

Student Manual

Restriction Digestion and Analysis of Lambda DNA Kit

Contents

Overview

Lesson 1 Introduction to Restriction Analysis

Restriction Digestion (Laboratory Procedure)

Review Questions

Lesson 2 Agarose Gel Electrophoresis (Laboratory Procedure)

Review Questions

Staining DNA with Fast Blast DNA Stain (Laboratory Procedure)

Quick Staining

Overnight Staining

Lesson 3 Analysis of Results

Introduction

Overview: How Can Pieces of DNA Solve a Puzzle?

DNA splicing, the cutting and linking of DNA molecules, is one of the basic tools of modern biotechnology. The basic concept behind DNA splicing is to remove a functional DNA fragment — let's say a gene — from the chromosome of one organism and to combine it with the DNA of another organism in order to study how the gene works. The desired result of gene splicing is for the recipient organism to carry out the genetic instructions provided by its newly acquired gene. For example, certain plants can be given the genes for resistance to pests or disease, and in a few cases to date, functional genes have been given to people with nonfunctional genes, such as those who have a genetic disease like cystic fibrosis.

In this laboratory activity, your task will be to cut (or digest) lambda DNA, the genomic DNA of a bacterial virus, and then determine the size of the DNA pieces using a procedure known as **gel electrophoresis**. This involves separating a mixture of the DNA fragments according to the size of the pieces. Once this is accomplished, you will compare your pieces of DNA with pieces of DNA whose size is already known.

Of the DNA fragments that are produced, imagine that one piece in particular represents a specific gene. This gene can code for any number of traits. But before it can be given to a recipient organism, you must first identify the gene by using gel electrophoresis.

Your tasks:

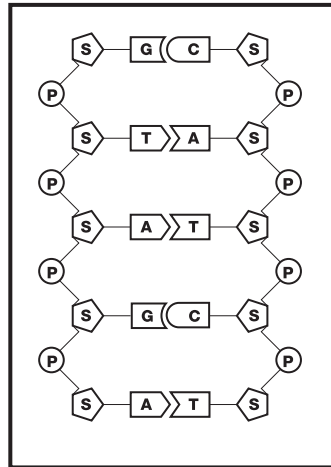
- **Cut lambda DNA into a series of fragments using restriction enzymes.**
- **To separate and sort a large group of DNA molecules according to their size.**
- **To determine the size of each molecule separated by gel electrophoresis.**

You will be provided with lambda DNA and three different restriction enzymes. The DNA restriction analysis that you are about to perform is fundamental to a variety of genetic engineering techniques, including gene splicing, DNA sequencing, gene localization, forensic DNA matching, or DNA fingerprinting. Before you begin, it might be helpful to review the structure of DNA and the activity of restriction enzymes.

Lesson 1 Introduction to Restriction Analysis

Consideration 1. How Does DNA Become Fragmented Into Pieces?

DNA consists of a series of nitrogenous base molecules held together by weak hydrogen bonds. These base pairs are in turn bonded to a sugar-phosphate backbone. The four nitrogenous bases are **adenine**, **thymine**, **guanine**, and **cytosine** (**A**, **T**, **G**, and **C**). Remember the base-pairing rule is **A - T** and **G - C**. Refer to the figure below of a DNA molecule.



In this representation of DNA, the symbols are as follows:

Backbone:

S = Five-carbon sugar molecule known as deoxyribose

P = phosphate group

Nitrogenous Bases:

A = adenine

C = cytosine

G = guanine

T = thymine

If a **segment** of DNA is diagrammed without the sugars and phosphates, a base-pair sequence might appear as:

Read toward the right → A C T C C G T A G A A T T C →
 ← T G A G G C A T C T T A A G ← Read toward the left

Look at the linear sequence of bases (**As**, **Ts**, etc.) on each of the strands.

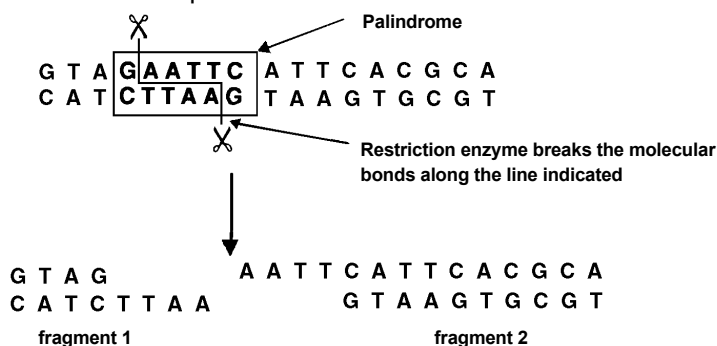
- Describe any pattern you might see in the upper sequence of bases.
- Compare the **bases** in the upper DNA strand to those in the lower strand. Can you discover any relationship between the upper and lower strands? Describe it.
- Now look at the upper **sequence** of bases and compare it to the lower. Do you notice any grouping of bases that when read toward the right on the upper strand and read toward the left on the bottom strand are exactly the same?

You may have discovered that the sequence of base pairs is seemingly random and that the two strands are complementary to each other; **A**s are paired with **T**s, etc. You may have also noticed that a portion of the top strand, **GAATTC** (read toward the right), has a counterpart in the lower strand, **CTTAAG** (read toward the left). Similar sequences are **AAGCTT** and **TTCGAA**, and **CTGCAG** and **GACGTC**. When such a sequence is looked at together with its complementary sequence, the group reads the same in both directions. These sequences, called **palindromes**, are fairly common along the DNA molecule.

Restriction Enzymes — Molecular Scissors

Viruses called bacteriophages are major enemies of bacteria. These viruses infect bacteria by injecting their own DNA into bacteria to force the bacteria to multiply the DNA. Bacteria have responded by evolving a natural defense, called restriction enzymes, to cut up and destroy the invading DNA. Bacteria prevent digestion of their own DNA by modifying certain DNA bases within the specific enzyme recognition sequence, which allows them to protect their own DNA while cutting up foreign DNA. This could be considered a very primitive immune system. Restriction enzymes search the viral DNA for specific palindromic sequences of base pairs, such as **GAATTC**, and cut the DNA at these sites. The actual sequence of DNA is called a **restriction site**. Some restriction enzymes may leave a short length of unpaired nucleotide bases, called a “sticky” end, at the DNA site where they cut, whereas other restriction enzymes make a cut across both strands creating double stranded DNA fragments with “blunt” ends.

Look at the DNA sequence below.



The restriction enzyme *EcoRI* cuts between **G** and **A** in the palindromic sequence **GAATTC**.

- How many base pairs are there to the left of the “cut”?
- How many base pairs are there to the right of the “cut”?
- Counting the number of base pairs, is the right fragment the same size as the left fragment?
- How could you describe the size of each fragment in terms of the number of base pairs in the fragment?

An important feature of restriction enzymes is that each enzyme only recognizes a specific palindrome and cuts the DNA only at that specific sequence of bases. A palindromic sequence can be repeated a number of times on a strand of DNA, and the specific restriction enzyme **will cut all those palindromes**, no matter what species the DNA comes from.

- If the **GAATTC** palindrome is repeated four times on the same piece of linear DNA, and the restriction enzyme that recognizes that base sequence is present and digests the DNA, how many DNA fragments will be produced?
- If the **GAATTC** palindrome repeats are randomly found along the DNA strand, then what can you say about the sizes of the fragments that will be produced when the DNA is digested with a restriction enzyme that recognizes that sequence?

The table below shows palindromic sequences that are recognized by the enzymes that are used to digest the DNA you will be analyzing in this activity.

Palindromic sequence	Name of restriction enzyme that recognizes the palindrome
GAATTC CTTAAG	<i>EcoRI</i>
AAGCTT TTCGAA	<i>HindIII</i>
CTGCAG GACGTC	<i>PstI</i>

Lesson 1 Restriction Digestion (Laboratory Procedure)

The DNA you will be provided with has been extracted from a bacteriophage — a bacterium-invading virus. The virus is known as lambda and is often written as “ λ ”. You will be working with three different restriction enzymes, also called endonucleases. These are referred to as *Pst*I, *Eco*RI, and *Hind*III.

Set up your restriction digest reactions:

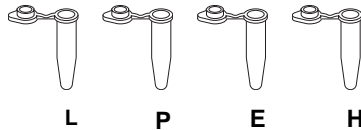
1. Obtain micro test tubes that contain each enzyme solution, lambda DNA, and restriction buffer from the common station. Keep all the stock solutions on ice.
2. Label four micro test tubes **L**, **P**, **E**, and **H** and place them in the foam micro test tube holder.

L = Uncut lambda DNA (yellow tube)

P = *Pst*I restriction digest of lambda DNA (violet tube)

E = *Eco*RI restriction digest of lambda DNA (green tube)

H = *Hind*III restriction digest of lambda DNA (orange tube)

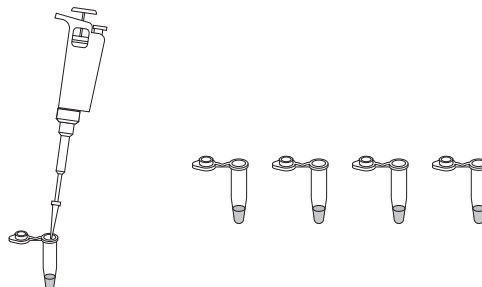


- Describe the appearance of the DNA in solution.
 - Is the DNA visible?
3. You will set up your digests in micro test tubes. To each tube, add 4 μ l of uncut lambda DNA, 5 μ l of restriction buffer and 1 μ l of enzyme. **Add only one kind of enzyme to a tube. Do not add enzyme into the tube labeled “L”.**

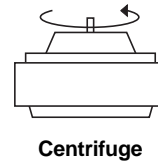
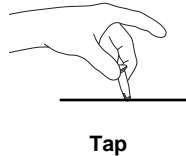
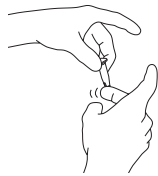
Important note: First add DNA, then restriction buffer, and then the enzymes to the tubes. Use a fresh pipet tip for restriction buffer and each enzyme.

Fill in this chart as you go.

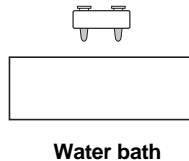
Tube	Lambda DNA	Restriction buffer	<i>Pst</i> I	<i>Eco</i> RI	<i>Hind</i> III
P	4 μ l	5 μ l	1 μ l	–	–
E					
H					
L					



- In which tube do you expect no changes to occur—that is, no DNA fragments produced.
 - What is missing in that tube that leads you to that decision?
4. Tightly cap each tube. In order to mix all reagents, hold the top of a micro test tube between the index finger and thumb of one hand and flick the bottom of the tube with the index finger of the other hand. Gently tap the bottom of the tube on the table to collect the liquid. If you are using a centrifuge, place the four tubes from your tube into the centrifuge, being sure that the tubes are in a balanced arrangement in the rotor. Have your teacher check before spinning the tubes. Pulse-spin the tubes (hold the button for a few seconds).



5. Place the sample tubes in a 37°C water bath for approximately 30 minutes or let them incubate at room temperature overnight. Restriction enzymes work best at 37°C since they were isolated from bacteria that live inside warm-blooded animals. After the incubation, place the samples in the refrigerator (4°C) until the next laboratory period, or proceed directly to step 2 of Lesson 2 if instructed by your teacher.



Note: While you are waiting, this a good time to cast your agarose gel, unless they have already been prepared for you. Check with your teacher for the proper procedure.

Review Questions

- Compare tube **P** to tube **L**; what do you expect to happen in the **P** tube compared to the **L** tube?
- Why do you expect this difference?
- If the DNA in the **L** tube becomes fragmented at the conclusion of the reaction, what can you conclude?
- Is there any visible change to the DNA after adding restriction enzymes?

Below is the summary of what we have learned so far:

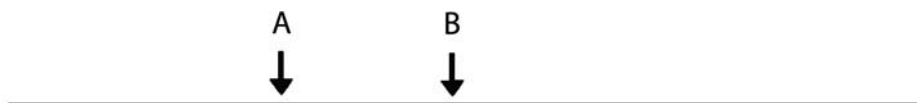
- A sequence on one strand of DNA and its complementary sequence on the other strand can form a palindrome i.e.,

G	A	A	T	C	
C	T	T	A	A	G
- Palindromes can be detected by restriction enzymes
- Restriction enzymes cut the palindromes at restriction sites
- Restriction enzymes recognize specific palindromes
- Cutting DNA at restriction sites will produce DNA fragments
- Fragment size can be described by the number of base pairs a fragment contains

Applying What You Have Learned

A linear DNA molecule is represented below. The DNA is represented by one line, although in actuality, DNA has two strands.

- If the DNA molecule has two restriction sites, A and B, for a specific restriction enzyme, how many fragments would be produced if the DNA is cut by that enzyme?



- Number each fragment.
- Which fragment would be the largest?
- Which fragment would be the smallest?

- Draw a DNA molecule that has five randomly spaced restriction sites for a specific palindrome. How many fragments would be produced if each site were cut by a restriction enzyme?
- Label each fragment.
- Rank them in order of size from largest to smallest.



In this diagram A and B are different palindrome sequences on a DNA strand. Only the restriction enzyme that recognizes site B is present.

- Explain why only two fragments would be produced.

Lesson 2 Agarose Gel Electrophoresis (Laboratory Procedure)

Part 1. Prepare Your Samples for Electrophoresis

Consideration 1. How Can Fragments of DNA Be Separated From One Another?

DNA is colorless so DNA fragments in the gel can't be seen during electrophoresis. A sample loading buffer containing two blue dyes is added to the DNA solution. The loading dyes do not stain the DNA itself but makes it easier to load the gels and monitor the progress of the DNA electrophoresis. The dye fronts migrate toward the positive end of the gel, just like the DNA fragments. The "faster" dye comigrates with DNA fragments of approximately 500 bp, while the "slower" dye comigrates with DNA fragments approximately 5 kb, or 5,000 bp, in size.

1. Following incubation, obtain your four micro test tubes **L**, **P**, **E**, and **H** and place them in the foam micro test tube holder at your laboratory desk.

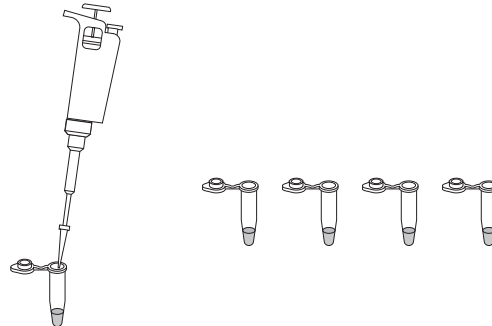
L = Uncut lambda DNA (yellow tube)

P = *Pst*I restriction digest of lambda DNA (violet tube)

E = *Eco*RI restriction digest of lambda DNA (green tube)

H = *Hind*III restriction digest of lambda DNA (orange tube)

2. Set the digital micropipet to 2.0 μ l and transfer this amount of sample loading dye to each of the tubes marked **L**, **P**, **E**, and **H** in the foam tube holder. Use a fresh tip with each sample to avoid contamination.



3. The DNA samples and the sample loading dye must be thoroughly mixed in each tube before placing the samples in the gel wells for electrophoresis. This is easily accomplished by holding the top of a microtube between the index finger and thumb of one hand and flicking the bottom of the tube gently with the index finger of the other hand.

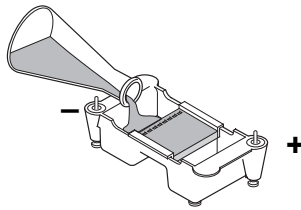


Collect the liquid to the bottom of the tube by tapping it gently on your laboratory bench. If you have access to a centrifuge, place the four tubes from your foam tube holder (these tubes now have DNA and loading dye) into the centrifuge, be sure that the tubes are in a balanced arrangement in the rotor. Have your teacher check before spinning the tubes. Pulse-spin the tubes (hold the button for a few seconds). This forces all of the components to the bottom of the tube.

- Obtain the DNA marker (M) from your teacher. Optional: Heat all samples at 65°C for 5 minutes and then place the samples on ice — this results in better separation of the DNA bands.

Part 2. Set Up Your Gel Electrophoresis Chamber

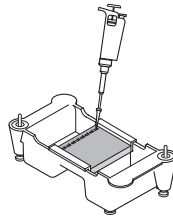
- Obtain an agarose gel from your teacher, or, if your teacher instructs you to do so, pour your own gel.
- Place the casting tray, with the solidified gel in it, onto the central platform in the gel box. The wells should be at the negative (cathode) end of the box where the black electrical lead is connected. Very carefully remove the comb from the gel by gently pulling it straight up.
- Pour about 275 ml of electrophoresis buffer into the electrophoresis chamber. Pour enough buffer into the box until it just covers the wells of the gel by 1–2 mm.



Part 3. Load your Samples and Run them by Electrophoresis

- Pipet 10 µl from each tube (**M**, **L**, **P**, **E**, and **H**) into separate wells in the gel chamber. Use a fresh tip for each tube. Gels are read from left to right. To keep things straight, the first sample is typically loaded in the well at the upper left-hand corner of the gel. For example

Lane	1	2	3	4	5
Sample	M	L	P	E	H



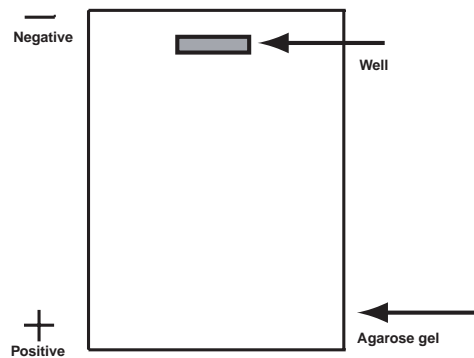
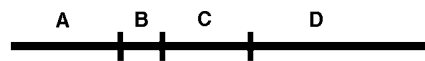
- Slide the cover of the chamber into place, and connect electrical leads to the power supply, anode to anode (red to red) and cathode to cathode (black to black). Make sure both electrical leads are attached to the same channel of the power supply.
- Electrophorese at 100 V for 30–40 minutes. Shortly after the current is applied, the loading dye can be seen moving through the gel toward the positive side of the gel chamber.
- When electrophoresis is complete, **turn off the power supply**, disconnect the leads from the inputs, and remove the top of gel chamber.
- Remove the casting tray from gel chamber. **The gel is very slippery. Hold the tray level.**
- Pour the excess buffer back into the original container for reuse, if desired.

Consideration 2. How Can Fragments of DNA Be Separated From One Another?

Agarose gel electrophoresis is a procedure used to separate DNA fragments based on their sizes. DNA is an acid and has many negative electrical charges. Scientists have used this fact to design a method that can be used to separate pieces of DNA. A solution containing a mixture of DNA fragments of variable sizes is placed into a small well formed in an agarose gel that has a texture similar to gelatin. An electric current causes the negatively-charged DNA molecules to move towards the positive electrode.

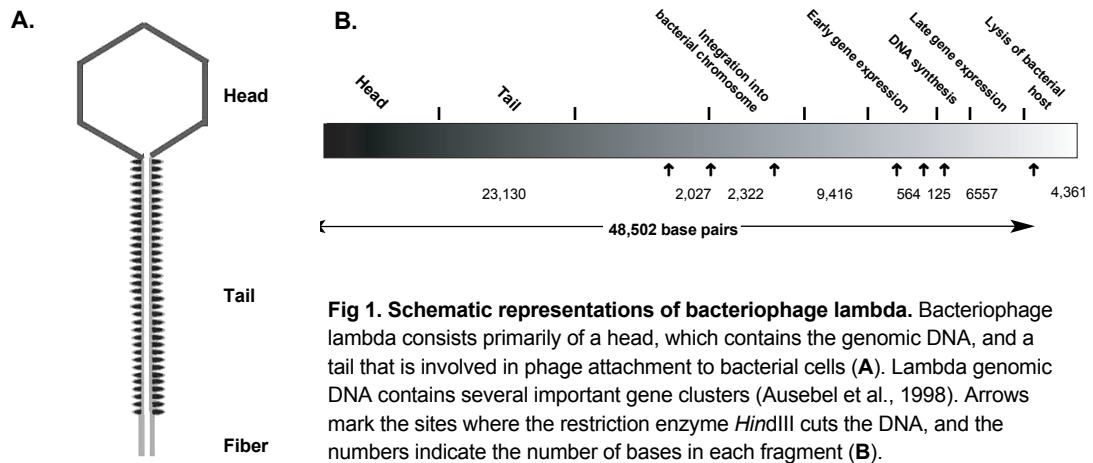
Imagine the gel as a strainer with tiny pores that allow small particles to move through it very quickly. The larger the size of the particles, however, the slower they are strained through the gel. After a period of exposure to the electrical current, the DNA fragments will sort themselves out by size. Fragments that are the same size will tend to move together through the gel and form bands.

A piece of DNA is cut into four fragments as shown in the diagram. A solution containing the four fragments is placed in a well in an agarose gel. Using the information given above, draw (to the right) how you think the fragments might be separated. Label each fragment with its corresponding letter.



- Have your teacher check your diagram before you proceed.
- Where would the larger fragments, those with the greater number of base pairs, be located, toward the top of the gel or the bottom? Why?
- Suppose you had 500 pieces of each of the four fragments, how would the gel appear?
- If it were possible to weigh each of the fragments, which one would be the heaviest? Why?
- Complete this rule for the movement of DNA fragments through an agarose gel.

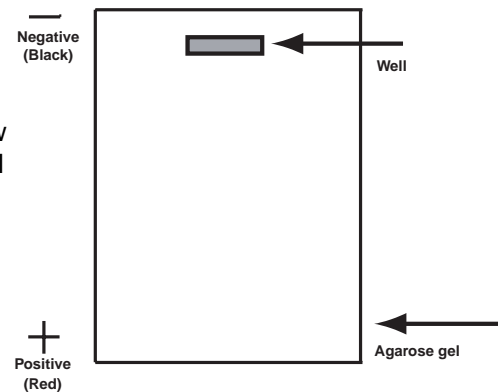
The larger the DNA fragment, the ...



- How many fragments were produced by the restriction enzyme *Hind*III?

On the gel diagram at the right, show how you believe these fragments will sort out during electrophoresis.

- Label each fragment with its correct number of base pairs.



Staining DNA with Fast Blast DNA Stain (Laboratory Procedure)

Consideration 3. How Can the DNA Be Made Visible?

- What color was the DNA before you added loading dye?

Since DNA is naturally colorless, it is not immediately visible in the gel. Unaided visual examination of the gel after electrophoresis indicates only the positions of the loading dyes and not the positions of the DNA fragments. DNA fragments are visualized by staining the gel with a blue stain called Fast Blast DNA stain. The blue stain molecules are positively charged and have a high affinity for the DNA. These blue stain molecules strongly bind to the DNA fragments and allow DNA to become visible. These visible bands of DNA may then be traced, photographed, sketched, or retained as a permanently dried gel for analysis.

There are two protocols for using Fast Blast DNA stain in the classroom. Use option 1 for quick staining of gels to visualize DNA bands in 12–15 minutes, and option 2 for overnight staining. Depending on the amount of time available, your teacher will decide which protocol to use. Two student teams will stain the gels per staining tray (you may want to notch gel corners for identification). Mark staining trays with initials and class period before beginning this activity.

WARNING

Although Fast Blast DNA stain is nontoxic and noncarcinogenic, latex or vinyl gloves should be worn while handling the stain or stained gels to keep hands from becoming stained blue. Lab coats or other protective clothing should be worn to avoid staining clothes.

Protocol 1: Quick Staining of Agarose Gels in 100x Fast Blast DNA Stain

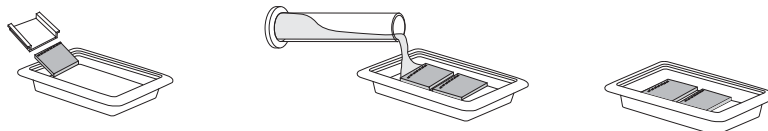
This protocol allows quick visualization of DNA bands in agarose gels within 15 minutes. For quick staining, Fast Blast DNA stain (500x) should be diluted to a **100x** concentration. We recommend using 120 ml of 100x Fast Blast to stain two 7 x 7 cm or 7 x 10 cm agarose gels in individual staining trays provided in Bio-Rad's education kits. If alternative staining trays are used, add a sufficient volume of staining solution to completely submerge the gels.

Following electrophoresis, agarose gels must be removed from their gel trays before being placed in the staining solution. This is easily accomplished by holding the base of the gel tray in one hand and gently pushing out the gel with the thumb of the other hand. Because the gel is fragile, special attention must be given when handling it. We highly recommend using a large spatula or other supportive surface to transfer the gel from one container to another. Destaining requires the use of at least one large-volume container, capable of holding at least 500 ml, at each student workstation. Each student team may utilize separate washing containers for each wash step, or simply use a single container that is emptied after each wash and refilled for the next wash.

1. Mark the staining trays with your initials and class period. You will stain 2 gels per tray.

2. Stain gels

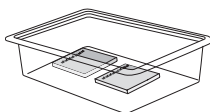
Remove each gel from the gel tray and carefully slide it into the staining tray. Pour approximately 120 ml of 100x stain into the staining tray. If necessary, add more 100x stain to completely submerge the gels. Stain the gels for 2–3 minutes, but not for more than 3 minutes. Using a funnel, pour the 100x stain into a storage bottle and save it for future use. **The stain can be reused at least 7 times.**



2–3 minutes

3. Rinse gels

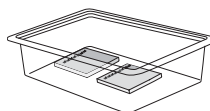
Transfer the gels into a large container containing 500–700 ml of clean, warm (40–55°C) tap water. Gently shake the gel in the water for ~10 seconds to rinse.



10 seconds

4. Wash gels

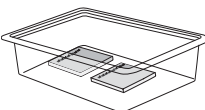
Transfer the gel into a large container with 500–700 ml of clean, warm tap water. Gently rock or shake the gel on a rocking platform for 5 minutes. If no rocking platform is available, move the gels gently in the water once every minute.



5 minutes

5. Wash gels

Perform a second wash as in step 4.



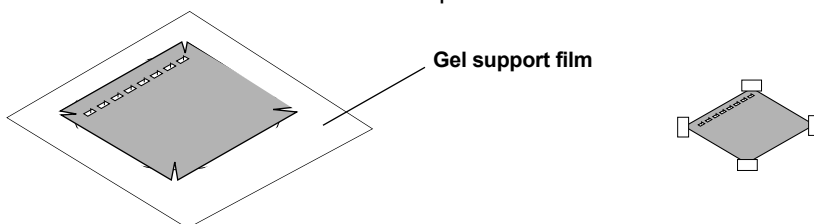
5 minutes

6. Analyze results

Pour off the water and examine the stained gels for expected DNA bands. The bands may appear fuzzy immediately after the second wash, but will begin to develop into sharper bands within 5–15 minutes after the second wash. This is due to Fast Blast stain molecules migrating into the gel and binding more tightly to the DNA.

To obtain maximum contrast, additional washes in warm water may be necessary. Destain to the desired level, but do not wash the gel in water overnight. If you cannot complete the destaining in the allocated time, you may transfer the gel to 1x Fast Blast stain for overnight staining. **See Protocol 2.**

- a. Place your gel on a light background and record your results by making a diagram as follows. Place a clear sheet of plastic sheet or acetate over the gel. With a permanent marker, trace the wells and band patterns onto the plastic sheet to make a replica picture of your gel. Remove the plastic sheet for later analysis. Alternatively, gels can be photocopied on a yellow piece of transparent film for optimal contrast.
- b. Dry the agarose gel as a permanent record of the experiment.
 - i. Trim away any unloaded lanes with a knife or razor blade. Cut your gel from top to bottom to remove the lanes that you did not load samples into, leaving only lanes 1–4.
 - ii. Place the gel directly upon the hydrophilic side of a piece of gel support film. (Water will form beads on the hydrophobic side of a piece of gel support film.) Center the gel on the film and remove bubbles that may form between the gel and film. Place the film on a paper towel and let the gel dry in a well-ventilated area, making sure to avoid direct exposure to light. As the gel dries it will bond to the film but will not shrink. If left undisturbed on the support film, the gel will dry completely at room temperature after 2–3 days. The result will be a flat, transparent, and durable record for the experiment.



Protocol 2: Overnight Staining of Agarose Gels in 1x Fast Blast DNA Stain

For overnight staining, Fast Blast DNA stain (500x) should be diluted to a **1x** concentration. We recommend using 120 ml of 1x Fast Blast to stain two 7 x 7 cm or 7 x 10 cm agarose gels in individual staining trays provided in Bio-Rad's education kits. If alternative staining trays are used, add a sufficient volume of staining solution to completely submerge the gels.

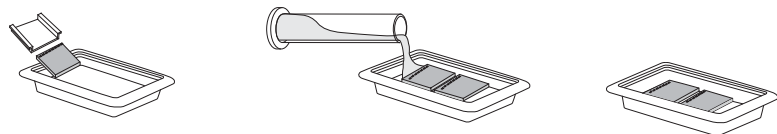
Following DNA electrophoresis, agarose gels must be removed from their gel trays before being placed in the staining solution. This is easily accomplished by holding the base of the gel tray in one hand and gently pushing out the gel with the thumb of the other hand. Because the gel is fragile, special attention must be given when handling it.

1. Mark the staining tray with your initials and class period. You will stain 2 gels per tray.

2. Stain gels (overnight)*

Pour **1x** stain into a gel staining tray. Remove the gel from the gel tray and carefully slide it into the staining tray containing the stain. If necessary, add more 1x staining solution to completely submerge the gels. Place the staining tray on a rocking platform and agitate overnight. If no rocking platform is available, agitate the gels staining tray a few times during the staining period. You should begin to see DNA bands after 2 hours, but at least 8 hours of staining is recommended for complete visibility of stained bands.

* It is crucial that you shake gels gently and intermittently while performing the overnight staining in 1x Fast Blast stain since smaller fragments tend to diffuse without shaking.

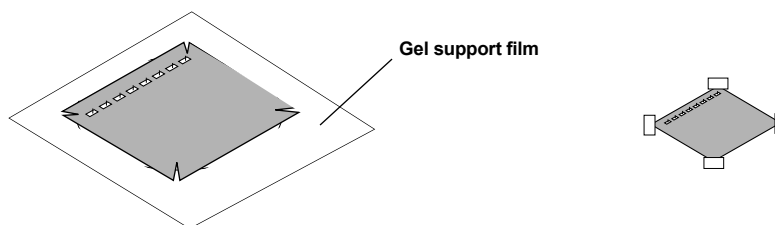


Stain overnight

2. Analyze results

No destaining is required after staining with 1x Fast Blast. The gels can be analyzed immediately after staining.

- a. Place your gel on a light background and record your results by making a diagram as follows. Place a clear sheet of plastic sheet or acetate over the gel. With a permanent marker, trace the wells and band patterns onto the plastic sheet to make a replica picture of your gel. Remove the plastic sheet for later analysis. Alternatively, gels can be photocopied on a yellow piece of transparent film for optimal contrast.
- b. Dry the agarose gel as a permanent record of the experiment.
 - i. Trim away any unloaded lanes with a knife or razor blade. Cut your gel from top to bottom to remove the lanes that you did not load samples into, leaving only lanes 1–4.
 - ii. Place the gel directly upon the hydrophilic side of a piece of gel support film. (Water will form beads on the hydrophobic side of a piece of gel support film.) Center the gel on the film on a paper towel and let the gel dry in a well-ventilated area, making sure to avoid direct exposure to light. As the gel dries it will bond to the film but will not shrink. If left undisturbed on the support film, the gel will dry completely at room temperature after 2–3 days. The result will be a flat, transparent, and durable record for the experiment.

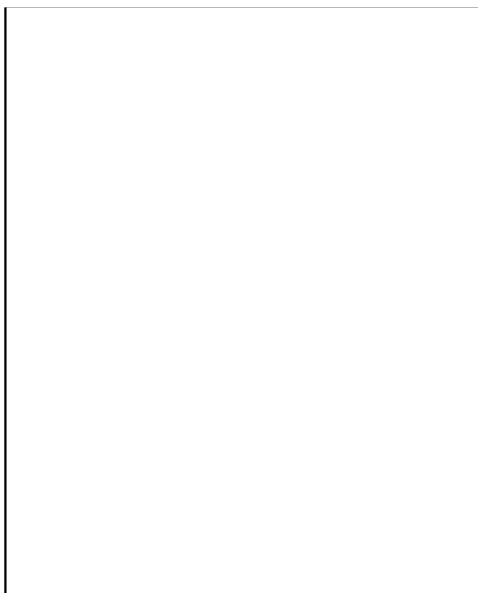


Note: Avoid extended exposure of dried gels to direct light to prevent band fading. However, DNA bands will reappear if the dried gels are stored in the dark for 2–3 weeks after fading.

Lesson 3: Analysis of Results

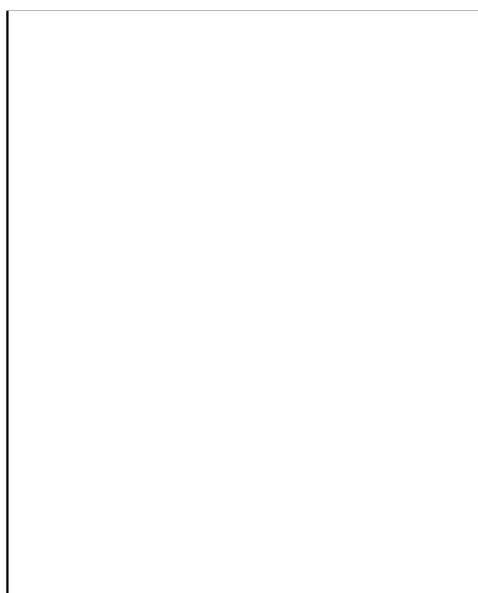
If the overnight staining protocol was used to stain gels, record your results and dry gels as described in the gel staining procedures in Lesson 2.

Attach the plastic sheet tracing of the banding patterns from the DNA electrophoresis below.



Tracing of electrophoresis gel

Attach the dried gel showing the banding patterns from the DNA electrophoresis below.



Dried electrophoresis gel

Organize Your Data

One of the first steps to analyze your data is to determine the approximate sizes of each of your restriction fragments. This can be done by comparing the DNA restriction fragments with DNA fragments of known sizes, or standards. You will use two methods to estimate the size of the fragments in the uncut lambda DNA, the *Pst*I lambda digest, and the *Eco*RI lambda digest lanes. The first method is based on visual estimation and is less precise than the second method, which involves creating a standard curve. Both methods rely on using the *Hind*III lambda digest as a DNA standard, or marker.

1. Using a ruler, measure the distance (in mm) that each of your DNA fragments or bands traveled from the well. Measure the distance from the bottom of the well to the bottom of each DNA band and record your numbers in the table on the next page.
2. Estimate the sizes, in base pairs (bp), of each of your restriction fragments. Hint: Compare the distance that the unknown bands (lambda DNA, *Pst*I digested, and *Eco*RI digested) traveled with those of the *Hind*III bands. Write the estimated sizes in the data table.
3. A more accurate way of estimating unknown DNA band sizes is to first construct a standard curve based upon the measurements obtained from the known *Hind*III lambda bands. Later in the analysis you will construct a standard curve and more accurately determine the size of each of the DNA bands.

Electrophoresis data: Measure the distance (in millimeters) that each fragment traveled from the well and record it in the table. Estimate its size, in base pairs, by comparing its position to the *HindIII* lambda DNA markers. **Remember: some lanes will have fewer than 6 fragments.**

Largest fragment first ↓	M = DNA marker		L = lambda DNA- no enzyme		P = <i>PstI</i> restriction digest of lambda DNA		E = <i>EcoRI</i> restriction digest of lambda DNA		H = <i>HindIII</i> restriction digest of lambda DNA	
	Distance in mm	Actual base pairs	Distance in mm	Estimated base pairs	Distance in mm	Estimated base pairs	Distance in mm	Estimated base pairs	Distance in mm	Estimated base pairs
Band 1		23,130								
Band 2		9,416								
Band 3		6,557								
Band 4		4,361								
Band 5		2,322								
Band 6		2,027								

Analysis of DNA Fragments

The data you entered for the lambda *Hind*III digest were the relative positions of DNA bands of known size. Since the exact size and position of these fragments are known, they can be used as standard reference points to estimate the size of unknown fragment bands. A set of fragments of known sizes is called a **molecular weight ruler** or **standards** or **marker** (or sometimes a **ladder** because of the bands' appearance).

Now look at the diagram of the agarose gel (below). It shows two **lanes**. A lane is the column below a well. The right lane contains a banding pattern from four fragments of known length (6,000, 5,000, 3,000, and 1,000 bp).

- Which lane contains the molecular weight standards? How do you know?

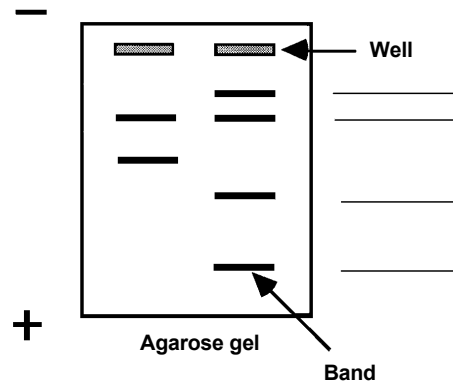
- Label each band in the right lane with its base-pair size.

- Compare the two lanes of bands. Estimate the size of the fragments in the left lane.

Upper band _____

Lower band _____

- How did you determine the sizes of the two bands in the left lane?



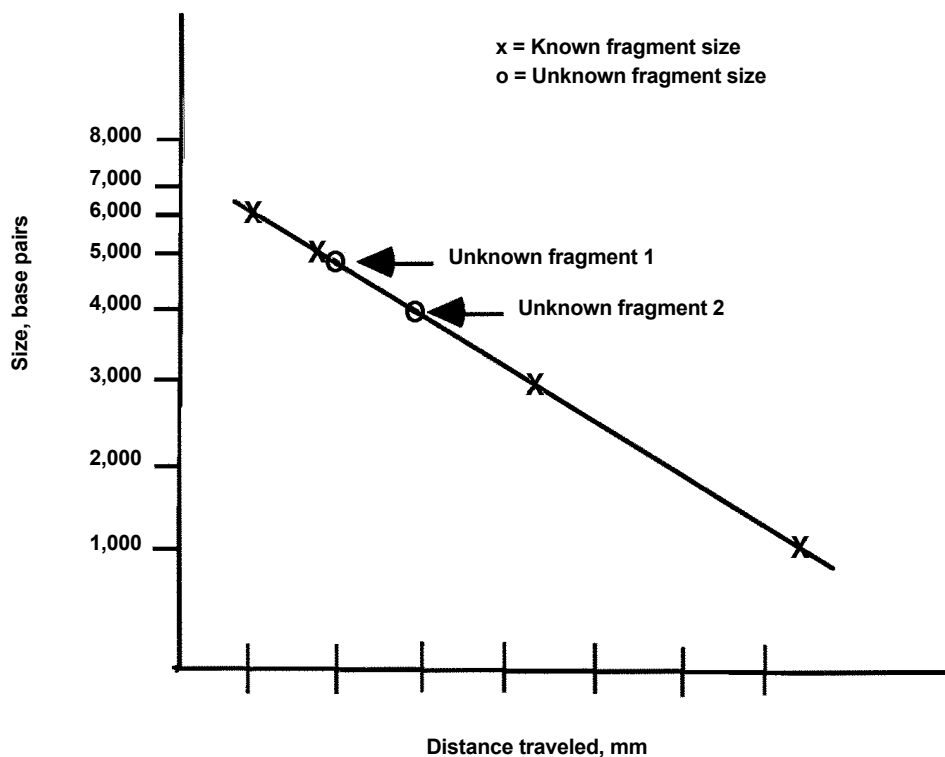
Examine the practice gel above.

- Measure the distance in millimeters (mm)** that each band moved.
Measure from the bottom edge of the well to the bottom edge of the band.
- Record the data in the table to the right, including the unit of measurement, mm.

Left lane		Right lane	
1		1	
2		2	
		3	
		4	

The number of base pairs in each of the DNA fragments on your gel can be determined using another method that can be more accurate. This involves graphing the size of the known fragments from the DNA standards against the distance each DNA band moved through the gel, to generate a standard curve. This is most conveniently done on semilog graph paper.

Look at the data from the practice gel on page 39. The fragments of known size were plotted on semilog graph paper, producing the standard curve below.



The distances migrated by two fragments of unknown length were also marked on the standard curve.

1. For each fragment, line up a ruler vertically from the distance traveled position on the horizontal x-axis to the line that you constructed.
2. From the point where your ruler intersected your line, place the ruler horizontally and note where it intersects with the vertical y-axis for fragment size. This will be your determination of the size for that fragment.

- How many base pairs does fragment 2 contain?
- How accurate is this estimation of size?

Determining the Size of the DNA Fragments by Creating a Standard Curve

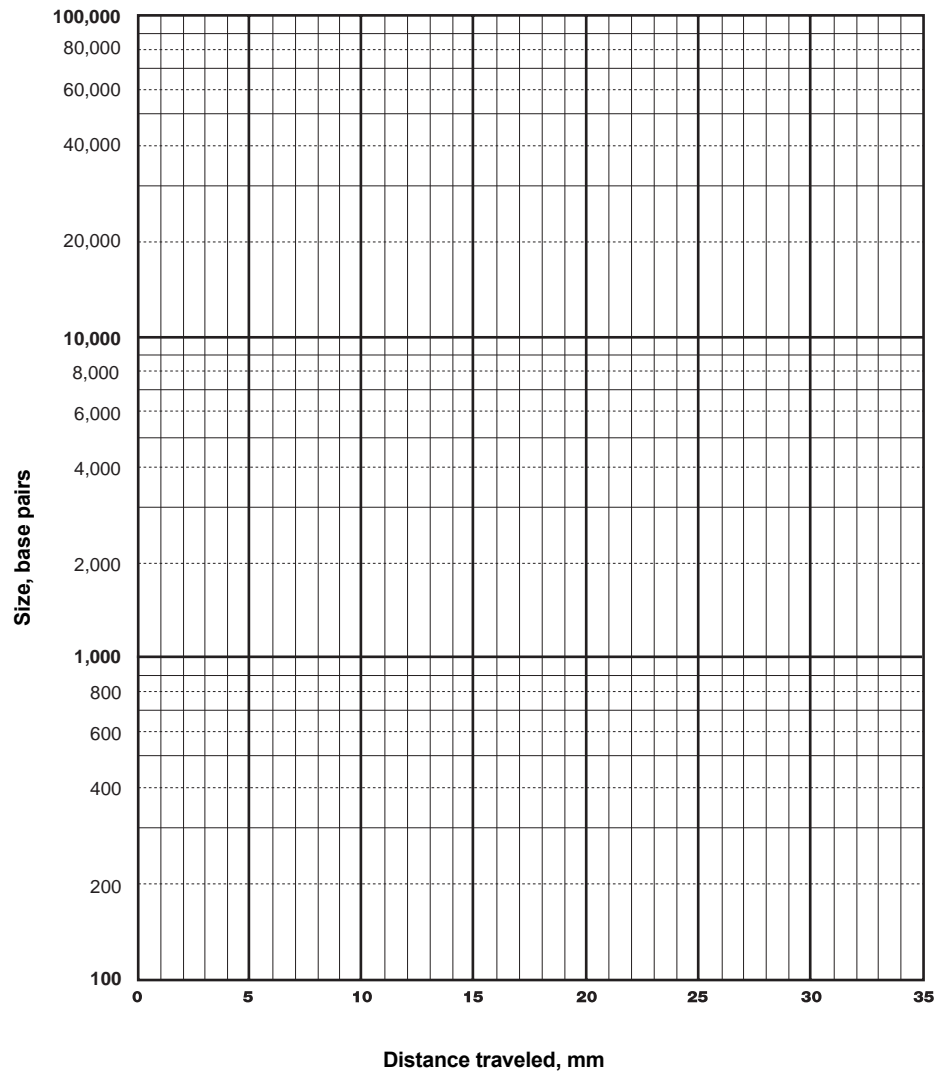
From your laboratory data, you were able to estimate the approximate size of each of the DNA fragments that you separated on your gel. This was done in terms of the number of base pairs.

- Explain how you made this determination.

You have been provided with three-cycle semilog graph paper.

1. Fragment size will be on the vertical (y) axis.
2. The horizontal (x) axis is your scale for distance traveled through the gel in millimeters.
3. Using the fragments from the *Hind*III lambda digest, plot the distance traveled in relationship to fragment size for each fragment. Connect as many of the points as you can by drawing a straight line through them. This will provide a standard curve with which you will be able to determine the size of your unknown fragments from the other three samples.
4. Determine the sizes of the fragments in your uncut and digested lambda DNA samples using the method described on the previous page.

Semilog Graph Paper



Base pair (size) determination based on standard curve. Construct your own table below to record the size of each unknown fragment as determined by the semilog graphing procedure. It might also be interesting to indicate on this same table the values you arrived at by comparing band positions in the original gel analysis. Compare the two sets of values.

Largest fragment first ↓	M = DNA marker		L = lambda DNA- no enzyme		P = <i>Pst</i> I restriction digest of lambda DNA		E = <i>Eco</i> RI restriction digest of lambda DNA		H = <i>Hind</i> III restriction digest of lambda DNA	
	Distance in mm	Actual base pairs	Distance in mm	Estimated base pairs	Distance in mm	Estimated base pairs	Distance in mm	Estimated base pairs	Distance in mm	Estimated base pairs
Band 1		23,130								
Band 2		9,416								
Band 3		6,557								
Band 4		4,361								
Band 5		2,322								
Band 6		2,027								

Remember that the three samples of DNA were originally the same size. Next, each sample was cut into pieces by the addition of three different restriction enzymes.

- What evidence do you have that each enzyme cuts the DNA at different locations?
- When this data table has been completed, describe what you have done to determine DNA fragment sizes in this investigation. Use no more than two sentences.
- Explain how you think you could make your DNA size estimation more accurate.
- Compare the two methods — direct gel examination and semilog graph — of determining the fragment size in base pairs. Which method seems to be more accurate? Explain your answer.

Appendix A: Teacher's Answer Guide

Lesson 1: Restriction Enzymes Consideration Questions

Look at the linear sequence of bases (As, Ts, etc.) on each of the strands.

- Describe any pattern you might see in the upper sequence of bases.

There is no specific type of pattern associated with the upper sequence of bases.

- Compare the bases in the upper DNA strand to those in the lower strand. Describe any relationship you can see.

A always pairs with T; G always pairs with C.

- Now look at the upper sequence of bases and compare it to the lower. Do you notice any grouping of bases that when read toward the right on the upper strand and read to the left on the bottom strand are exactly the same?

CTTAAG.

A restriction enzyme cuts between **G** and **A** in the palindromic sequence **GAATTC**.

- How many base pairs are there to the left of the cut?

4

- How many base pairs are there to the right of the cut?

10

- Counting the number of base pairs, is the right fragment the same size as the left fragment?

No, it is larger.

- How could you describe the fragment size in reference to the number of base pairs in the fragment?

Fragment 1 is a 4-base-pair fragment.

Fragment 2 is a 10-base-pair fragment.

- If the **GAATTC** palindrome is repeated four times on the same piece of linear DNA, and the restriction enzyme that recognizes that base sequence is present, how many DNA fragments will be produced?

5

- If the **GAATTC** palindrome repeats are randomly spaced along the DNA strand, then what can you say about the size of the fragments that will be produced when the DNA is digested with a restriction enzyme that recognizes that sequence?

Random sized fragments will be produced.

The Digestion

- Describe the appearance of the DNA in solution.

The DNA is colorless.

- Is the DNA visible?

No. DNA in solution is not visible.

Fill in this chart below as you go:

Tube	Lambda	Buffer	<i>Pst</i> I	<i>Eco</i> RI	<i>Hind</i> III
P	4 µl	5 µl	1 µl		
E	4 µl	5 µl		1 µl	
H	4 µl	5 µl			1 µl
L	4 µl	6 µl			

- In which tube do you expect no changes to occur—that is, no DNA fragments produced?

The DNA in the uncut lambda DNA tube, which gets no enzyme, should remain intact as a single band.

- What is missing in that tube that leads you to that decision?

No restriction enzymes are added to that tube, thus no digestion will occur, and no fragments will be produced.

This tube (L) is the control tube.

- Compare tube **P** to tube **L**; What do you expect to happen in the P tube compared to the L tube.

The P tube contains the restriction enzyme *Pst*I. There should be an enzymatic digestion that occurs in that tube, which results in the production of restriction fragments. The L tube only contains DNA and no enzyme and so should not produce any restriction fragments.

- Why do you expect this difference?

Restriction enzymes digest DNA at specific sites. If lambda DNA contains restriction sites for the enzyme *Pst*I, then the DNA should be cut into fragments. With no added enzyme, no digestion should occur.

- If the DNA in tube L becomes fragmented at the conclusion of the reaction, what can you conclude?

Most likely a restriction enzyme was inadvertently added to the L tube. This could have happened by accidentally adding an enzyme to the tube, or by not changing pipet tips, which could result in enzyme being carried over between tubes.

- Is there any visible change to the DNA after adding restriction enzymes?

No. The DNA still appears colorless.

Appendix B: Complete Lambda Genome Analysis

Some of the descriptions of the DNA fragment banding patterns produced in this kit have been simplified to facilitate student understanding of DNA restriction analysis and agarose gel electrophoresis. For teachers who would like to explore further the restriction analysis of the lambda genome, a few clarifications may be helpful. There are seven *HindIII* restriction sites in the lambda genome, so digestion of lambda DNA with *HindIII* produces eight DNA fragments. Six of these fragments are large enough for students to see because they contain sufficient amounts of DNA to be detected by Fast Blast stain. Digestion of lambda DNA with *EcoRI* generates six fragments, but two of them are so close in size that they cannot be separated under the gel conditions used. The *PstI* restriction enzyme produces 29 lambda DNA fragments! Some of these fragments migrate so closely together on a gel that they appear as one band, while other fragments are so small that they cannot be detected. Changing the agarose concentration, running the gels for longer time periods, and using a much more sensitive DNA stain would enable the detection of more DNA bands.

The following table lists the exact sizes of all the fragments produced when lambda DNA is digested with the indicated enzymes.

Uncut lambda DNA	<i>PstI</i> lambda digest	<i>EcoRI</i> lambda digest	<i>HindIII</i> lambda digest
48,502 bp	11,497 bp	21,225 bp	23,129 bp
	5,077	7,421	9,416
	4,749	5,804	6,557
	4,507	5,643	4,361
	2,838	4,878	2,322
	2,559	3,530	2,027
	2,459		564
	2,443		125
	2,140		
	1,986		
	1,700		
	1,159		
	1,093		
	805		
	514		
	468		
	448		
	339		
	264		
	247		
	216		
	211		
	200		
	164		
	150		
	94		
	87		
	72		
	15		

The complete lambda bacteriophage genomic DNA sequence can be found on the National Center for Biotechnology Information web site (ncbi.nlm.nih.gov/), under the accession number J02459.