Two electrophoresis experiments for freshmen in the health professions

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Two Electrophoresis Experiments for Freshmen in the Health Professions

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We report here on a traditional paper electrophoresis separation of amino acids, a state-of-the-art agarose gel electrophoresis separation of DNA fragments, and on the design of an electrophoresis tank which combines outstanding safety features with the ability to conduct both paper and gel electrophoresis experiments.

A drawing of the electrophoresis tank is shown in Figure 1. Like a commercially available tank,[2] our tank has a rotating cover; since the two electrical connections are made through holes in the cover, electric current cannot be applied to the tank unless the cover is closed. As an additional safety feature, banana jacks are used on the electrical leads, and mate with banana plugs in the tank. Although banana jacks can be purchased, we found that the commercially available jacks are not long enough to pass through the holes in the cover and reach the banana plugs. For this reason, we fabricated banana jacks with extra long "handles" by soldering each electrical lead to a conventional banana jack and then encapsulating this assembly with epoxy cement in a 1½-in. length of borosilicate glass tubing.

The tank is constructed of acrylic plastic. For paper electrophoresis experiments, the paper strips are laid directly on the support plate. Typically, we place two strips side by side and run two experiments simultaneously in the tank. For the agarose gel electrophoresis experiment, the gel tray is placed on the support plate.[3] A typical gel has 10 wells, thus permitting two or even three experiments to be run on each gel.

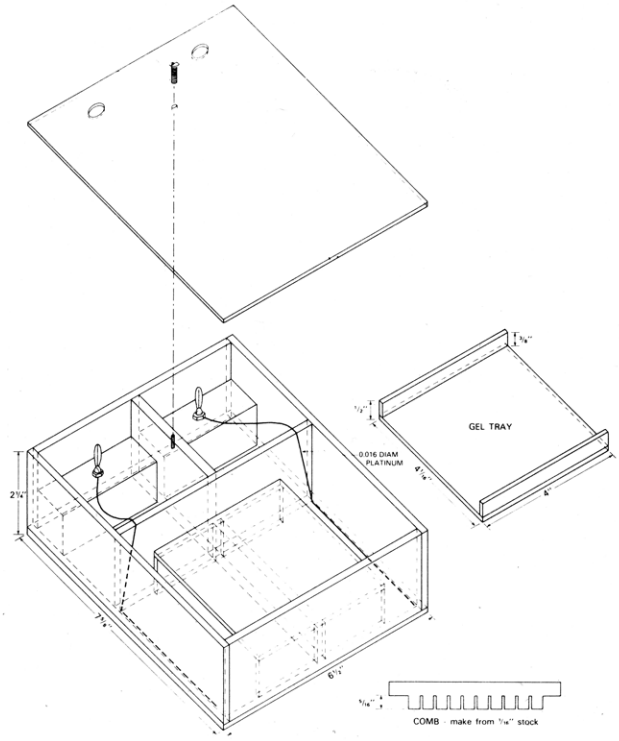

Figure 1. Electrophoresis tank.

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[2] Griffin & George, Ltd., Wembley, Middlesex, England.

[3] To assure maximum visibility of the DNA bands in the agarose gel experiment, be sure to construct the gel tray using a brand of acrylic plastic which does *not* fluoresce appreciably when exposed to UV light.

Composition of Restriction Digests

Tube	pBR322 DNA (0.8 μg/μL)	5X TAB Buffer	Water	Pst I (10 U/μL)	Pvu II (10 U/μL)	Bam HI (10 U/μL)
1	1 μL	4 μL	13 μL	1 μL	1 μL	...
2	1	4	13	1	...	1 μL
3	1	4	12	1	1	1

Separation of Amino Acids

The first objective of this experiment is to demonstrate graphically that the electrophoresis of an amino acid is a function both of its structure (the number and location of its –COOH and $-NH_2$ groups) and of the pH of the solution in which it is dissolved. To this end, we follow the suggestion of the manufacturer of our first electrophoresis tank[2] and provide 0.2 *M* solutions of three amino acids: L-alanine, L-glutamic acid, and L-lysine. The students are divided into three groups and each group uses a different buffer so that the students may compare and contrast their results as a function of pH. Group A discovers that, in an acidic (pH = 1.9) buffer, all the amino acids move toward the cathode because of protonation of all the $-NH_2$ groups, and that L-lysine (having two $-NH_2$ groups) moves twice as far as the other two amino acids. On closer inspection, the student discovers that, although L-alanine and L-glutamic acid move approximately the same distance in this buffer, the L-alanine moves a bit further than its companion. This result is precisely in accord with the Henderson-Hasselbalch equation,

$$\mathrm{pH} = \mathrm{p}K_a + \log_{10}\frac{[\mathrm{A}^-]}{[\mathrm{HA}]} \qquad (1)$$

Since $\mathrm{p}K_a(\alpha\text{-COOH}) = 2.35$ for L-alanine while $\mathrm{p}K_a(\alpha\text{-COOH}) = 2.19$ for L-glutamic acid, the fraction of protonated L-alanine molcules is larger than the fraction of protonated L-glutamic acid molecules, and the L-alanine molecules move farther toward the negatively charged cathode.

Group B uses a neutral buffer (pH = 6.5) and sighs with relief to note that the three amino acids separate cleanly, L-lysine moving smartly to the cathode and L-glutamic acid moving with equal alacrity to the anode, while L-alanine spectates on the midfield stripe.

Buffer C (pH = 10.5) provides the students with another opportunity to examine the results in terms of the Henderson-Hasselbalch equation. In terms of relative distances moved toward the anode, L-glutamic acid moves two units, L-alanine moves one unit, and L-lysine moves one-half unit. This result agrees with expectation in view of the fact that the $\mathrm{p}K_a(\epsilon\text{-}NH_3^+) = 10.53$ for L-lysine and thus the number of molecules with a charge of minus one is exactly equal to the number of molecules with a charge of zero in this buffer.

The second objective of the experiment with amino acids is to demonstrate the underlying principles of electrophoresis. The experiment yields a splendid example of electroosmotic flow. The student observes in Buffer B that the L-alanine moves perceptibly toward the cathode even though the Henderson-Hasselbalch equation predicts that this molecule should remain precisely on the "midfield stripe." This observation allows the instructor to point out that the fibers in the electrophoresis paper selectively adsorb negatively charged buffer ions, thus allowing a net flow of the electrolyte toward the cathode. The L-alanine molecules are swept along with the flow of electrolyte while the L-glutamic acid molecules are paddling upstream and the L-lysine molecules gain an extra push by moving with the current.

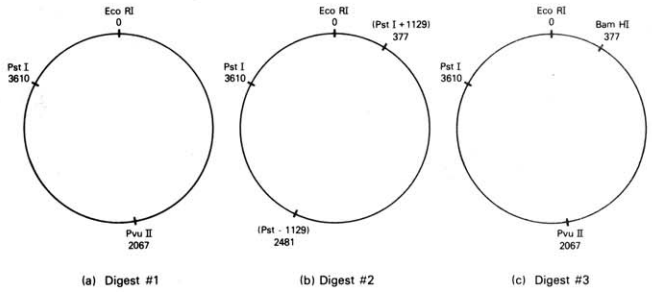

Figure 2. Restriction endonuclease maps. The coordinates refer to the centers of the recognition sites.

Separation of DNA Fragments

With the amino acid experiment completed, the student is ready to examine two of the key techniques of genetic engineering: the selective cleavage of DNA molecules by restriction endonucleases, and the separation and characterization of DNA fragments using agarose gel electrophoresis. We have devised a classic problem in which the student must use deductive reasoning to determine the location at which the restriction endonuclease Bam HI cuts a piece of DNA. We selected pBR322 DNA for this experiment and set up the problem as follows. The students are instructed to make three restriction digests and separate the resulting DNA fragments using agarose gel electrophoresis. The constituents of the three digests are listed in the table. As baseline information, we tell the students that pBR322 is a double-stranded, circular DNA consisting of 4362 base pairs, that each of the restriction endonucleases cuts the pBR322 only once, and that Pst I and Pvu II cut the pBR322 at 3610 and 2067, respectively.[4] The first digest confirms for the students the information they were given. Indeed, the DNA is broken into two fragments and the lengths of the fragments agree with the literature values (see Fig. 2a).

Examination of the results of the second digest (which also yields two fragments) allows the students to determine the distance between the Pst I and the Bam HI recognition sites but does not indicate to the students whether the Bam HI site is clockwise or counterclockwise with respect to the Pst I site (see Fig. 2b).

The ambiguity is resolved by the triple digest. When the student discovers that the longer (slower moving) fragment is Digest #1 is absent from Digest #3, it is immediately apparent that the Bam HI recognition site must be clockwise with respect to the Pst I site, as illustrated by Figure 2c.

Each of the three restriction digests occupies a separate lane in the agarose gel. It is important that the student be able to measure the distance traveled by each fragment in each digest and be able to estimate the length of each fragment from the distance it traveled. To enable these measurements, we place a mixture of DNA marker fragments of known lengths in a fourth lane. We instruct the students to measure the position of each band in each of the lanes occupied by the three restriction digests and then to measure the locations of six DNA marker fragments in the fourth lane. Using the data for the DNA marker fragments, the students prepare the traditional plot of distance traveled versus logarithm of fragment length (in number of base pairs (bp)), discover the inverse linear relationship between distance and log(bp), and then use this plot to estimate the lengths of the fragments responsible for the bands in the three digests.

[4] We have chosen to identify each recognition site by the coordinate of its center; as usual, the center of the Eco RI recognition site is taken as 0. See Figure 2. Note that catalogs typically list the location of a recognition sequence by referring to the coordinate of the nucleotide on the 5′ side of the cut.

Experimental: Separation of Amino Acids

Buffers: pH = 1.9 (50 mL 1 *M* formic acid + 150 mL glacial HOAc/L)
pH = 6.5 (5 mL glacial HOAc + 125 mL pyridine/L)
pH = 10.5 (50 mL diethylamine + 10 mL glacial HOAc/L)
Amino acids: 0.2 *M*
Marker Dye: 1% aq. Orange-G, Acid Fuchsin, Xylene Cyanol.
Ninhydrin: 0.25% in acetone.

Procedure

Five equally spaced pencil marks are made on a line drawn across the middle of a piece of Whatman #1 paper (47 mm × 175 mm). The paper is then saturated with buffer and placed on the support plate in the electrophoresis tank. Using micropipets made by drawing capillary tubes with the aid of a small Bunsen burner, 2- –3-mm diameter spots of reagents are made on the paper in the following order: marker dye, L-alanine, L-glutamate, L-lysine, and marker dye. The electrophoresis is conducted at 200 V for approximately 35 min. The students can observe the progress of the electrophoresis by watching the separation and migration of the marker dyes. At the conclusion of the electrophoresis, the paper is dried, and then sprayed with the ninhydrin solution to locate the amino acid spots. Best results are achieved if the purple spots are permitted to develop at room temperature; this takes only a few minutes.

Experimental: Separation of DNA Fragments

5X TAB Buffer[5]
pBR322 DNA (0.8 μg/μL)
Restriction endonucleases (10 U/μL)
10X Loading Dye: 0.1% Bromophenol Blue and Xylene Cyanol in 50:50 (V/V) glycerol:water
λ/ϕX Marker Solution: λ DNA Hind III Fragments + ϕX174 RF DNA Hae III Fragments and loading dye in TAB buffer
TRIS-Phosphate Buffer
Ethidium Bromide Solution

Procedure

The students prepare the three restriction digests in 1.5-mL polypropylene microcentrifuge tubes in accordance with the table using Pipetman[6] automatic micropipets. While the digestion is progressing (30 min at 37 °C), 0.25 g of agarose is dissolved in 50 mL of hot TRIS-phosphate buffer and then poured into the gel tray; adhesively backed paper tape is used to close the open ends of the gel tray during the casting process. Fifteen minutes after casting, the gel is ready, and the paper tape and the comb are removed. By this time, the digests are complete, and the students add 2 μL of loading dye to each tube. As a method for demonstrating the technique for loading the samples onto the gel, the instructor loads 10 μL of the marker fragment solution into one of the middle wells. The students then load their samples into the adjacent wells; the students use their entire samples (20 μL per well). Under our conditions (105 V and 110–150 ma), the electrophoresis takes about 45 min. The end of the electrophoresis is signaled when the faster moving dye (Bromothymol Blue) has moved 5 cm. The gel tray is removed from the electrophoresis tank and placed in the ethidium bromide solution for 5 min. Naturally, the students have been using latex gloves up to this point, but we make a special point of the importance of wearing the gloves during the staining process in the ethidium bromide solution because of the potential health hazard; nothing is lost if the instructor chooses to do this part of the procedure. After being thoroughly washed with distilled water, the gel (still on the gel tray) is dried with absorbent paper and placed in a long wavelength UV light box. The students measure the locations of six marker fragment bands (603, 872, 1078, 1353, 2000, and 2300 bp) and the restriction digest bands to the nearest 0.1 mm using a fluorescent ruler. To make this ruler, we started with a metal ruler that had engraved lines and numerals and filled the lines and numerals with a fluorescent acrylic paint.

Discussion

Because adequate separation is achieved quite rapidly, each experiment fits very nicely into a 3-h laboratory period. There is a period of 30–45 min, while each experiment is running, during which the students have little to do. We take advantage of this opportunity to gather the students at the blackboard and discuss what is happening and why. In the case of the amino acid experiment, we talk about the Henderson-Hasselbalch equation with particular emphasis on L-lysine in the pH = 10.5 buffer. While the DNA experiment is running, we lay out the conceptual framework for genetic engineering using a specific example such as the production of human growth hormone or human insulin.

Our experience with these experiments has been uniformly outstanding. In a sense, the DNA experiment is the capstone of our syllabus. The students respond enthusiastically, sometimes almost in awe, as they realize that they are using state-of-the-art techniques, just like the graduate students in nearby labs.

[5] Detailed instructions for preparation of the reagents will be forwarded on request.

[6] Trademark of Rainin Instrument Co.