Restriction Endonuclease Digestion of a Plasmid

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Abstract: Recombinant DNA technology is widely utilized and therefore, undergraduate laboratory courses incorporate these techniques. This reliable laboratory exercise introduces the student to plasmid vectors, restriction enzymes, and agarose gel electrophoresis. Restriction digestion of pBR325 with *Pst*I and *Hind*III, in single and double digests, was performed and analyzed on an agarose gel. Students constructed a standard curve based on the migration of fragments from a *Hind*III digest of bacteriophage λ DNA and used that curve to estimate the sizes of DNA fragments in the pBR325 digests. In this exercise, measurements on the largest λ fragment were included to illustrate the resolution limits of agarose gels and the proper use of standard curves.

Introduction

The plasmid cloning vector, pBR325, carries genes coding resistance to the antibiotics tetracycline, ampicillin and chloramphenicol. Unique restriction sites are located in each antibiotic resistance gene, for example, *Pst*I in ampicillin, *Bam*HI in tetracycline and *Eco*RI in chloramphenicol (Bolivar, 1978; Prentki et al., 1981) and at other positions around the plasmid, e.g., *Hind*III which is located in the promoter for the tetracycline resistance gene (Rodriguez et al., 1979). The location of unique restriction sites at various positions around the plasmid permits the formation of easily visualized and well separated restriction fragments. Thus, this vector is well suited for use in instructional restriction analysis protocols. Using this vector also introduces the student to the structure and utilization of the widely utilized plasmid cloning vector pBR322 and its derivatives. Students digest pBR325 with *Hind*III and *Pst*I, perform agarose gel electrophoresis, and estimate the fragment sizes from a standard curve generated from a λ *Hind*III digest. In this exercise, this plot includes the 23,130 base pair (bp) λ fragment to illustrate the limited resolution range of agarose gels and the necessity of having points on the standard curve bracket the experimental measurements.

Materials and Methods

Materials and Solutions

Restriction enzymes, restriction buffers, and λ DNA were from New England BioLabs; pBR325 was from Sigma-Aldrich. Plasmid DNA was resuspended in sterile TE buffer to 0.1 μg/μL, and λ DNA to 0.4 μg/μL. Electrophoresis reagents (agarose, loading dye, Tris-Borate-EDTA (TBE) buffer, ethidium bromide solution) and Tris-EDTA buffer can be purchased from Carolina Biological Supply Company or prepared according to Sambrook et al. (1989). Tris-EDTA (TE) is

10mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0); 5X TBE stock solution is 54 g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA (pH8.0); loading dye is 0.25% bromophenol blue, 0,25% xylene cyanol FF, 40% (w/v) sucrose in water (Sambrook et al., 1989).

Safety

Students were carefully supervised when they started the electrophoresis to prevent any safety hazards. Staining was with 1 μg/mL ethidium bromide which is a mutagen and suspect carcinogen. Therefore, to minimize risk, students were not allowed to handle the staining solution, aerosols were avoided, and a 5 mg/mL stock solution was purchased, not a powder. Ethidium bromide solution was chemically inactivated before disposal (Quillardet and Hofnung, 1988) or collected on commercially available filters which were discarded with hazardous waste. To protect the retina and skin from shorter wavelength ultraviolet light, an ultraviolet absorbing shield was always used when viewing gels.

Experimental Procedure

Students added all reagents as listed in Table 1 to digest plasmid (0.5 μg) with *Hind*III and *Pst*I in both single and double digests. A *Hind*III digest of λ DNA (2 μg) was also performed to provide molecular weight standards on the agarose gel. After incubation for 1 hour at 37°C, samples were heated to 60°C for 3 minutes to melt the λ cohesive ends before loading on a 0.8% agarose gel and performing electrophoresis at 80-100 volts for approximately 1 hour until the dye front was 1-2 cm from the end of the gel. If the experiment could not be completed in one laboratory session, samples were frozen after the restriction digestion and the 60°C incubation and electrophoresis was performed during a second session. The technician stained the gels in 1 μg/mL ethidium bromide for at least 1 hour and rinsed with water before viewing on an ultraviolet emitting transilluminator. The gels were photographed with Polaroid 667 film. The fragment migration distances were measured on an 8 X 11 inch print of the digitized photograph or alternatively, an expanded copy, or the Polaroid print itself.

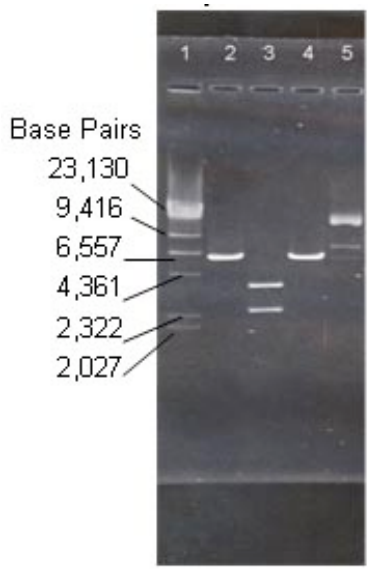
Table 1. Restriction reaction mixes

Tube #	Water	Buffer	DNA	Enzyme
1	10 μL	4 μL 5X *Hind*III buffer	5 μL λ	1 μL *Hind*III
2	10 μL	4 μL 5X *Pst*I buffer	5 μL plasmid	1 μL *Pst*I
3	9 μL	4 μL 5X *Hind*III buffer	5 μL plasmid	1 μL *Pst*I AND 1 μL *Hind*III
4	10 μL	4 μL 5X *Hind*III buffer	5 μL plasmid	1 μL *Hind*III
5	11 μL	4 μL 5X *Hind*III buffer	5 μL plasmid	---

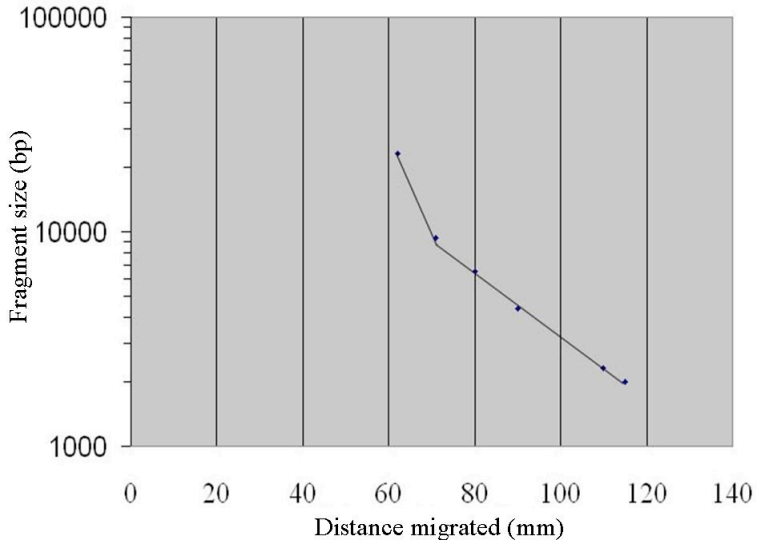
Results and Discussion

The results of an ideal gel are shown in Figure 1A. Comparison of either single digest with the λ ladder confirms the size of pBR325 which has 5,995 base pairs (bp). The double digest

produces fragments that are approximately 2,400 and 3,600 bp as expected. Clearly, *Hind*III and *Pst*I each cleave pBR325 only once and at different sites on the plasmid. Most undigested plasmid migrates closer to the wells than the linear pBR325 in the single digest lanes, confirming that both restriction enzymes cleaved pBR325 and emphasizing the need to compare like conformations in size determinations.



1A



1B

Figure 1A. Agarose gel electrophoresis of restriction digests. Lane 1, λ DNA digested with *Hind*III (the size of each fragment is indicated in base pairs); lane 2, pBR325 digested with *Pst*I; lane 3, pBR325 digested with *Pst*I and *Hind*III; lane 4, pBR325 digested with *Hind*III; and lane 5, undigested pBR325. 1B. Standard curve for fragment size determination. The distance each λ *Hind*III fragment migrated was measured and plotted (arithmetic X-axis) verses the size in base pairs (logarithmic Y-axis).

Figure 1B shows the standard curve semilog plot that was used to determine the fragment sizes. A 0.8% gel cannot resolve fragments in the 23,130 bp range and therefore, this λ fragment is not on the linear portion of the plot which extends from 2,027 to at most 9,416 bp. This can be used to illustrate the importance of bracketing the unknown points with the standard curve as a linear relationship might not continue beyond the measured range. In the case of the λ *Hind*III ladder, if the 2,027-9,416 bp linear portion of the curve were incorrectly extrapolated beyond the highest point, the apparent size of the 23,130 bp fragment would be 12,000 bp, nearly a two fold error. Clearly, it is inappropriate to extrapolate a standard curve beyond the measured range.

The 2,027-9,416 bp linear portion of the standard curve also reflects the fragment sizes that can be properly resolved; the steeper segment shows the size where the gel can no longer suitably separate fragments. The lack of resolution of larger DNA's can be emphasized by asking students to predict how far 23,000 and 22,700 bp fragments would migrate (both 62 mm) compared to the easily resolved 2,322 and 2,027 bp fragments (110 and 115 mm).

Assessment was from grading of student lab reports and a quiz. In this exercise, students gained knowledge of the pBR325 cloning vehicle, the restriction digestion procedure, the agarose gel electrophoresis procedure, the interpretation of agarose gel patterns, and certain dilution procedures. The resolution limits of agarose gels and the correct use of standard curves were emphasized by considering the data on the largest λ fragment. In addition, students acquired the information and practice required to properly use micropipettors, microcentrifuges, and electrophoresis equipment.

Literature Cited

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About the Author

Margaret M. Dooley earned her B.A. from Cornell University and her Ph.D. from Syracuse University. She received postdoctoral training at the University of California, Davis and Rutgers University before joining the faculty of the College of Staten Island where she teaches genetics, microbiology and general biology courses.