IN VITRO FOOTPRINTING METHODS

Preparation of labeled probes:

There are several choices available in the labeling of DNA termini for subsequent footprinting procedures-- here are two that work well:

The old way: Gel purification.

1. a.) A convenient subclone of the HSP70 promoter region is pKΔ3 which contains HSP70 promoter sequences from -188 bp to -4 bp. To single-end label the DNA 50 µg of pKΔ3 was digested with either Sal I (-188 bp) or Hind III (-4 bp). All of the DNA was treated with CIP as described by the manufacturer and the CIP was inactivated by heating to 65°C in the presence of 12.5 mM EGTA and organic extraction. After ethanol precipitation the DNAs were resuspended at 1 µg/µl.

b.) 5 µg of plasmid DNA linearized at either the Sal I or Hind III site was labeled with 10 units of T4 DNA kinase or E. coli Klenow fragment as described in *Maniatis* except that the reaction was performed in 10µl. Generally, 150-200 µCi were used per labeling reaction (5 µg of plasmid). Labeling proceeded for 30 min at 37°C and was terminated by the addition of EDTA and dilution to 100 µl with TE.

c.) The labeled DNA was then purified by spin chromatography over a Sephadex G-50 column. The DNA was then digested with a four-fold excess of the complementary enzyme (Sal I or Hind III whichever was appropriate for release of the single-end labeled fragment).

d.) The entire digest (≈120 µl) was loaded onto a 1.5 mm 4% 19:1 native acrylamide gel prepared in 0.5X TBE (use gel-shift size plates) and poured with the large 10 well BioRad prep comb. The entire 120 µl + tracking dye was loaded into one lane (yes, it's possible). Electrophoresis was at 150 - 200 V for approximately 2 - 3 hours depending on the size of the fragment.

e.) To visualize the position of the radioactive band of interest the gel was taken apart leaving the gel on one of the glass plates. It was quickly covered with SaranWrap and another clean glass plate (*work behind a shield as there is a fair amount of exposure at this point*). Take the covered gel and 8 x 10 X-ray film to the darkroom. Carefully overlay a sheet of film onto the gel (on top of the SaranWrap)-- I always line up the edges of the film with two edges of the glass plate -- put the clean glass plate back on for two minutes. Remove film and process. The desired band should be readily visible -- a dark black if you've done well.

f.) While the gel is still on the plate place the film underneath and align the film so that it indicates the position of the band in the gel (work behind shield). Use a clean razor blade to excise the band (remove SaranWrap before crushing). Place the gel slice in a 1.5 ml eppy tube and grind the gel fragment into a paste (I use a p200 pipette tip). Add 400 µl of 0.5 M Ammonium Acetate, 1 mM EDTA to the ground gel and incubate overnight with shaking at 37°C.

g.) Recover eluted DNA by centrifugation at high speed for 5 min. Remove as much of the supernatant as possible to a new tube. Add 150 µl of the elution buffer to the crushed gel pellet and vortex, spin again and combine the supernatant with the first. Using the Geiger counter you should find that 80-90% of the radioactivity is in the recovered supernatant. If the fragment is longer than 300 basepairs then recovery will decline.

h.) Spin the supernatant again for 15 min at high speed. This helps to remove small pieces of acrylamide. Transfer supernatant to new tube and ethanol precipitate with 3 volumes of 95%. No salt or carrier is necessary. After centrifugation the DNA was resuspended in approximately 100 µl and 0.5 to 1.0 µl were used for each footprinting reaction (5 - 10 fmoles, 10, 000 - 20,000 cpm). Purine ladders for markers were prepared as described previously (Maxam and Gilbert, 1980) with formic acid treatment and piperidine cleavage.

The newer (not necessarily better) way:

2. a.) The polymerase chain reaction can be used to prepare radioactive probes of high specific activity. Although any two primers could be used in this example the T7 and T3 promoter primers complementary to Bluescript KS- were used to prepare single end-labeled probes. The T7 primer was labeled to high specific activity with T4 DNA kinase as above except 1 mCi of g-ATP was used per 1 µg of primer as well as 20 units of T4 kinase.

b.) The amplification reaction was carried out in 50 µl with : 0.25 µM T7(labeled) and T3 primers 1.0 unit of Taq polymerase, 1X PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 6 mM $MgCl_2$, 1 mM DTT), 200µM dNTPs, and ≈1 ng of template (I use 5 µl of a 1:1000 dilution of miniprep DNA). The reaction was covered with oil and cycled 30 times at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min followed by 5 min 72°C.

c.) The labeled probe was purified of free primer and nucleotide by addition of 0.25 volumes of 8 M ammonium acetate, 5 µg of glycogen, and 1 volume of isopropanol. After 10 min at 25°C the labeled probe was recovered by centrifugation (12,000 x g, 10 min).

d.) The recovered DNA was resuspended in 400 µl. The integrity and purity can be checked by electrophoresis on a 10% (19:1) polyacrylamide gel (use the BioRad miniprotein gel rigs). The concentration of the labeled DNA is most accurately estimated by a direct spectrophotometric analysis of the entire sample (400 µL) at 260 nM. This necessitates adding the labeled DNA to the cuvettes so make sure you rinse in-between and clean at the end!

DNASE I FOOTPRINTING

Reactions are prepared and processed as follows:

a.) In a eppy tube at room temperature combine: single-end-labeled probe (1 x 10^{-10} M), competitor DNA (100 ng) poly (dI-dC)·(dI-dC), 50 µl of 2X transcription buffer (2X transcription buffer = 24 mM Hepes pH 7.9, 120 mM KCl, 24 % glycerol, 16 mM $MgCl_2$, 2mM DTT , and 1 mM EDTA) were mixed and brought to 100 µl. It is best to prepare one large reaction mix and then aliquot 100 µl into each sample tube.

b.) The mHSF1 or mHSF2 protein (amounts required for complete protection vary from 0.5 nM to 10 nM) is added and incubation proceeds at 23°C for 20 min.

c.) While the binding reaction occurs thaw the 10 mg/ml stock of DNase I (-20°C freezer, 50 µl aliquots) and dilute 1:100 in ice cold water. Keep this on ice at all times.

d.) Before adding DNase I <u>you must first</u> add 100 µl of 10 mM $MgCl_2$, 5mM $CaCl_2$. This should be done to 4 - 5 reactions at a time as that is all that can be reasonably processed at once.

e.) 4 µl of diluted DNase I (2 µg/ml final concentration) is added to the first tube (mix with pipette tip as adding) and 10-15 seconds later the next tube is started and so on. After 1 minute digestion (therefore only 4 - 5 tubes can be started at any one time) 200 µl of DNase I stop solution (1.0% SDS, 200 mM NaCl, 20 mM EDTA, 100 µg/ml yeast tRNA) is added.

f.) For optimal results a single phenol/chloroform extraction should be done followed by ethanol precipitation with 2 volumes 95% and placement at -20°C overnight or dry ice until solid. Spin in microfuge 15' and allow pellet to air dry (I do not recommend using the speed vac).

g.) Resuspend the sample completely in 4-5 µl of sequencing gel loading buffer (e.g. 80-90% formamide, 0.5x TBE, bromophenol blue, xylene cyanol) and boil sample for 90 sec. Quench on ice. Load onto a prerun 0.4 mm 6%, 8.3 M urea gel. I usually run gels at constant power to ensure constant heating. 40 -50 W gives 1600 -1800 V at the beginning of the run and closes at 2200-2300 V with even heating. Run as far as needed...the bromophenol blue in this gel will migrate at ≈ 25 bases and the xylene cyanol at ≈110 bases.

h.) For ^{32}P I do not soak the gels in acetic acid/methanol unless there is great difficulty in getting the gel to stick to one plate. Transfer the gel to 3MM or Whatman paper and cover with SaranWrap (my superstition --don't use Clingwrap unless you want little spots). Dry for 30 min with heat (80°C) and let cool on drier before breaking vacuum. Expose to XAR5 film with intensifying screen at -70°C. Depending on the strength of the probe exposure will be 1 - 3 days. If weak signal is seen overnight leave on for 4 days...usually looks better than you might think.

FE-EDTA OR HYDROXYL RADICAL FOOTPRINTING

Reactions are prepared and processed as follows:

a.) One major concern in hydroxyl radical footprinting with Fe-EDTA is that glycerol is extremely inhibitory (a potent free radical scavenger) so it must be eliminated from the binding buffer and the protein solution (pass mHSF1 or HSF2 over small desalting column e.g. G25 equilibrated with -glycerol storage buffer...still OK to freeze and thaw several times without apparent loss of activity).

b.) Binding reactions (this protocol uses a 25 µl volume) are established as in DNase I footprinting (minus glycerol) keeping the amount of protein (1 -10 nM) and DNA (0.1 -0.5 nM) the same. Since this is a rather harsh footprinting treatment it may be necessary to use higher concentrations of protein to reach saturation in solution. If there is any evidence of exonuclease activity in the HSF prep the binding reaction can be done on ice and brought to RT for the Fe-EDTA reaction.

Solutions needed for next step:

25.6 mM $Fe(NH_4)_2(SO_4)_2$ = 10 mg/ml (best if made fresh each time)

50 mM EDTA

20 mM Sodium Ascorbate -- make 100 mM and dilute (19.8 mg/ml = 100 mM)

0.3% H_2O_2

Stop solution: 5µl per 25 µl binding reaction composed of: 2 µl 100 mM thiourea, 1 µl 250 mM EDTA, and 2 µl of 3 M NaOAc.

c.) While the binding reaction is equilibrating mix 1.56 µl of 25.6 mM $Fe(NH_4)_2(SO_4)_2$ and 1.6 µl of 50 mM EDTA and dilute to 100 µl with water to create 400µM Fe/800µM EDTA. Dilute some of this 1:4 to make 100µM Fe/200 µM EDTA. Keep on ice until ready to use and always make fresh.

d.) For control reactions that contain no HSF protein and assuming a 25 µl binding reaction add components as follows: to the side of the tube add 2 µl of 100 mM Fe/200 mM EDTA, 2 µl of 20 mM Sodium Ascorbate, and 2 µl of 0.3 % hydrogen peroxide. To initiate reaction use pipette tip to mix components and push drop into binding reaction.

e.) For reactions that contain HSF protein in any amount add 2 µl of 400 µM Fe/800 µM EDTA, 2 µl of 20 mM Sodium Ascorbate, and 2 µl of 0.3 % hydrogen peroxide.

f.) Allow cleavage reaction to proceed for 3 minutes at room temperature.

g.) To stop the cleavage add 5 µl of stop solution and 60 µl of 95% ethanol.

h.) The gel and subsequent procedures are just as for DNase I footprinting.

METHIDIUMPROPYL-EDTA (MPE) FOOTPRINTING

MPE footprinting is a type of hydroxyl radical footprinting in which the MPE binds to the minor groove of the DNA and the hydroxyl radicals are produced in the minor groove. This has two advantages: the reaction is not very sensitive to glycerol and one can learn something about the structure of the DNA as intercalation is required for DNA cleavage.

The MPE is stored frozen at -70°C in one of my boxes...look for 3, 0.5 ml colored tubes wrapped in foil ---MPE is light sensitive and breaks down at RT --- so keep in freezer unless using immediately.

This reaction can be carried out essentially as originally described by Hertzberg and Dervan and O'Halloran et al.

a.) A binding reaction is established as in DNase I footprinting.

b.) After 20 minutes: the MPE, 2.5 µl of a 1.5 mM stock solution kept frozen at -70°C, was mixed with 4 µl of 4 mM Ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2$) and immediately diluted to 100 µl with cold H_2O.

c.) One µl of MPE-Fe(II) was added to the HSF binding reaction. After 3 min, 1 µl of 100 mM DTT was added and the cleavage reaction was allowed to proceed for an additional 2 min. The reaction was quenched and evaluated as for hydroxyl radical cleavage above.

DMS PROTECTION ANALYSIS

a.) Prepare 5X HSF binding mix with respect to protein and DNA only. That is, keep the volume small (25 µl) and raise the protein (≈ 10-20 nM) and DNA concentration (1- 2 nM).

b.) After 20 min add 1 µl of a fresh 1:100 dilution of stock DMS diluted in water (stock conc = 10.7 M -- working DMS concentration is ≈ 100 mM-- shake it up and don't worry about apparent insolubility).

c.) After 1 min add 4 µl of 100 mM DTT (to stop DMS) and load on 4% 40:1/0.25X TBE gel shift gel.

d.) Visualize bands as for preparing labeled probes and excise bands with clean razor blade.

e.) Cast acrylamide gel slices in a 1% agarose gel and electroelute onto S+S NA45 paper. Done according to manufacturers instructions.

f.) Precipitate eluted DNA and load on 6% denaturing gel as for other footprinting methods. If one wishes to visualize the "A" residues as well as the "G" residues one can first treat the eluted DNA with acid as follows:

Resuspend DNA in 20 µl of H_20.
Add 5 µl of 0.5 M HCl and leave at 0°C for 2 hours.
Add 200 µl of 0.3 M NaOAc pH 7.0 and 750 µl of 95% ethanol --put on dry ice.
Spin down DNA 15 min.
Resuspend in 10 µl of 0.1 M NaOH/1mM EDTA and place at 90°C for 30 min.
Add 1 µl of 1 M HCl, 100 µl of 0.3 M NaOAC, and 300 µl of 95 % ethanol.
Dry ice and spin.
Load on gel.

g.) process gel as for other methods.

MISSING NUCLEOSIDE ANALYSIS

To analyze the contribution of each nucleotide within the binding site of a protein one can take advantage of the Fe-EDTA reaction to prepare randomly gapped DNA that is then allowed to bind to the protein of interest which is in great excess. The bound and free DNAs are recovered and examined for the distribution of bands. Done as follows this method works well for HSF:

a.) The same probe DNA that is used for other footprinting reactions is cleaved in a control reaction with 100 µM Fe/200 µM EDTA as described for Fe-EDTA footprinting except that the reaction is scaled up:

1. Place 20 µl of labeled DNA in 70 µl of TE final volume.
2. Treat with 10 µl 100 µM Fe/200 µM EDTA, 10 µl 20 mM NaAscorbate, and 10 µl of 0.3 % hydrogen peroxide for 3 minutes.
3. Stop with 30 µl of thiourea, 10 µl of 0.2 M EDTA, and 20 µl of 3 M NaOAc.
4. Add 40 µl of TE to bring volume to 200 µl and precipitate with 2.5 volumes of 95% ethanol and dry ice.
5. Spin 15 min and resuspend in 20 µl TE.

b.) Prepare 50 µl binding reaction with 2X transcription buffer, 10 µl of gapped DNA, 10 µg poly IC, and 4 µg of HSF. Binding on ice for 20 minutes.

c.) Load onto 4% 40:1, 0.25XTBE gel --- 150 Volts for 3 hours.

d.) cover gel with SaranWrap and expose to film **wet** (may take 2 hours to overnight to see bands sufficiently well to cut them out).

e.) By aligning the film with the wet gel excise the **bound** and **unbound** fraction of each lane.

f.) Elute DNA by crush and soak in elution buffer (0.5 M Ammonium Acetate, 1 mM $MgCl_2$ with the addition of 0.1% SDS).

g.) Based on recovery, estimate how much of each sample should be loaded to give equivalent intensities on the resulting film (1 cpm/bp is good enough for overnight). Note that the amount of unbound DNA is very small and may be too little to detect, however, it is there and will show up nicely. The gel should include a G+A ladder and also a lane of each original DNA treated with Fe-EDTA --- called the input DNA. A standard denaturing sequencing gel can be used for these samples and processed as for other methods.

h.) Exposure times are generally several days to 1 week. Be patient.

i.) The interpretation: DNA that remained unbound even at high protein concentration contains gaps (missing bases) that are essential for the binding of the protein. The bound fraction is generally a very complementary picture to that of the unbound and reinforces the result. Sometimes the loss of a base can stimulate binding and may be observed in some bound fractions.