

21-1103

21-1106

DNA Restriction Analysis Kit

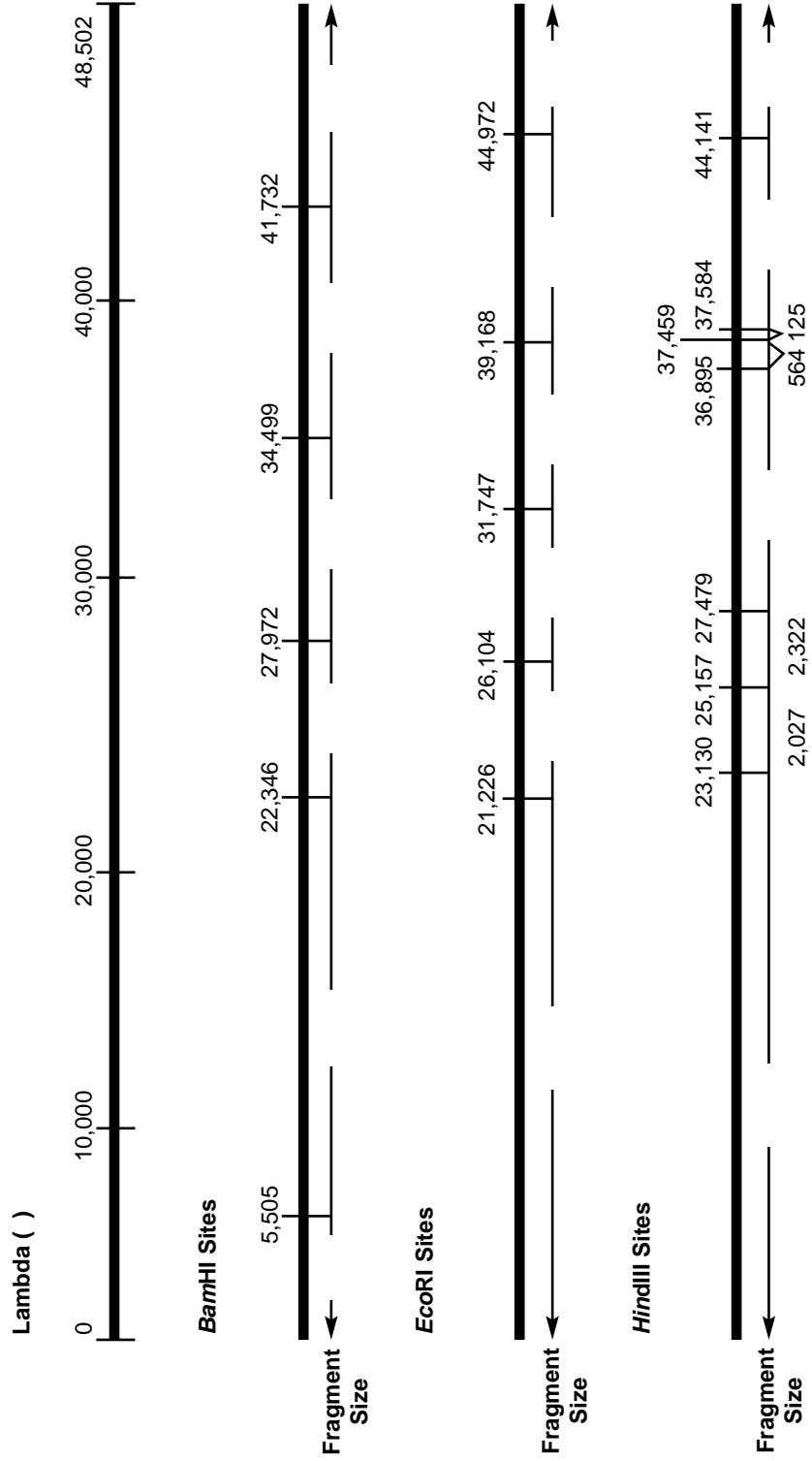
Instructor's Manual

This kit was developed in cooperation with the DNA Learning Center of Cold Spring Harbor Laboratory and has been tested over several years by thousands of teachers and students in 25 states. The experiment is adapted from *DNA Science: A First Course in Recombinant DNA Technology* by David A. Micklos and Greg A. Freyer, ©1995 Cold Spring Harbor Laboratory and Carolina Biological Supply Company.

Upon receipt of the kit, store restriction buffer, lambda DNA, and restriction enzymes (*Bam*HI, *Eco*RI, *Hind*III) in a freezer at approximately -20°C until use. Other materials may be stored at room temperature (approximately 25°C).

Warning: Individuals should use this kit only in accordance with prudent laboratory safety precautions and under the supervision of a person familiar with such precautions. **Use of this kit by unsupervised or improperly supervised individuals could result in serious injury.**

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Introduction

In this experiment, DNA from the bacteriophage lambda (48,502 base pairs in length) is cut with restriction enzymes and the resulting restriction fragments are separated using gel electrophoresis. Three samples of lambda DNA are incubated at 37°C, each with one of three restriction endonucleases: *Bam*HI, *Eco*RI, and *Hind*III. A fourth sample, the negative control, is incubated without an endonuclease.

The DNA samples are then loaded into wells of an agarose gel and electrophoresed. An electrical field applied across the gel causes the DNA fragments in the samples to move from their origins (sample wells) through the gel matrix toward the positive electrode. Smaller DNA fragments migrate faster than larger ones, so restriction fragments of differing sizes separate into distinct bands during electrophoresis. The characteristic number and pattern of bands produced by each restriction enzyme are, in effect, a “DNA fingerprint.” The restriction patterns are made visible by staining with a compound that binds to DNA.

Materials

The materials in the DNA Restriction Analysis Kit are sufficient for six complete setups of the experiment. The materials are supplied for use with the exercise in this kit only. Carolina Biological Supply Company disclaims all responsibility for any other uses of these materials. The kit includes:

agarose (3.2 g, for 400 mL)	6 vials loading dye (100 µL)
stain* (250 mL)	TBE buffer concentrate (150 mL)
40 reaction tubes, 1.5 mL	Instructor’s Manual
6 staining trays	15 2 Student Guides
2 pair disposable gloves	

**Perishables

6 vials 2 restriction buffer (50 µL)
6 vials lambda DNA* (25 µL)
vial <i>Bam</i> HI (15 µL)
vial <i>Eco</i> RI (15 µL)
vial <i>Hind</i> III (15 µL)

*Kit 21-1103 includes lambda DNA at 0.1 µg/µL, ethidium bromide stain at 1 µg/mL, and 250 mL 0.05 M KMnO₄ for decontamination. Kit 21-1106 (the *Carolina* BLU™ kit) contains lambda DNA at a concentration of 0.2 µg/µL, 7.5 mL of gel buffer stain, and 250 mL of final stain.

**The kits contain prepaid cards on which you specify the delivery date for the perishables.

Carolina BLU™ was developed as a significant advance over the methylene blue staining protocol and is provided at two concentrations. With this method, low concentrations of *Carolina* BLU™ gel and buffer stain are added to the agarose and electrophoresis buffer. During electrophoresis, *Carolina* BLU™ faintly stains the DNA, allowing for its immediate visualization. Students can immediately view their results, without the lengthy staining and destaining steps required for methylene blue.

Following electrophoresis, the DNA bands can be stained more intensely by soaking the gel in a dilute solution of *Carolina* BLU™ for 15–20 minutes. At this stage the DNA bands are stained more intensely. Background stain in the gel can be removed by several washes in deionized water over a period of 30–40 minutes. This additional staining and destaining intensifies the staining of all the fragments, making the smaller fragments more visible than is usually possible with methylene blue stain. If you do not wish to add stain to the agarose and buffer, the final stain will still work comparably as methylene blue. The advantages of the *Carolina* BLU™ gel system over methylene blue staining are:

- Immediate visualization of DNA.
- Greatly shortened staining and destaining times.
- Superior results. When used as described, *Carolina* BLU™ stains DNA more intensely, allowing for easier visualization of all DNA bands and visualization of smaller bands which are not usually seen with methylene blue staining.

Upon receipt of the kit, store restriction buffer, lambda DNA, and restriction enzymes (*Bam*HI, *Eco*RI, *Hind*III) in a freezer at approximately –20°C until use. Store enzymes in a NON frost-free freezer. A “frosty” freezer maintains stable temperature, while a frost-free freezer goes through freeze-thaw cycles that subject enzymes to repeated warming. If a frost-free freezer must be used, store enzymes in their styrofoam shipping container within the freezer. The container will help maintain constant temperature. Other materials may be stored at room temperature (approximately 25°C). Volumes of agarose solution and TBE buffer provided are sufficient for most “mini” gel systems.

Other materials needed but not included with this kit:

6 micropipettors (0–10 µL or 0–20 µL), 50 micropipet tips, 6 gel electrophoresis chambers and power supplies, masking tape for sealing gel-casting tray, 6 racks for 1.5-mL reaction tubes, a 1-L flask or beaker for agarose, a 600-mL beaker with cracked ice, a 3-L or larger carboy or container for electrophoresis buffer, distilled water, 6 permanent laboratory markers (felt tip or wax), toothpicks, boiling water bath or microwave oven,

water bath at 60°C, water bath at 37°C, and funnel. Students need semilog graph paper and metric rulers for Question 4 of Results and Discussion.

A white-light box is desirable for viewing blue-stained gels, although gels can also be viewed on an overhead projector. A Polaroid® “gun” camera or other camera is desirable for recording results. A mid- or long-wavelength UV light source, with protective screen or glasses, is needed to view ethidium bromide-stained gels. A 10% solution of household bleach for cleanup of lab surfaces, and 0.25 N HCl and 0.25 N NaOH for decontaminating ethidium bromide staining solution and gels.

Scheduling

DNA restriction analysis requires several different activities. Plan your time as follows. If a double period is available, Lab Day 1 and Lab Day 2 can be completed at once.

Day	Time Needed	Activity
Several days before lab	30 min	Pre-Lab: Mix TBE buffer. Aliquot distilled water.
Lab Day 1	30 min	Pre-Lab: Add stain to TBE buffer. Prepare agarose solution. Pool reagents into the bottom of their vials.
	15 min	Practice Pipetting
	15 min	Set Up Restriction Digest
	10 min	Cast Agarose Gel
	20+ min	Post-Lab: Incubate reactions
Lab Day 2	15 min	Practice Loading Gel
	15 min	Load Gel
	40+ min	Post-Lab: Electrophorese.
	40 min	Stain gels.
Lab Day 3	40 min	Results and Discussion

Pre-Lab Preparation

Mix (TBE) Buffer

Because tris-borate-EDTA (TBE) buffer solution is stable, it can be made ahead of time and stored in a carboy or other container until ready to use. Pour contents of 20 TBE concentrate into a 3-L flask or carboy. Add 2,850 mL of distilled or deionized water for a final volume of 3 L. If there is any

precipitate in the bottle containing the 20 TBE Buffer, rinse with a portion of the 2,850 mL of distilled water. Stir for 1–2 minutes.

Aliquot Distilled Water

Add approximately 1 mL distilled or deionized water to each of six 1.5-mL test tubes. These will be needed at student stations.

Prepare Agarose Solution

Before class on Lab Day 1, prepare 0.8% agarose solution. Add 3.2 g (entire bottle) of agarose to 400 mL TBE electrophoresis buffer in a clean 1-L flask or beaker. Cover with aluminum foil, and heat in a boiling water bath (double boiler) for 10–20 min. Solution will become clear as agarose dissolves. Swirl and observe bottom to insure that no undissolved agarose remains. Alternatively, heat solution at high setting of microwave oven for 7–10 min, **without aluminum foil**. Cool solution to approximately 60°C before use. Cover with aluminum foil and keep warm in 60°C water bath until ready to use. If using *Carolina* BLU™ stain, the following protocol is used for the addition of stain to the agarose and buffer.

The concentration of stain added to the agarose/buffer is dependent on the voltage used for electrophoresis. If electrophoresing at voltages less than 50 volts, a slightly lower concentration is utilized than if running at voltages greater than 50 volts. The stain may be added to the entire volume of agarose and distributed, or the agarose may be distributed to each lab station and the stain added by the students at the rates listed below:

Voltage	Agarose Volume	Stain Volume
< 50 Volts	30 mL	40 L (1 drop)
	60 mL	80 L (2 drops)
	400 mL	533 L (13 drops)
> 50 Volts	50 mL	80 L (2 drops)
	400 mL	640 L (16 drops)

After addition of the stain to the agarose, swirl to mix and immediately pour the gel. Gels may be prepared one day ahead of the lab day, if necessary. Gels stored longer tend to fade and lose their ability to stain bands during electrophoresis. Store covered with a small amount of buffer (leaving masking tape in place), or store covered in the gel box. **Do not try using more stain than recommended in your gel. This leads to precipitation of the DNA in the wells and can create artifactual aggregated DNA bands in the agarose gel.**

Addition of Stain to Buffer

Use the chart below for addition of the stain to 1 TBE electrophoresis buffer:

Electrophoresis Voltage	Buffer Volume	Stain Volume
< 50 Volts	500 mL	500 L (12 drops)
	2.6 liters	2.6 mL (65 drops)
> 50 Volts	500 mL	960 L (24 drops)
	2.6 liters	5 mL (125 drops)

The dropper bottle provided delivers 40 L/drop. If a calibrated pipet is available, the dropper tip can be removed for quicker addition of larger volumes of stain. The volume of buffer and agarose required for some gel box options are listed below:

Type Gel Box	Volume Buffer Required	Volume Agarose Required
Mini Gel System Box	200 mL	30 mL/casting tray
Carolina Gel Box, 1 tray	250 mL	50 mL
Carolina Gel Box, 2 trays	450 mL	100 mL

While *Carolina* BLU is not toxic, we recommend that the students wear gloves to prevent staining the skin. If reusing the buffer is important, we recommend using *Carolina* BLU in the gel and/or as final stain only.

Pool Reagents into the Bottoms of Their Vials

Because of the small volumes supplied, reagents often become spread in tiny droplets around storage tube wall or cap during shipping. Therefore, it is recommended that reagents be pooled at the bottom of their storage tubes prior to setting up workstations, using one of three methods:

1. Spin tubes briefly in a microfuge.
2. 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.
3. Tap tubes sharply on bench top.

Set Up Student Stations

1. Prepare six student stations, each with the following materials:

vial 2 restriction buffer (50 µL)	micropipet
vial lambda DNA (25 µL)	micropipet tips
tube distilled water (1 mL)	power supply
vial loading dye (100 µL)	gel electrophoresis chamber
5 reaction tubes, 1.5 mL	masking tape
rack for tubes	staining tray
permanent marker	2 sets of Student Guides
2. Groups must share the following materials: restriction enzymes *Bam*HI, *Eco*RI, and *Hind*III (on ice); agarose solution; TBE electrophoresis buffer; water bath at 37°C; and stain.
3. Store enzymes in a beaker of cracked ice during the experiment.
4. Hold agarose solution at 60°C in a water bath.
5. Set up a 37°C water bath for incubating restriction reactions. A constant-temperature water bath can be made by maintaining a trickle flow of tap water into a styrofoam box. Monitor temperature with a thermometer.

Student Lab Briefing

Principles of Restriction Enzymology and Gel Electrophoresis

Pipetting Tips

Train students in the proper use of the digital micropipet.

- Most digital micropipets have a two-position plunger with friction “stops.” Depressing to the first stop measures the desired volume. Depressing to the second stop introduces an additional volume of air to blow out any solution remaining in the tip. On many models, a third stop ejects the tip.
- When withdrawing or expelling fluid, always hold tube firmly between thumb and forefinger. Hold tube at nearly eye level, so you can observe fluid level change in pipet tip.
- Do not try to pipet with test tube in rack or try to pipet into tube held by lab partner.
- *To Withdraw Sample:* Depress plunger to first stop, and hold in this position. Then dip tip into solution to be pipetted, and draw fluid into tip

by releasing plunger. Slide pipet tip out along inside wall of tube to dislodge any excess droplets adhering to outside of tip.

- *To Expel Sample:* Touch pipet tip to inside wall of tube into which you want to empty sample. This creates a capillary effect that helps draw fluid out of tip. Slowly depress plunger to first stop. Depress to second stop to blow out last bit of fluid. Hold plunger in depressed position. Slide pipet out of tube with plunger depressed to avoid sucking liquid back into tip.
- To prevent cross-contaminating reagents, always add appropriate amounts of single reagent sequentially to all reaction tubes. Release each reagent drop onto new location on inside wall, near bottom of reaction tube. In this way, the same tip can be used to pipet reagent into each reaction tube. Use a fresh tip for each new reagent to be pipetted.

Practice Pipetting

Students should set up the following mock reaction to practice pipetting technique and check accuracy.

1. Use micropipet to sequentially add the following volumes of colored water to an empty 1.5-mL test tube: 4 μL , 5 μL , 1 μL . (Water may be colored with food coloring or loading dye.)
2. Pool and mix reagents by tapping the tube bottom on lab bench, or by giving the tube a short pulse in a microcentrifuge.
3. Set micropipet at 10 μL , and carefully withdraw contents of tube. Is there any air at the very end of the pipet tip? Is there any water left in the tube? If you answered yes to either of these questions, have you over- or under-pipetted?

Practice Gel Loading (Optional)

You may wish to have students practice loading a gel, according to directions in Procedure C. Practice gel-loading stations are available from Carolina Biological Supply (#21-1145).

Fine Points of Lab Procedure

Be alert to the following cautions when performing the experiments. Where appropriate, discuss fine points with students, and have them make annotations on their Student Worksheets.

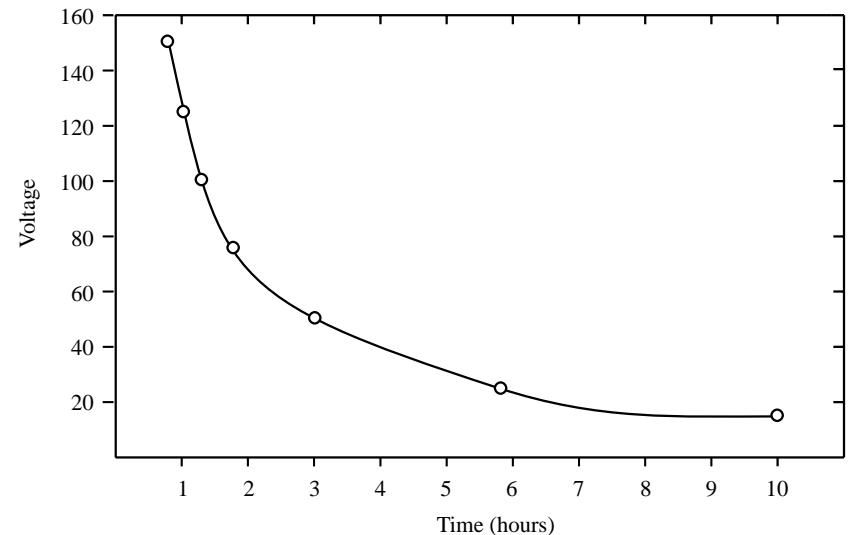
Incubating Restriction Reactions

Twenty minutes is the bare minimum incubation time for the restriction

reaction to go to completion. If you will be electrophoresing the following day, as recommended in the scheduling section, the reactions can be incubated 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 freezer (-20°C) until ready to continue. Thaw reactions before adding loading dye.

Storing Cast Agarose Gels

As recommended in the scheduling section, students may cast gels a day or two before use. If using the *Carolina* BLU™ stain, it is best not to make the gels more than one day in advance. Agarose with stain added fades with time, resulting in the inability to stain DNA fragments during electrophoresis. Keep gels covered with TBE electrophoresis buffer to prevent drying.



Graph plotting voltages versus time for 84 mm x 94 mm "mini-gel" system using 1.0% agarose.

Electrophoresing

The migration of DNA through the agarose gel is dependent upon voltage—the higher the voltage, the faster the rate of migration. Best separation is achieved when the bromophenol blue band (the faster-moving band of the loading dye) nears the end of the gel. Do not let bromophenol blue band run off the end of the gel. Refer to the chart above for approximate running times at various voltages. Times (hours) are for

“mini-gel” system with 84- 96-mm gel using 1.0% agarose; times will vary according to apparatus and the percentage of gel used. If using the mini-gel electrophoresis system, catalog #21-3650, gels are run for 18–22 hours. Loading dye diffuses from the agarose and will be absent. Continue staining as directed.

Carolina BLU Staining

Although students may stain gels in class, it saves time to stain gels after class, as recommended in the scheduling section. Destaining gels overnight improves results. **Wear disposable gloves during staining and cleanup.**

1. Flood gels with *Carolina BLU*TM Final Stain, and allow to stain for 15–20 min.
2. Following staining, use funnel to decant as much *Carolina BLU*TM solution as possible from staining tray back into storage container. Place stained gel on light box. DNA bands should be visible. If bands are faint, additional staining may be required.
3. Rinse gel in distilled or deionized water. Chlorinated water tends to bleach bands with time. Let gel soak for several minutes in several changes of fresh water. DNA bands will become increasingly distinct as gel destains. 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.

Ethidium Bromide Staining

Ethidium bromide, like many natural and man-made substances, is a mutagen by the Ames microsome assay and a suspected carcinogen. Stain gels after school or at other time when students are not present. With responsible handling, the dilute staining solution (1 µg/mL) used in this kit poses minimal risk:

1. Wear rubber gloves when staining gel, viewing gels, and cleaning up.
2. Confine all staining to sink area restricted from student use.
3. Flood gels with ethidium bromide solution, and allow to stain for 5 to 10 minutes. (Staining time depends on thickness of gel.)
4. Following staining, use funnel to decant as much ethidium bromide solution as possible from staining tray back into storage container. Stain may be reused to stain 15 or more gels. When staining time increases markedly, disable ethidium bromide solution as explained below.

5. Rinse gel and tray under running tap water to remove excess ethidium bromide solution. (Chlorine in water will largely inactivate trace amounts of residual ethidium bromide.)
6. If desired, gels can be destained in tap or distilled water for 5 or more minutes to remove background ethidium bromide.
7. Staining intensifies dramatically if rinsed gels set overnight. Stack staining trays, and cover top gel with plastic wrap to prevent dessication.
8. After viewing and photographing, disable stained gels and used staining solution:
 - a. Add 1 volume of 0.05 M KMnO₄ and *mix carefully*.
 - b. Add 1 volume of 0.25 N HCl and *mix carefully*.
 - c. Let stand at room temperature for several hours.
 - d. Add 1 volume of 0.25 N NaOH and *mix carefully*.
 - e. Discard disabled solution down sink drain. Drain disabled gels and discard in regular trash.

Viewing and Photographing Gels

Transillumination, where light passes up through gel, gives superior viewing of gels stained with either ethidium bromide or *Carolina BLU*TM.

- A fluorescent light box for viewing slides and negatives provides ideal illumination for *Carolina BLU*TM-stained gels. An overhead projector may also be used. Cover surface of light box or projector with plastic wrap to keep liquid off the apparatus.
- A mid-wavelength ultraviolet lamp emits in the optimum range for illuminating ethidium bromide-stained gels (260 to 360 nm). Avoid shortwave lamps, whose radiation is most dangerous. Long-wavelength (“black light”) lamps, though safe, give less intense illumination.

Caution: Ultraviolet light can damage the retina of the eye. Never look at unshielded UV light source with naked eye. Only view through filter or safety glasses that absorb harmful wavelengths.

A Polaroid® “gun” camera, equipped with a close-up diopter lens, can be 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.

Lab Procedure

Procedure A: Set Up Restriction Digest

1. Students label four 1.5-mL tubes, in which they will perform restriction reactions: *B* for *Bam*HI, *E* for *Eco*RI, *H* for *Hind*III, and – for no enzyme.
2. Students use the table 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. All groups share the same *Bam*HI, *Eco*RI, *Hind*III enzymes at a central station.

TUBE	DNA	Buffer	<i>Bam</i> HI	<i>Eco</i> RI	<i>Hind</i> III	H ₂ O
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. Reagents are pooled and mixed by tapping the tube bottom on lab bench, or with a short pulse in a microcentrifuge.
4. Incubate all reaction tubes for a minimum of 20 minutes at 37°C. You may instruct the students to incubate the reactions for a longer period.

Procedure B: Cast Agarose Gel

1. Students seal ends of gel-casting tray with tape, and insert well-forming comb. They should place gel-casting tray out of the way on lab bench, so that agarose poured in next step can set undisturbed.
2. Students carefully pour enough agarose solution into casting tray to fill to a depth of about 5 mm. Gel should cover only about ⅓ the height of comb teeth. While gel is still liquid, a pipet tip or toothpick is used to move large bubbles or solid debris to sides or end of tray.
3. Gel will become cloudy as it solidifies (about 10 minutes). Do not move or jar casting tray while agarose is solidifying.
4. When agarose has set, students unseal ends of casting tray and place tray on platform of gel box so that comb is at negative (black) end.
5. Students fill box with tris-borate-EDTA (TBE) buffer to a level that just covers entire surface of gel.
6. Students must gently remove comb, taking care not to rip wells.
7. Make certain that sample wells left by comb are completely

submerged. If “dimples” are noticed around wells, students slowly add buffer until dimples disappear.

8. The gel is now ready to load with DNA. If students will be loading the gel during another period, instruct your students to cover the electrophoresis tank to prevent drying of the gel.

Procedure C: Load Gel

1. Students add 1 μL loading dye to each reaction tube and mix dye with digested DNA by tapping tube on lab bench, or with a pulse in microcentrifuge.
2. Students use a micropipet to load contents of each reaction tube into a separate well in gel, as shown in the table on pg. 13. Students must use a fresh tip for each reaction tube.
 - 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.)
 - Dip pipet tip through surface of buffer, position it over the well, and slowly expel the mixture. Sucrose in the loading dye weighs down the sample, causing it to sink to the bottom of the well. *Be careful not to punch tip of pipet through the bottom of the gel.*

Procedure D: Electrophoresis

1. Students close top of electrophoresis chamber and connect electrical leads to an approved power supply, anode to anode (red-red) and cathode to cathode (black-black). Make sure both electrodes are connected to same channel of power supply.
2. Students turn power supply on, and set voltage as directed by the instructor. Shortly after current is applied, loading dye can be seen moving through gel toward positive pole of electrophoresis apparatus.
3. The loading dye will eventually resolve 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 the same rate as a DNA fragment approximately 300 base pairs long. Xylene cyanol migrates at a rate equivalent to approximately 2000 base pairs.
4. Allow the DNA to electrophorese until the bromophenol blue band nears the end of the gel. The instructor may monitor the progress of electrophoresis in the students’ absence; in that case, students omit Steps 5 and 6.

- Students turn off power supply, disconnect leads from the inputs, and remove top of electrophoresis chamber.
- Students should carefully remove casting tray and slide gel into staining tray labeled with their group name. The students bring their gels to the instructor for staining.

Results and Discussion

- Examine your stained gel on a light box or overhead projector. Compare your gel with the ideal gel shown below [pg. 19 in Instructor's Manual] and try to account for the fragments of lambda DNA in each lane.
- How can you account for differences in separation and band intensity between your gel and the ideal gel?
Bands on ideal gel are more spread out, because the gel electrophoresed for a longer period of time. Band intensity is dependent upon mass of DNA in the band—the greater the mass of DNA the more intensely stained the band.
- DNA fragments of similar size will not always resolve on a gel. This is seen in lane B in the Ideal Gel, where *EcoRI* 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 doublet fragments?
Change the concentration of agarose in the gel. A "tighter" gel matrix more effectively separates smaller DNA fragments, and a "looser" gel matrix more effectively separates larger fragments. Alternatively, cast a longer gel and let the DNA electrophorese a longer time.
- 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.
 - The matrix on the next page gives the actual size in base pairs (Act. bp) of lambda DNA fragments generated by a *HindIII* digest: (and *EcoRI* and *BamHI* digests, which you will share with the students at Step 4i in Student Guide 2):
 - Using the ideal gel shown on the front of this sheet [pg. 19 in Instructor's Manual] carefully measure distance (in mm) each *HindIII*, *EcoRI*, and *BamHI* fragment migrated from the origin.

Measure from front edge of well to front edge of each band. Enter distances into matrix. **Alternatively, have students measure distance directly from their Carolina BLU™-stained gel or from a photo of their ethidium stained gel.**

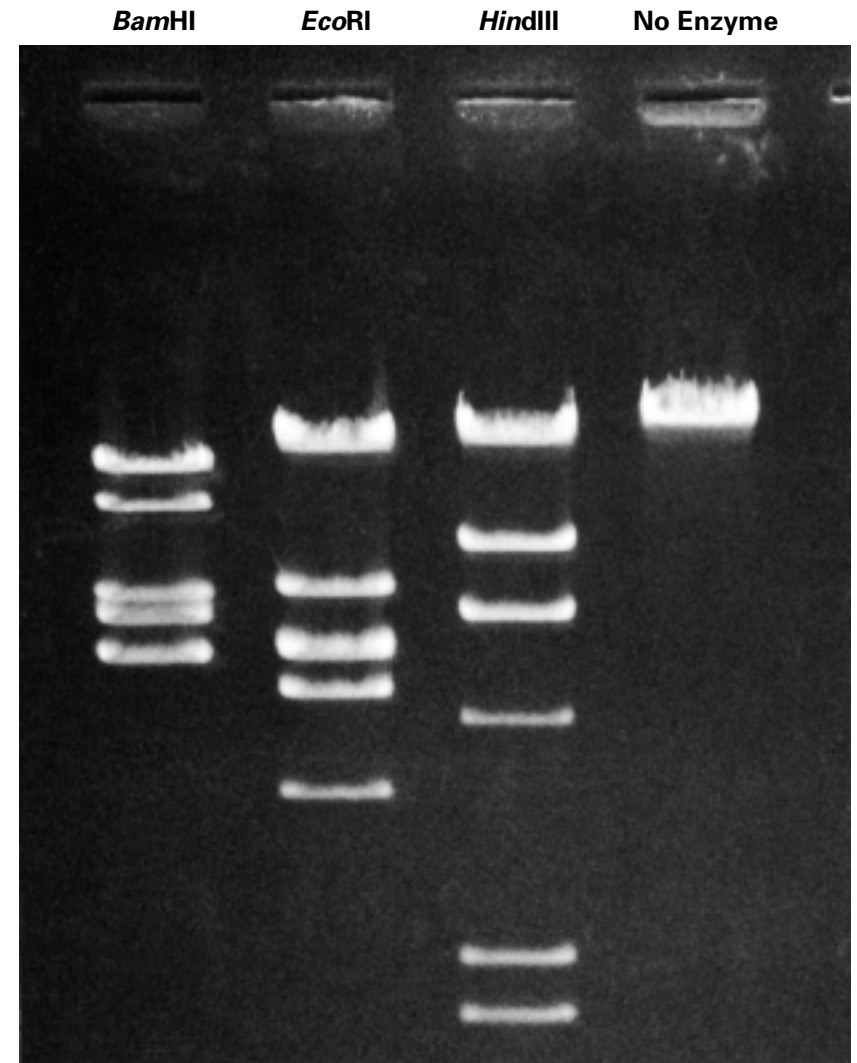
<i>HindIII</i>		<i>EcoRI</i>			<i>BamHI</i>		
Dis.	Act. bp	Dis.	Cal. bp	Act. bp	Dis.	Cal. bp	Act. bp
	*27,491			*24,756			16,841
	*23,130			*21,226			12,275
	9,416			7,421			7,233
	6,557			*5,804			*6,770
	4,361			*5,643			*6,527
	2,322			4,878			*5,626
	2,027			3,530			*5,505
	**564						
	**125						

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

- Match base-pair sizes of *HindIII* 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.
- 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 distance migrated versus base-pair length for each *HindIII* fragment.
- Connect data points with a best-fit line.
- Locate on *x* axis the distance migrated by the first *EcoRI* fragment. Using a ruler, draw a vertical line from this point to its intersection with the best-fit data line.
- Now extend a horizontal line from this point to the *y* axis. This gives the base-pair size of this *EcoRI* fragment.
- Repeat Steps f and g for each *EcoRI* and *BamHI* fragment. Enter results in the calculated base pairs (Cal. bp) columns for each digest.
- Enter the actual base-pair size of *EcoRI* and *BamHI* fragments, as provided by your instructor, into Act. bp columns.
- 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?

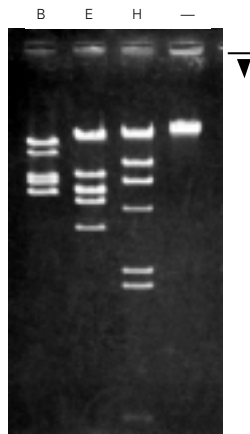
Extrapolations from the graph are most accurate for short to mid-size DNA fragments and least accurate for very large fragments. The 0.8% gel used in this experiment most effectively separates short to mid-size fragments.

Note: Lambda 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 of the linear form of lambda 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. This annealing of the right and left ends of lambda to each other explains the occasional to common appearance of a 27,491 bp band and the partial loss of the 4361 bp band seen in *Hind*III digests of lambda. The 23,130 bp left terminal fragment and the 4361 bp right terminal fragment anneal to each other to form the 27,491 bp fragment. The loss of the 23,130 fragment is not as obvious since the 27,491 bp and 23,130 bp are very difficult to separate from each other unless the correct percentage gel is run very slowly and for a long time.

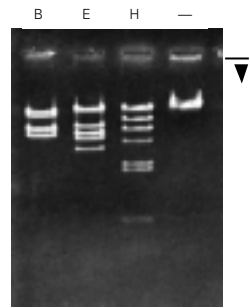


Ideal Restriction Digest of *lambda* DNA

Field Guide to Electrophoresis Effects

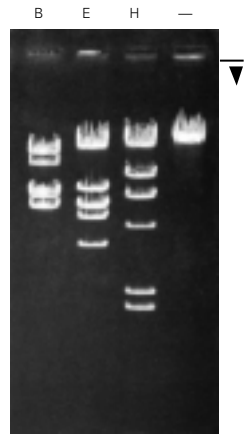


Ideal Gel



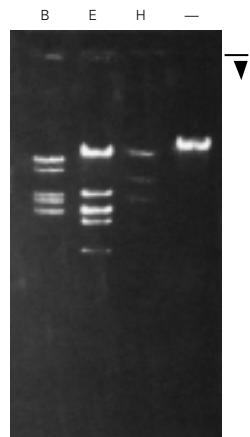
Short Run

Bands compressed. Short time electrophoresing.



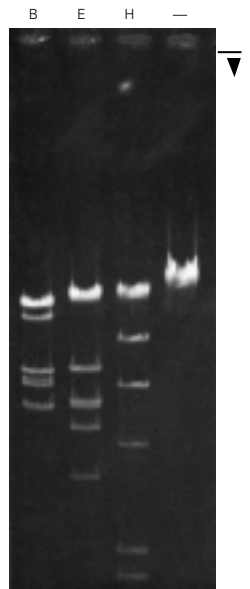
Overload

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



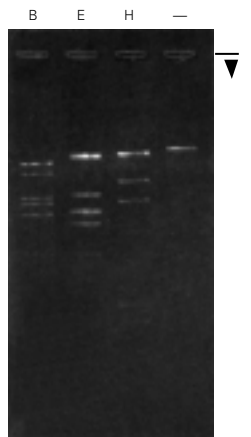
Punctured Wells

Bands faint in lanes B and H. DNA lost through hole punched in bottom of well with pipet tip.



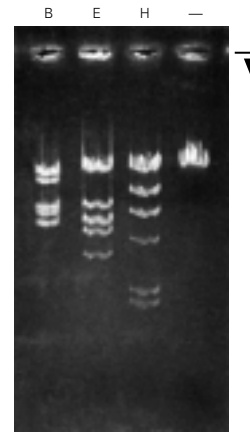
Long Run

Bands spread. Long time electrophoresing.



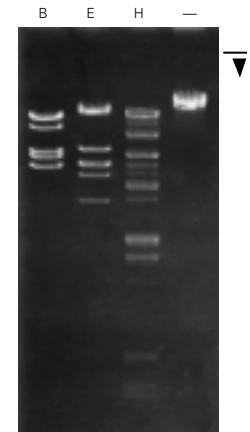
Underloaded

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



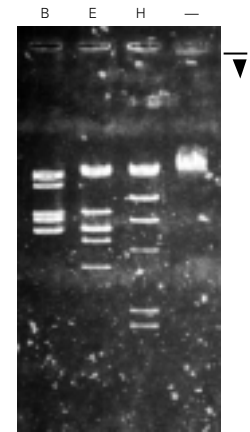
Poorly Formed Wells

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



Enzymes Mixed

Extra bands in lane H. *Bam*HI and *Hind*III mixed in digest.



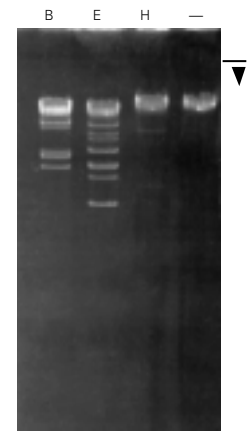
Precipitate

Precipitate in TBE buffer used to make gel.



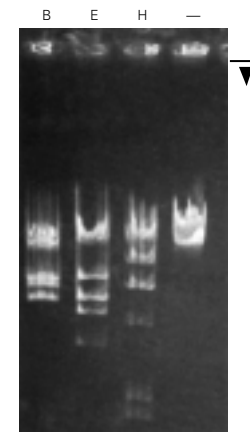
Bubble in Lane

Bump in band in lane B. Bubble in lane.



Incomplete Digest

Bands faint in lane H. Very little *Hind*III 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.

DNA Restriction Analysis

In this experiment, DNA from the bacteriophage lambda (48,502 base pairs in length) is cut with restriction enzymes and the resulting restriction fragments are separated using gel electrophoresis. Three samples of lambda DNA are incubated at 37°C, each with one of three restriction endonucleases: *Bam*HI, *Eco*RI, and *Hind*III. A fourth sample, the negative control, is incubated without an endonuclease.

The DNA samples are then loaded into wells of an agarose gel and electrophoresed. An electrical field applied across the gel causes the DNA fragments in the samples to move from their origins (sample wells) through the gel matrix toward the positive electrode. Smaller DNA fragments migrate faster than larger ones, so restriction fragments of differing sizes become concentrated into separate bands during electrophoresis. The characteristic number and pattern of bands produced by each restriction enzyme are, in effect, a "DNA fingerprint." The restriction patterns are made visible by staining with a compound that binds to DNA.

Procedure A: Set Up Restriction Digest

- Label four 1.5-mL tubes, in which you will perform restriction reactions: B for *Bam*HI, E for *Eco*RI, H for *Hind*III, and – for no enzyme.
- Use table 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. All groups share the same *Bam*HI, *Eco*RI, *Hind*III enzymes at a central station.

Tube	DNA	Buffer	<i>Bam</i> HI	<i>Eco</i> RI	<i>Hind</i> III	H ₂ O
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

- Pool and mix reagents by tapping the tube bottom on lab bench, or with a short pulse in a microcentrifuge.
- Incubate all reaction tubes for a minimum of 20 minutes at 37°C. Your teacher may instruct you to incubate the reactions for a longer period.

Procedure B: Cast Agarose Gel

- Seal ends of gel-casting tray with tape, and insert well-forming comb. Place gel-casting tray out of the way on lab bench, so that agarose poured in next step can set undisturbed.
- Carefully pour enough agarose solution into casting tray to fill to depth of about 5 mm. Gel should cover only about ½ the height of comb teeth. Use a pipet tip or toothpick to move large bubbles or solid debris to sides or end of tray, while gel is still liquid.
- Gel will become cloudy as it solidifies (about 10 min). Do not move or jar casting tray while agarose is solidifying.
- When agarose has set, unseal ends of casting tray. Place tray on platform of gel box, so that comb is at negative (black) end.
- Fill box with tris-borate-EDTA (TBE) buffer, to level that just covers entire surface of gel.
- Gently remove comb, taking care not to rip wells.

- Make certain that sample wells left by comb are completely submerged. If "dimples" are noticed around wells, slowly add buffer until they disappear.
- The gel is now ready to load with DNA. If you will be loading the gel during another period, your teacher will instruct you to cover the electrophoresis tank to prevent drying of the gel.

Procedure C: Load Gel

- Add 1 µL loading dye to each reaction tube. Mix dye with digested DNA by tapping tube on lab bench, or with a pulse in microcentrifuge.
- Use micropipet to load contents of each reaction tube into a separate well in gel, aligned as illustrated in **Ideal Restriction Digest of lambda DNA**. Use a fresh tip for each reaction tube.
 - 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.)
 - Dip pipet tip through surface of buffer, position it over the well, and slowly expel the mixture. Sucrose in the loading dye weighs down the sample, causing it to sink to the bottom of the well. **Be careful not to punch tip of pipet through bottom of gel.**

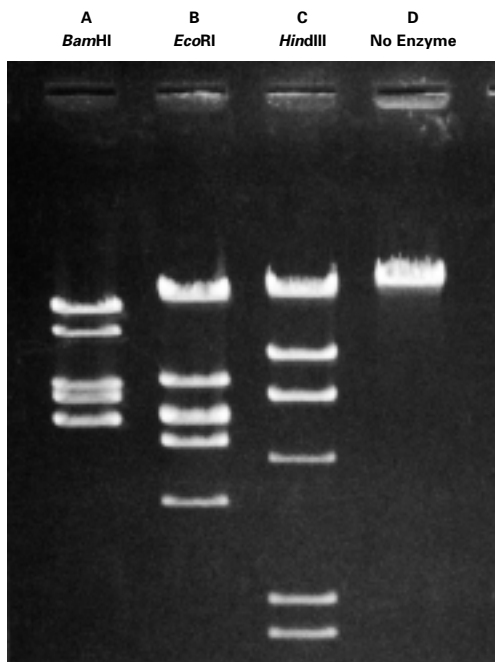
Procedure D: Electrophorese

- Close top of electrophoresis chamber and connect electrical leads to an approved power supply, anode to anode (red-red) and cathode to cathode (black-black). Make sure both electrodes are connected to same channel of power supply.
- Turn power supply on and set voltage as directed by your instructor. Shortly after current is applied, loading dye can be seen moving through gel toward positive pole of electrophoresis apparatus.
- The loading dye will eventually resolve 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 base pairs long. Xylene cyanol migrates at a rate equivalent to approximately 2000 base pairs.
- Allow the DNA to electrophorese until the bromophenol blue band nears the end of the gel. Your instructor may monitor the progress of electrophoresis in your absence; in that case, omit steps 5 and 6.
- Turn off power supply, disconnect leads from the inputs, and remove top of electrophoresis chamber.
- Carefully remove casting tray and slide gel into staining tray labeled with your group name. Take gel to your instructor for staining.

DNA Restriction Analysis

Results and Discussion

- Examine your stained gel on a light box or overhead projector. Compare your gel with the ideal gel shown below, and try to account for the fragments of lambda DNA in each lane.
- How can you account for differences in band separation and intensity between your gel and the ideal gel?
- DNA fragments of similar size will not always resolve on a gel. This is seen in lane B in the Ideal Gel, where *EcoRI* 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 doublet fragments?



Ideal Restriction Digest of Lambda DNA

- 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.
 - The matrix below gives the actual size in base pairs (Act. bp) of lambda DNA fragments generated by a *HindIII* digest.

<i>HindIII</i>		<i>EcoRI</i>			<i>BamHI</i>		
Dis.	Act. bp	Dis.	Cal. bp	Act. bp	Dis.	Cal. bp	Act. bp
	*27,491						
	*23,130						
	9,416						
	6,557						
	4,361						
	2,322						
	2,027						
	**564						
	**125						

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

- Using the ideal gel shown on the front of this sheet, carefully measure distance (in mm) each *HindIII*, *EcoRI*, and *BamHI* fragment migrated from the origin. Measure from front edge of well to front edge of each band. Enter distances into matrix.
- Match base-pair sizes of *HindIII* 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.
- 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 distance migrated versus base-pair length of each *HindIII* fragment.
- Connect data points with a best-fit line.
- Locate on x axis the distance migrated by the first *EcoRI* fragment. Using a ruler, draw a vertical line from this point to its intersection with the best-fit data line.
- Now extend a horizontal line from this point to the y axis. This gives the base-pair size of this *EcoRI* fragment.
- Repeat steps f and g for each *EcoRI* and *BamHI* fragment. Enter results in the calculated base pairs (Cal. bp) columns for each digest.
- Enter the actual base-pair size of *EcoRI* and *BamHI* fragments, as provided by your instructor, into Act. bp column.
- 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?

Note: Lambda 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 of the linear form of lambda 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. This annealing of the right and left ends of lambda to each other explains the occasional appearance of a 27,491 bp band and the partial loss of the 4361 bp band seen in *HindIII* digests of lambda. The 23,130 bp left terminal fragment and the 4361 bp right terminal fragment anneal to each other to form the 27,491 bp fragment. The loss of the 23,130 fragment is not as obvious since the 27,491 bp and 23,130 bp are very difficult to separate from each other unless the correct percentage gel is run very slowly and for a long time.

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