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FOOTPRINTING -**P2**

Procedure

A. Preparation of the labeled DNA fragment

1. Label the 5' end of one of the oligonucleotides to be used to make the fragment to footprint. Choose the oligo so that the suspected binding site is not too far from the labeled end. Normally the footprint should be performed on both strands of the DNA, *i.e.* using two DNA fragments labeled at either end. Use a 0.5 ml tube suitable for PCR. 3 µl Oligo 1, 10 pmoles/µl 2 µl 10x PNK buffer (supplied by manufacturer of PNK) 3 µl [γ- 32P] ATP (3,000 Ci/mmole 11 µl H2O1 µl (10 units) PNK

Incubate 30 min 37 °C *N.B. Take suitable precautions for use of radioactivity. Perform in approved location.*

2. Precipitate the labeled oligo. add

80 µl 0.1M Sodium acetate (natural pH about 9.0) 1 µl 10 mM Sodium phosphate buffer, pH 7.4 250 µl ethanol (96%) Incubate in dry ice for 30 min (or at -80 °C for > 1 h).

3. Centrifuge 15 min 4 °C 12,000 *x g*.

4. Carefully remove the supernatant.

5. Rinse the (tiny) pellet with 100 µl 96% ethanol (or 70% ethanol at -20 °C). Centrifuge 5 min 4 °C 12,000 *x g*.

6. Carefully remove the supernatant. Dry *in vacuo* 5 min.

7. Resuspend the labeled Oligo 1 in 35 µl H2O. Vortex well and give a quick centrifugation to place all labeled oligo in bottom of tube.

8. Add 5 µl Thermopol buffer (Biolabs or other suitable Taq polymerase buffer). 5 µl deoxyNTP mix containing 2.5 mM each dATP, dCTP, dGTP, DTTP. 4 µl Oligo 2 10 pmoles/µl (Oligo 2 corresponds to the other end of fragment to be amplified). 1 µl template DNA (*e.g.* plasmid DNA carrying the cloned region to be amplified, about 50 ng depending on size of plasmid. We usually use 1 µl 1/10 dilution of standard mini plasmid DNA preparation). Mix well, quick centrifugation and add 0.5 µl (2.5 units) Taq polymerase (5 units/µl) and immediately start PCR.

9. PCR cycling

a. Denature 94 °C for 2 min

b. 94 °C for 30 sec

c. 55 °C* for 30 sec

d. 72 °C for 30 sec*

e. Repeat b-d 25 times

f. Final extension 5 min 72 °C

* The temperature of annealing depends upon the oligonucleotides used and the extension time at 72 °C on the length of the fragment amplified. Footprints on fragments greater than 500 bp are not recommended and so 30 sec is usually good for most fragments.

10. Test 1-2 µl on small agarose gel (containing ethidium bromide or other suitable DNA detection reagent) for amplification of a fragment of the correct size and check you have a fragment of the correct size in good yield (*i.e.* most or all of the oligos have been used up) 30 pmoles of oligos can give maximally 2 µg of a fragment of 100 bp and 10 µg of a fragment 500 bp. Proceed to purification. In theory you can use the PCR as it is or after passage through a spin column. However any minor, shorter length contaminants will produce artifactual bands in the footprint and the presence of unincorporated radioactivity will not allow you to estimate the amount of radioactive DNA in the footprinting reactions. So we always proceed to purify the labeled DNA from an agarose gel. In exceptional cases *e.g.* to eliminate a close running contaminant band from a short (100-200 bp) fragment, the fragments can be purified on a native acrylamide gel (see step A-14 below).

11. Run the PCR mixture, mixed with 10 µl of loading dyes, on a small 1% agarose gel in 50 mM TBE buffer. Usually the whole 50 µl PCR can be loaded in 3 wells.

12. Visualize the gel on a long wavelength (365 nm) transilluminator (to minimize damage to the DNA by short wavelength) and cut out the agarose containing the radioactive fragment. Discard rest of gel as radioactive waste.

13. Extract the DNA from agarose using a gel purification kit (*e.g.* Machery-Nagel Nucleospin Gel and PCR clean-up). Elute in 50 µl elution buffer.

14. To purify the DNA from an acrylamide gel: run the PCR mix on a native acrylamide gel (5-8% depending upon size in 50 mM TBE at room temperature *i.e.* do not allow the gel to heat up. Depending upon the size of the apparatus used 100-200 volts should be adequate). Locate the radioactive DNA by short exposure of the wet gel wrapped in Saran wrap to a phosphorimager screen (or X-ray film). The piece of acrylamide containing the radioactive band is cut out and the radioactive DNA eluted by shaking overnight at 37 °C in 1 ml of 0.5 M ammonium acetate, 0.1% SDS, 1 mM EDTA. Separate the aqueous phase from the acrylamide gel piece by centrifugation and transfer to another tube. Extract with 0.5 ml phenol/CHCl3 and precipitate the DNA with 2.5 volumes ethanol in dry ice for at least 30 min. Centrifuge 10 min 4 °C, remove all the supernatant, dry *in vacuo* 2-3 min) Resuspend in 50 µl elution buffer (as for DNA eluted from agarose.).

15. Run 1 µl on a new small agarose gel and estimate the quantity by comparison to the staining intensity of marker DNAs (*e.g.* 100 bp ladder).

16. Count 1 µl by Kerenkov radiation in a scintillation counter or estimate using a Geiger counter. Expect to have 50,000-150,000 cpm/µl with about 5-50 ng DNA/µl (yield in range 10-50% of the moles of starting oligonucleotide.) You can calculate the molar concentration of the DNA fragment from the length of the fragment in bp and using 1 bp corresponds to a molecular mass of 660. B.