Laboratory Exercise – Sample Preparation

Sample Preparation

1. Label four microtubes as **L**, **P**, **E**, and **H**, and place them in the foam tube holder.

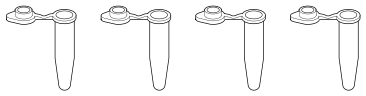
   L = Uncut lambda DNA

   P = *Pst*I restriction digest of lambda DNA

   E = *Eco*RI restriction digest of lambda DNA

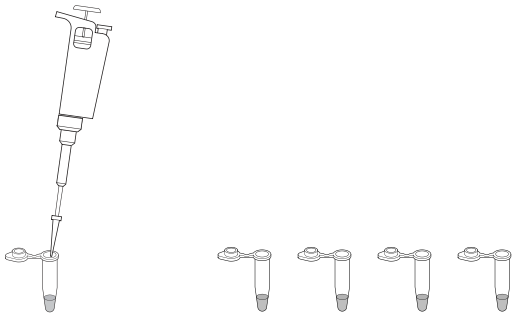
   H = *Hin*dIII restriction digest of lambda DNA

   Since the fragment sizes are **known** for the *Hin*dIII lambda digest, it will function as a DNA size standard.



2. Set the digital micropipet to 10 µl. Use a clean pipet tip, and transfer 10 µl of the uncut lambda DNA to the **L tube** in your foam tube holder. Alternatively, your teacher may give each team microtubes already containing DNA samples.

3. Repeat step 2 by transferring from the **P**, **E**, and **H** stock tubes into each of your appropriately labeled sample tubes. **Be sure to use 10 µl each time and change the pipet tip each time.**



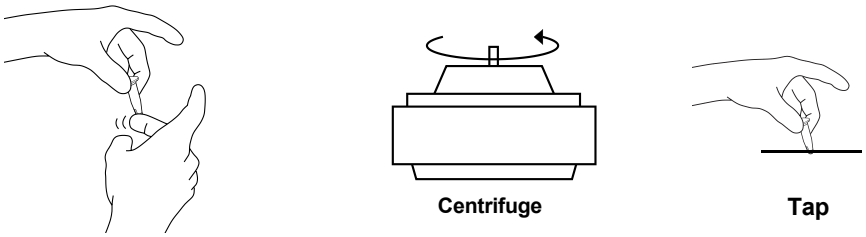
   - Is the DNA you added to these tubes visible?

   DNA is colorless so DNA fragments in the gel cannot be seen during electrophoresis. A blue loading dye, composed of two blue dyes, is added to the DNA solution. The loading dyes do not stain the DNA but make it easier to load your samples into the agarose 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 in size.

4. Redial 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 new pipet tip for all tubes.

5. The DNA and 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 closed micro test tube between the index finger and thumb of one hand and flicking the bottom of the tube with the index finger of the other hand.

   If you are using a microcentrifuge, transfer the four tubes containing digested DNA and loading buffer into the microcentrifuge. Be sure that the tubes are in a balanced arrangement in the rotor. Have your teacher check before spinning the tubes. Centrifuge the tubes by holding the button for a few seconds to collect all the liquid at the bottom of the tubes. If you don't have a centrifuge, collect the liquid to the bottom of each tube by gently tapping it upon your laboratory bench.



6. If possible, heat the samples at 65°C for 5 minutes, then chill on ice—this results in better separation of the DNA bands.

7. You have two options:

   Option one: Store the DNA samples in the refrigerator and run the agarose gel during the next class. Be sure to pulse-spin the tubes in the centrifuge or tap them gently on the bench to bring all of the liquid to the bottom prior to loading the samples on the gel.

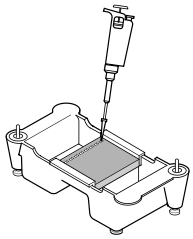
   Option two: If there is sufficient time, proceed to the next section.

Lession 2: Agarose Gel Electrophoresis

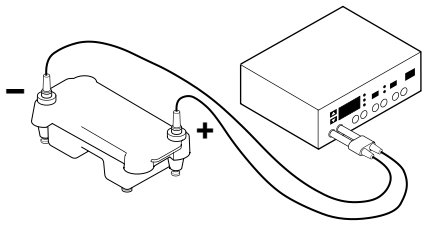
Loading the Gel and Setting Up the Gel Chamber for Electrophoresis

1. Using a fresh pipet tip for each sample, pipet 10 µl from the tubes labeled **L**, **P**, **E**, and **H** into separate wells in the gel. **Note**: Sample wells are often difficult to see. Visualization of the wells can be enhanced by placing black paper under the chamber. Load the gel in the following order:

Lane	Tube
1	L
2	P
3	E
4	H



2. Place the lid on the electrophoresis chamber. Do not disturb the samples. Connect the 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.



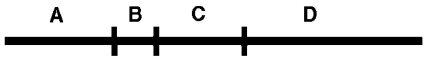
3. Electrophorese at 100 V for 30 minutes. Shortly after current is applied, the loading dye can be seen moving through the gel toward the positive side of the gel chamber.
4. When electrophoresis is complete, turn off the power supply, disconnect the leads from the power supply inputs, and remove the top of the electrophoresis chamber.
5. Remove the gel tray from the chamber. **The gel is very slippery. Hold the tray level.**
6. Pour excess buffer back into the original container for reuse, if desired.
7. Slide the gel into the staining tray. Proceed directly to the gel staining procedures on p. 30. Your instructor will determine whether to use the quick staining protocol (if there is sufficient time) or overnight staining protocol.

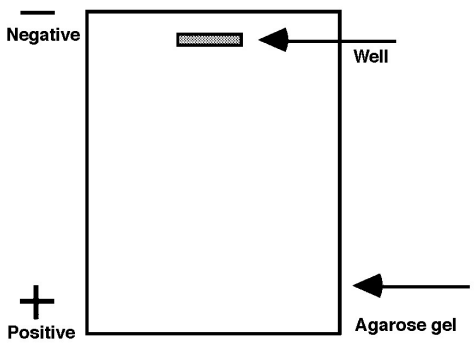
Consideration 2. How Can Fragments of DNA Be Separated From One Another?

Agarose gel electrophoresis is a procedure used to separate DNA fragments based on their sizes. DNA is 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 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.

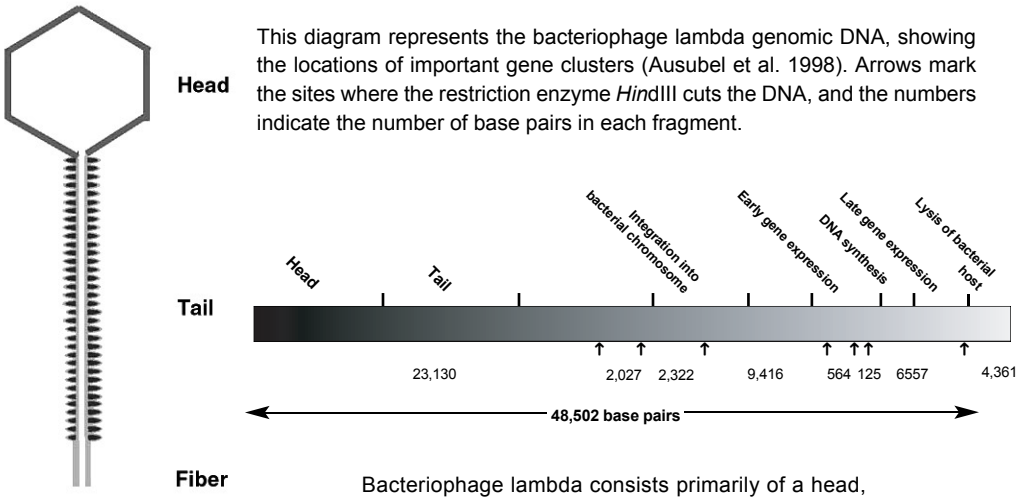
- Where would the larger fragments, those with the greater number of base pairs, be located, toward the top of the gel or the bottom? Why?

- 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 …



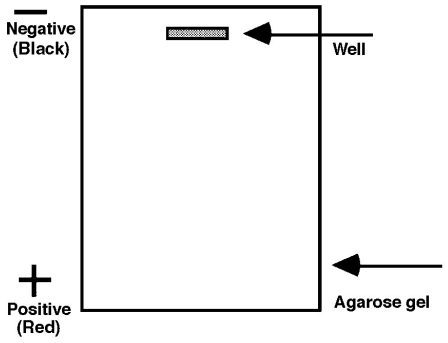
This diagram represents the bacteriophage lambda genomic DNA, showing the locations of important gene clusters (Ausubel et al. 1998). Arrows mark the sites where the restriction enzyme *Hin*dIII cuts the DNA, and the numbers indicate the number of base pairs in each fragment.

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.



Visualization of DNA Fragments

Consideration 3. How Can the DNA Be Made Visible?

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

Making DNA Fragments Visible

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 dye called Fast Blast DNA stain. The blue dye molecules are positively charged and have a high affinity for the DNA. These blue dye 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.

Laboratory Exercise – Staining with Fast Blast DNA Stain

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.

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

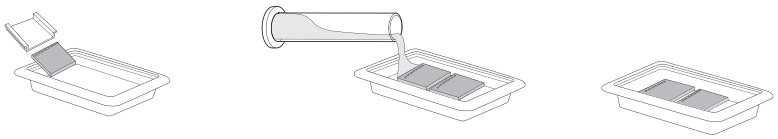
This protocol allows quick visualization of DNA bands in agarose gels within 15 minutes. For quick staining, Fast Blast DNA stain (500x) should be diluted to a 100x concentration. We recommend using 120 ml of 100x Fast Blast to stain two 7 x 7 cm or 7 x 10 cm agarose gels in individual staining trays 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

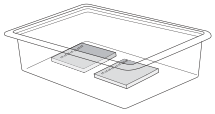
Remove each gel from the gel tray and carefully slide it into the staining tray. Pour approximately 120 ml of 100x stain into the staining tray. If necessary, add more 100x stain to completely submerge the gels. Stain the gels for 2–3 minutes, but not for more than 3 minutes. Using a funnel, pour the 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

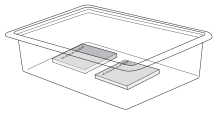
Transfer the gels into a large container containing 500–700 ml of clean, warm (40–55°C) tap water. Gently shake the gel in the water for ~10 seconds to rinse.



10 seconds

4. Wash gels

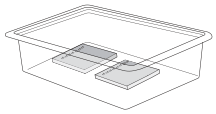
Transfer the gel into a large container with 500–700 ml of clean, warm tap water. Gently rock or shake the gel on a rocking platform for 5 minutes. If no rocking platform is available, move the gels gently in the water once every minute.



5 minutes

5. Wash gels

Perform a second wash as in step 4.



5 minutes

6. Record results

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 dye molecules migrating into the gel and binding more tightly to the DNA molecules.

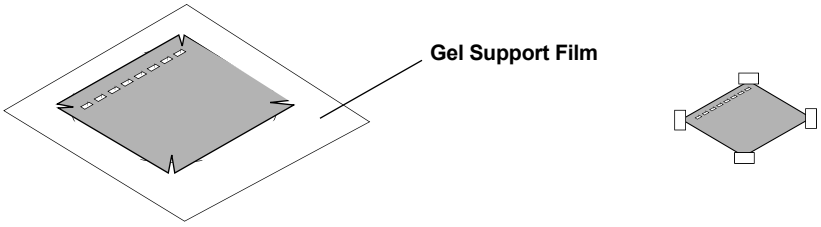
To obtain maximum contrast, additional washes in warm water may be necessary. Destain to the desired level, but do not wash the gel in water overnight. If you cannot complete the destaining in the allocated time, you may transfer the gel to 1x Fast Blast stain for overnight staining. **See Option 2**.

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 size 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.



Option 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.

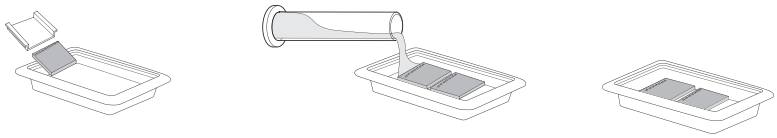
Following DNA electrophoresis, agarose gels must be removed from their gel trays before being placed in the staining solution. This is easily accomplished by holding the base of the gel tray in one hand and gently pushing out the gel with the thumb of the other hand. Because the gel is fragile, special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

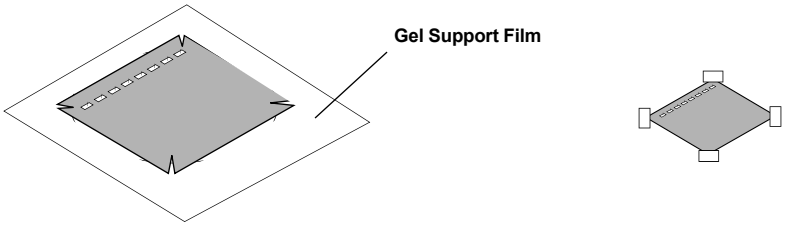
No destaining is required after staining with 1x Fast Blast. The gels can be analyzed immediately after staining.

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 size 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.

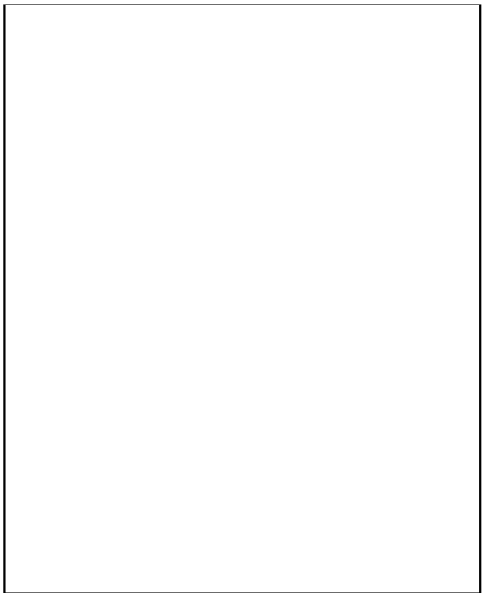


Note: Avoid extended exposure of dried gels to direct light to prevent band fading. However, DNA bands will reappear if the dried gels are stored in the dark for 2–3 weeks after fading.

Lesson 3: Analysis of Results

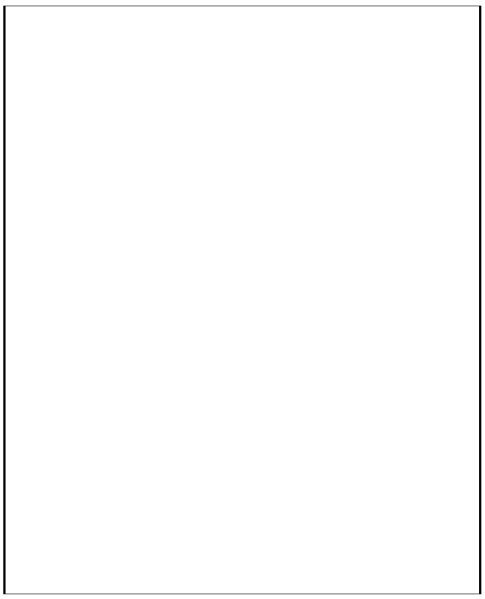
If the overnight staining protocol was used to stain gels, record your results and dry gels as described in the gel staining procedures in Lesson 2.

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 lambda *Hin*dIII 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 DNA *Hind*III 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 *Hind*III restriction digest (DNA standard or marker). Remember, some lanes will have fewer than 6 fragments.

Largest fragment first ↓	L = 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	
	Distance in mm	Estimated base pairs	Distance in mm	Estimated base pairs	Distance in mm	Estimated base pairs	Distance in mm	Actual base pairs
Band 1								23,130 bp
Band 2								9,416 bp
Band 3								6,557 bp
Band 4								4,361 bp
Band 5								2,322 bp
Band 6								2,027 bp

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 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 bp).

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

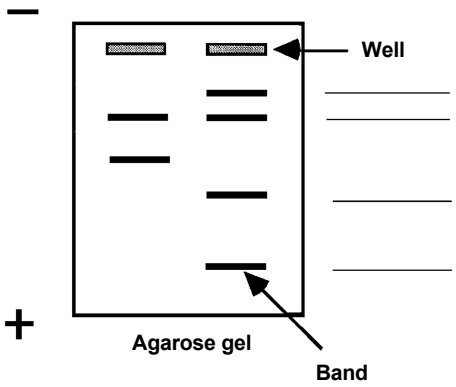
- Label each band in the right lane with its base-pair size.

- 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.

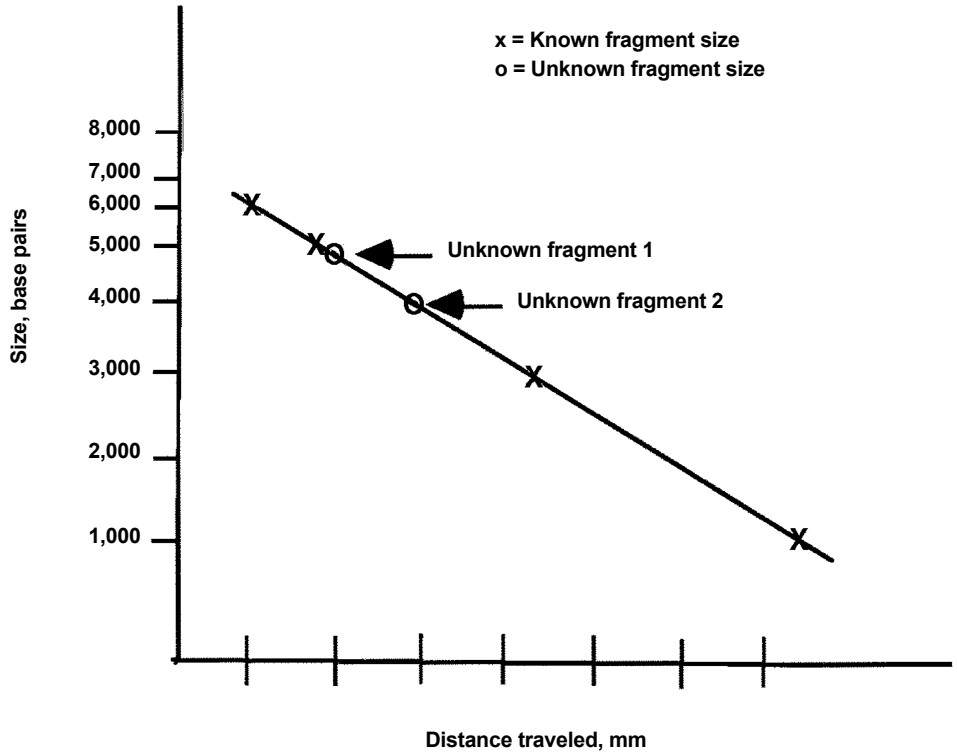
  Measure from the bottom edge of the well to the bottom edge of the band.

- 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 marker 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 37. 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 is fragment 2?

- How accurate is this estimation of size?

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

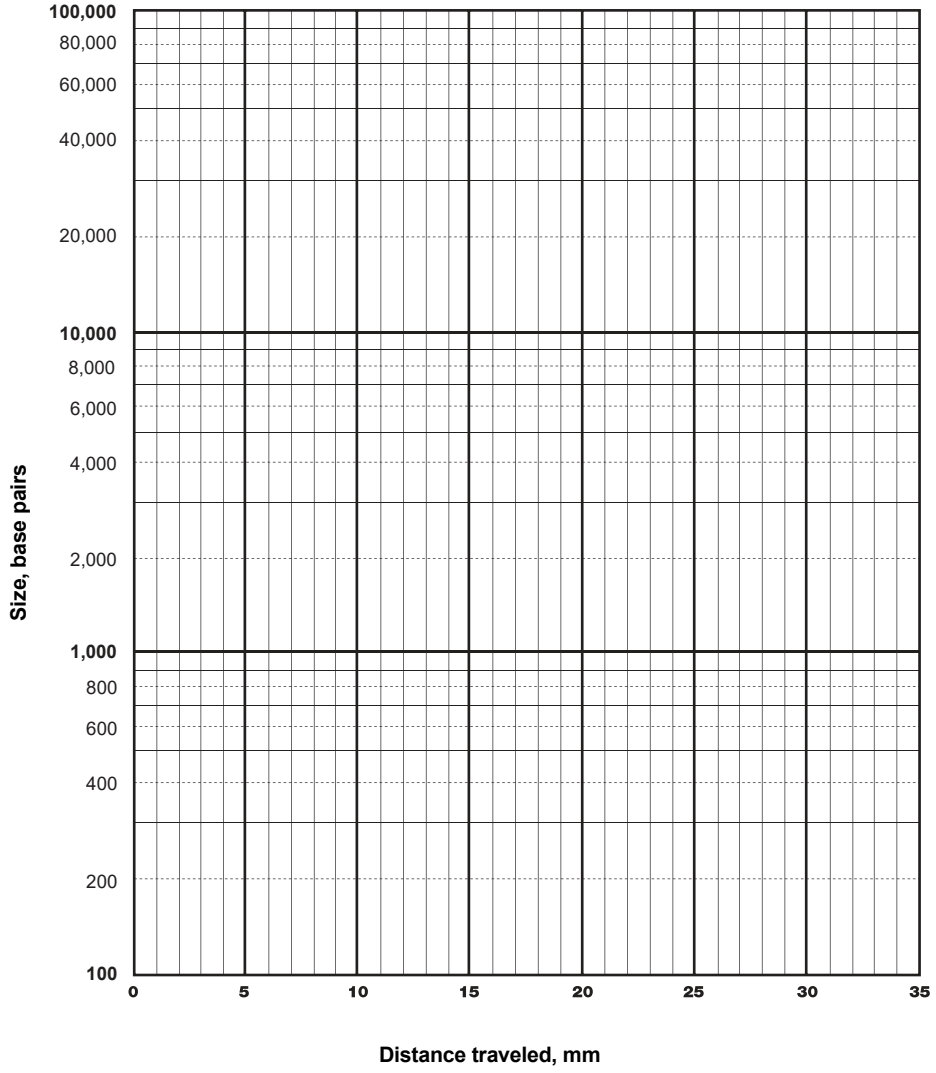
From your laboratory data, you were able to estimate the approximate size of each of the DNA fragments that you separated on your gel. This was done in terms of the number of base pairs.

- Explain how you made this determination.

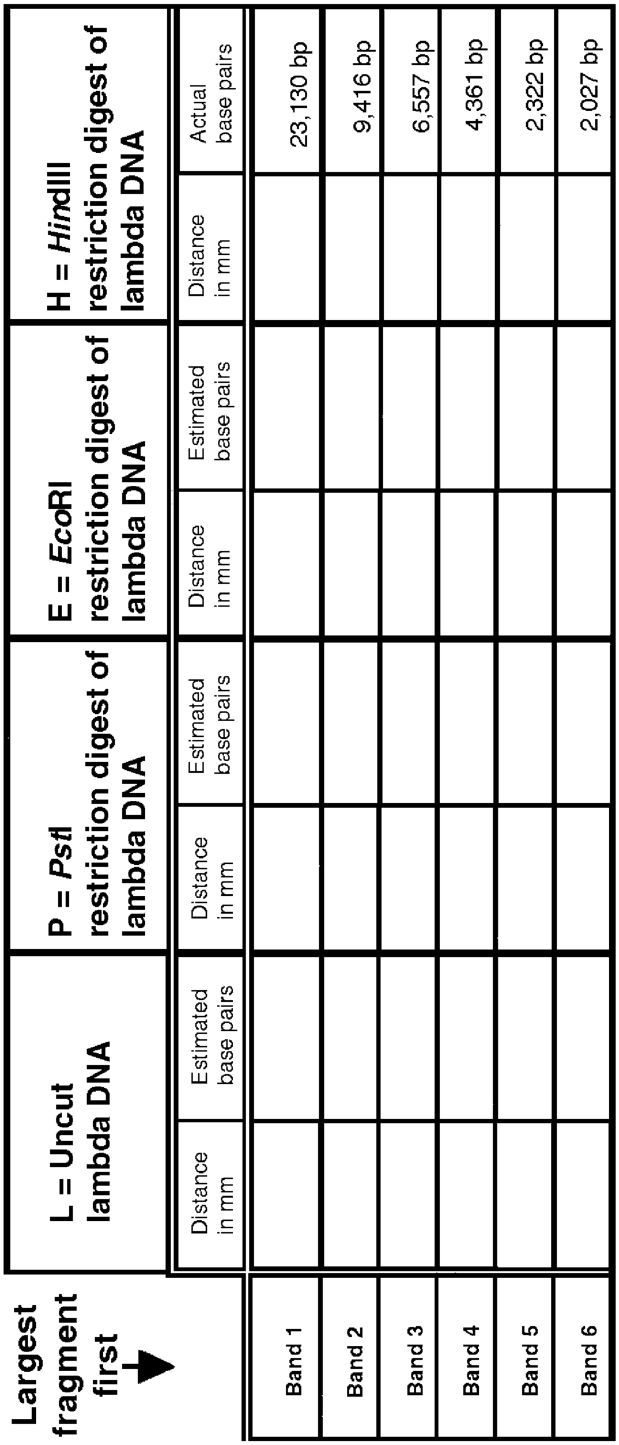
You have been provided with three-cycle, semilog graph paper.

1. Fragment size will be on 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 lambda *Hind*III 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 lambda (**L**), *Pst*I digest (**P**), and *Eco*RI digest (**E**), using the method described on the previous page.

Semilog Graph Paper



- 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 →	L = 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	
	Distance in mm	Estimated base pairs	Distance in mm	Estimated base pairs	Distance in mm	Estimated base pairs	Distance in mm	Actual base pairs
Band 1								23,130 bp
Band 2								9,416 bp
Band 3								6,557 bp
Band 4								4,361 bp
Band 5								2,322 bp
Band 6								2,027 bp

- 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.