

## MOLEBIO LAB #9: DNA Restriction Analysis

### Introduction:

DNA restriction analysis is at the heart of recombinant DNA technology and of the laboratories in this course. The ability to cut DNA predictably and precisely enables DNA molecules to be manipulated and recombined at will. The fact that discrete ends of like-sized DNA fragments are seen in one lane of an agarose gel shows that each of the more than 1 billion DNA molecules present in each restriction reaction was cut in precisely the same place.

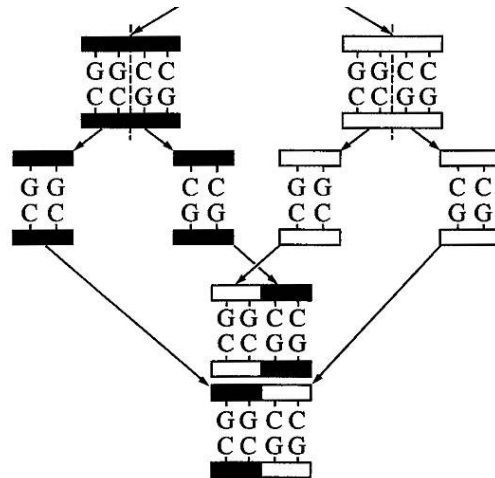
This protocol introduces the analysis of DNA using restriction enzymes. Three samples of purified DNA from bacteriophage  $\lambda$  (48,502 bp in length) are incubated at 37°C, each with one of three restriction endonucleases: *EcoRI*, *BamHI*, and *HindIII*. Each enzyme has five or more restriction sites in  $\lambda$  DNA and therefore produces six or more restriction fragments of varying lengths. A fourth sample of  $\lambda$  DNA, the negative control, is incubated without an endonuclease and remains intact.

Restriction endonucleases, or restriction enzymes, are essential tools in recombinant DNA methodology. Several hundred have been isolated from a variety of prokaryotic organisms. Restriction endonucleases are named according to a specific system of nomenclature. The letters refer to the organism from which the enzyme was isolated. The first letter of the name stands for the genus name of the organism. The next two letters represents the second word, or species name. The fourth letter (if there is one) represents the strain of the organism. Roman numerals indicate whether the particular enzyme was the first isolated, the second, or so on.

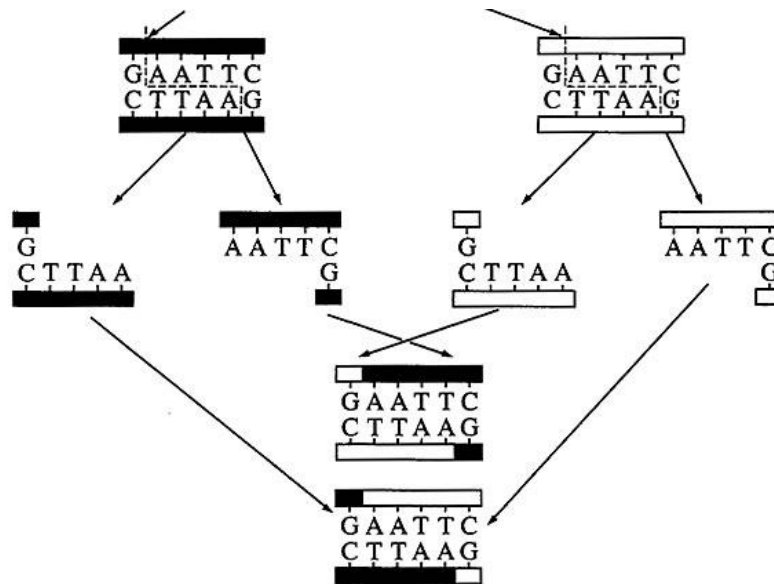
#### Examples:

<i>EcoRI</i> – <i>Escherichia coli</i>	strain RY13	first endonuclease isolated
<i>HaeIII</i> – <i>Haemophilus aegyptus</i>		second endonuclease isolated
<i>BamHI</i> – <i>Bacillus amyloli</i>		first endonuclease isolated
<i>HindIII</i> – <i>Haemophilus influenzae</i>		third endonuclease isolated
<i>PstI</i> – <i>Providencia stuartii</i>		first endonuclease isolated

Restriction endonucleases recognize specific DNA sequences in double-stranded DNA (usually a four to six base pair sequence of nucleotides) and digest the DNA at these sites. The result is the production of fragments of DNA of various lengths. Some restriction enzymes cut cleanly through the DNA helix at the same position on both strands to produce fragments with blunt ends (**Figure 1**). Other endonucleases cleave each strand off-center at specific nucleotides to produce fragments with "overhangs", or sticky ends (**Figure 2**). By using the same restriction enzyme to "cut" DNA from two different organisms, complementary sticky ends will be produced and can allow the DNA from two sources to be "recombined".



**Figure 1:** Cleavage by **HaeIII** produces blunt ends.



**Figure 2:** Cleavage by **EcoRI** produces sticky ends.

Groups of restriction enzymes operate under different conditions of salt and pH. For optimal activity, several different buffers are needed for the enzymes used in this course. To simplify procedures, we use a “compromise” restriction buffer – a universal buffer that is a compromise between the conditions preferred by various enzymes.

The “unit” is the standard measure of restriction enzyme activity and is defined as the amount of enzyme needed to digest to completion 1  $\mu$ L of  $\lambda$  DNA in a 50  $\mu$ L reaction in 1 hour. The unit concentration of various restriction enzymes varies from batch to batch and from manufacturer to manufacturer. Typical batches of commercially available enzymes have activities in the range of 5 – 120 units/ $\mu$ L.

## Gel Electrophoresis

When any molecule enters an electrical field, the mobility or speed at which it will move is influenced by the charge of the molecule, the strength of the electrical field, the size and shape of the molecule, and the density of the medium (in this case, agarose gel) through which it is migrating. When all molecules are positioned at a uniform starting site on a gel and the gel is placed in a chamber containing a buffer solution and electricity is applied, the molecules will migrate and appear as bands when stained. Nucleic acids, like DNA and RNA, move because of the charged phosphate groups in the backbone of the DNA molecule. Because the phosphates are negatively charged at neutral pH, the DNA will migrate through the gel towards the positive electrode. In this exercise, we will use an agarose gel. In agarose, the migration rate of linear fragments of DNA is inversely proportional to their size; the smaller the DNA molecule, the faster it migrates through the gel.

## Making DNA Visible

DNA is colorless, so DNA fragments in the gel cannot be seen during electrophoresis. A loading dye containing two blue dyes is added to the DNA solution. The loading dye does 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 about equals DNA fragments of approximately 500 bp, while the "slower" dye replicates the migration of DNA fragments about 5 kb (5000 bp) in size.

In this lab, we will use the SYBR Safe DNA stain to attach to the DNA trapped in the agarose gel when we are finished applying the current. This will be visible when viewed under a light source that emits blue light (our Blue Light Illuminator).

## Reliability of DNA Evidence

Two major factors affecting the reliability of DNA fingerprinting technology in forensics are population genetics and genetic statistics. In humans, there are thousands of RFLP loci or DNA segments that can be selected and used from fingerprinting analysis. Depending on demographic factors such as ethnicity or geographic isolation, some segments will show more variation than others.

Some populations show much less variation in particular DNA segments than others. The degree of variation will affect the statistical odds of more than one individual having the same sequence. If 90% of a given population has the same frequency in its DNA fingerprinting pattern for a certain DNA segment, then very little information will be attained. But if the frequency of a DNA pattern turning up in a population for a particular segment is extremely low, then this segment can serve as a powerful tool to discriminate between individuals in that population. Different populations show different patterns in their genotypes due to the contributions made to their individual gene pools over time.

Therefore, in analyzing how incriminating the DNA evidence is, one needs to ask the question: "Statistically, how many people in a population may have the same pattern as that taken from a crime scene: 1 in 1,000,000? 1 in 10,000? Or, 1 in 10?"

## DNA Used in this Simulation

Because it is inexpensive and readily available, purified DNA from bacteriophage  $\lambda$  is most suitable for demonstrating the concept of DNA restriction. Most commercially available  $\lambda$  is derived from a temperature-sensitive lysogen of *E. coli* called  $\lambda$ 857 and is 48,502 base pairs in length. Restriction of chromosomal DNA, even from a simple organism such as *E. coli*, will generate thousands of DNA fragments that appear as a smear in an agarose gel.

**Procedure:****PRELAB PREPARATION**

1. Prepare 1X Tris/Borate/EDTA (TBE) buffer and 25 mL 0.8% agarose – make sure to add SYBR Stain **AFTER** you dissolve the agarose but **BEFORE** you pour the gel in the gel tray!

**SET UP RESTRICTION DIGEST**

2. Use a permanent marker to label four 1.5 mL tubes, in which restriction reactions will be performed:
  - B = *Bam*HI
  - E = *Eco*RI
  - H = *Hind*III
  - – = no enzyme
3. Use the matrix below as a checklist while adding reagents to each reaction. Read down each column, adding the same reagent to all appropriate tubes. *Use a fresh tip for each reagent.* Refer to detailed directions that follow.

<b>Tube</b>	<b>λ DNA</b>	<b>Buffer</b>	<b><i>Bam</i>HI</b>	<b><i>Eco</i>RI</b>	<b><i>Hind</i>III</b>	<b>H<sub>2</sub>O</b>
B	4 μL	5 μL	1 μL	–	–	–
E	4 μL	5 μL	–	1 μL	–	–
H	4 μL	5 μL	–	–	1 μL	–
–	4 μL	5 μL	–	–	–	1 μL

4. Collect and place reagents in a test tube rack on the lab bench.
5. Add 4 μL of DNA to each reaction tube. Touch the pipette tip to the side of the reaction tube, as near to the bottom as possible, to create capillary action to pull the solution out of the tip.
6. Always add buffer to the reaction tubes *before* adding enzymes. Use a *fresh tip* to add 5 μL of restriction buffer to a clean spot on each reaction tube.
7. Use *fresh tips* to add 1 μL of *Eco*RI, *Bam*HI, and *Hind*III to the appropriate tubes.
8. Use a *fresh tip* to add 1 μL of deionized or distilled water to the tube labeled "–".
9. Close tube tops. Pool and mix reagents by pulsing in a microfuge or by sharply tapping the tube bottom on the lab bench.
10. Place the reaction tubes in a 37°C water bath, and incubate them for a minimum of 20 minutes. Reactions can be incubated for a longer period of time.
11. After incubation, freeze reactions at -20°C until ready to continue.

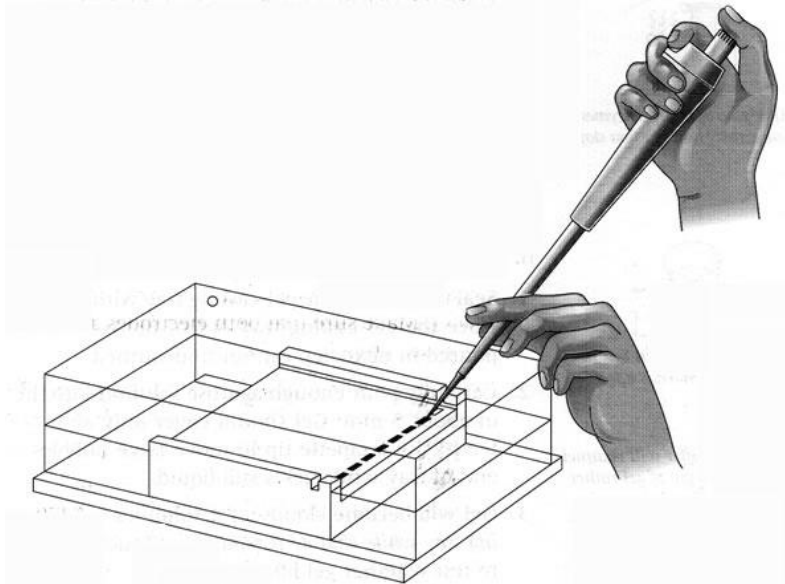
**Casting the Agarose Gels**

12. Seal the ends of a gel tray securely with strips of laboratory tape. Press the tape firmly to the edges of the gel tray to form a fluid-tight seal. Insert the well-forming comb.
13. Carefully pour the agarose (should be between 50°C – 60°C) into the casting tray to a depth of about 5 mm. Gel should only cover about one-third the height of the comb teeth.
14. The agarose gel will become cloudy as it solidifies between 5 and 10 minutes. Do not move or jar the casting tray while the agarose is solidifying. Touch corner of agarose away from the comb to test whether the gel has solidified.
15. When agarose has set, unseal ends of casting tray. Place tray on the platform of the gel box so that the comb is at the negative black electrode.

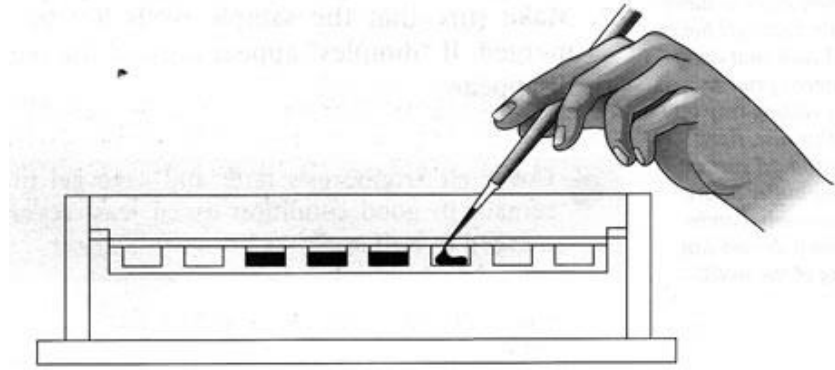
16. Fill box with 1X TBE buffer, to a level that just covers the entire surface of the gel. Too much buffer will channel current over top of the gel rather than through it, increasing the time required to separate DNA.
17. Gently remove comb, taking care not to rip the wells. This is sometimes done before adding buffer, but buffer solution helps to lubricate the comb. This generally avoids tearing the gel.
18. Make sure that the sample wells left by the comb are completely submerged. If "dimples" appear around the wells, slowly add buffer until they disappear.
19. If there is not enough time to continue, cover the electrophoresis tank and save gel until ready to continue. Gel will remain in good condition for at least several days if it is completely submerged in buffer.

### **Loading the Agarose Gels and Electrophoresis of Samples**

20. Add 1  $\mu\text{L}$  of loading dye into each reaction tube (not the 1kb DNA Ladder). Once completed, pulse the tubes in a balanced configuration in the microcentrifuge to make sure the samples are transferred to the bottom of the tubes.
21. Using a 1-10  $\mu\text{L}$  micropipette with a sterile tip for each sample, load 10  $\mu\text{L}$  of each reaction tube into a separate well in the gel (lanes 2 through 6, respectively – 1kb Ladder, E, B, H, and --) following the detailed steps on the next page.

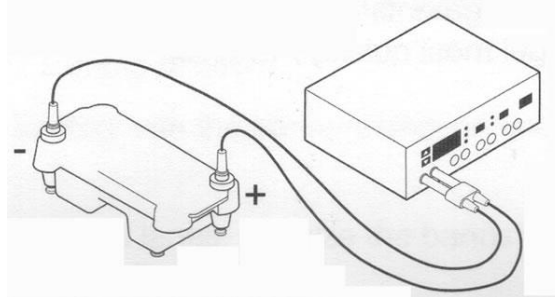


**Figure 3:** Hand Positioning For Loading an Agarose Gel



**Figure 4:** Loading the Gel

- a. Use two hands to steady the micropipettor over the well.
  - b. Before loading the sample, make sure that there are no bubbles in the wells. If bubbles exist, move them with a micropipette tip.
  - c. If there is air in the end of the tip, carefully depress plunger to push the sample to the end of the tip. (If an air bubble forms a "cap" over the well, DNA/loading dye will flow into buffer around edges of well.)
  - d. Dip micropipette tip through surface of buffer, center it over the well, and gently depress plunger to slowly expel sample. Sucrose in the loading dye weighs down the sample, causing it to sink to the bottom of the well. *Be careful not to punch the tip of the micropipette through the bottom of the gel.*
22. Carefully place the lid on the electrophoresis chamber. The lid should attach onto the base in only one orientation. Plug the electrodes into the power supply, red to red and black to black. Turn on the power and electrophorese your samples at 100 V for 30-60 minutes.



23. Good separation will have occurred when the bromophenol blue band has moved 4-7 cm from the wells. If time allows, carry out electrophoresis until the bromophenol blue band nears the end of the gel. *Stop* electrophoresis before the bromophenol blue band runs off the end of the gel.
24. Turn off the power supply, disconnect leads from the inputs, and remove the top of the electrophoresis box.
25. Carefully remove the tray from the gel box. **DO NOT DROP YOUR GEL OFF THE TRAY AS IT IS SLIPPERLY!** Slide the gel carefully (or use a spatula) onto the Blue Light Illuminator.

**PRE-LAB QUESTIONS**

1. The electrophoresis apparatus creates an electrical field with positive and negative poles at the ends of the gel. DNA molecules are negatively charged. To which electrode pole of the electrophoresis field would you expect DNA to migrate?

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2. What color of the electrophoresis apparatus represents the negative pole?

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3. After DNA samples are loaded into the sample wells, they are "forced" to move through the gel matrix. What size fragments (large vs. small) would you expect to move toward the opposite end of the gel most quickly? Explain.

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4. Which fragments (large vs. small) are expected to travel the shortest distance from the well? Explain.

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5. How can a mutation that alters a recognition site be detected by gel electrophoresis?

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6. A certain restriction enzyme digest results in DNA fragments of the following sizes: 4000 bp, 2500 bp, 2000 bp, and 400 bp. In the space below, sketch the resulting separation by electrophoresis. Show wells, starting point, positive and negative electrodes, and the resulting bands (labeled with their respective sizes, of course).



**POST-LAB QUESTIONS**

7. Why was 1  $\mu$ L of water added instead of restriction enzymes to the tube labeled “-”?

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8. What is the function of the compromise restriction buffer (NOT THE TBE)?

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9. What are **two** functions of the loading dye?

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10. Troubleshooting electrophoresis. What would occur...

a. ...if the gel box is filled with *distilled* water instead of TBE buffer?

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b. ...if the electrodes are reversed?

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**Quantitative Analysis of DNA Fragment Sizes**

11. Linear DNA Fragments migrate at rates inversely proportional to the  $\log_{10}$  of their molecular weights. For simplicity's sake, base-pair length is substituted for molecular weight. The table below gives the base-pair sizes of a 1kb ladder, and  $\lambda$  DNA fragments generated by single digests with the restriction enzymes *EcoRI*, *BamHI*, and *HindIII*.

1 kb DNA Ladder		<i>EcoRI</i>			<i>BamHI</i>			<i>HindIII</i>		
Distance (mm)	Actual bp	Distance (mm)	Calculated bp	Actual bp	Distance (mm)	Calculated bp	Actual bp	Distance (mm)	Calculated bp	Actual bp
	10000			<u>24756*</u>			<u>16841</u>			<u>27491</u>
	8000			21226*			<u>12275</u>			23130
	6000			7421			7233			9416
	5000			5804*			6770*			6557
	4000			5643*			6527*			4361
	3000			4878			5626*			2322
	2000			3530			5505*			2027
	1000									564
	500									125

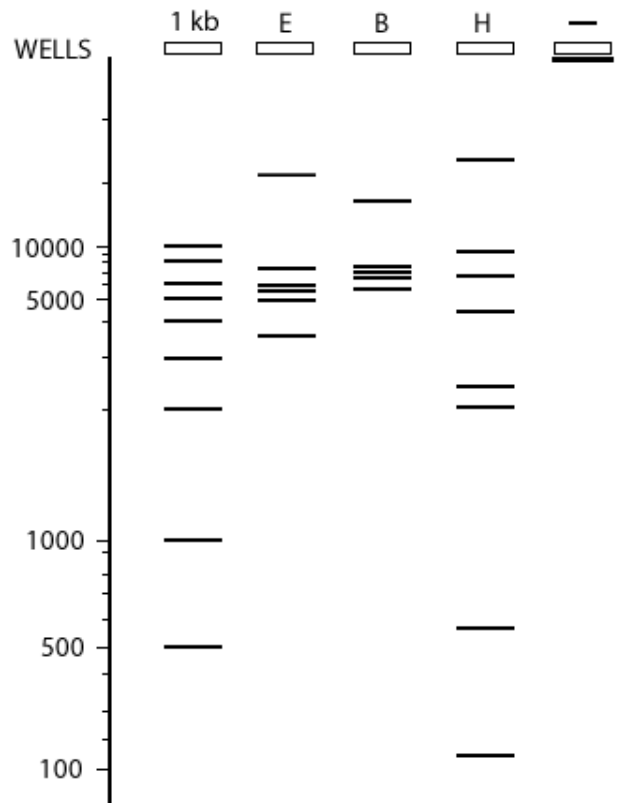
\*appears as one band; underlined fragments are "joined ends" see page 13 of the lab for a more detailed explanation

Using the ideal digest shown to the right, carefully measure the distance (in millimeters) of each *EcoRI*, *BamHI*, and *HindIII* fragment as it migrated from the origin. Measure from the 'front' edge of the well to the 'front' edge of each band. Enter the distances into the above table (in millimeters).

Match base-pair sizes of 1 kb Ladder fragments with bands that appear in the gel to the right. Measure the distance of these bands (in millimeters) and record them in the above table.

Set up the semi-log graph paper on the next page with distance migrated as the x-axis (linear scale) and log of base-pair length as the y-axis (logarithmic scale). Then plot the distance migrated versus the base-pair length for the 1 kb Ladder fragments as a "best-fit line".

Also set up a similar graph using the data above **using Excel!**



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12. Now, locate on the x-axis the distance migrated by the first *EcoRI* fragment. Use a ruler to draw a vertical line (lightly in pencil) from this point to its intersection with the best-fit data line. Extend a horizontal line (again, lightly in pencil) from this point on the best-fit line to the y axis. This gives the base-pair size of this *EcoRI* fragment.

Repeat the above steps for each remaining *EcoRI*, *BamHI*, and *HindIII* fragments on the ideal gel. Enter the results in the calculated base-pair columns for each digest. You can now compare them to the actual base pair data already in your table

For which fragment sizes was your graph most accurate? For which fragment sizes was it least accurate?

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13. DNA fragments of similar size will not always resolve on a gel. This is seen in lane B in the Ideal Gel, where *BamHI* fragments of 5626 bp and 5505 bp appear to migrate as a single heavy band. These are referred to as a doublet and can be recognized because they are brighter and thicker than similarly sized singlets. What could be done resolve the doublet fragments as single bands?

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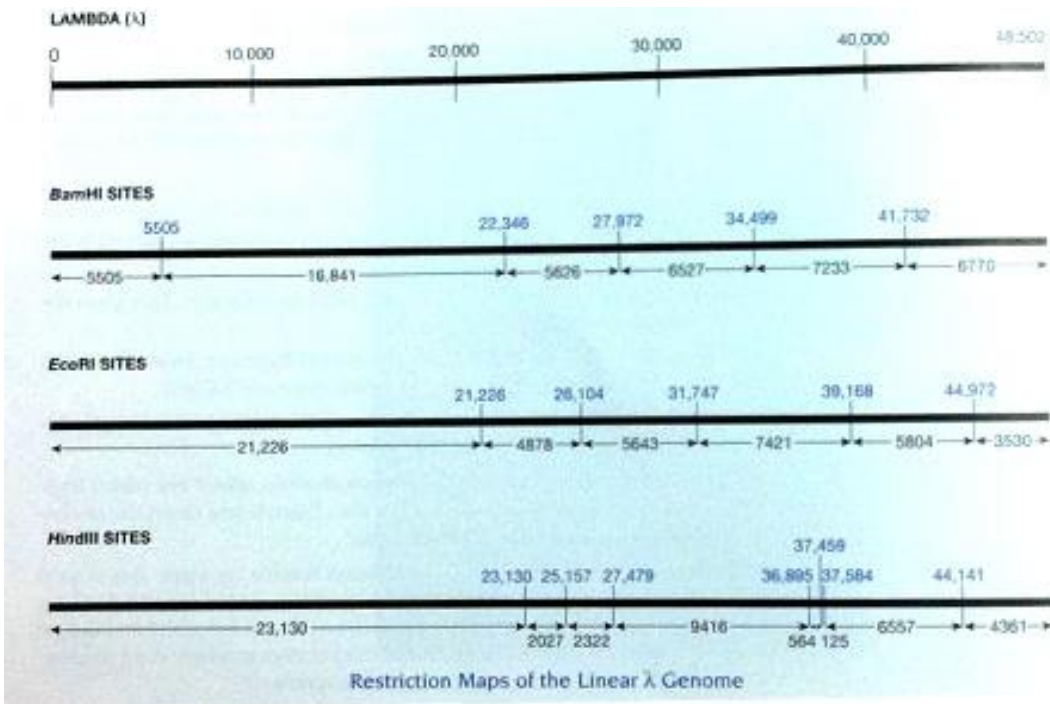
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14.  $\lambda$  DNA can exist both as a circular molecule and as a linear molecule. At each end of the linear molecule is a single-stranded sequence of 12 nucleotides, called a COS (or *cos*) site. The COS sites at each end are complementary to each other and thus can base pair to form a circular molecule. These complementary ends are analogous to the "sticky ends" created by some restriction enzymes. Commercially available  $\lambda$  DNA is likely to be a mixture of linear and circular molecules. This leads to the appearance of more bands on the gel than would be predicted from a homogeneous population of linear DNA molecules. This also causes the partial loss of other fragments. For example, the left-most *Hind*III site is 23,130 bp from the left end of the linear  $\lambda$  genome, and the right-most site is 4361 bp from the right end. The 4361 bp band is faint in comparison to other bands on the gel of similar size. This indicates that a percentage of the DNA molecules are circular – combining the 4361 bp terminal fragment with the 23,130 bp terminal fragment to produce a 27,491 bp fragment. However, the combined 27,491 bp fragment usually runs as a doublet along with the 23,130 bp fragment from the linear molecule.



On the next page, there are 3 circles. These represent  $\lambda$  DNA molecules with base-paired COS sites. Label a point at 12:00 on each circle 48/0 ( $\lambda$  DNA = 48.5 kbp). This marks the point where the COS sites are joined.

Use the data from the above restriction map of the linear  $\lambda$  genome to make a rough map of restriction sites for *Hind*III on the first circle. Next, make rough restriction maps of *Bam*HI and *Eco*RI sites on the remaining two circles.

What *Bam*HI and *Eco*RI fragments are created in the circular molecules? Why (or why not) can you locate each of these fragments on your gel or the ideal gel above?

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