BENCHMARKS

Benchmarks are brief communications that describe helpful hints, shortcuts, techniques or substantive modifications of existing methods.

*Mbo*II Endonuclease Heat Inactivation Before Agarose Gel Electrophoresis to Prevent Artifactual Bands in Restriction Patterns

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*Mbo*II restriction enzyme belongs to class-IIS endonucleases group (3–5). Like all of the enzymes belonging to this class, *Mbo*II cleaves DNA at a specific distance from its recognition sequence and still binds to its recognition sequence after DNA has been cleaved, because binding and cleaving domains have separate functions (5).

In our laboratory, we apply amplified ribosomal DNA restriction analysis (ARDRA) to bacterial identification following the technique of Garcìa-Arata et al. (2). *Mbo*II was retained, among other enzymes, because of appropriate frequency of the endonuclease recognition sequence on the amplified ribosomal RNA operon (*rrn*) of *Escherichia coli*. Since *E. coli* K-12 MG1655 strain has now been entirely sequenced (1), we could retrieve from the European Molecular Biology Laboratory (EMBL) database (GenBank® Accession No. U00096) the sequences corresponding to the amplified fragment in the seven *rrn* copies (A, B, C, D, E, G and H). Virtual *Mbo*II in silico restrictions could be performed on each of the seven sequences with the Geneman software (DNASTAR, Madison, WI, USA) on a Macintosh® (Apple Computer, Cupertino, CA, USA).

From the combination of all virtual restriction fragments from the seven operons, an in silico restriction pattern for *E. coli* K-12 MG1655 strain was obtained and could be compared to experimental patterns. Because the seven *rrn* operons in the *E. coli* chromosome differ in length and sequence, some of the fragments are contributed to by only one or a few operons. The amount of DNA contained in a single band will be proportional to the number of operons producing the band. Moreover, because the number of ethidium bromide molecules bound to double-stranded DNA fragments is proportional to fragment length, longer fragments will stain better than smaller ones even if they are produced by the same number of operons. Staining ability (S) of a band was calculated as $S = L \times N$, where L is the fragment length, and N is the number of operons contributing to the band. S values were considered as strong (above 1500), intermediate (600–1499) and weak (below 600).

Surprisingly, experimental patterns never entirely corresponded to the in silico one and, moreover, they seemed to vary according to enzyme concentration (Figure 1).

*Mbo*II endonuclease is heat-sensitive, and enzyme inactivation can be obtained after a 15-min incubation at 50°C (3). To verify enzyme-inactiva-

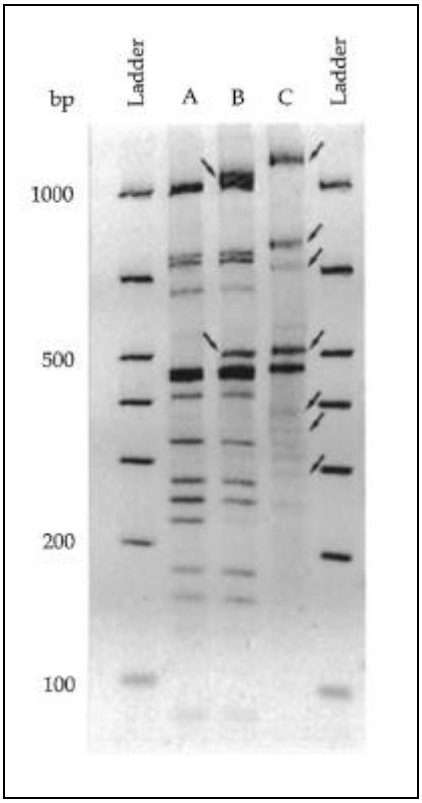

Figure 1. *E. coli* MG1655 strain restriction patterns. Ladder: AmpliSize; A: heat-inactivated 1.5 U/µL *Mbo*II; B: non-inactivated 0.6 U/µL *Mbo*II; C: non-inactivated 1.5 U/µL *Mbo*II. Artifactual bands are marked by arrows.

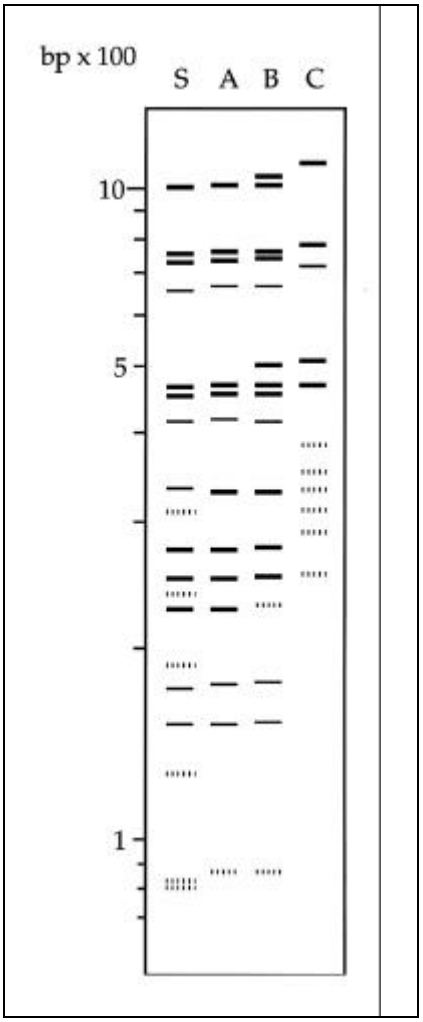

Figure 2. Schematic representation of *Mbo*II experimental patterns compared to in silico calculated pattern. S: in silico profile; A: heat-inactivated 1.5 U/µL *Mbo*II; B: non-inactivated 0.6 U/µL *Mbo*II; C: non-inactivated 1.5 U/µL *Mbo*II. Thin lines: weak bands at visual inspection in experimental patterns and bands with intermediate staining ability in an in silico profile. Dotted lines: bands barely detectable at visual inspection in experimental patterns and bands with weak staining ability in an in silico profile.

tion effect on fragment migration, experimental restriction mixtures were divided into two aliquots, and one of them was heated at 72°C for 10 min before placing it on the agarose gel for migration; the other was not heated.

Heat-inactivated profiles matched in silico migration except for some of the lowest amplification output bands (Figure 2). The size of profile bands was calculated with the Taxotron® software package (Taxolab®; Institut Pasteur, Paris, France) on the basis of AmpliSize molecular weight standard (Bio-Rad Laboratories, Hercules, CA, USA) using the Spline algorithm. The difference in fitting never exceeded 1.4% of the size of the corresponding in silico band. On the contrary, band artifacts appeared in non-heated samples. At lower enzyme concentration (0.6 U/μL), only two strong artifact bands were observed at approximately 1060 and 500 bp, while all the bands corresponding to the expected molecular weight sizes were still preserved except for the 240-bp one, which became barely detectable. At a higher enzyme concentration (1.5 U/μL), all but one of the expected molecular weight bands completely disappeared or became barely detectable, and three strong, one weak and many barely detectable band artifacts appeared (Figures 1 and 2). Artifact bands were assumed to be the result of cleavage-fragment retardation by bound enzyme molecules. The reason why some fragment bands were more prone to enzyme complexing and consequent shifting at a lower enzyme concentration remains unclear. We also observed that ionic concentration of the gel and migration buffer can affect the prevalence of one of the two forms (data not shown).

From our results, *Mbo*II inactivation is required for reproducible results when restriction fragments are smaller than 2 kbp. On the contrary, when longer fragments are studied (e.g., ribotyping), the increase in apparent size due to the complexed enzyme would be hard to appreciate, and heat inactivation is not required. The effects of heat inactivation procedure have been tested also on class-II endonucleases like *Hha*I or *Sau*3AI and, as expected, no change in the restriction profile could be observed (data not shown).

In conclusion, take care to inactivate *Mbo*II restriction endonuclease before electrophoresis of restriction fragments when the expected DNA fragments are smaller than 2 kbp. Because all endonucleases belonging to class-IIS (e.g., *Alw*I, *Bbv*II, *Hga*I, *Hph*I, *Fok*I, *Mnl*I, *Sfa*NI and *Taq*II) share common properties, it is best to heat inactivate restriction products obtained with any class-IIS endonucleases.

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Electrophoretic Mobility Shift Assay Coupled with Immunoblotting for the Identification of DNA-Binding Proteins

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Electrophoretic mobility shift assay (EMSA) is widely used to analyze and quantitate sequence-specific DNA-binding proteins in either cellular or nuclear extracts (4,7). Traditionally, compositional analysis is performed by the technique of "supershift," where antibodies against candidate proteins are added to the binding reaction before electrophoresis. If the antibody binds to a region of the protein not involved in DNA binding, a larger complex forms that migrates more slowly and appears supershifted in its mobility. Alternatively, the antibody might recognize the DNA binding portion of the protein and specifically inhibit protein:DNA complex formation, and a reduction in radiolabeled probe binding is observed. However, interpretation is compromised when an antibody to a candidate protein has no effect, and a false negative will result if the antibody fails to recognize the native form of a protein that is present.

Alternative techniques have been described in which the complex is analyzed in a second dimension by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (10) and where retardation gels have been directly subjected to immunoblotting after transfer to nitrocellulose (9). However, in both cases the protein of interest occupies a large area that diminishes sensitivity, and neither approach provides complete identification of the candidate protein in terms of both molecular mass and specific immunoreactivity. In this report, we describe a method for compositional analysis of specific proteins associated with protein:DNA complexes, which involves excision of shifted bands from EMSA gels followed by SDS-PAGE and immunoblotting.

We used as a model system activating protein-1 (AP-1), dimeric bZIP