SOUTHERN BLOT PROTOCOL

Genomic DNA Digestion:

Reagents	Vol		Reagents	Vol
gDNA (10ug)	20.7 µl		gDNA (10ug)	20.7 µl
Buffer (10x)	2.5 µl		Buffer (10x)	2.5 µl
Enzyme (20 U/µl)	1.8 µl	or	BSA (10mg/ml)	0.3 µl
*Hind*III			Enzyme (20 U/µl)	1.5 µl
*Eco*RI			*Pst*I	
*Sac*I				
*Kpn*I				
*BamH*I				
Total volume 25 µl			**Total volume 25 µl**	

-Buffer, BSA, and enzyme can be combined in a master mix, and 4.3 ul pipetted into genomic DNA for large sample sets.
-Digest 4 hours at 37°C in thermocycler
-Deactivate with appropriate heat and time in thermocycler and hold at 12˚C.

Gel Electrophoresis Separation:
-Run Digests on a 1% agarose, 1X TAE gel at 50V for 4hrs or 20V overnight. DO NOT use EtBr in the gel.
-Add 5ul of EZ vision loading buffer (Amresco) to total reaction volume and load total reaction on gel.
-Can load up to 44 samples (24-well combs) or 36 samples (20-well combs) per 12X14 gel*.
-Markers**:

1µl DIG-labelled Marker II and/or III (Roche), 1:50 dilution ***in TE buffer***.
6ul EZ vision loading buffer (Amresco)

*This is assuming two sets of wells/gel. Only use two sets if you have optimized digestion/probe/sample species with one set first.
**Load markers in different positions on different gels so that you can distinguish more than one gel

Capillary Transfer - Southern Blot:

1. Take the gels out of the gel box and put it in the plastic tray. Pay attention don't mix it up if you do multiple gels.
2. Visualize gel(s) and take photograph. Date and number photos, and place in your notebook.
3. Cut gels to size of membrane if membrane size is limiting (remove wells and bottom of gel, or cut gels with 2 sets of wells so you have 2 equal gels to transfer).
4. Gently rock the gel(s) in Depurination solution (0.25M HCl) once for 15 min, **wells down** (Note: Depurination is only necessary if the target DNA is >5 kb.) Rinse the gel with autoclaved mqH2O.
5. Gently rock the gel(s) in Denaturation solution (.5M NaOH, 1.5M NaCl) once for 30 minutes, **wells down**.
6. Gently rock the gel(s) in Neutralizing solution (.5M Tris pH 7.5, 1.5M NaCl) twice for 20 minutes each time, **wells down**.
7. Using a large glass Pyrex baking dish (one for two gels), fill with 10X SSC and place two glasses on the dish. Use Whatman paper as a bridge (use a laminated template to cut bridge width to the longest dimension of the membrane). Fill with more 10X SSC if needed (~1.5L total).
8. Wearing gloves, write the name of the gel (A, B, 1, 2, *etc*.) on the upper right corner of the membrane on the DNA side **with pencil. Cut the lower right corner for orientation reference**. Using a laminated template, cut 3 Whatman layers to the same size as the membrane (12cm x 10cm) for each gel to be blotted.
9. Pour ~50mL 2X SSC into a small rectangle glass baking dish.
10. Pour 10X SSC over the bridge with a transfer pipette to wet thoroughly- place gel(s) **wells down** on the Whatman paper and remove air bubbles by rolling a 10mL glass pipet across the gel.
11. Dip membrane(s) in 2X SSC, and place on gel(s) with **writing side down**. Remove air bubbles.
12. Dip 3 Whatman squares/gel in 2X SSC, and place them on top of each membrane.
13. Use strips of Parafilm (3 X 1 sections) to butt up against the gel(s) on all sides to create a barrier between the bridge and the paper towels on top (next step). This will maintain capillary flow through the gel instead of around it.
14. Unfold white paper towels to place on top of (both) gel(s). Use 3/4 pack of paper towels for 2 gels. Place a glass baking dish on top for weight.
15. Leave overnight.
16. The next day, break down blot carefully, discarding the paper towels and 2 Whatman layers. Gently pull off membrane(s) and dip in 2X SSC, then dry blot a little bit with Whatman paper that was on top of blotting paper stack. Use this to support the membrane during crosslinking. UV-crosslink both sides of membrane at "optimal crosslink" setting ("DNA-up" first).
17. Put membranes into the tubes to hybridize or store between damp (with 1X Maleic Acid Buffer or 2X SSC) Whatman paper wrapped in plastic wrap at 4°C.

SOUTHERN BLOT PROTOCOL (continued)

Preparation of Non-radioactive DIG-labelled probe:

1. Creating the Probe:
   a. PCR-amplify the (1) probe, (2) non-labeled control fragment, and (3) H_2O neg. control using the primers, template, PCR Master Mix recipe, and thermal-cycling parameters that have been optimized for the target with the following exception for the PROBE REACTION ONLY:
      i. Change the dNTPs to DIG-labelled dNTPs (5 ul/50 ul rxn) and make the appropriate reduction in H_2O in the reaction recipe.
      ii. When PCR is complete, DO NOT add loading dye to entire rxn!
   b. Take 5 ul samples of each reaction, add loading dye and run on a 1.5% agarose gel with 100bp ladder. (Try to run longer than usual to see the size shift in the DIG-labelled product).
   c. Take a picture and save in notebook.
   d. If you have a non-specifically amplified product, it can be removed by running the entire DIG-labelled reaction on a gel, excising the desired product and purifying from the gel using the kit described in the next step.
2. Clean up the probe with the NucleoSpin PCR Clean Up Kit according to the manufacturer's instructions. (Input 40ul of DIG-labelled PCR product, and elute with 30ul of NE buffer)

Non-radioactive DIG Hybridization:

1. Pre-Hybridize
   a. Pour DIG Easy Hyb into the hybridization tubes (7 mL for Short tubes, 15 mL for Long tubes) with the membrane you want to probe. Cap tightly and balance tubes as necessary in the oven and set temperature to 43°C. Rotate on speed **7** for at least **1 hour.**

2. Hybridization: (For one Hyb tube, double as needed)
   a. Thaw 20 mg/ml of **sheared** salmon sperm, kept in -20 ^{O}C.
   b. For short tubes: Add **0.5 µl salmon sperm and 0.5 µl probe** to 50 µl of sterile water per blot and heat shock in TC (100ºC for 5m, then 4ºC for 5m). The volume of probe added is variable: if using a new probe, use between 0.5-2 ul, based on band intensity on gel and length of probe.
   c. For long tubes: Add **1 µl salmon sperm and 1µl probe** to 50 µl of sterile water per blot and heat shock in TC (100ºC for 5m, then 4ºC for 5m). *See above note on probe volume.*
   d. **Spin down probe mixture** and add to 7mL/15 mL of DIG Easy Hyb (for one membrane) at room temperature. Vortex to mix.
   e. Pour off pre-hybridization solution, and add hybridization solution with probe and salmon sperm to hybridization tube. Cap tightly and rotate on speed **7** at 43°C overnight. Try not to probe more than 18 hours.

Stringency Washes

1. PREPARATION: Preheat **7mL/15mL** × **tube #** × **2 washes** of High Stringency wash (0.5X SSC/ 0.1% SDS) to 65ºC in the water bath. Melt **15 mL 10X Blocking Buffer** (per membrane) in the 65ºC in the water bath, but remove when melted.
2. Low stringency wash: Remove hybridization solution and replace with **2X SSC/ 0.1% SDS** for **5 minutes** at room temperature. **Repeat wash once.**
3. High stringency wash: Remove low stringency wash and replace with **0.5X SSC/ 0.1% SDS** for **30 minutes** at 65°C. **Repeat wash once.**

Detection:

1. Transfer membrane(s) to small glass dish(es), DNA side up. Cover with **1x Maleic Acid buffer** and shake at RT for **3 minutes**.
2. PREPARATION:
   a. Dilute10mL of **10X Blocking buffer** to 100mL with 90 mL **1X Maleic Acid buffer**, to make **Blocking buffer.** You need 100mL per dish, this is for **Blocking step 3**.
   b. Dilute 5mL of **10X Blocking buffer** to 50mL with 45mL **1X Maleic Acid buffer**, to make **Blocking buffer**. You need 50mL per dish, this is for **Antibody step 4.c.**
3. Pour off buffer and add ~**100 ml Blocking Buffer** (enough to cover blot) and shake at RT for **at least 2 hours.**

4. Prepare diluted DIG-Alkaline Phosphatase reagent as below:
    a. **Spin down antibody** ("Anti Digoxigenin AP Fab Fragments") for 5 minutes at speed 13,000 rpm
    b. Add 5 µl of antibody to 50 ml Blocking Buffer and vortex.
    c. Pour off Blocking Buffer and replace with antibody solution.
    d. Shake blots 30 minutes at RT.
5. Pour off antibody solution and replace with **1X Washing Buffer**. Rock for 15 minutes at RT, repeat twice.
6. Pour off Washing Buffer, and replace with 1X Detection Buffer. Rock for 3 minutes at RT.
7. For each membrane, prepare CDP-Star reagent: Add 5 µl CDP-Star to 1.5 ml of Detection Buffer per blot, final dilution of 5:1500, vortex
8. Place membrane(s) in plastic sheet protector (2 membranes/sheet protector), DNA side up. Squeeze air bubbles and excess Detection Buffer out.
9. Pipette 1.5 mL of CDP-Star solution onto each membrane in sheet protector. Allow to sit for 5 minutes.

To Expose:

1. Squeeze excess CDP-Star + detection buffer out of sheet protector.
2. Put membranes, DNA side up in sheet protector into Monotec cassette and go to dark room to put a film on membrane. **Make sure you don't move a film that is already on a membrane.**
3. Expose for 30m, 2h, and overnight. (If film/time is limiting, usually 1.5-2 hr exposure is sufficient.)
4. Turn Film Developer on 20 min before you need it (*see instructions on the wall in the 2nd floor media kitchen dark room*). Fold lower right corner of film up and place whole film into developer, upper edge first. Replace the film with a new sheet and expose the next time course. Turn the lights on after the developer beeps and the film and exposure cassette are packed away into a drawer.

Blot Stripping:

1. Place membrane in a glass dish and cover with 1x Stripping Buffer (.2N NaOH, 1% SDS). Shake at 65°C for 15 min. with gentle shaking three times.
2. Pour off wash and rock in 1x Maleic Acid buffer for 3 minutes at RT.
3. Membranes can now be stored at 4°C in plastic wrap with damp Whatman paper support for future re-probing, or hybridized with a new probe overnight. Begin with the pre-hybridization step.

Southern Blot buffers:

Depurination Solution:	1L
0.25N HCl	20.7mL 12N (37%) HCl 979.3mL mqH_2O Autoclave and store at RT

Denaturing Solution:	1L
0.5N NaOH	20g NaOH
1.5M NaCl	88g NaCl Store at 4˚C

Neutralizing Solution:	1L
0.5M Tris-HCl	60.6g Tris-HCl
1.5M NaCl	88g NaCl - Adjust to pH 7.5 Store at 4˚C

20X SSC:	2L
3M NaCl	350g NaCl
300mM Na-Citrate	176g Na-Citrate - Adjust to pH 7 Autoclave and store at RT

10X SSC:	2L
	1L 20X SSC 1L mqH_2O Autoclave and store at 4˚C

2X SSC:	1L
	100mL 20X SSC 900mL mqH_2O Store at RT

Low Stringency wash:	1L
2X SSC	100ml 20X SSC
0.1% SDS	5ml 20% SDS 895ml mqH_2O Store at RT

High Stringency wash:	1L
0.5X SSC	25ml 20X SSC
0.1% SDS	5ml 20% SDS 970ml mqH_2O Store at RT

Southern Blot buffers: (cont.)

10X Maleic Acid Buffer:	1L
1M Maleic Acid	116g Maleic Acid
1.5M NaCl	88g NaCl
	(Adjust to pH7.5 w/NaOH solid)
	Autoclave and store at RT

1X Maleic Acid Buffer:	1L
	900mL mqH_2O
	100mL 10X Maleic Acid Buffer
	Store at 4˚C

10X Blocking buffer:	100mL
	10g Blocking Reagent (Roche)
	100mL of 1X Maleic Acid buffer

- place on heat block, mix using stir bar while heating to less than or equal to 65°C until all powder goes into solution. Can be left overnight. Cover loosely to prevent evaporation.

OR

- place in 65˚C water bath for ~ 3hrs, removing every 30 min to stir.
- aliquot into 10ml and 5mL amounts and store in –20°C

1X Blocking buffer:

- thaw aliquots of 10X Blocking Buffer completely (in 65°C water bath)

Blocking step (100mL total):	Antibody step (50mL total):
10mL 10X Blocking buffer	5mL 10X Blocking buffer
90mL 1X Maleic Acid buffer	45mL 1X Maleic Acid buffer

- mix well and use immediately or store in 4°C

10X Wash buffer:	1L
	970ml 1X Maleic acid buffer
	30ml Tween 20
	Autoclave and store in 4°C

1X Wash buffer:	1L
	900mL mqH_2O
	100mL 10X Wash Buffer
	Store in 4˚C

10X Detection Buffer:	200mL
1M Tris-HCl	24.2g Tris-HCl
1M NaCl	11.6g NaCl
	(Adjust to pH 9.5 w/NaOH solid)
	Autoclave and store at room temperature

Southern Blot buffers: (cont.)

1X Detection Buffer: 1L

900ml mqH_2O
100ml 10X Detection Buffer
Store at room temperature