

PLASMIDS, RESTRICTION ANALYSIS AND LIGATION

(adapted from Amgen-Bruce Wallace Biotechnology Lab Program)

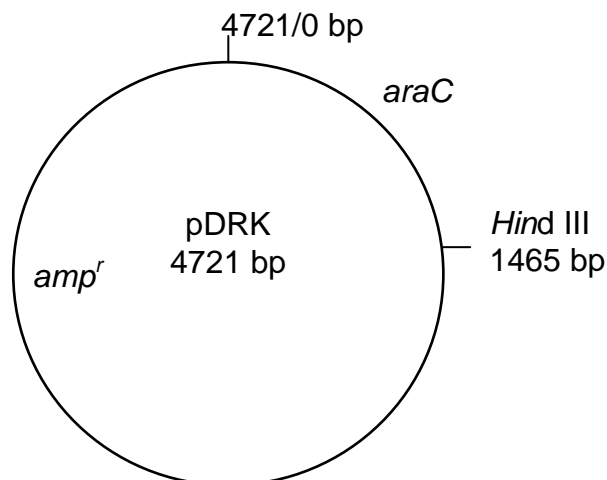
Lab Day 1 Restriction Analysis of pDRK and pGRN

Introduction

Plasmids are circular pieces of DNA that are naturally found in bacterial cells but have been modified through genetic engineering to facilitate gene cloning and protein production (*expression*) in bacteria. Antibiotic resistant genes have been engineered into these plasmids and function as *selectable markers*—that is, these genes allow us to *select* between bacteria that contain the plasmids from those that do not. If a bacterium carries a plasmid with an antibiotic resistant gene, the bacterium will be able to grow and reproduce in the presence of that antibiotic; those bacteria without the plasmid will not be able to grow. Thus, antibiotics can be used to select bacteria that are resistant, and presumably carry a plasmid with the resistant gene, from those bacteria that do not carry the plasmid. Two plasmids will be used in the laboratory: **pDRK** contains a gene for ampicillin resistance, *amp^r*, and **pGRN** contains a gene for kanamycin resistance, *kan^r*.

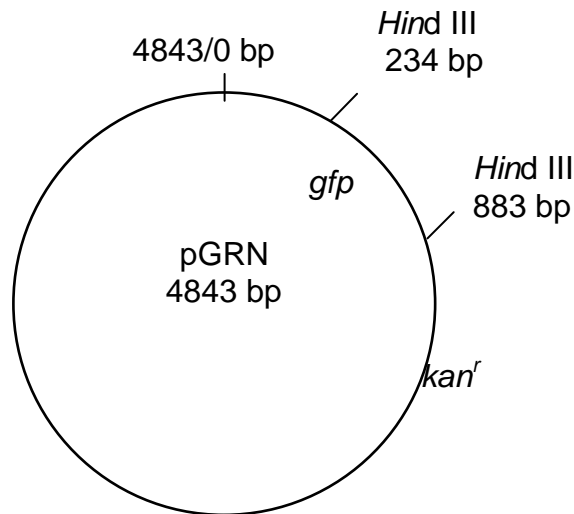
The purpose of the laboratory is fourfold: 1) to introduce a method commonly used to analyze the genetic elements of plasmid DNA, 2) to examine the role and nature of restriction enzymes, 3) to take the first steps in producing a recombinant DNA molecule, and 4) to practice basic biotechnology lab skills.

The plasmid pDRK is 4721 base pairs (bp) in size. A “base pair” would be adenine:thymine or guanine:cytosine and is the common method used to express the size of DNA molecules. The plasmid carries the *amp^r* gene, which encodes the protein beta lactamase, a protein that destroys ampicillin. Beta lactamase enables bacteria to reproduce in the presence of the antibiotic ampicillin. In addition, pDRK carries a gene for the Ara C protein; a protein that helps the bacterium make proteins encoded by genes inserted into this plasmid. A gene, even a foreign one, can be *expressed* (produced) if it is inserted into a specific location in this plasmid. Study the plasmid map below and locate these plasmid components. The plasmid can be read like a clock with 12:00 arbitrarily representing the beginning of the plasmid. If you count clockwise 1465 bp's, there is a *Hind* III restriction site located on the plasmid. Restriction sites will be discussed later.



The plasmid pGRN carries the kanamycin resistant gene, *kan^r*, that encodes a phosphotransferase, an enzyme that transfers a phosphate group to the kanamycin molecule. Kanamycin is an antibiotic that kills bacteria by preventing them from making proteins. This inhibition of protein synthesis is toxic to the cell. In addition to *kan^r*, the plasmid carries the gene (*gfp*) for green fluorescent protein.

The *gfp* gene was originally isolated from the marine jellyfish *Aequoria victoria*. The wild-type protein has been modified through a process called DNA shuffling. DNA shuffling results in a several-fold increase in fluorescence over the wild-type protein. The *gfp* gene has been engineered into the plasmid pGRN. Note that the gene for GFP has *Hind* III restriction sites on either side. A "restriction site" marks the specific location where an enzyme will cut the DNA molecule. If pGRN is digested with *Hind* III, the *gfp* gene will be physically cut from the plasmid. During the first laboratory, you will insert the *gfp* gene into pDRK producing a recombinant molecule.



Restriction Analysis of pDRK and pGRN

Pre-Laboratory Notes

1. Draw two circles, one representing pDRK and the other pGRN. Indicate the positions of *Hind* III restriction sites and other genes. Briefly describe the function of each of these genes: *amp^r*, *kan^r*, *araC* and *gfp*.

2. Which antibiotic resistant gene, is found in pDRK? In pGRN?

3. One of these plasmids has been engineered to express a protein if the gene for that protein is inserted into a specific location. Which of the plasmids has been engineered for protein expression?

4. From what organism did the gene for green fluorescent protein come?

5. Briefly outline, in your own words, the steps required to set-up plasmid restriction digest.

6. Why do you suppose you are asked to set up two tubes without *Hind* III?

Lab Day 1 Materials:**Reagents**

pDRK (10 ng/ μ L)
 pGRN (80 ng/ μ L)
 Restriction enzyme (*Hind* III)
 2.5x restriction buffer
 distilled water

Equipment and supplies

p 20 micropipette and tips
 1.5 mL microfuge tubes
 Minicentrifuge
 37° water bath
 permanent marker
 crushed ice (optional)

Methods:

This laboratory protocol uses the restriction enzyme *Hind* III to digest the plasmids pDRK and pGRN. This is the first step in making a recombinant DNA molecule.

Preparing the pDRK restriction digest

1. Obtain the following three microfuge tubes from your teacher: **pDRK**, **pGRN** and **2.5x buffer**.
2. Obtain four clean 1.5 mL microfuge tubes and use a marker to label a set of these four tubes as follows:
 - pDRK+** = pDRK + *Hind* III
 - pDRK--** = uncut pDRK (pDRK without enzyme)
 - pGRN+** = pGRN + *Hind* III
 - pGRN--** = uncut pGRN (pGRN without enzyme)

Include whatever other identifying information as instructed by your teacher so that you can locate your tubes for the next lab period.

3. Use a fresh tip and add 4 μ L of 2.5-x restriction buffer to **all** four tubes.
4. Add 2 μ L of dH₂O to tubes labeled pDRK— and pGRN—.
5. Using a fresh tip for each addition, add 4 μ L of pDRK to tubes labeled pDRK+ and pDRK—.
6. Using a fresh tip for each addition, add 4 μ L of pGRN to tubes labeled pGRN+ and pGRN—.
7. Bring the pDRK+ and pGRN+ tubes to your teacher who will dispense (or watch you dispense) 2 μ L of *Hind* III enzyme into each tube. You will need to use a clean tip for each addition and pipette up and down to mix the reagents. After mixing, be sure that all liquid in the tip is dispensed into the mixture. Cap the tubes and flick gently to mix again.
8. Set the tubes into the mini-centrifuge, making sure that the load is balanced, and spin for 5 seconds. This will pool all the reagents at the bottom of the tube.

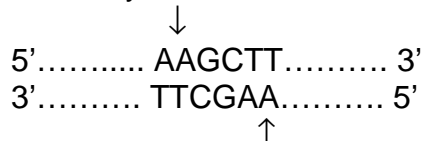
The reaction matrix below summarizes the reagents used in the restriction digest:

Tube	2.5X buffer	dH ₂ O	pDRK	pGRN	<i>Hind</i> III	Total Volume
pDRK+	4 μ L	----	4 μ L	----	2 μ L	10 μ l
pDRK--	4 μ L	2 μ L	4 μ L	----	----	10 μ l
pGRN+	4 μ L	----	----	4 μ L	2 μ L	10 μ l
pGRN--	4 μ L	2 μ L	----	4 μ L	----	10 μ l

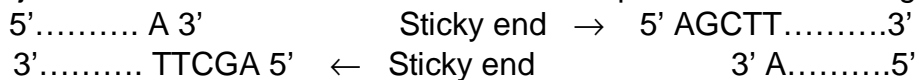
9. Place all of your tubes into the 37°C water bath, and incubate for at least 60 minutes.
10. Your teacher will remove the tubes from the water bath and place them in the freezer.

Questions/Conclusions:

Review the restriction maps of plasmids, pDRK and pGRN. Because *Hind* III is a specific restriction endonuclease, it 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. Thus, the two strands that make-up a DNA molecule are said to be “anti-parallel.” For convenience, we can 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; that is to say, it reads the same on both strands when read in a 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.”



1 a. What is the *recognition sequence* for *Hind* III?

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

2 a. Examine the pDRK and pGRN plasmid maps and fill-in the following:

b. pDRK digestion will yield _____ fragment which will be _____ base pairs in length.

c. pGRN digestion will yield _____ fragments and will be _____ bp and _____ bp in length.

3. Assume your teacher gave you a culture of bacteria carrying one or both of these plasmids. Design a simple experiment that you could use to determine which of these plasmids, pDRK, pGRN or both, the bacteria in the culture were carrying.

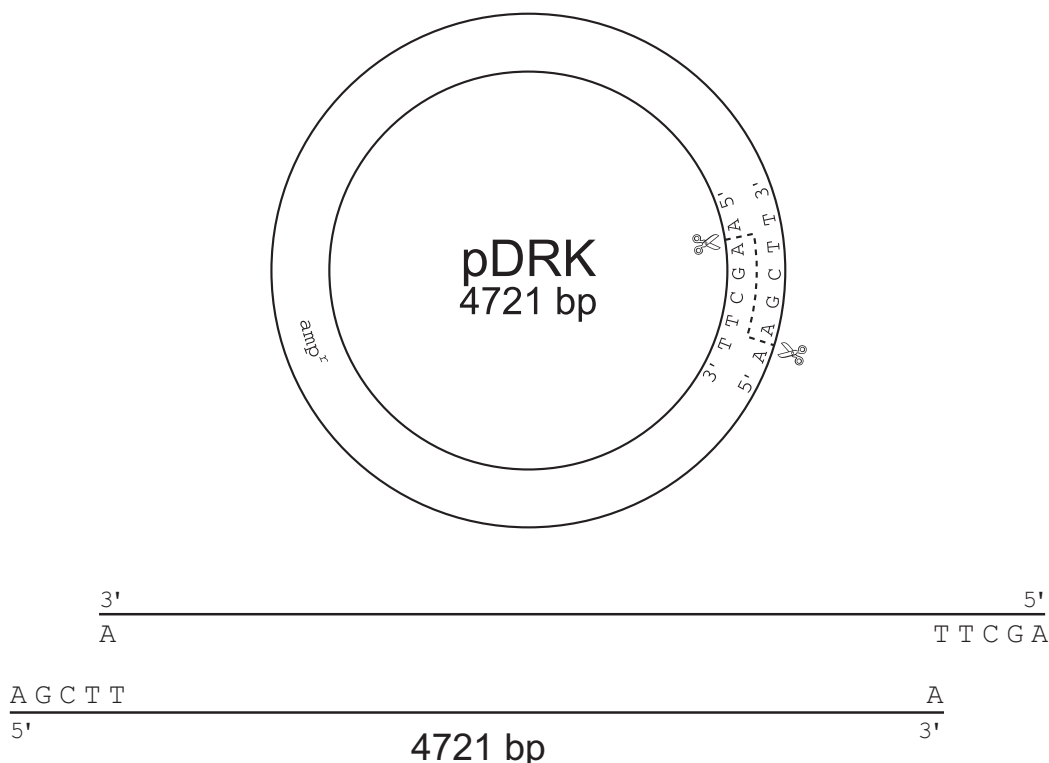
Lab Day 2 Ligation of pDRK/pGRN Restriction Fragments

Producing a Recombinant Plasmid, rpGLO

INTRODUCTION

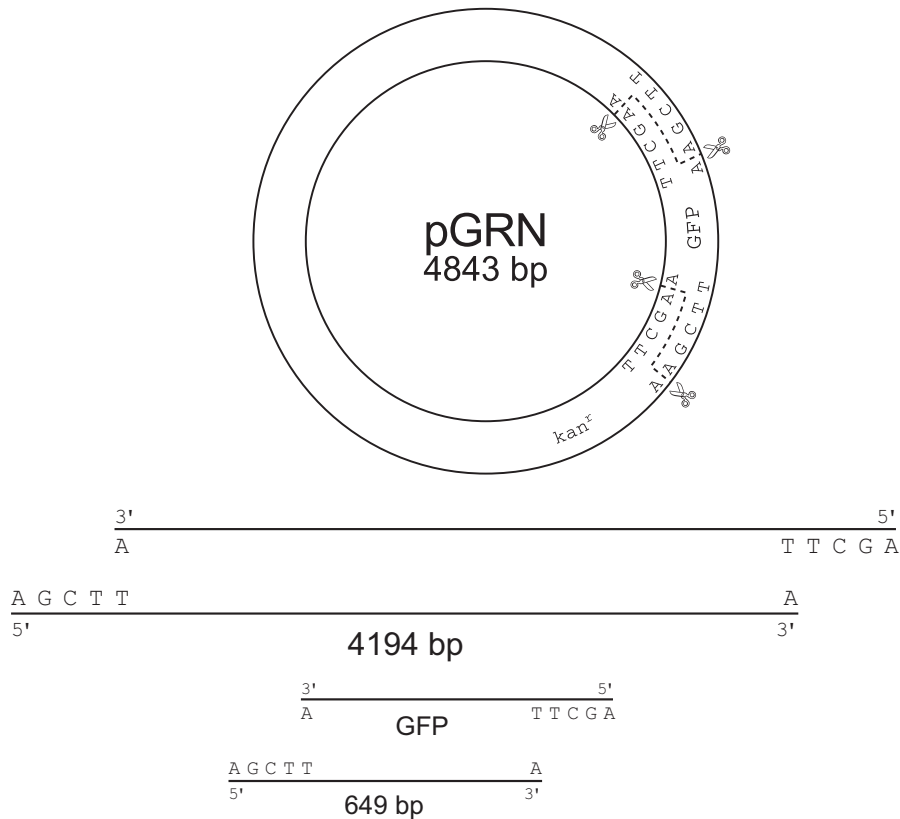
In this laboratory the restriction fragments produced during the previous lab will be *ligated* or bonded together using the enzyme DNA ligase to make new recombinant plasmids. Many of these newly formed plasmids will represent *recombinant* DNA molecules because the three restriction fragments have been recombined in different ways to produce new plasmids. For example, assume that the three plasmid fragments were represented by the letter **A**, **K** and **G**, where **A** represents the pDRK fragment and **K** and **G** represent the two fragments resulting from the pGRN digest. Plasmids could be represented by any single letter, like A or K and any combination of two or more letters, like AAK, AGK, KKG, AK and so forth. As you can see, there are many kinds of recombinant molecules that could result from mixing together these restriction fragments.

As you will remember, the restriction enzyme we are using is *Hind* III. Cutting the plasmids at the *Hind* III restriction site leaves “sticky ends.” The sticky ends on the cut DNA can be ligated to any other fragment of DNA with a complementary sticky end. Examine the pDRK plasmid map, below, to see the location of the *Hind* III restriction site and the sticky ends that form on the 5'-ends of its restriction fragment.

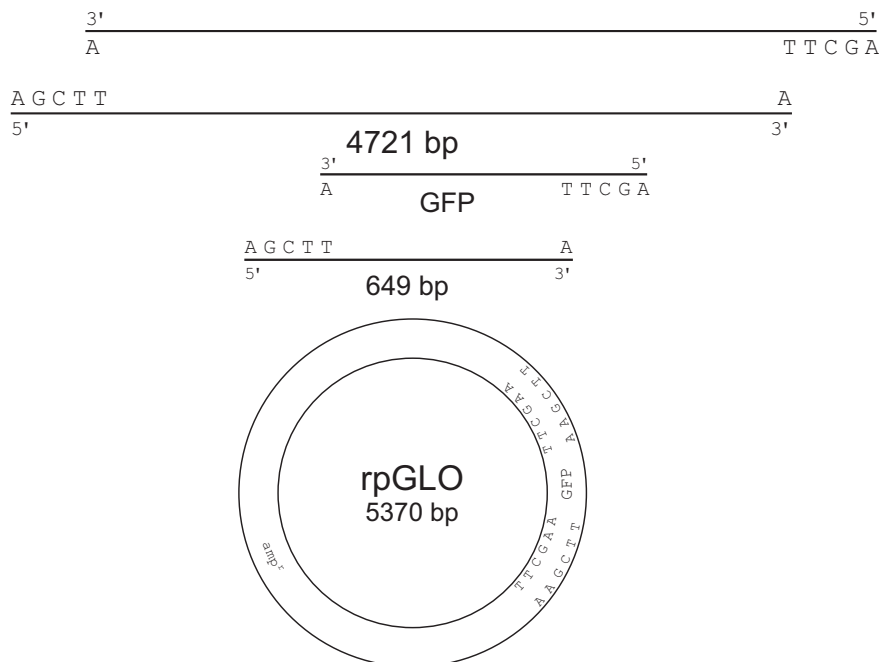


Since pDRK has only one *Hind* III restriction site, the digest will leave only one fragment. The length of the linearized restriction fragment is 4721 bp. It is important to remember that this restriction fragment carries the *amp^r* gene, the gene that provides resistance to ampicillin.

The plasmid pGRN has two *Hind* III restriction sites, one on either side of the *gfp* gene. The digestion of pGRN will leave two fragments, one will be 4194 bp and the other will be 649 bp.

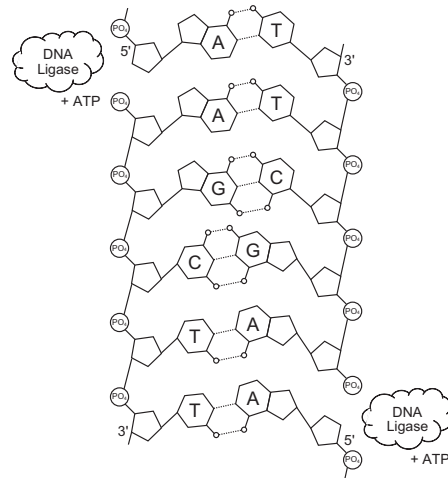


Ligation will bond any *Hind* III sticky end to any other *Hind* III sticky end. You should be able to see that many different combinations of ligation are possible bonding 2,3,4, etc. fragments. The combination of interest to us is the 4721 bp pDRK fragment recombined with the 649 bp pGRN fragment. The combination of these two fragments will yield a recombinant plasmid we will call **rpGLO** (= "*recombinant*" pGLO).

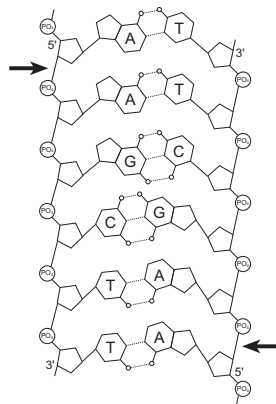


The ligation of the 649 bp pGRN fragment will place the *gfp* gene into the plasmid at a location that will allow a bacterium to synthesize (*express* or *produce*) GFP.

The restriction fragments are initially held together by the hydrogen bonding between the nucleotide bases that make-up the sticky ends. You may recall that adenine and thymine share two hydrogen bonds while cytosine and guanine share three. This helps to ensure that only complementary sticky ends will match up.



Hydrogen bonds are weak chemical bonds and they are inadequate to hold the sticky ends together permanently. The enzyme DNA ligase, with energy supplied by ATP, will form covalent bonds between the sugar and phosphate groups of the DNA backbone. In the diagram below, you can see the positions of these bonds on each side of the DNA molecule. When the covalent bonds are formed, the bonds complete the phosphodiester linkage between the two sugars and the phosphate group on each strand. The resulting chemical bonds are relatively strong bond.



Lab Day 2 Materials

Reagents

Digested pDRK (pDRK + from Lab 2)
 Digested pGRN (pGRN + from Lab 2)
 5x Ligation buffer with ATP
 T4 DNA ligase
 Distilled water

Equipment and supplies

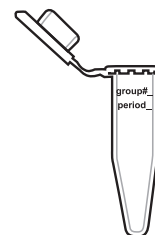
P-20 micropipettor and tips
 70°C water bath
 Plastic tube rack
 Permanent marker

Methods

1. Obtain your pDRK+ and pGRN+ from the rack at the front of the class. **Place the two tubes in the 70°C water bath for 15 minutes.** This heat exposure will denature (inactivate) any *Hind* III that might be active. Why is this important?
2. While your tubes are in the water bath, obtain the 5x buffer from your teacher and a clean 1.5 mL microfuge tube.
3. Label the 1.5 mL microfuge tube "**LIG**" and include your group number and class period (or whatever identifiers your teacher instructs) so that you can locate it later.
4. After the 15 minute 70°C incubation step, add 4 μ L of pDRK + to the *LIG* tube. **Save the pDRK + tube** which should contain 6 μ L of digested pDRK.
5. Using a new tip, add 4 μ L of pGRN+ to the *LIG* tube. **Save the pGRN+ tube** which should contain 6 μ L of digested pGRN.
6. Using a new tip, add 3 μ L of 10x ligation buffer to the *LIG* tube.
7. Finally, add 18 μ L of dH₂O to the *LIG* tube, using a clean tip, and *gently* pump the plunger in and out to mix the reagents. Do this without splashing the solution onto the sides of the microfuge tube. The table below summarizes the contents of the *LIG* tube.

Digested pDRK	Digested pGRN	10x ligation buffer	dH ₂ O	Total volume
4 μ L	4 μ L	3 μ L	18 μ L	29 μ L

8. If you have droplets of liquid clinging to the sides of the tube, ask your teacher to briefly centrifuge the tube to pool the reagents.
9. Have your teacher add 1 μ L of DNA ligase to the reaction mix.
10. Place your *LIG* tube in the rack designated for your class. The *LIG* tube will be incubated at room temperature overnight. Be certain that you have placed your group number and class period on this tube *before* leaving it in the rack. **Also return the pDRK+ and pGRN+ microfuge tubes to your teacher. You will need all of these tubes for the next lab.**



Ligation of pDRK/pGRN Restriction Fragments Producing a Recombinant Plasmid, rpGLO

Pre Laboratory Notes

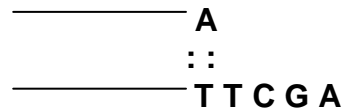
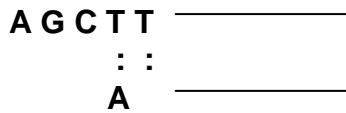
1. Briefly define the term “recombinant DNA.”
2. What does the term ligation indicate?
3. In order for two sticky ends to join together, what relationship needs to exist between them?
4. What properties of the restriction fragments produced from the previous lab are needed to permit ligation of these fragments?
5. What is the importance of **step 1** in this protocol?
6. Draw three possible recombinant plasmids resulting from pDRK and pGRN fragments. Include any genes these fragments carry.

Questions/Conclusions

1 a. Why was it important to place the pDRK+ and pGRN+ tubes in the 70°water bath before setting-up the ligation reaction?

b. What do you think might have happened if this step were omitted?

2. Make a diagram to show how the following sticky ends would join together.
 (“:” = hydrogen bonding)



3. Although many recombinant plasmids are possible, draw *three* possible recombinant plasmids. Include as one of the three the combination in which we are most interested- the one that combines pDRK with the pGRN fragment carrying the *gfp* gene.

4. What would be the *smallest* circular molecule that could form in the LIG tube?

5. In the DNA molecule, there are two kinds of chemical bonds: covalent chemical bonds and hydrogen bonds. Briefly describe how these bonds differ in strength and where, in the DNA molecule, you would find them.

6 a. During ligation, which of the bonds: hydrogen or covalent, form first? Where do they form? Which bonds are next to form and where?

b. DNA ligase is required to form which bond?

Lab Day 3: Confirmation of Restriction and Ligation Using Agarose Gel Electrophoresis

Introduction

It is important, at this stage of our experimental procedure, that we confirm that *Hind* III has digested the original pDRK and pGRN plasmids and the restriction fragments have been ligated together by DNA ligase. This lab will provide evidence that we have recombinant DNA molecules.

Gel electrophoresis is a procedure commonly used to separate fragments of DNA according to molecular size or number of base pairs. DNA fragments will migrate through the agarose maze. DNA, because of the phosphate groups, is negatively charged and will move towards the positive (red) electrode. Because it is easier for small molecules to move through the agarose matrix, they will migrate faster than the larger fragments. Smaller DNA fragments will move through the tangle of agarose molecules faster than the longer fragments.

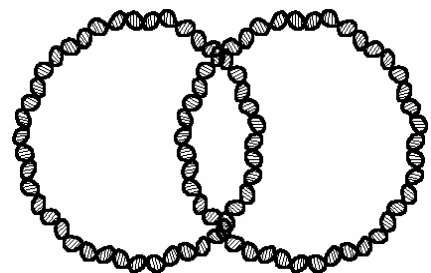
We'll take all of our plasmid samples: digested, undigested and ligated, and use electrophoresis to separate these pieces. You might have predicted that your uncut plasmids would produce only a single DNA band; there's no reason why you would think otherwise. However, it is likely that two or three bands will appear in the undigested plasmid lanes. Here is the reason for this: 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 a "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 agarose 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 plasmids, the plasmids are often replicated 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. Your pDRK – and pGRN – samples, then, may each 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.



We will use a special staining technique that permits us to see the fragments embedded within the gel, then make a photographic record of your gel to document this important step.

Lab Day 3 Materials

Reagents

Plasmid samples:
 pDRK -, pDRK +
 pGRN -, pGRN +
 Ligated plasmid ("LIG" tube)
 0.8% agarose gel
 5x loading dye
 0.5x TBE
 DNA size marker (25 ng/ μ L)

Equipment and supplies

P-20 micropipettor and tips
 1.5 mL microfuge tubes
 Electrophoresis apparatus
 Power supply
 Marker pen
 Plastic microfuge tube rack

Methods

1. Collect the 5 plasmid samples and the DNA marker from your teacher and place them in your plastic tube rack. You should have *six* tubes.
2. Obtain five clean 1.5 mL microfuge tubes and label them as follows: *D +*, *D-*, *G+*, *G-*, and *L*. The microfuge tube with the marker should already be labeled.
3. The following table summarizes plasmid sample preparation for electrophoresis. See "Hints" **before** setting-up these tubes.

Tube	dH ₂ O	Loading dye	pDRK +	pDR K -	pGRN +	pGR N -	LIG	Total volume
D+	7 μ L	3 μ L	5 μ L	-	-	-	-	15 μ L
D-	7 μ L	3 μ L	-	5 μ L	-	-	-	15 μ L
G+	10 μ L	3 μ L	-	-	2 μ L	-	-	15 μ L
G-	10 μ L	3 μ L	-	-	-	2 μ L	-	15 μ L
L	-	4 μ L	-	-	-	-	16 μ L	20 μ L

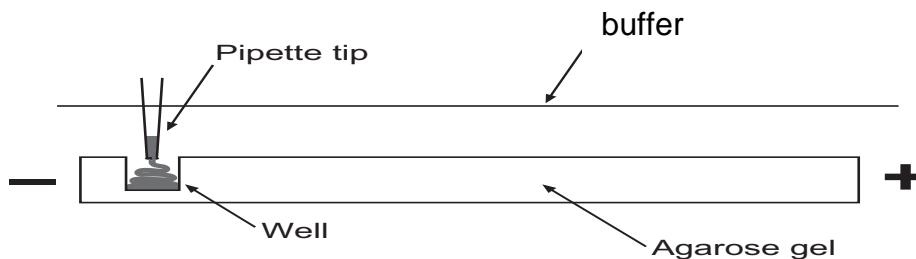
Hints:

- For example, to the tube labeled "D+," add 5 μ L of pDRK+, 7 μ L of dH₂O and 3 μ L of loading dye. The loading dye should be located in your plastic microfuge tube rack next to the dH₂O tube.
- If you study this table, you'll see that you can add water to the first four tubes, omitting the "L" tube, then add the loading dye to all of the tubes without changing the tip. Then, **dispense the plasmid sample into each tube, changing the tip each time to avoid contamination.**
- **Save the "LIG" tube** that contains your ligated plasmid; there should be about 14 μ L remaining in this tube. **IMPORTANT:** Return the "LIG" tube to the collection rack, at the front of the room, as you will need it for the next lab.

- Centrifuge all samples to pool the reagents at the bottom of each tube. Be certain that the tubes are placed in a balanced configuration.
4. Prepare the gel and electrophoresis box to receive these plasmid samples.
 - Be certain the gel wells are oriented closest to the negative (black) electrode.
 - Pour the 0.5x TBE over the gel until there are no visible “dimples” breaking the surface of the buffer over the wells. It’s important that the gel be completely under the buffer but you don’t want so much buffer in the box that it will spill over.
 5. Take your plasmid samples and marker to the gel, along with your pipettor and tips. You may be asked share this gel with another group.
 6. Unless your teacher has you load your samples in a different pattern load your samples in the order indicated below. Follow the loading directions that begin with step seven. If you load your sample in a different order, *be certain to record it in your notebook for later reference.*



7. Using a clean tip and set your P-20 micropipettor to 10 μ L. Aspirate 10 μ L of your “DNA size marker” and *slowly* dispense it into the well.
 - 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.
8. Continue this procedure with the plasmid samples following the order indicated above. For your “D” and “G” samples, you will need to reset the micropipettor to 15 μ L. For your “L” (*ligated*) sample, you will be loading 20 μ L. Remember to use a new tip for each sample to avoid contamination. If you choose to load your samples in a different order, be certain to record the sample order in your notebook.

9. Close the gel box lid tightly over the electrophoresis chamber. Connect the electrical leads to the power supply. Be certain that both leads are connected to the same channel (same side) with the negative (black) to negative (black) and positive (red) to positive (red).

10. On the power supply, set the voltage to 130-140v.

11. After two or three minutes, look at your gel and be certain that the purple dye (bromophenol blue) is moving towards to positive electrode. If it's moving in the other direction- towards the negative (black) electrode, check the electrical leads to see if they are plugged into the power supply correctly.

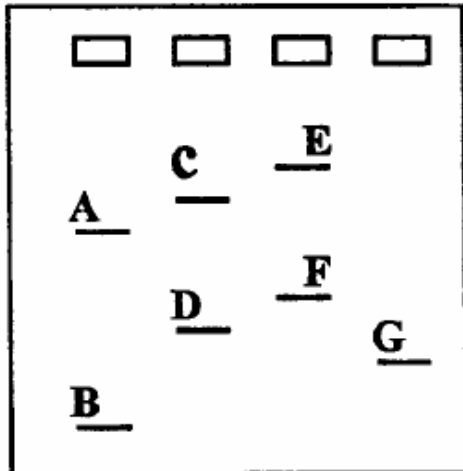
12. Be certain that you return your "LIG" tube to the front of the room. This tube should contain your recombinant plasmids and will be used for the next lab.

13. Your teacher will explain what to do with your gels **so listen carefully**. If your lab time is short, you may not have sufficient time to complete the electrophoresis. The purple dye will need to run just to the end of the gel, about 30 minutes.

Confirmation of Restriction and Ligation Using Agarose Gel Electrophoresis

Pre Laboratory Notes

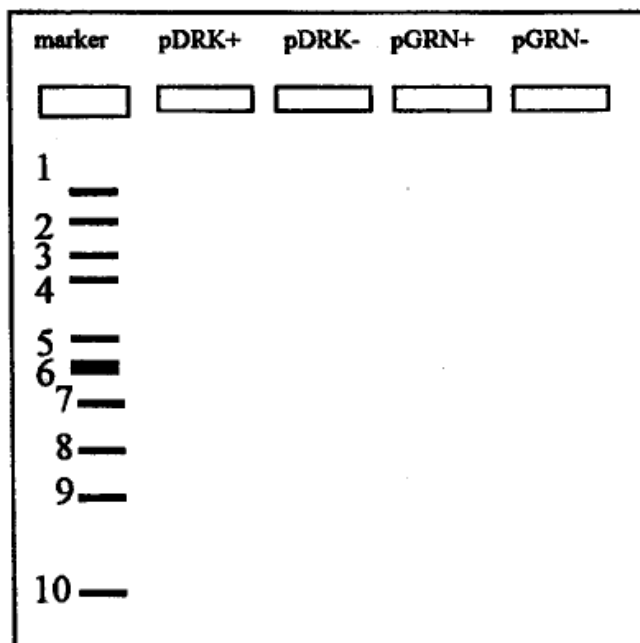
1. Using the location of each DNA “band” in the gel, the relative size of each DNA fragment can be determined. For example, examine the figure below and arrange the letters, identifying each band, from smallest to largest.



Besides using electrophoresis to separate DNA fragments according to their sizes, it can be used to estimate the actual size, in base pairs, of each fragment. For example, we might be looking for a gene and we suspect it is of a certain size; electrophoresis can be used to locate fragments in that size range. In order to do this, we would need to run a gel with a mixture of DNA fragments of known sizes. This mixture called a “marker” or “ladder” serves as a control or a standard to which we can compare the positions of other DNA bands in the same gel.

In the following diagram, the “marker” lane contains 10 DNA bands of known sizes. These sizes are given below. Using this information and the plasmid maps of pDRK and pGRN, **predict** the positions of DNA bands produced by the pDRK -, pDRK +, pGRN -, pGRN + samples. Hint: first determine how many fragments should appear in each sample, and then determine the size(s) of each fragment. We will omit the “LIG” sample. Draw them on the gel.

- Marker fragments:**
1. 10.0 kilobase pair
 2. 8.0
 3. 6.0
 4. 5.0
 5. 4.0
 6. **3.0 (thick band)**
 7. 2.0
 8. 1.5
 9. 1.0
 10. 0.5



Lab Day 3 Questions/Conclusions

These questions are to be answered after you've had an opportunity to analyze your gel photograph.

1. How did your actual gel results compare to your gel predictions?

2. a) Are there any bands, appearing in your gel photo, that are not expected?

b) What could explain the origin of these unexpected bands?

- 3 a) Do you see evidence of the three plasmid forms in the uncut lanes? Explain.

b) Is there evidence of more than one form of multimer? How do you know?

4. Why are the ligated plasmids so close to the well?

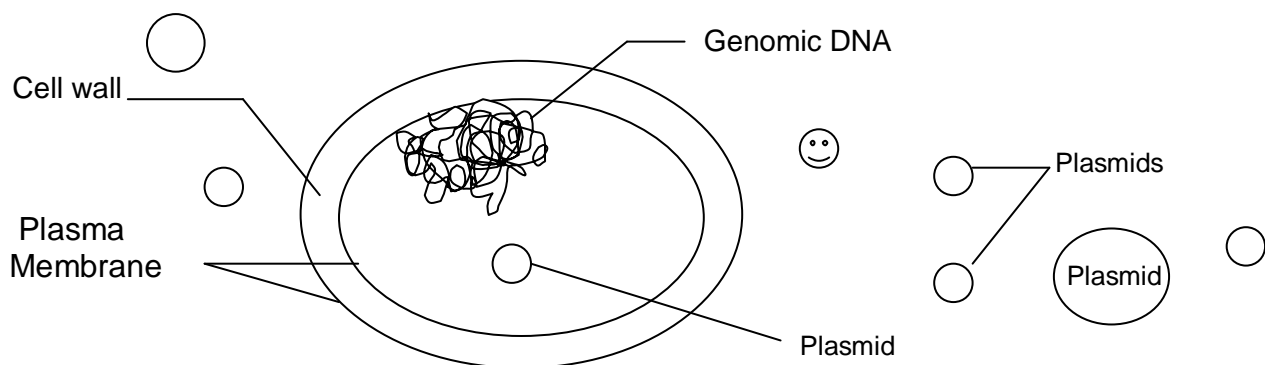
5. The 649 bp pGRN fragment, carrying the *gfp* gene, may form a circularized fragment since each end of the fragment terminates in a *Hind* III sticky end. Is there evidence of a circularized 649 bp fragment in the ligated lane? Explain.

Introduction to Transformation: (more complete explanation for pGLO lab)

Thus far, you've produced ligated, recombinant plasmids. Hopefully, some of these DNA recombinants will have the small pGRN fragment, the one representing the gene for green fluorescent protein, ligated into the pDRK plasmid in the correct orientation. This plasmid is referred to as rpGLO ("r" referring to *recombinant*). Now, we want to get these recombinant plasmids into bacterial cells so that we can get the cells to express this gene and make green fluorescent protein.

The process of taking up foreign pieces of DNA, like a plasmid, into a bacterial cell is called *transformation*. Transformation is a process that occurs in nature although it is probably somewhat rare. A British medical officer, Frederick Griffith first studied the process, in 1928. Bacteria usually pass on extra chromosomal genetic material, like plasmids, during conjugation (bacterial sex) rather than relying on luck. But taking up plasmids can provide bacteria with certain genes that confer selective advantage, for example antibiotic resistance. Under experimental conditions, however, it is possible to prepare cells so that about one cell in a thousand will take in a plasmid from the surrounding environment.

There are several factors that determine transformation efficiency. Two of these are related directly to the plasmid used for transformation. The larger the plasmid, the less likely it will be taken up by the bacterium. Remember, in order for the bacterium to take in foreign DNA, the plasmid must pass through bacteria's plasma membrane and cell wall.



Therefore, small plasmids are more likely to pass through the bacterium's plasma membranes (*E. coli* has two) and its cell wall than large plasmids.

Plasmids can assume different shapes. The supercoiled form is the easiest to get into the cell while the nicked-circle or the multimer, two or more plasmids linked together, are more difficult. The ligation tube, containing the recombinant plasmids you prepared, does not contain any supercoiled plasmids. This form requires an enzyme that is found only in the bacterial cell. The recombinant plasmids you prepared are primarily nicked-circled but there is a wide variation in sizes.

In nature, transformation is a relatively rare event. To increase our chances of getting our recombinant plasmids into bacterial cells we will use "competent" cells. When cells are "competent," it means that they are ready to receive plasmids. For the most part, you don't find competent cells in nature; instead, cells have to be made competent in the laboratory. One common way this is done is by soaking the cells in calcium chloride.

Although the *E. coli* strain that you are using in these labs, HB101, is relatively benign, it is important that you use proper techniques when handling them.

Remember that DNA is negatively charged. Do you remember why? The plasma membranes surrounding the bacterial cell also contain phosphate groups and are negatively charged. The problem of trying to get negatively charged DNA past a negatively charged membrane is that *like* electrical charges tend to *repel* each other. When cells are made competent, they are suspended in a solution of calcium chloride because calcium ions (positively charged atoms of calcium, Ca^{++}) help to neutralize the negative electrical charges of the plasma membrane and the plasmid. With these repulsive charges neutralized by the calcium ions, the plasmid DNA has an easier time passing by the plasma membrane of the bacterial cell. The cells have already been made competent for you and your teacher will give you an aliquot. You will, however, need to do the next step.

Now that we have the negative charges on the DNA and the plasma membranes neutralized, we need to create a bit of a pressure difference between the inside and the outside of the bacterial cell. This is done by first getting the bacteria really cold and then quickly putting them into warm water. This is called “heat shock” and it creates a situation where the pressure outside the cell is a tiny bit higher than inside the cell. This pressure gradient will help to move the plasmid DNA from the outside to the inside of the bacterial cell. Following this brutal treatment, we’ll need to feed our bacteria and let recover for a few minutes before we spread them onto agar plates.

Once the cells have recovered, you’ll take samples of these cells and spread them on a series of sterile agar plates. One of these plates will contain only bacterial food; it contains no antibiotic. This plate is marked “**LB.**” A second plate contains LB and ampicillin; this plate is marked “**amp.**” The third plate contains LB, ampicillin and a simple sugar called arabinose; this plate is marked “**ara.**”

Ampicillin is an antibiotic that prevents bacteria from fully forming its cell wall. Cells that are not ampicillin resistant cannot grow in its presence; the new cells simply rupture or lyse. If a cell receives an ampicillin resistant gene, *amp^r*, it will produce a protein that will chemically decompose ampicillin and, therefore, will be able to grow with ampicillin in its environment.

Arabinose, a simple sugar, is needed by the bacterium to express the green fluorescent protein gene. If a bacterium takes up rpGLO, arabinose helps the enzyme needed to transcribe the *gfp* gene to align itself correctly on the gene. This relationship will be discussed in the next lab.