

INTRODUCTION TO PLASMIDS AND RESTRICTION ENZYMES

(adapted from Amgen-Bruce Wallace Biotechnology Lab Program)

Student Guide

Introduction and background:

Two powerful and fundamental tools used in biotechnology are restriction enzymes and bacterial plasmids. Restriction enzymes allow molecular biologists to cut the DNA molecules from different organisms and recombine the molecular pieces to produce *recombinant* DNA molecules. Plasmids are circular pieces of DNA that are naturally found in bacteria. Through recombinant DNA technology and restriction enzymes, recombinant DNA plasmids can be engineered to clone (copy) genes or to express proteins encoded by genes..

Restriction enzymes evolved in bacteria as a defense mechanism, similar to a primitive immune system. When viruses infect a host cell, they inject their own nucleic acids (DNA or RNA) into the host. If a bacterial host cell has restriction enzymes, those proteins can cut the invading viral DNA before it takes over the cell, and the bacterium has successfully defended itself against infection. These enzymes work by breaking a bond in the sugar (deoxyribose)-phosphate backbone of the invading DNA and cut the viral DNA into small fragments.

The restriction enzymes now used in the lab cut the sugar-phosphate backbone within a specific nucleotide sequence, usually four to six nucleotides in length. Table 1 identifies some of these specific restriction enzymes, their source bacterium, and the nucleotide sequences each recognizes. The first letters of the name of the restriction enzyme come from the name of the bacteria from where the enzyme was found.

Source	Restriction Enzyme	Recognition sequence
<i>Bacillus amyloliquefaciens</i>	<i>Bam</i> H I	↓ 5' GGATCC 3' 3' CCTAGG 5' ↑
<i>Escherichia coli</i>	<i>Eco</i> R I	↓ 5' GAATTC 3' 3' CTTAAG 5' ↑
<i>Haemophilus influenzae</i>	<i>Hind</i> III	↓ 5' AAGCTT 3' 3' TTCGAA 5' ↑

Table 1. Common restriction enzymes. ↑ ↓ indicate sites where the sugar-phosphate backbone is cut by the enzyme.

When restriction enzymes cut or digest DNA, the fragments that result are called restriction fragments, and they have several unpaired bases extending from their cut ends. These are called “sticky ends.” If DNA from different sources is digested using the same restriction enzyme, the unpaired bases from each piece should be able to join (or anneal) together as the unpaired bases at the sticky ends will be complimentary (A:T and G:C). It is this unique quality of restriction enzymes that enable genetic engineers to combine DNA fragments from different organisms to produce recombinant DNA molecules.

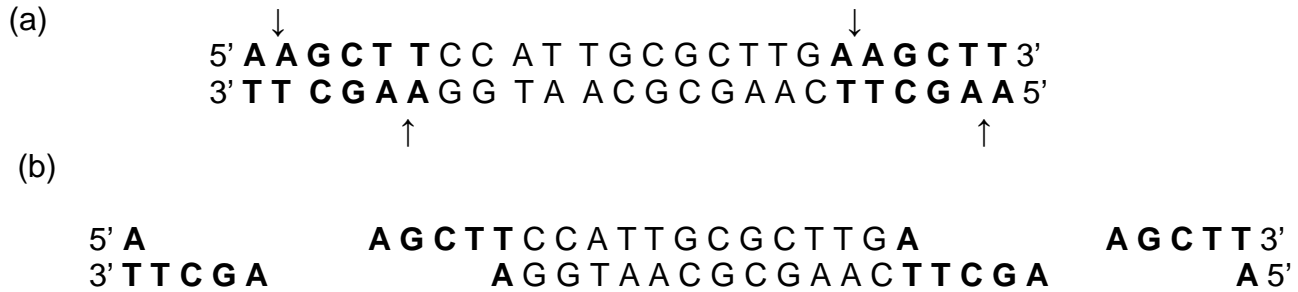
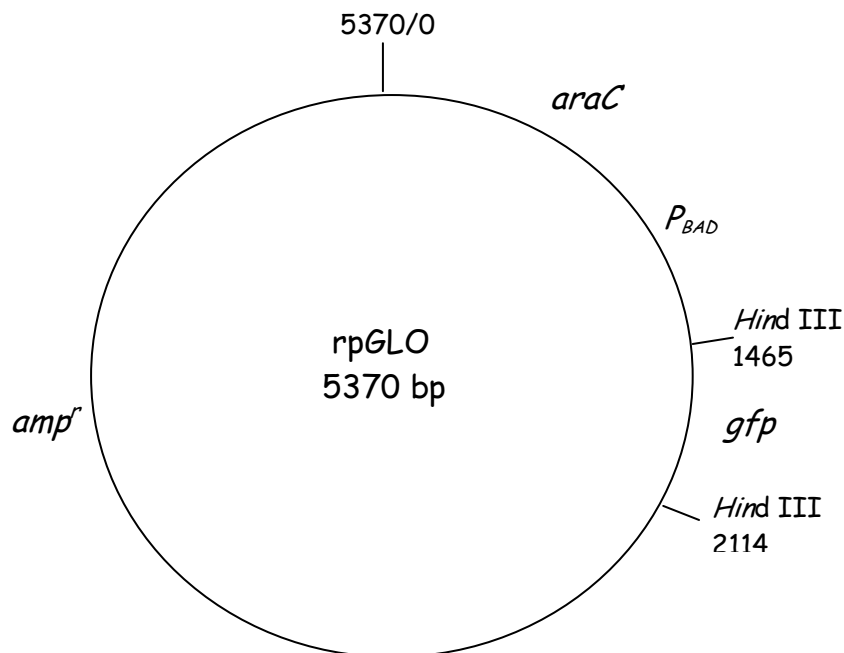


Figure 1. (a) DNA molecule with two *Hind* III restriction sites (bold). The arrows indicate sites where *Hind* III will cut the sugar-phosphate backbone of the DNA molecule. (b) The lower DNA molecule indicates the location of the “sticky-ends” (bold).

Bacterial plasmids are relatively small, circular pieces of DNA that bacteria can have in addition to their genomic DNA (a single chromosome that is also circular, but much larger). In nature, the plasmid DNA frequently carries one to several genes that help the bacterium survive in special environmental conditions—perhaps by providing a protein that will allow the bacterium to disable an antibiotic before it can kill the cell, or a protein that will allow it to digest an uncommon sugar molecule. Bacteria can easily share plasmids with one another during conjugation (mating).

Naturally occurring plasmids have been engineered to perform specific functions. This laboratory examines *rpGLO*, a recombinant DNA plasmid that has been engineered to express the GFP gene to produce **Green Fluorescent Protein**. The plasmid contains various control elements that allow a bacterium carrying this plasmid to express this foreign gene. The gene was originally obtained from the genome of *Aquoria victoria*, a marine jellyfish.

The plasmid map that follows indicates some of the important control regions, *araC* and *P_{BAD}* and the location of the GFP gene. In addition, the map indicates the location of two *Hind*III restriction sites: one located at 1465 base pairs from an arbitrary position indicated at top of the plasmid and the other located at 2114 base pairs from the same position. How might you go about cutting out the GFP gene? Also note, the plasmid carries an antibiotic resistance gene, *amp^r*. This gene will enable a bacterium carrying this plasmid to live in an environment containing the antibiotic ampicillin.



Lab Day 1—Restriction Digest

Purpose: The purpose of this laboratory is (1) to examine the role of restriction enzymes and their importance in genetic engineering, (2) to examine a bacterial plasmid and how it is used in biotechnology, (3) to practice basic biotechnology lab skills.

Materials:

Reagents

rpGLO (20ng/ μ L)
 Restriction enzyme (Hind III)
 2.5x restriction buffer
 Distilled water
 Tube with 10 μ L water for comparison

Equipment and supplies

p20 micropipettes and tips
 1.5 mL microfuge tubes (eppendorf)
 Minicentrifuge
 37° water bath
 Permanent marker

Methods:

- Obtain the following three 1.5ml microfuge tubes from your teacher:
rpGLO, Hind III and 2.5x restriction buffer
- Obtain two clean 1.5ml microfuge tubes and use a marker to label the tubes as follows: “rpGLO+” and “rpGLO—” Include any other identifiers as instructed by your teacher so that you can find these tubes for the next lab period.
- Use a fresh tip on a p20 pipette, and add 4 μ L of 2.5x restriction buffer to BOTH tubes.
- Using the p20 pipette and another clean tip, add 2 μ L of distilled H₂O to tube *rpGLO--* .
- Use another fresh tip and add 4 μ L of rpGLO to tubes labeled rpGLO+ and rpGLO--.
- Carefully add 2 μ L of the Hind III enzyme directly into the solution in the pGLO + tube. Slowly pipette up and down to mix. Be sure that all liquid is expelled back into the tube. Gently flick the tube to mix.
- Set tubes into the mini-centrifuge, being careful that tubes are in a balanced pattern and spin the tubes for 5 seconds. This will pool all the reagents into the bottom of the tube.
- Compare the volume in your tube with a 10 μ L standard, or set a pipette to 10 μ L and take up what is in your tube to make sure you have the correct volume.
- Place both tubes into the 37°C water bath and incubate for at least 60 minutes. Your teacher may freeze the tubes after the incubation until you are ready for electrophoresis.

Summary of reagents used in the restriction digest:

Tube	2.5x buffer	rpGLO	H ₂ O	Hind III	Total Volume
rpGLO+	4 μ L	4 μ L	-----	2 μ L	10 μ L
rpGLO--	4 μ L	4 μ L	2 μ L	----	10 μ L

Lab day 2—Electrophoresis of Restriction fragments

Purpose: To examine the restriction fragments that result from the digestion of rpGLO by *Hind* III.

Materials:

Reagents

Plasmid samples from Day 1
0.8% agarose gel
loading dye
0.5x TBE
DNA size marker (ladder)

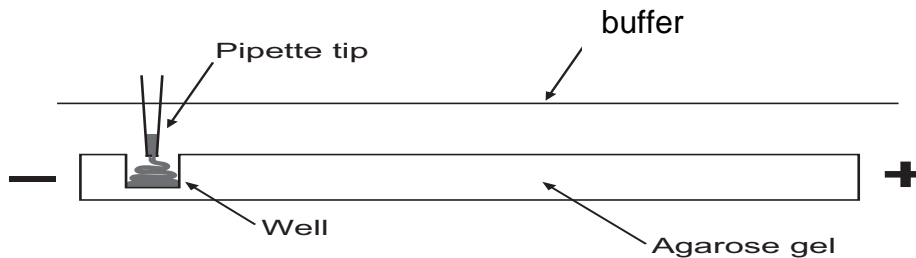
Equipment and supplies

p20 micropipette and tips
1.5 ml microfuge tubes
electrophoresis box
power supply
microfuge tube rack
Marking pen

Methods:

1. Collect both plasmid samples and the DNA marker from your teach and place them in your plastic tube rack. You should have three tubes.
2. Add 3 μ L of loading dye to the rpGLO+ and rpGLO—tubes. Use a clean plastic tip for each addition to prevent contamination between the tubes. Gently pipette up and down to mix the sample without creating bubbles. The DNA size marker already contains loading dye.
3. Prepare the gel and electrophoresis chamber to receive the three samples.
 - a. Be sure the gel wells are closest to the negative (black) electrode.
 - b. Pour the 0.5x TBE buffer into the gel box until there are not visible “dimples” breaking the surface of the buffer over the wells in the gel. It’s important that the gel be completely submerged in the buffer, but you don’t want more buffer than necessary either.
4. Take your plasmid samples and marker to the gel, along with your pipette and tips. You may share this gel with another group if necessary.
5. Follow the directions below (steps 6-8) for loading your gel. You will be loading your samples from left to right on the gel in this order:
Marker rpGLO--- rpGLO+
Be sure to make a map of your gel showing which samples are loaded in which wells. (Depending on the number of wells in use for this lab, you may want to keep the wells on the edge of the gel empty for better results.)
6. Using a clean tip, set your p20 micropipette to 10 μ L. Pick up 10 μ L of your DNA size marker and slowly dispense it into the well. Reminder: lower the pipette tip below the surface of the buffer directly over, *but not into*, the well (see picture below).

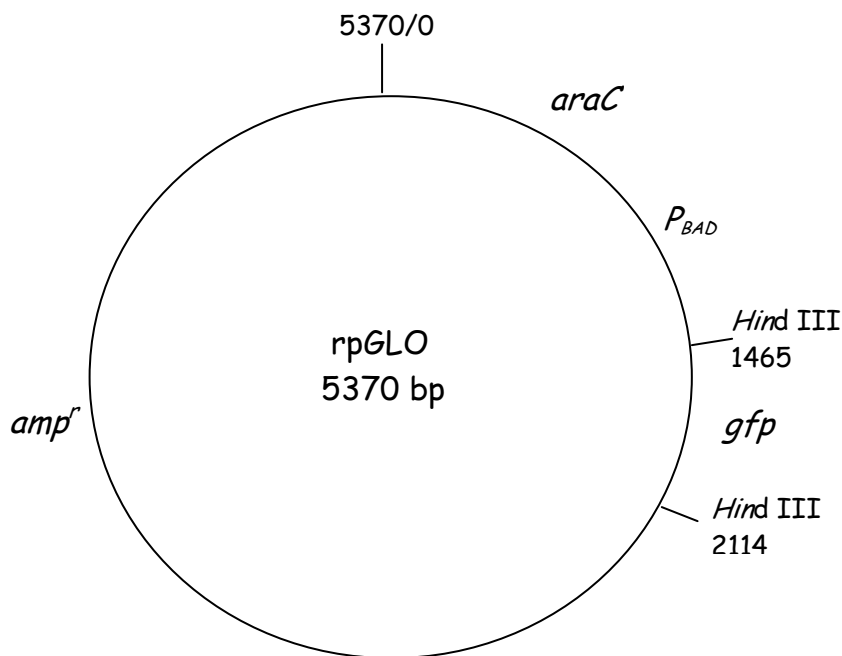
- As you do this, slowly lower the pipette tip below the surface of the buffer directly over, *but not into*, the well. Putting the tip into the well can damage the wall of the well or puncture the bottom of the well. These are not good things to do.



- Use two hands to steady the pipettor. *Slowly* dispense the sample by pushing to the second stop of the pipettor. Because of the loading dye, the sample will have a greater density than the electrophoresis buffer. This will allow the sample to *sink* into the well.
 - **Important:** While holding the button on the second stop, slowly remove the pipette tip from the gel box. If you've loaded your sample correctly, the well will be filled with a blue colored solution.
7. Using a clean pipette tip, load 12 μ L of your rpGLO—sample into the next well.
 8. Change the pipette tip and load 12 μ L of your rpGLO+ sample into the next well.
 9. Cover the gel box and connect the electrical leads to the power supply. Be sure to match the red (positive) electrode to the red plug, and the black (negative) electrode to the black plug.
 10. Set the power supply to 130-140 Volts.
 11. After 2-3 minutes, check your gel and be certain that the purple dye (bromophenol blue) is moving towards the positive electrode. If it's moving in the other direction (toward the black negative electrode) check the connections to be sure they are set up correctly.
 12. The electrophoresis needs to run for around 30 minutes, until the purple dye runs just about to the end of the gel. If your lab time is short, your teacher will explain what you need to do with your gels for staining and photographing to observe later.

Student Worksheet for Lab Day 1

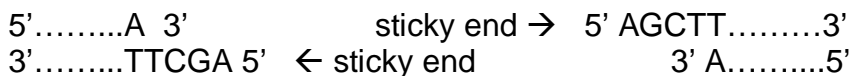
This lab protocol uses the restriction enzyme *Hind* III to digest the recombinant plasmid, rpGLO. The restriction digest will isolate, from rpGLO, the GFP (*gfp*) gene from the larger fragment of the plasmid that contains *amp^r*, *araC* and P_{BAD}. The protocol uses a control, undigested rpGLO along with a DNA size marker or ladder that will help you identify and confirm the sizes of the restriction fragments.



Review the restriction map of the rpGLO plasmid above. *Hind* III is a *specific* restriction enzyme and will consistently cut DNA wherever it encounters the six-base *recognition sequence* indicated below. The precise location that is cut is called its *restriction site*. The DNA molecule consists of two strands of nucleotide building blocks. These building blocks are oriented in the opposite direction on each strand (we call this *anti-parallel*). For convenience, we say that one strand is oriented in a 5' (five prime) to 3' (three prime) direction while the other strand is oriented 3' to 5'. Careful examination of the restriction sequence will reveal that the sequence of nucleotides is a palindrome (it reads the same on both strands when read in the 5' → 3' direction).



Therefore, whenever *Hind* III encounters this six-base sequence, it will cut the DNA helix between the adjacent adenine bases. This leaves four unpaired bases forming a “sticky end.”



Questions:

1. What is the purpose of adding distilled water to the rpGLO—tube in this lab?

2. What is the recognition sequence for *Hind* III?

3. In a 5' → 3' direction, what sequence of bases represents the “sticky-ends?”

4. Examine the rpGLO plasmid map and fill in the following:
 - a. How many restriction fragments will result from the digestion of rpGLO with *Hind* III?

 - b. What will be the lengths, in base pairs, of these restriction fragments?

 - c. Which restriction fragment will carry the *amp^r* gene?

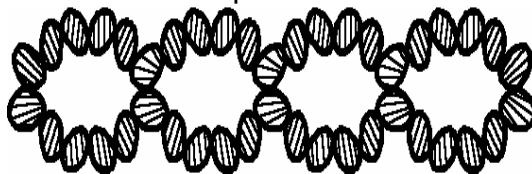
 - d. Which restriction fragment will carry the *gfp* gene?

5. Let's say you were given a culture of bacteria. This culture could be one containing bacteria carrying the plasmid rpGLO or a culture containing bacteria without the plasmid. What are some tests you can do to determine which of these cultures you were given? Design an experiment to test your cultures.

Student Background for Lab Day 2

In this part of the lab, we used gel electrophoresis to separate the fragments of DNA according to number of base pairs. Remember that DNA is negatively charged (because of all the phosphate groups), and will move through the gel toward the positive (red) electrode. Because it is easier for smaller molecules to move through the “maze” of the gel, the shorter pieces of DNA will move faster down the gel than the longer pieces.

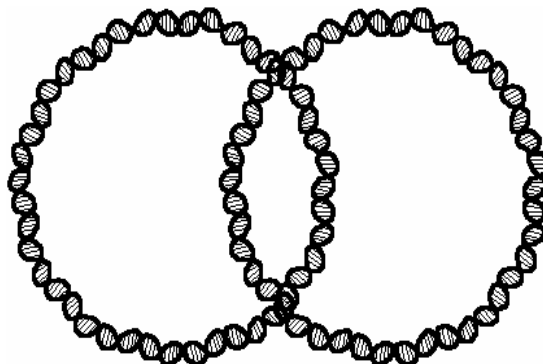
We loaded both plasmid samples, digested and undigested, onto the gel. You might have predicted that your uncut plasmids would produce only a single DNA band; there’s no reason why you would think otherwise. However, it is likely that three or four bands will appear in the undigested plasmid lane (the control). The reason for this is that plasmids isolated from cells exist in several forms. One form of plasmid is called “**supercoiled**.” You can visualize this form by thinking of a circular piece of plastic tubing that is twisted. This twisting or supercoiling results in a very compact molecule; one that will move through the gel very quickly for its size.



A second plasmid form is called a “**nicked-circle**” or an open circle. Often a plasmid will experience a break in one of the covalent bonds located in its sugar-phosphate backbone along one of the two nucleotide strands. Repeated freezing and thawing of the plasmid or other rough treatment can cause the break. When this break occurs, the tension stored in the supercoiled plasmid is released as the twisted plasmid unwinds. This circular plasmid form will not move through the gel as easily as the supercoiled form; although it is the same size, in terms of base pairs, it will be located closer to the well than the supercoiled form.



The last plasmid form we are likely to see is called the “**multimer**.” When bacteria replicate (copy) plasmids, the plasmids are often copied so fast that they end up linked together like links in a chain. If two plasmids are linked, the multimer will be twice as large as a single plasmid and will migrate very slowly through the gel. In fact, it will move slower than the nicked-circle. The undigested plasmid rpGLO sample may have three bands that appear in the gel. Starting closest to the well, you might observe a multimer, followed by a nicked-circle band and, finally, a fast traveling supercoiled band.



Since DNA is colorless, we will use a special staining technique that permits us to visualize the fragments embedded within the gel. This technique involves a stain that cannot be used by students so your teacher will do the actual staining for you. After the gel is stained, your teacher will make a photographic record of your gel to document your results.

Questions Lab Day 2:

1. Compare your gel photo with your prediction. Do you see any unexpected DNA bands?
2. Relative to the DNA ladder, between what 2 bands is the *gfp* gene located? Is this where you predicted it to be located?
3. In the rpGLO—lane, do you see evidence of different plasmid forms? Which conformation migrates the fastest?

Which is the slowest?

4. Does the rpGLO+ lane indicate complete digestion? Explain your answer.
5. Which DNA fragment contains the *amp^r* gene? What is the size of this DNA restriction fragment?

Assessment questions:

1. What is the role of a restriction enzyme and what term is used to describe the ends of the molecular fragments that result from its action?

2. Name the restriction enzyme used in this lab and indicate from what organism this enzyme is obtained.

3. In the space provided, briefly describe a bacterial “plasmid.” Include the name of the plasmid used in this protocol and the jellyfish gene it carries.

4. What is the purpose of this laboratory protocol?

5. Using the location of each DNA “band” in the gel, the relative size of each DNA fragment can be determined. For example, examine the figure below and arrange the letters, identifying each band, from smallest to largest. List them on the line below:

