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Solid phase DNase I footprinting: quick and versatile

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DNase I footprinting is often the method of choice to characterise the targets of sequence-specific DNA binding proteins (1). DNase I footprinting is labor intensive and the time required to prepare samples for gel electrophoresis is in the order of 3 hours (2). Here we describe an adaptation of the standard procedure to solid phase technology with many unique advantages over conventional footprinting assays. Immobilisation of the target DNA onto paramagnetic beads allows the quick and efficient purification of nicked fragments prior to electrophoresis. Since organic extractions as well as precipitations are avoided, ten samples can be processed in about 30 minutes starting from the DNase I digestion of protein–DNA complexes to the loading of the gel. The separation of the recovered DNA fragments on sequencing gels is optimal since impurities that affect the single nucleotide resolution of DNA adversely are efficiently removed.

DNA fragments containing the putative protein binding site with one biotinylated and one radioactively labelled end are prepared according to standard procedures. This is either achieved by PCR using one biotinylated primer and one oligonucleotide kinased with $\gamma[^{32}P]$ATP, or, if phosphatase activity is obvious in the protein sample, by filling in the 5′ overhangs of suitable restriction fragments with biotinylated and $\alpha[^{32}P]$-labelled dNTPs and Klenow polymerase (2, 3). The labelled, biotinylated fragments are immobilised on streptavidin-coated paramagnetic beads (Dynabeads M280-Streptavidin, Dynal, Oslo) according to the manufacturer's specifications (time required: 15–30 minutes). Concentrations of beads in the magnetic field are performed with a magnetic particle concentrator (MPC, Dynal, Oslo) which takes about 30–60 seconds. Unincorporated radioactive primer or free nucleotides are quantitatively removed during subsequent washes of the DNA beads. As a result the amount of radioactivity that is handled in all following steps is drastically reduced, minimizing the exposure of the researcher to radiation. Bead-DNA is stored at 4°C in 2 M NaCl/10 mM Tris–Cl, pH 7.5/1 mM EDTA. For each footprinting reaction 10,000–15,000 cpm of DNA beads are added to a 1.5 ml reaction tube, concentrated and equilibrated by one wash with protein binding buffer. Finally beads are concentrated, the supernatant is removed and the complete binding reaction containing the protein sample and competitor DNA is added. The beads are resuspended and incubated for the desired period of time at the appropriate temperature using either an Eppendorf thermomixer or an ordinary waterbath. If binding reactions take longer than 5 minutes occasional agitation of the tube by gentle tapping is required to keep the beads in suspension.

The amount of DNase I added to produce on average about 1 nick per DNA molecule needs to be determined in analogy to reactions in solution. DNase I digestion is terminated by addition of an equal volume of 4 M NaCl/100 mM EDTA. The nicked fragment on the beads is washed once with 100 µl of 2 M NaCl, 20 mM EDTA and 100 µl 10 mM Tris–HCl pH 8.0, 1 mM EDTA. Finally the supernatant is removed completely and the beads are mixed with 4 µl loading buffer (96% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue, 10 mM EDTA; mixed 3:1 with freshly diluted 150 mM NaOH). The sample is denatured for 5 min at 76°C and loaded on a prerun sequencing gel. Beads do not interfere with the electrophoresis and so it is unnecessary to remove them before loading.

Figure 1 shows an exemplary result of a footprinting reaction assaying the binding of *Drosophila* Heat Shock Factor (HSF, Ref. 4) in a crude extract from HSF expressing *E.coli* to heat shock elements in the hsp70 promoter (Lanes 4,5). To emphasize the usefulness of our approach for crude extracts we mixed into the reactions shown in lanes 2 and 3 crude nuclear extract from *Drosophila* nuclei (5).

Our protocol should be applicable to other kinds of footprinting reactions, provided that the reagent used cleaves only one strand of the DNA. The extraordinary stability of the streptavidin–biotin interaction assures the immobilisation of the DNA under a wide range of reaction conditions.

The immobilisation expands the options for experimental design as reaction conditions can be changed conveniently after the formation of stable protein–DNA complexes. Protein binding can be promoted under optimal conditions (such as high protein concentration or presence of reagents that promote molecular crowding), and challenged by removal of the binding reaction containing excess protein. Hence, off-rates can be determined under a variety of experimental conditions. The solid phase approach may also facilitate the analysis of factors in crude protein mixtures since unspecific protein–DNA complexes can be disrupted by washing the immobilised probe prior to DNase I addition.

In conclusion, solid phase footprinting not only accelerates and facilitates footprinting analyses, but also increases the flexibility and versatility in experimental design.

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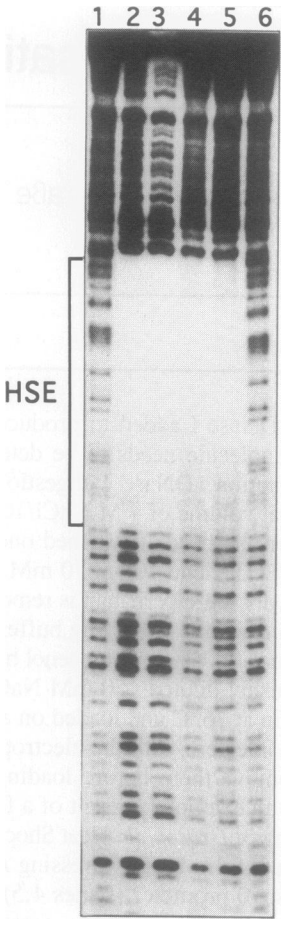

Figure 1. Solid phase DNase I footprint of *Drosophila* HSF on the hsp70 gene promoter. A fragment containing the hsp70 gene promoter was generated by PCR (1 ng template, 25 cycles, Ref. 4) with primer A (positions −185 to −156 upstream of the hsp70 cap site, biotinylated at its 5′ end during the synthesis on a ABI 394 DNA synthesizer via incorporation of a biotin-2-o-propyl phosphoramidite) and radioactively labelled primer B (positions +19 to +37). PCR products were immobilized without prior purification on 3 mg Dynabeads (Dynal, Oslo) according to the manufacturer's specifications. Bead-DNA was washed twice with PBS, 0.01% BSA, 0.05% Nonidet P40 and resuspended in 200 μl of binding buffer (BB: 12.5 mM Hepes pH 7.6, 0.05 mM EDTA, 50 mM KCl, 5% glycerol, 5 mM β-glycerophosphate, 0.5 mM DTT, 0.1 mM PMSF). For each reaction 5 μl DNA beads (15,000 cpm) were concentrated and resuspended in 25 μl of BB. To this 25 μl of a premix containing 3 μg pUC DNA and 2 μg of crude extract protein from *E.coli* expressing HSF (Ref. 4) in BB was added. Reactions shown in lanes 2, 3 also contained 7.5 μg of crude NUN extract (5) from *Drosophila* embryo nuclei, dialysed against BB. After incubation for 10 min at 26°C, 0.04 (lanes 2,4) or 0.06 (lanes 3,5) units of DNase I in 25 μl 5 mM $CaCl_2$/10 mM $MgCl_2$ were added and after 1 min at room temperature the reaction was quenched and processed as described in the text. Control digestions of bead DNA without protein (lanes 1,6) were treated with 0.005 units of DNase I.

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