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A recommended workflow for DNase I footprinting using a capillary electrophoresis genetic analyzer

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Abstract

Fragment analysis was developed to determine the sizes of DNA fragments relative to size standards of known lengths using a capillary electrophoresis genetic analyzer. This approach has since been adapted for use in DNA footprinting. However, DNA footprinting requires accurate determination of both fragment length and intensity, imposing specific demands on the experimental design. We delineate here essential considerations involved in optimizing the fragment analysis workflow for use in DNase I footprinting to ensure that changes in DNase I cleavage patterns may be reliably identified.

Key words: Capillary electrophoresis; DNase I footprinting; fragment analysis; single hit

Fragment analysis, in which fluorescently labeled DNA is analyzed on a capillary electrophoresis genetic analyzer, was developed for applications such as single nucleotide polymorphism and microsatellite analyses. In these cases, DNA fragment length is primarily probed. However, when applying this approach to DNA footprinting, where peak intensity is of the utmost importance, it is critical to optimize procedures to avoid artifacts that may confound data interpretation. The following outlines an optimized workflow.

DNase I footprinting is widely used to identify binding sites for DNA-binding proteins. In addition to protection from cleavage, protein binding may induce hyper-sensitive sites at which DNase I cleavage is enhanced due to protein-induced DNA distortions that facilitate enzyme access. In its original inception, the technique involves labeling of double-stranded DNA at one end of one strand with ^{32}P for visualization purposes [1]. Mild DNase I digestion results in fragments that may be separated on a sequencing gel, and comparison of samples with and without protein of interest reveals protected and hypersensitive sites.

The advent of automated DNA sequencers that can analyze patterns of fluorescent DNA fragments (fragment analysis) has led to the development of an alternative approach to DNA footprinting [2, 3]. For this approach, DNA is labeled with a fluorophore such as 6-carboxyfluorescein (6-FAM), following which cleavage reactions are performed. Separation of fragments on a DNA analyzer allows comparison of samples with and without protein of interest. This approach has some advantages in that it circumvents handling of large slab gels and radioisotopes, and analysis times are shorter. Since 6-FAM-labeled DNA is typically not limiting, the lower sensitivity compared to ^{32}P is usually not an issue. However, optimizing procedures to ensure consistent signal intensity is critical, as interaction with protein may result

in subtle, yet functionally important differences in cleavage efficiency that may otherwise be missed.

The first step is to PCR amplify a DNA fragment of interest using one 5'-fluorescently labeled primer and one unlabeled primer such that the resulting DNA duplex is labeled at one end of one strand. Internal labeling is not recommended due to interference with protein binding and DNase I cleavage. Since labeled DNA fragments will be detected along with a labeled size standard, the two labels must differ, yet be detectable on an automated capillary sequence analyzer without spectral overlap. We used 6-FAM labeled primer for DNA amplification and LIZ®-labeled standards (LIZ-500; Life Technologies). The labeled DNA duplex must include at least 50 bp flanking the expected binding site. The reason is the loss of short fragments that occurs during analysis; the extent of this loss is dependent on the length of the capillaries and may be as much as 40 nt. It is essential to design the DNA such that not only protected regions, but also flanking sequence (at least 10 bp) is not lost. In the example shown here (Figure 1), the promoter region (270 bp) of a gene encoding the *Streptomyces coelicolor* transcription factor XdhR [4] was amplified and analyzed using an ABI 3130 automated capillary sequence analyzer and GeneMapper® 4.1 software

DNA fragments are separated by capillary electrophoresis. Electrokinetic injection of samples forces negatively charged molecules into the capillaries, where they are separated based on total charge. As a result, other ionic compounds (from buffers, distilled water) are also injected, resulting in artifactual signals. Negatively charged ions will also compete with DNA for entry into the capillaries (the highly mobile ions will be preferentially injected), significantly reducing signal intensity. We routinely purify digested DNA samples by phenol:chloroform extraction and ethanol precipitation. Precipitated DNA should be dried and dissolved in highly

deionized (Hi-Di™) formamide. If dissolution of DNA in water cannot be avoided due to issues with solubilizing the DNA directly in formamide, water should be evaporated immediately after addition of formamide. Exposing Hi-Di formamide to water will result in production of formic acid, which will compete with DNA fragments during sample injection. Since consistent signal intensity is critical for reliable data analysis, it is essential that the ionic strength of all samples be identical.

Using Hi-Di formamide as the diluent, samples must be mixed with the size standard and run together in the same capillary. The software uses the size standard to create a standard curve for each sample to determine the relative size of each unknown fragment; noting that the called size may differ from the actual size, it is essential to use the same size standard and instrument conditions for all samples. In addition, the size standard is also used to determine precision of the instrument by ensuring reproducible, uniform and precise spacing of the size standards. The size standards must appear as well resolved, sharp peaks and conform to the pattern specified by the manufacturer, and they must be called precisely by the software for identification of unknown fragments to be meaningful. The size standard should be vortexed thoroughly prior to use to resuspend fragments, diluted in Hi-Di formamide, and used immediately following dilution.

The fluorescence intensity of both DNA fragments and size standards must be optimized. Intensity of samples should be within the acceptable range of the analyzer, which for the ABI 3130 is 150-4,000 rfu. If the signal intensity is too high, samples must be diluted or sample injection time and/or voltage reduced until intensity is within range. When the signal from an individual fragment is too high, the instrument software cannot correct for spectral overlap with other dyes. This leads to so-called pull-up peaks of different color to appear under the position of the strong peak, precluding data interpretation. The intensity of size standards should be equal to

or less than the sample peaks (30-100%). We had reliable results when 1 µL of a 1:10 dilution of LIZ-500 size standards was mixed with 0.5 ng undigested DNA in a 10 µL reaction volume. Samples were heat-denatured and injected using an injection voltage of 1.6 kV for 15 seconds.

Total DNA in samples containing uncut DNA may have to be lower than in digested samples; using the same DNA concentration may result either in saturation of the signal for uncut DNA with the attendant pull-up peaks precluding data analysis or in insufficient signal-to-noise ratio for digested fragments; under our experimental conditions, a 4-fold lower concentration of uncut DNA compared to DNase I-digested samples yielded satisfactory results. Analysis of uncut DNA is critical, however, to ensure that no DNA degradation has occurred. Signal intensities near or above the saturation limit may also cause 'bleeding' of a high intensity signal from one capillary to adjacent capillaries, causing the appearance of spurious peaks. This occurs because the instrument detects the emitted fluorescence of samples from all capillaries at once; this may lead to assignment of a strong signal from one capillary to neighboring capillaries as well. It is therefore advisable to leave blank capillaries between samples if such bleeding is suspected.

The optimal concentration of DNase I and time of cleavage depends on solution conditions and the activity of the enzyme and must be empirically determined. Ideally, the average number of nicks per DNA fragment should be one, as over-digestion results in higher molecular weight fragments being less represented (Figure 1A) and a biased distribution of digested fragments [5]. In addition, when the protected area is large relative to total fragment length, unprotected regions may receive more nicks, increasing the proportion of shorter fragments.

We have found that a strict one-hit regime in which few DNA molecules receive multiple nicks is incompatible with the fragment analysis procedure, as a suitable signal-to-noise ratio for digested fragments requires a total DNA concentration at which the signal for uncut DNA is saturated. Slightly over-digesting the DNA resulted in signal intensity of both uncut DNA and cleavage fragments to be within a range commensurate with the instrument. Fluorescently labeled DNA (50 ng) was incubated with or without protein for 10 min in binding buffer (25 mM Tris pH 8, 0.1 mM EDTA, 100 mM NaCl, 0.05% Brij, 10 mM DTT, 2% glycerol), following which 2 μL 10x DNase I reaction buffer (New England BioLabs) and 2 μL 25 mM $MgCl_2$ was added and the sample was incubated with 0.08 units of DNase I for 3.5 minutes at room temperature in a 20 μL reaction volume. Each cleavage reaction was terminated after precisely the same incubation time by bringing the concentration of EDTA to 7.5 mM, vortexing, and placing the samples on ice. DNA was extracted with phenol:chloroform after bringing the total volume to 100 μL with TE' (10 mM Tris pH 8.0, 0.1 mM EDTA), ethanol precipitated, and dissolved in Hi-Di formamide. Two ng of DNA was mixed with 0.4 μL of 1:10 diluted LIZ-500 size standards in a 10 μL reaction volume and used for fragment analysis.

Due to electrokinetic injection of samples, an increase in peak height with injection time is more evident for smaller fragments, whereas larger fragments are more sensitive to the accompanying reduction in resolution. This bias in sample injection, combined with the biased distribution of DNA fragments resulting from over-digestion, necessitates that conditions and time of DNase I cleavage and DNA purification be maintained strictly constant for all samples, as minor variations may be amplified during analysis. Figure 1B illustrates the relatively uniform intensity of DNA fragments obtained in absence of protein (green), with variations in peak intensity arising from sequence-dependent differences in DNase I cleavage efficiency; incorrect

levels of DNase I cleavage would result in systematic differences in peak intensity as a function of fragment length (e.g., excessive over-digestion, Figure 1A; [3]).

Electropherograms corresponding to DNA digested in absence and presence of protein must be overlaid to allow rigorous data interpretation. It is essential that sequence blocks be identified on either side of the protected area where signal intensity is unaffected by protein (black electropherogram trace in Figure 1B); if such areas cannot be identified, variations in DNase I cleavage cannot be reliably assigned to the protein of interest. A side-by-side comparison of electropherograms precludes accurate validation that no systematic or global differences in cleavage intensity exist between samples; such systematic differences arising, for example, from injection bias or inconsistent sample preparation would confound accurate identification of protected regions. In addition, global or systematic changes in signal intensity between samples are likely to obscure identification of sites at which partial protection or enhanced cleavage occurs, such as the enhanced cleavage at the center of the protected region in our example (position -50; Figure 1B). DNase I is exquisitely sensitive to protein-mediated changes in DNA conformation that alter its accessibility; valuable data that offer insight into modes of protein binding may therefore be lost if electropherograms are not carefully compared.

Because the called fragment sizes do not correspond to actual sizes, the protected sequence must be identified by comparing with fragments generated using a dideoxy sequencing reaction. The DNA used for footprinting was used as the template with the same 6-FAM-labelled primer and Thermo Sequenase in a PCR cycle sequencing reaction according to the manufacturer's protocol (Affymetrix). The PCR product was purified using phenol:chloroform extraction and ethanol precipitation and the DNA dissolved in formamide. Purified DNA was mixed with LIZ-500 standards and used for fragment analysis, as described above. Overlaying

the electropherogram traces of different sequencing reactions with that of the DNase I-digested fragments such that size standards overlap precisely allows accurate assignment of fragments to the DNA sequence (Figure 1C; [3]).

Through fastidious adherence to consistency in sample preparation and analysis, automated capillary sequencers afford a reliable alternative to traditional slab-gel electrophoresis for determination of DNA footprints. However, the need to maintain signal intensities of all fragments within a limited range necessitates that the DNA be over-digested. A thorough appreciation of caveats associated with sample preparation and analysis is therefore critical to avoid artifacts that may confound data interpretation.

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References

[1] D.J. Galas, A. Schmitz, DNAse footprinting: a simple method for the detection of protein-DNA binding specificity, Nucleic Acids Res, 5 (1978) 3157-3170.

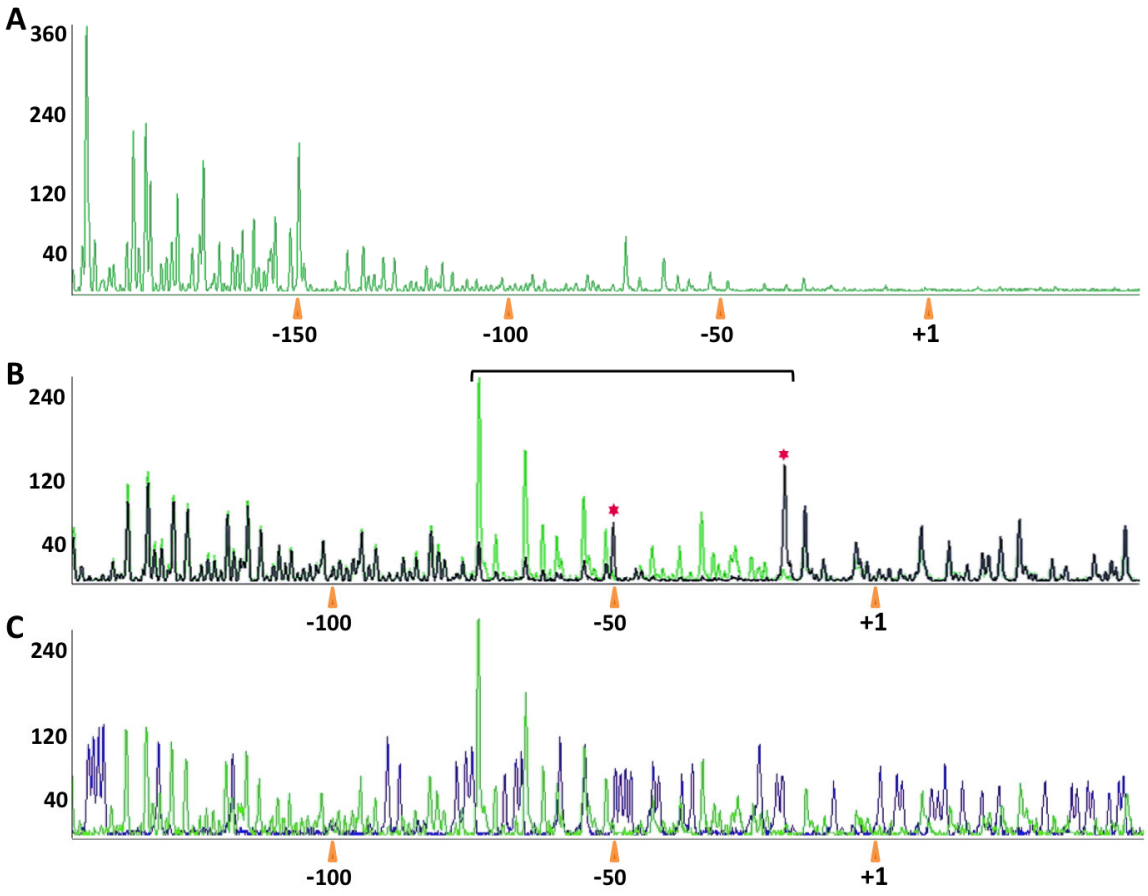
[2] W. Yindeeyoungyeon, M.A. Schell, Footprinting with an automated capillary DNA sequencer, Biotechniques, 29 (2000) 1034-1036, 1038, 1040-1031.

[3] M. Zianni, K. Tessanne, M. Merighi, R. Laguna, F.R. Tabita, Identification of the DNA bases of a DNase I footprint by the use of dye primer sequencing on an automated capillary DNA analysis instrument, J Biomol Tech, 17 (2006) 103-113.

[4] B. Hillerich, J. Westpheling, A new TetR family transcriptional regulator required for morphogenesis in *Streptomyces coelicolor*, J Bacteriol, 190 (2008) 61-67.

[5] M. Brenowitz, D.F. Senear, M.A. Shea, G.K. Ackers, Quantitative DNase footprint titration: a method for studying protein-DNA interactions, Methods Enzymol, 130 (1986) 132-181.

Figure 1. Analysis of DNase I-digested DNA. The y-axes are in rfu (ABI 3130). Sequence positions are marked with reference to the transcription start site of the *xdhR* gene [4]. A. Over-digested DNA revealing an over-representation of smaller fragments. B. Overlay of electropherograms of DNase I-digested DNA in the presence of XdhR protein (black) and without protein (green). Bracket identifies region of protein-mediated changes in DNase I digestion; hypersensitive sites are indicated with pink asterisks. Note that digestion of flanking sequence is unaffected by XdhR protein. C. Overlay of DNase I-digested DNA with dideoxy sequencing reaction terminating with dideoxy-adenosine performed using the same 6-FAM-labeled primer. Precise alignment of size standards allows assignment of DNA fragments to specific bases. In GeneMapper 4.1, overlay of electropherograms in different colors may be accomplished as follows: Select samples, click ‘view’, click ‘legend’, double-click the colored box that appears to change color of one electropherogram, go back to ‘view’, click on ‘overlay’. We have found it more convenient to export traces to PowerPoint or similar applications for purposes of overlaying the traces.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