Chapter 9

A Laboratory Introduction to DNA Restriction Analysis*

David A. Micklos [1] ***and*** **Greg *A.* Freyer** [2]

[1] DNA Learning Center of Cold Spring Harbor Laboratory
[2] Columbia University College of Physicians and Surgeons

David Micklos is director of the DNA Learning Center of Cold Spring Harbor Laboratory. He received a bachelor's degree in biology from Salisbury State College, Maryland and a master's degree in science journalism from the University of Maryland. Prior to joining Cold Spring Harbor Laboratory, he worked as a survey researcher for Hill & Knowlton, Inc. and a Peace Corps Volunteer in Botswana Africa. He has taught at both the high school and college levels.

Greg Freyer is an assistant professor in the radiation oncology department of Columbia University College of Physicians and Surgeons. He received a bachelor's degree in biology from the University of Cincinnati and a doctorate in biochemistry from the University of Missouri. He did postdoctoral work at Cold Spring Harbor Laboratory and was associate researcher at Memorial Sloan Kettering Cancer Center.

In 1985, they founded the DNA Literacy Program as a means to train instructors to better teach recombinant-DNA technology at the college and advanced high school levels. The most visible aspect of the program has been the Vector DNA Science Workshop that has now been taken be more than 600 high school and college educators from New York to California, and from Wisconsin to Alabama. The week-long course gives teachers practical experience with recombinant-DNA techniques and addresses the practical aspect of implementing DNA science labs. The program is now based in the DNA Learning Center, the nation's first "museum" devoted entirely to biotechnology education.

For more information about the DNA Literacy Program and the Vector DNA Science Workshops, contact the DNA Learning Center at (516) 367-7240.

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A LABORATORY INTRODUCTION TO DNA RESTRICTION ANALYSIS*

David A. Micklos
DNA Learning Center of Cold Spring Harbor Laboratory

Greg A. Freyer
Columbia University College of Physicians and Surgeons

PURPOSE

This laboratory demonstrates that the DNA molecule can be precisely manipulated and that it behaves as predicted by the structure determined by James Watson and Francis Crick in 1953. The genotypic analysis performed in this experiment is the basis for DNA fingerprinting and the starting point in the construction of recombinant-DNA molecules.

In this protocol, purified DNA from the bacteriophage lambda is digested with the restriction endonucleases EcoRI, BamHI, and HindIII. Each enzyme has five or more recognition sites in lambda DNA and, therefore, produces six or more restriction fragments.

The digested DNA is loaded into a 1% agarose gel. An electric field applied across the gel separates the DNA fragments according to size. The bands of DNA within the gel are made visible by staining with either ethidium bromide or methylene blue.

BACKGROUND INFORMATION

Restriction Endonucleases

Restriction endonucleases, or restriction enzymes, are used as molecular scalpels to cut DNA in a precise and predictable manner. They are members of the class of nucleases that display the general property of breaking the phosphodiester bonds that link adjacent nucleotides in DNA and RNA molecules. Endonucleases cleave nucleic acids at internal positions, while exonucleases progressively digest from the ends of nucleic acid molecules.

There are three major classes of restriction endonucleases. Type-I and Type-III enzymes have both restriction (cutting) and modification (methylating) activity. Both types cut at sites some distance from their recognition sequences; ATP is required to provide energy for movement of the enzyme along the DNA molecule from recognition to cleavage site.

*Abridged from DNA Science: A First Laboratory Course in Recombinant-DNA Technology, now in preparation. Presented as a workshop by William Gette.

Type-II restriction enzymes are for several reasons most useful to molecular biologists:

1) Each has only restriction activity; modification activity is carried by a separate enzyme.
2) Each cuts in a predictable and consistent manner at a site within or adjacent to the recognition sequence.
3) They do not require ATP for cutting activity.

Today, more than 900 Type-II enzymes have been isolated from a variety of prokaryotic organisms. Enzymes have been identified that recognize 130 different nucleotide sequences; over 70 types are commercially available. To avoid confusion, restriction endonucleases are named according to the following nomenclature:

1) The first letter is the initial letter of the genus name of the organism from which the enzyme is isolated.
2) The second and third letters are the initial letters of the organism's species name. (Since they are derived from scientific names, the first three letters of the endonuclease name are italicized.)
3) A fourth letter, if any, indicates a particular strain of organism.
4) According to most recent nomenclature, a Roman numeral indicates the order in which enzymes, isolated from the same organism and strain, are eluted from a chromatography column. However, an earlier nomenclature, in which Roman numerals indicate the order of discovery, is still used.

Each restriction endonuclease scans along a DNA molecule, stopping only when it recognizes a specific sequence of nucleotides. Most restriction enzymes recognize a four- or six-nucleotide sequence. Assuming that the four component nucleotides (A,C,T,G) are distributed randomly within a DNA molecule, then any four-nucleotide recognition site will occur, on average, every 256 nucleotides (4 x 4 x 4 x 4). A six-nucleotide recognition site is likely to occur every 4,096 nucleotides (4 x 4 x 4 x 4 x 4 x 4).

Many restriction enzymes have recognition sites that are composed of symmetrical, or palindromic, nucleotide sequences. This means that the recognition sequence read forward on one DNA strand is identical to the sequence read backward on its complementary strand Put another way, the 5'-to-3' sequence is identical on each DNA strand.

In a general sense, the terms 5' and 3' refer to either end of a single DNA strand. Specifically, they designate carbon atoms on opposite sides of the deoxyribose ring that are joined to form a single strand DNA polymer. The 5' carbon is linked, through ester bonds with an intervening phosphate, to the 3' carbon of the adjacent nucleotide. By convention, the nucleotide sequence is "read from the 5' end to the 3' end of the DNA strand. The situation is confused in duplex DNA, where the strands are "antiparallel;" that is, 5'-to-3' reads in opposite directions on the two complementary strands.

To carry out a restriction reaction, solubilized DNA is incubated at 37ºC with one or more endonucleases. The reaction takes place in a buffered solution that provides salt conditions necessary for optimum enzyme activity. Within or very near the recognition site, the enzyme catalyzes a hydrolysis reaction that uses water to break a specific phosphodiester linkage on each strand of the DNA helix. Two DNA fragments are produced, each with a phosphate group at the 5' end and a hydroxyl group at the 3' end.

Some endonucleases, such as HindII, cut cleanly through the DNA helix by cleaving both complementary strands at the same nucleotide position, typically in the center of the recognition site. These enzymes leave flush- or blunt-ended fragments.

Other endonucleases cleave each strand off-center in the recognition site, at positions two to four nucleotides apart. This creates fragments with exposed ends of short, single-stranded sequences. Various enzymes leave single-stranded "overhangs" on either the 5' or 3' ends of the DNA fragments. EcoRI, BamHI, and HindIII, for example, each leave 5' overhangs of four nucleotides.

Single-stranded overhangs, also called cohesive or "sticky" ends, are extremely useful in making recombinant-DNA molecules. These exposed nucleotides serve as templates for realignment, allowing complementary nucleotides to hydrogen bond to one another. A given restriction enzyme cuts all DNA in exactly the same fashion, regardless of whether the source is a bacterium, a plant, or a human being. Thus, any sticky-ended fragment can be recombined with any other fragment generated by the same restriction enzyme.

For each restriction Type-II endonuclease there is a corresponding modifying enzyme that blocks restriction activity by methylating DNA within the recognition sequence. The protruding methyl group presumably prevents binding by interfering with the close molecular interaction between the restriction enzyme and its recognition site. EcoRI methylase, for example, adds a methyl group to the second adenine residue within the EcoRI recognition site.

Nomenclature, Recognition Sequences, and Cutting Action of *EcoRI*, *BamHI*, and *HindIII*

EcoRI	E	= genus Escherichia	Recognizes	G↓A A T T C C T T A A↑G
	co	= species coli		
	R	= strain RY13		
	I	= first endonuclease isolated		
BamHI	B	= genus Bacillus	Recognizes	G↓G A T C C C C T A G↑G
	am	= species amyloliquifaciens		
	H	= strain H		
	I	= first endonuclease isolated		
HindIII	H	= genus Haemophilus	Recognizes	A↓A G C T T T T C G A↑A
	in	= species influenzae		
	d	= strain Rd		
	III	= third endonuclease isolated		

Agarose Gel Electrophoresis

Electrophoresis means literally "to carry with electricity." The method takes advantage of the fact that, as an organic acid, DNA is negatively charged. DNA owes its acidity to phosphate groups that alternate with deoxyribose to form the rails of the double helix. In solution, hydrogen ions are liberated from hydroxyl groups, leaving negatively-charged oxygen ions that radiate from phosphates on the outside of the DNA molecule. When placed in an electrical field, DNA molecules are attracted toward the positive pole and repelled from the negative pole.

Sorting of differently-sized molecules is achieved by electrophoresing DNA fragments through a gel-like matrix that acts as a molecular sieve through which smaller molecules can move more quickly than larger ones. The distance moved by a DNA fragment is

inversely proportional to the logarithm of its molecular weight. Thus, in a given period of time, smaller restriction fragments migrate relatively far from the origin compared to larger fragments.

A research team at Cold Spring Harbor Laboratory, led by Joseph Sambrook, introduced two important refinements to electrophoresis that made possible rapid analysis of DNA restriction fragments.

First, they used a matrix composed of agarose, a highly-purified form of agar. Agarose efficiently separates larger DNA fragments ranging in size from 100 to more than 50,000 nucleotides. DNA fragments in different size ranges are separated by adjusting the agarose concentration. A low concentration, down to 0.3% produces a looser gel that separates larger fragments. A high concentration, up to 2%, produces a stiffer gel that resolves small fragments.

Second, they used a fluorescent dye, ethidium bromide, to stain DNA bands in agarose gels. Following a brief staining step, the fragment pattern is viewed directly under ultraviolet light. As little as 10 nanograms (0.01 micrograms) of DNA can be detected in a band. (An earlier method was laborious and required the use of radio-labeling.)

Currently used electrophoresis methods are essentially identical to those published by the Cold Spring Harbor team in 1973. Molten agarose is poured into a casting tray in which a "comb" is suspended. As it cools, the agarose hardens to form a jello-like substance consisting of a dense network of cross-linked molecules. The solidified gel slab is immersed in a chamber filled with buffer solution, which contains ions needed to conduct electricity. The comb is removed, leaving wells into which DNA samples are loaded.

Just prior to loading, the digested DNA is mixed with a "loading dye" consisting of sucrose and one or more visible dyes. The dense sucrose solution weights the DNA sample, helping it to sink into the well when loaded. The negatively-charged dye molecules do not interact with the DNA, but migrate independently toward the positive pole. For example, the commonly used marker bromophenol blue migrates at a rate equivalent to a DNA fragment of approximately 300 nucleotides (in a 1% gel). Thus, the visible movement of the dye allows one to monitor the relative migration of the unseen DNA bands.

Current supplied through electrodes at either end of the chamber creates an electrical field across the gel. The negatively-charged DNA fragments move from the wells into the gel, migrating through the pores in the matrix toward the positive pole of the electrical field.

Following electrophoresis, the gel is soaked in a dilute solution of ethidium bromide. The stain diffuses throughout the gel, becoming concentrated in regions where it binds to DNA fragments. (Alternately, ethidium bromide is incorporated into the gel and electrophoresis buffer prior to electrophoresis.) A planar group of the ethidium bromide molecule intercalates between the stacked nucleotides of the DNA helix, staining DNA bands in the gel.

The stained gel is then exposed to medium wavelength ultraviolet (UV) light. The DNA/ethidium bromide complex strongly absorbs UV light at 300 nm, retains some of the energy, and reemits visible light at 590 nm. Thus, the stained restriction fragments appear as fluorescent orange bands in the gel, when exposed to ultraviolet light.

It is important to understand that a band of DNA seen in a gel is not a single DNA molecule. Rather, the band is a collection of millions of DNA molecules, all of the same nucleotide length.

REAGENTS, SUPPLIES , AND EQUIPMENT

0.1 µg/µl *lambda* DNA
restriction enzymes
EcoRI
BamHI
HindIII
2X restriction buffer
loading dye
distilled water
1% agarose solution
1X TBE buffer
1µg/ml ethidium bromide solution
(or 0.025% methylene blue solution)
0-10 µl micropipetor + tips
electrophoresis box
power supply
1.5 ml tubes
permanent marker
test tube rack
37ºC incubator
microfuge (optional)
transilluminator/camera
(optional)
parafilm (optional)

PRELAB PREPARATION

1) Aliquot for each lab group:

20 µl 0.1 µg/µl *lambda* DNA (store on ice)
25 µl 2x restriction buffer (store on ice)
2 µl each of *BamHI, EcoRI,* and *HindIII* (store on ice)
distilled water
loading dye

(NOTE: The volumes and concentrations of DNA have been optimized for ethidium bromide staining, which is the most rapid and sensitive method. If you prefer to use methylene blue staining, increase DNA concentration to 0.3-0.4 µg/µl Volumes used remain as stated.)

2) Prepare 1% agarose solution (40-50 ml per lab group). Keep agarose liquid in a hot water bath (about 55ºC) throughout lab period.

3) Prepare 1X Tris-Borate-EDTA (TBE) buffer for electrophoresis (400-500 ml per lab group).

(NOTE: The volumes of agarose solution and TBE buffer needed vary according to electrophoresis apparatus used. The volumes used here are based on typical "mini-gel" systems.)

4) Set water bath to 37ºC.

(NOTE: An aquarium heater makes a very good constant-temperature bath.)

LABORATORY PROTOCOL

I. Prepare Restriction Digest (30 minutes, including incubation)

1) Label four 1.5 ml tubes, in which you will perform restriction reactions:

B *BamHI*
E *EcoRI*
H *HindIII*
-- no enzyme

2) Set up matrix to use as checklist as reagents are added to each reaction:

	DNA	2X buffer	BamHI	EcoRI	HindIII	H20
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

3) Collect reagents, and set in test tube rack on lab bench.

4) Set micropipetor to 4 µl, and add DNA to each reaction tube. Touch pipet tip to side of reaction tube, as near bottom as possible, to create capillary action to pull solution out of tip. (It is not necessary to change tips when adding same reagent.)

5) Buffer should be added to reaction tubes before enzymes. Use fresh tip to add 5 µl restriction buffer to clean spot on each reaction tube. (Same tip may be used for all tubes, provided tip is not touched to solution already in tubes.)

6) Use fresh tips to add 1 µl *EcoRI*, *BamHI*, and *HindIII* to appropriate tubes.

7) Use fresh tip to add 1 µl deionized water to -- Tube.

8) Pool and mix reagents:

-- by sharply tapping tube bottom on lab bench.

OR

-- with a short, several-second pulse in microfuge. (Make sure tubes are placed in balanced configuration in rotor.)

9) Place reaction tubes in 37ºC water bath.

10) Let reactions incubate for minimum 20 minutes. (Reactions can be incubated for longer periods of time. After several hours, enzymes lose activity and reaction stops.)

** OPTIONAL STOP POINT: Following incubation, reactions can be frozen at -10 to -20°C until ready to continue. Thaw reaction before continuing to Step III-1.

II. Cast 1% Agarose Gel (20 minutes)

1) Seal ends of gel-casting tray and insert comb. Place gelcasting tray out of the way on lab bench, so that agarose poured in next step can set undisturbed. (Gel is cast directly in box in some electrophoresis apparatuses.)

2) Carefully pour enough agarose solution, *at approximately* 55ºC, into casting tray to fill to depth of about 5 mm. (Liquid agarose container should be just cool enough to hold comfortably in the hand.) Gel should cover only about 2-4 mm height of comb teeth. Large bubbles or solid debris can be moved to sides or ends of tray, while gel is still liquid, using toothpick or pipet tip.

   Do not move or jar casting tray whileagarose is solidifiing. As it polymerizes -- in about 10-15 minutes -- agarose will change from clear to cloudy. Touch corner of agarose away from comb to test if gel has solidified.

3) When agarose is set, unseal ends of casting tray. Place tray on platform of gel box, so that comb is at negative end.

4) Fill box with 1X Tris-Borate-EDTA (TBE)buffer to level that just covers entire surface of gel. Too much buffer will channel current over top rather than through gel, increasing time required to separate DNA. (TBE buffer can be used several times; do not discard until told to do so.)

5) Gently remove comb, taking care not to rip gel. (Buffer solution helps lubricate comb teeth.)

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

** OPTIONAL STOP POINT: Cover electrophoresis tank and save gel until ready to continue. Gel will remain in good condition for at least several days, so long as it is completely submerged in buffer. (Gels can be cast and sealed in a small volume of buffer inside zip-lock plastic or "seal-a-meal" bags. Before sealing, squeeze out as much air as possible. Include enough buffer to completely surround gel, and refrigerate.)

III. Load Gel and Electrophorese (40-60 minutes)

1) Add 1-2 µl loading dye to each sample:

   -- Set up four individual droplets of loading dye (1-2 µl each) on small square of parafilm or wax paper. Withdraw contents from reaction tube, and mix with a loading dye droplet by pipeting in and out. Immediately load dye mixture according to Step 3. Repeat successively, with clean tip, for each reaction.

   OR

   -- Add 1-2 µl loading dye to each reaction tube. Mix by tapping on lab bench, pipeting in and out, or spinning tubes for short several-second pulse in microfuge. *(Make sure tubes are placed in a balanced configuration in rotor.)* This mixes loading dye with reactants.

3) Use micropipetor to load entire contents of each reaction tube into separate well in gel, as shown in diagram below. (A piece of dark construction paper beneath the gel box or a piece of black electrical tape affixed to gel box bottom makes wells easily visible.)

- Steady pipet over well using two hands.
- Be careful to expel any air in micropipet tip end before loading gel. (If air bubble forms "cap" over well, DNA/loading dye will flow into buffer around edges of well.)
- *Be careful not to punch tip of pipet through bottom of gel.*
- Gently depress pipet plunger to slowly expel sample into appropriate well. If tip is centered over well, reaction solution will sink to bottom of well.

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

5) Set power supply at 100-150 volts, and turn unit on. (Alternately, set power source on lower voltage and let gel run for several hours. Check to monitor progress of bromophenol blue band.)

6) Ammeter should register approximately 50-100 milliamps. (Current through two gels is double that for single gel at same voltage.) This confirms that current is flowing through gel. If no current is detected, check connections, and try again.

7) Shortly after current is applied, loading dye can be seen moving through gel toward positive side of electrophoresis apparatus. It will appear as a blue band -- eventually resolving into two bands of color.

The faster-moving, purplish band is the dye bromophenol blue. The slower-moving, aqua band is xylene cyanol. Bromophenol blue migrates through gel at same rate as a DNA fragment approximately 300 basepairs long.

8) Electrophorese for 20-60 minutes. Good separation will have occurred when the bromophenol blue band has moved 4-8 cm from wells. *CAUTION: If gel is allowed to run for too long, dye and DNA samples will electrophorese out end of gel into buffer.*

9) Turn off power, and disconnect leads.

10) Carefully remove gel from electrophoresis chamber, and place in disposable weigh boat or other shallow tray.

** OPTIONAL STOP POINT: Gel can be stored in a zip-lock plastic bag for viewing and photographing the next day. However, over time DNA will diffuse through gel, and bands become indistinct.

11) Stain and view gel using one of following methods.

IVA. Stain with Ethidium Bromide and View (10-15 minutes)

INSTRUCTOR: Before using this method, familiarize yourself with *Safety Note: Responsible Handling of Ethidium Bromide* at the end of this laboratory.

Your instructor may complete staining.

RUBBER GLOVES MUST BE WORN DURING STAINING, PHOTOGRAPHY, AND CLEANUP.

1) Flood gel with ethidium bromide solution (1 μg/ml).

2) Allow to stain for 5-10 minutes. (Staining time depends on thickness of gel. Gentle rocking of staining tray will aid diffusion of ethidium bromide into gel.)

3) Use funnel to drain off ethidium bromide solution into storage container for reuse.

4) Rinse gel and tray under running tap water to remove excess ethidium bromide solution. (Chlorine in water will inactivate traces of ethidium bromide.)

5) If desired, gels can be destained in tap or distilled water for 5 or more minutes to remove background ethidium bromide.

** OPTIONAL STOP POINT: Staining intensifies dramatically if rinsed gels set overnight. Cover or wrap gel in plastic wrap to prevent dessication. (Weigh boats containing stained and rinsed gels simply can be stacked together overnight.)

6) View under ultraviolet transilluminator or other W source.

CAUTION: Ultraviolet light can damage your eyes. Never look at unshielded W light source with naked eyes. Only view through filter or safety glasses that absorb harmful wavelengths.

IVB. Stain with Methylene Blue and View (30+ minutes)

1) Flood gel with 0.025% methylene blue.

2) Allow to stain for approximately 15 minutes.

3) Retrieve stain and rinse gel in tap water. Let gel soak for several minutes in several changes of fresh water. DNA bands will become increasingly distinct as gel destains. (Heat and agitation facilitate rapid destaining.)

OPTIONAL STOP POINT: For best results, continue to destain overnight in small volume of water. Cover container to retard evaporation. (Gel may destain too much if left overnight in large volume of water.)

4) View gel over light box.

V. Photograph(10-15 minutes)

1) FOR W PHOTOGRAPHY..

- Use Polaroid high-speed film Type 667 (ASA 3,000).

- Set camera aperture to f/8 and shutter speed to B (bulb). Make a 2-4 second exposure by depressing shutter release for desired length of time. (Shutter remains open as long as release is depressed.)

FOR WHITE-LIGHT PHOTOGRAPHY:

- For Polaroid film Type 667, set aperture to f/8 and shutter speed to 1/125 second.

Exposure times vary according to mass of DNA in lanes, level of staining, degree of background staining, thickness of gel, and density of filter. Experiment to determine best exposure. When possible, stop lens down (higher f/number) to increase depth of field and sharpness of bands.

2) ***Place left hand firmly on top of camera to steady.*** Firmly grasp small white tab, and pull straight out from camera. This causes a large yellow tab to appear.

3) Grip yellow tab in center, and in one steady motion, pull straight out from camera. This starts development.

4) Let develop for recommended time -- 45 seconds at room temperature. Do not disturb print while developing.

5) After full development time has elapsed, separate print from negative. Begin peeling back at end nearest yellow tab.

CAUTION: Avoid getting caustic developing jelly on skin or clothes. If jelly gets on skin, flush immediately with plenty of water.

DISCUSSION

1) Troubleshooting Electrophoresis

What electrophoresis effect would occur:

a) If gel box is filled with water instead of TBE buffer?

b) If water is used to prepare gel instead of TBE buffer?

What effect would be observed in the stained bands of DNA in an agarose gel:

C) If casting tray is moved or jarred while agarose is solidifying in step II.2?

d) If gel is run at very high voltage?

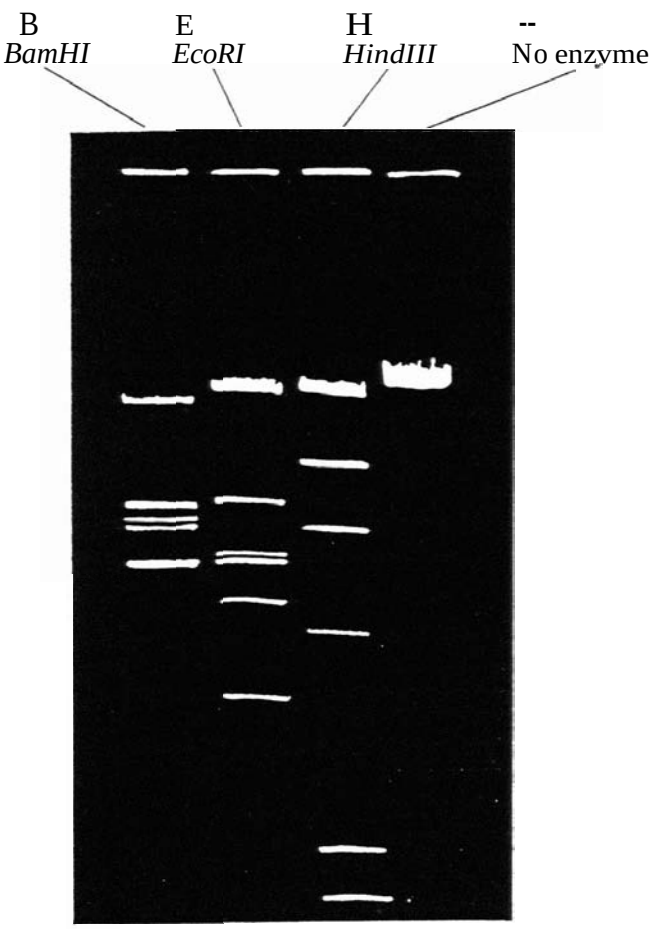
e) If a large air bubble or clump is allowed to set in agarose?

f) If too much DNA is loaded in a lane?

2) Two small restriction fragments of nearly the same size appear as a single band, even when sample is run to very end of the gel. What could be done to resolve the fragments? Why would it work?

3) Shown below is an ideal fragment pattern for a digest of *lambda* DNA with *BamHI*, *EcoRI*, and *HindIII*. Using the ideal gel as a reference, how many fragments can be identified on the photograph of gel obtained in this laboratory?

Account for differences in separation and band intensity between the experimental gel and the ideal gel.



4) Linear DNA fragments migrate at rates inversely proportional to the log10 of their molecular weights. For simplicity's sake, basepair length is substituted for molecular weight.

a) Construct a matrix like the one shown below, which gives the size in basepairs (bp) of lambda DNA fragments generated by BamHI and HindIII digests:

BamHI		EcoRI		HindIII	
bp	dis	bp	dis	bp	dis
16,841				*23,130	
7,233				9,416	
6,770				6,682	
6,527				4,361	
*5,626				2,322	
*5,505				2,027	
				**564	
				**125	

*Pair appears as single band. **Does not appear on this gel.

b) Use ideal gel shown above. For each of the three digests, carefully measure distance (in mm) each fragment migrated from the origin. Measure from front edge of well to front edge of each band. Enter distances into matrix.

c) Match sizes of known BamHI and HindIII fragments with bands that appear on the ideal digest. Label each band with kilobasepair (kbp) size. For example 16,841 bp equals 16.8 kbp.

d) Set up semi-log graph paper with distance migrated as the X (arithmetic) axis and log basepair length as the Y (logarithmic) axis. Then plot distance migrated vs. basepair length for each BamHI and HindIII fragment.

(Alternately, calculate log bp and plot directly on the Y axis of standard graph paper. To finish problem, compute anti-logs in step g.)

e) Connect data points with a best-fit line.

f) Locate on X axis the distance migrated by one EcoRI fragment. Using a ruler, draw a vertical line from this point to its intersection with the best-fit data line.

g) Now extend a horizontal from this point to the Y axis. This gives the basepair size of this EcoRI fragment.

h) Repeat steps f and g for each EcoRI fragment.

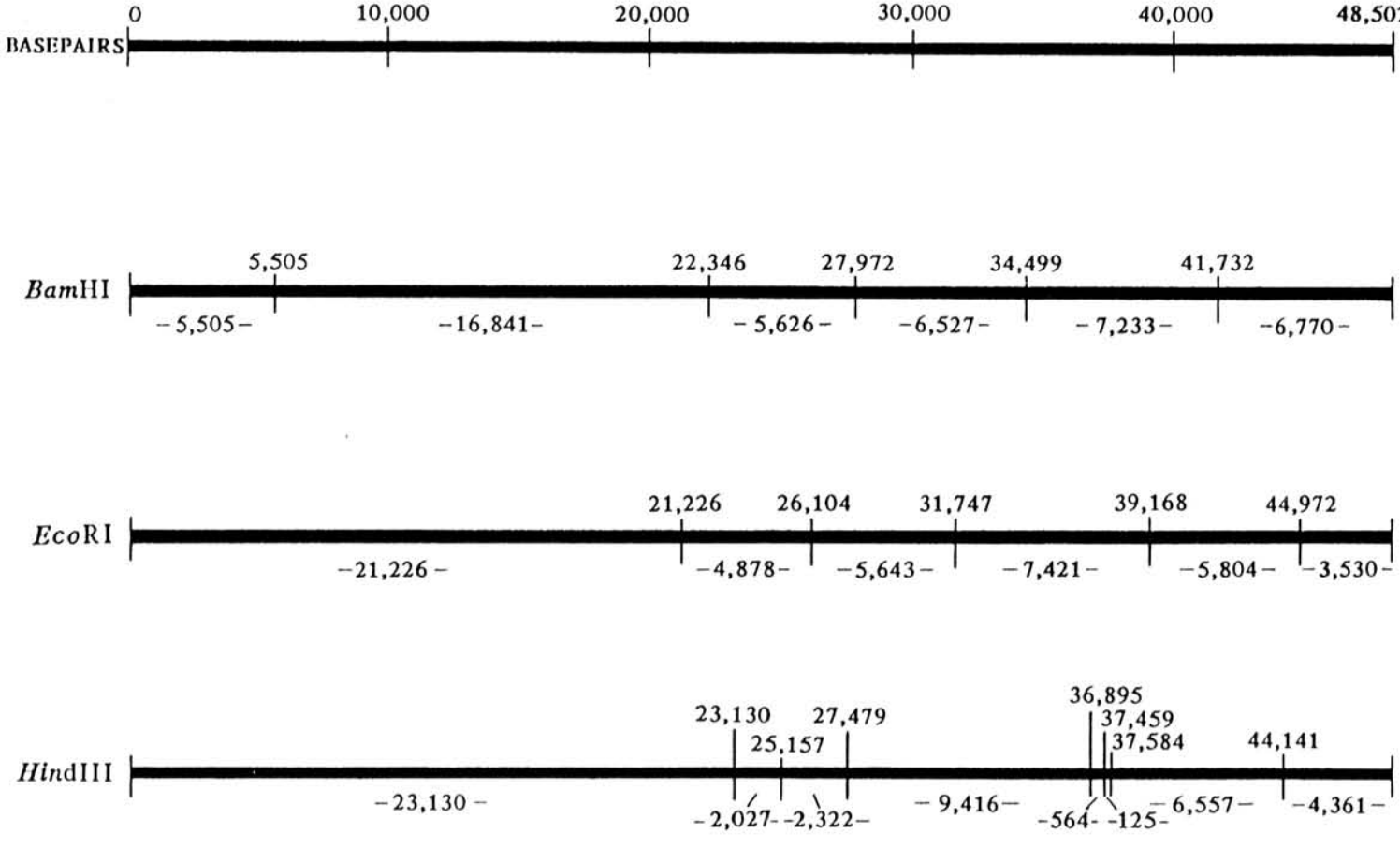
For Further Experimentation

5) Design and carry out a series of experiments to study the kinetics of a restriction reaction. Determine approximate percentage of digested DNA at various time points. Repeat experiments with several enzyme dilutions and several DNA dilutions. In each case, at what time point does reaction appear complete?

6) Design and test an assay to determine the relative stability of BamHI, EcoRI, and HindIII at room temperature.

BACTERIOPHAGE LAMBDA DNA (48,502 BASEPAIRS)

Restriction Sites (above) and Fragment Sizes (below)
of *Bam*HI, *Eco*RI, and *Hin*dIII



SAFETY NOTE: RESPONSIBLE HANDLING OF ETHIDIUM BROMIDE

Like many natural and man-made substances, ethidium bromide is a mutagen and cancer suspect agent. Therefore, this protocol has been written to limit its use to a single step that can be performed by the instructor in a controlled area. With responsible handling, the dilute ethidium bromide solution used in this experiment poses minimal risk.

The greatest risk is the posssibility of inhaling ethidium. bromide powder when mixing a 5 mg/ml stock solution. Therefore, we suggest purchasing ready-mixed stock solution from a supplier.

The stock solution is then diluted by the instructor to make a staining solution with a final concentration of 1 µg/ml. This is near the threshold of detection of ethidium bromide in mutagenicity assays -- approximately 0.5 µg/ml.

Experimental data suggest that treatment with 5,000 ppm available chlorine (10% bleach solution) inactivates 95 percent or more of ethidium bromide in solution. Thus, treatment of fresh staining solution should bring ethidium bromide concentration to a maximum of 0.05 µg/ml- well below the threshold of detection. Following bleach treatment, ethidium bromide concentration in stained gels and in reused staining solution is considerably less.

a) Always wear gloves when working with ethidium bromide solutions and stained gels.

b) Limit ethidium bromide use to a restricted area located next to a sink with running water.

c) Following gel staining, use funnel to retrieve as much of the ethidium bromide solution for reuse or disposal as outlined below.

d) Following lab, wipe down camera, transilluminator, and staining area with 10% bleach solution.

e) Flood stained gels with 10% bleach solution and let stand for 15 or more minutes. Drain, and collect stained gels in plastic bag. Discard in regular trash.

f) To discard staining solution, mix with equal part 10% bleach solution. Let stand for one or more hours, and discard down drain.