INTRODUCTION

In this experiment, samples of DNA from bacteriophage *Lambda* (48,502 base pairs in length) are cut with two different restriction enzymes, *Eco*RI and *Hin*dIII. Each enzyme recognizes and cuts at a different DNA sequence. A third DNA sample is the undigested control, while a fourth sample is cut with an unknown enzyme (either *Eco*RI or *Hin*dIII). Following incubation at 37° C the resulting restriction fragments are loaded into wells of an agarose gel and electrophoresed. An electric field applied across the gel causes the negatively charged DNA fragments to move from their origin (the sample well) through the gel matrix toward the positive electrode. The gel matrix acts as a sieve through which smaller DNA molecules migrate faster than larger ones; thus, restriction fragments of differing sizes separate into distinct bands during electrophoresis.

Following electrophoresis, the gel is exposed to medium-wavelength ultraviolet (UV) light. The stained DNA strongly absorbs UV light, loses some energy, and reemits visible light in the orange part of the spectrum. Under UV illumination, stained DNA fragments of identical size migrate to the same position in the gel and appear as a discrete orange band. By comparing the band pattern from DNA cut with the unknown enzyme to patterns obtained with *Eco*RI and *Hin*dIII, the identity of the unknown enzyme can be determined.

PROCEDURE

I. Set Up Restriction Digest

1. Use permanent marker to label four 1.5-ml tubes, in which restriction reactions will be performed: "E" *Eco*RI, "H" *Hin*dIII, "–" no enzyme, and "?" unknown enzyme.

   Use matrix below as a checklist while adding reagents to each reaction tube. Read down each column, adding the same reagent to all appropriate tubes; *use a fresh tip for each reagent.*

Tube	DNA	Buffer	*Eco*RI	*Hin*dIII	H_2O	unknown
"E"	4 µl	5 µl	1 µl	---	---	---
"H"	4 µl	5 µl	---	1 µl	---	---
"–"	4 µl	5 µl	---	---	1 µl	---
"?"	4 µl	5 µl	---	---	---	1 µl

2. Close tube tops. *Pool* and mix reagents by pulsing in a microfuge. Make sure tubes are placed in a balanced configuration in the rotor.

3. Place reaction tubes in 37°C water bath, and incubate for a minimum of 20 minutes. Reactions can be incubated for a longer period of time.

II. Cast 0.8 % Agarose Gel

1. Seal ends of gel-casting tray with tape, and insert well-forming comb in the notches over the black band. Place gel-casting tray out of the way on your lab bench, so that agarose poured in next step can set undisturbed.

2. Carefully pour enough agarose solution into casting tray to fill to depth of about 5 mm. Gel solution should cover only about 1/3 the height of comb teeth. Use a pipet tip to move large bubbles or solid debris to sides or end of tray, while gel is still liquid.

3. Gel will become cloudy as it solidifies (about 10 minutes). *Be careful not to move or jar* casting *tray while agarose is solidifying*. Touch corner of agarose away from comb to test whether gel has solidified.

4. When agarose has set, unseal ends of casting tray. Place tray between pegs in the gel box. The comb should be at negative end (black dot).

5. Fill box with 1X Tris-Borate-EDTA (TBE) buffer, until it just covers the entire surface of gel. Too much buffer will channel the current over the top rather than through the gel, increasing time required to separate DNA.

6. Gently remove comb, taking care not to rip wells. Do not wiggle the comb as you pull it out.

7. Make certain that sample wells left by comb are completely submerged. If "dimples" are noticed around wells, slowly add buffer until they disappear.

III. Load Gel and Electrophorese

1. Add 1 µl of loading dye with SYBR Green to each reaction tube. Mix by pulsing in a microfuge. Make sure tubes are placed in a *balanced* configuration in rotor.

2. Use micropipette to load 10 µl of each reaction tube into separate well. *Use fresh tip for each reaction*.
   a. Steady pipet over well using two hands.
   b. Dip pipet tip through surface of buffer, center it over the well, and gently depress pipet plunger to slowly expel sample. Sucrose in the loading dye weighs down the sample, causing it to sink to the bottom of the well. *Be careful not to punch tip of pipet through bottom of the gel*.

3. Close top of electrophoresis chamber, and connect electrical leads to a power supply, anode to anode (red-red) and cathode to cathode (black-black). Make sure both electrodes are connected to same channel of power supply.

4. Turn power supply on, and set to 140 volts.

5. Electrophorese for approximately 45 minutes. Shortly after current is applied, loading dye should be moving toward the positive side of gel. It appears as a blue band, eventually resolving into two bands of color. The faster-moving, purple band is bromophenol blue. The slower-moving, blue band is xylene cyanol. In a 0.8 % gel, bromophenol blue migrates through the gel at same rate as a DNA fragment of approximately 300 bp. Xylene cyanol migrates at a rate equivalent to approximately 9000 bp.

   Good separation will have occurred when the bromophenol blue band has moved 6-8 cm from wells. If time allows, electrophorese until the bromophenol blue band nears the end of the gel.

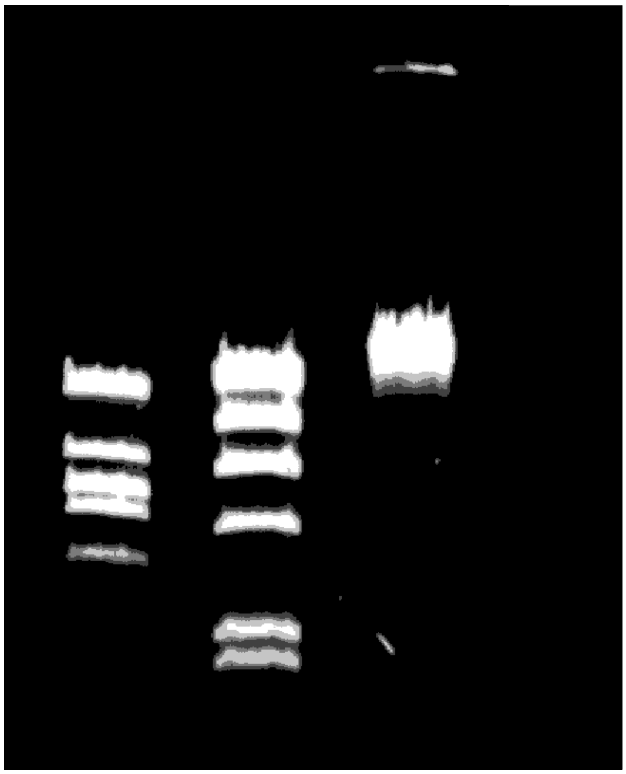
6. Turn off power supply, disconnect leads from the inputs, and slide off top of electrophoresis chamber.

7. Remove casting tray from the gel box, pour off excess buffer, and slide gel into disposable tray labeled with your initials. The gel is slippery, so be sure to hold the casting tray carefully.

8. View the gel with a UV transilluminator. DNA fragments of identical size in the same lane will appear as a discrete band.

IDEAL GEL

*Eco*RI *Hin*dIII — ?



RESULTS AND DISCUSSION

1. Why is water added to tube "–" in Part 1, Step 2?

2. What is the function of restriction buffer?

3. What are the two functions of loading dye?

4. What is the effect of run time on the observed pattern of restriction fragments? How would the gel concentration affect the observed pattern?

5. Examine the photograph of your stained gel. Compare your gel with the ideal gel shown below, and try to account for the fragments of *Lambda* DNA in each lane. How can you account for differences in separation and band intensity between your gel and the ideal gel?

6. What is the identity of the unknown restriction enzyme?