Southern Blotting

1) Digest genomic DNA
Digest genomic DNA by appropriate restriction enzyme(s).

H_2O	77 µl
Buffer#	10 µl
Genomic DNA (1~10 ug)	10 µl
Enzyme	3 µl
- total -	100 µl

Incubate at 37˚C for O/N.
(Do not use block incubator, use air incubator. For over night, water get evaporate A LOT in block incubator!)

2) Ethanol precipitation (No rinse is required)
Add 10 ul (1/10 volume of the reaction mix) of 5M NaCl, and mix well by tapping
Add 250 ul (2.5 volume of the reaction mix) of 100% EtOH, and mix well by inversion
Incubate samples at -20 ˚C for 30 min.
Precipitate DNA by centrifugation (13000 rpm, 5 min).
Discard supernatant by decanting.
Spin down (13000 rpm, 10 sec) and remove all supernatant by pipetting. (DO NOT disturb the DNA pellet)
Dry the precipitation for 5 to 10 min in the hood, until you don't smell EtOH. Don't dry too much.
Dissolve DNA pellet in 18 µl of TE + 2 ul of 10 x dye (20 ul total)
(Prepare master mix in advance to avoid pipetting error; DNA might be stuck on the wall of the tube)

3) Agarose electrophoresis
Prepare agarose gel with 1 x TAE
Load: Lane 1; size marker
Lane 2 ~; Digested DNA (20 ul)
Running condition: 1 x TAE, 20V ~ 50V constant voltage (2h ~ overnight, depending of the agarose percentage, voltage and gel size)

4) Take a picture
Take a picture of the gel on UV light. *Important: Place the ruler for UV light next to the gel.

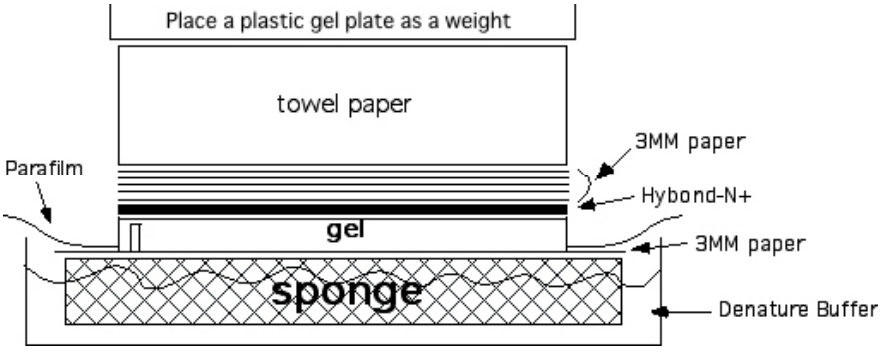
5) (optional) Remove buffer from the surface of the gel and exposed to UV in the UV linker.
Optimal crosslink but stop at 500 to avoid excessive DNA damage

6) Denaturalization
Soak the gel in Denature/Transfer Buffer in glass container.
Shake it slowly on the shaker until the lower dye (Xylene cyanole) turn to green. (about 15~30 min)

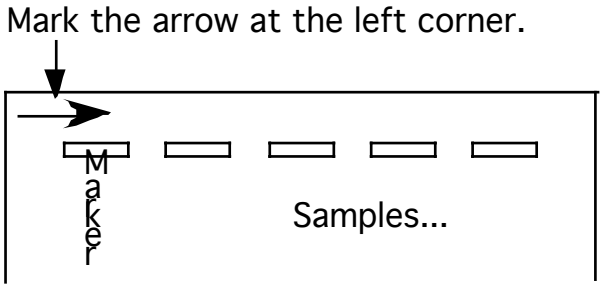
While doing denaturalization, prepare:
1 sheet of Hybond-XL
5 sheets of 3MM paper
A lot of towel paper
*Important: Cut these membrane and paper to the exact size of gel.

7) Transfer
Set up transfer sandwich. (See picture below.)

Place the special sponge in a glass container.
Pour denature/transfer buffer below sponge's surface.
Place a sheet of 3MM paper (larger than the gel size) on the sponge
Pour some denature/transfer buffer on the 3MM sheet, roll a glass tube to get rid of bubbles between 3MM and the sponge
Place the denatured gel "up side down". (Remove bubbles under the gel)
Soak Hybond-XL membrane in water, and soak it in denature buffer. Then place it on top of the gel. It is important that the top edge of the gel and the top edge of the membrane are aligned perfectly. Remove bubbles between gel and membrane. If you put some denature buffer between them, it is easy to remove all the bubbles.
Soak 3MM paper in denature buffer, then place it on top, one by one.
Remove all the bubbles between the 3MM paper by rolling a glass test tube.
Then place a stuck of towel paper. "BUT" before that, place Parafilm (or saran wrap) at each side of the gel to cover the surface of sponge. You don't want the towel paper to touch the sponge.
Place something on top of the towel paper as a weight. Don't put a heavy thing or you'll squish the gel, and the transfer does not work efficiently. A plastic gel plate works well. Check the level by a plastic round level vial.
Leave the transfer set O/N in a safe place.



8) UV cross-link
After the overnight transfer, take the membrane out with forceps (DNA side up). DO NOT touch the side that DNA is attached. Soak the membrane in 5x SSC.
Pick up the membrane using forceps and drain off the 5x SSC buffer. Place the membrane on 3MM paper to remove liquid. DO NOT dry the membrane.
Mark an arrow at the top left corner of the membrane with a ballpoint pen (or a water-resistant marker)



Place the membrane on a new piece of 3MM paper, and place it in the UV linker.
Expose the membrane to UV (Auto, 1200 J/m^2 but stop at 500).
(After this UV link, dry the membrane completely on 3MM paper, and store the membrane between 2 sheets of 3MM paper, in a safe place, such as in your drawer. But do not touch the surface that DNA is attached.)

9) Pre-hybridization
Warm up Southern Church buffer at 65˚C.
Soak the membrane in 5x SSC buffer.
Discard buffer.
Roll the membrane (DNA side in) and insert it in a hybridization bottle.

Put 20 ml of warmed Southern Church buffer and incubate it at 65˚C rotor oven for 2 hour ~ O/N (Pre-hybridization). *Important: Make a balance.

10) DNA probe labeling (Roche, Random Primed DNA Labeling Kit, modified protocol)
After 1 hour of pre-hybridization, put the entire [α-^{32}P]dCTP blue container on the bench and allow 30 min to thaw the content (***do not remove the actual vial from the blue container)
Start DNA labeling, after 1.5 hour you started the pre-hybridization (by this time [α-^{32}P]dCTP is thawed).
Prepare a screw-top 1.5ml tube with probe DNA: 10 μl (20~100 ng)
Boil for 5 min.
On ice for 5 min.
Centrifuge the tube briefly to collect all DNA at the bottom of the tube
Add the following reagents in the following order

- 1 ul of dATP (vial 2)
- 1 ul of dGTP (vial 4)
- 1 ul of dTTP (vial 5)
- 2 ul of reaction mixture (vial 6)
- 4 ul of [α-^{32}P]dCTP (40 uCi)
- 1 ul of Klenow enzyme (vial 7)

Mix gently but thoroughly by tapping
Centrifuge the tube briefly to collect the content at the bottom of the tube
Incubate at 37˚C for 30 min.
Centrifuge the tube briefly again to collect the content at the bottom of the tube
On ice to stop the reaction.
Add 30 ul of STE buffer (see the instruction for Probe Quant G-50 Micro Column) to make it 50 ul

11) Purification of labeled probe DNA (Probe Quant G-50 Micro Column)
Mix the content of the G-50 column by vortex
Loosen the top cap, and snap off the bottom cap
Put the column in a screw-top 1.5ml tube, and centrifuge for 1 min at 3000 rpm, toss the tube
Put the column in a new screw-top 1.5 ml tube
Load the 50 ul of labeled DNA at the center of the G-50 resin, do not disturb the resin
Centrifuge for 2 min at 3000 rpm, purified probe will be collected in the 1.5 ml tube.
Keep the tube on ice.

12) Hybridization
Get the hybridization bottle from the 65˚C oven, discard the Southern Church buffer that you used for pre-hybridization.
Add 5~10 ml of fresh Southern Church buffer (pre-warmed at 65˚C) to the bottle, put it back to the oven.
Then, boil the purified probe (see above) for 5 min, on ice for 5 min.
Centrifuge the tube briefly to collect the content (purified probe) at the bottom of the tube.
Add the purified probe (25 ul) to the hybridization bottle, store the rest of the probe at -20˚C
Rotate the hybridization bottle in the 65˚C oven for O/N.

13) Washing
Warm Wash 1 and 2 at 50˚C
Rinse membrane with Wash1 once
Wash membrane with Wash1 at 50˚C for 10min twice in the bottle
If the background is high, wash membrane with Wash2 at 50˚C for 10min once or twice in the bottle

**When washing, it is important to get buffer between the bottle and membrane, remove bubble by rotating the bottle, then put the bottle back to the oven

Take the membrane out of the bottle, and wrap the membrane well
**If you plan to strip the probe for another hybridization, do not dry the membrane

14) Imaging
Expose membrane to phosphoimager screen for 2 hr – 3 days
Analyze the blot with Storm 840/ImageQuant

15) Stripping the probe
Stripping buffer
0.1% SDS/2mM EDTA
Put the membrane in the glass container
Pour boiling Stripping buffer to the membrane, agitate for a min, and pour off the buffer to the sink (radioactive!)
Repeat this wash more as necessary
Every wash, check radioactivity
The membrane is now ready for re-hybridization

Reagent:
Denature / Transfer Buffer

Final Conc.	Ingredient	Stock Conc.	For 1 L	
0.5 M	NaOH	pellet	20 g	
1.5 M	NaCl	5 M	300 ml	
	Milli Q Water		700 ml	

Southern Church buffer (7% SDS, 1% BSA, 1mM EDTA pH8.0, 250mM NaPO4 pH7.2)

MilliQ Water	199 mL
20% SDS	175 mL
BSA	5 g
0.5M EDTA	1 mL
1M NaPO4 pH7.2	125 mL
	500mL

Roche, Random Primed DNA Labeling Kit
[α-^{32}P]dCTP
Probe Quant G-50 Micro Column
STE (see the instruction for Probe Quant G-50 Micro Column)

Wash 1 (0.5L) (2 x SSC, 0.1% SDS), at 50˚C

20 x SSC	50mL
20% SDS	2.5mL
H2O	447.5mL

Wash 2 (0.5L) (0.2 x SSC, 0.2% SDS), at 50˚C

20 x SSC	5mL
20% SDS	5mL
H2O	490mL