

# Activity #5b. Plasmid DNA Isolation, Restriction Enzyme Digestion & Bacterial Transformation

## Learning Goals:

- To learn about bacterial plasmids, one of the basic tools of genetic engineering
- To purify plasmid DNA from a bacterial culture
- To learn to measure small volumes of liquids using micropipettes
- To prepare competent cells and conduct transformation with plasmid DNA
- To confirm the identity of plasmid DNA using restriction enzyme digestion

## Lab Background and Procedures:

We have a lot to do today. You should be sure you've read ALL of the background sections before you set foot in the lab. We will be completing 5 procedures today:

Procedure A. Use of Micropipettes

Procedure B. Preparation of competent bacteria and transformation

Procedure C. Purification of pGLO plasmid DNA from bacteria

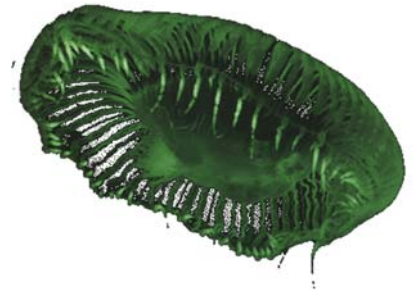
Procedure D. Digestion of plasmid DNA with restriction enzymes

To save time, we will begin with Procedure A, then Procedure B. During Procedure B there are several lengthy incubation steps. While you are waiting for these steps, you will do Procedures C and D, returning to parts of Procedure B at the appropriate time intervals. PLEASE BE SURE TO CHECK OFF EACH STEP AS YOU COMPLETE IT, SO THAT AS I MOVE BETWEEN LAB GROUPS I CAN SEE AT A GLANCE WHICH STEP YOU ARE SUPPOSED TO BE ON!!!!

## A1. Introduction

In this experiment, you will explore several fundamental techniques that have revolutionized biology! While incomplete, the recently acquired understanding of how organisms function at the subcellular level has changed the way scientists approach biological questions. Molecular Cell Biology has touched every corner of biology. Specific examples include the use of pre-implantation diagnosis and gene therapy in the medical field; the design of new, more specific drugs with fewer side effects; engineering of food crops to confer disease, frost, and drought resistance; genetic modification of bacteria for bio-remediation of polluted sites; and genetic analysis to determine evolutionary relationships between organisms or to track the migration of specific populations over time.

We will begin the exercise by learning **how to isolate and manipulate the genetic material, DNA**. The specific DNA we are interested in comes from the bioluminescent jellyfish *Aequorea victoria*. This DNA contains the jellyfish gene that encodes the instructions for a green fluorescent protein (GFP) that causes the jellyfish to fluoresce and glow in the dark.



The easiest way to work with DNA is to let bacteria do all the work for you. It is impractical to chemically synthesize large amounts of a specific DNA molecule, although it can be done. A better strategy is to place the specific DNA molecule into bacteria and provide nutrients that allow the bacteria to reproduce themselves and the foreign DNA to a density of  $>10^9$ /mL. One small complication, however, is that the jellyfish GFP gene does not have the right signals to be copied by bacteria. This problem can be overcome by inserting the GFP gene into another piece of DNA, a plasmid, that can be copied because it contains a bacterial origin of replication (ori).



In addition to one large circular chromosome, bacteria often contain one or more small, circular pieces of DNA called **plasmids**. Plasmid DNA usually contains genes for one or more traits that are beneficial to bacterial survival. In nature, bacteria can transfer plasmids back and forth to share these beneficial genes. This natural mechanism allows bacteria to adapt to new environments. The recent occurrence and spread of bacterial resistance to multiple antibiotics is due to the transmission of plasmids.

In this laboratory exercise, we will purify plasmid DNA from a bacterial culture. We will then analyze the structure and function of the plasmid DNA in the next few labs.

**Discuss the following questions within your group (4).**

What are the major components (macromolecules) of cells?

How is DNA chemically different from most of the other components of cells?

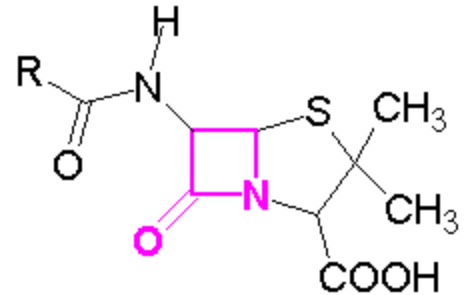
How could these differences be used to purify plasmid DNA from other cellular constituents?

What are the three functions we hypothesized the pGLO plasmid might encode?

## A2. Introduction to Bacterial Transformation

Restriction enzyme digestion and gel electrophoresis can provide information about physical characteristics of a particular DNA such as the size and relative position of specific base sequences. However, these do not provide information on the function of the DNA. To examine the function of a specific DNA, it must be studied *in vivo* (in life). What is the function of DNA?

DNA contains genes encoding proteins (usually) that do something for the organism. Two genes present on the pGLO plasmid encode a **beta-lactamase enzyme** and the **green fluorescent protein**. Beta-lactamase is an enzyme that breaks down members of beta-lactam family of antibiotics, which includes ampicillin, penicillin and amoxicillin. These antibiotics are used to treat infections because they normally kill *E.coli* and other bacteria. However, when a plasmid such as pGLO is introduced to bacteria, either intentionally or unintentionally, the bacteria express the beta-lactamase gene, breakdown the antibiotic, and therefore are not killed – they are now resistant to the antibiotic!



**Figure 1. Beta-lactam antibiotic structure.**

The process by which free DNA is taken up by and is incorporated into an organism is called **genetic transformation**. An organism with such DNA is referred to as **transgenic**. Transgenic plants, animals, yeast and bacteria have become critical tools for the pharmaceutical, agricultural, and biomedical industries.

Transforming plasmid DNA into bacteria is relatively simple. While the bacterial cell wall is porous, so that DNA can pass through it easily, the plasma membrane is more of a challenge. **Preparation of cells for transformation involves incubation with calcium ( $\text{Ca}^{2+}$ ) to neutralize the negative charges that would cause repulsion of the negatively-charged DNA.** Bacteria are also incubated **on ice** to induce the low temperature adaptation of increasing membrane permeability. Such cells are now **“competent”** to take up DNA. At this stage, a brief **heat shock** opens holes in the membrane, which allows the plasmid DNA to enter the cell. Fewer than 1% of the bacterial cells will actually take up DNA, so a method is necessary to identify those that have done so. Spreading the bacteria onto an agar plate containing the antibiotic **ampicillin** kills those cells that have not acquired the plasmid with the beta lactamase gene. Only the bacteria that have taken up the plasmid will be able to multiply. Since they are on an agar surface, this multiplication will form a visible “pile” or **colony** of bacteria on the plate.

A final concept demonstrated by this lab exercise is gene regulation. If all of the cells in your body have the same set of genes, why don't your brain cells produce the same digestive enzymes that your stomach cells produce?

All organisms regulate the expression of genes, in other words, they are able to control when specific genes are turned on and when they are turned off. In higher organisms, such as plants and animals, the primary function of gene regulation is cell and tissue specialization. Different types of cells express different sets of genes (and therefore produce different proteins) at different stages of development. In bacteria, the primary function of gene regulation is adaptation to the environment. The production of proteins is an energetically expensive process. It would be a huge waste of energy for a cell to produce the proteins necessary to breakdown a particular nutrient if the nutrient was not available. The expression of genes is generally controlled by the binding of proteins to a region in front of the gene called a promoter.

When the jellyfish green fluorescent protein gene was inserted into the plasmid, it was placed after a **promoter** that normally controls the expression of genes involved in metabolizing the sugar **arabinose**. When arabinose is absent, the promoter is “off” and the GFP gene is not expressed, i.e. the protein is not produced, or is produced only at very low levels. **In the presence of arabinose, the promoter is “on”, which allows the GFP gene to be transcribed into RNA very efficiently.** The RNA is translated to produce the protein, which causes the bacteria to glow green when exposed to an ultraviolet light source.

## Procedure A. Use of Micropipettes (Review from previous labs)

Compared to many other areas of biology, the technical skills required to do molecular biology are very simple; the key is to get comfortable working with small volumes. These small volumes are measured out using **micropipettors**. A “set” consists of three pipettors that together can accurately measure and dispense volumes from 0.5  $\mu\text{L}$  to 1000  $\mu\text{L}$  (1 mL).

- Volumes between 0.5  $\mu\text{L}$  and 20  $\mu\text{L}$  are measured using the smallest micropipette, the **I-20**. The yellow digit on the bottom of the setting display indicates tenths of a microliter, e.g. setting of “0 - 5 - 3” corresponds to 5.3  $\mu\text{L}$ .
- Some sets have **I-100’s** and some have **I-200’s** for the intermediate volumes between 20  $\mu\text{L}$  and 100 or 200  $\mu\text{L}$ . The 3 digits on the display show hundreds, tens and ones; e.g. “0 – 7 – 5” corresponds to 75  $\mu\text{L}$ .
- The largest micropipette, a **I-1000**, is used for volumes between 100 and 1000  $\mu\text{L}$ . The yellow digit at the top of the setting display indicates thousands of  $\mu\text{L}$ ; e.g. “0 – 8 – 0” corresponds to 800  $\mu\text{L}$  (or 0.80 mL).

Review the use of the pipettes, summarized below, with your lab partner.

8. **Set dial to desired volume.** Do not try to turn volume setting dial beyond the maximum volume for the pipettor. This is how students most frequently damage the pipettors.
9. **Attach a pipette tip.** The small yellow or white tips are used on the I-20, I-100 and I-200’s; the large blue tips are used with the I-1000 pipettors. Be sure tip is attached securely to pipettor, liquid will drip out if seal is not tight.
10. **Press plunger down to 1<sup>st</sup> stop. Place tip into liquid**
11. **Slowly release plunger so that liquid is drawn into pipette tip.** Always hold pipettors with the shaft pointing down, especially when liquid is in the tip. If liquid does get into the shaft, please tell your instructor immediately, so that it can be cleaned out. Damage and corrosion can occur if salt solutions dry inside pipettor.
12. **Dispense liquid into desired tube by pushing down plunger to the second stop. Remove tip from liquid before releasing plunger,** otherwise you will draw liquid back into tip.
13. **Eject used tips into coffee cans at your bench.**

Practice using the pipettors to measure the following volumes of water; 7  $\mu\text{L}$ , 50  $\mu\text{L}$ , 250  $\mu\text{L}$ , 350  $\mu\text{L}$ , 900  $\mu\text{L}$ . **How could you tell whether your measurements were accurate?** (Hint: the density of water is 1gram per mL, and we have balances for measuring weights very accurately).

Remember that 1.0 mL = 1000  $\mu\text{L}$ .

## Procedure B. Bacterial Transformation & Phenotype Confirmation

For a group of 4 students, except where noted.

NOTE: After a spin in the centrifuge, the **pellet** is solid material on the bottom or side of the tube. The **supernatant** is the fluid on top.

### B1. Begin preparation of competent *E. coli* cells

- a. Within your group of 4 students: each *pair* of students should process *one* 1.5 mL tube of *E. coli* strain TB-1 bacterial culture (So there are 2 tubes for each group of 4 students). **Keep the bacteria on ice whenever possible during this procedure.** Centrifuge at 7000 rpm for 30 seconds.
- b. Pour off the supernatant into the waste can, **save the pellets which contain the bacteria.**
- c. Resuspend each **cell pellet** in 1 mL ice-cold 0.1 M CaCl<sub>2</sub> by vortexing. Incubate cells on ice for 30 min. The 0.1 M CaCl<sub>2</sub> neutralizes negative charges on the surface of the bacteria that would repel the negatively charged DNA backbone, and the cold temperature increases the permeability of the bacterial cell membrane.

While you are waiting for this step, purify the pGLO plasmid DNA using **Procedure C** below, and come back to continue this transformation protocol at the appropriate time.

### B2. Complete preparation of competent *E. coli* cells, begin transformation.

*Do not continue with this part of the procedure until you have finished Procedure C.*

- a. Centrifuge cells suspended in CaCl<sub>2</sub> at 7000 rpm for 30 seconds, decant supernatant, and **save the cell pellets.** Resuspend each **cell pellet** in 100  $\mu$ L ice-cold 0.1 M CaCl<sub>2</sub> by vortexing.
- b. Your group of 4 students now has 2 tubes of competent bacteria. One of these tubes will get DNA added to it: label this tube "+DNA" The other tube will have no DNA added, and will serve as a negative control for the bacterial transformation; label this tube "no DNA".
  - Add 3  $\mu$ L of each student pair's DNA preparation to the **same tube** of resuspended cells (the "+ DNA" tube; this first tube gets a total of 6  $\mu$ l of DNA, 3  $\mu$ l from each pair of students).
  - The second, "no DNA" tube of bacteria does not get any DNA at all; this is a negative control for the transformation.
  - Incubate both tubes on ice for 30 min.

### **B3. Heat shock cells for transformation.**

- a. Place *both* transformation tubes in 42°C heating block for EXACTLY 2 minutes.

Note: If you leave the bacteria at 42°C for too long, it will kill them!

- b. Add 1 mL LB liquid, mix by *gently* inverting tubes several times. This is a nutrient growth medium that allows your bacteria to recover from the rigors of exposure to CaCl<sub>2</sub>, cold temperature, and heat shock.

- c. Lay tube on its side in 37°C incubator for 30 min.

While you are waiting for this step, familiarize yourself with the procedure for spreading bacteria onto a Petri plate (B4 below). Your Professor or TA will demonstrate. You may also set up your restriction digests as in Procedure E below while waiting for this incubation step.

### **B4. Plate out transformation**

- a. After incubating for 30 min at 37°C, invert transformation tubes several times to suspend cells.

- b. Pipette 100 µL of transformation mix onto each of the following plates:

LB + arabinose,

LB + ampicillin,

LB + ampicillin + arabinose.

Light bunsen burner, remove glass spreader from beaker of ethanol, hold with your hand higher than the spreader but not directly over it, pass through flame to ignite ethanol. After ethanol has burned off spreader, spread cells over plates. Incubate plates in the inverted position overnight at 37°C.

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### Procedure C. pGLO plasmid DNA Isolation from *E. coli*

Each pair of students will be provided with a culture of *E. coli* carrying the pGLO plasmid: this is the yellowish fluid in the 1.5 mL tubes. Use the following procedure to purify the plasmid DNA from the *E. coli* cells.

NOTE: After a spin in the centrifuge, the **pellet** is solid material on the bottom or side of the tube. The **supernatant** is the fluid on top.

C1. Label two 1.5 mL tubes with pGLO and your initials. Add 1.5 mL of culture to each tube. Centrifuge at high speed (13,000 rpm) for 30 seconds. This will cause the bacteria to pellet.

C2. Decant (pour off) the supernatant into your liquid waste container.  
(Ensure that RNase A has been added to Buffer P1—Your Professor or TA will probably have done this for you already)

Resuspend *both* pellets of bacterial cells in a *total* of 250  $\mu$ l Buffer P1:  
--put the 250  $\mu$ l Buffer P1 into **one** tube, vortex until the pellet resuspends, then -  
----transfer the **same** liquid from the first tube into the **second** tube,  
and vortex again until the second pellet also resuspends,  
--Discard the empty first tube in the biohazard waste can.  
*Buffer P1 contains enzymes that begin to break down the bacterial cell walls.*  
No cell clumps should be visible after resuspension of the pellet.

C3. Add 250  $\mu$ l Buffer P2 and gently invert the tube 4–6 times to mix. Mix *gently* by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. *Buffer P2 has an alkaline pH, and chemicals that will lyse the bacterial cells.* If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.

C4. Add 350  $\mu$ l Buffer N3 and invert the tube immediately but gently 4–6 times. To avoid localized precipitation, mix the solution gently but thoroughly, immediately after addition of Buffer N3. The solution should become cloudy. *Buffer N3 neutralizes the pH of the mixture, and adjusts the salt content so that the DNA will later be able to bind to the column.*

C5. Centrifuge for 10 min at 13,000 rpm ( $\sim 17,900 \times g$ ) in a table-top microcentrifuge. A compact white pellet will form. This pellet contains pieces of bacterial cell walls, membranes, and many proteins that you do not want to purify. The pGLO plasmid DNA you want to purify will remain in the **supernatant** (liquid on top).

C6. Make sure that you have a QIAprep Spin Column (small blue plastic cylinder with white resin inside) sitting on a round-bottomed 2 ml centrifuge tube. Apply the **supernatant** from step 5 to the QIAprep Spin Column by decanting (pouring) or pipetting.

- C7. Centrifuge for 30–60 s at 13,000 rpm. The **pGLO plasmid DNA is now bound onto the column**, along with some contaminating proteins.
- C8. Discard the fluid flow-through from the bottom of the tube; this contains undesirable cell components.
- C9. Wash the QIAprep Spin Column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s at 13,000 rpm. *This removes more unwanted proteins from the column.*
- C10. Discard the liquid flow-through.  
*Your pGLO plasmid DNA is still bound onto the column.*  
(This C9-C10 step is necessary to remove trace nuclease activity when using *endA*<sup>+</sup> strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5 $\alpha$  do not require this additional wash step.)
- C11. Wash QIAprep Spin Column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s at 13,000 rpm. *This removes even more unwanted proteins from the column.*
- C12. Discard the flow-through. *Your pGLO plasmid DNA is still bound onto the column.*
- C13. Centrifuge for an additional 1 min to remove residual wash buffer. **IMPORTANT:** Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.
- C14. Place the QIAprep column with DNA bound onto it in a clean 1.5 ml microcentrifuge tube.  
To elute DNA (release it from the column and let it flow out), add 50  $\mu$ l Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of each QIAprep Spin Column, let stand for 1 min, and then centrifuge for 1 min at 13,000 rpm. *The low pH of this buffer will allow the DNA to come off of the column and into the tube.*
- C15. Clearly label your tube “pGLO DNA”, add your initials, and place on ice.

Return to Procedure B at the appropriate time—If your plasmid DNA is not ready in time, finish purifying it and *then* continue with Procedure B.

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## D. Introduction to Restriction Enzymes

You have just purified plasmid DNA from bacterial cultures. **How do you know whether you have DNA in the tube? How do you know whether it's the right DNA?** These questions can best be answered by physical analysis. How do DNA molecules differ? How is the DNA in your cells different from that of your lab partner?

The previous DNA sequence analysis revealed the functional characteristics of DNA—you were able to make predictions about the functions encoded on the pGLO plasmid based on its DNA sequence. One can also examine the physical characteristics of the DNA such as the size, sequence, and base composition. Determining the entire sequence is a relatively cumbersome and time-consuming process. However, to test for a limited number of sequence characteristics, one can use **restriction enzymes** that recognize specific sequences and cut the DNA at those sequences. For example, the sequence GGCC is recognized by the restriction enzyme *HaeIII* (see diagram below). The *HaeIII* enzyme will cleave the phosphate backbone of the DNA between the GG and the CC, leaving “blunt-ended” fragments of DNA. The sequence GAATTC is recognized by the restriction enzyme *EcoRI*, which cuts the phosphate backbone in a staggered fashion, leaving “sticky” ends on the DNA fragments.

The sequence AAGCTT is cut by the enzyme *HindIII*. If that sequence occurs three times on a specific circular DNA molecule such as pGLO, then cutting that molecule with *HindIII* will produce 3 fragments with characteristic sizes. The combination of sizes can be used to identify a DNA molecule.

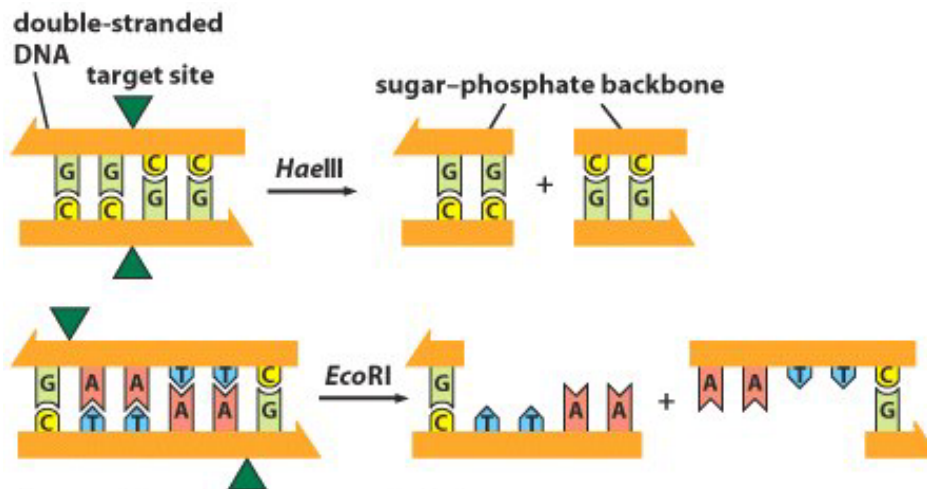


Figure 4-1 Biology Today, 3/e (© 2004 Garland Science)

To determine whether we have the right DNA, we must know what to look for. In the introduction to this experiment, it was pointed out that the jellyfish GFP gene was present within a circular bacterial plasmid. How did it get there? **Genetic Engineering!** This powerful technology allows scientists to cut separate pieces of DNA, such as the GFP gene and the plasmid, and paste them together to make a recombinant DNA

molecule. By re-cutting the DNA at the sites where it was originally pasted, we can examine the fragments to see if they are same DNAs that were put together.

To identify specific DNA fragments, one must separate the fragments based on their size. This is accomplished by **gel electrophoresis**. Because DNA is negatively charged (due to the phosphate groups in its backbone) it is attracted to the positive electrode when subjected to an electric field. If the DNA is present within a gel, its movement toward the positive electrode will be hindered to different extents, depending on the size of the DNA molecule (would a fish swim better in water or jello?). Thus, one can use the distance migrated by a particular DNA fragment to determine the size of the fragment. This week, we will set up restriction enzyme digests of our plasmid DNA, and next week we will run the restriction fragments out on agarose gels using electrophoresis to separate the DNA fragments.

#### **Procedure D. Restriction enzyme digestion of pGLO plasmid DNA.**

Each pair of students should do the following with their plasmid DNA prep:

- E1. Add 10  $\mu\text{L}$  of your plasmid DNA to each of the 3 tubes provided to you. Mix the DNA into the liquid by pipetting up and down gently 2-3 times. One tube (B) contains the restriction enzyme BamHI, another (X) contains the enzyme XhoI, and the third (T) contains only TE buffer, as a negative control.
  
- E2. Label the tubes with your initials and incubate in the 37°C heating block for at least 1 hour, preferably for 2 hours. After this time, store at -20°C. Your Professor or TA will transfer your digests to the freezer for you.

Return to the bacterial transformation protocol B at the appropriate time.

#### **Summary Questions**

1. When using micropipettes, how could you know if your micropipette was working properly and measuring the expected amount of fluid? (Remember that the density of water is 1 g per mL)
  
2. Why does the GFP gene need to be inserted into a plasmid for growth in bacteria? Why can't we just take the GFP gene alone and put it into bacteria to grow up more copies?

3. What was the function of each step of the DNA purification protocol?

Buffer P1

Buffer P2

Buffer N3

Centrifugation of the lysate

Passage over the Qiagen column

Buffer PB

Buffer PE

Buffer EB

4. What is a restriction enzyme?

5. Give an example of how one restriction enzyme cuts DNA.

6. If you start with a circular plasmid DNA, and cut it with a restriction enzyme that recognizes 4 sites on the plasmid, how many DNA fragments will be generated? (Draw it out!)

7. a. What is DNA transformation?

b. What function do calcium ions serve in DNA transformations?

8. What is beta-lactamase/what properties should it confer on bacteria receiving a plasmid that encodes it?

9. a. What are the DNA sequences that control the expression of a gene called?

b. What DNA sequences control the expression of the GFP gene in pGLO?

c. What 2 conditions would allow you to see the GFP gene being expressed?