution (1L)**

<u>Solution</u>	<u>Stock</u>	<u>Volume (ml)</u>
Tris-Cl pH 7.5	1 M	200
SSC	20x	5
SDS	20%	5
Water		790

Methods

⌘ **DNA digestion**

Digest 10 µg of each genomic DNA sample with an appropriate enzyme at 37°C, overnight. Negative genomic DNA spiked with linearized vector DNA served as positive

control. The amount of vector DNA added into the control can be calculated as followed:
 $1 \text{ copy DNA control (pg)} = 10^7 [\text{size of vector(bp)}] / [\text{genome size (bp)}].$

⌘ **Gel electrophoresis**

DNA samples are electrophoresed overnight on a 0.8%-1% agarose gel at constant voltage of 17-35 v.

⌘ **Gel wash**

1. Photograph the gel to check for proper migration and trim away any area with no DNA. Notch the top left corner for orientation purposes.
2. Depurinate (nick) the DNA by soaking the gel in depurination solution (0.2 M HCl) with shaking at RT for 15 minutes or until the blue loading dye turns yellow.
2. Rinse in dH₂O.
3. Denature the DNA 30 minutes (or until the dye turns blue again) with shaking in denaturation solution (0.5 M NaOH, 1 M NaCl).
4. Rinse in dH₂O.
5. Neutralize the gel by soaking 2 x 15 minutes with shaking in neutralization solution (0.5 M Tris.HCl, 3 M NaCl).
6. Rinse in dH₂O.

⌘ **Membrane blotting- gravity**

1. Lay 2 pieces of plastic wrap directly on the bench in the shape of a plus sign. Make sure they are big enough to wrap around the apparatus when assembled. Place a plexiglass plate on top of the plastic wrap.
2. Cut a stack of paper towel (about 7 cm thick) to a size that is about 1-2 mm wider and longer than the gel and put onto the plexiglass.
3. Briefly dip 2 layers of filter paper (Whatman 3MM) that are slightly larger than the gel in water and place on top of the paper towels. Remove any bubbles by rolling a plastic pipet over the paper.
4. Cut the nylon membrane to the exact size of the gel and notch the upper left corner for orientation purposes. Label the membrane with pencil and briefly soak in water. Lay the membrane on top of the filter paper and remove any bubbles.
5. Place the gel on top of the membrane so that the bottom of the gel is in contact with the membrane. Start by touching the bottom edge of the gel to the membrane and slowly allowing more of the gel to come into contact with the membrane from bottom-up. This reduces the amount of bubbles.
6. Remove any bubbles and fold the plastic wrap over the gel. Seal the edges of the wrap and place a plexiglass plate on top with 1 kg of weight on the plexiglass. Leave overnight.
7. Dismantle the apparatus. Mark membrane with a pencil for wells and orientation.
8. Wash membrane in 5x SSC for 5 min.

9. Blot membrane in filter paper and cross-link (still inside the filter paper). Set the UV transilluminator to Auto and press start. Make sure to crosslink both sides of the membrane.
10. Soak the remaining gel in 1X TAE with ethidium bromide for 10 min and take a picture to ensure complete transfer of the DNA to the membrane.

⌘ Membrane blotting- capillary

1. Pre-cut membranes so that it is slightly larger, but almost the exact size of the gel. Cut the top left corner of the membrane for orientation purposes. Label the membrane name with pencil and pre-wet in 10x SSC.
2. Set up capillary blot: place a raised gel support into a dish containing 10x SSC.
3. Saturate 2 layers of filter paper (Whatmann 3MM) with 10x SSC and cover the support. The filter paper should be wide enough to hang into the reservoirs. Remove any bubbles by rolling a plastic pipet over the paper.
4. Place gel onto the filter paper and smooth out bubbles with the pipet. Place the membrane upon the gel (the bottom side of the gel should be in contact with the membrane). Smooth out any bubbles.
5. Pre-cut 2 sheets of 3MM filter paper to the same size as the gel, pre-wet in 10x SSC and place on top of membranes. Smooth out bubbles.
6. Put a layer of plastic wrap with a membrane size hole on the top of the filter paper. Stack paper towels (about 5 cm) on top of filter paper. Place a plexiglass plate on top of paper towels and another weight of about 1 kg on top of that. Leave overnight.
7. Dismantle the apparatus. Mark membrane with a pencil for wells and orientation.
8. Wash membrane in 5x SSC for 5 min.
9. Blot membrane in filter paper and cross-link (still inside the filter paper). Set the UV transilluminator to Auto and press start. Make sure to crosslink both sides of the membrane.
10. Soak the remaining gel in 1X TAE with ethidium bromide for 10 min and take a picture to ensure complete transfer of the DNA to the membrane.

⌘ Probe labeling

Follow the instructions on the labeling Kit (Prime-It II Random Primer labeling Kit, Stratagene)

2. Add the following components to the bottom of a clean microcentrifuge tube:
 - Sample: 25 ng (1-23 μ l) of DNA template to be labeled
 - 0-23 μ l of high quality H₂O
 - 10 μ l random oligonucleotide primers (total reaction volume should be 34 μ l)
3. Heat the reaction tubes in a boiling water bath for 5 minutes and then centrifuge briefly at room temperature to collect the liquid, which may have condensed on the cap of the tubes.

4. Add 10 μ l of 5 \times primer buffer: The 5 \times *dCTP primer buffer contains dATP, dGTP and dTTP, and it should be used when [α -32P]dCTP. The 5 \times *dATP primer buffer contains dCTP, dGTP and dTTP and it should be used when [α -32P]dATP or a dATP analog is to be incorporated.
5. Add 5 μ l of labeled nucleotide: Use either [α -32P]dCTP at 3000 Ci/mmol or [α -32P]dATP at 3000 Ci/mmol. Mix the contents of the tube thoroughly with your pipet tip.
6. Add 1 μ l Exo(-) Klenow enzyme (5 U/ μ l).
7. Mix the reaction components thoroughly with your pipet tip.
8. Incubate the reactions at 37–40°C for 20 minutes
9. Add 2 μ l of the stop mix to stop the labeling.

⌘ Pre-hybridization

1. Soak membranes in 5x SSC for 5 min, place in hybridization bottles. Add 10 ml pre-hybridization solution per membrane with an additional 1ml per additional membrane.
2. Place hybridization bottle in the rotating hybridizer (65°C) for 3 hr at speed 10.

⌘ Hybridization

1. Heat the labeled probe at 100°C for 5 min and chill it in ice.
2. Add 10-50 μ l of the probe directly to the hybridization solution in the hybridization bottles. Do not touch the probe directly to the membrane or you will see very high background when developed.
3. Set up overnight hybridization in the hybridizer at 65°C at speed of 10.
4. Place the washing solutions in the 65°C oven for the next-day washing.

⌘ Membrane wash

1. Add 50 ml Rinse solution and wash membranes for 20 minutes at 65°C.
2. Add 50 ml Washing 1 solution and wash membranes for 20 minutes at 65°C.
3. Add 50 ml Washing 2 solution and wash membranes for 15 minutes at 65°C.
4. Blot the membrane dry with paper towels and place inside plastic wrap. Try to get the wrap as smooth as possible. Cut off the excess and fold over the edges.

⌘ Detection

1. Expose membranes to X-ray film in a darkroom with only the safe light on. Inside a film cassette, place the saran wrapped membranes at the bottom. Place a sheet of X-ray film on the top of the film, and place an intensifying screen face down on top of the film. Close the cassette.
2. Place the cassette at -80°C for 1-14 days, depending on the signal strength. The intensifying screen acts at low temperatures to enhance the efficiency of detection of

radioactivity. This occurs when the photon emitting intensifying screen absorbs beta particles that pass through the film.

⌘ **Probe removal**

1. Shake membranes in Probe Removal Solution for 5-10 minutes at RT.
2. Rinse in ddH₂O for 1-2 minutes.
3. Neutralize membrane in Probe Removal Neutralization Solution for 20 minutes.
4. Dry membranes on 3MM filter paper.

References

1. Sambrook J, Fritsch EF, Maniatis, editors (1989) Molecular Cloning: A Laboratory Manual (2nd ed) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, Chapter 9-31 to 9-62