SOUTHERN BLOTTING OF GENOMIC DNA

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1. Digest DNA:

- 1.5 µg DNA in a 25 µl reaction containing BSA
- 3 to 5 units for 5 to 3 hours (aim for 10-fold overdigestion; remember many enzymes will not survive well for more than 3 hr).
- Also prepare molecular weight standard (Lambda plus HindIII); avoid plasmid-derived markers (1 kb ladder, etc.) since it might cross-hybridize with the probe!

2. Run gel:

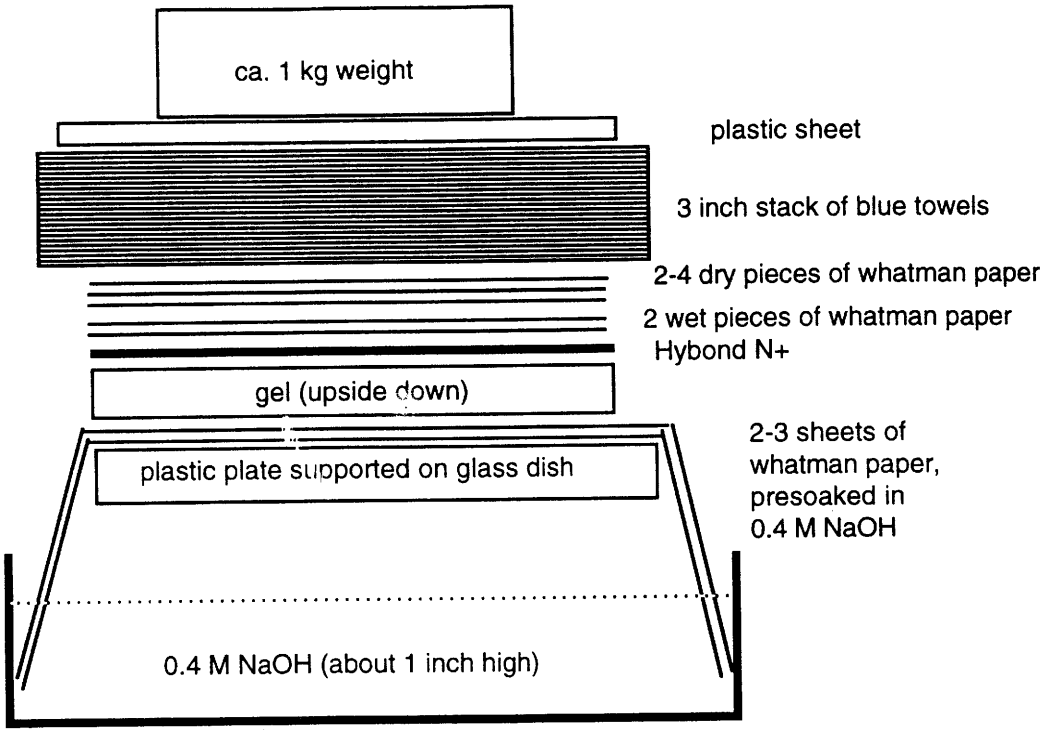
- load samples onto agarose gel (typically 0.8 to 1.0% agarose in TBE, depending on desired size range).
- run gel at maximum rate of 5 volts per cm.
- run until the bromophenol blue has run ca. 9 cm (some DNA will run 1-2 cm below the dye).
- Using gloves, place in staining solution (0.5 to 1 µg/ml ethidium bromide in water, 20-40 min. Be careful not to break the gel.

3. Nicking of DNA (to improve transfer of high molecular weight DNA):

- Place the gel (on a plastic plate) in a UV oven.
- Irradiate ca. 1200 µJoules.
- Destain 10-30 minutes in water (optional) and photograph.

4. Transfer to nylon membrane:

- WEAR GLOVES when handling blotting membrane, wicks, etc., since oils on skin will inhibit transfer. WASH OFF powder from gloves before use.
- Soak gel 20-40 minutes in denaturation solution (0.4 M NaOH, 0.8 M NaCl)
- Set up capillary blot apparatus (this is one of several transfer methods that can be used for transfer of DNA) as shown on the next page.
- Cut out wicks from Whatman 3 paper using the paper cutter. Setting up the blot will be easiest if one dimension of the wick equals that of the gel. Soak the wick in denaturing solution. Place on plastic sheet suspended on a giass pyrex dish. Use a pipette to rub out any air bubbles.
- CAREFULLY turn over gel and place on top of wick apparatus (transfer is sometimes inhibited by the hardened top surface of gels). Use plastic sheets to help turn over the gel. Use a wet, gloved finger to press out any air bubbies.



- Place a sheet of Hybond N+ membrane on the gel. Use a piece the same size as the gel. Do not prewet the membrane. Be careful to align the membrane with the gel. Use a pipette to rub out any bubbles.
- Cover with 2 wet sheets of Whatman paper, cut to exactly the same size as the nylon. Cover with 2-4 sheets of dry paper.
- Add "short circuit" prevention device (to make sure that liquid will not bypass the gel and membrane). Use either saran wrap, nescofilm, etc. to cover parts of the wick that might later come into contact with the paper towels.
- Cover with a few inches of blue paper towels, followed by a piastic sheet and weight (1 kg for a 5 x 10 inch gel).

5. Final preparation of membrane (post-blot treatment):

- Let the transfer proceed for 12-18 hr (do not let go longer as the quality of the biot will degrade with time.
- Remove the paper towels and whatman paper from the transfer apparatus, until the membrane is exposed.
- Use a clean blade to notch one side of the membrane to help orient it to the gel (I make a notch on the top of the gel by lane #1). Label the membrane using a pencil.
- Carefully remove the membrane and place, DNA side up, in a dish containing ca. 200 ml of 3X SSPE (20X SSPE is 3.6M NaCI, 0.2M sodium phosphate, 0.02M EDTA pH 7.7). Rock back and forth 10 seconds. Repeat two more times to neutralize the membrane (the alkali transfer covalently bonds the DNA to the membrane; baking or drying is not needed).
- Air dry in a clean and protected piace. Store at room temperature until needed for hybridization.

MAKING PROBES FOR DNA (or RNA) BLOTS

A. Labelling the DNA

The lab currently uses a kit from Amersham. Here is a brief protocol:

1. In screw-cap 1.5 ml tube, place 25 ng DNA in 30 µl water plus 5 µl primer/BSA mix.
2. Boil 5 min; place on ice.
3. Add 10 µl labelling mix.
4. Using a filter-tip pipette tip, add 5 µl (50 µCi) ^{32}P-alpha-dCTP
6. Add 2 uI Klenow.
7. Close the tube. Mix gently by agitating with finger. Spin 10 seconds.
8. Incubate 37°C 10-30 minutes (for Megaprime kit).

B. Removing unincorporated label

(this helps to eliminates speckle-causing debris, confirms that the probe was OK, and reduces background)

1. Make a minicolumn in a narrow-tipped plastic transfer pipette.
   - Cut off the bulb (top)
   - Plug the bottom using a small chunk of sterile, siliconized glass wool.
   - Support the column in a 2 ml screw-cap tube.
   - Add gel slurry (Biogel P-60 100-200 mesh in TE [10 mM Tris 8.0, 1 mM EDTA] plus 0.2 % SDS).
   - Add about 2 inch of 66% slurry for a typical column.
   - Wait for the resin to settle and for the liquid to drain out; discard the liquid.
2. To the DNA labelling reaction (50 µl) add about 20 µl of column loading buffer:
   - 50% glycerol
   - 0.1% bromophenol blue (dark blue)
   - 1% blue dextran (light blue-aquamarine)
   - Store buffer at 4°C (aliquots can be kept at room temp for 2+ months)
3. Load the reaction. For the greatest efficiency, try to squirt it down to the top of the resin, along the side of the column.
4. Once the reaction has entered the resin, fill the column with TE-0.2% SDS.
5. The labelled DNA will elute with the blue dextran. Just before the bromophenol blue elutes, remove the column. Cap the tube.
6. On the geiger counter a "good" probe will register >2x105 cpm.

NUCLEIC ACID HYBRIDIZATION (BLOTS, COLONY LIFTS, ETC).

1. Prehybridization:

- Place the membrane in a glass hybridization tube. If you are using multiple membranes it is OK to stack them up.
- Add hybridization buffer: 0.5 M $NaHPO_4$ pH 7.2, 7% SDS, 0.25 mM EDTA (made from 1 M, 28%, and 0.5M stock solutions). Add about 10 ml for a 10 x 20 cm membrane.
- Screw on the cap (not too tight or else it will crack!) and rotate at 65°C for 10-60 min at speed 4. Make sure you balance the tubes.

2. Hybridization:

- Remove "excess" liquid (the blot will go faster in a small volume). Generally, I leave about 2-3 mls of "free" liquid in the tube (4-5 mls for the large tubes), i.e. beyond that needed to wet the membrane.
- Boil the probe for five minutes, and then cool on ice.
- Add the probe.
- Seal the tube and rotate 12-24 hr at 65C. Maximum hybridization is generally obtained after about 16 hrs (about 3 Cot). Of course, when very strong signals are expected, shorter times can be employed.
- Make sure that the tubes rock back and forth and aren't leaking!

3. Washing:

- IMPORTANT: Never let the membrane dry out, which will fix the probe to the membrane!
- The following procedure is adequate for most applications (up to 5 blots with <250 uCi of probe). If an unusually large amount of radioactivity or blots are being washed, more washes or time may be required.
- Prepare about 1.8 liters of each wash solutions at 65C on the hot plate:

Watch the temperatures! Don't let them get too hot, esp. the final wash.

First washes: 1X SSPE, 0.2% SDS, 0.1% sodium pyrophosphate

Final wash: 0.2X SSPE, 0.2% SDS, 0.1% sodium pyrophosphate

- Pour off the probe into the liquid radioactive waste. Add 20-30 ml of 1X solution. You can dip a little beaker into the large beaker to get the 20-30 ml. Rotate about 5 minutes at speed 4 to 8.
- Repeat for a total of 3 washes. These steps remove most of the radioactivity, so that the next washes (ca. 3.5 liters) are "nonradioactive" [i.e. background counts] and can be poured down the sink.
- Using gloved fingers, carefully remove the membrane from the tube and place in the 1 X solution at 65C. Stir for 10 minutes.
- Transfer the membrane(s) to the 0.2X solution at 65C (watch the temperature!!!). Stir for 10 minutes.
- Remove the filters and carefully wrap in plastic wrap. Do not let them dry out!. Expose to Xray film using intensifying screens at -80C, or to a phosphorimager screen at room temperature.
- If the 1 X and 0.2X wash solutions in the beakers are close to background, they can be poured down the sink. Rinse out the beakers, being careful not to shock them with cold water (which might crack them): you should dump out the hot solution, wait a few minutes and then rinse out the beakers.

4. Stripping off probe:

- When done with the blot remove the probe, so that the membrane can be used again.
- Heat 0.1 % SDS, 0.1X SSPE (or SSC) to 95C on the hotplate. Turn off the heat. Add the membranes. Incubate 20 minutes.
- Remove the membranes and blot dry. Store dry at room temperature.