Optimizing and troubleshooting DNase I footprinting reactions

ARTICLES

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DNase I footprinting is a powerful technique for the characterization of protein-DNA interactions, but several reaction parameters must be optimized before definitive results can be obtained. This report describes the parameters that must be optimized for DNase I footprinting and provides a guide to troubleshooting these reactions.

Introduction

DNase I footprinting has become a standard technique for the identification of protein binding sites on specific DNA sequences (1-6). To perform a DNA footprinting reaction, a DNA probe labeled at only one end is prepared and incubated with purified DNA binding proteins, or with crude or fractionated cellular extracts. After this binding step, DNase I is added at a concentration sufficient to introduce, on average, a single random nick per molecule. The reaction then is terminated by addition of EDTA and SDS. The reaction products are visualized by denaturing gel electrophoresis and autoradiography, and the protein binding region is identified by comparing the ladder of DNase I digestion products with that generated in the absence of protein. Bands missing from the ladder result from bound protein protecting the DNA molecule from DNase I nicking. Darker bands or hypersensitive sites appear when the bound protein alters the DNA conformation to allow preferential DNase I nicking.

The choice of a fragment to be used in footprinting experiments can be made in two ways. If the binding sites for *novel* DNA binding proteins are sought (or if your promoter sequence is unknown), then gel shift assays may be used initially to screen for the new activity and localize the binding site to a particular fragment (7-13). Footprinting assays then are used to further characterize the binding site.

Another option is to determine if the promoter carries a consensus sequence to common transcription factors, such as SP1, AP1, AP2, NF-κB, OCT 1, CTF/NF1 and SRF. (For recent reviews, see references 14 and 15.) If these sites are present, then DNase I footprinting can be used directly to provide the most rapid and definitive binding results.

Optimization of footprinting parameters

Virtually all published photographs of DNase I footprints result from numerous preliminary experiments in which the investigators optimized wide ranges of DNA binding conditions, DNase I concentrations and levels of specific and nonspecific competitor DNA. The purpose of this paper is to provide a general guide for optimizing and troubleshooting DNase I footprinting reactions using Promega's purified transcription factors, other purified DNA binding proteins and HeLa nuclear extracts.

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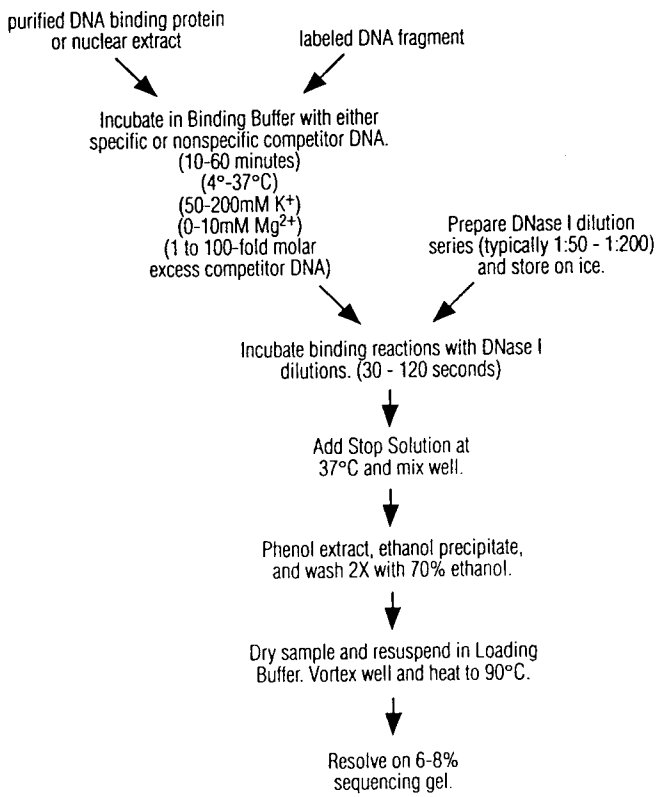

Figure 1. Standard DNA footprinting procedure. Parameters to be optimized are shown in parentheses.

The single most important requirement for optimizing footprinting is an ample supply of clean, high specific activity probe.

DNase I footprinting requires simultaneous optimization of a number of parameters (Figure 1). The single most important requirement for optimizing footprinting reactions is an ample supply of singly end-labeled, double-stranded DNA probe of high specific activity and impeccable cleanliness. Sufficient probe must be generated to perform many experiments with a single batch. In addition, the probe produced should be of similar specific activity each time it is made.

Strategy for selection of DNA fragments for footprinting

Singly end-labeled probes from the promoter of interest can be generated using a number of labeling methods. The most common strategy is to cut a plasmid containing the promoter with an appropriate restriction enzyme, label the ends, cut with a second restriction enzyme, and then gel-purify the resulting singly end-labeled fragment. The optimal DNA fragment size is between 100 and 700bp, with the protein binding site positioned approximately 50-150bp from the labeled end and the second restriction site approximately 150-600bp beyond the binding site (5).

Once a binding site has been identified, the researcher must look for unique restriction sites which bracket it. A restriction enzyme generating 5′ overhanging ends is preferable for the first cut site, since this provides the most labeling options. Computer searches can be helpful in identifying these restriction sites, especially since many new restriction enzymes have become commercially available within the last two years. When no appropriate restriction sites are available, it may be necessary to subclone a DNA fragment into a plasmid polylinker. This step ultimately may save time by making probe production more convenient.

Considerations for labeling DNA probes

Because DNA binding proteins make specific contacts with a three-dimensional helix, the bases protected by DNase footprints may vary when using different labeled strands. The footprints of DNA binding proteins should be examined using both DNA strands, as one strand may show a more sharply defined footprint. Alternative strategies for producing singly labeled double-stranded DNA probes are outlined in Figure 2 and are discussed below in more detail.

End-fill labeling

DNA fragments with 5′ overhanging ends can be end-filled using the 5′ to 3′ polymerase activity of the Klenow fragment of DNA polymerase I (13,16,17). End-filling with Klenow fragment initially labels both DNA strands. One labeled end is released following cleavage with a second restriction enzyme and gel purification of the appropriate DNA fragment (see below and Figure 2, Standard Procedure). Because more than one radioactive nucleotide can be incorporated at each end, this method can generate the highest specific activity probes. High specific activity [α-^{32}P]dNTPs (≥3,000Ci/mM at 10mCi/ml) should be used for end-filling. Higher specific activity probes may be obtained when the radioactive dNTP is not the 3′-most nucleotide added by Klenow fragment (11).

Because Klenow fragment is not stable at 37°C, the initial 30-minute labeling reaction may be enhanced by the addition of a second aliquot of Klenow fragment followed by a second 30-minute incubation. This labeling step should be followed by a 15-minute chase containing Klenow fragment and *all four* cold dNTPs to counteract the 3′ to 5′ exonuclease activity of Klenow. This chase step is important to ensure a completely blunt-ended fragment because any heterogeneity in fragment length will produce an uninterpretable DNase I footprint. Addition of a dideoxynucleotide (either radioactive or non-radioactive) at the final position can be advantageous in the 15-minute chase, because the 3′ to 5′ exonuclease activity of Klenow fragment does not use this analog as a substrate (18).

Kinase labeling

The radioactive phosphate group from [γ-^{32}P]ATP can be transferred to the 5′ end of DNA fragments by bacteriophage T4 Polynucleotide Kinase (PNK) if the 5′ end has been previously dephosphorylated with Calf Intestinal Alkaline Phosphatase (CIAP) (see Figure 2, Alternative Procedure). This reaction is most efficient with protruding 5′ termini, but can be used for dephosphorylating recessed 5′ termini or blunt ends (13,16,19).

Care must be taken to completely inactivate the CIAP by phenol extraction or with Promega's Magic™ DNA Clean-Up System. DNA to be kinase-treated should not be dissolved in or precipitated from buffers containing ammonium ions, because ammonium is a strong inhibitor of T4 PNK. High specific activity [γ-^{32}P]ATP (≥3,000Ci/mM at 10mCi/ml) also should be used. The concentration of ATP in the reaction should be at least 1mM. As for end-fill labeled fragments, singly labeled probe of either strand is generated by cleavage with a second restriction enzyme and gel purification of the desired fragment.

One advantage of this kinase end-labeling procedure is that it allows the preparation of large amounts of CIAP-treated, unlabeled probe for future use. To prepare probe in this way, cut the probe DNA with the first restriction enzyme (generating the end to be labeled), dephosphorylate the ends using CIAP and then purify the DNA by phenol extraction and ethanol precipitation or using the Magic DNA Clean-Up System. Perform the second restriction enzyme digestion, following the manufacturer's instructions, and gel-purify the appropriate unlabeled fragment as described below. This DNA fragment can be kinase-labeled and used for footprinting after phenol extraction and ethanol precipitation but without further gel purification, because only one end is dephosphorylated.

T4 PNK is capable of labeling the phosphorylated 5′ end via an exchange reaction, but the efficiency of kinase labeling the dephosphorylated end is at least 100-fold greater. In this method, DNA fragments may be purified on a large scale (50µg plasmid or more) for high-efficiency recovery, and aliquots then may be labeled as needed to a consistent specific radioactivity. This method avoids gel purification of radioactive DNA fragments and subsequent handling of large volumes of radioactive gel buffer.

Terminal transferase labeling

Terminal deoxynucleotide transferase can be used to label 3′ overhang restriction sites (e.g., *Pst* I, *Sst* I and *Sph* I) by transfer of [α-^{32}P]dATP to the end of the DNA molecule (6). The labeled DNA must be purified as described above for kinase end-labeled probes. A singly end-labeled DNA probe then is generated by a second restriction enzyme digestion followed by gel purification.

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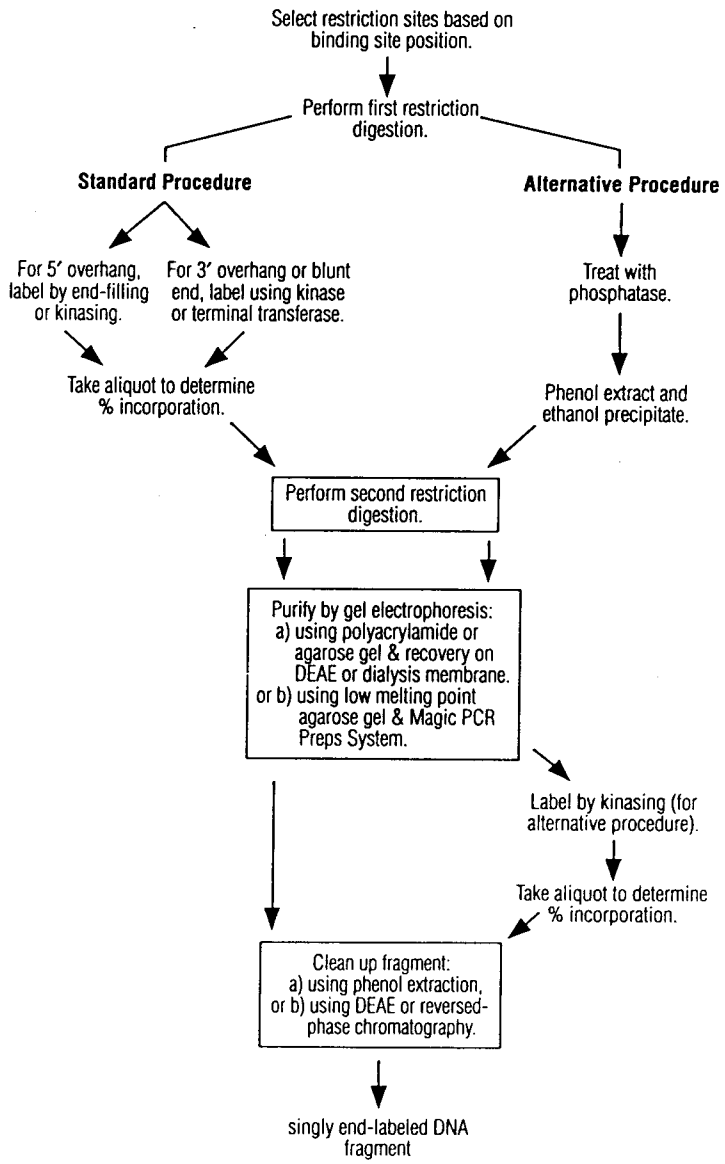

Figure 2. Options for DNA fragment labeling and purification.

Handling of labeled probe

When using any of these labeling protocols, calculate the percent of label incorporated and the specific activity of the probe using the DE81 filter binding assay or TCA precipitation (13,16). Probe should be labeled to a high specific activity (about 10,000-20,000cpm per 1-10pmol of binding site DNA) and a labeling reaction should produce enough purified DNA to perform over 100 footprinting reactions. Labeled probe may be stored for up to 2 weeks before excessive radioactive nicking occurs.

Labeled probe alone should be run as a control on gels. The probe can be used to generate a number of other size markers by cutting with restriction enzymes. For the most precise identification of the footprint location, the labeled probe can be sequenced using the Maxam-Gilbert method (12) and run next to the footprinting reactions on a gel.

Gel purification and clean-up of DNA fragments for footprinting

Gel purification is required for fragments labeled by any of the methods described. We recommend labeling a minimum of 10µg of DNA to ensure efficient recoveries from gels and during later clean-up steps. Fragments may be purified using either polyacrylamide or agarose gels.

Polyacrylamide gel purification

Both labeled and unlabeled DNA fragments can be separated on 1.5mm thick, 5% nondenaturing polyacrylamide gels. For unlabeled DNA fragments, stain the gel in 250ml of 1X TBE buffer (89mM Tris-HCl, pH 7.8, 89mM borate, 1mM EDTA) containing 1µg/ml ethidium bromide for up to 1 hour with shaking, then rinse the gel in water. Visualize the bands by UV illumination and excise the desired bands. Radioactive fragments can be visualized by a brief (1-3 minute) exposure to X-ray film. Fluorescent paint or radioactive ink markers may be used to orient the film precisely with respect to the gel. The appropriate labeled fragment then can be excised from the gel with a razor blade. DNA can be electroeluted from the acrylamide slice into dialysis tubing (20-22).

Agarose gel purification

Alternatively, DNA fragments may be purified from low melting point agarose gels using Promega's Magic™ PCR Preps DNA Purification System (23) followed by phenol extraction and ethanol precipitation. Another option is to separate the DNA fragments on a standard agarose gel or nondenaturing polyacrylamide gel and recover the desired DNA fragment by electrophoresis of the band onto a DEAE membrane inserted into a slit in the gel. The DNA can be eluted from the DEAE membrane using a high salt buffer and recovered by ethanol precipitation (5).

However the DNA is gel-purified, the DNA probe used in footprinting reactions must be free from enzyme and gel contaminants and may require further clean-up by ion exchange (DEAE-cellulose) (13) or reversed phase (Elutip™-D) microchromatography, or by using the Magic DNA Clean-Up System.

Optimizing DNA binding conditions

Figure 1 illustrates the steps involved in optimization of DNA binding conditions and DNase I footprinting. More detailed footprinting protocols are available (5, 6, 20-22). The footprinting reaction volume can range from 20-200µl, depending on the concentration of the protein added. Many parameters can affect DNA-protein interactions, including KCl, NaCl, $MgCl_2$ and $CaCl_2$ concentrations pH; presence or absence of nonionic detergents; protein and DNA concentrations; type and concentration of DNA competitor; and the length and temperature of the incubation period. While the optimal values for these parameters are best determined empirically, we offer some suggestions for optimization ranges in Figure 1 and Chart 1.

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Chart 1. Critical Parameters to Optimize in DNase I Footprinting

Parameter	Variable Optimized	Comments
DNA probe	High specific activity	10,000-20,000cpm per 1-10pmol DNA per assay.
	Purity	Agarose or acrylamide gel electrophoresis, followed by elution and clean-up (phenol extraction and reversed phase or ion-exchange chromatography, or Magic PCR Preps DNA Purification System).
	Size of DNA probe and location of DNA binding site	100-700bp, with the protein binding site no closer than 25bp from the labeled end. Protein binding sites as far as 400bp from the labeled end can be used, but require longer electrophoresis and bands are not as sharp.
	Labeling	DNA probe must be labeled at only one site. End-fill, kinase or terminal transferase labeling and second enzyme digestion must be complete.
Protein	Purity	Only *relative* binding affinity can be measured in crude extracts. High purity is required for quantitation of binding kinetics.
	Concentration	Protein titrations should fully saturate DNA probe. In crude HeLa extracts, suggested titrations are 10, 20, 40, 80 and 160µg protein. Promega's purified transcription factors are calibrated in footprint units (fpu), the amount of protein required to give full footprinting activity.
DNA/protein binding conditions	Temperature	4°-37°C.
	Time	10-60 minutes.
	pH	pH 5-9, with most reactions at pH 7-8.
	K^+	50-200mM. For most DNA binding proteins, specific binding affinity decreases with increasing K^+ concentration while specificity of binding increases.
	Mg^{2+}	0-10mM. DNase I requires some divalent metal ions, either Mg^{2+} or Ca^{2+}.
	Ca^{2+}	0-1mM. DNase I requires some divalent metal ions, either Mg^{2+} or Ca^{2+}.
	PEG	0-5%. Use only high quality PEG (8,000 MW). PEG may change the rate at which DNase I cuts.
	Nonionic detergents	0-0.1% Triton X-100.
DNase I	Concentration	DNase I concentration such that 1 nick occurs per molecule of DNA probe.
	Exposure time	30-120 seconds.
Nonspecific competitor DNA	Type	Alternating copolymers such as dIdC or dAdT present a large number of nonspecific sites and fewer sequence-specific sites than a heterogeneous competitor such as herring sperm or *E. coli* DNA.
	Order of addition	Preincubate or add at same time as probe DNA.
	Concentration	1- to 100-fold molar excess.
Specific competitor DNA	Concentration	1- to 100-fold molar excess. Either the natural DNA binding site or a mutant site can be used to indicate specificity of binding.

When purifying a novel DNA binding protein from extracts, conditions developed for gel shift assays may be used as a first step in optimizing binding conditions for footprinting. Binding conditions for common transcription factors may be found in the literature. Purified transcription factors (SP1, AP1, AP2) in Promega's footprinting systems are supplied with optimized binding buffers and clearly defined footprinting conditions. Promega's recently introduced Core Footprinting System contains a CIAP-treated DNA fragment of the SV40 Early promoter/enhancer which can be used to footprint all three purified factors. This control

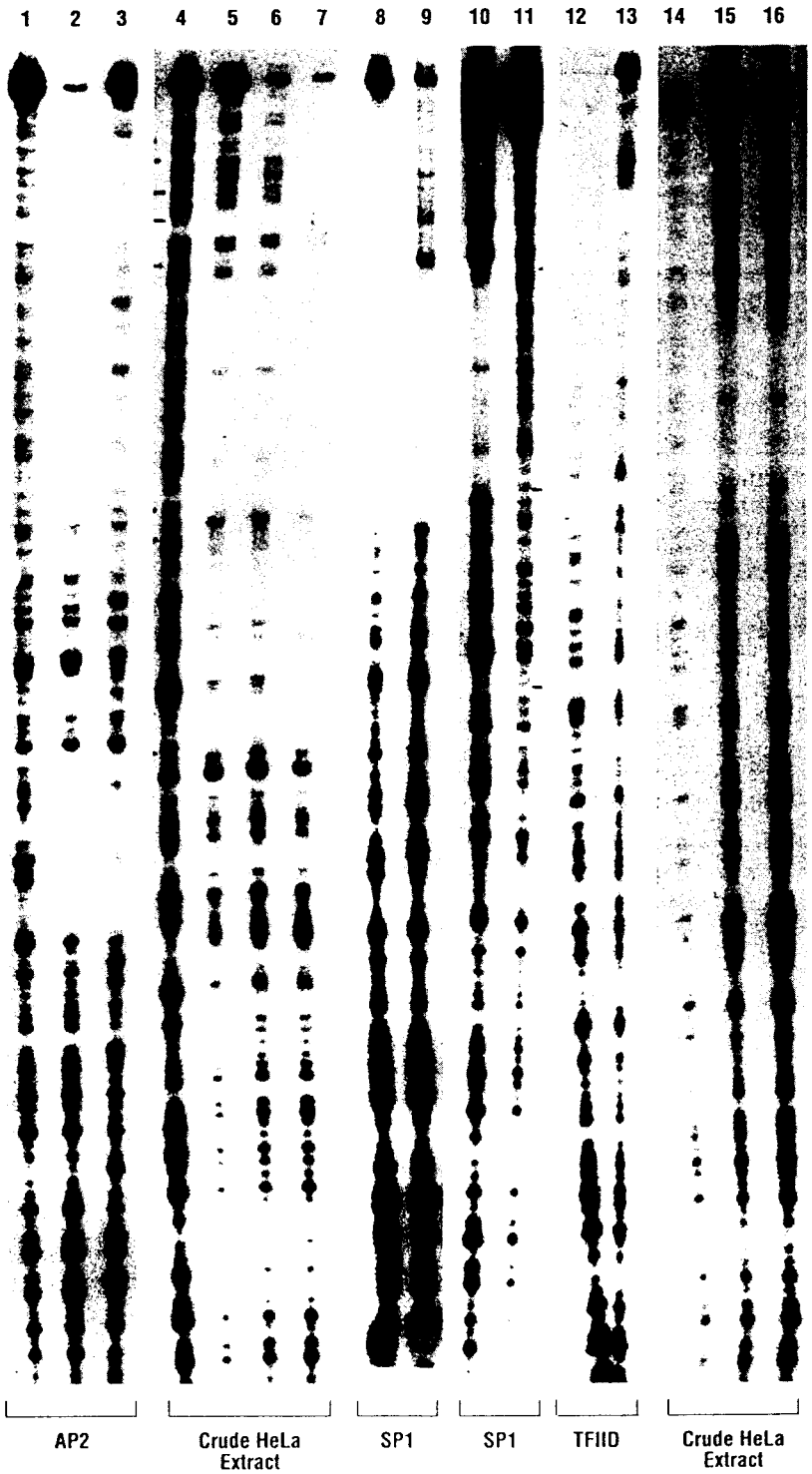

Figure 3. Examples of common footprinting results. The factor being footprinted is indicated below each lane. In all cases, the probe was the SV40 Early promoter/enhancer 300bp *Kpn* I-*Hin*d III fragment singly end-labeled by T4 PNK. Refer to the Figure Key for a description of conditions used in each lane. One fpu = one footprint unit (the amount of protein required to give full DNase I protection on a standard template).

Figure Key

Representative AP2 footprint
1 No protein control, 0.15u RQ1 DNase I.
2 Purified AP2 protein (1.5fpu), 0.15u RQ1 DNase I.
3 Partially purified AP2 protein in crude bacterial extract (2µl), 0.15u RQ1 DNase I.

DNase I titration
4 No protein control, 0.15u RQ1 DNase.
5 Crude HeLa nuclear extract (125µg protein), 1u RQ1 DNase I.
6 Crude HeLa nuclear extract (125µg protein), 5u RQ1 DNase I.
7 Crude HeLa nuclear extract (125µg protein), 10u RQ1 DNase I.

Overdigestion of no protein control
8 No protein control, 0.15u RQ1 DNase I.
9 SP1 (1fpu), 0.15u RQ1 DNase I.

Underdigestion with DNase I in lane 11
10 SP1 (0.67fpu), 0.1u RQ1 DNase I.
11 No protein control, 0.1u RQ1 DNase I.

Residual salt in both sample lanes
12 No protein control, 0.15u RQ1 DNase I.
13 TFIID (1fpu), 0.15u RQ1 DNase I.

Incomplete sample resuspension in lane 14
14 No protein control, 0.15u RQ1 DNase I.
15 Crude HeLa nuclear extract (125µg protein), 1u RQ1 DNase I.
16 Flowthrough from AP1 binding site oligo-affinity chromatography column without DNA competitor (25µl), 1u RQ1 DNase I.

probe allows binding conditions and other steps to be optimized before working with the DNA fragment of interest. Figure 3, lanes 1-3 illustrates an AP2 footprint using this probe.

Footprinting reactions also may be performed using HeLa nuclear extract rather than purified factors. In Figure 3, lanes 4-7, we illustrate the footprint of SV40 Early promoter probe in HeLaScribe™ *in vitro* Transcription Grade Nuclear Extract. We have observed a positive correlation between the transcription competence of nuclear extracts and their ability to footprint a wide variety of previously identified DNA-binding proteins (K. Lewis, unpublished results).

When using crude HeLa nuclear extracts, the amount of protein per reaction must be optimized for footprinting [10, 20, 40, 80 and 160µg are suggested ranges (24)], and it may be necessary to include competitor DNA to obtain footprints for some DNA binding proteins. The amount of RQ1 DNase I used with crude nuclear extract was higher than the amount used to footprint purified protein (1.0 unit vs. 0.15 units) (Figure 3, lanes 4-7). The concentrations of some DNA binding proteins may be too low in crude extracts to obtain footprints, so these extracts will require further purification before use.

Ensuring adequate DNase I activity

DNase I activity is influenced by many of the parameters being optimized in the DNA-protein binding reactions, as well as by the particular purification used for the binding protein. For example, Promega's AP1 (c-jun) transcription factor is purified from cDNA clones expressed in bacteria and is renatured in guanidine-HCl. As a result of these purification conditions, higher concentrations of DNase I are needed to produce a good footprint.

Because DNase I activity is reduced by repeated freezing and thawing, DNase I is best stored at –20°C in small aliquots which are used for only 1 or 2 experiments. Diluted DNase I should be discarded immediately after use. DNase I is supplied by vendors as a lyophilized powder or as a stable enzyme solution in glycerol. Lyophilized enzyme must be resuspended using gentle inversion, because DNase I is unusually sensitive to denaturation by shaking. Enzyme activity should be determined after resuspension.

Two unit definitions of DNase I are used by vendors: 1) "Kunitz" units, in which 1 unit increases the absorbance of high molecular weight DNA solution by 0.001 A_{260} units/min/ml at 25°C in pH 5.0 reaction buffer; and 2) "molecular biology units," in which 1 unit degrades 1µg of plasmid DNA to completion in 10 minutes at 37°C in pH 7.9 buffer. Promega's RQ1 RNase-Free DNase is provided in solution in molecular biology units, because these reaction conditions are more typical in molecular biology applications. One "molecular biology" unit equals approximately 0.3 "Kunitz" units.

Optimizing DNase I digestion

Once the binding reaction conditions are optimized, the amount of DNase I required to obtain a good footprint also must be determined empirically. DNase I should be added at a concentration which allows approximately one random nick per molecule. Under these conditions, approximately 50% of the fragments will contain nicks and 50% will remain intact. The ratio of unnicked to nicked DNA in an experiment can be determined by comparing the largest band seen after electrophoresis with a control band of intact probe. The relative amounts of DNA in these bands can be estimated using autoradiography (with short exposures) and densitometry (5).

Diagnosing over/underdigestion

Figure 3 (lanes 4-7) shows a titration of DNase I concentrations. Overdigestion by DNase I results in a very faint top band of unnicked probe and an uneven ladder of bands, with more intense bands appearing at the bottom of the gel (lane 7). The footprint is gradually lost as the DNase I concentration is increased. Underdigestion by DNase I results in an uneven ladder with less intense bands at the bottom of the gel and a more intense band of intact probe at the top (Figure 3, lane 11).

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Comparison to probe-only control

To identify a footprint, the DNase I digestion pattern of the probe alone is compared with that of probe which has been allowed to interact with DNA binding protein for a defined period of time. The bands in the DNA ladder above and below the footprint should be of the same intensity as those in the ladder generated in the absence of protein. At a given DNase I concentration, the digestion pattern of the probe-only sample often differs from that of probe incubated with protein due to differences between the protein extract and the protein storage buffer. Figure 3 (lanes 8-9) illustrates a commonly observed phenomenon, where an apparently good SP1 footprint is obtained under conditions in which the probe-only control is overdigested (lane 8). If this is observed, the probe-only reactions require a separate titration of DNase I.

Digestion time

DNase I titrations must be carefully performed, with all reactions carried out in the same order for the same length of time. Variations in the exposure of sample to DNase I and other handling conditions can result in differences in the DNase I ladder. Failure to stop the DNase I reaction promptly can lead to overdigestion of the probe. We recommend that, during the DNase I addition step, no more than 1 or 2 samples be handled at a time, and that pipet tips and pipettors be ready for immediate addition of Stop Solution. Working with a large number of samples can lead to variation in DNase I digestion times, resulting in over- or underdigestion of samples. Stop Solution should be prewarmed to 37°C and the samples vortexed well after addition to ensure uniform termination of the DNase I reactions.

Resuspension and gel loading of footprinting samples

After terminating reactions with Stop Solution, samples may be extracted once with phenol/chloroform followed by precipitation with ethanol, or simply ethanol precipitated directly. After DNA precipitation, care should be taken to wash each sample *2 times* with cold 70% ethanol to remove residual EDTA and salts in the Stop Solution. If salts are not removed at this stage, bands in the ladder can become compressed (Figure 3, lanes 12 and 13).

Care also must be taken to completely dissolve the dried sample in the small Loading Buffer volume. We recommend precipitating the DNA in commercially silanized microcentrifuge tubes, vortexing the sample well and centrifuging the microcentrifuge tube to bring the sample to the bottom of the tube prior to heating the sample. If a sample is not properly dissolved, the entire lane exhibits a weaker signal upon autoradiography of the gel (Figure 3, lane 14). Chart 2 (page 10) is a troubleshooting guide which summarizes these and other common problems experienced in DNase I footprinting.

Summary

DNase I footprinting and gel shift techniques are the primary assays used during transcription factor purification and characterization of the *cis*-acting elements to which they bind. The power of DNase I footprinting is its use for identification of protein binding sites. Once sequence elements are identified, interactions between different factors can be characterized further through DNA competition and protein add-back experiments. Finally, calculations of intrinsic binding and cooperativity constants for systems of repeated, interacting sites of purified DNA-binding proteins can be made using quantitative DNase I footprinting (3-5). ■

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Several products for studying eukaryotic transcription regulation are available from Promega, including gel shift and footprinting systems. Ordering information for products mentioned in this article is provided below and on pages 22 and 24 of this issue of Promega Notes.

Ordering Information:

Product	Size	Cat.#
Footprinting System, AP1 (c-jun, human)	50 Reactions	E3040
Footprinting System, AP2	50 Reactions	E3180
Footprinting System, SP1	50 Reactions	E3320
Core Footprinting System	50 Reactions	E3730
HeLaScribe™ Nuclear Extract, *in vitro* Transcription Grade	40 Reactions	E3091
	160 Reactions	E3092
AP1 (c-jun, human)	50fpu	E3061
AP2 (human)	50fpu	E3071
TFIID (human), TATA Binding Protein	50fpu	E3081
SP1 (human)	50fpu	E3391
Magic™ PCR Preps DNA Purification System	50 Purifications	A7170
Magic™ DNA Clean-Up System	100 Purifications	A7280
DNA 5′-End Labeling System	10 Reactions	U2010
Alkaline Phosphatase, Calf Intestinal	1,000u	M1821
	5,000u (5 x 1,000u)	M1822
Klenow Fragment	150u	M2201
	300u (1 x 300u)	M2205
	750u (5 x 150u)	M2202
RQ1 RNase-Free DNase	1,000u	M6101
	5,000u (5 x 1,000u)	M6102
T4 Polynucleotide Kinase	100u	M4101
	500u (5 x 100u)	M4102
Terminal Transferase	300u	M1871
	1,500u (5 x 300u)	M1872

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Chart 2. Troubleshooting DNase I Footprinting.

Symptoms	Possible Causes	Comments
DNase I ladder uneven, with more intense bands at top of gel and less intense bands at bottom of gel	Underdigestion by DNase I	Optimize reaction using a range of DNase I concentrations.
	DNase I not mixed adequately	Mix DNase I thoroughly but gently with sample by pipetting up and down, stirring or vortexing at lowest setting for 3 seconds.
	Conditions used in binding assay do not favor DNase I activity	AP1 storage buffer contains guanidine-HCl which inhibits DNase I. The no protein control should include the AP1 storage buffer provided.
DNase I ladder uneven, with more intense bands at bottom of gel and less intense bands at top of gel	Overdigestion by DNase I	Optimize reaction using a range of DNase I concentrations.
	Specific activity of DNA probe too low	DNA probe concentration should be low relative to protein concentration, and labeling efficiency should be 40-50%.
	DNase I not inhibited by Stop Solution	Stop Solution should be heated and mixed well. It should be kept at 37°C until immediately prior to use, and samples should be vortexed after Stop Solution addition.
Faint signal in some lanes even when same probe worked well in other lanes under same conditions	Sample not completely dissolved in loading buffer	Vortex sample well and spin down prior to heating and loading on gel. Use silanized microcentrifuge tubes.
	Pellet lost during ethanol precipitation	Place tubes in microcentrifuge with cap hinge at top and note position of pellet. Draw ethanol off gently so as not to disturb pellet.
Some lanes over-digest while others underdigest under same DNase I concentrations	Variation in handling reactions	Time the exposure of sample to DNase I, and handle all samples in identical fashion while mixing DNase I. Treat samples in groups of 1 or 2 to facilitate handling.
No protein control overdigests while protein-containing reactions work well	DNase I digestion conditions altered by addition of protein	Optimize no protein control with different, usually lower, DNase I dilutions than the sample containing DNA binding proteins.
Doublet bands in undigested probe and digestion pattern	Partial end-filling by Klenow fragment led to 2 DNA fragments with a 1-2bp size difference	Chase with cold nucleotides and additional Klenow fragment to completely end-fill probe.
	Probe has been stored too long and is beginning to degrade	Only store labeled probe for 1-2 weeks.

Symptoms	Possible Causes	Comments
No DNase I ladder on gel, but probe signal is strong	Inactive or underactive DNase I	Perform activity assay for DNase I. Avoid freezing and thawing DNase I. Store in small aliquots.
	Insufficient DNase I used	Optimize using a range of DNase I concentrations.
	Probe contains contaminants from agarose or acrylamide gel electrophoresis	Clean up probe using reversed phase or ion exchange (DEAE cellulose) chromatography, or Magic PCR Preps DNA Purification System.
	DNase I inactivated during handling	Vortex DNase I for 3 seconds only at lowest setting or mix manually by stirring or pipetting up and down.
	Not enough protein added	Protein concentration should fully saturate DNA probe. Titrate protein amounts.
	Too much competitor DNA added with purified protein	Titrate amount of competitor DNA in reactions.
	Too little competitor DNA added in crude extracts	Titrate amount of competitor DNA in reactions.
	High concentrations of $(NH_4)SO_4$ or NaCl in protein fraction inhibit DNase I	Dialyze fractions against storage buffer at 4°C.
DNase I ladder narrows as samples run to bottom of gel	Excess salt or EDTA in sample	Wash samples 2 times in 70% ethanol before drying and resuspending sample. Reduce amount of EDTA in Stop Solution.
DNA bands fuzzy in gel	Intensifying screen and film not in direct contact with gel	Repeat film exposure with tighter cassette or add extra layers of paper or exposed film for tighter contact.
	Impurities in acrylamide or gel buffer	Reagents should be ultrapure.
	Inadequate buffering during gel run	Check TBE concentration. Don't run gel at excessive voltage to avoid buffer breakdown during gel run.
	Excess nucleic acid in sample	Reduce or eliminate carrier tRNA and competitor DNA.
Signal weak	DNA probe will not phosphorylate well with T4 PNK following CIAP treatment	Ammonium ions are strong inhibitors of T4 PNK. DNA should not be dissolved in or precipitated from buffers containing ammonium salts prior to kinasing.
	High TCA-precipitable label incorporation but low amount of labeled probe at gel purification step	Sample may be contaminated with low MW RNA or bacterial genomic DNA, which can compete for kinase labeling. Prepare DNA for kinasing by CsCl gradient centrifugation.