Southern Blots by Lee

1. DNA extraction

DNA extraction should yield HMW clean DNA.
I like a lysis buffer recipe given to me by Dr. Tsujimoto (sp) which can be made up as follows:
100ml:
5ml 1M Tris pH8.3
4ml 0.5 EDTA pH 8.0
10ml 10% SDS
2ml 5M NaCl
79 ml HOH

Cells are lysed in this buffer, and >500ug/ml proteinase K is added. Pro K is made up in HOH as a 50mg/ml stock. The lysate is then incubated ON at 55'c.
Extraction involves mixing the lysate with equal volumes of Tris saturated phenol, twice (or more if there is evidence of lysed RBCs), followed by an extraction with an equal volume of CHCl3. After each extraction spin and transfer the supernatant to a new tube. Do not shear the DNA by rough treatment or vortexing.
DNA is salt precipitated with 0.1 vol. 3M NaOAc pH5.2 and two volumes cold ETOH. The precipitate is transferred ASAP to an epi tube. This excludes some of the LMW DNA and RNA which precipitate slowly. The excess ETOH is removed by a micro pipette tip (like a yellow tip, p200). Do not dry or pellet HMW DNA. Resuspend in TE at RT. ON.

2. Restriction Digestion

Completely digest 10ug of genomic DNA with 5U of enzyme per microgram of DNA ON.

Note: both HMW DNA and complete digestion are important to increase the number of DNA species of a single MW upon fractionation. These factors can affect your signal (maybe 10-20%).

3. Gel electrophoresis

I usually run a 0.8% TAE agarose gel. I add 1ug/ml EB (stock 10mg/ml in HOH) to the buffer before melting the agarose. TAE resolves and transfers large DNA fragments more efficiently than TBE.
The DNA is usually digested in a volume that can be directly added to the agarose gel well, this often necessitates the use of high concentration enzymes. Alternatively, you can precipitate your digest to decrease the volume.

50 X TAE:
1L:
242g Tris Base
100ml 0.5M EDTA pH 8.0
57ml gAcetic Acid
HOH to 1L

Run the gels slowly as the TAE buffer will heat up over time, ~35 volts may be OK, depending on your apparatus. A long ON -24 hour run usually works well. If you wish to run longer than ON you may need to replace the buffer. Recirculation helps but is often problematic on long runs.
Photograph the gel using the 302nM WL transilluminator, photograph the gel with a ruler.

4. Gel processing

After DNA size fractionation you can nick the top half of the gel by covering the 2kb or smaller fragments with foil and irradiating at 254nM; one auto-crosslink on the strata linker. This will improve the transfer of HMW restriction fragments. Small fragments should be protected as they may transfer through the membrane if nicked. The pore size of the membrane becomes a factor when transferring small fragments. I like to use nylon charged membranes, these membranes are not as brittle as nitrocellulose,
and reprobe easily.
At this point the DNA needs to be denatured, this allows probe annealing. Denature, with gentle agitation, for 30 minutes in a sodium hydroxide NaCl solution (DEN).
Neutralize the gel with a Tris NaCl solution (NEUT) in the same manner.

DEN: 0.2M NaOH, 0.6M NaCl

NEUT: 0.5 M Tris pH 7.4, 1.5M NaCl

Recipes vary greatly, they all seem to work well so use the cheapest. For DEN this means using less NaOH and NaCl.

5. Blotting

Transfer the denatured and neutralized DNA from the inverted gel (bottom of well up) to nylon by capillary blotting ON (overnight) as follows:

10 ml Hyb: 5 ml formamide
1.5ml 20XSSC
1ml 50X Denhardts
0.2ml 10% tetrasodium pyrophosphate
1ml 10% SDS
0.1ml 10mg/ml SSS (add freshly boiled)
0.3ml HOH
0.8ml 50% dextran sulfate Do not use : Causes background problems
0.1-.2ml labeled probe vol.

Note: a high dextran sulfate concentration can greatly improve hybridization signal but can also increase background. You may wish to adjust this concentration to your liking.

Probe: label 25-100ng of your probe DNA by Feinberg and Vogelstein method for random hexamer primer extension. Generally speaking the more probe DNA the greater the signal, however, this can also increase the non specific background. You may wish to vary this on occasion

Lee's Random Primer Kit

1) Boil 25-50ng linear probe DNA, chill on ice.

2) For a standard blot mix the following:

Xul 25-50ng DNA
Xul HOH
1ul A (0.5mM)
1ul T (0.5mM)
1ul G (0.5mM)
2.5ul alpha dCTP (3000-6000 Ci/mM)
2.5ul 10x klenow buffer
1ul random hexamer (1ug/ul)
0.5-1ul Klenow

total volume = 25ul

Notes: The klenow enzyme is labile, if things are not working well the enzyme is suspect. Klenow likes to be stored at a stable -20'c. Do not remove the enzyme from it's freezer box. The hexamer/target DNA ratio is also important for good incorporation, you may wish to vary this. Up to a

point, less is better. The dNTPs and other reagents should be thawed briefly, used, and placed back in the freezer; they can deteriorate.

3) Incubate at RT. or 37'c.
Notes: You can anneal and partially extend the primers at RT. for 5mins followed by a more efficient elongation at 37'c. You can also leave at RT. ON.

4) Purify over a G50 spin column. Spin column can be saturated in TE with SSS DNA to block non-specific binding to the column.

Notes: After boiling the probe the SSS also contributes as a blocking agent and reduces the background slightly. The probe should have at least 50% incorporation of the labeled nucleotide. This can be crudely measured by scanning the counts remaining at the top of the spin column and the counts that spin out. Use the small window Geiger counter for this reading. The specific activity of the probe is very important, by increasing your specific signal you can shorten your exposure and thereby decrease your background.

5) Boil the probe, chill, and to your hyb solution.

Drain prehyb and add hyb.
Incubate at 42'c ON or for the weekend for stringent probing.

8. Washing blot

Rinse the membrane in 500ml 2x SSC twice.
Wash the membrane with 500ml of a 0.1x SSC, 0.5% SDS solution for 30 minutes, bringing the wash buffer up to 65'c while washing
Repeat wash.

9. Autoradiography

Wrap the damp (blot dry) membrane in saran wrap.
Expose the autorad with two lightening plus screens and XAR-5 film ON.
The type of screen and film are important for a rapid exposure, other films may give a sharper band.

PCR labeling alternative:

Amplify a PCR product (0.1ul) 25X as usual, with two exceptions: Reduce your dCTP concentration to 20uM and add 2.5 ul of ^{32}PalphadCTP (6000Ci/mM), in a 25ul volume. If you end in a denaturing cycle, you can do a spin column ASAP and add the probe to the hyb solution with out boiling.