To close the yellow note, click once to select it and then click the box in the upper left corner.
To open the note, double click (Mac OS) or right click (Windows) on the note icon.

Restriction Analysis of Lambda DNA

Miriam Golbert, College of the Canyons, Santa Clarita, CO

INTRODUCTION

Description

This lab is separated into two different sections due to lab time allowed for each session over a two-week period. During the first lab session, students will prepare their first digests and the control, using the three different restriction enzymes. During the second lab period, the students will get their digests from the instructor; cast the agarose gel; set up and run the electrophoresis; and photograph, analyze, and graph the results.

Student Audience

This laboratory exercise is suitable for college biology majors and non-majors in an introductory biology course. It may also be used in an Advanced Placement/International Baccalaureate or advanced biology course, or in an anatomy, physiology, genetics, or biotechnology course.

Goals for the Activity

Students will learn

- the importance of restriction enzymes in DNA research and biotechnology,
- how to handle microvolumes using the different equipment used by researchers and the biotechnology industry,
- the importance of using controls in DNA science,
- how to cast an agarose gel and set up the electrophoresis chamber,
- how to use ethidium bromide to visualize DNA in the agarose gel,
- how to analyze a photograph and interpret their results, and
- how to construct a standard curve and compare it to their results.

Recommended Placement in the Curriculum

This laboratory exercise is recommended for the ninth and tenth week of an 18-week semester because that is the time at which the lecture introduces the material to the students.

STUDENT HANDOUT
Restriction Analysis of Lambda DNA

Restriction endonucleases, or restriction enzymes, are important classes of enzymes used by biotechnologists. Scientists discovered restriction enzymes in 1962 while studying bacteria that appeared to possess a primitive immune system that prevented viral DNA from replicating within the infected host bacterium. Later, it was found that this immune response involved enzymes that would cut up the viral DNA at specific recognition sites and render it ineffective at causing infection. Since 1962, over 100 different restriction enzymes have been isolated from different types of bacteria. Each specific restriction enzyme is named for the bacteria it was isolated from. The first letter in a specific name is capitalized and represents the genus of the bacteria. The second two letters indicate the species of the bacteria, and are lower case. The strain of this species is indicated by the fourth letter, usually capitalized. The Roman numeral that ends the name of a particular restriction enzyme indicates the order in which this particular enzyme was isolated from this strain of bacteria.

Consider the name of one of the enzymes you will use in lab: *Eco*R I. This enzyme was isolated from *Escherichia coli*, strain RY13, and it was the first restriction enzyme isolated from this strain of *E. coli*. Table 1 identifies the restriction enzymes you will use during this lab.

When it is mixed with a restriction enzyme, DNA is sliced up into fragments. The lengths of these fragments will vary, depending on the particular enzyme used. Most restriction enzymes recognize and cleave DNA at sites where two strands of the DNA molecule exhibit two-fold symmetry. Table 1 identifies the recognition sequence and cleavage or restriction sites for the enzymes you will use in lab. Some enzymes cut both strands of DNA at exactly the same axis of symmetry to generate blunt-ended DNA fragments. Others cleave each strand at the same location on opposite sides of the axis of symmetry, thereby creating single-stranded "sticky-ends" or COS sites.

Table 1: Restriction Enzymes Used in This Laboratory (Arrows indicate cut sites.)

Source	Restriction Enzyme	Recognition Site
Escherichia coli	*Eco*R I	5' G↓AATTC 3' 3' CTTAA↑G 5'
Haemophilus influenzae	*Hin*d III	5' A↓AGCTT 3' 3' TTCGA↑A 5'
Bacillus amyloliquefaciens	*Bam*H I	5' G↓GATCC 3' 3' CCTAG↑G 5'

Purpose

This laboratory introduces 1) the importance of restriction enzymes in DNA research and biotechnology, 2) the mechanism of separating and analyzing DNA using gel electrophoresis and the use of ethidium bromide to visualize DNA in the agarose gel, and 3) the use and importance of controls in DNA science.

Materials

Reagents

- lambda DNA (0.45 µg/µL)
- DNA size marker
- restriction enzymes
  - *Eco*R I
  - *Hin*d III
  - *Bam*H I
- 10X buffer
- distilled water
- agarose (0.8%)
- 0.5%X TBE buffer
- 5X loading dye
- ethidium bromide (1µg/mL)

Equipment and supplies

- P-20-µL micropipet and tips
- 1.5-mL microcentrifuge tubes
- microcentrifuge
- 37°C and 60°C water baths
- floating tube holder
- electrophoresis apparatus
- UV transilluminator
- instant-print camera and film
- permanent marker
- waste container
- staining tray
- crushed ice in plastic cup
- UV safety glasses
- latex gloves
- vortex apparatus
- (optional) plastic wrap

Safety, Handling, and Disposal

It is your responsibility to specifically follow your institution's standard operating procedures (SOPs) and all local, state, and national guidelines on safe handling and storage of all chemicals and equipment you may use in this activity. This includes determining and using the appropriate personal protective equipment (e.g., goggles, gloves, apron). If you are at any time unsure about an SOP or other regulation, check with your instructor.

Visualization of DNA fragments uses ethidium bromide, which is a mutagen and suspected carcinogen. Although it is used in very dilute concentrations, handle carefully as directed by the instructor. To dispose of ethidium bromide, return it to the biohazard liquids container according to local ordinances.

When dealing with biological materials, take particular precautions as called for by the kit manufacturer or supplier.

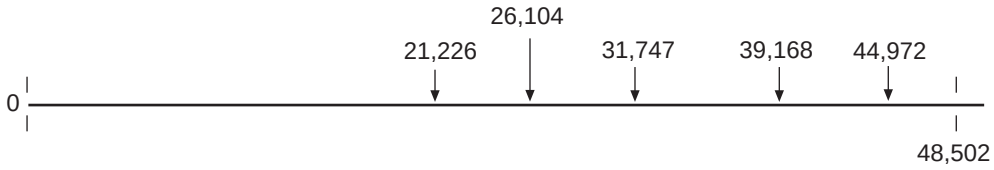
The UV radiation used to view DNA fragments stained with ethidium bromide can cause eye damage. View stained gels only with the UV blocking cover in place and UV safety glasses.

Wear gloves while handling the staining tray and the gel after staining.

Procedure

Restriction maps for Lambda DNA

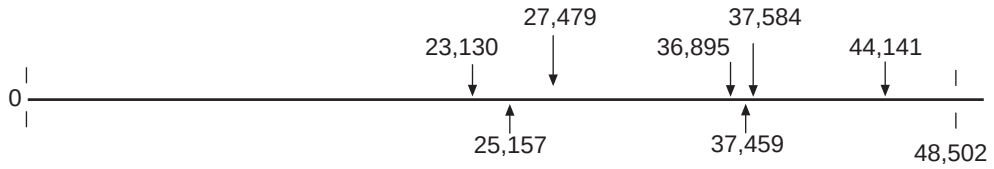
Lambda DNA may exist as a linear or circular molecule. The linear Lambda DNA is 48,502 base pairs in length. It contains five *Eco*R I restriction sites or cleavage sites. These restriction sites are illustrated below. Each number indicates the distance, in base pairs, that the restriction site is located from the end of the linear Lambda DNA molecule. *Hin*d III recognizes seven restriction sites. *Bam*H I recognizes five sites. Figures 1–3 show the restriction sites for the enzymes we will use. In the spaces provided below each map, calculate the size, in base pairs, of the individual fragments produced by each enzyme.



Number of l DNA fragments produced by digestion with *Eco*R I: _____

Size, in base pairs (bp), of fragments produced by *Eco*R I digestion: _____

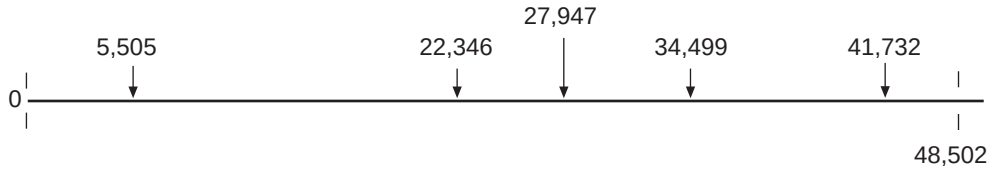
Figure 1: Restriction sites for Eco*R I*



Number of fragments produced: _____

Size of fragments produced: _____

Figure 2: Restriction sites for Hin*d III*



Number of fragments produced: _____

Size of fragments produced: _____

Figure 3: Restriction sites for Bam*H I*

Lambda DNA/*Hin*d III fragments are suitable for sizing linear double-stranded DNA fragments ranging from 125 bp to 23 kbp. They are prepared by digesting lambda DNA completely with *Hin*d III, running the fragments on an agarose gel, and staining them with ethidium bromide.

A. Restriction enzyme digestion

1. Collect the reagents from the instructor. Keep all of the enzymes and the DNA in "wet ice." Be certain to push the tubes into the crushed ice.

2. Place the microcentrifuge tube containing the lambda DNA in a floating tube holder, then place the holder in a 37°C water bath for 10 minutes. Identify your tubes with your group number.

3. Use the minicentrifuge to centrifuge this tube for 30 seconds to collect the solution at the bottom of the tube. Place the lambda DNA in the test tube rack at room temperature.

4. Label four 1.5-mL centrifuge tubes 1 through 4 with a marking pen. Identify these also with your group number.

5. If you are not familiar with the correct way to introduce microvolumes into microcentrifuge tubes and then mix by centrifugation after all the volumes have been added, ask your instructor. Only AFTER all the other substances have been added to each tube should you add the enzyme. ALWAYS ADD THE ENZYME LAST!

   Using your micropipet, prepare the restriction enzyme digestion mixtures shown in Table 2:

Table 2: Restriction Enzyme Digestion Mixtures

Tube #	Lambda DNA	Distilled Water	BSA 1 mg/mL	10X Buffer	Enzyme
1	1 µL	15 µL	2 µL	2 µL	0 µL
2	1 µL	14 µL	2 µL	2 µL	1 µL *Eco*R I
3	1 µL	14 µL	2 µL	2 µL	1 µL *Hin*d III
4	1 µL	14 µL	2 µL	2 µL	2 µL *Bam*H I

6. Mix each tube using a vortex mixer for 5 seconds.

7. Centrifuge these tubes using the minicentrifuge for 30 seconds.

8. Immediately place the tubes in a floating tube holder and place the holder in a 37°C water bath for 1 hour.

9. After the 1-hour incubation, centrifuge the tubes for 30 seconds in the minicentrifuge. Place the tubes in a microcentrifuge tube rack at room temperature.

10. Label a set of centrifuge tubes #1 to #4, then transfer 5 μL of each of the digestion mixtures to the new tube of the SAME NUMBER. Immediately add 11 μL distilled water and 4 μL loading dye to each tube. Identify your tubes with your group number.

11. Vortex the tubes briefly and then centrifuge them for 30 seconds in the minicentrifuge. Store these samples in the freezer until needed. You may also store your remaining digests in the freezer.

B. Agarose gel preparation and gel electrophoresis

1. Prepare the gel tray by removing it from the electrophoresis chamber and pushing the gates into the "UP" position. Check to make sure that the screws that secure the gate are snug but not too tight; if they are too tight, the gates will leak.

2. Position the comb in the slots nearest the end of the tray. Make sure that the comb is sealed on both sides of the gel tray and that the tray is positioned on a flat surface.

3. Obtain a centrifuge tube containing liquid agarose from the 60°C water bath.

4. Pour the liquid agarose into the gel tray SLOWLY to avoid creating bubbles in the gel. Bubbles in the gel may interfere with the movement of the DNA fragments within the gel.

5. Allow the gel to solidify for approximately 10–20 minutes. After the gel is solid, it may be wrapped in plastic wrap and stored in the refrigerator or used immediately.

6. To run the gel, place the gel tray in the electrophoresis chamber so that the comb end is nearest the black electrode or cathode. Fill the chamber with 0.5X TBE buffer until the gel is covered by approximately 1–2 mm of buffer. Using both hands, pull the comb straight out of the gel, being gentle to avoid tearing the wells. Check the buffer level to be sure it is still above the wells and that there are no observable "dimples" above the wells. Add buffer if necessary.

7. Label a microcentrifuge tube #5. Introduce 1.0 μL of DNA size marker into this tube. This has been digested by *Hin*d III. Add 15 μL distilled water and 4 μL loading dye.

8. Obtain your digestion samples from the instructor. Heat these digests and the DNA marker prepared in step 7 in a 37°C water bath for 5 minutes.

9. Rapidly cool these samples in a "wet ice" bath for 10 minutes.

10. Centrifuge these samples for 30 seconds in the microcentrifuge.

11. Carefully pipet 20 μL of each of the above solutions, 1–5, into the wells of the submerged agarose gel. Be sure to CENTER THE PIPET TIP over the well and GENTLY depress the plunger to expel the sample. Be careful not to tear the well. Use Table 3 to position the samples in the gel.

Table 3: Gel Preparation	
Gel well number from left to right or near to far	Sample description # indicates tube #
2	20 µL digest #1
3	20 µL digest #2
4	20 µL digest #3
5	20 µL digest #4
6	20 µL DNA marker #5

12. Close the cover of the electrophoresis chamber. Connect the electrical leads to the power supply. Be sure that both leads are connected to the same channel with the cathode (-) to cathode, (black to black), and the anode (+) to anode, (red to red).

13. Before turning the power supply on, rotate the voltage adjustment to set the voltage to zero. Turn on the power supply and set the voltage between 90 and 100 volts.

14. When the visible dye is within 1–2 cm of the far edge of the gel, set the voltage back to zero and turn the power supply off. Unplug the electrical leads by pulling the plugs, not the cords. Remove the electrophoresis chamber.

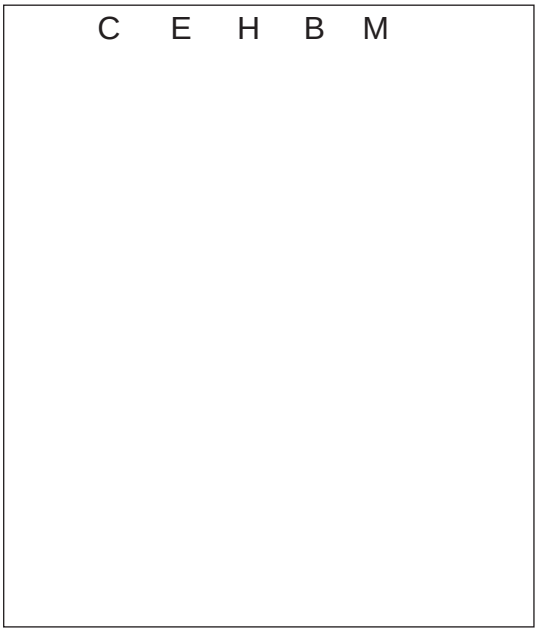
15. Carefully remove the gel by sliding it into the plastic staining tray. Pour enough ethidium bromide into the staining tray to cover the gel, and then rotate the tray on the counter or agitate for 15 minutes. After the 15 minutes, recycle the ethidium bromide into an appropriate container, and wash the gel in distilled water for 10 minutes, agitating or rotating on the counter top. **Use caution when working with the ethidium bromide.**

16. To visualize the gel, wear UV protective glasses. Place the gel on a photoilluminator, turn the light on, and observe.

17. Place an instant-print camera on the photoilluminator and take the picture.

Questions

Attach your photo of the gel:



C: control
E: *Eco*R I digestion
H: *Hin*d III digestion
B: *Bam*H I digestion
M: DNA marker

Lambda DNA Marker Restriction Sizes:

1.	8,454 bp	8.	2,323 bp
2.	7,242 bp	9.	1,929 bp
3.	6,369 bp	10.	1,371 bp
4.	5,686 bp	11.	1,264 bp
5.	4,822 bp	12.	702 bp
6.	4,324 bp	13.	224 bp (may not be visible)
7.	3,675 bp	14.	117 bp (may not be visible)

1. What could account for any differences between the sample and your photograph?

2. Why is tube #1 considered to be a negative control?

3. What is the purpose of the lambda DNA marker?

4. Considering the direction the DNA migrated in the gel, what can be concluded about the electrical charge of DNA? What part of the DNA molecule is responsible for this charge?

5. Using the photo of your gel, measure the distance in millimeters of each of the bands that appear in the M lane. Measure from the front edge of the well to the front edge of each lane. Using semi-log graph paper or a spreadsheet program, label the *x* axis “distance migrated (mm),” and the *y* axis “fragment size (bp).” Plot the data collected for the M lane. This will produce a standard curve to which you can compare other restriction digests.

Follow the same procedure for the other restriction enzymes and compare the measurement of each band to the size predicted by the standard curve and the size according to the restriction maps on your handout.

Suggested Reading

Bloom, M.; Fryer, G.; Micklos, D. *Laboratory DNA Science: An Introduction to Recombinant DNA Techniques and Methods of Genome Analysis;* Benjamin/Cummings: Menlo Park, CA, 1996; p 31.

Raven, P.; Johnson, G. *Biology,* 4th ed.; Wm. C. Brown: Dubuque, IA, 1996; Chapter 19; p 434.

INSTRUCTOR NOTES
Restriction Analysis of Lambda DNA

Time Required

First week: A lab period of 3 hours, where the students will prepare their first digests and then freeze them until the following week.

Second week: A lab period of 3 hours, where the students will cast an agarose gel, set up and run the electrophoresis, and then photograph and analyze the results.

Group Size

This lab is suitable for a class of 25–27, with groups of two or three students each.

Materials

Per group

- P-20-µL micropipet and tips
- 5–6, 1.5-mL microcentrifuge tubes
- permanent marker
- staining tray (plastic container)
- plastic cup to hold crushed ice
- The following reagents:
  - restriction enzymes in aliquots of 2 µL each of *Eco*R I, *Hin*d III, and *Bam*H I
  - 70 µL distilled water
  - 10 µL BSA (1 mg/mL)
  - 10 µL 10X buffer
  - 5 µL lambda DNA
  - 1.5 µL DNA size marker
  - 20 µL loading dye
  - 45 mL agarose (liquid form)
  - 0.5%X TBE buffer

Per table of 4 groups

- 2 microcentrifuges
- 2 electrophoresis power supplies (with 2 jacks each)
- 2 vortex apparatuses

Per class

- 37°C and 60°C water baths with floating microcentrifuge tube holders
- ice chest with crushed ice
- waste container
- UV transilluminator
- ethidium bromide (1 µg/mL)
- instant-print camera and film
- UV safety glasses
- latex gloves
- (optional) plastic wrap

Safety, Handling, and Disposal

As the instructor you are expected to provide students with access to SOPs, MSDSs, and other resources they need to safely work in the laboratory while meeting all regulatory requirements. Before doing this activity or activities from other sources, you should regularly review special handling issues with students, allow time for questions, and then assess student understanding of these issues.

Ethidium bromide is a mutagen and a possible carcinogen. Although it is used in very dilute concentrations, it must be disposed of in a special biohazard container, according to local ordinances.

Dispose of stained gels in a biohazard container, following local ordinances. Use protective UV glasses to visualize the stained gels. Use latex gloves when handling the stained trays.

When dealing with biological materials, take particular precautions as called for by the kit manufacturer or supplier.

Points to Cover in the Pre-Lab Discussion

A pre-lab introductory mini-lecture should explain to students what restriction enzymes are and how they work. Explain the importance of restriction enzymes in DNA research and biotechnology, and give examples of other uses (e.g., paternity suits, crime scenes, etc.). Students will also need a detailed explanation of the steps in the procedure.

It is important that you provide detailed specific information about biohazards and safety precautions.

Procedural Tips and Suggestions

If students are given precise detailed information during the pre-lab, this experiment should run smoothly. Students tend to forget the addition of the enzymes when preparing the digests, so it is important to reinforce this step during the pre-lab. Students become very involved when this laboratory lesson is related to everyday life, court actions of the past, and resolution of crimes by DNA technology.

Plausible Answers to Questions

1. What could account for any differences between the sample and your photograph?
   The differences between the sample and the photograph could be due to many different reasons, including the following:
   - *overload (too much DNA)*
   - *underload (too little DNA)*
   - *bands not spreading enough due to shorter run time*
   - *bands spreading too much because they ran for longer time than required*
   - *wavy bands because of poorly made wells, or maybe the comb was taken out before the gel was completely set*
   - *extra bands due to mixing of the enzymes*
   - *faint bands, showing an incomplete digestion*

2. Why is tube #1 considered to be a negative control?
   Tube #1 is considered to be a negative control because it does not contain any enzyme; it represents just the entire lambda DNA with no digestion (seen as one very thick band).

3. What is the purpose of the lambda DNA marker?
   The purpose of the lambda DNA marker is to have a positive control of the different fragments after DNA has been digested with Hin*d III, to compare with our individual samples.*

4. Considering the direction the DNA migrated in the gel, what can be concluded about the electrical charge of DNA? What part of the DNA molecule is responsible for this charge?
   The electrical charge of the DNA molecule is negative (-). The phosphate groups of the nucleotides are responsible for this charge.

Extensions and Variations

- This activity can be used at various levels. In most high schools it will probably be best suited as an advanced biology lab. If time is a factor, it can also be conducted as a dry lab, analyzing photographs of gels. A good extension activity could be involving the students in real research, especially college students. A human cDNA clone can be purchased on-line from Research Genetics (www.resgen.com). The students use the Internet to research information and order the clone. They can contact a scientist at Lawrence Livermore National Laboratory who will help the students design an experiment to determine the sequence of the given clone. Students can do gel quantitation to roughly determine the human insert size and quantity of the insert. They can do a restriction analysis on the insert to see if the information that they obtain is possibly correct, given the proper fragments. Students can use the Internet to upload the data gathered.

- Another variation of this experiment is to use a spectrophotometer to quantitate more accurately and to determine exact amounts.

References

Bloom, M.; Fryer, G.; Micklos, D. *Laboratory DNA Science: An Introduction to Recombinant DNA Techniques and Methods of Genome Analysis;* Benjamin/Cummings: Menlo Park, CA, 1996; p 31.

Raven, P.; Johnson, G. *Biology*, 4th ed.; Wm. C. Brown: Dubuque, IA, 1996; Chapter 19; p 434.