Southern Hybridization - Genomic ES Cell DNA

1) Digestions

Genomic DNA (from ES prep)	15 - 20 µL
Enzyme	2-3 µL
10X Buffer	4 µL
100X BSA	0.4 µL
100 mM Spermidine	1.6 µL
Water	X µL
Total	*40 µL*

Spermadine can be purchased from Amersham, #US21760

It is always a good idea to run a control. If using plasmid DNA or BAC DNA, use 1 ng or LESS. Too much will overwhelm the signal of the real bands.

Digest 6 hours to overnight (usually ON is the easiest/best).

This is a good time to make 20xSSPE, 1M Tris pH 7.5 and TAE if you don't have them already. Recipes at the bottom of the protocol.

2) The Gel

Pour low percentage (usually 0.6% to 0.8%) agarose gel in 1X TAE. TAE is better than TBE for resolving larger fragments. The gel should be very long (25-40cm) to give good separation of bands. Add ethidium to gel and all running buffer.

Run gel 5-6 hours at 40 to 60 volts. You can also run overnight at 20 to 25 volts. Once gel is finished running, be sure to take a picture with a ruler that glows under UV!!! (Line-up zero on ruler with wells). Cut off top, left corner of gel for future orientation.

3) After the gel

Add 500 mL of 1:50 dilution of HCL (490 mL H_2O plus 10 mL HCl) to depurinate. Shake/rock gently for 15 minutes. BE CAREFUL! LOWER % GELS LOVE TO CRACK.

Add 500 mL of 0.4 N NaOH to neutralize (480 mL H_2O plus 20 mL 10 N NaOH).
(Make a big batch of this!) Shake gently 15 minutes. Watch for the dye front color to change.

4) Transfer

The sandwich - in a big tray set up the following from the bottom up....
a. Big sponges or paper towels (2-3 inches in height) in 0.4 N NaOH.
Sponges/towels must be larger than gel.
b. 2 sheets of Whatman filter paper cut slightly larger than gel size.
c. Gel, open wells face down. (Now the cut corner is on the right.)
d. Hybond-N+ membrane (Amersham) (pre-soaked in 0.4 N NaOH, one corner removed for orientation). Line up top of the membrane to the wells of the gel, with cut corners in the same quadrant. For further orientation, you can mark the membrane with pencil or VWR marker. Do not move the membrane around once settled on gel.
e. Sheets of Parafilm to surround gel like a frame and prevent wicking
f. 2 sheets of Whatman filter paper cut slightly larger than gel size.
g. Big stack of paper towels. 4-5 inches is good.

h. Weight - a smooth piece of plastic with a heavy book (or 2)on top works well.

Add plenty of 0.4 N NaOH to the tray. Let go for at least 4-6 hours, though overnight is great.

5) Rapid Hybridization

Neutralize blot in 100 mL neutralization buffer - 15' or so.

Neutralization Buffer	Amount	Final Concentration
20X SSPE	10 mL	2X
1.0 M Tris pH 7.5	20 mL	0.2 M
H_2O	70 mL	X

Dry membrane - putting it between two pieces of Whatman paper is good. This isn't important, but it helps if the membrane is a little dry to get into hybridization oven tube. Membrane can also be stored in saran wrap in a refrigerator for a while at this stage. Also, no need to crosslink! Base transfer takes care of that.

Make up FBI buffer! (also called pre-hybridization buffer)

FBI (Hyb)	Amount		Final Concentration
20X SSPE	3 mL	75mL	1.5X
PEG 8000	4 grams	100g	10%
SDS	2.8 grams	70g	7%
H_2O	About 35mL		
Total	*40 mL (for one blot)*	*1 Liter*	

Heat 55-65 ˚C. Mix often (it takes a while to get in to solution).
Once in solution, add 20 mL FBI buffer to 30 cm tube. Prehyb for at least 1 hour in 65 ˚C oven.
Now is a good time to make probe. Always a good idea to make probe fresh!

6) Hybridization

Add 1×10^6 cpm/mL of **denatured probe** (100 ˚C for 5', secure the top of tube with parafilm or cap lock) to 5-10 mL of pre-heated FBI buffer (5×10^6 to 1×10^7 cpm total) to make hyb buffer.

Discard "old" prehyb buffer, add hyb buffer and incubate at 65˚C for at least 6 hours (ON good).

7.) Washing!

All washes done at 65 ˚C.

Remove Hyb buffer (discard in radioactive waste).

Washes 1-3 in 1X SPPE, 1% SDS, 30' each. Wash 1 & 2 - 20 mL. Wash 3 - 100 mL (or more). Discard first wash in radioactive waste, after that, down the sink. First two washes done in tube, then tupperware (makes for better washing).

Wash 4 in 0.5X SPPE, 1% SDS. 30' (100 mL or more).

Wash 5 in 0.1X SPPE, 1% SDS. 30'(100 mL or more). Gently polish membrane with kimwipe after last wash to lower background; discard kimwipe in radioactive waste.

8) Develop!

Mount membrane on filter paper (tape it down). Cover with saran wrap and pop it in film cassette with intensifying screen. Store at -80˚C ON (at least 15 hours) - may take longer for good exposure.

Recipes:

1. For 1 Liter of 20xSSPE
   600mL Water
   175.3g NaCl sodium chloride
   27.6g NaH_2PO_4 sodium phosphate monobasic
   9.4g EDTA powder FW=372

2. bring up to 800mls with H_2O

3. add NaOH to pH 7.4 (~27mls/liter of 10N NaOH)

4. autoclave for 20 min

5. Add H_2O to bring final volume to 1 Liter

1. For 1 Liter of 50xTAE
   600mL water
   242 g Tris base
   57.1mls acetic acid
   1/10 mole of EDTA ie 37.2g of Na2EDTA 2H2O

2. bring up to 800mls with H_2O

3. autoclave for 20 min

4. Add H_2O to bring final volume to 1 Liter