LABORATORY 6. MOLECULAR BIOLOGY

OVERVIEW

In this laboratory, you will investigate some basic principles of genetic engineering. Plasmids containing specific fragments of foreign DNA will be used to transform *Escherichia coli* cells, conferring antibiotic (ampicillin) resistance. Restriction enzyme digests of phage lambda DNA will also be used to demonstrate techniques for separating and identifying DNA fragments using gel electrophoresis.

OBJECTIVES

Before doing this laboratory you should understand:

- how gel electrophoresis separates DNA molecules present in a mixture;
- the principles of bacterial transformation;
- the conditions under which cells can be transformed;
- the process of competent cell preparation;
- how a plasmid can be engineered to include a piece of foreign DNA;
- how plasmid vectors are used to transfer genes;
- how antibiotic resistance is transferred between cells;
- how restriction endonucleases function; and
- the importance of restriction enzymes to genetic engineering experiments.

After doing this laboratory you should be able to:

- use plasmids as vectors to transform bacteria with a gene for antibiotic resistance in a controlled experiment;
- demonstrate how restriction enzymes are used in genetic engineering;
- use electrophoresis to separate DNA fragments;
- describe the biological process of transformation in bacteria;
- calculate transformation efficiency;
- be able to use multiple experimental controls;
- design a procedure to select positively for antibiotic resistant transformed cells; and
- determine unknown DNA fragment sizes when given DNA fragments of known size.

INTRODUCTION

The bacterium *Escherichia coli* (or *E. coli*) is an ideal organism for the molecular geneticist to manipulate and has been used extensively in recombinant DNA research. It is a common inhabitant of the human colon and can easily be grown in suspension culture in a nutrient medium such as Luria broth, or in a petri dish of Luria broth mixed with agar (LB agar) or nutrient agar.

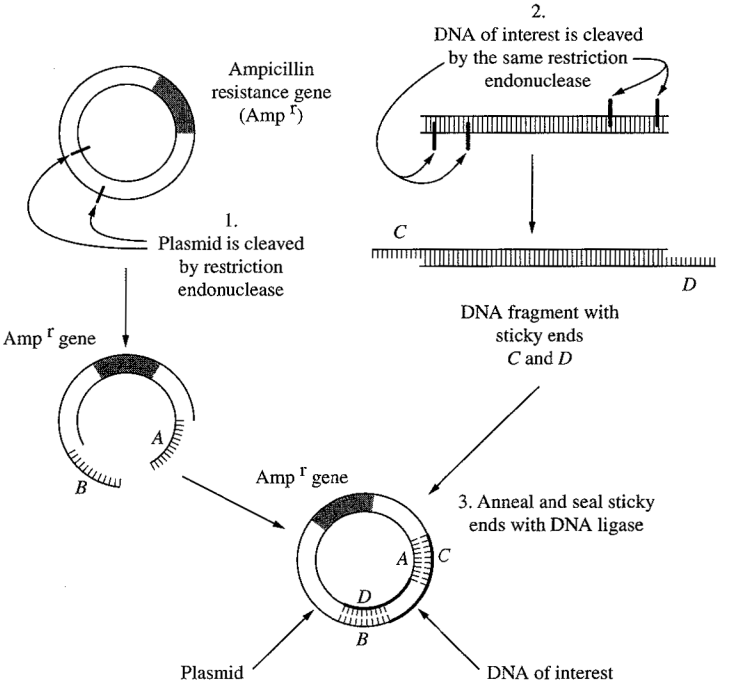
The single circular chromosome of *E. coli* contains about five million DNA base pairs, only 1/600th the haploid amount of DNA in a human cell. In addition, the *E. coli* cell may contain small circular DNA molecules (1,000 to 200,000 base pairs) called **plasmids**, which also carry genetic information. The plasmids are extrachromosomal; they exist separately from the chromosome. Some plasmids replicate only when the bacterial chromosome replicates, and usually exist only as single copies within the bacterial cell. Others replicate autonomously and often occur in as many as 10 to 200 copies within a single bacterial cell. Certain plasmids, called R plasmids, carry genes for resistance to antibiotics such as ampicillin, kanamycin, or tetracycline.

In nature, genes can be transferred between bacteria in three ways: conjugation, transduction, or transformation. **Conjugation** is a mating process during which genetic material is transferred from one bacterium to another of a different mating type. **Transduction** requires the presence of a virus to act as a vector (carrier) to transfer small pieces of DNA from one bacterium to another. **Bacterial transformation** involves transfer of genetic information into a cell by direct uptake of the DNA. During gene transfer, the uptake and expression of foreign DNA by a recipient bacterium can result in conferring a particular trait to a recipient lacking that trait. Transformation can occur naturally but the incidence is extremely low and is limited to relatively few bacterial strains. These bacteria can take up DNA only during the period at the end of logarithmic growth. At this time the cells are said to be **competent**. Competence can be induced in *E. coli* with carefully controlled chemical growth conditions. Once competent, the cells are ready to accept DNA that is introduced from another source.

Plasmids can transfer genes (such as those for antibiotic resistance) that occur naturally within them, or plasmids can act as carriers (vectors) for introducing foreign DNA from other bacteria, plasmids, or even eukaryotes into recipient bacterial cells. Restriction endonucleases can be used to cut and insert pieces of foreign DNA into the plasmid vectors (Figure 6.1). If these plasmid vectors also carry genes for antibiotic resistance, transformed cells containing plasmids that carry the foreign DNA of interest in addition to the antibiotic resistance gene can be easily selected from other cells that do not carry the gene for antibiotic resistance.

Figure 6.1: Bacterial Transformation Using a Restriction Endonuclease

I. Create plasmid with gene of interest.



II. Transform recipient cells with plasmid DNA.

III. Plate recipients on ampicillin plates and select for resistant colonies.

IV. Isolate colonies carrying the plasmid.

EXERCISE 6A: Bacterial Transformation - Ampicillin Resistance*

Background Information

You will insert a plasmid that contains a gene for resistance to ampicillin, an antibiotic that is lethal to many bacteria, into competent *E. coli* cells.

Transformed bacteria can be selected based on their resistance to ampicillin by spreading the transformed cells on nutrient medium that contains ampicillin. Any cells that grow on this medium have been transformed.

Procedure

1. Mark one sterile 15-mL tube "+"; this tube will have the plasmid added to it. Mark another tube "–"; this tube will have no plasmid added.

2. Use a sterile micropipette to add 250 microliters (µL) of **ice cold** 0.05*M* $CaCl_2$ to each tube.

3. Transfer a large (3mm) colony of *E. coli* from a starter plate to **each** of the tubes using a sterile inoculating loop. Try to get the same amount of bacteria into each tube. Be careful not to transfer any agar.

4. Vigorously tap the loop against the wall of the tube to dislodge the cell mass.

5. Mix the suspension by repeatedly drawing in and emptying a sterile micropipette with the suspension.

6. Add 10 µL of pAMP solution (0.005 µg/µL) directly into the cell suspension in tube "+". Mix by tapping the tube with your finger. This solution contains the antibiotic resistance plasmid.

7. Keep both tubes on ice for 15 minutes.

8. While the tubes are on ice, obtain two LB agar plates and two LB/Amp agar (LB agar containing ampicillin) plates. Label each plate on the bottom as follows: one LB agar plate "LB+" and the other "LB–." Label one LB/Amp plate "LB/Amp+" and the other "LB/Amp–."

9. A brief pulse of heat facilitates entry of foreign DNA into the *E. coli* cells. Heat-shock cells in both the "+" and "-" tubes by holding the tubes in a 42°C water bath for 90 seconds. It is essential that cells be given a sharp and distinct shock, so take the tubes directly from the ice to the 42°C water bath.

10. Immediately return cells to ice for two minutes.

11. Use a sterile micropipette to add 250 µL of Luria broth to each tube. Mix by tapping with your finger and set at room temperature. Any transformed cells are now resistant to ampicillin because they possess the gene whose product renders the antibiotic ineffective.

* Exercise 6A is adapted with permission from *DNA Science: A First Course in Recombinant-DNA Technology* by David A. Micklos, DNA Learning Center of Cold Spring Harbor Laboratory, and Greg A. Freyer, Columbia University College of Physicians and Surgeons, Copyright 1990 Cold Spring Harbor Laboratory Press and Carolina Biological Supply Company. It is based on a protocol published by Douglas Hanahan, University of California, San Francisco.

12. Place 100 µL of "+" cells on the "LB+" plate and on the "LB/Amp+" plate. Place 100 µL of "–" cells on the "LB–" plate and on the "LB/Amp–" plate.

13. Immediately spread the cells using a sterile spreading rod. (Remove the spreading rod from alcohol and briefly pass it through a flame. Cool by touching it to the agar on a part of the dish away from the bacteria. Spread the cells and once again immerse the rod in alcohol and flame it.) Repeat the procedure for each plate.

14. Allow plates to set for several minutes. Tape your plates together and incubate **inverted** overnight at 37°C.

Analysis of Results

1. Observe the colonies through the bottom of the culture plate. **Do not open the plates.** Count the number of individual colonies; use a permanent marker to mark each colony as it is counted. If cell growth is too dense to count individual colonies, record "lawn."

   LB +(Positive Control)_____ LB –(Positive Control) _____

   LB/Amp + (Experimental)_____ LB/Amp – (Negative Control) _____

2. Compare and contrast the number of colonies on each of the following pairs of plates. What does each pair of results tell you about the experiment?

   a. LB + and LB – _____
   _____

   b. LB/Amp – and LB/Amp + _____
   _____

   c. LB/Amp + and LB + _____
   _____

3. Transformation efficiency is expressed as the number of antibiotic-resistant colonies per microgram of pAMP. Because transformation is limited to only those cells that are competent, increasing the amount of plasmid used does not necessarily increase the probability that a cell will be transformed. A sample of competent cells is usually saturated with small amounts of plasmid and excess DNA may actually interfere with the transformation process.

   a. Determine the total mass of pAMP used. _____
      (You used 10 µL of pAMP at a concentration of 0.005 µg/µL.)
      Total Mass = volume X concentration.
   b. Calculate the total volume of cell suspension prepared. _____
   c. Now calculate the fraction of the total cell suspension that was spread on the plate.
      (Number of µL spread/total volume) _____
   d. Determine the mass of pAMP in cell suspension. _____
      (Total mass of pAMP X fraction spread.)
   e. Determine the number of colonies per µg of plasmid. Express in scientific notation.

(Number of colonies observed/mass pAMP spread (from calculation in step (d)) = transformation efficiency.)

4. This is the **transformation efficiency.** What factors might influence transformation efficiency? Explain the effect of each you mention.

EXERCISE 6B: Restriction Enzyme Cleavage of DNA and Electrophoresis

Background Information

Restriction enzymes or **restriction endonucleases** 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 represent the second word or the 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:

EcoRI E = genus *Escherichia*

co = species *coli*

R = strain RY13

I = first endonuclease isolated

HaeII H = *Haemophilus*

ae = *aegyptus*

II = second 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 6.2a). Other endonucleases cleave each strand off-center at specific nucleotides to produce fragments with "overhangs" or sticky ends (Figure 6.2b). By using the same restriction enzyme to "cut" DNA from two different organisms, complementary "overhangs" or sticky ends will be produced and can allow the DNA from two sources to be "recombined."

Figure 6.2a

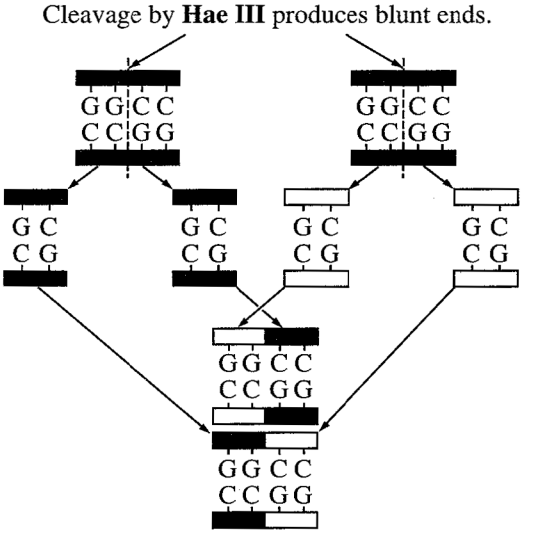
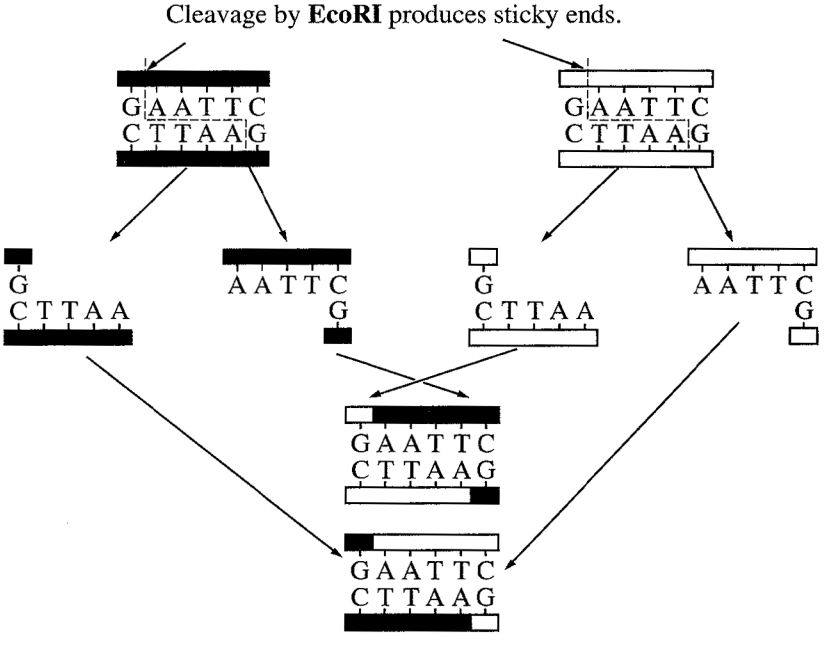


Figure 6.2b



In this exercise, samples of DNA obtained from the bacteriophage *lambda* have been incubated with different restriction enzymes. The resulting fragments of DNA will be separated by using gel electrophoresis. One sample has been digested with the restriction endonuclease **EcoRI**, one with the restriction endonuclease **HindIII**, and the third sample is uncut. The DNA samples will be loaded into wells of an agarose gel and separated by the process of electrophoresis. After migration of the DNA through an electrical field, the gel will be stained with methylene blue, a dye which binds to DNA.

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 (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. 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 toward 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.

General Procedure

A: Preparing the Gel

1. Prepare the agarose gel for electrophoresis according to the directions given by your teacher or in the kit.

2. Obtain the phage lambda DNA digested with **EcoRI** endonuclease. The DNA is mixed with a gel-loading solution containing a tracking dye, bromophenol blue, that will make it possible to "track" the processes of its migration in the agarose gel.

3. Obtain the phage lambda DNA digested with **HindIII** endonuclease. The DNA fragments are of a known size and will serve as a "standard" for measuring the size of the **EcoRI** fragments from step 2. It also contains the tracking dye.

4. Obtain the undigested phage lambda DNA to use as a control. It also contains the tracking dye.

B: Loading the Gel

Helpful Hints for Gel Loading

Pull a small amount of gel-loading solution into the end of a micropipette. (Do not allow the solution to move up into the pipette, or bubbles will be introduced into the well of the agarose gel during loading.)

Hold the tip of the pipette **above** the gel and gently dispense the solution. The loading dye is denser than the buffer and will move into the well. (Do *not* place the tip of the pipette into the well or you might puncture the gel).

1. Pour enough buffer gently over the gel to cover it.

2. Load 5-10 µL of undigested lambda phage DNA (control) into a well.

3. Load 5-10 µL of the **HindIII** digest into a second well.

4. Load 5-10 µL of the **EcoRI** digest into a third well.

C: Electrophoresis

1. Place the top on the electrophoresis chamber and carefully connect the electrical leads to an approved power supply (black to black and red to red). Set the voltage to the appropriate level for your apparatus. When the current is flowing, you should see bubbles on the electrodes.

2. Allow electrophoresis to proceed until the tracking dye has moved nearly to the end of the gel.

3. After electrophoresis is completed, **turn off the power**, disconnect the leads, and remove the cover of the electrophoresis chamber.

D: Staining and Visualization

Note: Wear gloves

1. Carefully remove the gel bed from the chamber and gently transfer the gel to a staining tray for staining. Use the scooper provided with your kit or keep your hands under the gel during the transfer. You may wish to remove a small piece of gel from the upper right-hand corner to keep track of the gel's orientation. **Do not stain in the electrophoresis chamber.**

2. Label the staining tray with your name and take it to your teacher for staining.

3. Examine your stained gel. Compare your gel with the sample gel shown in Figure 6.3.

Figure 6.3: Sample Restriction Digest of Lambda DNA

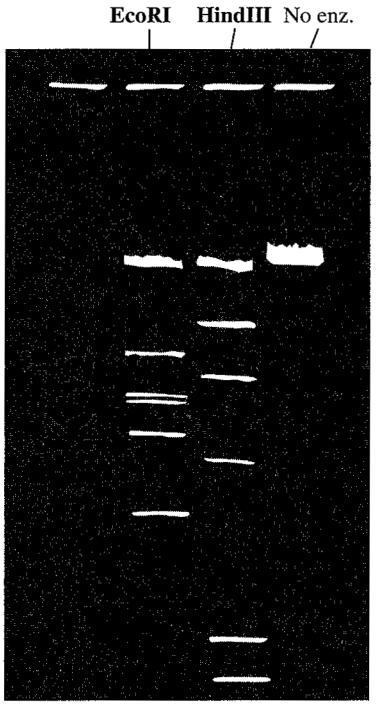


Figure not to scale.

E: Determining Fragment Size

1. After observing the gel, carefully wrap it in plastic wrap and smooth out all wrinkles.

2. Using a marking pen, trace the outlines of the sample wells and the location of the bands.

3. Remove the plastic wrap and flatten it out on a white piece of paper on the laboratory bench. Save the gel in a Ziplock® plastic bag. Add several drops of buffer. Store at 4°C. You can make your measurements directly from the marked plastic wrap.

Analysis and Results

Background Information

The size of the fragments produced by a specific endonuclease (**EcoRI** in this exercise) can be determined by using standard fragments of known size (fragments produced by **HindIII** in this case). When you plot the data on semilog graph paper, the size of the fragments is expressed as the log of the number of base-pairs they contain. This allows data to be plotted on a straight line. The migration distance of the unknown fragments, plotted on the x-axis, will allow their size to be determined on the standard curve.

Graphing

A. Standard Curve for HindIII

1. Measure the migration distance (in cm) for each **HindIII** band on your gel. Measure from the bottom of the sample well to the bottom of the band. The migration distance for the largest standard fragment (approximately 23,120 base-pairs) nearest to the origin does not need to be measured. Record these measurements in Table 6.1 (page 73).

2. Plot the measured migration distance for each band of the standard **HindIII** digest against the actual base pair (bp) fragment sizes given in Table 6.1 using the semilog graph paper. Draw the best-fit line to your points. This will serve as a **standard curve**.

B. Interpolated Calculations for EcoRI

From the standard curve for **HindIII**, made from known fragment sizes, you can calculate fragment sizes resulting from a digest with **EcoRI**. The procedure is as follows.

1. Measure the migration distances in cm for each **EcoRI** band. Record the data in Table 6.1.

2. Determine the sizes of the fragments of phage lambda DNA digested with **EcoRI**. Locate on the X axis the distance migrated by the first **EcoRI** fragment. Using a ruler, draw a vertical line from this point to its intersection with the best-fit data line. Now extend a horizontal line from the intersection point to the Y axis. This point gives the base-pair size of this **EcoRI** fragment. Repeat this procedure and determine the remaining **EcoRI** fragments. Enter your interpolated data in Table 6.1, in the interpolated bp column.

3. Your teacher will provide you with the actual bp data.

Compare your results to these actual sizes. Note: This interpolation technique is not exact. You should expect as much as 10% to 15% error.

Table 6.1: Distance *Hind*III produced fragments migrate in agarose gel (cm)

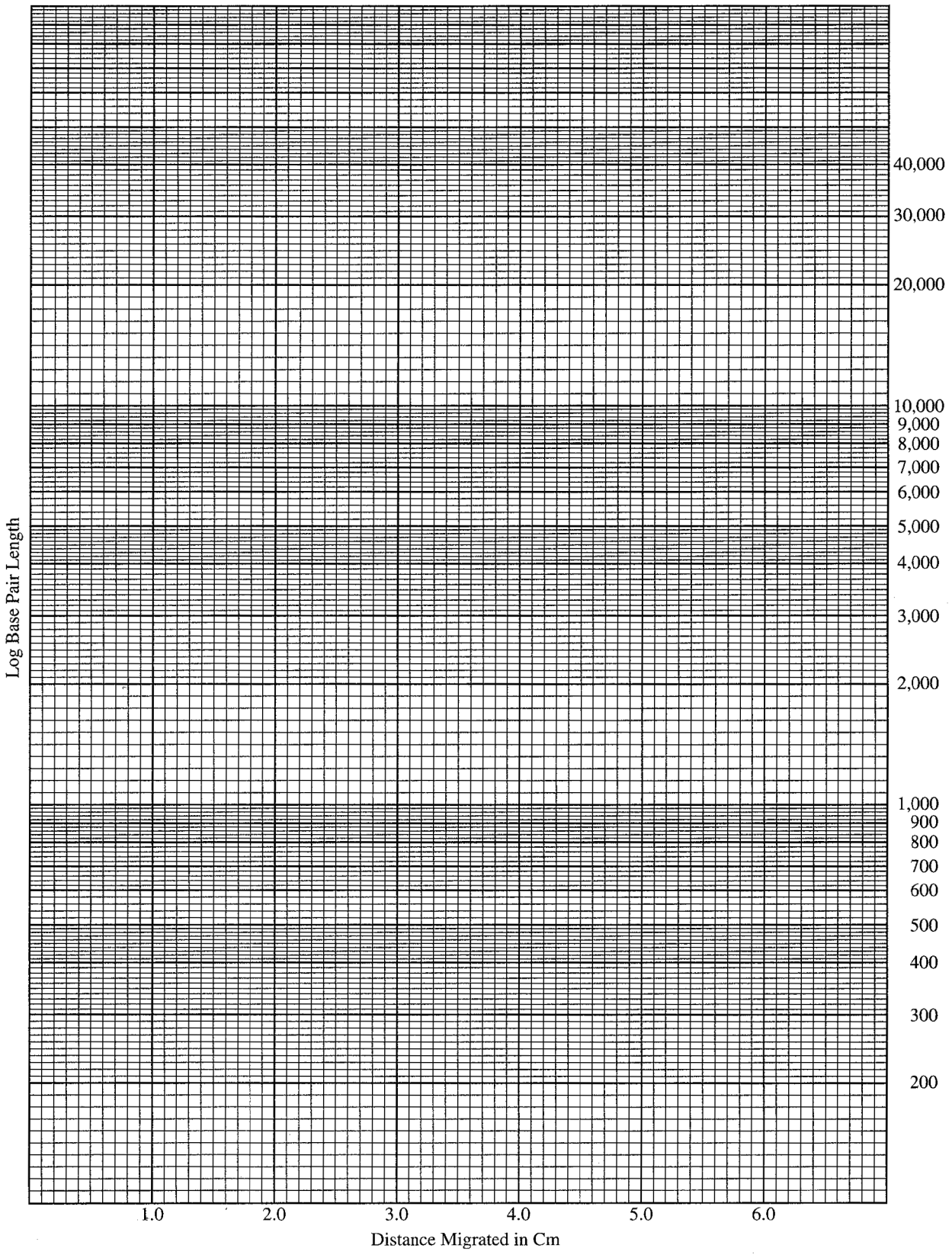
*Hind*III	
Actual bp	Measured Distance (cm)
23,130	–
9,416	_____
6,557	_____
4,361	_____
2,322 †	_____
2,027 †	_____
570 *†	_____
125 *	_____

* may not be detected
† may form a single band

Table 6.2: Distance *Eco*RI produced fragments migrate in agarose gel (cm)

	*Eco*RI		
	Measured Distance (cm)	Interpolated bp	Actual bp
Band 1	_____	_____	_____
Band 2	_____	_____	_____
Band 3	_____	_____	_____
Band 4	_____	_____	_____
Band 5	_____	_____	_____
Band 6	_____	_____	_____

Graph 6.1 **Graph Title:** _____



4. For which fragment sizes was your graph most accurate? For which fragment sizes was it least accurate? What does this tell you about the resolving ability of agarose-gel electrophoresis?

Analysis

1. Discuss how each of the following factors would affect the results of electrophoresis:

   a. Voltage used

   b. Running time

   c. Amount of DNA used

   d. Reversal of polarity

2. Two small restriction fragments of nearly the same base-pair size appear as a single band, even when the sample is run to the very end of the gel. What could be done to resolve the fragments? Why would it work?

Questions

1. What is a plasmid? How are plasmids used in genetic engineering?

2. What are restriction enzymes? How do they work? What are recognition sites?

3. What is the source of restriction enzymes? What is their function in nature?

4. Describe the function of electricity and the agarose gel in electrophoresis.

5. If a restriction enzyme digest resulted in DNA fragments of the following sizes: 4,000 base pairs, 2,500 base pairs, 2,000 base pairs, 400 base pairs, sketch the resulting separation by electrophoresis. Show starting point, positive and negative electrodes, and the resulting bands.

6. What are the functions of the loading dye in electrophoresis? How can DNA be prepared for visualization?

7. Use the graph you prepared from your lab data to predict how far (in cm) a fragment of 8,000 bp would migrate.

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