PROBE LABELLING AND BLOT PROCESSING

To detect specific restriction fragments on the genomic Southern blot you prepared earlier, you will radioactively label a fragment of the enhancer trap transposon that you are studying (*lacZ* gene sequence) by random prime labelling with ^{32}P. This probe will then be hybridized to the Southern blot, followed by high stringency washing of the blot and autoradiography to detect bands corresponding to the probe.

Random prime labeling

This procedure requires limited exposure to ^{32}P irradiation and requires extreme caution. The greatest risks arise from inadvertent spills and aerosols of radioactive reagents. All steps will be demonstrated and closely supervised by the lab instructor and assistants and will be performed using maximum shielding to minimize radiation exposure. Avoiding contamination requires careful attention to the protocol, constant attention to radioactive sources, and minimizing exposure.

We will use the NEBlot kit from New England BioLabs for random primed labeling. Consult the kit manual online for details of the components and protocols (http://www.neb.com/neb/frame_tech.html).

1. Add 2 μl probe (~40 ng DNA) to 36 μl H_2O in microtube. Denature the DNA by placing in a boiling waterbath for 5 min. Quickly place the tube in ice for 5 min., then pulse it in a microcentrifuge for a few seconds, to accumulate the moisture in the bottom of the tube.

2. In a shielded ice bucket, add the following ingredients to the microtube in order, mixing each by gentle aspiration with the micropipet tip:

    a. 5 μl of 10X labeling buffer (includes random primers)
    b. 6 μl of dNTP mixture (2 μl each of dATP, dTTP, dGTP)
    c. 5 μl of [α-^{32}P]dCTP (3000 Ci/mmol, 50 μCi total)
    d. 1 μl Klenow Fragment of DNA Polymerase I (5 u).

    Incubate for 1 hr at 37°C in a shielded waterbath. Terminate the reaction by adding 5 μl 0.2 M EDTA, pH 8.

3. Measure the incorporation of radioactivity into your probe as follows:

    a. Dilute 1 μl of the probe to 100 μl in TE buffer. Spot 5 μl of the dilution onto each of two pieces of Whatman DE81 paper, one labeled "+" and one labeled "-" with a pencil. Allow to dry.
    b. Add the "+" filter to a beaker containing ~50 ml of 0.5 M Na_2HPO_4. Swirl for 1 min, then decant the liquid into the radioactive waste container. Repeat this washing step three more times.

c. Add 50 ml of H_2O to the beaker, swirl, and decant. Add 20 ml EtOH to the beaker. Add the "-" filter to the beaker and swirl for 1 min. Remove the two squares, dry them, and perform liquid scintillation counting of each square.
d. Compute your incorporation values as follows:

Percent of incorporation of label into DNA = "+" cpm ÷ "-" cpm

Total incorporation = "+" cpm X 100 (dilution) X 50 (reaction vol.)

cpm/μg DNA = total incorporation ÷ 0.04 (μg DNA in reaction)

Prehybridization & Hybridization of a Southern blot

Probing a blot requires incubating the blot (solid-phase DNA target) with single-stranded, labeled probe (solution-phase DNA probe) in an appropriate solution and temperature to favor formation of duplex hybrids between the solid-phase and solution phase molecules. Because nitrocellulose will react with the DNA probe itself, it is first necessary to "pre-hybridize" the blot in the hybridization solution, minus probe, so as to block all reactive sites on the membrane that might bind the probe. *All steps involving probe involve exposure to and potential accidents with radioactive materials. Before such treatments, you must be very familiar with the protocols and take precautions to avoid contamination with the radioactivity.*

4. Carefully place your blot in a hybridization tube and add 25 ml of prehybridization solution. Place the tube in the rotator/incubator and allow to prehybridize for at least two hrs at 42°C.

5. Denature your radioactive probe by placing it in a boiling waterbath for 5 min, then immediately putting in on ice for 5 min.

6. Add the probe to 10 ml of ice-cold prehybridization solution. Decant the prehybridization solution from the hybridization tube (leaving the blot in place), then carefully replace it with the 10 ml of hybridization solution containing the probe.

7. Tightly cap the hybridization tube and gently mix the contents by inverting the tube. Place it in the rotator and incubate overnight at 42°C.

Washing and autoradiography of the blot

To remove unhybridized probe from the blot, it is necessary to carry out a series of washes that will leave only stably attached radioactive probe on the membrane. After washing, the moist membrane can be placed against a piece of X-ray film to permit autoradiography of the membrane; the developed film can be analyzed to determine the location on the gel of bands that hybridized to the probe.

8. Carefully decant and discard the radioactive hybridization mixture from the hybridization tube. Add 50 ml of 2xSSC + 0.1% SDS to the tube, cap tightly, and return to the rotator for 15 min.

9. Decant and discard the solution in the tube. Replace it with another 50 ml of 2xSSC + 0.1% SDS and repeat the washing. Repeat this washing a third time.

10. Remove the blot from the tube and carefully place it into a sealable bag. Add 50 ml of 2xSSC + 0.1% SDS to the bag, seal it, and place it in a 42°C waterbath for 15 min.

11. Decant and discard the solution in the bag. Replace it with another 50 ml of 2xSSC + 0.1% SDS and repeat the 42°C washing (15 min.).

12. Remove the membrane from the bag and lightly blot it. Mount it, cover it with Saran wrap, and place it in a cassette against X-ray film. These steps will be demonstrated. Autoradiograph at -80°C using an appropriate amplifier screen.

13. Develop and analyze the exposed film.

Prehybridization solution:
5x SSC
50% formamide
4x Denhardt's solution
100 μg/ml denatured DNA
0.1% SDS

50x Denhardt's solution:
1% polyvinylpyrrolidone 25
1% Ficoll 400
1% nuclease-free bovine serum albumin