Biotechnology Explorer™

Restriction Digestion and Analysis of Lambda DNA

Instruction Manual

Catalog #
166-0002-EDU

explorer.bio-rad.com

Note: Kit contains temperature–sensitive reagents. Open immediately upon arrival and store components at -20°C as indicated.

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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 *Hin*dIII restriction sites in the lambda genome, so digestion of lambda DNA with *Hin*dIII 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 the Bio-Safe staining solution. Digestion of lambda DNA with *Eco*RI generates six fragments, but two of them are so close in size that they cannot be separated under the gel conditions used. The *Pst*I 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 can not be detected. Changing the concentration of agarose gel, 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	*Pst*I lambda digest	*Eco*RI lambda digest	*Hin*dIII 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 (www.ncbi.nlm.nih.gov/), under the accession number J02459.

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?

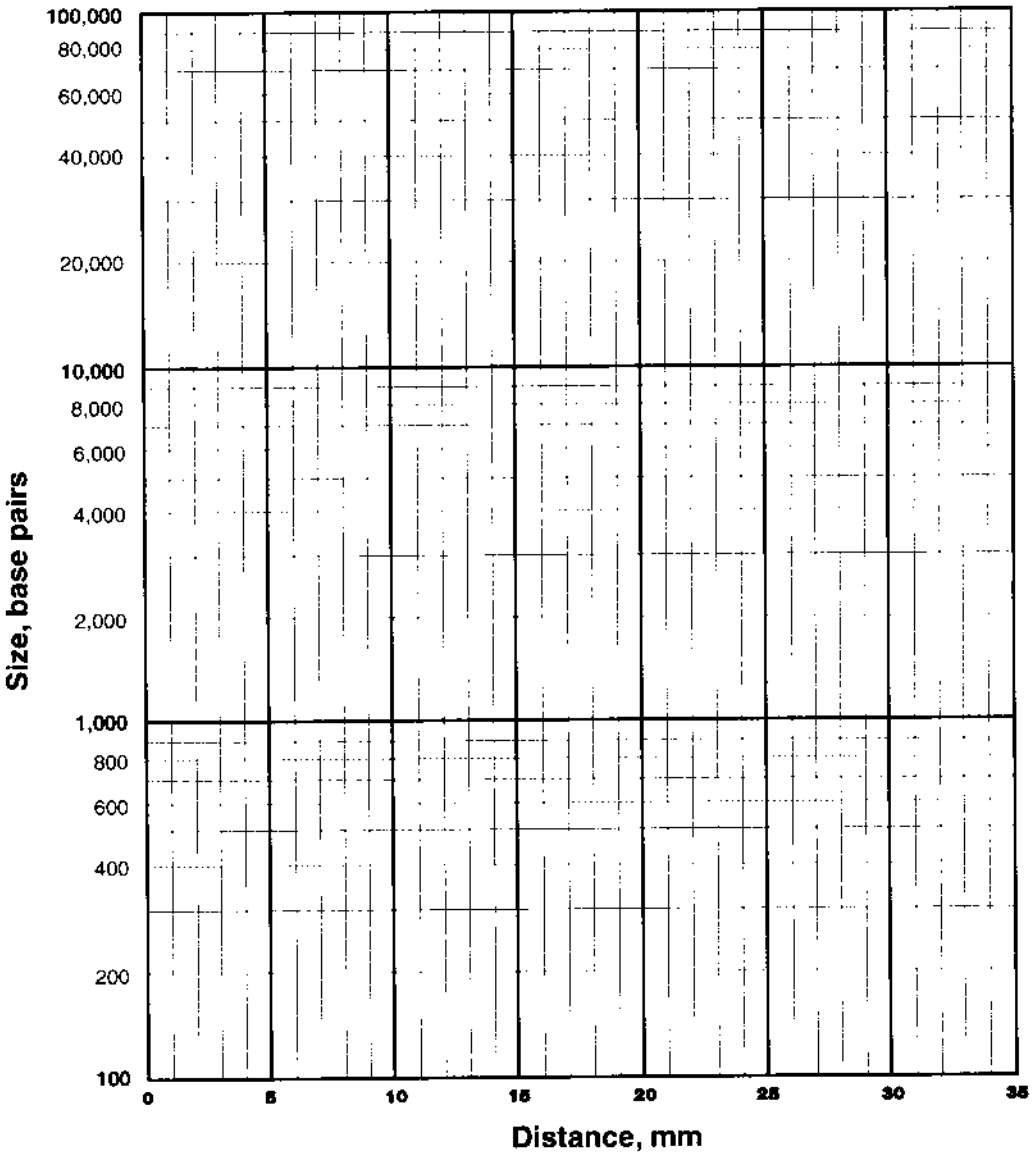
- When this activity has been completed, describe what you have done in no more than two sentences.

- Compare the two methods—direct gel examination and semi-log graph—of determining the fragment size. Which method seems to be more precise? Explain your answer.

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 semi-log 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 = *Pst*I restriction digest of lambda DNA		E = *Eco*RI restriction digest of lambda DNA		H = *Hin*dIII 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								

Semi-Log Graph Paper



Part 4. 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. The estimate was in terms of the number of base pairs.

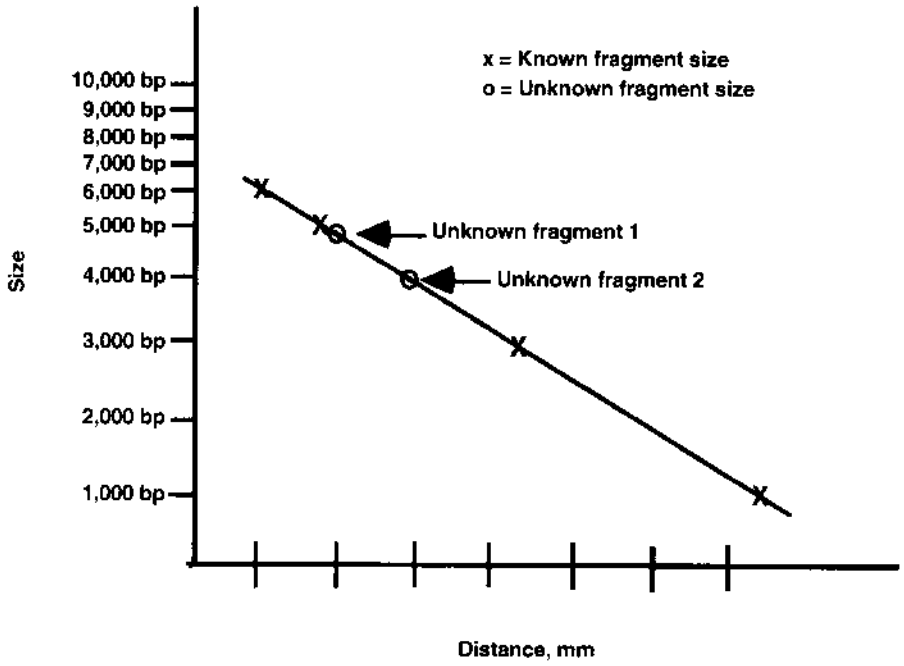
- Explain how you made this determination.

You have been provided semi-log graph paper.

1. Fragment size will be on the vertical (Y) axis.
2. The horizontal (X) axis should have your scale for distance traveled through the gel in millimeters.
3. Using the fragments from the DNA markers, plot the distance traveled versus 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 two samples.
4. Determine the sizes of the DNA fragments in your uncut and digested lambda samples.

The number of base pairs in each of the DNA fragments on your gel can be determined using another method that sometimes can be more accurate. This involves graphing the size of the known fragments from the DNA marker against the distance each DNA band moved through the gel. This is most conveniently done on semi-log graph paper.

Look at the data from the practice gel above. The fragments of known size were plotted on semi-log graph paper producing the curve shown below.



The distance migrated by the fragments of unknown length were also marked on the standard curve. To determine the size of an unknown fragment, line up a ruler vertically from the distance-traveled position on the X axis to the line that you constructed. From the point where your ruler intersected your line, place the ruler horizontally and note where it intersects the Y axis. This value on the Y axis is the predicted size for that fragment.

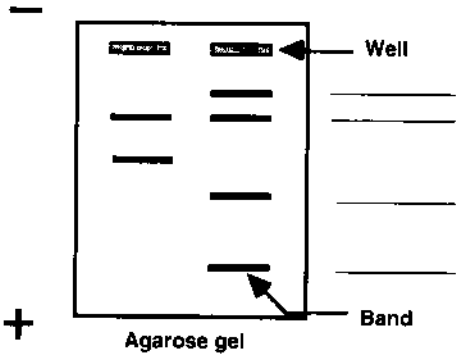
- How many base pairs does fragment 2?

- How accurate is this estimation of size?

Part 3. Analysis of DNA Fragments

The data you entered for the DNA marker represents the relative position of the bands of fragments produced by digesting a piece of DNA of known size. This kind of digest is often called a **DNA marker**, **ladder,** or **ruler**. Since you know the exact size and position of these fragments, they can be used as standard reference points to estimate the size of unknown fragment bands.

Now look at the diagram of the agarose gel (below). It shows two **lanes,** or columns of bands 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 base pairs).



- Which lane is the reference ladder? How do you know?

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

- Compare the two columns 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** that each band moved 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, millimeters (mm).

Left Lane		Right Lane	
1		1	
2		2	
		3	
		4	

Data Table Directions. 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 DNA marker. Remember, some lanes will have fewer than 6 fragments.

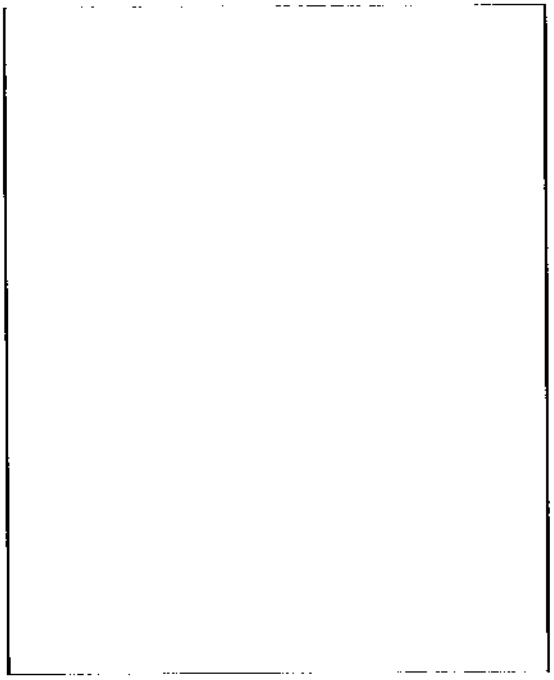
Largest fragment first ↓	M = DNA marker		L = lambda DNA- no enzyme		P = *Pst*I restriction digest of lambda DNA		E = *Eco*RI restriction digest of lambda DNA		H = *Hin*dIII restriction digest of lambda DNA	
	Distance in mm	Actual base pairs	Distance in mm	Estimated base pairs	Distance in mm	Estimated 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								

Part 2. Organize Your Data

One of the first ways of analyzing your data is to determine the approximate sizes of each of your restriction fragments. This can be done by comparing the DNA restriction fragments to DNA fragments of known sizes, or a DNA marker. You will use two methods to estimate the size of the fragments in the uncut and digested lambda DNA samples. The first method is based on visual estimation and is less precise than the second method, which involves creating a standard curve.

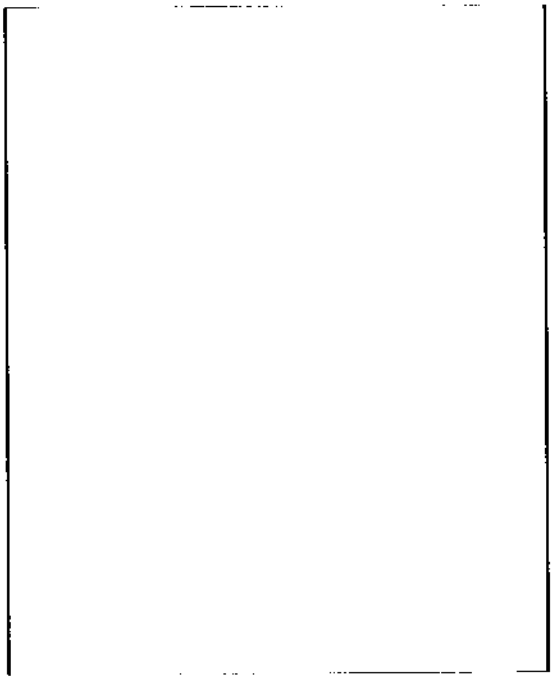
1. Measure the distance (in mm) that each of your DNA fragments traveled from the bottom of the loading well to the bottom of each DNA band. Record your numbers in the table on the next page.
2. Using the sizes given for the fragments in the marker sample, estimate the sizes, in base pairs, for each of your restriction fragments. Compare the distance traveled by the unknown bands (lambda DNA, *Pst*I digest, *Eco*RI digest and *Hin*dIII digest) to those of the DNA marker. Write the estimated sizes in the data table.
3. A more accurate way of estimating unknown DNA band sizes is to construct a standard curve based upon the measurements obtained from the DNA marker. Later in the analysis you will construct a standard curve and more accurately determine the sizes of your DNA bands.

Attach the plastic sheet that is the replica of the banding patterns from the DNA electrophoresis below.



Replica of electrophoresis gel

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



Dried electrophoresis gel

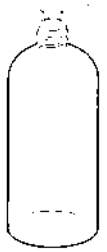
Lesson 3: Data Analysis

The DNA staining solution you used in the last period has bound to the DNA fragments in the gel. At this stage you will remove the excess DNA stain from the gel. Some stain will remain in the gel itself but the DNA in the gel will be stained dark blue.

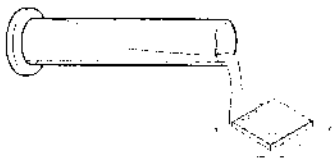
After destaining the gel, your task will be to analyze the DNA banding patterns in the gel.

Part 1. Destain Your Gel

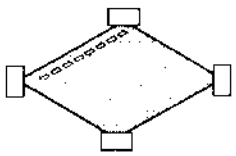
1. Pour the DNA staining solution in your staining tray back into the container; it is recyclable. To destain your gel, rinse the gel several times with water.



2. Fill your staining tray with enough water to cover the gel. Let stand or shake for 10–20 minutes, then pour off the excess water. Examine DNA banding patterns in each lane. Better contrast between the DNA bands and the agarose gel will occur if the gel is allowed to sit in fresh water overnight.



3. Place your gel on a light background and record your results by making a trace as follows. Place a clear sheet of plastic over the gel. With a permanent marker, trace the wells and band patterns onto the plastic sheet to make a replica 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.
4. Trim your gel with a knife or razor blade. Trim to remove the lanes that you did not load samples into, leaving only lanes 1–5.
5. Place the gel on the **hydrophilic side** of a sheet of gel support film or back into the plastic staining tray to air dry at room temperature. The gel will stick to the film when dry. Save the dried gel; it is part of your experimental data.



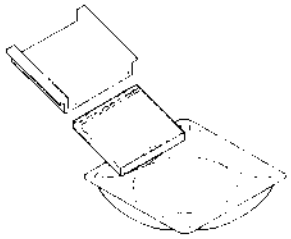
Part 4. Stain the DNA in Your Gel

Consideration—How Can DNA Be Made Visible?

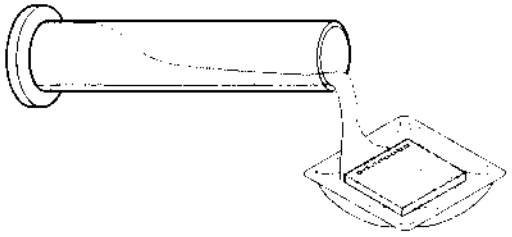
- What color was the DNA before you added loading dye?

Since DNA is not naturally colored, it is not immediately visible in a gel. Following electrophoresis, the DNA in the gel must be placed in a staining solution in order to visualize the bands.

1. Locate the plastic staining tray at your laboratory station. Mark staining trays with your initials and class period.
2. Carefully place the gel into this tray by very carefully sliding it off the end of the gel tray.

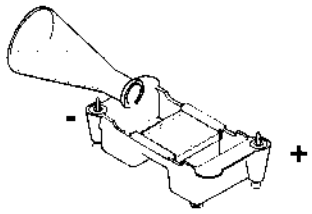


3. Add enough diluted DNA staining solution to just cover the gel. That's about 60 ml per tray. Cover the tray with plastic wrap. Allow your gel to soak in the staining solution overnight, with gentle shaking if possible.



Part 2. Set Up Your Gel Electrophoresis Chamber

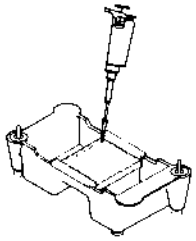
1. Obtain an agarose gel from your teacher, or, if your teacher instructs you to do so, pour your own gel.
2. Place the casting tray, with the solidified gel in it, onto the central platform in the gel box. The wells should be at the negative (cathode) end of the box where the black electrical lead is connected. Very carefully remove the comb from the gel by pulling it straight up.
3. 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

1. Pipet 10 mm 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**



2. 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.
3. 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.
4. When electrophoresis is complete, **turn off the power supply**, disconnect the leads from the inputs, and remove the top of gel chamber.
5. Remove the casting tray from gel chamber. **The gel is very slippery. Hold the tray level.**
6. Pour the excess buffer back into the original container. (It's recyclable.)

Lesson 2: Laboratory Exercise – DNA Fragment Separation Procedure

Part 1. Prepare Your Samples for Electrophoresis

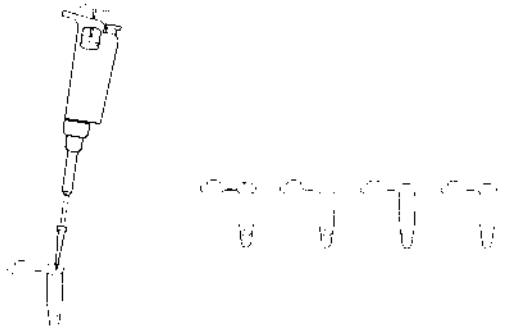
Consideration—How Can the Pieces of DNA Be Separated from One Another?

DNA is colorless so DNA fragments in the gel can't be seen during electrophoresis. A blue loading buffer, containing two blue dyes, is added to the DNA solution. The loading dyes do not stain the DNA but make 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 microtubes **L**, **P**, **E**, and **H** and place them in the foam microtube rack at your laboratory desk.

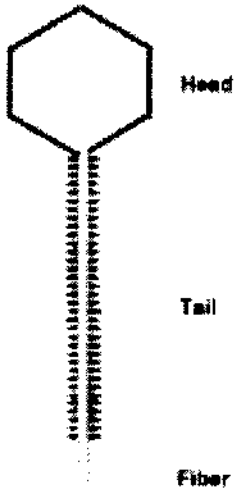
   L = No restriction enzyme—just uncut lambda DNA
   P = *Pst*I restriction digest of lambda DNA
   E = *Eco*RI restriction digest of lambda DNA
   H = *Hin*dIII restriction digest of lambda DNA

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

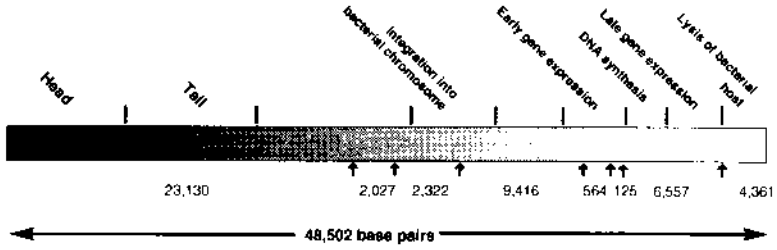


2. The DNA samples and the sample loading dye must be thoroughly mixed in each tube before placing the samples in the gel wells for electrophoresis. This is easily accomplished by holding the top of a microtube between the index finger and thumb of one hand and flicking the bottom of the tube gently 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 microcentrifuge, place the four tubes from your microtube holder (these tubes now have DNA and loading dye) into the microcentrifuge, being sure to space them evenly around the inside. 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.



This diagram represents the bacteriophage lambda genomic DNA, showing the locations of important gene clusters (Ausubel et al. 1998). Arrows mark the sites where the restriction enzyme *Hin*dIII cuts the DNA, and the numbers indicate the number of base pairs in each fragment.

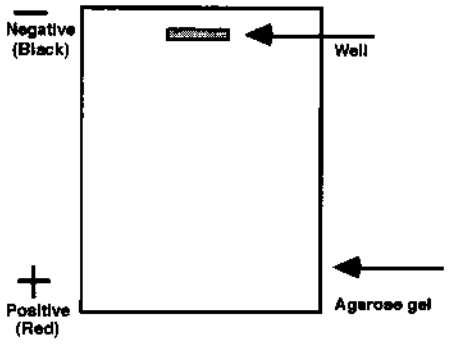


Bacteriophage lambda consists primarily of a head, which contains the genomic DNA, and a tail that is involved in phage attachment to bacterial cells.

- How many fragments were produced by the restriction enzyme *Hin*dIII?

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.



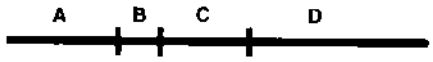
Lesson 2: Agarose Gel Electrophoresis—A Molecular Strainer

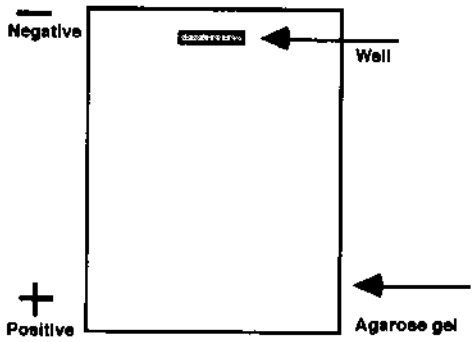
Consideration—How Can Fragments of DNA Be Separated From One Another?

Agarose gel electrophoresis is a procedure used to separate DNA fragments based on their sizes. DNA is a molecule that contains **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 is placed in a small well formed in a gel, which has a consistency similar to gelatin dessert. Any 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 electricity, the DNA fragments will sort themselves out on the agarose gel by size. **Fragments that are the same size will move together** through the gel and form **bands**.

A piece of DNA is cut into four fragments as shown in the diagram. A solution of the four fragments is placed in a well in an agarose gel. Using the information given above, draw on the diagram how you think the fragments might separate themselves. Label each fragment





- Have your teacher check your diagram before you proceed.

- Where would the larger fragments with the greater number of base pairs be located, toward the top of the gel or the bottom? Why?

- If 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...**

- 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 1 Review

Let's summarize what have we learned so far

- The base sequence in one strand of DNA can have a palindrome in the other strand (**GAATTC** and **CTTAAG**).
- Palindromes can be detected by restriction enzymes.
- Restriction enzymes cut the palindromes at restriction sites.
- A restriction enzyme only recognizes one specific kind of palindrome.
- Cutting DNA at restriction sites will produce DNA fragments.
- Fragment sizes can be described by the number of base pairs they contain.

Apply what you have learned

A linear DNA molecule is shown below. The DNA is represented by one dark line, but 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 it is cut by that enzyme?

- Number each fragment.

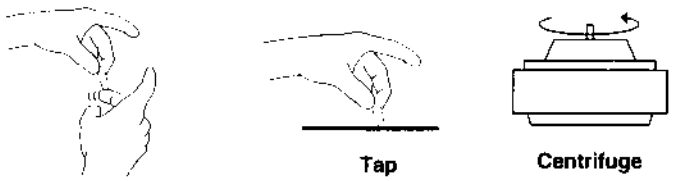
- Which fragment would be the largest?

- Which fragment would be the smallest?

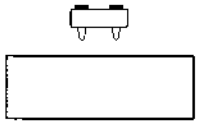
- In which tube do you expect no changes to occur—that is, no DNA fragments produced.

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

3. Securely fasten the cap on each tube. In order to mix all reagents, hold the top of a microtube 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 tub on the table to collect the liquid. If you are using a microcentrifuge, place the four tubes from your microtube into the microcentrifuge, being sure to space them evenly around the inside. Have your teacher check before spinning the tubes. Pulse-spin the tubes (hold the button for a few seconds).



4. 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.



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.

- 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?

Lesson 1: Laboratory Exercise – The Digestion

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

Set up your restriction digest reactions:

1. Label four microtubes L, P, E, and H and place them in the foam microtube rack.

L = No restriction enzyme—just uncut lambda DNA
P = *Pst*I restriction digest of lambda DNA
E = *Eco*RI restriction digest of lambda DNA
H = *Hin*dIII restriction digest of lambda DNA

- Describe the appearance of the DNA in solution.

- Is the DNA visible?

2. You will set up your digests in small tubes. To each tube, add 4 µl of uncut lambda DNA, 5 µl of restriction buffer and 1 µl of enzyme. **Add only one kind of enzyme to a tube.**

   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	*Pst*I	*Eco*RI	*Hin*dIII
P	4 µl	5 µl	1 µl	–	–
E					
H					
L					

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 palindrome can be repeated a number of times on a strand of DNA, and the specific restriction enzymes **will cut all those palindromes** at their restriction sites.

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?

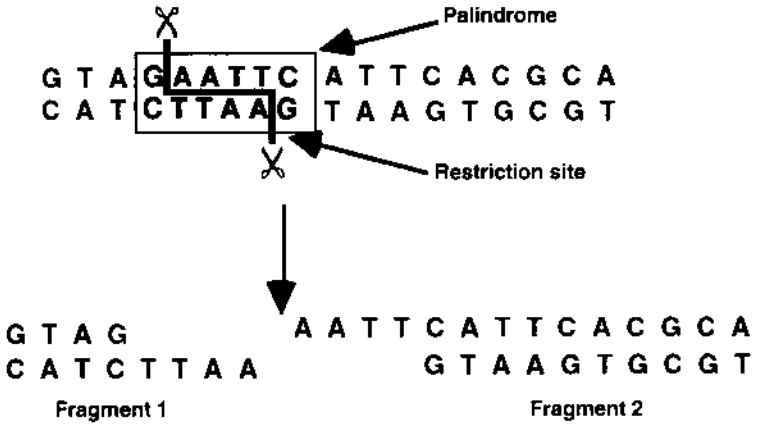
- If the **GAATTC** palindrome repeats are randomly spaced along the DNA strand, then what can you say about the size of the fragments that will be produced?

Lesson 1: Introduction to Restriction Analysis

Part 3. 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 viral DNA. Bacteria have responded by evolving a natural defense, called restriction enzymes, to cut up and destroy the invading DNA. These enzymes search the viral DNA for specific palindromic sequences of base pairs, such as (GAATTCs), and cut up the DNA into pieces at these sites. The actual place in the palindrome where the DNA is cut is called a **restriction site**.

Look at the DNA sequence below:



The *Eco*RI enzyme cut the DNA between the **G** and the **A** in a **GAATTC** palindrome.

- 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 fragment size with respect to the number of base pairs in the fragment?

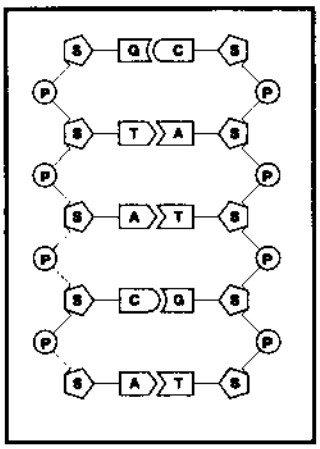
You may have discovered that the sequence of base pairs is random and that the two strands are complementary to each other; **A**s are paired with **T**s, etc. You may have also noticed that a portion of the top strand **GAATTC** (read to the right) has a counterpart in the lower strand **CTTAAG** (read to the left). Similar examples are **AAGCTT** and **TTCGAA**, and **CTGCAG** and **GACGTC**. These sequences, called **palindromes**, are quite common in DNA molecules.

Lesson 1: Introduction to Restriction Analysis

Part 2. The Structure of DNA

Consideration 1. How Can DNA Be Cut into Pieces?

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



In this representation of DNA the symbol system is as follows:

Side Chains

S = Five-carbon **Sugar** molecule known as deoxyribose

P = **Phosphate** molecule composed of a phosphorus and oxygen atoms

DNA Nucleotide Bases:

A = adenine **C** = cytosine **G** = guanine **T** = thymine

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

Read to 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 to the left**

Look at the linear sequence of bases on each of the strands.

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

- Compare the **bases** in the upper portion of the molecule to those in the lower portion. Describe any relationship you can see.

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

Lesson 1: Introduction to Restriction Analysis

Part 1. Molecular Biology Background

One of the basic tools of modern biotechnology is DNA splicing; cutting DNA and linking it to other DNA molecules. 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 can be given to people with nonfunctional or mutated genes, such as those who have a genetic disease like cystic fibrosis.

In this laboratory, the DNA you will be working with is the entire genome from a bacterial virus that has been cut into pieces with enzymes. Your task will be to determine the size of the DNA pieces by performing a procedure known as gel electrophoresis. This involves separating a mixture of the molecules by the size of the pieces and comparing your pieces to DNA pieces of known sizes.

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 agarose gel electrophoresis.

Your tasks:

- **Cut lambda DNA into a series of fragments using restriction enzymes.**
- **Separate a large group of DNA molecules by their sizes using agarose gel electrophoresis.**
- **Determine the size of each molecule separated.**

You will be provided with a quantity of 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.