
INTRODUCTION TO PLASMIDS AND RESTRICTION ENZYMES

(adapted from AMGEN – Bruce Wallace Biotechnology Lab Program)

Background information:

This is an alternate lab that has been written to reduce your preparation time and to shorten the lab sequence for your students. Rather than prepare the recombinant plasmid, rpGLO, students are given rpGLO directly. This plasmid has been engineered by BioRad for gene expression with the *gfp* gene already positioned within the plasmid for expression. The plasmid sample the students receive carries a significant amount of supercoiled rpGLO. This will make transformation many times more efficient than using ligated recombinants prepared in the last lab of the series “Plasmids, Restriction Analysis and Ligation.”

Students MUST have completed both the pipetting and electrophoresis introductory labs to be successful in this lab.

In this lab, students will be digesting rpGLO, using *Hind* III, instead of pDRK and pGRN as in the other lab series. The restriction map of rpGLO indicates two *Hind* III restriction sites—one on either side of the *gfp* gene. The plasmid digest will result in two restriction fragments—one will be 4721 base pairs (bp) and the other will be 649 bp.

The restriction enzymes used in biotechnology, typically are those that leave “sticky ends” when digested. These sticky ends allow annealing and recombination of DNA fragments from different sources—plants and animals, animals and bacteria, etc. The discovery of these molecular biology workhorses has been a cornerstone in recombinant DNA technology.

Prior to the discovery of restriction enzymes, it was observed that certain strains of bacteria seemed to be immune to bacteriophages. These bacteria were producing an unknown molecular “agent” that appeared to function as a primitive immune system; the agent *restricted* the growth of viruses. In the early 1970’s, Hamilton Smith and Daniel Nathans were able to purify some of these immune agents. They were discovered to be enzymes that the bacterium produced and used to cut-up the viral DNA as it was injected into its cell. When the virus introduced its DNA into the bacterial cell, the enzymes cleaved the viral DNA into fragments, thus preventing or restricting the growth of the virus. Smith, Nathans, along with Werner Arber, received the Nobel Prize for their discovery and characterization of these important molecules.

There are several classes of restriction enzymes, but the ones that have been most useful are the *specific* endonucleases. These endonucleases cut the DNA molecule internally, not from the ends as *exonucleases* will cut. Because many of these endonucleases are *specific*, they consistently recognize a specific nucleotide base sequence, the **recognition sequence**. Some recognize a four-base sequence; others recognize a five- or six-base sequence. The important feature is that a given restriction endonuclease will always recognize the same nucleotide sequence and cut the DNA at its **restriction site**. The recognition sequences are palindromes. This is an important concept that you’ll want to emphasize to your students. Here are some examples of palindromes: “radar,” “Madam, I’m Adam,” and “yreka bakery.”

See accompanying activities for examples to give students

Some restriction enzymes will make a “blunt cut,” leaving no overhanging bases. Other enzymes will leave overhanging bases creating “sticky ends.” These enzymes are particularly useful; since sticky ends make recombining DNA fragments a fairly simple procedure. The “stickiness” is the result of the extraordinary affinity of complimentary nucleotides to form hydrogen bonds between them.

The nomenclature used to identify restriction enzymes is fairly straightforward. The first letter of the enzyme’s name is derived from the genus of bacterium from which the enzymes was isolated. The next two letters come from the first two letters of the bacterium’s specific epithet. Because these three letters represent the *species* of the bacterium, they are italicized. Often there is a letter following the first three, this represents the *strain* or type of bacterium. Because some strains of bacteria produce several restriction enzymes, there is a Roman numeral that identifies the order in which the enzymes was isolated. Examine the table below to see some examples.

Source	Restriction Enzyme	Recognition sequence
<i>Bacillus amyloliquefaciens</i>	<i>Bam</i> H I	↓ 5' GGATCC 3' 3' CCTAGG 5' ↑
<i>Bacillus stearothermophilus</i>	<i>Bst</i> E II	↓ 5' GGTNACC 3' 3' CCANTGG 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' ↑

*Note: The ↑↓ indicate the locations of the restriction sites. “N” refers to any nucleotide.

Hind III will leave a four-base, 5' overhang as a sticky end. The overhang, reading 5' to 3', is A-G-C-T-. This is an important idea since it is this overhang of four unpaired bases that is available for hydrogen bonding with fragments with a complementary 5' overhang, -T-C-G-A. When rpGLO is digested (cut) with *Hind* III, two (restriction) fragments will result: one end of each will have a 5' sticky end A-G-C-T- and the other end will the complementary 5' sticky end -T-C-G-A.

s*There are several overhead transparencies to help you with presenting this background information to your students.

Laboratory Day 1 Restriction Digest

MATERIALS:

Reagents

- rpGLO (30ng/μL)
- Restriction enzyme (*Hind* III 3 unit/μL)
- 2.5x restriction buffer
- Distilled water
- Tube with 10 μL water for comparison

Equipment and supplies

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

The major work you will need to do prior to lab will be to aliquot the rpGLO, *Hind* III and 2x restriction buffer. An aliquot table appears below to help you with this task. Before you aliquot the *Hind* III and 2x restriction buffer, you might want to finger vortex to thoroughly mix each reagent. After you have aliquoted these reagents, you can place them into the freezer (-20°C) for several days until you are ready to run the lab. Each group will also need a tube of dH₂O.

The day of the lab, you need to turn on the water bath and set it at 37°C. This is the optimum temperature for *Hind* III. Check the temperature before the lab to be sure it is correct. It is important that the temperature not be above 37°C as this will lead to denaturation of the enzyme. It would be safer to err on the low side if necessary. The digests can sit in the water bath for two hours without significant problems but should not be incubated longer. One hour is generally sufficient to digest all of the plasmid.

Size of tube	Label tube	Contents of tube	Aliquot	Actually used
0.5 mL	rpGLO	rpGLO (30ng/μL)	9 μL	8 μL
1.5 mL	2.5x buffer	2.5x restriction buffer	12 μL	10 μL
1.5 mL	*dH ₂ O	Distilled water	1000 μL	2 μL

*This tube is used for both labs

Student aliquotting instructions (FYI):

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

Answers to student lab questions:

Questions:

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

To make the volumes of both tubes equal.

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

5' AAGCTT 3'

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

5' AGCT.....3' These are the 4 bases that remain unpaired. The four bases sequence is sometimes referred to as the 5 prime overhang.

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?

Two

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

4721 bp and 649 bp

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

The 4721 bp fragment

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

The 649 bp fragment.

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.

You would need two plates of liquid media—an LB plate and an LB/amp plate. Streak or inoculate each plate with the unknown culture to see where growth occurred. If the bacteria grew on the LB plate but not the LB/amp plate, the bacteria would not be carrying the rpGLO plasmid. If the bacteria grew on both plates, the culture is amp resistant and is likely carrying rpGLO. If the bacteria did not grow on either plate, they are probably dead.

Laboratory Day 2 Confirmation of rpGLO Restriction Digest

The purpose of this laboratory is to confirm that the plasmid, rpGLO, was digested by *Hind* III. The plasmid map in the student guide indicates there are two *Hind* III restriction sites in rpGLO. Because these sites appear on either side of the *gfp* gene, digestion of rpGLO with this restriction enzyme will produce two fragments—a **4721 bp fragment** containing the *amp^r* gene and the control regions needed for gene expression and a **649 bp fragment** representing the *gfp* gene.

The first lab has students set up a negative control tube—an enzyme control. Although one might expect to see only one DNA band in the agarose gel, recall that plasmids that are replicated inside a bacterial cell will generally be found in several forms—supercoiled, relax circle, and one or more multimers. Therefore, in the rpGLO lane, you should expect to see three or more DNA bands. Remember, however, that the supercoiled and relax circle are equal in size—5370 bp. Their differential movement through the gel is simply the result of shape, with the supercoiled shape being more compact.

A good concept to discuss with students is the fact that almost any gene could have been placed in the location occupied by the *gfp* gene, for example, a gene for human insulin or human growth hormone. Once the plasmid is inserted into a bacterium, that protein will be produced regardless of what organism it originally came from.

Preparation

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

Perhaps the most significant preparation concern in this lab related to the agarose gels, It is suggested that you prepare the gels ahead of time for your class. This can be done several days in advance as long as you keep them in 0.5x TBE and refrigerated. It is best to make the gels, gently slide them into a weigh boat and put in a plastic bag for storage. On the day of the lab, carefully place the gels into the gel trays and cover with TBE buffer.

The plasmid samples the students will be loading into their gels were prepared in the previous lab. You will need to aliquot 10 μ L of 1 Kb Ladder (= marker) for each group. Aliquotting the ladder can be done several days in advance and stored in the freezer.

TEACHER Aliquot table

Tube size	Label Tube	Contents of tube	Aliquot	Actually used
1.5 mL	Marker	1 kb ladder	10 μ L	10 μ L
1.5 mL	Loading dye	5x loading dye	20 μ L	16 μ L

Running and staining gels:

Gels should run at 120v to 140v for 20 minutes. It's important that you or your students check the gels to make certain that the purple tracking dye is moving and that it is moving towards the positive (red) electrode. If you observe a gel where the dye is not moving, check the buffer level, then the connections. (With Bio-Rad large power supplies, make sure the start button has been pressed – it is the one with icon of the running figure).

By the time your students load their gels, you may discover that you do not have sufficient time to make a complete run; this will take around 30 minutes even using a half gel. In order to resolve these fragments sufficiently to analyze fragments, the purple tracking dye (Bromophenol blue) should run to the middle of a full length gel. Here are some suggestions:

1. Loaded gels must be run at least 5 minutes to insure the DNA has been drawn into the gel from the well. Partially completed gels can be removed from the electrophoresis box and carefully transferred to a zip lock baggie. Mark the baggie using a piece of tape with the group number and class period to identify to which the gel belongs. The DNA fragments are sufficiently large that diffusion in the gel will be fairly minimal.
2. Complete the electrophoresis when time permits by placing the half run gel back into the casting tray and placing it into the electrophoresis chamber.
3. Electrophoresis buffer can remain in the chamber and reused throughout the day. **It is important to have several plastic bottles around the lab with extra 0.5x TBE to allow students to cover their gels properly, that is, deep enough to just cover the well “dimples.”**

Staining the gels will be time consuming and can **only be done by the teacher.**

Ethidium bromide is a mutagen and suspected carcinogen. It is important that you wear gloves and safety glasses when staining gels and handling gels that have been stained.

Here are some staining suggestions:

1. Designate an area of the lab that will be used for staining only.
2. Have each group label their group number and class period on the outside of a plastic weigh boat. Have them carefully slide their gel from the casting tray into the weight boat and have them bring their gel over to the staining area and then return to their lab area or seats.
3. **The teacher** will then pour sufficient ethidium bromide to just cover the top of the gel. Allow the gel to stain around 10-20 minutes. Pour the ethidium bromide back into its container using a funnel. The ethidium bromide solution can be reused many times before it is depleted.
4. Destain the gels by covering the gels with tap water. Allow the gels to remain in tap water for a few minutes. Rinse water can be poured down the sink.
5. Gels are now ready to photograph. If your students are not in the lab during this documentation, be certain to save one of the “ideal” gels so that you can show them the fluorescence of the ethidium bromide – DNA complex. This generally gets the students excited. Save the gel labeled and in plastic wrap or zip lock baggie. Store the gel in the refrigerator (4°C) until ready to view.

Answers to Questions Lab Day 2:

1. Compare your gel photo with your prediction. Do you see any unexpected DNA bands?

Answers will vary. Some common results: 1) no plasmid garments are seen in lanes 2 and/or 3 (they forgot to add rpGLO to the digest) 2) Both lanes 2 and 3 appear identical (They added Hind III to both lanes; they forgot to add Hind III to rp GLO) The gel generally provides one of the best “teaching moments” as it truly requires some understanding to trouble-shoot gel problems.

2. Relative to the DNA ladder, between what 2 bands is the *gfp* gene located? Is this where you predicted it to be located?

The *gfp* gene is 649 bp. Therefore, the band is located between the 1 Kb and the 0.5 Kb bands. The *gfp* may appear to be faint and not too obvious. The reason is that it is small, relative to the other plasmid bands, and therefore does not retain much ethidium bromide.

3. In the rpGLO—lane, do you see evidence of different plasmid forms? Which conformation migrates the fastest?

The answer should be “yes.” The supercoiled should move the fastest, appearing as the lowest fragment in lane 2. The supercoiled form is generally the form in highest concentration and therefore should appear as the brightest band.

Which is the slowest?

One of the multimers should appear to be the slowest moving band in lane 2. There is a possibility that more than one multimer can appear in this lane.

4. Does the rpGLO+ lane indicate complete digestion? Explain your answer.

The answer will vary since the digestion is dependent upon several variables. If more than two bands appear in lane 3, in addition to the 4721 and 549 bp fragments, this would be an indication of incomplete digestion.

The most common preparation error that produces this sort of result is insufficient incubation. It is suggested that a plasmid be digested between 60-90 minutes. It is also possible that buffer conditions (pH and salt balance) were not optimal.

5. Which DNA fragment contains the *amp^r* gene? What is the size of this DNA restriction fragment?

The 4721 bp fragment carries the *amp^r* gene, along with the other genetic elements needed for gene expression.

Answers to 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?

Restriction enzymes are used to cut or break the DNA molecules within the sugar-phosphate backbone. The fragments that result from this chemical breakage terminate in “sticky ends” or unpaired nucleotide bases.

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

The enzyme used in this lab is called *Hind III*. It is purified from a bacterium called *Haemophilus influenzae*

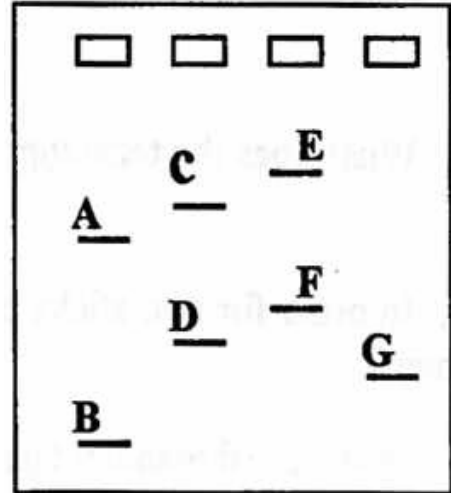
3. In the space provided, briefly describe a bacterial “plasmid.” Include the name of the plasmid used in this protocol and the jellyfish gene it carries.

A plasmid is a circular piece of DNA that is independent of the bacteria’s chromosome. Plasmids can carry important genes that help the bacterium to survive. The plasmid used in this protocol is rpGLO and the gene is for green fluorescent protein, *gfp*.

4. What is the purpose of this laboratory protocol?

The purpose is to better understand some of the properties of plasmids and the use of restriction enzymes in biotechnology.

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:



Answer: B,G,D,F,A,C,E (small to large)

6. What lab procedure does this protocol utilize to separate plasmid fragments?

Agarose gel electrophoresis is used to separate plasmid fragments.

7. On what basis do the DNA fragments move through the gel?

Since DNA fragments are negatively charged, all fragments will migrate towards the positive (red) electrode. The major factor affecting migration through the gel is size. This is particularly true of linear fragments. Intact plasmids, however, are also influenced by shape/

8. What plasmid forms might you expect to see in an undigested plasmid sample?

Generally, there will be at least 3 plasmid forms: supercoiled, relaxed circle and multimer. Occasionally, more than one multimer may appear.

- b. What two factors will determine the rate these plasmid forms migrate through the gel?

The two factors affecting the rate of migration are shape and size.

9. What are two reasons why loading dye is added to the samples?

Loading dye is added to all of the DNA samples to increase the density of the sample and to enable us to see the progress of the samples moving through the gel.