

# EXERCISE 9A

Name \_\_\_\_\_

## How are plasmids used in recombinant DNA technology?

### Day One: *Transformation of E. coli and digestion of plasmid DNA with restriction enzymes*

#### Objectives

After completing this exercise, you should be able to:

- ◆ Define gene cloning, recombinant DNA technology, and genetic engineering.
- ◆ Describe how genes are cloned, and explain the usefulness of gene cloning.
- ◆ Explain the role of vectors, restriction enzymes, and DNA ligase in genetic engineering.
- ◆ Give examples of different types of vectors used in genetic engineering.
- ◆ Describe the process of transformation and carry out this procedure in the laboratory.
- ◆ Inoculate agar plates with bacteria.
- ◆ Describe the function of a selectable marker in the isolation of transformants.
- ◆ Perform a restriction digest of plasmids.
- ◆ Pour and run an agarose gel to separate DNA fragments produced by a restriction digest.

#### Prelab

**Before you come to lab, read this entire exercise. You must also answer all questions and complete all assignments on the first 8 pages of this exercise. Your instructor will give you directions on when and where to turn in your work.**

**Gene cloning** is a method for making many identical copies of a gene. This technique permits biologists to produce a sufficient quantity of a single gene so that they can study the gene in detail—determining not only its DNA sequence, but also how the gene functions. Biologists can also induce cells containing cloned genes to produce large quantities of the protein coded for by the cloned gene. By making large amounts of “proteins to order” in this way, scientists can study the protein in detail. For example, the study of a particular enzyme allows scientists to learn a great deal about its function in the cell, its kinetics, whether cofactors are required, etc.

Gene cloning also provides a way for scientists to produce large quantities of proteins that have commercial value. For example, scientists can produce large quantities of proteins that are used as enzymes in commercial manufacturing and food processing techniques. Other proteins produced by gene cloning, such as human growth hormone, are medically useful. Before these techniques were developed, children deficient in human growth hormone had no hope of attaining normal height unless they had access to the small amount of the hormone made by extracting it from the pituitary glands of human cadavers. Growth hormone made in this way was rare, expensive, and carried the risk of contamination. Now this hormone, produced from cloned genes, is widely available, less expensive, and safer.

Perhaps the simplest way to clone a gene is to induce bacteria to take up the gene you wish to clone. Under the appropriate conditions, the bacterial cells will replicate the “foreign” DNA when they replicate their own DNA. With each bacterial division, the number of copies of the “foreign” gene doubles. Because bacteria can replicate so rapidly, this generates huge numbers of copies of the foreign gene quickly and easily.

**Vectors** are tools used by molecular biologists to insert pieces of foreign DNA into host cells. Two naturally occurring vectors are **bacteriophages** and **plasmids**. Bacteriophages (or phages, for short) are viruses that infect bacterial cells by injecting their genetic material into the bacterial cell. **Plasmids** are small circles of DNA that are sometimes present in bacteria in addition to the larger circle of DNA that constitutes the main bacterial genome. Many bacteria readily absorb plasmids from the environment. Like the main bacterial genome, plasmid DNA contains genes that code for specific proteins. Since plasmids are optional, so to speak (some members of a particular bacterial species may have them and some may not), the proteins they code for are not necessary for the bacterium's survival under normal conditions. However, these proteins may confer an advantage under certain conditions. For example, genes for antibiotic resistance are often found in plasmids.

One way scientists can insert foreign DNA into host cells is by combining the foreign DNA with the DNA of a vector. When scientists combine DNA from 2 different sources, the resulting DNA is called **recombinant DNA**. If the recombinant DNA gets inside a host cell, it can replicate along with the DNA of the host cell. This means that every time the host bacterium undergoes cell division, each new daughter cell receives a copy of the recombinant DNA, thus amplifying the recombinant DNA with each cell division.

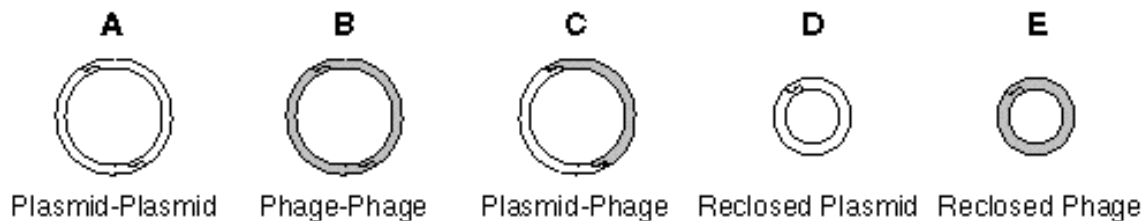
Although many different phages and plasmids occur naturally, the phages and plasmids used for gene cloning are usually artificial constructs. Often they include pieces of foreign DNA that have added to the original DNA of the vector. For example, some plasmids contain pieces of phage DNA that make them especially versatile. These plasmids are called **phagemids**. Another type of plasmid vector with both plasmid and phage components is the **cosmid**, which allows the cloning of especially large stretches of DNA.

The following procedure describes how scientists can combine phage DNA with a plasmid called **pUC18** to form a recombinant vector. It also explains one way scientists can screen *E. coli* cells (a bacterium commonly used in genetic engineering experiments) to determine which cells have absorbed the recombinant vector:

1. The plasmid pUC18 includes 2 genes. One gene confers resistance to the antibiotic ampicillin. Bacteria that contain this gene are able to grow in the presence of ampicillin, while bacteria that lack this gene are not. The second gene is called the *lacZ* gene. The *lacZ* gene codes for the enzyme  $\beta$ -galactosidase. This enzyme is normally used by *E. coli* cells to digest lactose. However, when bacteria with this gene are grown in a medium containing a synthetic analog of lactose called Xgal, the enzyme digests Xgal and releases compound "X", which is blue. Therefore, bacteria with a functional *lacZ* gene will produce a blue color when grown in the presence of Xgal.
2. A restriction enzyme is used to linearize the pUC18 plasmids. This means each circular plasmid is cut at a single location to produce a linear piece of DNA. The restriction enzyme that is used is called EcoRI. This enzyme will cut the plasmid at a single point, somewhere within the *lacZ* gene. The same restriction enzyme is used to cut the phage DNA into fragments:



3. The linearized plasmids and the phage DNA fragments are combined. Complementary sticky ends allow the pieces of DNA to anneal in five possible combinations.



Note that only combination "C" contains **recombinant DNA**. Recombinant DNA is made by joining DNA from 2 different sources, in this case, the plasmid and the phage. All the other combinations (A, B, D, and E) contain **non-recombinant DNA** (DNA from one source only.)

4. *E. coli* cells are incubated with the DNA vectors. During incubation, some *E. coli* cells will absorb no DNA, some will absorb non-recombinant DNA (either plasmid DNA alone or phage DNA alone), and some will absorb a recombinant plasmid. How do scientists know which bacterial cells absorbed a recombinant plasmid?
5. To screen for bacterial cells that contain a recombinant plasmid, the bacteria are placed on growth medium that contains ampicillin and Xgal. There are 3 possible outcomes:
  - a. *E. coli* cells that absorbed no DNA at all, as well as those that absorbed only phage DNA (combinations B and E above) will lack the gene for ampicillin resistance (present only on the plasmid.) Therefore, these bacteria will not grow and no bacterial colonies will be formed.
  - b. *E. coli* cells that absorbed only plasmid DNA (combinations A and D above) will have the gene for ampicillin resistance and the *lacZ* gene. The gene for ampicillin resistance allows them to grow and form colonies. The *lacZ* gene produces  $\beta$ -galactosidase which digests Xgal and releases compound "X", which is blue. Therefore, these *E. coli* cells will produce blue colonies.
  - c. *E. coli* cells that absorbed a recombinant plasmid (combinations C above) will have the gene for ampicillin resistance (present on the plasmid.) Therefore they will grow and produce colonies. However, the *lacZ* gene on the plasmid has been cut into 2 pieces by the restriction enzyme and a piece of phage DNA has been inserted between the 2 parts of the *lacZ* gene. This inactivates the *lacZ* gene, meaning no  $\beta$ -galactosidase will be produced. Therefore, Xgal is not digested, no blue color is produced, and the colonies remain white.

### ***How do I work safely with bacterial cultures?***

The bacterial strains used by molecular biologists are generally safe to work with. *E. coli*, in particular, is a common species that is present in the digestive tract of all warm-blooded animals. It is usually harmless. When working with bacteria, however, it is wise to remember that mutations may occur in any cell. For this reason, it is important to handle any bacterial culture *as if* it were potentially pathogenic. The reasons for using safe practices when working with any bacteria are similar to the reasons why universal precautions are taken in hospitals when handling any body fluids. Although the majority of hospital patients do not have a blood borne disease like HIV or hepatitis, all body fluids are handled *as if* they were contaminated.

### **Safety procedures for handling bacteria:**

1. Clear your work area of all non-essential items such as purses, clothing, backpacks, and books you will not need during lab.
2. Wipe off your lab table with disinfectant solution before you begin.
3. Remove dangling jewelry and make sure long hair is pinned or tied out of the way.
4. Wear gloves when working with bacterial cultures and dispose of the gloves in a BIOHAZARD bag.
5. **Food and beverages are NOT allowed in the lab room.** Never place pens, pencils, fingers, labels, or any other items in your mouth while in the lab.
6. Avoid spilling solutions that contain bacteria. In case of an accidental spill, inform your instructor. Wipe up as much of the spilled material as you can with paper towels and dispose of them properly. Thoroughly soak the area where the spill occurred with disinfectant solution. Wait 20 minutes before wiping up the disinfectant solution.

7. Dispose of materials and supplies used during the lab as follows:
  - Plastic and paper disposable items (e.g. paper towels, gloves) should be placed in a BIOHAZARD bag.
  - Any sharp disposable item that could puncture a biohazard bag and pose a danger to the person who must handle the bag should be placed in a hard-sided plastic or cardboard “sharps” container.
  - Reusable items should be submerged and left in a dilute bleach solution.
  - If you have any doubt about how to dispose of an item, ask your instructor. **Don’t guess!**
8. When you have completed the lab activity, remove all items from your lab table and wipe it with disinfectant solution.
9. Before you leave the lab, wash your hands thoroughly with soap and warm water.

***How efficient is gene cloning for producing many copies of a gene?***

In this lab exercise, you will use a plasmid called pUC18 as a vector to introduce foreign DNA into *E. coli* cells. Under ideal conditions, *E. coli* can undergo binary fission every 20 minutes. Imagine that one *E. coli* cell with a single plasmid containing a foreign gene was inoculated into a flask of nutrient broth and incubated for 12 hours. **How many copies of the foreign gene could it produce?**

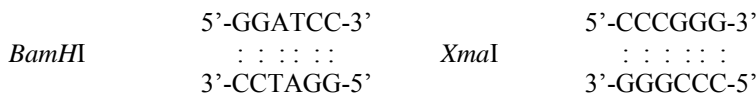
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***How are restriction enzymes and DNA ligase used to combine foreign DNA with a plasmid?***

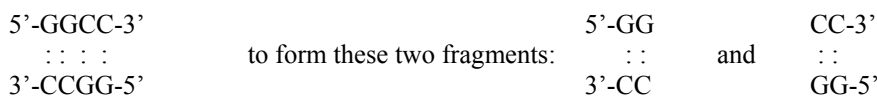
In order to use a plasmid to insert “foreign” DNA into a bacterial cell, two steps are required:

- 1) The “foreign” DNA must be combined with a plasmid. When DNA from 2 different sources is combined, the resulting DNA is called **recombinant DNA**. The procedure used to make recombinant DNA is called **recombinant DNA technology**.
- 2) A bacterial cell must absorb the recombinant plasmid

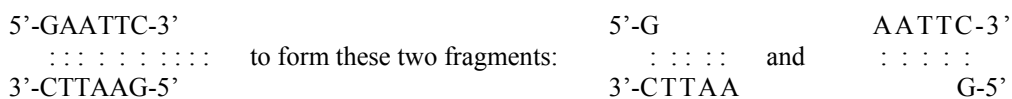
For the first step, restriction enzymes and DNA ligase are used. **Restriction enzymes** are naturally occurring enzymes that cut DNA. Many restriction enzymes have been isolated from bacteria, providing a valuable tool for molecular biologists. Each restriction enzyme cuts DNA only where a specific sequence of base pairs occurs. For example, these are the sequences cut by the restriction enzymes *Bam*HI and *Xma*I:



An important difference in restriction enzyme types is whether the cuts they make produce “blunt ends” or “sticky ends.” *Hae*III cuts this sequence:

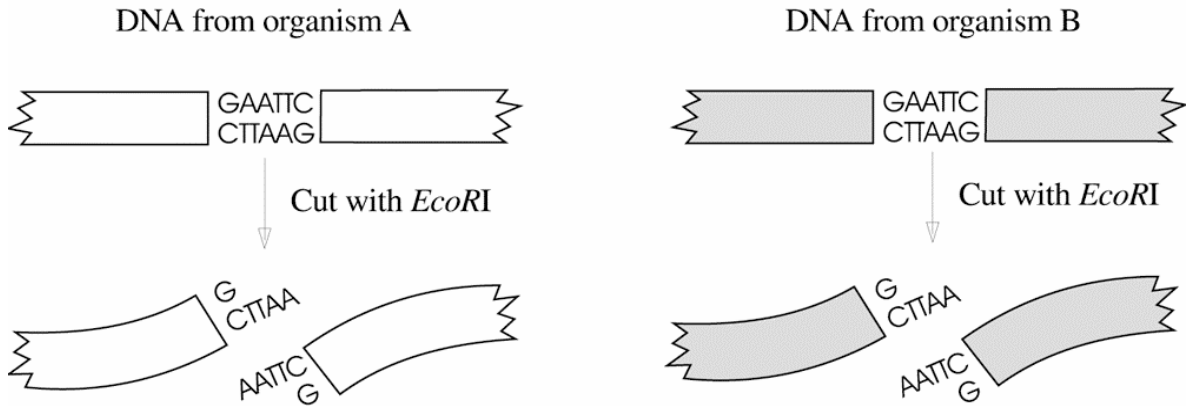


Because these 2 fragments do not have any unpaired bases, we say they have “**blunt ends**.” *Eco*RI, on the other hand, cuts this sequence:

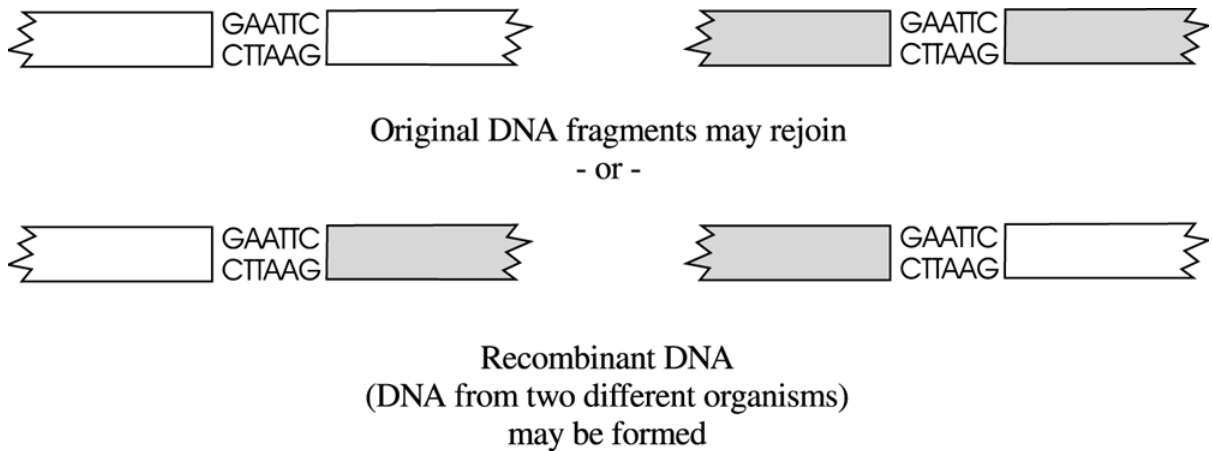


Because these 2 fragments have unpaired bases which can potentially pair with complementary bases, we say they have “**sticky ends**.”

By cutting DNA from two different sources with the same restriction enzyme, DNA fragments with complementary sticky ends are formed. When these fragments are mixed together, the complementary single-stranded ends may join together to form double stranded DNA by hydrogen bonding between the bases. **Add lines to the illustration below to link each DNA fragment with the two other fragments that it could join with, based on complementary base-pairing:**



If the DNA fragments are mixed together, they will join together at random, provided their unpaired bases match. Therefore, sometimes pieces of DNA from the same source will join back together, and sometimes pieces of DNA from different sources will join together, as shown below.

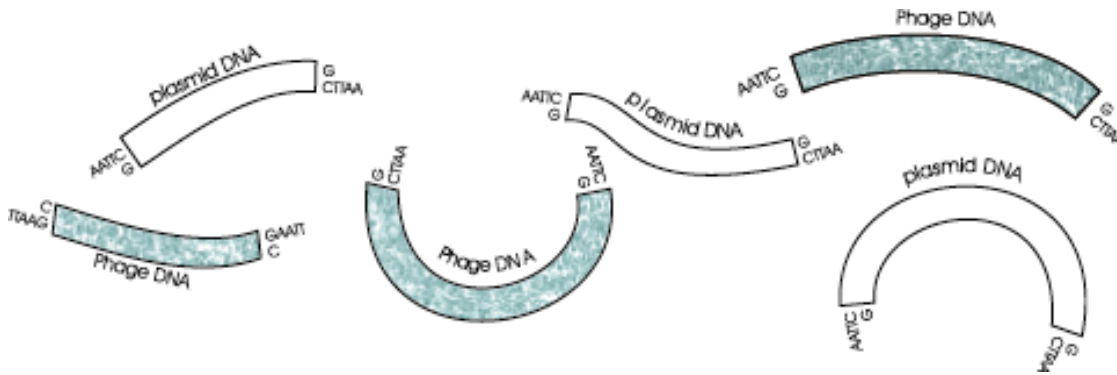


Hydrogen-bonding between complementary bases is not sufficient to completely rejoin the DNA fragments. The broken bonds between the deoxyribose and phosphate groups that form the “side-rails” of the DNA double helix (the phosphodiester linkages) must also be repaired. **DNA ligase** is the enzyme, also isolated from bacteria, that catalyzes this reaction.

*EcoRI* is the restriction enzyme you will use to cut pUC18 plasmids during this exercise. If a pUC18 plasmid is cut in only one location by the restriction enzyme *EcoRI*, what will happen to the overall shape of this plasmid?

**It will be changed from a circular form to a \_\_\_\_\_ form.**

If pUC18 plasmids that have been cut with *EcoRI* are mixed with fragments of phage DNA that have also been cut with *EcoRI*, there are several ways the DNA pieces may join together. Color the cut plasmids and the phage DNA fragments illustrated below with two different colors to represent DNA from the two different sources:



Now, use the space below to draw 5 circles that show the five possible ways circular pieces of DNA can be formed by joining the ends of either one or two of the DNA fragments above. Note that the 2 ends of one piece of DNA can join to form a circle, or two different pieces of DNA can join to make a circle. Color the circles using the same colors that you used above to distinguish between plasmid DNA and phage DNA. Label your 5 drawings A through E.

Which of your drawings (A-E) above represent a recombinant plasmid? \_\_\_\_\_

Which drawings represent plasmids that are not recombinant? \_\_\_\_\_

Which drawings represent circular pieces of DNA that do not include a plasmid? \_\_\_\_\_

## ***How can I induce E. coli cells to absorb plasmids?***

Remember that a plasmid is only a small circle of DNA. It does not contain the enzymes, nucleotides, and other molecules needed to copy itself. Therefore, in order to replicate, the plasmid must be inserted into a living host cell. To get the plasmid into a host cell, molecular biologists exploit the natural tendency of many bacteria to absorb fragments of DNA from the surrounding medium. This process is called bacterial **transformation**.

At first glance, *E. coli* might seem like a poor choice for use in genetic engineering experiments because it does not naturally or easily undergo transformation. However, *E. coli* has many other advantages--its genetics and life cycle are probably better understood than any other organism, it is extremely easy to grow, and there are many different strains available for the molecular biologist to choose from. When it was discovered that cold and the presence of calcium ions could induce *E. coli* to absorb plasmids, scientists were able to devise a simple procedure for transforming *E. coli* cells. Through the use of cold and calcium ions, *E. coli* cells are said to be rendered "competent," that is, competent to take in plasmids by transformation.

The basic procedure for transforming *E. coli* cells is described below:

- \_\_\_\_\_ First, the *E. coli* cells are suspended in a CaCl<sub>2</sub> solution and placed on ice.
- \_\_\_\_\_ After about 20-30 minutes, plasmids and the *E. coli*/CaCl<sub>2</sub> suspension are mixed together.
- \_\_\_\_\_ The plasmid/*E. coli* mixture is incubated on ice for another 30 minutes.
- \_\_\_\_\_ The plasmid/*E. coli* mixture is given a heat shock at 37-45° for a few minutes, which improves the efficiency of the transformation.
- \_\_\_\_\_ The plasmid/*E. coli* mixture is mixed with nutrient broth and placed in a warm water bath for 30 minutes to an hour. During this time, the *E. coli* cells will proceed with their normal metabolism. This gives the cells a chance to produce some of the proteins encoded by the plasmid DNA, which will allow us to determine which *E. coli* cells did in fact take in a plasmid.

**Read through the Lab Procedures for Exercise 9A. Then, in the spaces provided, write down which of the steps listed above will be performed by you and which will be performed by your instructor.**

## ***How do I know which E. coli cells have absorbed a recombinant plasmid?***

Using the procedure described above, you will attempt to transform *E. coli* cells by mixing them with 3 different unknown solutions. The strain of *E. coli* that you will be using (DH5) is killed by ampicillin and does not have the lacZ gene. The 3 unknown solutions will be labeled A, B, and C:

- One unknown solution contains no DNA at all.
- One unknown solution contains normal pUC18 plasmids. These plasmids contain a gene for ampicillin resistance, and the lacZ gene. Recall that the lacZ gene codes for the enzyme β-galactosidase. This enzyme is normally used by *E. coli* cells to digest lactose. However, when bacteria with this gene are grown in a medium containing a synthetic analog of lactose called Xgal, the enzyme digests Xgal and releases compound "X", which is blue.
- One unknown solution contains recombinant pUC18 plasmids. Like the normal pUC18 plasmids, the recombinant pUC18 plasmids have the lacZ gene and the gene for ampicillin resistance. However, the recombinant pUC18 plasmids also contain a piece of foreign DNA from a phage named λ. In order to insert the phage DNA into pUC18, pUC18 was cut with a restriction enzyme called EcoRI. EcoRI cuts pUC18 at a site located within the lacZ gene. Therefore, the extra piece of phage DNA was inserted into the lacZ gene and inactivated it. This means no β-galactosidase will be produced. Therefore, Xgal is not digested, no blue color is produced, and any colonies that grow will remain white.

After incubating competent *E. coli* cells with the 3 unknown solutions described above, the cells will be inoculated on 3 separate Petri dishes containing nutrient agar with ampicillin and Xgal. The Petri dishes will be incubated for several days at 37° C to allow bacterial growth to occur. "Bacterial growth" refers to an increase in the number of cells present, not an increase in the size of an individual cell. When placed on nutrient agar, one bacterial cell can divide over and over again until there are millions of descendants on the plate. Although one bacterium is not visible to the naked eye, the millions of descendants piled together appear as a shiny dot, called a bacterial colony, on the surface of the agar.

After you have incubated your Petri dishes at 37° C for several days, what do you expect to see in the dish inoculated with *E. coli* that were mixed with the unknown solution that contained no DNA at all? Explain your answer.

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After you have incubated your Petri dishes at 37° C for several days, what do you expect to see in the dish inoculated with *E. coli* that were mixed with the unknown solution that contained the normal pUC18 plasmids? Explain your answer.

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After you have incubated your Petri dishes at 37° C for several days, what do you expect to see in the dish inoculated with *E. coli* that were mixed with the unknown solution that contained the recombinant pUC18 plasmids? Explain your answer.

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## Lab Procedures:

### I. Treat *E. coli* cells so they become competent to take up plasmids

These 4 steps will be performed by your instructor. After the cells are competent, all student groups will obtain bacteria from this sample.

#### Instructor:

1. Locate the Eppendorf tube labeled *E. coli* and the vial of CaCl<sub>2</sub> in the ice bath.
2. Using a sterile transfer pipet, add about 0.5 mL of the CaCl<sub>2</sub> solution to the Eppendorf tube containing the *E. coli*. Mix by gently suctioning and expelling the solution into the Eppendorf tube with the pipet a few times.
3. Using the same pipet, transfer the *E. coli*/CaCl<sub>2</sub> mixture back to the vial containing the remainder of the CaCl<sub>2</sub> solution. Gently swirl or tap the vial to mix.
4. Place the vial containing the *E. coli* suspended in CaCl<sub>2</sub> in the ice bath and leave it there for at least 20 minutes. (Note: The suspension can remain in the ice bath as long as 12 hours.)

### II. Transform the *E. coli* with the 3 unknown plasmid samples

In this part of the lab, you will attempt to transform the competent *E. coli* cells with 3 unknown plasmid samples. The unknown samples are in Eppendorf tubes labeled A, B, and C:

- One sample contains no DNA at all
  - One sample contains normal pUC18 plasmids with functional lacZ and ampicillin resistance genes.
  - One sample contains recombinant pUC18 plasmids that have a segment of λ DNA inserted at the EcoRI site. The λ DNA disrupts the lacZ gene, but the ampicillin resistance gene is unaffected.
1. Obtain three empty, sterile Eppendorf tubes and label them A, B, and C. Using tape, label a floating Eppendorf tube holder with your group name and “transformation.” Place your A, B, and C Eppendorf tubes in it. You will be adding unknown plasmid samples to these tubes, but before you do, carefully read the three reminders below:
    - ⇒ The 3 Eppendorf tubes that contain the unknown plasmid samples will be available at the instructor’s desk or at the general supply area for your class. The small amounts of sample in each tube must supply all the groups in your class. Therefore, it is essential that you withdraw the proper amount and no more, so that there will be enough for everyone.
    - ⇒ Be sure to use the micropipettor correctly. Remember, in Exercise 1 you saw that using it incorrectly could result in withdrawing as much as twice as much solution as you intended. Also, check to make sure you are using the correct micropipettor and that it is set to 10 μL.
    - ⇒ It is essential not to cross-contaminate these samples. Use a new sterile tip *every time* you withdraw a sample. If one group is careless and mixes the different unknowns by not using a new tip each time, then every group to obtain sample afterwards will be attempting to perform this exercise with a contaminated sample. (Odds of obtaining decent results when using a contaminated sample are very poor.)

- Using a sterile tip for the 2-20  $\mu\text{L}$  micropipettor, withdraw 10  $\mu\text{L}$  of the “A” plasmid unknown. Check to make sure you are measuring correctly; 10  $\mu\text{L}$  is a very small amount, so the liquid should fill only about the last cm of a yellow tip

Touch the tip of your micropipettor to the side of your sterile “A” tube near the bottom. After expelling the solution, you should see a small droplet on the side of the tube. Discard the tip and obtain a new sterile tip.

- Transfer 10  $\mu\text{L}$  of “B” plasmid unknown to your tube marked B. Replace the tip and transfer 10  $\mu\text{L}$  of “C” plasmid unknown to your tube marked C.
- Next you will add competent *E. coli* cells to your 3 unknown plasmid samples. Take your Eppendorf tubes to the ice bath containing the vial with *E. coli* in  $\text{CaCl}_2$ .

**The *E. coli* cells tend to settle to the bottom of the  $\text{CaCl}_2$  solution, so gently swirl the vial each time before withdrawing the solution.**

Using a 100-1000  $\mu\text{L}$  micropipettor with a sterile tip, add 100  $\mu\text{L}$  of the *E. coli* suspension to your Eppendorf tube labeled A. Using a new sterile tip each time, add 100  $\mu\text{L}$  of the *E. coli* suspension to your B and C tubes. Be sure to mix the plasmid DNA with the *E. coli* suspensions by tapping the tube. Dispose of your pipet tips immediately in a biohazard bag.

- Place your *E. coli*/plasmid mixtures in an ice bath and leave them there for at least 30 minutes. It is okay for them to remain in the ice for up to 90 minutes. **During this time, begin Part III, below.**
- Remove the tubes from the ice and place them in a 42° C water bath for exactly 90 seconds. This is a heat shock step that favors the uptake of the plasmids by the *E. coli*. If a 42°C water bath is not available, place your tubes into a 37°C water bath for 5 minutes, instead.
- Remove the tubes from the water bath and add 0.7 mL of nutrient broth to each tube, using a clean sterile micropipettor tip for each tube. Return the tubes to a 37° water bath for 30 minutes. **During this time, pour your agarose electrophoresis gels as described in Part IV.**
- Obtain 3 Petri plates containing nutrient agar with Xgal and ampicillin, and label them. When labeling Petri plates, always label the bottom of the plate (the part that contains the agar), not the lid. Use small print, along the outer edge of the plate, so your writing does not interfere with viewing the contents of the plate later on. Label the three plates A, B, and C. In addition, write the following information on each plate:
  - “transformed *E. coli* cells”
  - your instructor’s name
  - lab day and time
  - your group name
  - today’s date
- When the 30 minute incubation is complete, remove your “transformation” tubes from the 37° C water bath.
- Using a micropipettor and sterile tip, transfer 0.25 mL of the contents of your Eppendorf tube A to the surface of the agar plate labeled A. Dispose of your pipet tip immediately in a biohazard bag.
- With an inoculating loop, **gently** spread the liquid broth over the **surface** of the agar. Do not pierce or stab the surface of the agar. Dispose of your inoculating loop immediately in a biohazard bag.
- Repeat steps 10 and 11 with your B and C samples, using a clean pipet tip and a new sterile inoculating loop each time.
- Tape the plates closed and give them to your instructor for incubation. **You are now ready to finish Part IV, beginning with step 3.**

### III. Digest the plasmids in the 3 unknown samples with the restriction enzyme *EcoRI*

In this part of the lab, you will cut the plasmids in the 3 unknown samples with the restriction enzyme *EcoRI*. In the next part of the lab, you will use agarose gel electrophoresis to separate the resulting DNA fragments by size.

1. Label 3 empty, sterile Eppendorf tubes A, B, and C. Place the tubes in a floating Eppendorf tube holder labeled with your group name and the words “restriction digest.”
2. Place 10  $\mu\text{L}$  of *EcoRI* solution in the bottom of each of the 3 Eppendorf tubes. Since you are measuring the same solution into three empty, sterile tubes, you can use the same tip for all three transfers.

Remember to use the micropipettor correctly and measure carefully, so there is enough *EcoRI* solution for all groups.

3. Take your 3 Eppendorf tubes to the area where the A, B, and C plasmid unknown stock solutions are located. With a new, sterile micropipettor tip, add 5  $\mu\text{L}$  of the “A” plasmid unknown to your A tube. Using a clean tip each time, add 5  $\mu\text{L}$  of the “B” plasmid unknown to your B tube and 5  $\mu\text{L}$  of the “C” plasmid unknown to your C tube.
4. Tap the tubes gently to mix the plasmids with the restriction enzyme and then spin them for a few seconds in the microcentrifuge to make sure all of the solution is at the bottom of the tube.
5. Place your 3 Eppendorf tubes in the 37° C water bath and leave them there for 60 minutes. ***During this time, return to Part II, step 6.***

### IV. Separate the DNA fragments using agarose gel electrophoresis

1. Obtain a bottle of melted agarose from the water bath and pour the contents into the gel casting tray fitted with a 6-well comb as explained by your instructor.
2. Allow the gel to set until it has solidified (about 20 minutes.) ***While the gel sets, return to Part II, step 8.***
3. Plug in your power supply (which is not yet connected to the electrophoresis apparatus). Turn it on and set it to 100 volts. Then turn it off and unplug it.
4. After your gel is set, lower the 2 adjustable sides of the casting tray. Place your gel, in its casting tray, into the electrophoresis apparatus. Make sure that the wells of the gel are at the side where the cathode (black electrode) will connect.
5. Gently pour electrophoresis running buffer into the sides of the apparatus (not onto the gel itself) until the buffer is 2-3 mm above the gel. Ask your instructor to check your set-up before you proceed.
6. When the 60 minute incubation is complete (Part III, step 5), remove your 3 “restriction digest” tubes from the 37° C water bath. Add 5  $\mu\text{L}$  of Electrophoresis Sample Buffer to each tube. Locate the Eppendorf tube labeled “DNA markers”, and spin all 4 tubes in the microcentrifuge for a few seconds.
7. Load 15  $\mu\text{L}$  of sample into each well of your gel according to the plan below. To assign numbers to the lanes, view the gel with the wells oriented towards the top, then count from left to right.

- Lane 1-- Unknown sample A + *EcoRI*
- Lane 2-- DNA markers
- Lane 3-- Unknown sample B + *EcoRI*
- Lane 4-- DNA markers
- Lane 5-- Unknown sample C + *EcoRI*
- Lane 6-- leave empty

- Place the lid on the electrophoresis apparatus and connect the power supply to it. Have your instructor check your set-up and then plug in the power supply and turn it on.
- Allow the gel to run until the blue tracking dye is at the end of the gel. This should take about 30 minutes.
- While the gel is running, use tape to label the bottom of a large Petri dish with your instructor's name, your lab day and time, your group name, and the date. Later, you will use this Petri dish to stain your gel.
- When the blue tracking dye reaches the end of your gel, turn off the power supply and unplug it.

**NEVER REMOVE THE COVER FROM THE ELECTROPHORESIS APPARATUS WHEN IT IS PLUGGED IN! SEVERE ELECTRICAL SHOCK COULD OCCUR!**

*After unplugging the apparatus*, remove its cover. The buffer solution will be hot! Use a transfer pipet to withdraw and discard buffer until it is possible to remove the gel in its casting tray without burning yourself. Make sure you wear gloves when you remove the gel and casting tray from the electrophoresis apparatus.

- Slide the gel out of the casting tray and into the labeled Petri dish. With the wells oriented towards the top of the gel, use a spatula to cut and discard a small triangular piece (approximately 5 mm on a side) from the lower right corner of the gel. When you analyze your gel, this will allow you to identify the lower right corner.
- Pour enough stain into the Petri dish to cover the gel. Give the dish to your instructor who will store it in the refrigerator until the next lab period.

*When you return to lab next week, you will examine and analyze your stained gel for bands of DNA, and check the Petri plates inoculated with E. coli for bacterial growth.*

## Clean up

Dispose of any materials that had contact with *E. coli* cells in a **biohazard bag**.

Be careful not to lose any parts of the gel apparatus, including the casting tray and the gel comb. Wash them in soapy water, rinse with tap water and dH<sub>2</sub>O, and leave on absorbent toweling to dry.

Dispose of your excess solutions as directed by instructor.

Remove label tape and any marks made with a marking pen from all glassware. Wash and rinse all glassware, give it a final rinse with dH<sub>2</sub>O, and leave it inverted at your work area in order to drain.

All disposable glassware goes into the special glass disposal receptacle.

All instruments should be turned off and unplugged.

Wipe off your workspace with a damp paper towel.

Make sure everything that you have used is clean, put away, or discarded. Leave your work area in the same order that you found it in.

Wash your hands and ask your instructor to check your work area before you leave.

## Postlab:

1. The DNA sequences that are recognized by restriction enzymes are referred to as palindromic. Explain why.
2. Synthetic plasmids like pUC18 are often designed to have an MCS (multiple cloning site) region, also called a “polylinker” region. This region of the plasmid contains single (unique) recognition sites for several restriction enzymes. For example, pUC18’s MCS region contains one recognition site each for the following restriction enzymes: *EcoRI*, *SacI*, *KpnI*, *SmaI*, *BamHI*, *XbaI*, *Sall*, *BspMI*, *PstI*, *SphI* and *HindIII*. Why is a plasmid more useful if it has only one site for a particular restriction enzyme, rather than several sites for the same restriction enzyme?
3. The plasmid pUC18 is 2686 base pairs in size. Draw a circular diagram, or map, of pUC18 that shows the location of the features in the table below. **Note:** Number your diagram clockwise.

- bp 861--Replication origin. (This is the site where DNA copying of the plasmid is initiated.)
- bp 236-469--lac-Z gene
- bp 399-450--MCS region (Multiple Cloning Sites-where many restriction sites have been inserted)
- bp 396--*EcoRI* site
- bp 1626-2486--ampicillin resistance gene (for  $\beta$ -lactamase)

4. Draw another diagram, similar to the one you drew for question 3, that represents the pUC18 plasmid with a segment of phage  $\lambda$  DNA inserted into it.
5. Draw the plasmids you diagrammed for questions 3 and 4 after an *EcoRI* digest. Use the same style or color coding that you used above.
6. The DNA sequence of the pUC18 plasmid is shown below, along with information about its engineering and publication history. This information is typical of the information available on the Internet about vectors, organisms, and DNA sequences used in molecular biology

Using the information from question 3, locate the six base sequence recognized by *EcoRI*. Underline or highlight this sequence and draw a vertical line where the DNA is cut when digested by *EcoRI*. (Note: The base sequence of only one strand of the double helix is shown, and the sequence should be read from left to right across the page, as in a page of printed text.)

### Accessed through GenBank at [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov):

[L09137](#). Cloning vector pUC18...[gi:20141090]

```
LOCUS       SYNPU19CV                2686 bp    DNA     circular SYN 22-MAY-2002
DEFINITION Cloning vector pUC18, complete sequence.
ACCESSION   L09137 X02514
VERSION     L09137.2  GI:20141090
SOURCE      Cloning vector pUC18
REFERENCE   1  (bases 1 to 2686)
AUTHORS     Yanisch-Perron,C., Vieira,J. and Messing,J.
TITLE       Improved M13 phage cloning vectors and host strains: nucleotide
            sequences of the M13mp18 and pUC19 vectors
JOURNAL     Gene 33 (1), 103-119 (1985)
MEDLINE     85180545
PUBMED     2985470
REFERENCE   2  (bases 1 to 2686)
AUTHORS     Chambers,S.P., Prior,S.E., Barstow,D.A. and Minton,N.P.
TITLE       The pMTL nic- cloning vectors. I. Improved pUC polylinker regions
            to facilitate the use of sonicated DNA for nucleotide sequencing
JOURNAL     Gene 68 (1), 139-149 (1988)
MEDLINE     89121486
PUBMED     2851488
REFERENCE   3  (bases 1 to 2686)
AUTHORS     Gilbert,W.
TITLE       Obtained from VecBase 3.0
JOURNAL     Unpublished
```

REFERENCE 4 (bases 1 to 2686)  
 AUTHORS Messing,J.  
 TITLE Direct Submission  
 JOURNAL Submitted (27-APR-1993) Department of Biochemistry, University of Minnesota, St. Paul, MN 55108, USA

REFERENCE 5 (bases 1 to 2686)  
 AUTHORS Messing,J.  
 TITLE Direct Submission  
 JOURNAL Submitted (11-APR-2002) Rutgers, The State University of New Jersey, Waksman Institute of Microbiology, 190 Frelinghuysen Road, Piscataway, NJ 08854-8020, USA

REMARK Sequence update by submitter  
 COMMENT On Apr 11, 2002 this sequence version replaced gi:[209213](#). These data and their annotation were supplied to GenBank by Will Gilbert under the auspices of the GenBank Curator Program. pUC19c - Cloning vector (beta-galactosidase mRNA on complementary strand)  
 ENTRY PUC18 #TYPE DNA CIRCULAR TITLE pUC19c - Cloning vector (beta-galactosidase mRNA on complementary strand)

DATE 03-FEB-1986  
 #sequence 16-DEC-1986  
 ACCESSION VB0033  
 SOURCE artificial  
 COLLECTION ATCC 37254

REFERENCE  
 #number  
 #authors Norrander J., Kempe T., Messing J.  
 #journal Gene (1983) 26: 101-106

REFERENCE  
 #number 1  
 #authors Yanisch-Perron C., Vieira J., Messing J.  
 #journal Gene (1985) 33: 103-119  
 #comment shows the complete compiled sequence

REFERENCE  
 #number 2  
 #authors Chambers,S.P., et al.  
 #journal Gene (1988) 68: 139-149  
 #describes mutation at nt1308 and its effect on copy number

REFERENCE  
 #number  
 #authors Pouwels P.H., Enger-Valk B.E., Brammar W.J.  
 #book Cloning Vectors, Elsevier 1985 and supplements  
 #comment vector I-A-iv-20

COMMENT  
 This Sequence was obtained 3-MAR-1986 from J. Messing, Waksman Institute, NJ on floppy disk.  
 Revised 16-DEC-1986 by F. Pfeiffer:  
 1062/3 'AT' to 'TA' to match revised sequence of PBR322  
 The beta-galactosidase mRNA sequence including the multiple cloning site of M13mp19 is on the strand complementary to that shown.

KEYWORDS  
 CROSSREFERENCE  
 #complement  
 VecBase(3):pUC19  
 #prerevised  
 GenBank(50):M11662, EMBL(11):ARPuc19  
 #parent  
 VecBase(3):pUC13, VecBase(3):M13mp19, VecSource(3):bGal19

PARENT  
 Features of pUC18 (2686 bp)  
 residue source  
 1- 137 2074-2210 pBR322  
 138- 237 2252-2351 pBR322  
 238- 395 1461-1304 (c) Lac-Operon  
 396- 452 57- 1 (c) polylinker of M13mp19  
 455- 682 1298-1071 (c) Lac-Operon

683-2686 2352-4355 pBR322

Conflict (cfl) and Mutations (mut):

pUC19 source

mut 1308 A G 2977 pBR322 linked to increased copy number

mut 1942 A G 3611 pBR322

mut 2243 T C 3912 pBR322

FEATURE

1629-2417 789-1 (c) Ap-R; b-lactamase

POLYLINKER HindIII-SphI-PstI-SalI-XbaI-BamHI-SmaI-KpnI-SacI-EcoRI

SELECTION

#resistance Ap

#indicator beta-galactosidase

SUMMARY pUC18 #length 2686 #checksum 4465.

FEATURES

source

Location/Qualifiers

1..2686

/organism="Cloning vector pUC19c"

/mol\_type="genomic DNA"

/db\_xref="taxon:174689"

ORIGIN

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61 cagcttgtct gtaagcggat gccggggagca gacaagcccg tcagggcgcg tcagcgggtg
121 ttggcgggtg tcggggctgg cttactatg cggcatcaga gcagattgta ctgagagtgc
181 accatattcg gtgtgaaata ccgcacagat gcgtaaggag aaaataccgc atcagcgccc
241 attcgccatt caggctgctc aactgttggg aagggcgatc ggtgcggggc tcttcgctat
301 tacgccagct ggcgaaaggg ggatgtgctg caaggcgatt aagttgggta acgccagggg
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