DNA Restriction Analysis

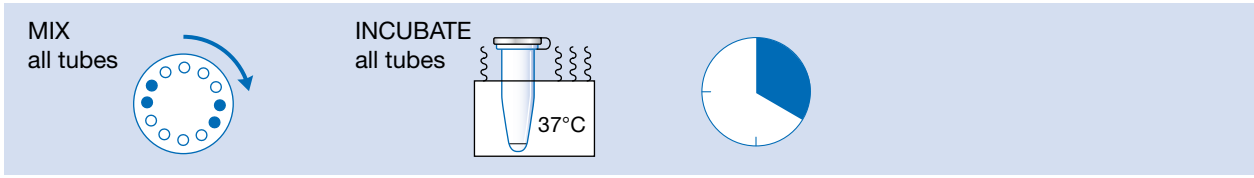
Laboratory 3 introduces the analysis of DNA using restriction enzymes and gel electrophoresis. Three samples of purified DNA from bacteriophage λ (48,502 bp in length) are incubated at 37°C, each with one of three restriction endonucleases: *Eco*RI, *Bam*HI, and *Hin*dIII. Each enzyme has five or more 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.

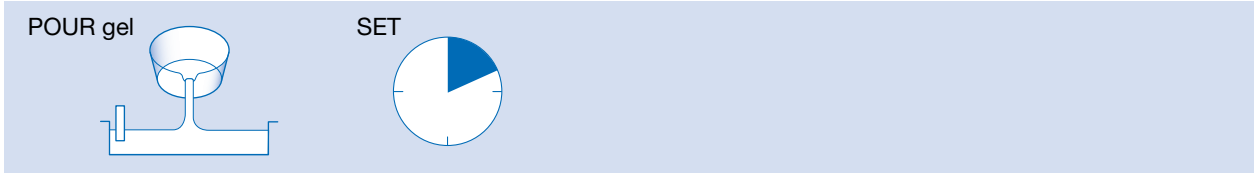
Kits based on this laboratory are available from the Carolina Biological Supply Company.

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

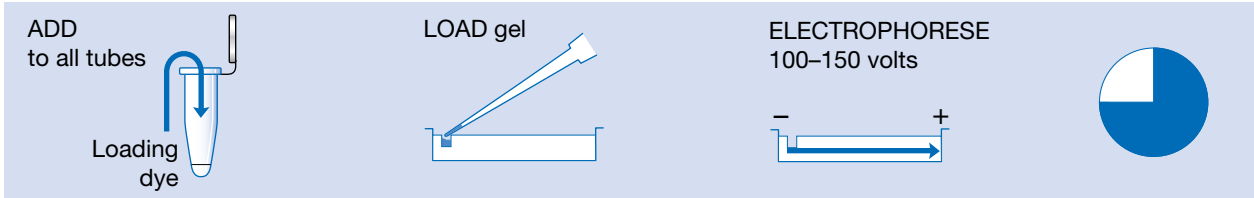
I. Set Up Restriction Digest



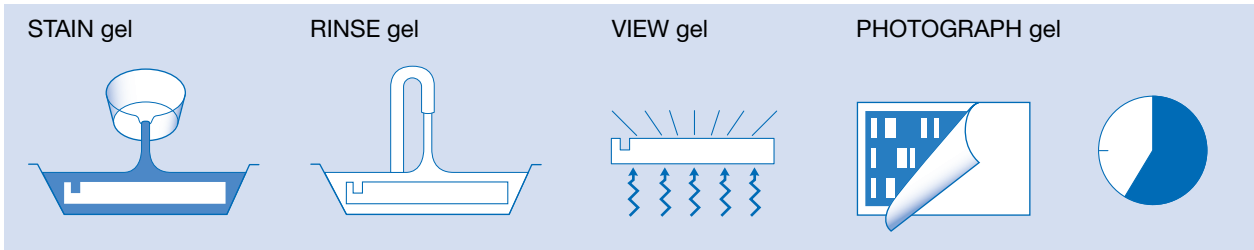
II. Cast 0.8% Agarose Gel



III. Load Gel and Separate by Electrophoresis



IV. Stain Gel and View (Photograph)



PRELAB NOTES

Storing and Handling Restriction Enzymes

Restriction enzymes, like many enzymes, are most stable at cold temperatures and lose activity if warmed for any length of time. Since maintaining these enzymes in good condition is critical to the success of experiments in this course, follow the guidelines listed below for handling.

1. Always store enzymes in a NON-frost-free freezer that maintains a constant temperature between –10°C and –20°C. NON-frost-free freezers develop a layer of frost around the chamber that acts as an efficient insulator. Frost-free freezers go through freeze-thaw cycles that subject enzymes to repeated warming and subsequent loss of enzymatic activity. However, there are commercially available storage containers that are essentially plastic freezer packs with holes for tubes. Restriction enzymes stored in these containers (even in a frost-free freezer) are much more stable because the freezer packs hold the temperature for several hours. Even in a NON-frost-free freezer, enzymes can warm up when the freezer is opened or when enzymes are used. Storage in these containers maintains constant temperature regardless of freezer type.
2. When a large shipment of an enzyme is received, split it into several smaller aliquots of 50–100 µl in 1.5-ml tubes. Use a permanent marker on tape to clearly identify aliquots with enzyme type, concentration in units/µl, and date received. Use up one aliquot before starting another.
3. Remove restriction enzymes from the freezer directly onto crushed or cracked ice in an insulated ice bucket or cooler. Make sure that the tubes are pushed down into the ice and not just sitting on top. Keep enzymes on ice at all times during preparation, and return to the freezer immediately after use.
4. Keep aliquots of enzymes, buffer, and DNA in a cooler filled with ice while in use to ensure that the unused aliquots remain fresh.
5. Although it is good technique to set up restriction digests on ice, it is much simpler to set up reactions in a test tube rack at room temperature. Little loss of enzyme activity occurs during the brief time it takes to set up the reaction.

But take heart—the enzymes used in this course are all remarkably stable. Those used in training workshops presented by us have survived multiple day-long shipments on ice, freezer power failures, and various abuse by student interns. In 15 years and more than 100 training workshops, we have yet to experience enzyme failure. Most restriction enzymes can survive several hours if left out on the lab bench, but do not take any chances!

Storing DNA and Restriction Buffer

Purified DNA is generally stored in the refrigerator (~4°C). DNA can be kept at –10°C to –20°C for long-term storage of several months or more. However, repeated freezing and thawing damages DNA. Restriction buffer is best kept frozen; freezing-thawing does not affect restriction buffer.

Buffers

Several types of buffers are used in this course: restriction buffer, electrophoresis buffer, and ligation buffer. Each has a different chemical composition and use. Always double-check to ensure that the proper buffer is being used.

Tris/Borate/EDTA (TBE) electrophoresis buffer can be reused several times. Collect used buffer and store in large carboy. If different gels are to be run over a period of several days, store the buffer in the electrophoresis box with the cover in place to retard evaporation. Prior to reusing buffer stored in the electrophoresis box, rock the box back and forth to mix the buffer at either end. This reequilibrates ions that differentially accumulate at either end during electrophoresis.

Groups of restriction enzymes operate under different conditions of salt and pH. For optimal activity, several different buffers are needed for the enzymes used in this course. To simplify procedures, we use a "compromise" restriction buffer—a universal buffer that is a compromise between the conditions preferred by various enzymes.

All buffers are used at a final concentration of 1x. Rely on the standard $C_1V_1 = C_2V_2$ formula to determine how much buffer to add to obtain a 1x solution:

(vol. buffer)	(conc. of buffer)	=	(total vol. of reaction)	(1x buffer)
(5 µl)	(2x)	=	10 µl	(1x)
(1 µl)	(10x)	=	10 µl	(1x)

For convenience, use 2x restriction buffer whenever possible—it can save a pipetting step to add water to bring a reaction up to 10 µl total volume. It is also easier and more accurate to pipette 5 µl than to pipette 1 µl. Compare a typical restriction reaction using 2x versus 10x restriction buffer:

	2x Buffer	10x Buffer
DNA	4 µl	4 µl
Enzyme	1 µl	1 µl
Buffer	5 µl	1 µl
Water	–	4 µl
Total Solution	10 µl	10 µl

Bacteriophage λ DNA

Because it is inexpensive and readily available, purified DNA from bacteriophage λ is most suitable for demonstrating the concept of DNA restriction. λ DNA costs approximately $0.10 per microgram, compared to plasmid DNA which ranges typically from $2.00 to $3.00 per microgram. Most commercially available λ is derived from a temperature-sensitive lysogen of *E. coli* called *c*I857 and is 48,502 bp in length. Restriction of chromosomal DNA, even from a simple organism such as *E. coli*, will generate thousands of DNA fragments that appear as a smear in an agarose gel.

Diluting DNA

DNA for near-term use can be diluted with distilled or deionized water. However, dilute the DNA with Tris-EDTA (TE) buffer for long-term storage.

EDTA in the buffer binds divalent cations, such as Mg^{++}, that are necessary cofactors for DNA-degrading nucleases. Always dilute DNA to the concentration specified by the protocol.

1. Determine the total volume of DNA required by multiplying the number of experiments times the total volume of DNA per experiment, including overage.

   (10 experiments) (20 µl DNA) = 200 µl DNA

2. Use this number in the $C_1V_1 = C_2V_2$ formula, along with the desired final DNA concentration and the concentration of the stock DNA. Solve for V_1, the volume of stock DNA needed in the dilution.

   (C_1 stock DNA) (V_1) = (C_2 final DNA) (V_2 total volume)
   (0.5 µg/µl) (V_1) = (0.1 µg/µl) (200 µl)
   (V_1) = (0.1 µg/µl) (200 µl)/(0.5 µg/µl) = 40 µl stock DNA

3. Add water or TE to make total volume of final solution.

   40 µl stock DNA + 160 µl H_2O or TE = 200 µl final solution

Making Aliquots of Reagents

1. We find that it is safest to prepare separate aliquots of enzyme, DNA, and buffer in 1.5-ml tubes for each experiment. Each aliquot should contain slightly more than is required for the lab. Following the experiment, discard the tubes, and make new aliquots for the next experiment. Although this procedure appears to be wasteful, it avoids cross-contamination that invariably occurs if aliquots are reused or shared between groups. It is a small price to pay for consistent results.
2. For aliquots of restriction enzymes, add 1 µl extra when 1–3 µl is called for and 2 µl extra when 4–6 µl is actually needed. The overage aids in visualizing the reagent in the tube and allows for small pipetting errors.
3. It is probably unwise to make small aliquots of enzymes more than 1 or 2 days in advance. A several-microliter droplet, clinging to the side of a 1.5-ml tube, has a large surface-to-volume ratio. For this reason, it may be affected by temperature fluctuations. If aliquots are made in advance, store them in the freezer until needed. When setting up, remove aliquots from the freezer and place on ice, making sure that the tubes are fully submerged in ice while awaiting use.
4. Aliquots of DNA and buffer should be approximately 20% larger than the volume actually needed in the experiment. This allows for overpipetting and other mishaps. Considering that DNA is generally the most expensive component of an experiment, making aliquots of the exact amount and adding other reagents directly to the DNA tube may be more cost-effective.
5. Reagent volumes listed in the Prelab Preparation section of each laboratory have been scaled to include the overage suggested here.
6. Large aliquots of distilled water and loading dye can be used for several experiments.
7. Colored 1.5-ml tubes are very handy for color-coding each reagent aliquot.

Pooling Reagents

During aliquot preparation and movement to and from the freezer or refrigerator and ice bucket, reagent aliquots often become spread in a film around the sides or caps of the 1.5-ml tubes. Use one of the following methods to pool reagent droplets to make them easier to find in the tube.

- Spin tubes briefly in a microfuge.

  or

- Spin tubes briefly in a preparatory centrifuge, using adaptor collars for 1.5-ml tubes. Alternately, spin tubes within 15-ml tube, and remove carefully.

  or

- Tap tubes sharply on bench top.

Restriction Enzyme Activity

The "unit" is the standard measure of restriction enzyme activity and is defined as the amount of enzyme needed to digest to completion 1 μg of λ DNA in a 50-μl reaction in 1 hour. The unit concentration of various restriction enzymes varies from batch to batch and from manufacturer to manufacturer. Typical batches of commercially available enzymes have activities in the range of 5–20 units/μl.

We suggest using enzymes at full strength as supplied—a working concentration of approximately 1 unit per microliter of reaction mix. Although this is technically far more enzyme than is required, such "overkill" assures complete digestion by compensating for the following experimental conditions:

1. As a time saver, reaction times for restriction digests have been shortened to 20 minutes. Complete digestion of the DNA would not occur during an abbreviated incubation if the restriction enzyme was used at the standard condition of 1 unit/μg of DNA.
2. Many enzymes do not exhibit 100% activity in a compromise buffer.
3. Enzymes lose activity over time, due to imperfect handling.
4. It is easy enough to underpipette when measuring 1 μl of enzyme, especially considering that the micropipettor's mechanical error is greatest at the low end of its volume range.

Incubating Restriction Reactions

A constant-temperature water bath for incubating reactions can be made by maintaining a trickle flow of tap water into a Styrofoam box. Monitor temperature with a thermometer. An aquarium heater can be used to maintain constant temperature.

Twenty minutes is the bare minimum incubation time for the restriction reaction to go to completion. If electrophoresis is to be done the following day, incubate the reactions for 1–24 hours. After several hours, enzymes lose their activity, and the reaction simply stops. Stop incubation whenever it is convenient; reactions may be stored in a freezer (–20°C) until ready to continue. Thaw reactions before adding loading dye.

Casting Agarose Gels

Remarkably, the enzyme digest of DNA is not the main determining factor of good results in restriction analysis. Enzymes rarely fail, and λ DNA is inexpensive enough that there is no need to scrimp. Measurements are not extremely critical—restriction reactions come out fine as long as some DNA, some enzyme, and some buffer make it into the test tube. Most unsatisfactory results in restriction analysis can be traced to the problems in casting agarose gels.

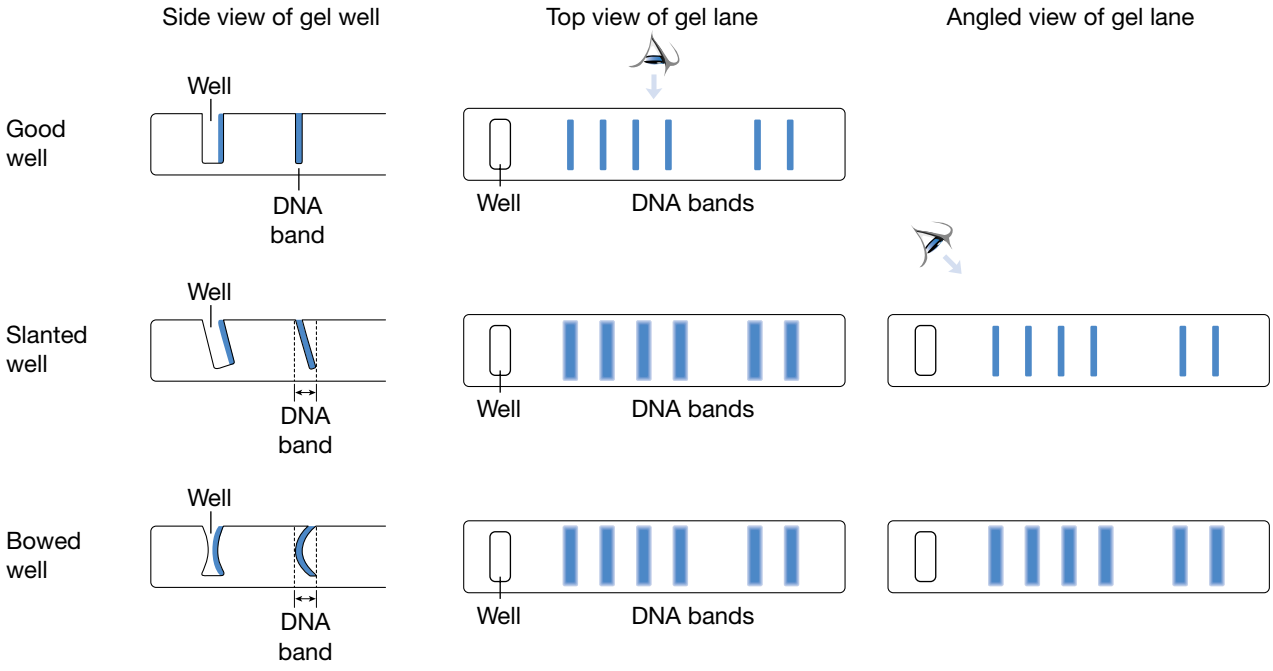
- Thin gels yield dramatically better results than thick gels, so cast gels only thick enough to contain the volume of DNA that will be loaded. A thin gel stains quickly and concentrates the DNA in a shorter vertical distance. This improves contrast, and the stained DNA appears brighter. The reason is that, in addition to binding to DNA, stain also accumulates in the gel itself. This unbound stain creates a background that decreases contrast, and relative brightness, of the stained DNA. Thick gels inherently have less contrast, because the DNA is viewed through a thicker background of unbound stain. In addition, thick gels distribute the DNA over a larger vertical distance, decreasing the width of the band. Considering that thick gels consume more agarose and take longer to set and stain, there is no reason to cast them.

  Although casting thin gels is the desirable way to go, remember that they are relatively fragile! For all intents and purposes, the least concentrated gel that can be handled without great fear of breaking is 0.8%. If problems are encountered, increase the concentration to 1.0%.

- Clean, properly shaped wells are key to producing technically excellent gels—those with straight, well-focused bands. The front edge of the well determines how the DNA enters the gel and what kinds of bands are formed as the DNA sorts by size. Ideally, the front edge of the well should be smooth and perpendicular to the gel. This forces the DNA to enter the gel as a single vertical front. Given relatively constant current, the DNA will retain this focused configuration as it moves down the gel and resolves into bands. However, a loose or bent comb may not rest perpendicular to the casting tray, which produces a slanted well, allowing the DNA to enter the gel over a broad front. Viewed from above, the DNA is spread out along the slanted front. This effectively dilutes the DNA, producing a diffuse, or "fuzzy," band. This effect can be seen by moving your head in an arc when viewing a stained gel. The fuzzy bands produced by an angled well will become sharply focused when your viewing angle aligns with the angle of the slanted wells.

  Even more insidious is the effect of removing the comb when the gel is not completely set. In this case, the weight of the gel compresses the partially set gel into the well, causing the edges to bow inward. This destroys the perpendicular edge, and the DNA enters the well over a broader front, which produces fuzzy bands that cannot be resolved by changing the viewing angle of the stained gel. Changes in the shape of the well, due to partially set gel, are almost impossible to detect ahead of time. However, they are likely the major cause of chronically fuzzy bands—especially when short lab periods impose a need for speed. So it pays to cast gels well in advance of when they will be used.

  The problems described above offer good arguments for immersing a gel in buffer prior to removing the comb. The buffer aids in cooling a recently cast gel, helping to ensure that it has solidified throughout. In addition, the buffer



Problems with Casting Gels

helps to lubricates the comb, reducing the chance of damaging the front edge of the well when the comb is withdrawn.

- It is a sad fact that the design of some commercially available combs and casting trays makes it difficult to cast an excellent gel. Poorly molded combs may have burrs that nick or scrape the well edge; combs molded of nonrigid materials may bend; and ill-fitting combs may "wobble" in the casting case. At least these problems are relatively easy to fix. A difficult problem to fix is a comb whose teeth are set too far above the bed of the casting tray. The distance between the comb and the tray surface should be about 1 mm. Any more distance produces an overly thick gel, with the attendant problems of staining and contrast. The only remedy is to file the comb or casting tray to allow the comb to come to rest closer to the tray surface.

Storing Cast Agarose Gels

Gels can be cast 1 or 2 days before use. Keep gels covered with TBE electrophoresis buffer or wrapped in plastic wrap to prevent drying.

Separating by Electrophoresis

Shortly after the current is applied to the electrophoresis system, the loading dye should be seen moving through the gel toward the positive side of the gel apparatus. It will appear as a blue band, eventually resolving into two bands of color. The faster-moving, purplish band is bromophenol blue. The slower-moving,

aqua band is xylene cyanol. In a 0.8% gel, bromophenol blue migrates through the gel at the same rate as a DNA fragment of approximately 300 bp. Xylene cyanol migrates at a rate equivalent to approximately 9000 bp. Best separation for analysis of plasmid DNA is achieved when the bromophenol blue migrates 4–7 cm or more from the origin. However, be careful not to let the bromophenol blue band run off the end of the gel.

The migration of DNA through the agarose gel is dependent on voltage—the higher the voltage, the faster the rate of migration. Refer to the chart below for approximate running times at various voltages. The times below are for a "minigel" system with a 84 x 96-mm gel; times will vary according to apparatus.

Voltage	150	125	100	75	50	25	12.5
Time	0:40	0:50	1:20	1:40	3:20	6:40	13:00

Responsible Handling of Ethidium Bromide

The protocols in this manual limit the use of ethidium bromide to a single procedure that can be performed by the instructor in a controlled area. With responsible handling, the dilute solution (1 μg/ml) used for gel staining poses minimal risk. Ethidium bromide, like many natural and man-made substances, is a mutagen by the Ames microsome assay and a suspected carcinogen.

CAUTION/HANDLING AND DECONTAMINATION OF ETHIDIUM BROMIDE

1. Always wear gloves while working with ethidium bromide solutions or stained gels.
2. Limit ethidium bromide use to areas covered with bench paper.
3. Following gel staining, use a funnel to decant as much of the ethidium bromide solution as possible into a storage container for reuse or decontamination and final disposal.
4. Dispose of stained gels and used staining solution according to current chemical waste disposal regulations. Consult your local safety office for these regulations. For further information, see Appendix 4.

The greatest risk is to inhale ethidium bromide powder when mixing a 5 mg/ml stock solution. Therefore, we suggest purchasing a ready-mixed stock solution from a supplier. The stock solution is diluted to make a staining solution with a final concentration of 1 μg/ml.

DNA Staining with Methylene Blue

The volumes and concentrations of DNA used in these experiments have been optimized for staining with ethidium bromide, which is the most rapid and sensitive method. If methylene blue staining (or other proprietory staining procedure) is preferred, increase stated concentrations 4–5 times for λ DNA and 2 times for plasmid DNA. If DNA *concentration* is increased, volumes used in laboratories remain as stated.

Viewing Stained Gels

Transillumination, where light passes up through gel, gives superior viewing of gels stained with either ethidium bromide or methylene blue.

A mid-wavelength (260–360 nm) ultraviolet (UV) lamp emits in the optimum range for illuminating ethidium-bromide-stained gels. Commercially available gel illumination systems are designed for optimal illumination of ethidium bromide. These systems use UV light at a specific wavelength that does not contain the most harmful rays. However, use great caution to ensure that skin is not exposed and that eye protection is used.

> **CAUTION**
> Ultraviolet light can damage the retina of the eye. Never look at an 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.

A fluorescent light box for viewing slides and negatives provides ideal illumination for methylene-blue-stained gels. An overhead projector may also be used. Cover the surface of the light box or projector with plastic wrap to protect the apparatus from liquid spills.

Photographing Gels

Photographs of DNA gels provide a permanent record of the experiment, allowing time to analyze results critically, to discover subtleties of gel interpretation, and to correct mistakes. Furthermore, time exposure can record bands that are faint or invisible to the unaided eye.

A Polaroid "gun" camera, equipped with a close-up diopter lens, is used to photograph gels on either a UV or white-light transilluminator. A plastic hood extending from the front of the camera forms a mini-darkroom and provides correct lens-to-subject distance. Alternatively, a close-focusing 35-mm camera can be used. For UV photography, two filters are placed in front of the lens: a 23A orange filter is closest to the camera and a 2B UV-blocking filter (clear) is closest to the subject. Any yellow or orange filter will intensify contrast in gels stained with methylene blue. The UV filter set described above works well and can be left in place for both ethidium bromide and methylene blue photography.

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

For UV photography of ethidium-bromide-stained gels, use Polaroid high-speed film Type 667 (ASA 3000), black and white. Set camera aperture to f/8 and shutter speed to B. Depress shutter for a 2–3-second time exposure. For white-light photography of methylene-blue-stained gels, use Polaroid film Type 667, with an aperture of f/8 and shutter speed of 1/125 second.

Digital photography is becoming increasingly popular for gel documentation. Digital cameras, especially those with close focus capability, can provide high-quality images. The addition of a hood (extending from the lens) will block ambient light and increase contrast. Image files can be opened and edited in any photo-editing program, such as Microsoft Photo Editor and Adobe Photoshop. A hard copy is printed on regular or glossy printer paper, saving the cost of Polaroid or photographic film/printing. The digital images can also be distributed via e-mail or an Internet site.

For Further Information

The protocol presented here is based on the following published methods:

Aaij C. and Borst P. 1972. The gel electrophoresis of DNA. *Biochim. Biophys. Acta* **269:** 192–200.

Helling R.B., Goodman H.M., and Boyer H.W. 1974. Analysis of R-*Eco*RI fragments of DNA from lamboid bacteriophages and other viruses by agarose-gel electrophoresis. *J. Virol.* **14:** 1235–1244.

Sharp P.A., Sugden B., and Sambrook J. 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. *Biochemistry* **12:** 3055–3063.

PRELAB PREPARATION

Before performing this Prelab Preparation, please refer to the cautions indicated on the Laboratory Materials list.

The volumes of agarose solution and Tris/Borate/EDTA (TBE) buffer needed vary according to the electrophoresis apparatus used. The volumes quoted here are based on typical "minigel" systems.

1. Prepare aliquots for each experiment:

   20 µl of 0.1 µg/µl λ DNA (store on ice)
   25 µl of 2× restriction buffer (store on ice)
   2 µl each of *Bam*HI, *Eco*RI, and *Hin*dIII (store on ice)
   500 µl of distilled water
   500 µl of loading dye

2. Prepare 0.8% agarose solution (40–50 ml per experiment). Keep agarose liquid in a hot-water bath (at ~60°C) throughout the lab. Cover the solution with aluminum foil to retard evaporation.
3. Prepare 1× TBE buffer for electrophoresis (400–500 ml per experiment).
4. Prepare ethidium bromide or methylene blue staining solution (100 ml per experiment).
5. Adjust water bath to 37°C.

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
2× Restriction buffer
Restriction enzymes
*Eco*RI
*Bam*HI
*Hin*dIII
1× 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)

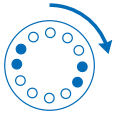
1. Use a permanent marker to label four 1.5-ml tubes, in which restriction reactions will be performed:

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

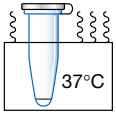
2. Use the matrix below as a checklist while adding reagents to each reaction. Read down each column, adding the same reagent to all appropriate tubes. *Use a fresh tip for each reagent.* Refer to detailed directions that follow.

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

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 b 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 mir imum of 20 minutes. Reactions can be incubated for a longer period time.

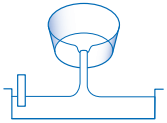


After several hours, enzymes lose activity and reaction stops.

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

II. Cast 0.8% Agarose Gel

(15 minute



Gel is cast directly in box in some electrophoresis apparatuses.

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 lubri-

1. Seal the ends of the gel-casting tray with tape, and insert well-forming com Place the gel-casting tray out of the way on the lab bench so that agaros poured in next step can set undisturbed.
2. Carefully pour enough agarose solution into the casting tray to fill to a dept of about 5 mm. Gel should cover only about one-third the height of com teeth. Use a pipette tip to move large bubbles or solid debris to the sides end of tray while gel is still liquid.
3. Gel will become cloudy as it solidifies (~10 minutes). *Do not move or jar cas ing tray while agarose is solidifying*. Touch corner of agarose away from com to test whether gel has solidified.
4. When agarose has set, unseal ends of casting tray. Place tray on the platfor 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 sub

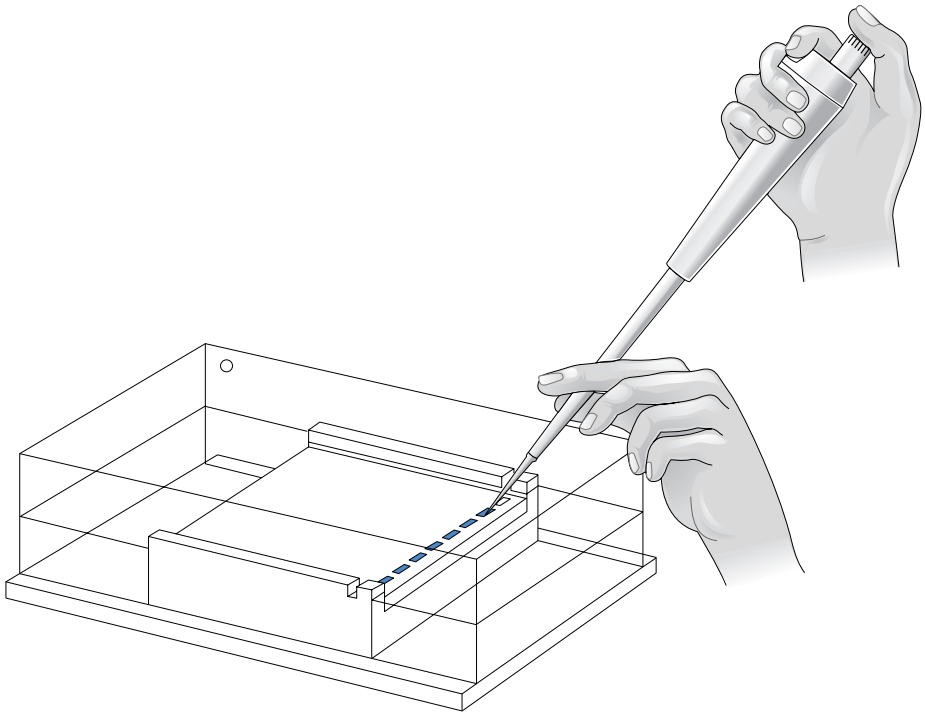
III. Load Gel and Separate by Electrophoresis (50–70 minutes)

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

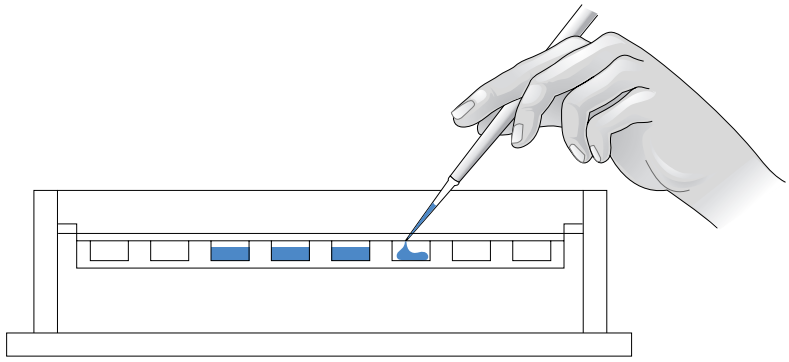
   b. Place four individual droplets of loading dye (1 µl each) on a small square of Parafilm or wax paper. Withdraw contents from the reaction tube and mix with a loading dye droplet by pipetting in and out. Immediately load dye mixture according to Step 2. Repeat successively, *with a clean tip*, for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



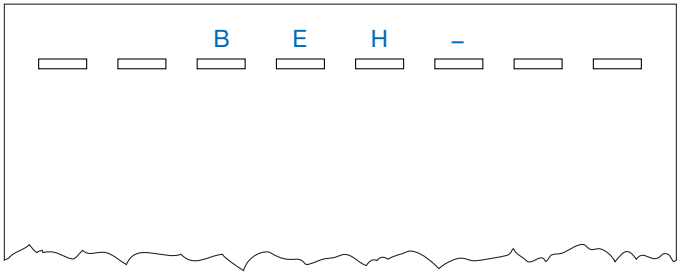
Hand Positions for Loading an Agarose Gel (Step 2)

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.*



and gently depress micropipettor plunger to slowly expel sample. Sucros
in the loading dye weighs down the sample, causing it to sink to the bo
tom of the well. *Be careful not to punch the tip of the micropipettor through th
bottom of the gel.*

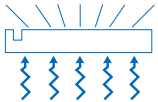


Alternately, set power supply on lower voltage, and run gel for several hours. When running two gels from the same power supply, the current is double that for a single gel at the same voltage.

3. Close the top of the electrophoresis box, and connect electrical leads to
power supply, anode to anode (red-red) and cathode to cathode (blac
black). Make sure that both electrodes are connected to the same channel
the power supply.
4. Turn the power supply on, and set to 100–150 volts. The ammeter shou
register approximately 50–100 milliamperes. If current is not detected, che
connections and try again.
5. Separate by electrophoresis for 40–60 minutes. Good separation will ha
occurred when the bromophenol blue band has moved 4–7 cm from th
wells. If time allows, carry out electrophoresis until the bromophenol blu
band nears the end of the gel. *Stop* electrophoresis before the bromophen
blue band runs off the end of the gel.
6. Turn off power supply, disconnect leads from the inputs, and remove top
electrophoresis box.
7. Carefully remove the casting tray from the electrophoresis box, and slide th
gel into a disposable weigh boat or other shallow tray. Label staining tra
with your name.

aining time increases arkedly for thicker gels. Do ot be tempted to use a higher ncentration of ethidium bro- ide in the staining solution. his will not enhance the NA bands; it only increases e background staining of the arose gel itself.

hidium bromide solution ay be reused to stain 15 or ore gels. When staining time creases markedly, dispose of hidium bromide solution as plained 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)

1. Wear latex gloves during staining and cleanup.

RESULTS AND DISCUSSION

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

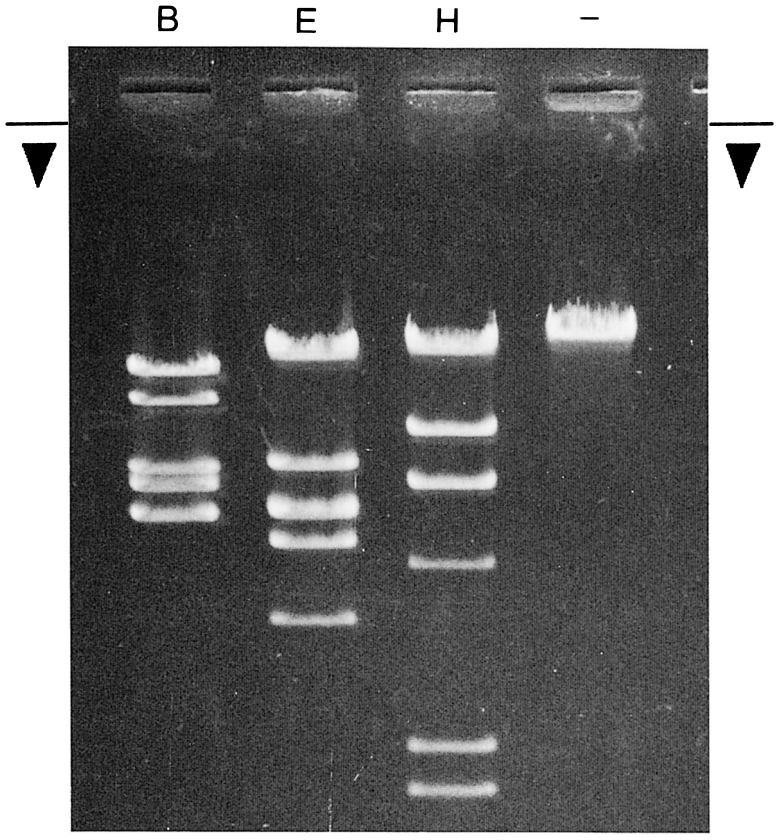
Electrophoresis using a polyacrylamide gel in a glass tube was an improve ment, but it could only be used to separate small DNA molecules of up to 200 bp. Another drawback was that the DNA had to be radioactively labeled prior t electrophoresis. Following electrophoresis, the polyacrylamide gel was cut int thin slices, and the radioactivity in each slice was determined. The amount o radioactivity detected in each slice was plotted versus distance migrated, pro ducing a series of radioactive peaks representing each DNA fragment.

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

By convention, DNA gels are "read" from left to right, with the sample wel oriented at the top. The area extending from the well down the gel is termed "lane." Thus, reading down a lane identifies fragments generated by a particula restriction reaction. Scanning across lanes identifies fragments that have com grated 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?

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:

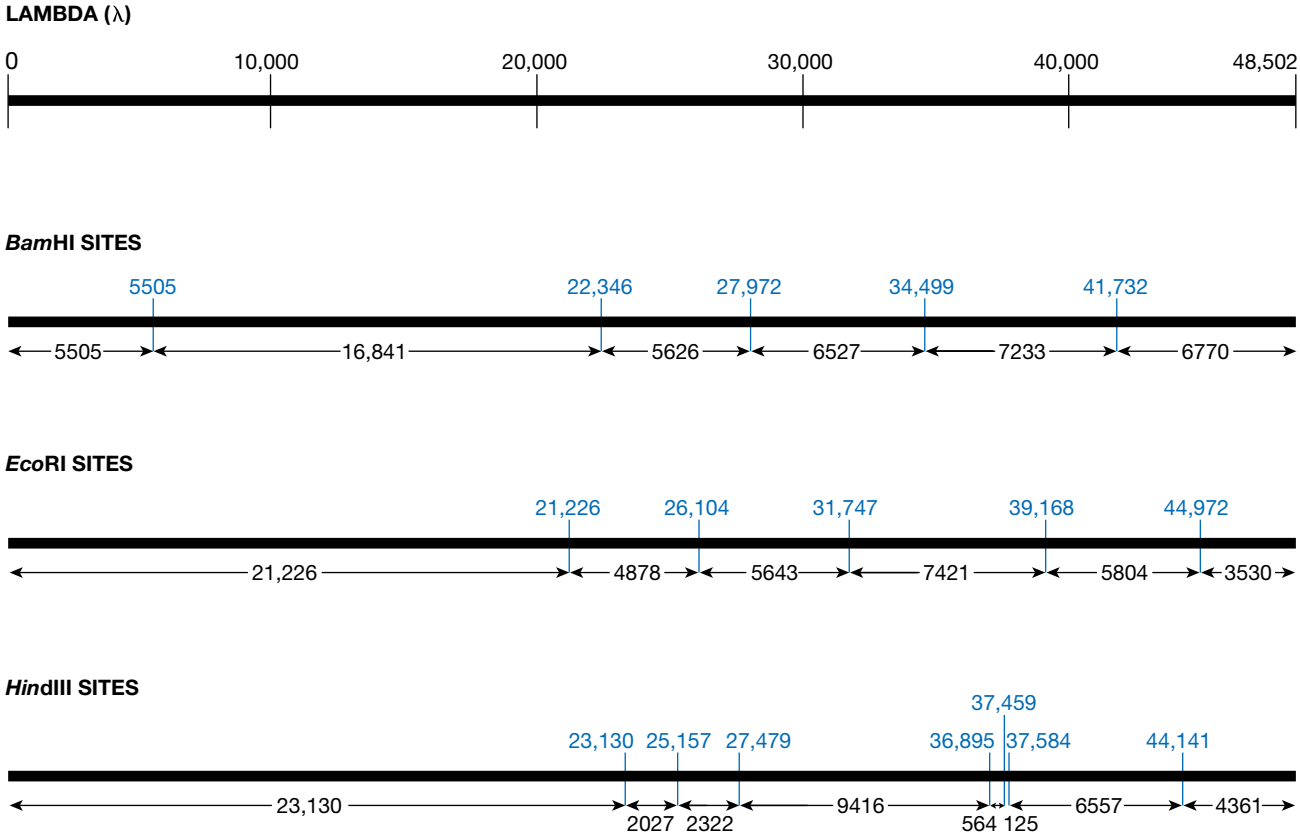
$$\frac{\text{number of bp in fragment} \times (\text{conc. of DNA}) \times (\text{vol. of DNA})}{\text{number of bp in } \lambda \text{ DNA}}$$

For example:

$$\frac{24{,}251 \text{ bp } (0.1 \text{ μg/μl}) \ (4 \text{ μl})}{48{,}502 \text{ bp}} = 0.2 \text{ μg}$$

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.

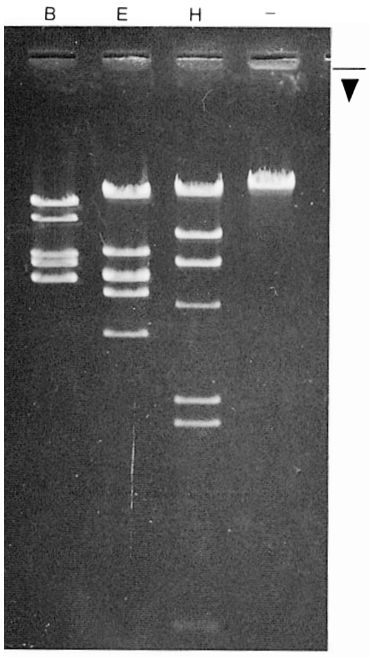
   a. Determine approximate percentage of digested DNA at various time points.

   b. Repeat experiments with several enzyme dilutions and several DNA dilutions.

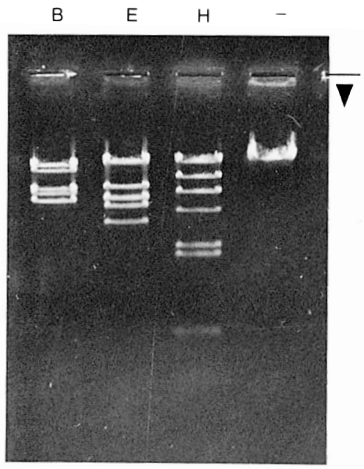
   c. In each case, at what time point does the reaction appear to be complete?

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

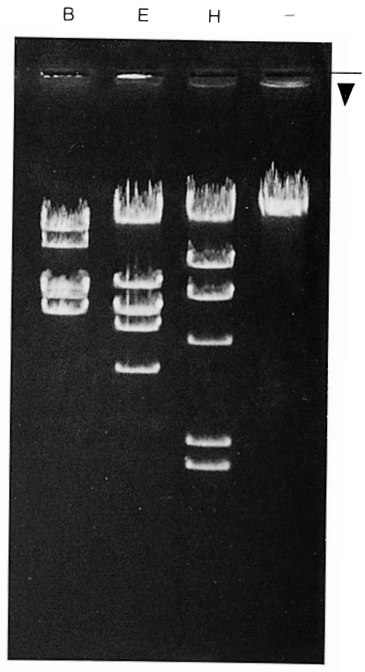


Ideal Gel



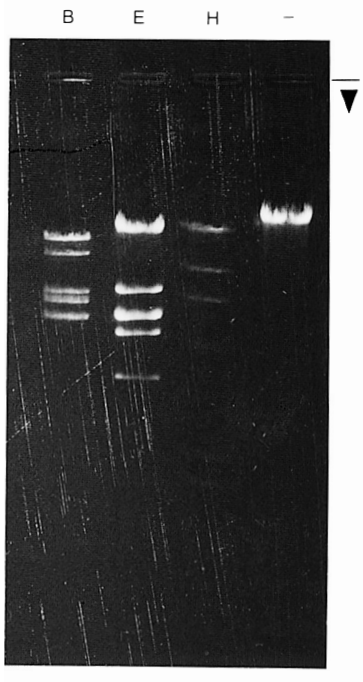
Short Run

Bands compressed. Short time electrophoresing.



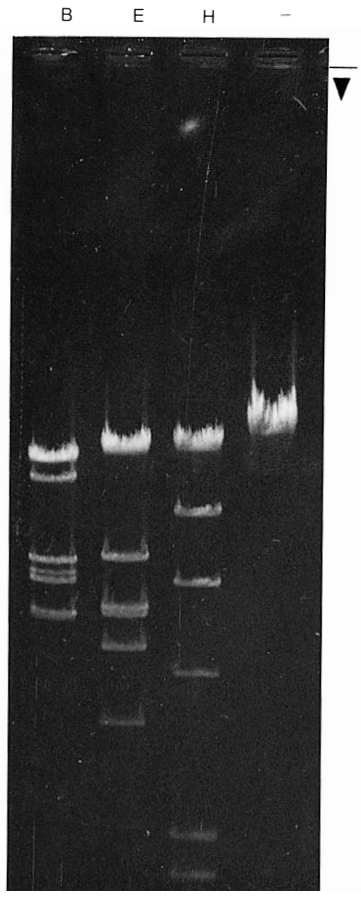
Overloaded

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



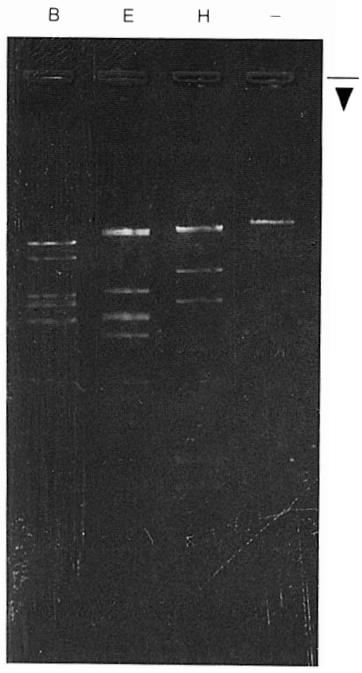
Punctured Wells

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.

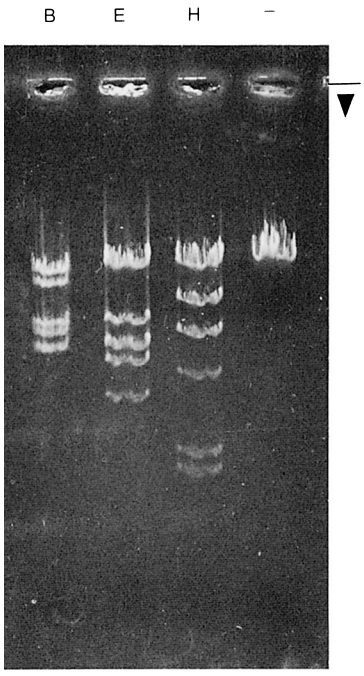


Underloaded

Bands faint in all lanes. Too little DNA in digests.

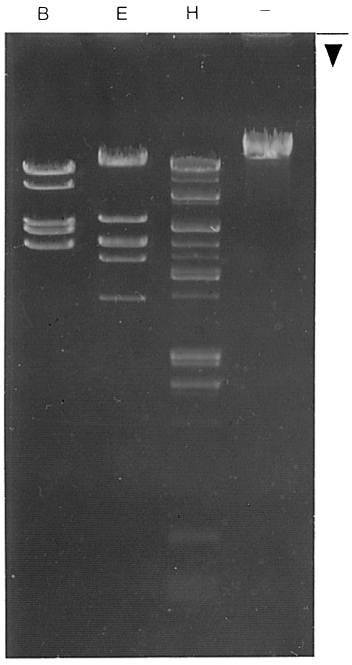
(Field Guide is continued on next page.)

Field Guide to Electrophoresis Effects (*continued*)



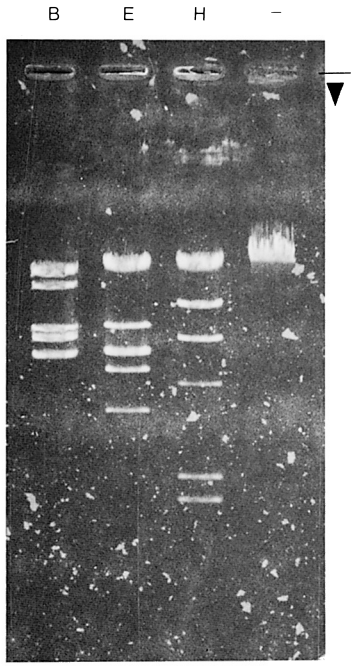
Poorly Formed Wells

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



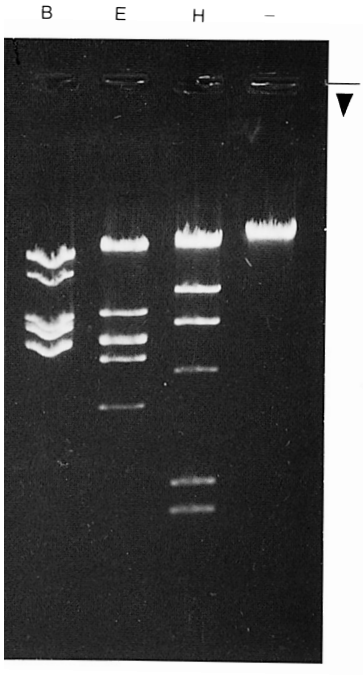
Enzymes Mixed

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



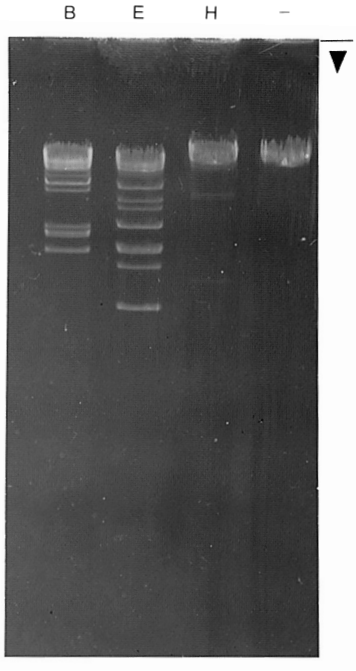
Precipitate

Precipitate in TBE buffer used to make gel.



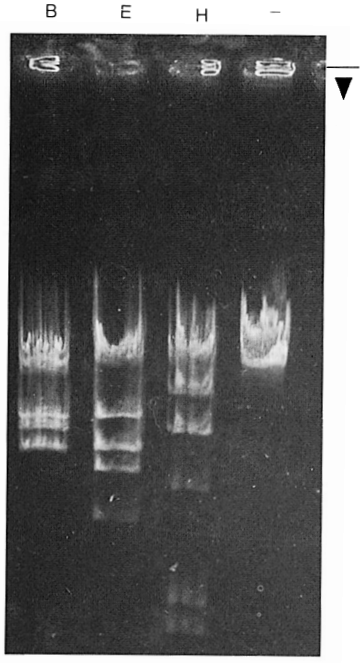
Bubble in Lane

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 lanes. Gel made with water or wrong concentration of TBE buffer.