DNA Restriction Analysis

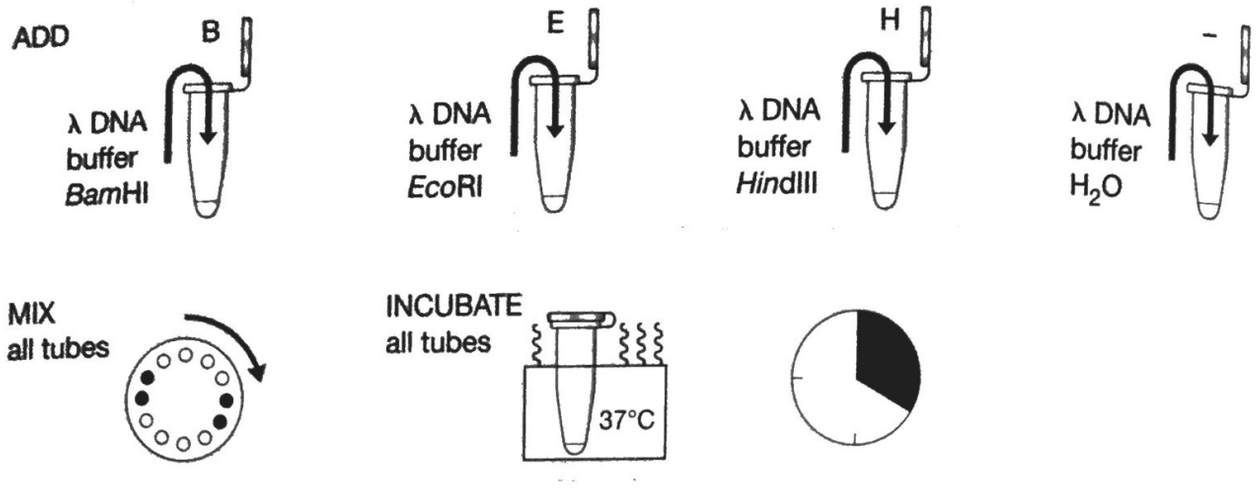
LABORATORY 3 INTRODUCES THE ANALYSIS OF DNA using restriction enzymes and gel electrophoresis. Three samples of purified DNA from bacteriophage λ (48,502 bp in length) are incubated at 37°C, each with one of three restriction endonucleases: *Eco*RI, *Bam*HI, and *Hin*dIII. Each enzyme has five or more restriction sites in λ DNA and therefore produces six or more restriction fragments of varying lengths. A fourth sample of λ DNA, the negative control, is incubated without an endonuclease and remains intact.

The digested DNA samples are then loaded into wells of a 0.8% agarose gel. An electrical field applied across the gel causes the 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 molecules; restriction fragments of differing sizes separate into distinct bands during electrophoresis. The characteristic pattern of bands produced by each restriction enzyme is made visible by staining with a dye that binds to the DNA molecule.

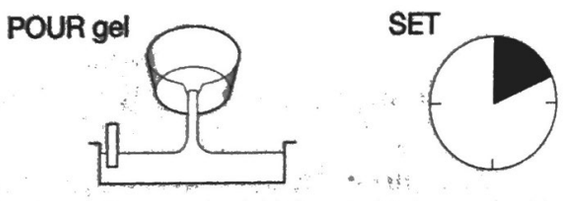
Kits based on this laboratory are available from the Carolina Biological Supply Company.

- Catalog no. 21-1103 (with ethidium bromide stain)
- Catalog no. 21-1104 (with *Carolina*Blu™ stain)

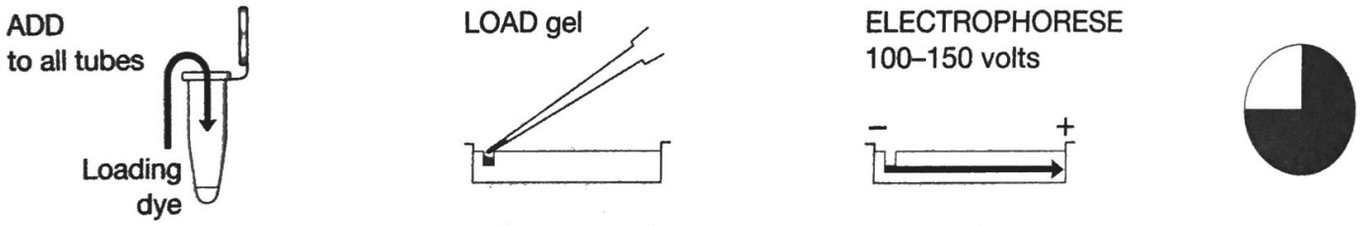
I. Set Up Restriction Digest



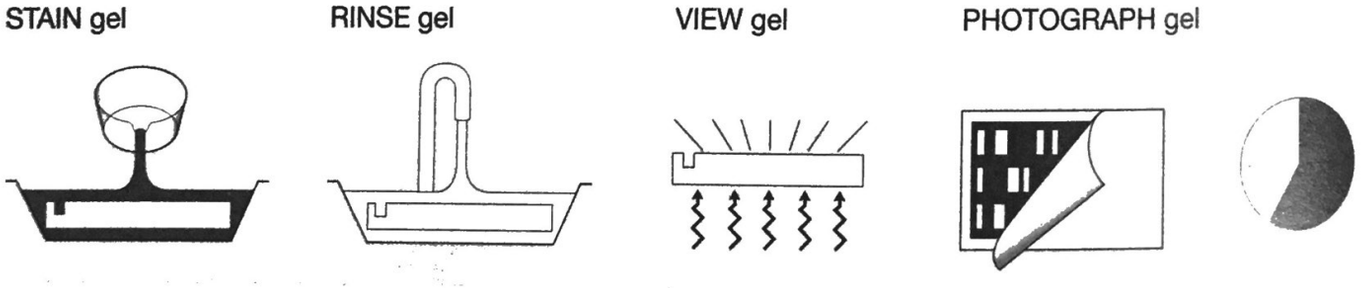
II. Cast 0.8% Agarose Gel



III. Load Gel and Separate by Electrophoresis



IV. Stain Gel and View (Photograph)



MATERIALS

REAGENTS

Agarose (0.8%)
Distilled water
Ethidium bromide▼(1 μg/ml) (or 0.025% methylene blue▼)
λ DNA (0.1 μg/μl)
Loading dye
2x Restriction buffer
Restriction enzymes
*Eco*RI
*Bam*HI
*Hin*dIII
1x Tris▼/Borate/EDTA (TBE) buffer

SUPPLIES AND EQUIPMENT

Aluminum foil
Beakers for agarose, for waste/used tips, and for TBE buffer
Camera and film (optional)
Electrophoresis box
Latex gloves
Masking tape
Microfuge (optional)
Micropipettor (0.5–10 μl) + tips
Parafilm or wax paper (optional)
Permanent marker
Plastic wrap (optional)
Power supply
Test tube rack
Transilluminator (optional)▼
Tubes (1.5-ml)
Water bath (37°C)

▼ See Appendix 4 for Cautions list.

METHODS

I. Set Up Restriction Digest

(30 minutes, including incubation)

1. Use a permanent marker to label four 1.5-ml tubes, in which restriction reactions will be performed:

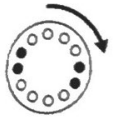
   B = *Bam*HI
   E = *Eco*RI
   H = *Hin*dIII
   – = no enzyme

2. Use the matrix below as a checklist while adding reagents to each reaction. Read down each column, adding the same reagent to all appropriate tubes. *Use a fresh tip for each reagent.* Refer to detailed directions that follow.

Tube	λ DNA	Buffer	*Bam*HI	*Eco*RI	*Hin*dIII	H_2O
B	4 μl	5 μl	1 μl	—	—	—
E	4 μl	5 μl	—	1 μl	—	—
H	4 μl	5 μl	—	—	1 μl	—
–	4 μl	5 μl	—	—	—	1 μl

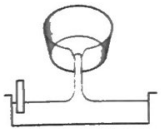
It is not necessary to change tips when adding the same reagent. The same tip may be used for all tubes, provided the tip has not touched solution already in tubes.

3. Collect and place reagents in a test tube rack on the lab bench.
4. Add 4 µl of DNA to each reaction tube. Touch the pipette tip to the side of the reaction tube, as near to the bottom as possible, to create capillary action to pull the solution out of the tip.
5. Always add buffer to the reaction tubes *before* adding enzymes. Use a *fresh tip* to add 5 µl of restriction buffer to a clean spot on each reaction tube.
6. Use *fresh tips* to add 1 µl of *Eco*RI, *Bam*HI, and *Hin*dIII to the appropriate tubes.
7. Use a *fresh tip* to add 1 µl of deionized water to tube labeled "–."
8. Close tube tops. Pool and mix reagents by pulsing in a microfuge or by sharply tapping the tube bottom on the lab bench.
9. Place the reaction tubes in a 37°C water bath, and incubate them for a minimum of 20 minutes. Reactions can be incubated for a longer period of time.



After several hours, enzymes lose activity and reaction stops.

Following incubation, freeze reactions at –20°C until ready to continue. Thaw reactions before continuing to Section III, Step 1.



Gel is cast directly in box in some electrophoresis apparatuses.

II. Cast 0.8% Agarose Gel

(15 minutes)

1. Seal the ends of the gel-casting tray with tape, and insert well-forming comb. Place the gel-casting tray out of the way on the lab bench so that agarose poured in next step can set undisturbed.
2. Carefully pour enough agarose solution into the casting tray to fill to a depth of about 5 mm. Gel should cover only about one-third the height of comb teeth. Use a pipette tip to move large bubbles or solid debris to the sides or end of tray while gel is still liquid.
3. Gel will become cloudy as it solidifies (~10 minutes). *Do not 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 on the platform of the gel box so that comb is at negative black electrode (cathode).
5. Fill box with TBE buffer, to a level that just covers entire surface of gel.
6. Gently remove comb, taking care not to rip the wells.
7. Make sure that the sample wells left by the comb are completely submerged. If "dimples" appear around the wells, slowly add buffer until they disappear.

Too much buffer will channel current over top of gel rather than through gel, increasing the time required to separate DNA. TBE buffer can be used several times; do not discard. If using buffer remaining in electrophoresis box from a previous experiment, rock chamber back and forth to remix ions that have accumulated at either end.

Buffer solution helps to lubricate the comb. Some gel boxes are designed such that comb must be removed prior to inserting the casting tray into the box. In this case, flood casting tray and gel surface with running buffer before removing the comb. Combs removed from a dry gel can cause tearing of the wells.

Cover electrophoresis tank and save gel until ready to continue. Gel will remain in good condition for at least several days if it is completely submerged in buffer.

III. Load Gel and Separate by Electrophoresis

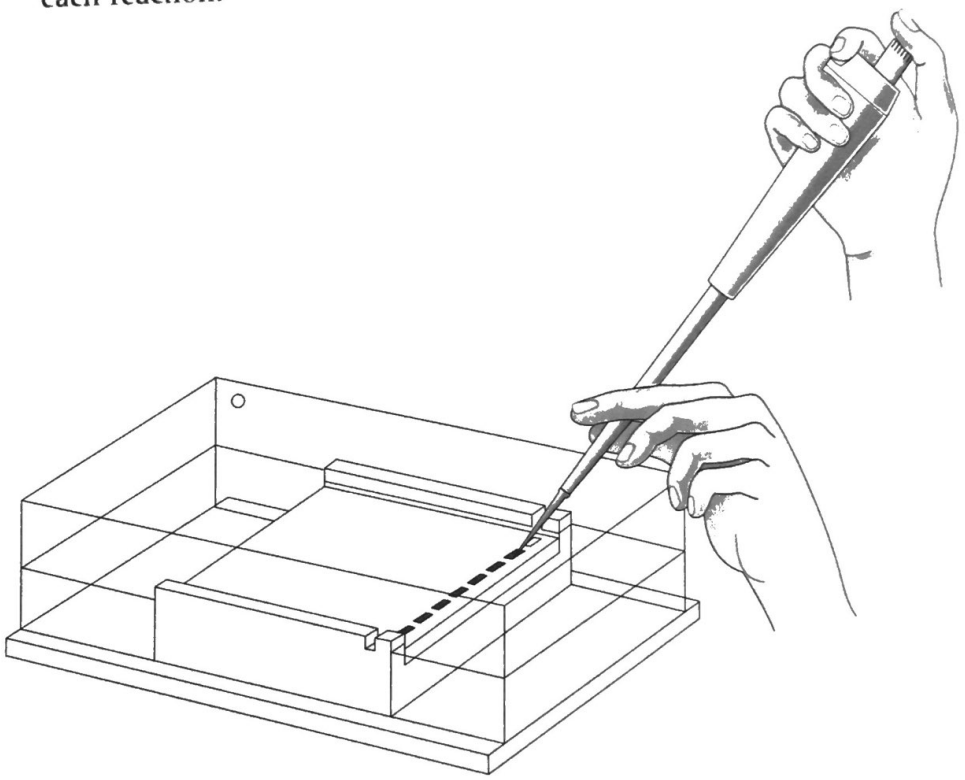
(50–70 minutes)

1. Add loading dye to each reaction. Either

   a. Add 1 µl of loading dye to each reaction tube. Close tube tops, and mix by tapping the tube bottom on the lab bench, pipetting in and out, or pulsing in a microfuge. Make sure that the tubes are placed in a *balanced* configuration in the rotor.

   or

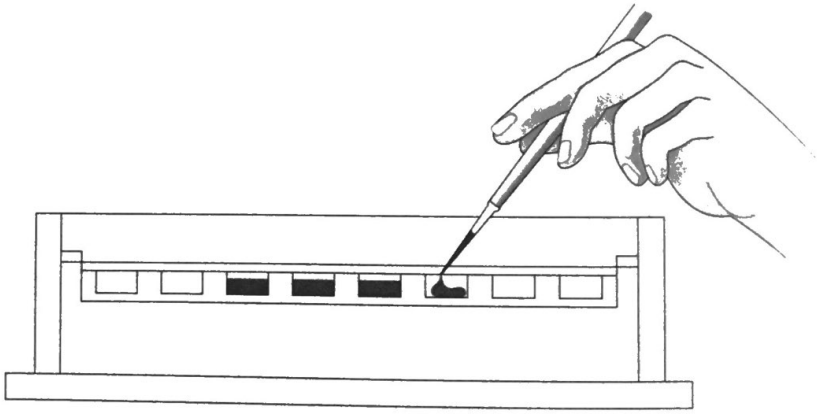
   b. Place four individual droplets of loading dye (1 µl each) on a small square of Parafilm or wax paper. Withdraw contents from the reaction tube and mix with a loading dye droplet by pipetting in and out. Immediately load dye mixture according to Step 2. Repeat successively, *with a clean tip*, for each reaction.



Hand Positions for Loading an Agarose Gel (Step 2)

A piece of dark construction paper beneath the gel box will make the wells more visible.

2. Use a micropipettor to load 10 µl of each reaction tube into a separate well in the gel, as shown in the diagrams. *Use a fresh tip for each reaction.*

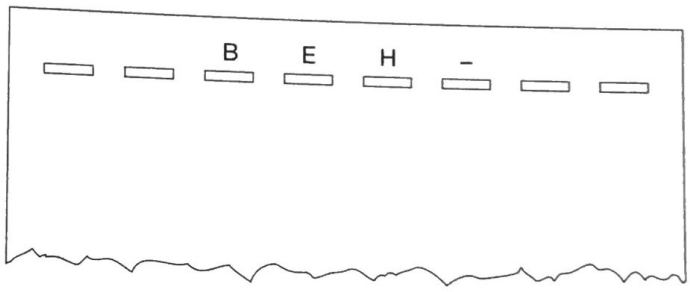


a. Use two hands to steady the micropipettor over the well.

b. Before loading the sample, make sure that there are no bubbles in the wells. If bubbles exist, move them with a micropipettor tip.

c. If there is air in the end of the tip, carefully depress plunger to push the sample to the end of the tip. (If an air bubble forms a "cap" over the well, DNA/loading dye will flow into buffer around edges of well.)

d. Dip micropipettor tip through surface of buffer, center it over the well, and gently depress micropipettor 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 the tip of the micropipettor through the bottom of the gel.*



Alternately, set power supply on lower voltage, and run gel for several hours. When running two gels from the same power supply, the current is double that for a single gel at the same voltage.

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

4. Turn the power supply on, and set to 100–150 volts. The ammeter should register approximately 50–100 milliamperes. If current is not detected, check connections and try again.

5. Separate by electrophoresis for 40–60 minutes. Good separation will have occurred when the bromophenol blue band has moved 4–7 cm from the wells. If time allows, carry out electrophoresis until the bromophenol blue band nears the end of the gel. *Stop* electrophoresis before the bromophenol blue band runs off the end of the gel.

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

7. Carefully remove the casting tray from the electrophoresis box, and slide the gel into a disposable weigh boat or other shallow tray. Label staining tray with your name.

STOP Cover electrophoresis tank and save gel until ready to continue. Gel can be stored in a zip-lock plastic bag and refrigerated overnight for viewing/photographing the next day. However, over longer periods of time, the DNA will diffuse through the gel, and the bands will become indistinct or disappear entirely.

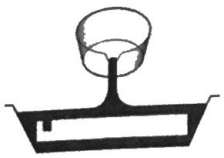
Staining may be performed by an instructor in a controlled area when students are not present.

8. Stain and view gel using one of the methods described in Sections IVA–IVB.

IVA. Stain Gel with Ethidium Bromide and View (Photograph)

(10–15 minutes)

CAUTION
Review Responsible Handling of Ethidium Bromide in the Prelab Notes. Wear latex gloves when staining, viewing, and photographing gel and during clean up. Confine all staining to a restricted sink area. For further information, see Appendix 4.



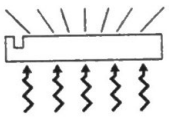
Staining time increases markedly for thicker gels. Do not be tempted to use a higher concentration of ethidium bromide in the staining solution. This will not enhance the DNA bands; it only increases the background staining of the agarose gel itself.

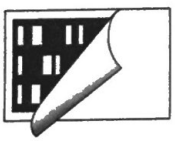
Ethidium bromide solution may be reused to stain 15 or more gels. When staining time increases markedly, dispose of ethidium bromide solution as explained in the Prelab Notes.

1. Flood gel with ethidium bromide solution (1 µg/ml), and allow to stain for 5–10 minutes.
2. Following staining, use a funnel to decant as much ethidium bromide solution as possible from the staining tray back into the storage container.
3. Rinse gel and tray under running tap water.
4. If desired, the gel can be destained in tap water or distilled water for 5 minutes or more to help remove background ethidium bromide from the gel.

STOP Staining intensifies dramatically if rinsed gels set overnight at room temperature. Stack staining trays, and cover top gel with plastic wrap to prevent desiccation.

5. View under UV transilluminator or other UV source.





CAUTION
UV light can damage eyes. Never look at unshielded UV light source with naked eyes. View only through a filter or safety glasses that absorb harmful wavelengths. For further information, see Appendix 4.

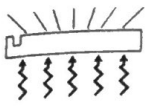
6. Photograph with a Polaroid or digital camera.
7. Take time for responsible cleanup.
   a. Wipe down camera, transilluminator, and staining area.
   b. Decontaminate gels and any staining solution not to be reused.
   c. Wash hands before leaving lab.

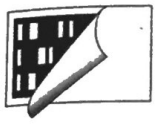
IVB. Stain Gel with Methylene Blue and View (Photograph)

(30- minutes)

Destaining time is decreased by agitating and rinsing in warm water.

1. Wear latex gloves during staining and cleanup.
2. Flood gels with 0.025% methylene blue, and allow to stain for 2· min-utes.
3. Following staining, use a funnel to decant as much methylene bl· ·ion as possible from the staining tray back into the storage containe·.
4. Rinse the gel in running tap water. Let the gel soak for several m· in several changes of fresh water. DNA bands will become increasing· ·ct as the gel destains.





STOP For best results, continue to destain overnight in a *small volume* of water. (Gel may destain too much if left overnight in large volume of water.) Cover staining tray to retard evaporation.

5. View gel over light box; cover the surface with plastic wrap to prevent staining.
6. Photograph with a Polaroid or digital camera.

RESULTS AND DISCUSSION

Agarose gel electrophoresis combined with ethidium bromide staining allows the rapid analysis of DNA fragments. However, prior to the introduction of this method in 1973, analysis of DNA molecules was a laborious task. The original separation method, involving ultracentifugation of DNA in a sucrose gradient, gave only crude size approximations and took more than 24 hours to complete.

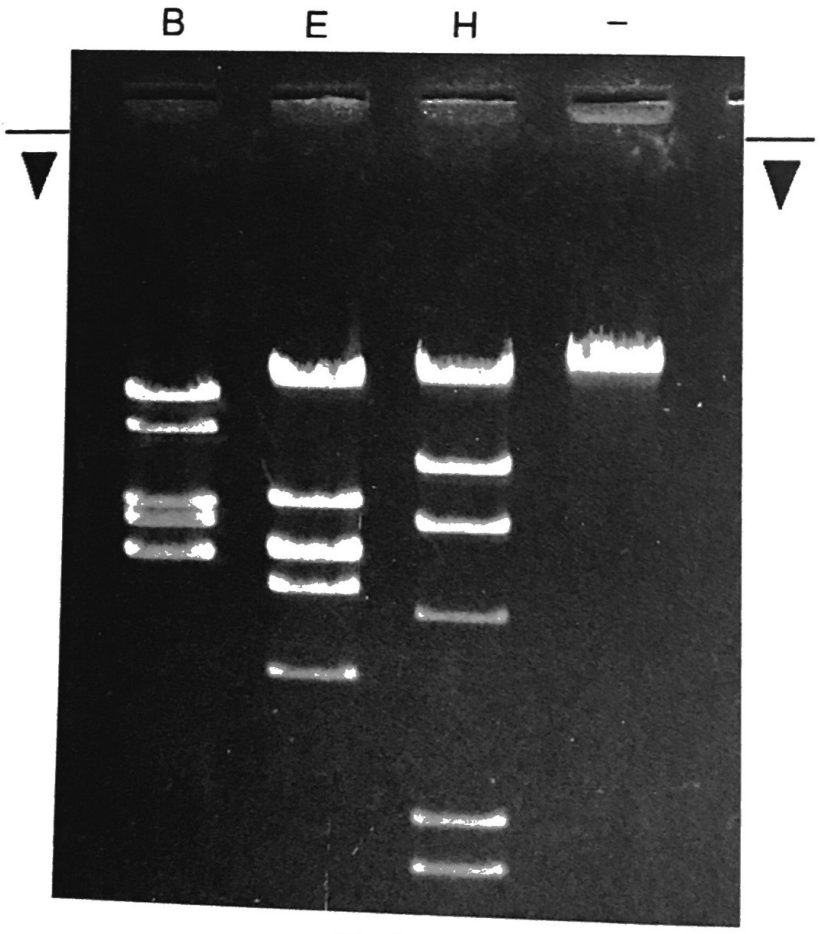
Electrophoresis using a polyacrylamide gel in a glass tube was an improvement, but it could only be used to separate small DNA molecules of up to 2000 bp. Another drawback was that the DNA had to be radioactively labeled prior to electrophoresis. Following electrophoresis, the polyacrylamide gel was cut into thin slices, and the radioactivity in each slice was determined. The amount of radioactivity detected in each slice was plotted versus distance migrated, producing a series of radioactive peaks representing each DNA fragment.

DNA restriction analysis is at the heart of recombinant DNA technology and of the laboratories in this course. The ability to cut DNA predictably and precisely enables DNA molecules to be manipulated and recombined at will. The fact that discrete bands of like-sized DNA fragments are seen in one lane of an agarose gel shows that each of the more than 1 billion λ DNA molecules present in each restriction reaction was cut in precisely the same place.

By convention, DNA gels are "read" from left to right, with the sample wells oriented at the top. The area extending from the well down the gel is termed a "lane." Thus, reading down a lane identifies fragments generated by a particular restriction reaction. Scanning across lanes identifies fragments that have comigrated the same distance down the gel and are thus of like size.

1. Why is water added to tube labeled "–" in Part I, Step 7?
2. What is the function of compromise restriction buffer?
3. What are the two functions of loading dye?
4. How does ethidium bromide stain DNA? How does this relate to the need to minimize exposure to humans?
5. Troubleshooting electrophoresis. What would occur
   a. if the gel box is filled with water instead of TBE buffer?
   b. if water is used to prepare the gel instead of TBE buffer?
   c. if the electrodes are reversed?

6. Examine the photograph of your stained gel (or view on a light box or overhead projector). Compare your gel with the ideal gel shown below and try to account for the fragments of λ DNA in each lane. How can you account for differences in separation and band intensity between your gel and the ideal gel?
7. Troubleshooting gels. What effect will be observed in the stained bands of DNA in an agarose gel
   a. if the casting tray is moved or jarred while agarose is solidifying in Part II, Step 3?
   b. if the gel is run at very high voltage?
   c. if a large air bubble or clump is allowed to set in agarose?
   d. if too much DNA is loaded in a lane?
8. Linear DNA fragments migrate at rates inversely proportional to the $\log_{10}$ of their molecular weights. For simplicity's sake, base-pair length is substituted for molecular weight.
   a. The matrix on the facing page gives the base-pair size of λ DNA fragments generated by a *Hin*dIII digest.



Ideal Gel

*Hin*dIII		*Eco*RI			*Bam*HI		
Dis.	Act. bp	Dis.	Cal. bp	Act. bp	Dis.	Cal. bp	Act. bp
	27,491[a]						
	23,130[a]						
	9,416						
	6,557						
	4,361						
	2,322						
	2,027						
	564[b]						
	125[c]						

[a]Pair appears as a single band on the gel.
[b]Band may not be visible in methylene-blue-stained gel.
[c]Band runs off the end of the gel when bromophenol blue is approximately 2 cm from the end of the gel. When present on the gel, the band is not detected by methylene blue and is usually difficult to detect with ethidium bromide staining.

b. Using the ideal gel shown on the facing page, carefully measure the distance (in millimeters) each *Hin*dIII, *Eco*RI, and *Bam*HI fragment migrated from the origin. Measure from the front edge of the well to the front edge of each band. Enter distances into the matrix. Alternatively, measure the distances on the overhead-projected image of the methylene-blue-stained gel.

c. Match base-pair sizes of *Hin*dIII fragments with bands that appear in the ideal digest. Label each band with kilobase pair (kbp) size. For example, 27,491 bp equals 27.5 kbp.

d. Set up semilog graph paper with distance migrated as the *x* (arithmetic) axis and log of base-pair length as the *y* (logarithmic) axis. Then, plot the distance migrated versus the base-pair length for each *Hin*dIII fragment.

e. Connect data points with a line.

f. Locate on the *x* axis the distance migrated by the first *Eco*RI fragment. Use a ruler to draw a vertical line from this point to its intersection with the best-fit data line.

g. Now extend a horizontal line from this point to the *y* axis. This gives the base-pair size of this *Eco*RI fragment.

h. Repeat Steps f and g for each *Eco*RI and *Bam*HI fragment. Enter the results in the calculated base-pair (Cal. bp) columns for each digest.

i. Enter the actual base-pair size of *Eco*RI and *Bam*HI fragments (as provided by your instructor) into Act. bp column.

j. For which fragment sizes was your graph most accurate? For which fragment sizes was it least accurate? What does this tell you about the resolving ability of agarose gel electrophoresis?

9. DNA fragments of similar size will not always resolve on a gel. This is seen in lane E in the Ideal Gel, where *Eco*RI fragments of 5804 bp and 5643 bp migrate as a single heavy band. These are referred to as a doublet and can be recognized because they are brighter and thicker than similarly sized singlets. What could be done to resolve the doublet fragments?

10. Determine a range of sensitivity of DNA detection by ethidium bromide by comparing the mass of DNA in the bands of the largest and smallest detectable fragments on the gel. To determine the mass of DNA in a given band:

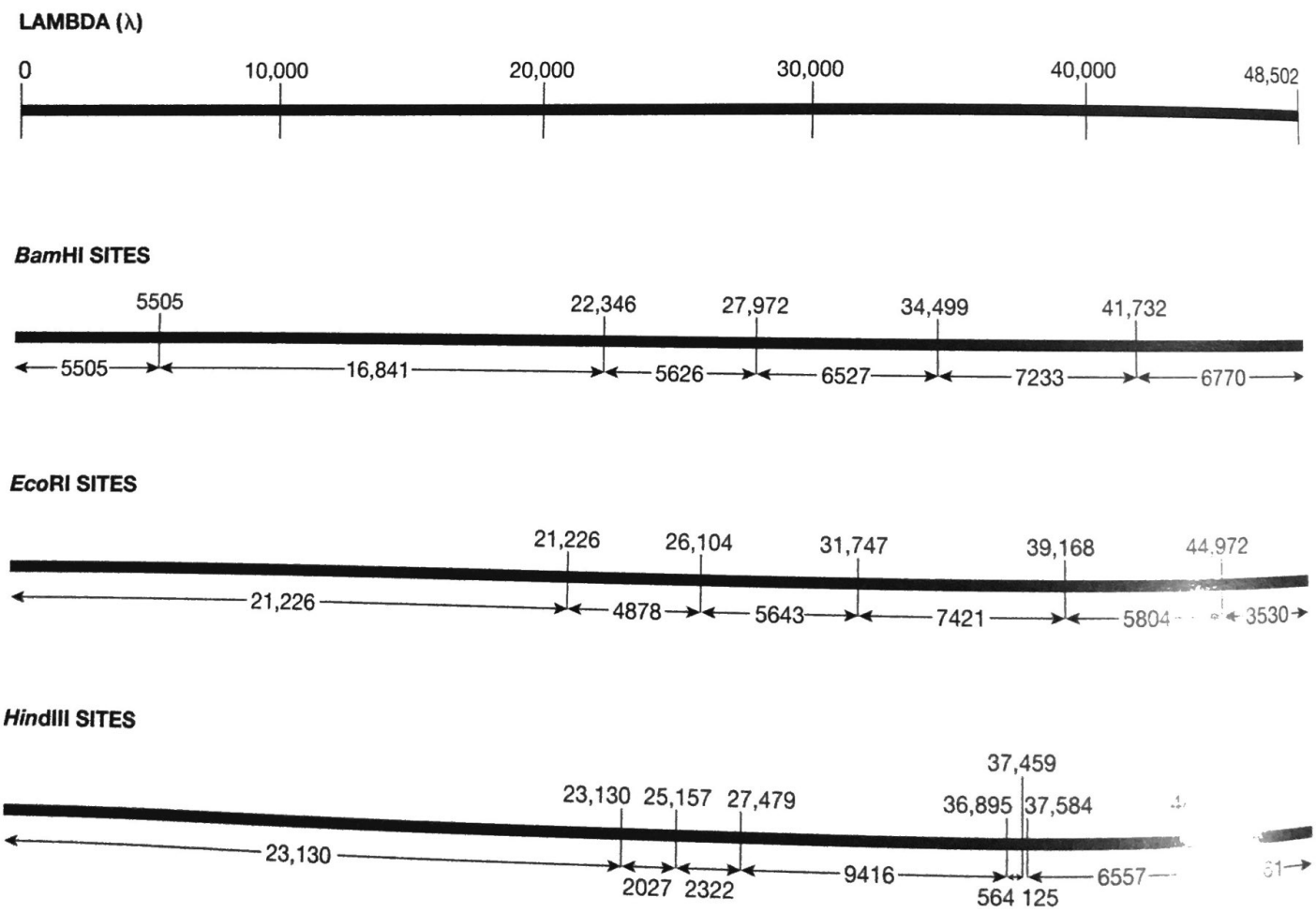
$$\frac{\text{number of bp in fragment} \times (\text{conc. of DNA}) \times (\text{vol. of DNA})}{\text{number of bp in } \lambda \text{ DNA}}$$

For example:

$$\frac{24{,}251 \text{ bp } (0.1 \text{ } \mu g/\mu l) \text{ } (4 \text{ } \mu l)}{48{,}502 \text{ bp}} = 0.2 \text{ } \mu g$$

Now, compute the mass of DNA in the largest and smallest *singlet* fragments on the gel.

11. λ DNA can exist both as a circular molecule and as a linear molecule. At each end of the linear molecule is a single-stranded sequence of 12 nucleotides, called a COS site. The COS sites at each end are complementary to each other and thus can base pair to form a circular molecule. These complementary ends are analogous to the "sticky ends" created by some restriction enzymes. Commercially available λ DNA is likely to be a mixture of linear and circular molecules. This leads to the appearance of more bands on the



Restriction Maps of the Linear λ Genome

gel than would be predicted from a homogeneous population of linear DNA molecules. This also causes the partial loss of other fragments. For example, the left-most *Hin*dIII site is 23,130 bp from the left end of the linear λ genome, and the right-most site is 4361 bp from the right end. The 4361-bp band is faint in comparison to other bands on the gel of similar size. This indicates that a percentage of the DNA molecules are circular—combining the 4361-bp terminal fragment with the 23,130-bp terminal fragment to produce a 27,491-bp fragment. However, the combined 27,491-bp fragment usually runs as a doublet along with the 23,130-bp fragment from the linear molecule.

a. Use a protractor to draw three circles about 3 inches in diameter. These represent λ DNA molecules with base-paired COS sites.

b. Label a point at 12:00 on each circlet 48/0. This marks the point where the COS sites are joined.

c. Use data from the restriction maps of the linear λ genome to make a rough map of restriction sites for *Hin*dIII on one of the circles. Note the situation described above.

d. Next make rough restriction maps of *Bam*HI and *Eco*RI sites on the remaining two circles.

e. What *Bam*HI and *Eco*RI fragments are created in the circular molecules? Why (or why not) can you locate each of these fragments on your gel or the ideal gel above?

FOR FURTHER RESEARCH

1. Some of the circular λ molecules are covalently linked at the COS sites. Other circles are only hydrogen-bonded and can dissociate to form linear molecules. Heating λ DNA to 65°C for 10 minutes linearizes any noncovalent COS circles in the preparation by breaking hydrogen bonds that hold the complementary COS sites together.

   a. Set up duplicate restriction digests of λ DNA with several enzymes. Then heat one reaction from each set at 65°C for 10 minutes, while holding the duplicates on ice. After 10 minutes, immediately place the heated tubes on ice. Relate changes in restriction patterns of heated versus unheated DNA to a restriction map of the circular λ genome as in Question 11 in Results and Discussion.

   b. How can the data generated by this experiment be used to quantify the approximate percentage of circular DNA in your preparation?

2. Design and carry out a series of experiments to study the kinetics of a restriction reaction.

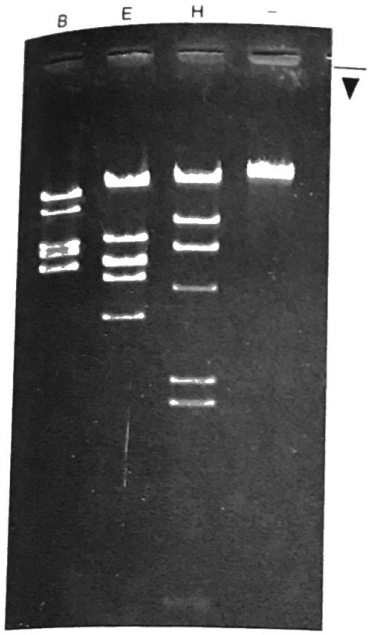
   a. Determine approximate percentage of digested DNA at various time points.

   b. Repeat experiments with several enzyme dilutions and several [illegible] tions.

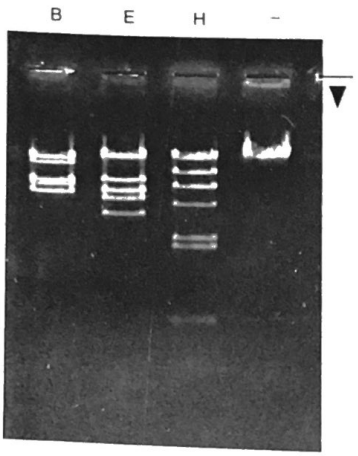
   c. In each case, at what time point does the reaction appear to be comp[illegible]

3. Design and test an assay to determine the relative stabilities of *Bam*HI, *Eco*RI, and *Hin*dIII at room temperature.
4. Determine the identity of an unknown restriction enzyme.
   a. Perform single digests of λ DNA with the unknown enzyme, as well as with several known restriction enzymes. Run the restriction fragments in an agarose gel at 50 volts to produce well-spread and well-focused bands.
   b. For each fragment, plot distance migrated *versus* base-pair size, as in Question 8 in Results and Discussion. Use the graph to determine the base-pair lengths of the unknown fragments and compare with restriction maps of commercially available enzymes.
5. Research the steps needed to purify a restriction enzyme from *E. coli* and characterize its recognition sequence.

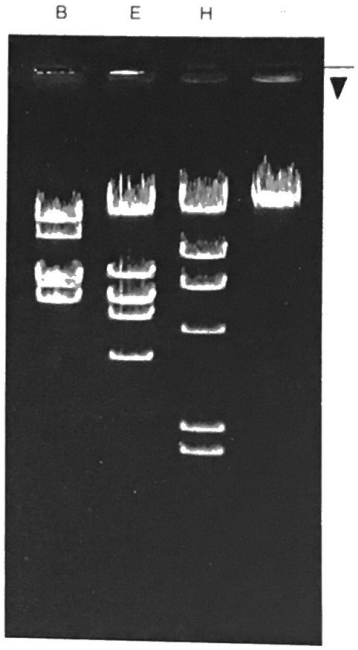
Field Guide to Electrophoresis Effects



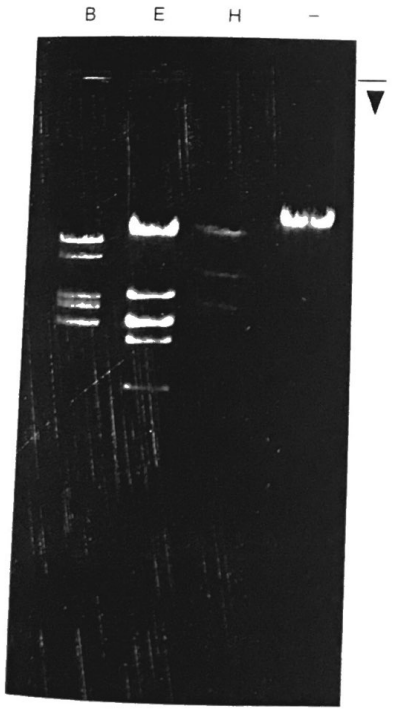
Ideal Gel



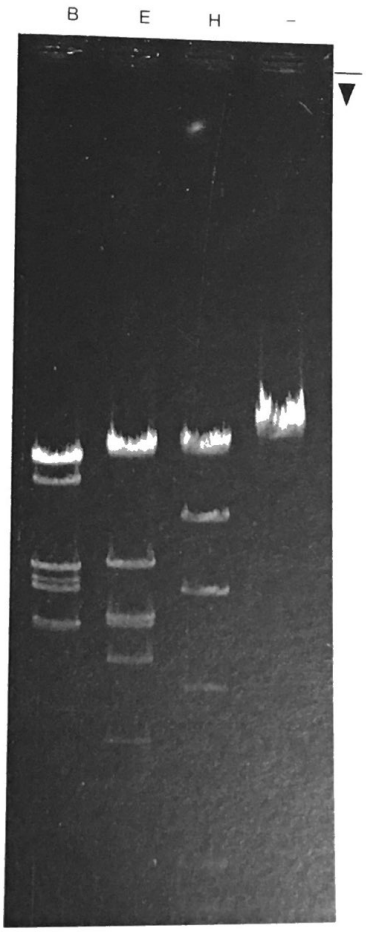
Short Run
Bands compressed. Short time electrophoresing.



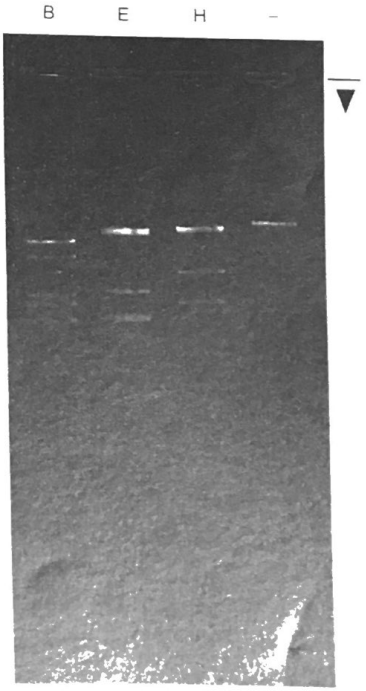
Overloaded
Bands smeared in all lanes. too much DNA in digests.



Punctured Wells
Bands faint in Lanes B and H. DNA lost through hole punched in bottom of well with pipette tip.



Long Run
Bands spread. Long time electrophoresing.



Unde[illegible]
[illegible] lanes. Too
sis.

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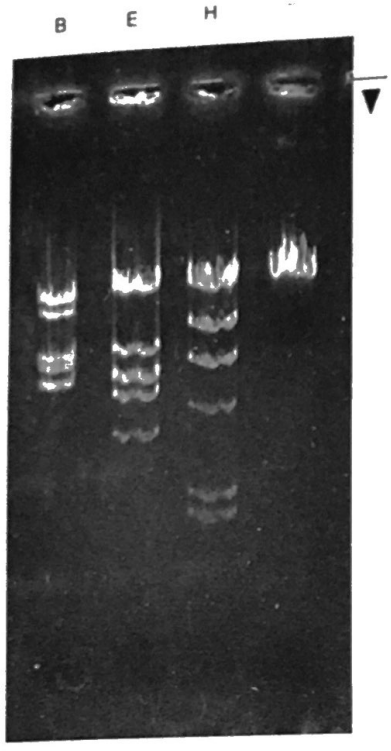
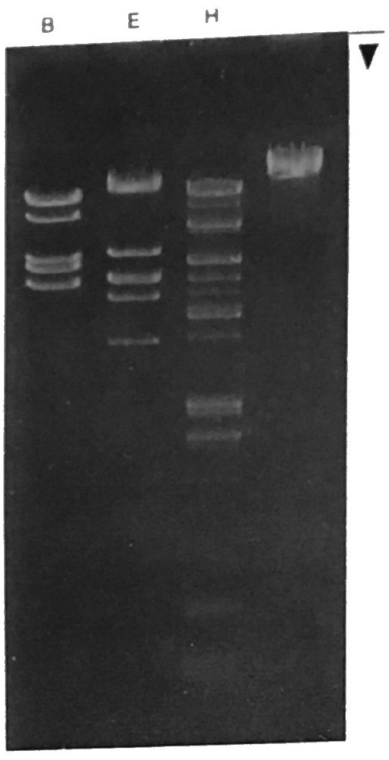
Field Guide to Electrophoresis Effects *(continued)*



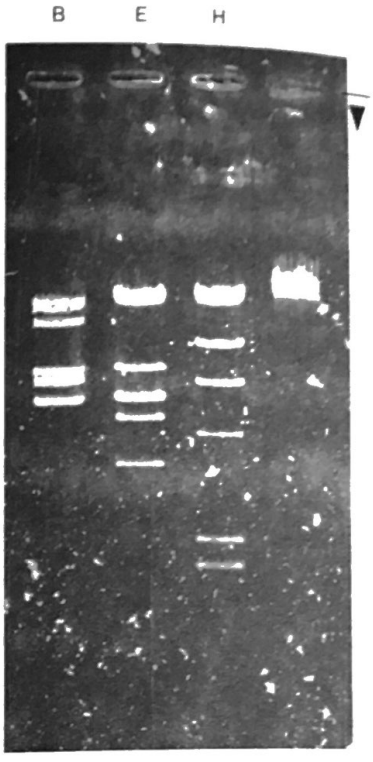
Poorly Formed Wells

Wavy bands in all lanes. Comb removed before gel was completely set.



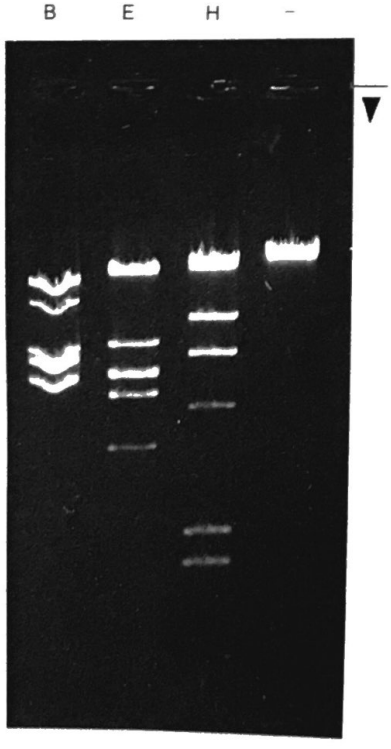
Enzymes Mixed

Extra bands in Lane H. *Bam*HI and *Hin*dIII mixed in digest.



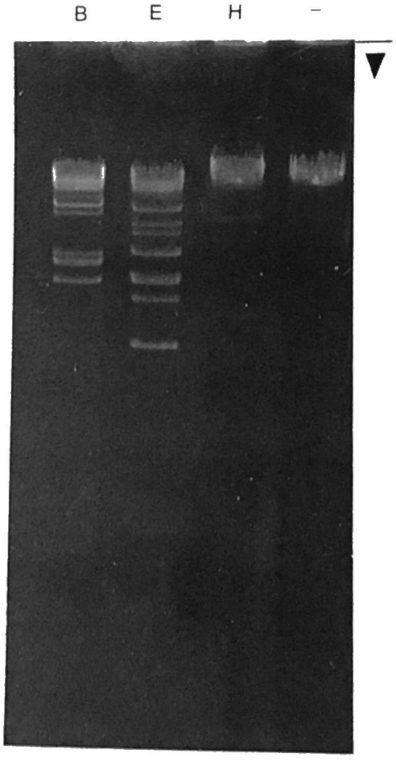
Precipitate

Precipitate in TBE buffer used to make gel.



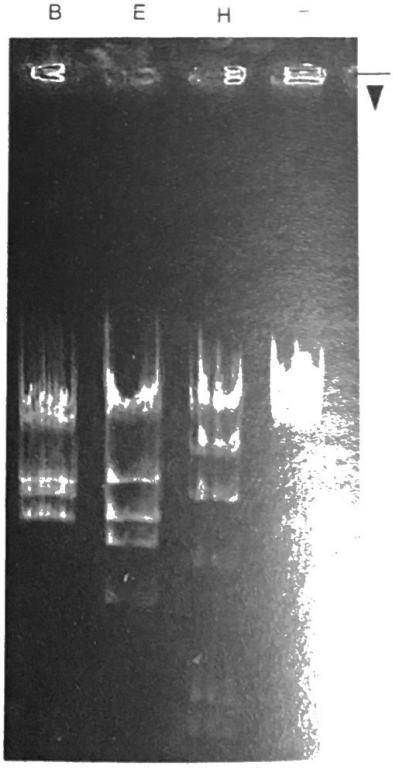
Bubble in Lane

Bump in band in Lane B. Bubble in lane.



Incomplete Digest

Bands faint in Lane H. Very little *Hin*dIII in digest. Also, extra bands are present in Lanes B and E.



Gel Made with Water

Bands smeared in all l
Gel made with wate
wrong concentration of
buffer.