

PLASMIDS, RESTRICTION ANALYSIS AND LIGATION

(adapted from AMGEN – Bruce Wallace Biotechnology Lab Program)

Day 1: Restriction Analysis of pDRK and pGRN

Background Information

The purpose of this laboratory is threefold:

1. to examine the role of plasmids in molecular biology
2. to examine the role and significance of restriction enzymes (endonucleases) in molecular biology
3. to initiate the first step in producing a recombinant DNA molecule

The two plasmids used in this lab have been modified from two commercially available plasmids, **pGLO™** (Bio-Rad) and **pKAN** (DNA Learning Center at Cold Spring Harbor Laboratory). Each plasmid carries a different antibiotic resistant gene that can be used as a selectable marker to facilitate separate cloning. Selectable markers, like antibiotic resistant genes engineered into plasmids, allow us to discriminate between bacteria harboring a plasmid from those that are not. By simply growing the bacteria on an agar plate containing the antibiotic, for example ampicillin, only those that are resistant to the antibiotic will grow. Thus, the cells containing the antibiotic resistant gene and presumably the plasmid are easily marked.

This lab uses ***Hind* III**, a restriction endonuclease, to cut two plasmids, pDRK and pGRN. This is the initial step in producing a recombinant molecule. By cutting the two plasmids with the **same restriction enzyme**, the resulting molecular fragments terminate with complementary “sticky ends.” This feature makes it relatively easy to recombine the fragments into recombinant DNA molecules using a second enzyme **T4 DNA ligase**. Although several recombinant constructs will result from this procedure, we are looking for the pDRK recombinant plasmid that has incorporated the gene for green fluorescent protein, originally in pGRN. While the gene being recombined into pDRK was cut from pGRN and originally isolated from a jellyfish, it is important that your students understand that this gene could have come from a human, plant, or any other organism. Because both ends of the *gfp* gene are cut with *Hind* III, the gene can be inserted in two orientations into the pDRK plasmid. However, only one orientation will produce a functional protein.

Restriction enzymes play an essential role in recombinant DNA technology. The significance of these enzymes cannot be overstated since, to large extent, the biotechnology industry has been built upon these enzymes.

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 pDRK is digested (cut) with *Hind* III, one (restriction) fragment will result: one end will have a 5' sticky end A-G-C-T- and the other end will the complementary 5' sticky end -T-C-G-A. The size of this pDRK fragment will be the same size as the intact plasmid: 4, 721 base pairs.

Hind III will digest pGRN leaving two restriction fragments: one will carry the gene for green fluorescent protein and will be 649 base pairs; the second fragment will be 4, 194 base pairs and will carry the gene for kanamycin resistance. Each fragment will carry the *Hind* III 5' sticky ends.

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

Laboratory Preparation:

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

MATERIALS:

Reagents

pDRK (10 ng/μL)
 pGRN (80 ng/μL)
 Restriction enzyme (*Hind* III 3 unit/μL)
 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)

Your major work, for this protocol, involves aliquoting solutions. The protocol has been written to have the students label three tubes: pDRK, pGRN and 2x buffer. You might ask three of your better students to aliquot the plasmids and buffers as a member from each group brings the labeled tubes to them. This will save you aliquoting and tube labeling time. If you decide to have students get their reagents from you, “assembly line” in the manner we recommend for our PCR labs, then you can cut back on the individual aliquotting you do for the class. This is particularly important for the restriction enzyme, but less important for the plasmids.

Because *Hind* III is expensive, it is recommended that you do this aliquoting for each group yourself (or use the assembly-line). There is a table that will summarize the reagents and tubes you will need to prepare.

Here are a few problems that *will* arise as your students work through this protocol:

1. When your students are aspirating solution, they will often push to the second stop, on the pipettor instead of the first. This results in aspirating more solution than is set on the pipettor. For the most part, this will not interfere with the success of the plasmid restriction but they will run short of reagents. A better way of handling this is to hold the stock tube yourself, and have students do the aliquotting in front of you so you can be sure they are getting the proper amount. If you do this “assembly-line” fashion, it takes very little time and you can cut back on the individual aliquotting mentioned above. It is a good idea to have some extra reagents handy, if possible.
2. *Hind* III is the costly reagent in this lab. There is extra enzyme included but remind students to push only to the first stop when aspirating and that they should look carefully to be certain they see the enzyme at the end of the pipette tip. Because each group receives only a 8 μL aliquot, it is recommended that you centrifuge the enzyme tubes before handing them out to the students. This will help insure that all 8 μL are pushed to the bottom of the microfuge tube.
3. You'll note that crushed ice is listed as *optional*. A recently published paper suggest that *Hind* III is far more stable at room temperature than was commonly believed. Although we recommend that you keep it in the freezer (-20°C) until you're ready to use it in class ; you DO NOT have to keep it in ice during the lab.
4. Be certain that the students have **marked their tubes with their group number and class period**, as they will need to locate them during the next lab day.
5. Remember to remove the restriction digests from the 37°C water bath. Leaving the digest in the water bath for a couple of hours (one to two hours is recommended) will not interfere with the restriction. Place them at 20°(freezer) until you are ready to run the ligation protocol. The samples are fine in the freezer for up to 1 month.

***Before aliquoting these reagents, be certain to vortex the stock microfuge tube before dispensing.**

TEACHER Aliquot table: (for each group of students, or each student, depending on your setup)

Size of tube	Label tube	Contents of tube	Aliquot	Actually used
0.5 mL	pDRK	pDRK (10ng/ μ L)	8 μ L	8 μ L
0.5 mL	pGRN	pGRN (80ng/ μ L)	8 μ L	8 μ L
1.5 mL	2.5x Buffer	2.5x restriction buffer	16 μ L	16 μ L
1.5 mL	**dH ₂ O	Distilled water	1000 μ L	4 μ L

**This tube, containing distilled water, is used in several lab protocols and should be available for student use each day.

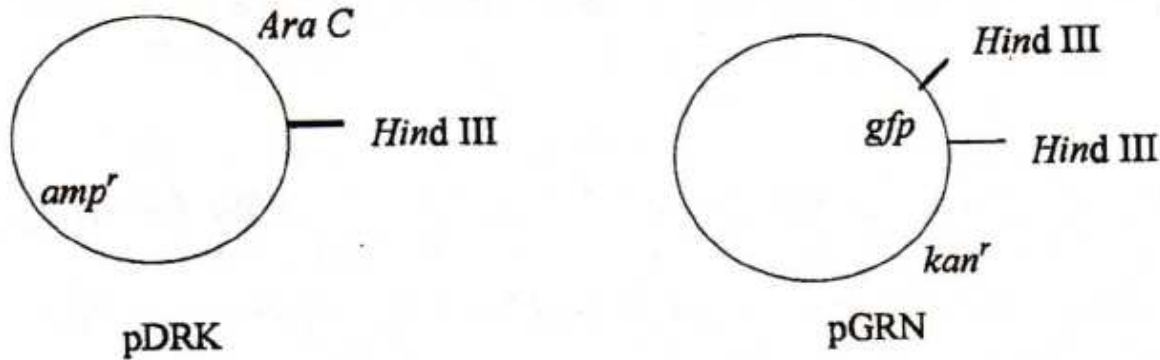
The students are required to get the enzyme aliquot directly from the teacher (step 8 of the student protocol). This will give you an opportunity to “eyeball” each tube to help ensure they have about the correct volume of reaction mix.

STUDENT Aliquot table (FYI)

Tube	2.5X buffer	dH ₂ O	pDRK	pGRN	Hind 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

Answers to Student Pre-Lab Questions:

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*.



pDRK: Contains only a single *Hind* III restriction site, located at 1,465 bp from “0” position. There are two genes: *Ara C* is a gene that encodes a protein that is needed for protein expression and *amp^r* is a gene for ampicillin resistance.

pGRN: Contains two *Hind* III restriction sites, one on either side of the *gfp* gene. The *gfp* gene carries the code for green fluorescent protein. The plasmid also carries a gene for kanamycin resistance.

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

pDRK carries the gene for ampicillin resistance while pGRN carries the gene for kanamycin resistance.

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?

pDRK is engineered as an expression vector. A gene, inserted into this vector, can be expressed.

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

A marine jellyfish, *Aequoria Victoria*

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

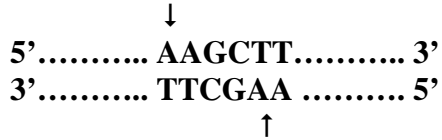
Answers will vary. It’s important that the students look at the reaction matrix and think about how they will label tubes and aliquot reagents before they do it in the lab.

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

These are control tubes- technically, enzyme controls. They are used for Comparison, to determine the effect of the enzyme on the restriction digests. They will, also, let us know if the enzyme is not working.

Answers to Protocol Questions (Conclusions):

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



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

AGCT-

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

pDRK digestion will yield 1 fragment which will be 4721 base pairs in length.

pGRN digestion will yield 2 fragments that will be 4194 bp and 649 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.

Answers will vary. Look for an answer related to growth of the bacteria in the presence of antibiotics. Those that can grow in the presence of ampicillin carry pDRK while those that can grow in kanamycin carry pGRN. If both plasmids are being carried, the bacteria can be challenged with both antibiotics. Another possibility would be to grow the bacterium and purify the plasmid from the cells. The former answer is more likely to be used.

DAY 2: Ligation of pDRK and pGRN Restriction Fragments

Background Information:

This laboratory protocol takes the restriction fragments generated from the digestion of pDRK and pGRN and ligates them to produce numerous species of recombinant plasmids. Such recombinants are often called “chimeras,” after the Greek mythological monster having a lion’s head, a goat’s body, and a serpent’s tail.

Because both plasmids were cut with *Hind* III, each of the three restriction fragments will carry two *Hind* III sticky ends. For this reason, any single fragment can simply circularize and form a single DNA plasmid. It is also likely that two or more fragments will join (ligate) together to yield larger plasmids. The plasmid we hope to produce is one that contains the small pGRN fragment, containing the *gfp* gene, inserted into the pDRK fragment. The resulting plasmid we will identify as rpGLO, where “r” refers to recombinant and it represents a true chimera as it consists of two *different* pieces of DNA.

It is important, then, for your students to understand there are several ways fragments can come together; the only criterion being there must be a 5’ overhang hydrogen bonding to a complementary 5’ overhang. Here are a few possibilities:

1. The pDRK fragment can close up without incorporating another fragment.
2. The pDRK fragment and the long pGRN fragment can ligate together.
3. The small pGRN fragment can circularize and ligate with itself.
4. The pDRK fragment can incorporate the small pGRN fragment carrying the GFP gene.

Although there are many more possibilities, it is the latter example is important. Note that there are two ways this can occur: the small pGRN fragment can be incorporated in the correct direction (for expressing green fluorescent protein) or it could be inserted backwards. If the fragment is incorporated in the latter direction, the gene cannot be properly expressed and the protein will not be synthesized. In our protocol, students will be able to visualize the bacteria that have incorporated the plasmid with the *gfp* gene, in the proper orientation, as the bacteria will release a green color when exposed to ultraviolet light. However, the students will NOT be able to tell this on the gel.

There is a template included that you can use to prepare some paper plasmids. 3M makes a RESTICKABLE glue stick with the adhesive properties of “sticky notes.” It’s important to avoid regular glue sticks, as the plasmid pieces glued with them will not come apart easily. For many students, the incorporation of these paper plasmids into the curriculum will help them visualize sticky-ends and how many recombinant constructs can form from the three restriction fragments.

Laboratory preparation:

Materials

Reagents

Digested pDRK (pDRK + from Lab 2)
Digested pGRN (pGRN + from Lab 2)
5x Ligation buffer with ATP (diluted with water)
T4 DNA ligase (1 unit/ μ L)
Distilled water

Equipment and supplies

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

For the student, this is a fairly simple lab. Problems arise if they skip the first step. Pipetting errors and throwing away microfuge tubes containing samples they should have saved can also lead to difficulties. The protocol has

been designed in a way that allows students to recover from mistakes by borrowing reaction mixes from students who have followed directions.

1. Remember to set the water bath to 70°C first thing in the morning.
2. Frozen restriction digest should be removed from the -20°C freezer before class. The digests can sit at room temperature or many hours without degradation. Spin down any condensation by using the microfuge.
3. Remind students to **denature** the *Hind* III by placing their pDRK + and pGRN + tubes in the 70°C water bath before setting up their ligations. It is not uncommon for students to forget this step; it's not a step to omit.
4. Because of its expense, you should aliquot the 1 µL of ligase directly into their ligation mix. Students are instructed to get their ligase from you (step 9 of the student protocol). This is a good check point as you can "eyeball" the volume in each ligation tube; there should be about 29 µL.
5. When your students have completed setting up their ligations, **be certain to collect ALL of their tubes:** pDRK +, pGRN + and LIG microfuge tubes. They should check to make certain that all three tubes carry their group number and class period so they can locate them for the next lab.

NOTE:

The LIG tubes will be kept at room temperature overnight.

The pDRK + and pGRN + tubes should be put into the refrigerator (or freezer). But room temp is OK if you forget. Store the pDRK- and pGRN- tubes the same way.

Samples from all of these tubes, including the LIG tube will be used for electrophoresis in the next lab.

Aliquot table: (for each group of students, or each student, depending on your setup)

Size of tube	Label tube	Contents of tube	Aliquot	Actually used
1.5 mL	*Lig Buf	5x ligation buffer	6 µL	6 µL
1.5 mL	**dH ₂ O	Distilled water	1000 µL	18 µL

*After aliquoting the ligation buffer, return to freezer (-20°C) until class.

**This tube, containing distilled water, is used in several lab protocols and should be in the plastic microfuge rack from the last lab.

Student Aliquot table (FYI)

Digested pDRK	Digested pGRN	5x ligation buffer	dH ₂ O	Total volume
4 µL	4 µL	3 µL	18 µL	29 µL

Then you add the 1µL of ligase to make the total volume 30 µL.

Answers to Student Pre-Lab Questions:

1. Briefly define the term “recombinant DNA.”

A DNA molecule composed of ligated fragments taken from other DNA molecules.

2. What does the term ligation indicate?

Ligate means to bind together.

3. In order for two sticky ends to join together, what relationship needs to exist between them?

The nucleotide bases that comprise the sticky ends must be complementary.

4. What properties of the restriction fragments produced from the previous lab are needed to permit ligation of these fragments?

Because both plasmids were cut or digested with the same restriction enzyme, *Hind* III, the resulting sticky ends must be complementary to each other.

5. What is the importance of **step 1** in this protocol?

The restriction digests, from the previous lab, must be heated to denature *Hind* III. If the enzyme is not denatured and remains active, the restriction fragments cannot be ligated together; *Hind* III would simply cut them apart as soon as they were ligated.

6. Draw three possible recombinant plasmids resulting from pDRK and pGRN fragments. Include any genes these fragments carry.

These will vary; any combination is possible. However, not all of the combinations can be used to transform bacteria. Some will be too large; others will not carry an antibiotic resistant gene and would not survive when challenged by ampicillin or kanamycin.

Answers to Protocol Questions (Conclusions):

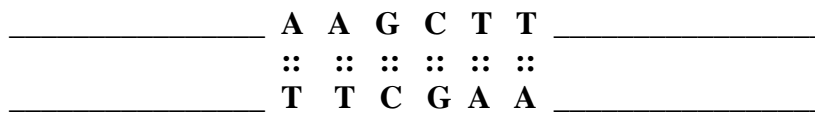
1. Why was it important to place the pDRK + and pGRN + tubes in the 70°C water bath before setting up the ligation reaction?

It is essential that *Hind* III be denatured before the ligation step.

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

No recombinant molecules could be formed since the restriction enzyme would digest them about as fast as they were ligated by DNA ligase.

2. Show how the following sticky ends would join together. Label the 5' and 3' ends of both DNA fragments. (":" = hydrogen bonding).



3. Although many recombinant plasmids are possible, the combination of greatest interest combines pDRK with the pGRN fragment carrying the *gfp* gene. In terms of base pairs, what should be the size of this recombinant plasmid?

4,721 bp + 649 bp = 4,370 bp

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

The *gfp* gene could simple form a circle joining its two *Hind* III sticky ends. This circularized molecule would be 649 bp in size. (You will actually see this construct in the confirmation gel).

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.

The covalent chemical bond is stronger than the hydrogen bond. Covalent bonds can be found along the sugar-phosphate backbone, in addition to all of the atoms found in the bases. Hydrogen bonds are located between the base pairs: two between A and T and three between G and C.

6. During ligation, which of the bonds: hydrogen or covalent forms first? Where do they form? When and where do the other bonds form?

Hydrogen bonds first form between complementary bases. Covalent bonds will form next along the sugar-phosphate backbone.

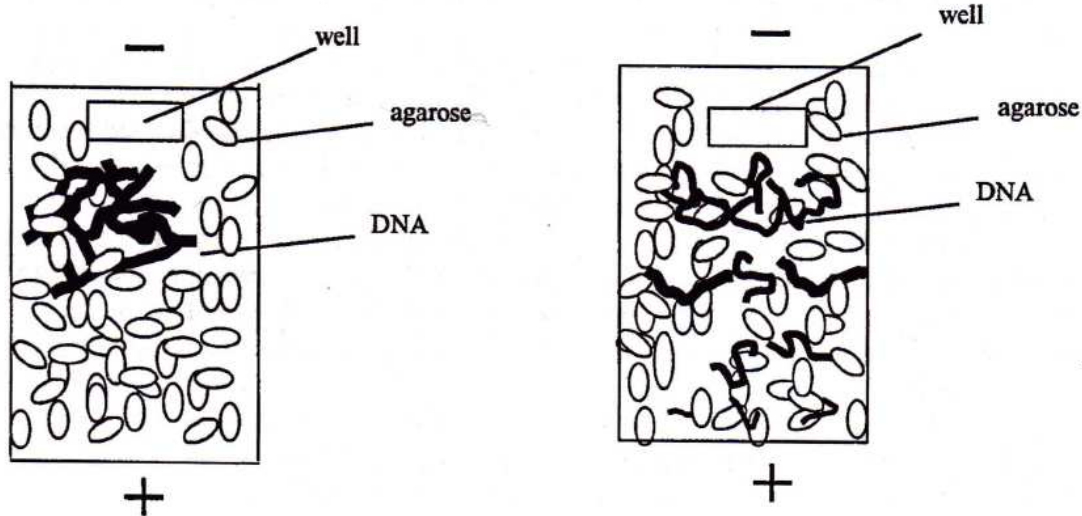
DNA ligase is required to form which bond?

Covalent chemical bonds require DNA liagase.

LAB DAY 3: Confirmation of Restriction and Ligation

Background Information:

This laboratory uses agarose gel electrophoresis to examine plasmid samples from the two previous labs to confirm restriction digestion and ligation. The student protocol provides some theoretical aspects of separating DNA fragments based on molecule size. You may want to use the overhead of the diagram below to help students visualize this process.



In the student protocol, there is a brief explanation of different plasmid forms: supercoiled, nicked-circle and multimers. In the student pre-lab, students are asked to predict what DNA fragment(s) will appear in each gel lane. One suggestion is to show your students these plasmid visual aids BEFORE they try to answer the pre-lab questions. This will help them predict, with greater accuracy, the plasmid patterns produced in the pDRK – and pGRN – lanes. Another approach would be to discuss this AFTER they run their samples and analyze their results. In either case, these plasmid models are helpful in explaining why supercoiled or nicked-circle plasmids can be the same sizes yet migrate at different rates. Overheads are also included.

DNA is colorless and in order to detect the fragments in the gel, we need to stain the gel with a molecule that we can visualize. Staining of DNA is done with ethidium bromide. Ethidium bromide is a flat, planer molecule that slips in between the nucleotide base pairs along the DNA double helix. The larger the DNA fragment or the greater the concentration of DNA in the gel, the greater will be the amount of ethidium bromide intercalating into the molecule. When exposed to ultraviolet light, ethidium bromide will fluoresce an orange color. The greater the amount of DNA present, the brighter will be the fluorescence. When you document agarose gels with the Polaroid camera, the camera has an orange filter mounted in front of the lens. This filter is transparent to orange light but eliminates other wavelengths from passing through to the film. The result is a pattern of bands on a gel photo illustrating where the ethidium bromide-stained DNA is concentrated in the gel.

Have the students load different volumes of plasmid into the gel because the plasmid concentrations are different. The initial pDRK concentration was 10 ng/μL and pGRN was 80 ng/μL. The concentration of pDRK is low because it is important that the ampicillin/arabinose plates are not overrun with amp resistant bacteria. If too many bacteria are growing on this plate, it may be difficult to select a clean sample from one of the green colonies without contaminating the sample with non-green bacteria. Using a higher pGRN concentration increases the likelihood that the *gfp* fragment will be incorporated into a cut pDRK.

If the same volume of each plasmid were loaded into the gel, the pGRN restriction fragments would be too bright as a result of overloading the gel lane with DNA. Therefore, a smaller volume of this plasmid is used to prepare the electrophoresis sample.

Fifteen microliters of ligated plasmid is used to prepare the last sample. Again the reason is partially related to concentration; the plasmid concentration in the LIG tube is low. Because many different plasmid constructs are formed, no single band appears. This will explain why the DNA in this lane is very faint and does not appear in a single band (there is simply not enough of a single fragment to form a distinct band).

Although an attempt is made to produce bands that stain and fluoresce with similar intensities, there will be a difference with some bands appearing brighter than others. The more DNA you have in a band, the greater the band will fluoresce. Therefore, if time permits, you may want to discuss the relationship of brightness to quantity of DNA.

Laboratory Preparation:

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

There isn't much to aliquot for this lab. Locate the 1 kb ladder or marker. This ladder is used as a size marker so we can compare DNA bands in our gels with bands of known size in this marker. It's similar to having a molecular ruler running in our gel. By comparing the position (= distance from the well) of our DNA fragments with those in the size marker, we can get an estimate of sizes, in base pairs. There are 10 different size fragments in the 1 kb ladder; they are given to you in the "answers to the Conclusions" section. If a 1Kb extended ladder has been substituted, it will have 20 or so size fragments. The plasmid fragments, however, will be obvious and your students should be able to determine which ladder fragments correlate to the digested plasmid bands.

Once aliquoted, the markers should be placed into the refrigerator (4°C) until ready to use. The marker can remain at this temperature for several weeks.

Aliquot table (for each group of students, or each student, depending on your setup)

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

Student preparation table (FYI)

Tube	dH ₂ O	Loading dye	pDRK +	pDRK -	pGRN +	pGRN -	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

The most difficult part of this lab is preparing the gels. Here are some ways you could reduce time:

1. Prepare gels several days before you need them. Each gel will take around 30 mL of 0.8% agarose dissolved in 0.5X TBE. If they are kept in the refrigerator (4°C), they will keep for several days if stored in a weigh boat inside zip lock baggies.

2. Each comb either forms 8 or 12 wells, depending on the size. Each group only needs 6 wells. Therefore, you could have two groups loading each of their six samples into a single gel. This creates somewhat of a bottleneck around the electrophoresis chamber but it will reduce the number of gels you will need to prepare and stain. Students are asked to check with their teacher regarding how they are to load their gels.

Running and staining the 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.”**

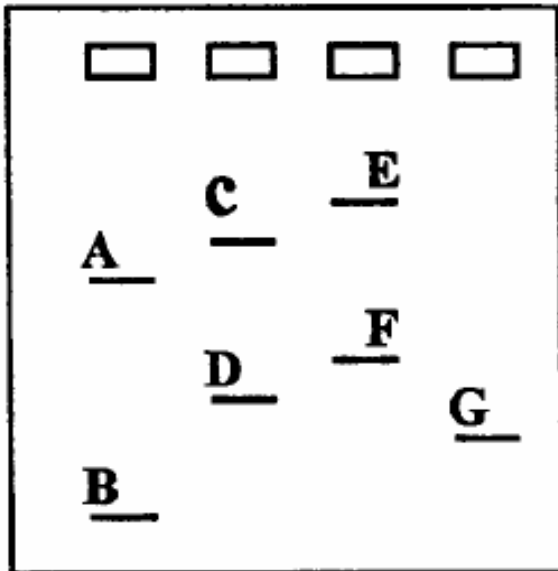
Staining the gels will be time consuming and can **only be done by the teacher.**

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 Student Pre-Lab Questions:

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.



Answer:

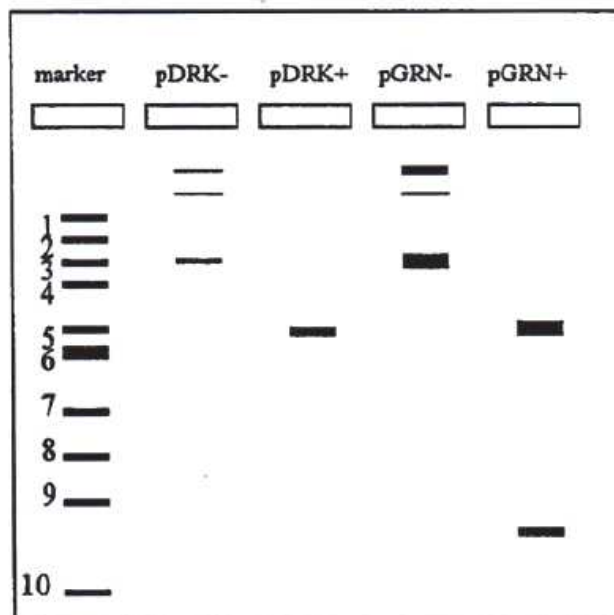
B,G,D,F,A,C,E (small to large)

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.

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



Description of lanes:

Lane 1. Marker (=1 kb ladder). Numbers are given in kilobases so the upper band is actually 10,000 base pairs. The smallest band is 500 base pairs.

Lane 2. This is uncut pDRK and three bands may be visible although they will not be very bright. The lowest band is supercoiled plasmid, the next band (toward the well) is nicked-circle and the upper bands(s) are a multimer(s) (there may be several).

Lane 3. This is the digested pDRK. Since this plasmid only has one *Hind* III restriction site, only one fragment is produced.

Lane 4. This lane is uncut pGRN. It's brighter than the pDRK lane because its concentration is much higher. Supercoiled, nicked-circled and multimer(s) may be present.

Lane 5. This is the digested pGRN. Because this plasmid contains two *Hind* III restriction sites, two fragments will appear. The smaller of the two represents the *gfp* gene.

Answers to Protocol Questions (Conclusions):

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

Answers will vary. It is not uncommon for students to not add an enzyme, but that's why we run load controls. Also, they may not load the gels in the order suggested in the protocol. This is okay as long as they've kept a record or can figure out what is in each lane by making reference to the control plasmids.

2. Are there any bands that appear in your gel photo that are not expected?

Again, answer will vary.

What could explain the origin of these unexpected bands?

Using control uncut plasmids as controls and comparing their gels with others in the class, they should be able to figure out what they did or did not do.

3. Do you see evidence of the three plasmid forms in the uncut lanes?

The pDRK plasmid bands will be faint but you should be able to find the three bands on one of the gels in your class. In the pGRN lane, you will definitely see at least three bands.

Is there evidence of more than one form of multimer?

There is a possibility that two multimers will be present in the pGRN lane.

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

There are two reasons: First of all the recombinant plasmids are not supercoiled but open circles. Secondly, many contain numerous inserts so they are quite large in terms of base pairs.

5. The 649 bp of pGRN fragment, carrying the *gfp* gene, will 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?

If you look carefully in the "L" lane, at a distance just above the 0.5 kb fragment, you should see a faint band. This is the circularized 649 bp fragment that represents the *gfp* gene. It does not migrate as far as the same fragment in the pGRN + lane because it is circularized.

Suggestions for transformation lab:

In these labs, students have (hopefully) produced the pGLO plasmid that is used in the transformation lab that EBBEP teachers have done for many years. You now have some decisions to make regarding how you wish to handle the transformation.

Although students may have produced the pGLO plasmid, the concentration of this plasmid in the mix is going to be relatively low compared to what we usually use for the transformation lab. Our suggestions are the following: (1) you use the concentrated provided pGLO plasmid for transformation as you have in the past, (2) you use the concentrated provided pGLO plasmid for most of your class while using the plasmid mixture from the labs in one lab group to see if there are any transformants from this, (3) you use the plasmid mixture from the labs in all but one lab group, and provide only one lab group with the purified pGLO plasmid, (4) you “spike” the plasmid mixture from the labs with the purified pGLO plasmid provided.

Each of these alternatives presents some problems and some benefits.