Southern Blotting

Blotting

Assemble the LKB Vacugene vacuum blotting unit on a leveling table. Place the white plastic backing sheet smooth side up on the base unit with a washed plastic mask with appropriately sized centre cutout on top

To prepare the membrane wear washed gloves and wash a ruler with water to avoid dirtying the membrane. For most purposes use BioRad ZetaProbe nylon membrane. Cut an appropriate sized piece of membrane (approximately 7 x 13cm for medium gel, 5.5 x 7.5cm for mini gel) so that the membrane is slightly larger than the cutout. Soak the membrane briefly in Milli-Q H_2O and place under the plastic mask. Ensure that there are no air bubbles or dirt under the membrane.

After electrophoresis photograph the gel on the UV transilluminator. Note: For a southern blot to detect mtDNA deletions UV-crosslink on transilluminator table for 2-3 minutes. Cut a slice off the gel through the wells with a scalpel or spatula. Slide the gel off its tray onto the blotting unit leaving at least a 0.5-0.75cm of overlap on all four sides of the cutout.

Replace the top half of the blotting unit and screw down the lid. Turn on the vacuum pump and set to 40 cm H_2O of vacuum pressure.

Depurination

Pour 0.25M HCl onto the gel surface and leave for 20 minutes. Check every few minutes that the gel surface is still covered, adding more 0.25M HCl with a transfer pipette if necessary. At the end of 20 minutes, remove the HCl with a transfer pipette.

Denaturation	1.5M NaCl	87.66g
	0.5M NaOH	20.00g

In 1L Milli-Q H_2O.

Increase vacuum to 50cm H_2O. Pour onto gel and denature for 20 minutes. Remove the solution with a transfer pipette

Neutralization	1.0M Tris (base)	121.10g
	2.0M NaCl	116.88g

In 1L Milli-Q H_2O. Adjust to pH 5.0

Pour onto gel and neutralize for 20 minutes. Remove the solution with a transfer pipette.

Transfer

Flood gel with 10x SSC (approximately 500mL) and transfer for one hour. Near the end of the hour place 2 layers of paper in a shallow bath and soak them in lOX SSC. Remove them to a glass plate. Prepare 100mL of 2x SSC in a shallow bath.

Preparation of Radioactive DNA Probe by Random Priming

All work must be performed in the hot room. This step can be performed up to three days in advance and the probe stored at –20°C.
Radiochemicals are found at 4°C or -20°C in the fridge/freezer in hot room or at -80°C in the freezer in the hallway closet. For most routine Southern hybridizations ∞^{32}P dCTP is used. (Amersham Biosciences AA005) It is stored at 4°C.
Boil water in beaker with floating tube rack and watch glass cover on heating stirrer.
From Amersham MegaPrime DNA Labeling Kit (RPN 1606/7) kit at -20°C remove labeling buffer and the primer solution. Allow to thaw.

Combine in an 1.5mL tube:	5µL primer solution
	25ng DNA(2.5-25ng/µL)
	Milli-Q up to 38µL – volume of ∞32P dCTP

Boil for 5 minutes. Spin down briefly and keep at room temperature.

Add the following:	10µL labeling buffer.
	2µL enzyme.
	Labeled isotope - 50µCi ∞32P dCTP (5µL at reference date)

Mix gently. Incubate at 37°C for 10 minutes. Stop reaction with 5µL 0.2M EDTA.

Probe purification and counting

Prepare a Nick Column (Amersham 17-0855-01/02) by removing the cap and pouring off any excess buffer in the column. Rinse once with equilibration buffer. Set up a retort stand with a clamp in the "HOT" area. Attach the column and place a beaker or Falcon tube underneath the column to collect the flow through. Equilibrate column with 3mL of buffer. Allow the buffer to completely enter the gel bed. Place an 1.5mL tube in a rack underneath it. Pipet the labeled and denatured probe onto the column and allow to enter the gel bed. Add 400µL buffer and let it enter the gel bead. Add another 400µL of buffer to the column and collect the flow through in the 1.5mL tube. This fraction contains the purified probe.
Pipet 4µL ($^{1}/_{100}$ of the elution volume) of the purified probe into a large scintillation vial with 10mL scintillation fluid. Count with protocol 5 - Joan. Retain the scintillation count printout. The total count should be approximately 1.0-7.0 X 10^{7} cpm.

Pre-Hybridization and Hybridization

Solution	13.0mL	MilliQ
	1.5mL	20x SSC
	2.0mL	10% SDS
	3.0mL	50% dextran sulphate
	0.5mL	Salmon sperm DNA 5mg/mL

20 ml

Leave solution in the water bath by the culture hood to warm.

When the transfer is complete turn off vacuum. Remove the transfer solution from the blotting apparatus. Check that the transfer worked by gently lifting the edge of the gel and shining the hand-held UV light on the membrane. Bands should be seen on the membrane. Using a pencil mark the bands of the DNA marker. Remove the gel and discard. Disassemble the vacuum blotter and remove the membrane. Cut off the bottom right hand corner of the membrane. Place the membrane in the shallow 2x SSC bath and rinse gently for 30 seconds. Remove the membrane to the presoaked sheets of filter paper.
Place blot in the UV Stratalinker 1800 to cross-link the DNA to the membrane (Power - Auto CrossLink - Start - Reset - Power). Place membrane in a hybridization tube and pour in the pre-hybridization solution. Incubate for 2 hours at 65°C.
While the blot is pre-hybing, boil the probe for 10 minutes to denature the DNA then place on ice immediately for about 2 minutes. Add the probe to the pre-hyb solution
Hybridize overnight at 65°C.

Washing

Draw off hybridization solution with pipette, place in 50 ml centrifuge tube and discard in radioactive waste container.
Wash membrane 4x for 5 minutes at room temperature in 2x SSC/0.1%.
Wash membrane 1x for 15 minutes at 65°C in 0.5x SSC/0.1%.
Wash membrane 1x for 15 minutes at 65°C in 0.1x SSC/0.1%.
Monitor signal with survey meter. When washed sufficiently remove blot from tube and place on filter paper. Wrap well with plastic film. NOTE: It is important that no moisture be allowed to leak out of the blot wrapping because this will damage the phospho-imager screens.

Imaging

Phosphoimager: Clean screen with intensifying screen cleaner. Blank screen on light box in for 30 minutes. Place wrapped blot in cassette and place screen over top. Close. Expose and then scan on Storm.