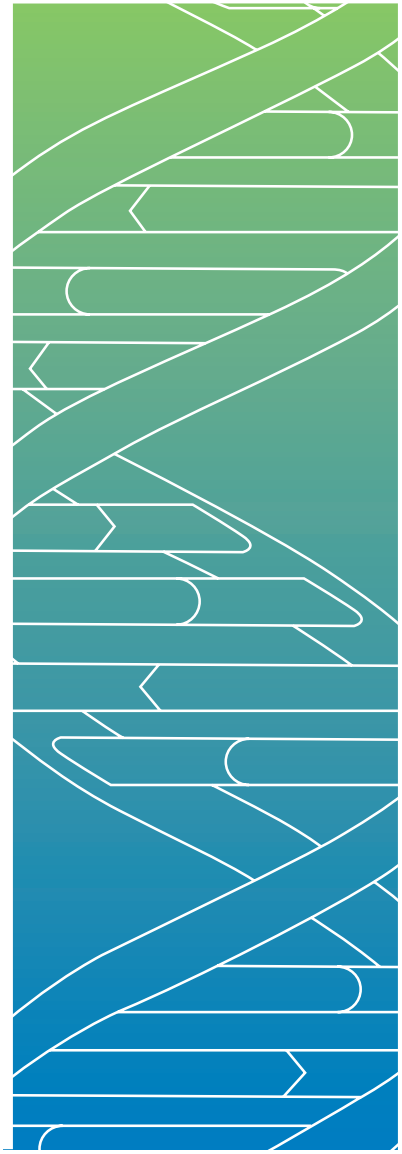
AMGEN® Biotech Experience

Scientific Discovery for the Classroom

FOUNDATIONS OF BIOTECH



STUDENT LAB MANUAL

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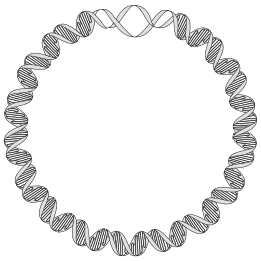


CHAPTER 1

SOME TOOLS OF THE TRADE

INTRODUCTION

The year 1978 marked a major breakthrough in medicine. For the first time ever, scientists were able to engineer bacteria capable of producing human proteins. They achieved this by strategically inserting small pieces of human DNA into bacterial cells. This new technology, termed genetic engineering, can be used to make proteins that treat the symptoms of certain genetic diseases (those caused by a change in DNA, often inherited from parents). Genetic engineering, also called genetic modification, is the direct manipulation of an organism's genes using biotechnology.

To carry out genetic engineering, you need good laboratory skills. In this chapter, you'll focus on gaining practice in the use of micropipettes (instruments used to transfer small volumes of liquid) and gel electrophoresis (a technique for separating and identifying biomolecules)—two critical skills for biotechnology. You will complete two labs, using instruments and supplies that are identical to the ones used in biotechnology research laboratories. These labs are the first step in building the skills you'll need to be successful in biotechnology.

CHAPTER 1 GOALS

By the end of this chapter, you will be able to do the following:

- Correctly use micropipettes and the technique of gel electrophoresis
- Explain the importance of micropipettes and gel electrophoresis in genetic engineering
- Describe how gel electrophoresis separates DNA
- Explain how genetic engineering can be used to treat some genetic diseases

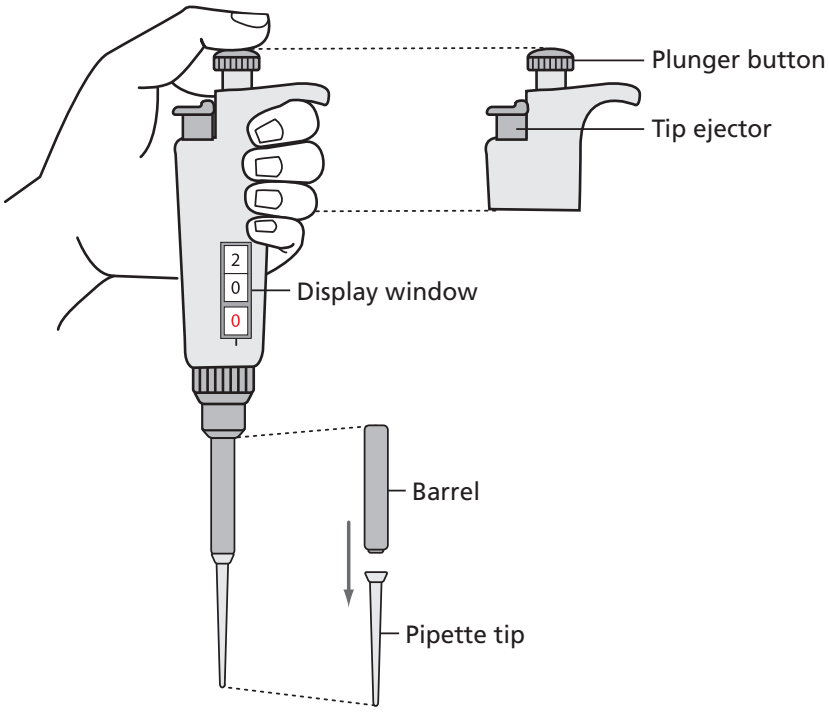
WHAT DO YOU ALREADY KNOW?

Discuss the following questions with your partner, and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these questions will help you think about what you already know about biotechnology.

1. What tools and techniques of biotechnology have you used before? What did you use them for?
2. Why is precision important when you are carrying out biotechnology procedures?

LABORATORY 1.1: HOW TO USE A MICROPIPETTE

The purpose of this laboratory is to introduce you to an important tool used in genetic engineering: the micropipette, shown in **Figure 1.1**. A micropipette is used to transfer very small and exact volumes of liquids in either milliliters (mL, thousandths of a liter) or microliters (µL, millionths of a liter), which are the measurements of volume most often used in genetic engineering. This laboratory will give you the chance to learn how to use the micropipette and to see the relative size of different amounts of solution measured by this very precise tool and how precise the amounts that you can measure with it are.



A P-20 micropipette

BEFORE THE LAB

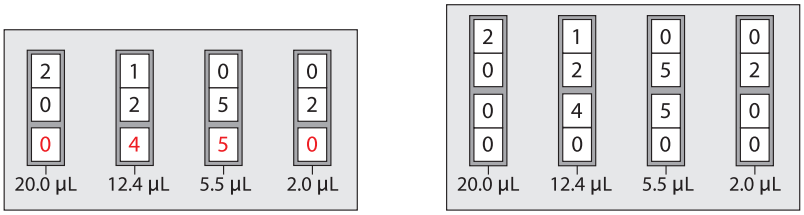
Respond to the following with your group and be prepared to share your responses with the class.

1. Why do you think it is necessary to use very small and exact volumes of material in biotechnology?
2. Read through the *Methods* section and briefly outline the steps, using words and a flowchart.

METHODS

1. Practice setting the micropipette to the volumes shown **below**.

SAFETY: Never set a micropipette above or below its range or you could damage the equipment.



Four micropipette volumes shown on two different micropipettors

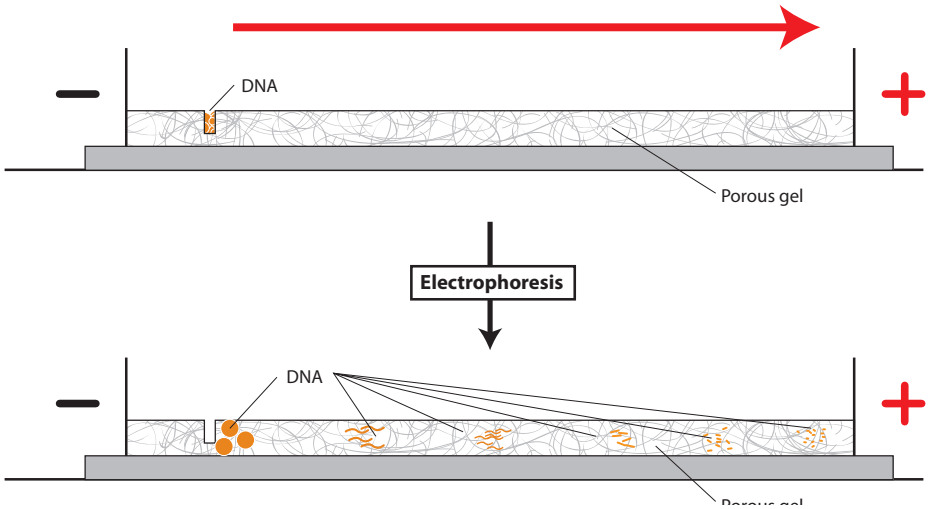
2. Review the laminated micropipette practice sheet. Each group member will pipette five drops of different volumes onto the sheet. Pipetting consists of two parts: loading the liquid into the micropipette and dispensing the liquid from the micropipette.
3. Following your teacher's directions, load the micropipette with 20.0 µL of red dye (RD).
4. Dispense RD onto the space labeled 20.0 µL on the laminated sheet.
5. Using the same micropipette tip, reload and dispense RD on each of the remaining spaces by adjusting the volume between each aliquot: 15.0 µL, 10.0 µL, 5.0 µL, and 2.0 µL.
6. After filling each of the spaces, use the tip ejector to place your micropipette tip into the waste container. When everyone in your group has had a chance to dispense RD onto the practice sheet, draw the approximate sizes of each drop in your notebook (or take a photograph and tape it into your notebook), and label each drop with the amount it represents.

STOP AND THINK

- **When loading or dispensing a solution, why is it important to actually see the solution enter or leave the micropipette tip?**
- **You were instructed to avoid contact with the micropipette tips—for example, you were asked to put the micropipette tip on without using your hands to avoid setting the micropipette down, to use the ejector button to remove the tip, and to keep the tip box closed. If you were working with plasmids and bacterial cells, why would these precautions be important?**

LABORATORY 1.2: GEL ELECTROPHORESIS

The purpose of this laboratory is to give you experience with gel electrophoresis, which is used to separate and identify a mixture of biomolecules; the components of each mixture can then be identified by their location in the gel. Gel electrophoresis works based on the fact that some biomolecules, such as DNA, have a negative charge, which means that they will move in response to an electric current. The biomolecules move through a gel composed of agarose, a polysaccharide (complex sugar) found in seaweed. Its structure is a porous matrix (like a sponge) with lots of holes through which the solution and biomolecules flow (see **below**). The speed of the biomolecules varies primarily according to their molecular size, although shape and degree of charge also influence their movement. In the genetic engineering process, gel electrophoresis is used to separate and identify plasmids and short linear pieces of DNA.



How biomolecules, including DNA, move through the agarose gel matrix in gel electrophoresis

The electrophoresis setup consists of a box containing an agarose gel and two electrodes that create an electric field across the gel when the box is attached to a power supply. The negative electrode is black, and the positive electrode is red. Samples of biomolecules are pipetted into wells near the negative (black) electrode. The samples move through the gel toward the positive (red) electrode.

BEFORE THE LAB

Respond to the following items with your group, and be prepared to share your responses with the class.

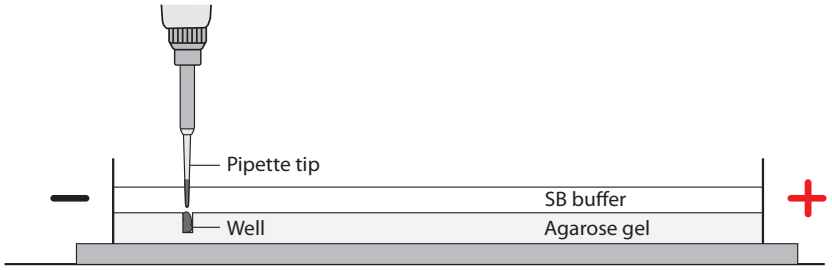
1. In what circumstances might it be important to use gel electrophoresis to separate and identify plasmids and short linear pieces of DNA?
2. Read through the *Methods* section, and briefly outline the steps for Part A and for Part B using words and a flowchart.

PART A: PIPETTING INTO WELLS

METHODS

You will practice pipetting RD into preformed wells in an agarose gel within a Petri plate.

1. Fill the two pipetting practice dishes with 1x sodium borate (SB buffer) to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more buffer.
2. Set the P-20 micropipette to 10.0 µL, put on a micropipette tip, and load 10.0 µL of RD into the micropipette.
3. Dispense the RD into a well in one of the practice dishes by doing the following:
   a. Place your elbow on the table to steady your hand. If needed, also use your other hand to support the hand holding the micropipette.
   b. Lower the micropipette tip until it is under the buffer but just above the well, as shown **below**.



   c. Gently press the plunger to slowly dispense the RD. To avoid getting air in the RD, do not go past the first stop. Keep the plunger depressed as you pull the tip out of the buffer.
4. Repeat steps 2 and 3 until all the practice plate wells have been filled. Everyone in your group should get an opportunity to practice pipetting into the wells.

PART B: SEPARATING DYES WITH GEL ELECTROPHORESIS

METHODS

Now you will use gel electrophoresis to separate different dyes. First you will add dyes to the wells in the gel electrophoresis unit. You will then turn the unit on to create an electric current and move the negatively charged dyes through the gel.

1. Check your rack to make sure that you have the three dye solutions (labeled S1, S2, and S3).
2. Check to make sure that the wells in the gel are located near the negative (black) electrode of the gel electrophoresis chamber (unit).
3. Fill the box with 1x SB buffer to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more buffer.
4. Briefly pulse centrifuge the S1, S2, and S3 tubes, making sure they are distributed evenly in the microcentrifuge.
5. Make a drawing in your notebook that shows the location of the wells. Record which solution you will place in each well.
6. Set the P-20 micropipette to 10.0 µL, put on a micropipette tip, and load 10.0 µL of S1 into the micropipette.
7. Dispense the S1 into the well you've designated for that solution by doing the following:
    a. Place your elbow on the table to steady your hand. If needed, also use your other hand to support the hand holding the micropipette.
    b. Lower the micropipette tip until it is under the buffer but just above the well.
    c. Gently press the plunger to slowly dispense the sample. To avoid getting air in the sample, do not go past the first stop. Keep plunger depressed as you pull the tip out of the buffer.
8. Using a new micropipette tip for each solution, repeat steps 6 and 7 for loading S2 and S3.
9. When all the samples have been loaded, close the cover tightly over the electrophoresis box.
10. If your machine requires external power, attach the electrical leads to the power supply.
11. Turn on the power supply, and set it to the correct voltage for your machine. (You will see bubbles form in the buffer at the positive [red] end of the electrophoresis unit.)
12. After two or three minutes, check to see if the dyes are moving toward the positive (red) electrode. You should begin to see the dyes separate.
13. In approximately 10 minutes, or when you can distinguish all three dyes, turn off the power and unplug.

14. Carefully remove the cover from the gel box and observe the dyes in the gel.
15. Take a picture of your gel or draw one in your notebook showing the relative location and color of the bands in each of the lanes.
16. Leave the gels in the gel box.

STOP AND THINK

- **Study your gel electrophoresis results. Which solution sample contained a single dye: S1, S2,or S3? How do you know?**
- **What electric charge do the dyes have? Explain your reasoning.**
- **The dyes that you are separating are orange G (yellow), bromophenol blue (purple), and xylene cyanol (blue). If the molecular shape and electric charge of all three dyes are similar, what is the order of the dyes from heaviest to lightest molecules, based on your initial results? Why do you think this is the correct order?**

SB

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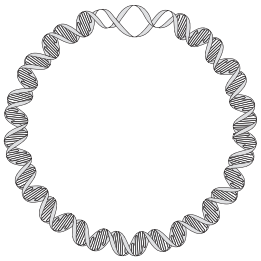


CHAPTER 2

HOW DO YOU BEGIN TO CLONE A GENE?

INTRODUCTION

In the Program Introduction, you learned about the development of biopharmaceuticals and were introduced to the techniques used in developing these therapeutics. One of these techniques—bacterial transformation—allows human genes to be inserted into bacteria, enabling the bacteria to produce the human therapeutic proteins. Chapter 1 gave you a chance to work with two physical tools and techniques of genetic engineering that are used to clone a gene: the micropipette and gel electrophoresis. In this chapter you will work with two other important genetic engineering tools—plasmids and restriction enzymes. These "tools" are actually biomolecules found in many bacteria, and their discovery was crucial to genetic engineering. With these tools, scientists can modify microorganisms to make human proteins. You will now learn more about these tools and will then carry out the first steps in your quest to clone a gene.

CHAPTER 2 GOALS

By the end of this chapter, you will be able to do the following:

- Describe the characteristics of plasmids
- Explain how plasmids are used in cloning a gene
- Describe the function of restriction enzymes
- Explain how to use restriction enzymes to create a recombinant plasmid

WHAT DO YOU ALREADY KNOW?

Discuss the following with your partner, and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these questions will help you think about what you already know about DNA, plasmids, and restriction enzymes.

1. What is the structure and function of DNA? Describe in words or a drawing the structure of a DNA molecule. Be as detailed as possible.

2. All living organisms contain DNA. In what ways is DNA from different organisms the same, and in what ways does it vary?

3. Using your understanding of genes and how they are expressed (information encoded in a gene is converted first into messenger RNA and then to a protein), explain why it is possible for a bacterial cell to make a human protein from the instructions encoded in a human gene.

4. Scientists use two biological tools to engineer organisms to make new proteins: plasmids and restriction enzymes. How might each of these be useful in creating a new protein?

CLONE THAT GENE

You now know about two biological tools for cloning a gene: plasmids and restriction enzymes.

1. Plasmids have several important features:
    - A sequence for the initiation of DNA replication, called the *ori* site, which allows the plasmid to replicate in the bacteria using the host DNA synthesis enzymes
    - A promoter for initiating transcription of the inserted gene
    - A gene encoding a protein for antibiotic resistance, which allows for identification of bacteria that have taken in the plasmid
2. Restriction enzymes digest both the plasmid and the human DNA containing the gene of interest (such as insulin) to be cloned.

How do scientists use these two tools to create a recombinant plasmid, which contains a human gene inserted into a bacterial plasmid? One important step is choosing a restriction enzyme (or enzymes) that cuts the plasmid and the human DNA. The restriction enzyme(s) must do all of the following:

- Cut the plasmid at a site (or sites) that allows for the insertion of the new gene.
- Cut the plasmid at an appropriate site to ensure that no important genes or sequences are disrupted, including the *ori* site, the promoter, and at least one of the genes encoding antibiotic resistance.
- Cut the plasmid near the promoter so that the inserted gene can be expressed.
- Cut the human DNA as close as possible to both ends of the gene of interest so that it can be inserted into the appropriate site in the plasmid DNA, without cutting within the gene.

STOP AND THINK: Why is it important that the same enzyme or enzymes be used to cut both the plasmid and the gene from the human DNA?

In this activity, you will make a paper model of a recombinant plasmid that contains a gene for a human therapeutic protein—in this case, insulin. You have three tasks:

1. Cut the plasmid and the human DNA with the appropriate restriction enzyme
2. Insert the human insulin gene into the plasmid DNA
3. Determine which antibiotic you would use to identify bacteria that have taken in the plasmid

PROCEDURE

1. On the **Plasmid Diagram (RM 2)**:
   - Use scissors to cut out the plasmid sequence, and tape the ends together to make a paper model of the plasmid.
   - Locate the positions of the *ori* site, the promoter, and the genes for antibiotic resistance.
   - Locate the positions of each restriction enzyme restriction site.

4. Choose the restriction enzyme that should be used to cut the plasmid. Verify that the restriction enzyme meets all the following criteria:
   - It leaves the *ori* site, the promoter, and at least one antibiotic-resistance gene intact.
   - It cuts the plasmid only once.
   - The cut is close to the promoter.

3. Review the **table below**, and use scissors to cut the plasmid at the recognition site exactly as the restriction enzyme would cut it. Write the sequences of the nucleotides that are left on each end of the plasmid.

Restriction enzymes used in this laboratory

Source	Restriction enzyme	Recognition site
Escherichia coli	*Eco*RI	5′ G↓AATTC 3′ 3′ CTTAA↑G 5′
Bacillus amyloliquefaciens	*Bam*HI	5′ G↓GATCC 3′ 3′ CCTAG↑G 5′
Haemophilus influenzae	*Hin*dIII	5′ A↓AGCTT 3′ 3′ TTCGA↑A 5′

The symbols ↑ and ↓ indicate where the DNA is cut.

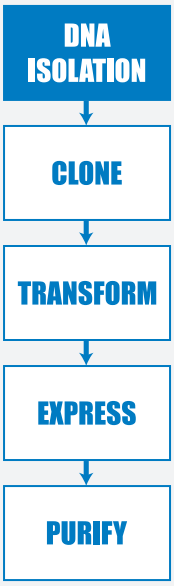
4. On the **Human DNA Sequence (RM 3)**, scan the human DNA sequence and determine where the three restriction enzymes, *Bam*HI, *Eco*RI, and *Hin*dIII, would cut the DNA.

5. Determine whether the restriction enzyme you chose in step 2 is a good choice for cutting out the insulin gene from the human DNA by verifying that it meets all the following criteria:
   - It does not cut within the insulin gene.
   - It cuts very close to the beginning and end of the gene.
   - It will allow the insulin gene to be inserted into the cut plasmid.

6. Review the **table on the previous page,** and use scissors to cut the human DNA at the recognition site exactly as the restriction enzyme would cut it. Write the sequences of the nucleotides that are left on each end of the insulin gene after it is cut from the human DNA.
7. Use tape to insert the insulin gene into the cut plasmid. Verify that the sticky ends will connect in the correct orientation. (In the lab, a third biological tool, DNA ligase, is used to permanently connect the sticky ends together.) You now have a paper model of a recombinant plasmid that contains an insulin gene. Once the plasmid replicates (copies) itself, the insulin gene is also copied, or cloned!

ACTIVITY QUESTIONS

1. Which restriction enzyme did you choose? Why did you choose that one?
2. Where would you insert the insulin gene, and why?
3. Which antibiotic would you use to determine if the recombinant DNA was taken in?



LABORATORY 2: PREPARING TO CLONE THE *rfp* GENE: DIGESTING THE pKAN-R AND pARA

The purpose of this lab is to use restriction enzymes to produce the DNA fragments that will be joined to make the recombinant plasmid, pARA-R; the recombinant plasmid can then make the red fluorescent protein (RFP) in bacteria. Cutting DNA with restriction enzymes is known as a restriction digest, and the fragment lengths can be determined by gel electrophoresis (which you may do in Chapter 4).

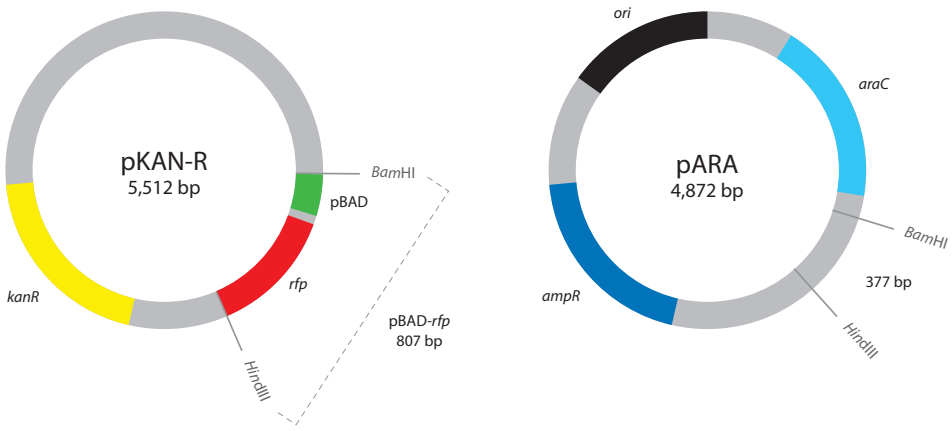
To clone the *rfp* gene, you will need DNA from two different plasmids, pKAN-R and pARA (see the **table below** for more information), to generate the recombinant DNA pARA-R.

pKAN-R and pARA Plasmids Comparison

pKAN-R contains ...	pARA contains ...
• gene that makes bacteria resistant to antibiotic **kanamycin** • the *rfp* gene • a promoter sequence	• gene that makes bacteria resistant to the antibiotic **ampicillin** • the *ori* site for initiating DNA replication • the DNA sequence (*araC*) that activates the promoter when the bacteria are grown in the presence of the five-carbon sugar arabinose

If arabinose is not present, the promoter will not bind RNA polymerase and transcription will not occur.

The **figure below** shows the size of the plasmid (the number in the center, which indicates the number of base pairs [bp]) and the sequences where it can be cut by the restriction enzymes "*Bam*HI" and "*Hin*dIII."



The pKAN-R and pARA plasmids

BEFORE THE LAB

Respond to the following items with your group, and be prepared to share your responses with the class.

1. Review the **figure on the previous page**. If pKAN-R is digested with *Bam*HI and *Hin*dIII, what fragments are produced? If pARA is digested with *Bam*HI and *Hin*dIII, what fragments are produced? Record the nucleotide sequence of the sticky ends and the length of each fragment (bp), and indicate the genes and other important sequences present on each fragment.
2. To create a plasmid that can produce the red fluorescent protein in bacteria, what components are needed in the plasmid?
3. Bacteria can be killed by an antibiotic unless they carry a plasmid that has the gene for resistance to that antibiotic. These genes are known as selectable markers because only bacteria that carry the gene will survive an antibiotic. If the uptake of DNA by bacteria is inefficient, why is a selectable marker critical in cloning a gene in bacteria?
4. Read through the *Methods* section below, and briefly outline the steps in a flowchart.

METHODS

1. With a marker, label the caps of four clean microfuge tubes with your group identifier and K+, K–, A+, or A–.
2. **To avoid contamination, use a new micropipette tip for each reagent,** and add the following:

	K+ tube	K– tube	A+ tube	A– tube
a. Restriction buffer (2.5xB)	4.0 µL	4.0 µL	4.0 µL	4.0 µL
b. pKAN-R plasmid (K)	4.0 µL	4.0 µL		
c. pARA plasmid (A)			4.0 µL	4.0 µL
d. *Bam*HI and *Hin*dIII (RE)	2.0 µL		2.0 µL	
e. Distilled water (dH_2O)		2.0 µL		2.0 µL

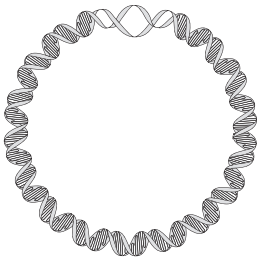
* For d and e: Add directly into the solution at the bottom of the microfuge tube, and gently pump the solution in and out to mix the reagents. Cap the tubes when done.

3. Distribute the four microfuge tubes (K+, A+, K–, and A–) evenly in the microcentrifuge, and spin them for several seconds to pool the reagents at the bottom of each tube.

   Place all four tubes into a floating microfuge rack, and then place in the 37°C water bath and note your start time. Incubate for at least 5 minutes, but no longer than 15 minutes. After the incubation is complete, place all four tubes in the freezer at –20°C. You will use the contents of the tubes in Laboratory 3.

STOP AND THINK

- **In step 2, you are asked to set up two tubes without the restriction enzymes *Bam*HI and *Hin*dIII. What is the purpose of this step, and why is it important?**
- **Why might the enzymes work best at 37°C? (*Hint:* The normal human body temperature is approximately 37°C.) Why should the enzymes then be placed in the freezer?**



CHAPTER 3

BUILDING A RECOMBINANT PLASMID

INTRODUCTION

In Chapters 1 and 2, you learned about four important tools of genetic engineering: the micropipette, gel electrophoresis, plasmids, and restriction enzymes. Chapter 2 focused on cutting DNA into fragments that could be combined into a recombinant plasmid. In this chapter, you will ligate (paste together) the fragments using a fifth tool that is needed to clone genes—DNA ligase. DNA ligase is an enzyme that catalyzes (increases the rate of a reaction) the joining of DNA fragments; it is one of several enzymes involved in DNA replication in all cells. This same process is used to make recombinant plasmids that contain the gene for human insulin, human growth hormone, blood clotting factors, and other human therapeutic proteins

CHAPTER 3 GOALS

By the end of this chapter, you will be able to do the following:

- Describe the role of a DNA ligase in replication
- Explain how DNA ligase is used to create a recombinant plasmid
- Describe possible recombinant plasmids that form when ligating a restriction digest

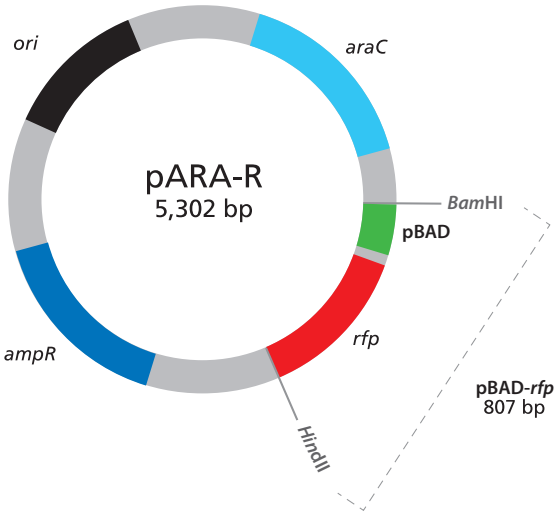
WHAT DO YOU ALREADY KNOW?

Discuss the following items with your partner, and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these items will help you think about what you already know about enzymes, DNA replication, and DNA ligation.

1. What is the function of enzymes in reactions?
2. How does DNA replication occur?
3. Why is replication of DNA essential in all cells?
4. What happens when two DNA fragments with complementary sticky ends join? How does the activity of DNA ligase ensure that the join is permanent?

LABORATORY 3: BUILDING THE pARA-R PLASMID

In this laboratory, you will ligate the DNA fragments you produced during Laboratory 2 using DNA ligase to make new recombinant plasmids. These recombinant plasmids will contain the four restriction fragments from Laboratory 2 recombined in different ways to produce new sets of DNA. The ligation process will result in several different plasmids, but the plasmid that you are interested in will contain the gene for ampicillin resistance (*ampR*), the red fluorescent protein (*rfp*) gene, a promoter sequence for initiating transcription (pBAD), the arabinose activator sequence (*araC*), and the *ori* sequence for the initiation of DNA replication. This desired recombinant DNA plasmid is called the "pARA-R plasmid" (see the **figure below**).



The pARA-R plasmid

During this lab, you will mix together the DNA fragments from your restriction digest in Laboratory 2 and the DNA ligase, but you will not be able to observe anything until Laboratory 4, when you will have the opportunity to separate and identify your DNA molecules using gel electrophoresis. However, you will prepare for what you might observe by determining the possible plasmids that can occur and drawing diagrams of these plasmids. In this work, you are modeling the process of ligation.

BEFORE THE LAB

Discuss the following items with your group, record your answers, and be prepared to share your responses with the class.

1. Review your answer to question 1 in *Before the Lab* for Laboratory 2, in which you described the fragments that formed from the digestion of pKAN-R and pARA with *Bam*HI and *Hin*dIII. Using this information, draw three possible recombinant plasmids resulting from the joining of two pKAN-R and pARA fragments. For each plasmid, identify the genes, other important sequences, and the number of base pairs each has.
2. Read through the *Methods* section, and briefly outline the steps using words and a flowchart.

METHODS

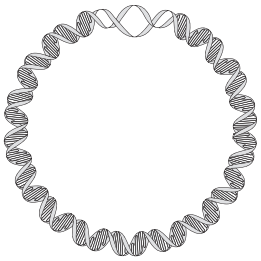
1. Place your labeled K+ and A+ tubes from Laboratory 2 into a floating microfuge rack and into the 80°C water bath for 20 minutes to denature (inactivate) the restriction enzymes.

   NOTE: During the incubation, share and discuss your answers to question 1 in *Before the Lab*. Also share and discuss your answer to the *STOP AND THINK* questions that follow.

STOP AND THINK

Why is it important to inactivate the *Bam*HI and *Hin*dIII restriction enzymes before ligating the fragments? What might happen if you did not perform this step?

2. After 20 minutes, remove the K+ and A+ tubes from the water bath and place them in your rack.
3. Label the pre-aliquoted LIG tube containing 2 µL of ligase enzyme with your group identifier. Using a new micropipette tip for each reagent, add the following directly into the solution at the bottom of the tube, and mix after adding the dH_2O:
   a. 4.0 µL of A+
   b. 4.0 µL of K+
   c. 3.0 µL of 5xB
   d. 2.0 µL of dH_2O
4. Spin the LIG tube in a balanced microcentrifuge for several seconds to pool the reagents at the bottom of the tube.
5. Place your LIG tube in a rack to incubate at room temperature for 15 minutes. Return your A+ and K+ tubes to the –20°C freezer for use in Laboratory 4.



CHAPTER 4

MAKING SURE YOU'VE CREATED A RECOMBINANT PLASMID

INTRODUCTION

When scientists clone a gene in order to produce a human therapeutic protein, they create a recombinant plasmid that includes the human gene of interest. To do so, they use restriction enzymes to create DNA fragments that contain the plasmid components (Chapter 2) and then use DNA ligase to join those fragments together (Chapter 3). As part of the gene cloning process, scientists have to verify (confirm) that they have created the recombinant plasmid they need—that is, the one with the gene of interest (which will make the therapeutic human protein) and all the necessary components for that protein to be made. In this chapter, you will continue to work with the tools of genetic engineering as you verify that you have the recombinant plasmid you need in order to produce RFP.

CHAPTER 4 GOALS

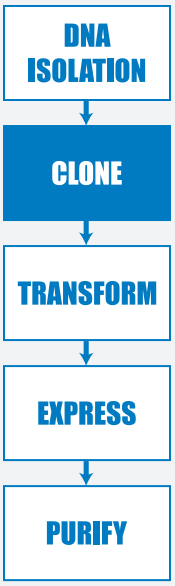
By the end of this chapter, you will be able to do the following:

- Describe why it is important to verify products created in the genetic engineering process
- Predict the relative speed of DNA restriction fragments and plasmids through a gel during gel electrophoresis
- Separate and identify DNA restriction fragments and plasmids using gel electrophoresis

WHAT DO YOU ALREADY KNOW?

Discuss the following items with your partner, and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these items will help you think about what you already know about gel electrophoresis, verification in the lab, and ligation.

1. Why do DNA restriction fragments and plasmids separate when analyzed by gel electrophoresis?
2. Why is it important to identify and verify a recombinant plasmid?
3. When DNA fragments are joined with a DNA ligase, an array of products is created. How does this happen?



LABORATORY 4: VERIFICATION OF RESTRICTION AND LIGATION USING GEL ELECTROPHORESIS

In this laboratory, you will use gel electrophoresis to examine the products from the restriction digest of the pKAN-R and pARA plasmids (Laboratory 2) and verify the products from the ligation (Laboratory 3). It is important to verify work in the lab; otherwise, there are many sources of potential error in any procedure, including the procedures used in cloning a gene. In gene cloning, there is also the problem that some procedures are not selective. For example, when a DNA ligase is used to ligate (bind together) DNA fragments, many different combinations result from the ligation process. Unless you verify your work, you do not know if you have made the needed recombinant plasmid.

You can determine the sizes of the DNA fragments by comparing them to a DNA ladder—a mixture of DNA fragments with known sizes. The DNA ladder is loaded adjacent to other DNA samples in order to make it easy to compare the bands in the samples with the bands in the ladder. Whereas short, linear pieces of DNA move as expected when run on gel electrophoresis, the movement of plasmids is not as straightforward. This is because a plasmid can exist in different configurations that move at different rates through the gel. There are three plasmid configurations—supercoiled, nicked, and multimer—so you may see several bands for the uncut plasmid where you would expect to see a single band.

The results from the gel electrophoresis will provide evidence that your restriction and ligation procedures were successful and that you have created the pARA-R recombinant plasmid that contains the *rfp* gene.

BEFORE THE LAB

Discuss the following items with your group, and be prepared to share your responses with the class:

1. The pKAN-R and the pARA plasmids you digested in Laboratory 2 were replicated in a bacterial cell. What configurations—supercoiled, nicked circle, and multimer—might these two plasmids have before digestion?

2. The ligation you carried out in Laboratory 3 can result in a number of plasmids, but none of these have been replicated in a bacterial cell. What configurations—supercoiled, nicked circle, and multimer—might the ligated plasmids have?

3. You need to predict all the products you might see, including the different plasmid configurations. Review your work in Laboratories 2 and 3. What

products might you expect to see in the K–, K+, A–, A+, and LIG tubes? Create a table that shows all the possible fragments and plasmids by tube. Include the length (bp size) of each fragment or plasmid, and arrange the products found in each microfuge tube by size, from smallest to largest. Include any possible plasmid configurations, and arrange them first by size and next by speed through the gel, from fastest to slowest.

4. Read through the *Methods* section, and briefly outline the steps, using words and a flowchart.

METHODS

1. Obtain necessary samples from your teacher.
2. Use the marker to label five clean microfuge tubes with your group identifier and "geA–," "geA+," "geK–," "geK+," and "geLIG."

   NOTE: ge = gel electrophoresis

3. Use a new micropipette tip for each reagent. To verify the restriction and ligation procedures, add reagents to each of the tubes using the **table below as a guide**.

Sequence	geK– tube	geK+ tube	geA– tube	geA+ tube	geLIG tube
a. Distilled water (dH_2O)	4.0 µL	4.0 µL	4.0 µL	4.0 µL	3.0 µL
b. Loading dye (LD)	2.0 µL	2.0 µL	2.0 µL	2.0 µL	2.0 µL
c. Nondigested pKAN-R (K–)	4.0 µL				
d. Digested pKAN-R (K+)		4.0 µL			
e. Nondigested pARA (A–)			4.0 µL		
f. Digested pARA (A+)				4.0 µL	
g. Ligated plasmid (LIG)					5.0 µL

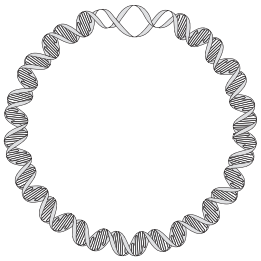
4. After adding all of the reagents, return the "LIG" tube to your teacher for use in the next lab.
5. Arrange the five microfuge tubes evenly in the microcentrifuge, and spin for several seconds.
6. Make sure that the wells in your gel electrophoresis unit are located near the negative (black) electrode.
7. Fill the box with 1x SB buffer to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more buffer.

8. Make a drawing in your notebook that shows the location of the wells in the electrophoresis box. The order of the samples in each well is shown **below**.

9. Using a new micropipette tip for each sample, dispense 10.0 µL of the DNA marker/ladder (M), geK–, geK+, geA–, geA+, and geLIG into their designated wells. When all the samples have been loaded, close the cover tightly over the electrophoresis box and turn on the power supply to the voltage indicated by your teacher.
10. After two or three minutes, check to see if the loading dye is moving toward the positive (red) electrode. If it's moving in the other direction—toward the negative (black) electrode—check the electrical leads to see whether they are plugged into the power supply correctly.
11. Your teacher will explain what to do with your gel. You may not have sufficient time to complete the electrophoresis. The smallest loading dye molecules (yellow/orange) will need to run just near the end of the gel, about 30–50 minutes (depending on your machine). After the gel has finished running, it will need to be imaged to show the location of the DNA fragments and plasmids.

STOP AND THINK

- **The DNA ladder serves as a standard because it contains a mixture of DNA molecules of known sizes. By running your samples and the DNA ladder side by side in your gel, you can estimate the actual size in base pairs of unknown DNA molecules. The DNA Ladder Diagram (RM 4) shows 10 DNA bands of known sizes. Using this information, can you predict the positions of DNA bands produced by the possible products found in the K–, K+, A–, A+, and LIG tubes by indicating their position on the DNA Ladder Diagram?**
- **The DNA samples and the DNA ladder are not visible on the gel. How might the DNA be made visible once the gel electrophoresis is complete?**



CHAPTER 5

GETTING RECOMBINANT PLASMIDS IN BACTERIA

INTRODUCTION

Inserting a gene into a plasmid vector is an important first step in the gene cloning process. However, if the ultimate goal is to produce a large amount of a particular protein, what is your next step? The plasmid must replicate to make sure that there are many copies of the gene, and the gene must be expressed—and both activities can only occur inside a cell. Therefore, your next step in the gene cloning process is to put the recombinant plasmid into *E. coli* bacteria through a process that is called "transformation." In this chapter, you will carry out the transformation of *E. coli* bacteria using the recombinant plasmid that contains the *rfp* gene. If you were making a human therapeutic protein, the bacteria that you transform would contain the human gene and would be capable of producing the desired human therapeutic protein.

A plasmid is an ideal vector for carrying DNA sequences from one organism to another. The plasmid is equipped with (1) a promoter that enables gene transcription, (2) a sequence for the initiation of DNA replication, and (3) an antibiotic resistance gene. The plasmid can be taken up by bacteria where it replicates, and its genes are expressed using the bacterial cellular machinery. If a gene of interest has been inserted into the vector, the bacteria produces the product encoded by that gene, in this case the RFP protein (in industry, the protein may be therapeutic, such as insulin).

To increase the efficiency of plasmid uptake, bacteria are treated in two ways to make them "competent." First, exposure to positive calcium ions neutralizes the negative charge on the cells' outer membranes, enabling DNA molecules to cross the plasma membranes and enter the cell. Next, a sudden increase in temperature, called a "heat shock," causes a pressure difference and enables the plasmid DNA to enter the bacterial cell from the outside. With this treatment only about 1 in 10,000 bacterial cells takes up a plasmid in its environment. By including a gene for antibiotic resistance in your recombinant plasmid, you can select for the transformed cells by growing them in the presence of the antibiotic.

CHAPTER 5 GOALS

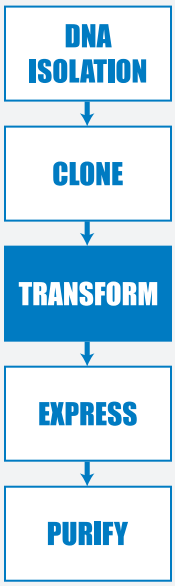
By the end of this chapter, you will be able to do the following:

- Describe the role of transformation in the gene cloning process
- Explain the purpose of each control in the transformation experiment
- Explain how the information encoded in a gene is expressed as a trait

WHAT DO YOU ALREADY KNOW?

Discuss the following items with your partner, and write your ideas in your notebook. Be prepared to discuss your responses with the class.

1. Do you think that bacterial uptake of a plasmid from the environment is a common event? Why or why not?
2. What are the steps involved in transcription and translation of a gene?
3. What is the relationship between genes, proteins, and traits (or observable characteristics)?
4. What do bacteria and humans have in common that makes it possible for a human gene to be expressed in bacteria?



LABORATORY 5: TRANSFORMING BACTERIA WITH THE LIGATION PRODUCTS

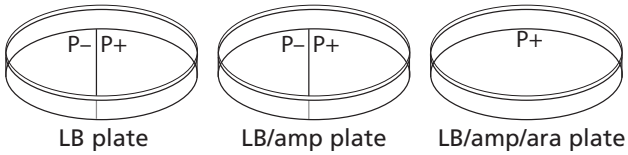
Once a recombinant plasmid that includes the gene of interest has been created, the next step is to replicate the plasmid and allow the bacteria to produce the protein. Both replication and protein expression (the way that proteins are synthesized, modified, and regulated in living organisms) can occur only inside a cell. Therefore, your next step in the gene cloning process is to put the recombinant plasmid into *E. coli* bacteria through a process called transformation, which changes the DNA content of the bacteria. In this chapter, you will carry out the transformation of *E. coli* bacteria using a recombinant plasmid that contains the *rfp* gene. If you were making a human therapeutic protein, the bacteria that you transform would contain the human gene and would be capable of producing the desired human therapeutic protein.

By examining the growth of bacteria under these conditions, you can verify that your transformation worked. How will you know if you were successful? The bacteria that took up the pARA-R plasmid from Laboratory 3 will glow red because they are producing the red fluorescent protein.

BEFORE THE LAB

Discuss the following items with your group, and be prepared to share your responses with the class.

1. Ampicillin is an antibiotic that kills bacterial cells by disrupting the formation of cell walls. However, the pARA-R plasmid has the ampicillin resistance gene, which produces a protein that breaks down ampicillin. What is the purpose of growing bacteria that have been transformed in the presence of ampicillin?
2. What will happen when bacterial cells that contain the pARA-R plasmid are not given arabinose?
3. In the lab, you will add samples of the control group P– and the treatment group P+ to plates that contain various combinations of LB, ampicillin, and the sugar arabinose. The plates will be arranged as shown **below.**



LB plate LB/amp plate LB/amp/ara plate

Using the key on **Bacterial Growth Predictions (RM 5)**, show your predictions for the growth you would expect for each combination. Then fill in **Table 1** and **Table 2** in the handout by describing the conclusions that can be drawn if the predicted growth occurs or does not occur.

4. Read through the *Methods* section, and briefly outline the steps using words and a flowchart.

METHODS

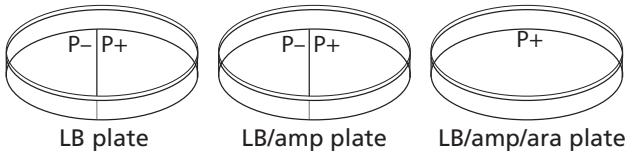
SAFETY: Use caution when handling *E. coli* bacteria, and use aseptic technique as instructed by your teacher. Aseptic technique is a set of procedures that ensure protection of the lab worker and ensure your bacteria cultures are not contaminated with unwanted microbes, both of which are necessary for the experiment to be successful.

1. Check that the LIG tube from Laboratory 3 is labeled with your group identifier.
2. Obtain a tube of competent cells (CC) and keep it cold by picking it up by the upper rim and placing it immediately on ice. Label your CC tube with your group identifier.
3. Label two clean microfuge tubes with "P–" and "P+" and your group identifier.
4. Place the P– and P+ tubes on ice with the CC tube.
5. Obtain a P-200 micropipette. This will be used to add the competent cells from the CC tube to the P– and P+ tubes as directed below:
    a. Set the P-200 micropipette to 50 µL.
    b. Very carefully resuspend the bacterial cells in the CC tube by gently pumping two times in the solution. Expel all of the cells back into the tube before moving on.
    c. Add 50 µL of CC to the chilled P– tube. Using a new micropipette tip, repeat for the P+ tube. Be sure to hold each tube at its rim to keep it cold, returning each tube quickly to the ice.
6. Obtain a P-20 micropipette to add LIG to the tube labeled "P+" according to directions below:
    a. Set the P-20 micropipette to 10 µL.
    b. Hold the chilled P+ tube by the upper rim, and add 10 µL of LIG. Mix the solutions by pumping two times in the liquids, and return the P+ tube to the ice.
7. Keep the P– and P+ tubes on ice for 15 minutes.

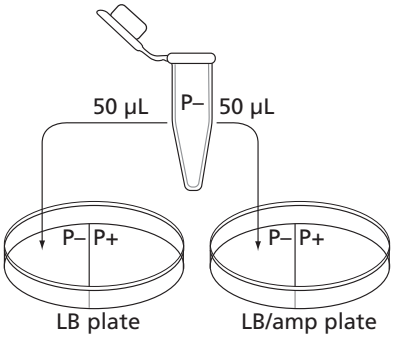
    NOTE: During the 15-minute interval, share and discuss your answers to item 3 in *Before the Lab* and complete step 8.
8. While the cells are on ice, prepare your three agar plates—one plate each of LB, LB/amp, and LB/amp/ara:
    a. Label the bottom of each plate (the part that contains the agar) with your group identifier.

NOTE: Write small and on the edge of the plate.

b. With the plates closed, draw a line on the LB plate and the LB/amp plate that divides each plate in the middle. Label half of each plate "P–" and the other half "P+." Label the LB/amp/ara plate "P+." The plates will be arranged as shown **below**.



9. Following the 15-minute incubation on ice, bring the P– and P+ tubes **on ice** to the water bath. Place the two tubes in the floating microfuge tube rack in the 42°C water bath **for exactly 45 seconds**.
10. After the 45-second heat shock, **immediately** place the tubes back on ice and leave them there for at least one minute.
11. Obtain a P-200 micropipette, and add LB to the P– and P+ tubes using the following steps:
    a. Set the P-200 micropipette to 150 µL.
    b. Add 150 µL of LB to the P– tube, and gently pump up and down two or three times to mix. Cap the tube.
    c. Using a new micropipette tip, add 150 µL of LB to the P+ tube, and gently pump up and down two or three times to mix. Cap the tube.
12. If time permits, allow the cells in the P– and P+ tubes to incubate at room temperature for 15 minutes.
13. Following directions below, add cells from the P– tube onto your LB and LB/amp plates:
    a. Set the P-200 micropipette to 50 µL.
    b. Gently resuspend the cells in the P– tube, and load 50 µL of the P– cells.
    c. Open the lid of the LB plate like a "clamshell," and add 50 µL of cells from the P– tube to the section marked "P–." Close the lid.



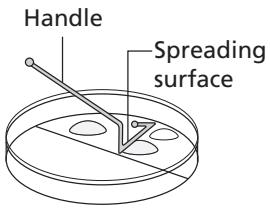
14. Using a new micropipette tip, repeat steps 13b and c, adding 50 µL of cells from the P– tube to the LB/amp plate.
15. Spread the cells from the P– tube on your LB and LB/amp plates:

LAB TECHNIQUE: Hold the spreader by the handle and do not allow the bent end to touch any surface, as this will contaminate the spreader. Place the used spreader in the biohazard bag.

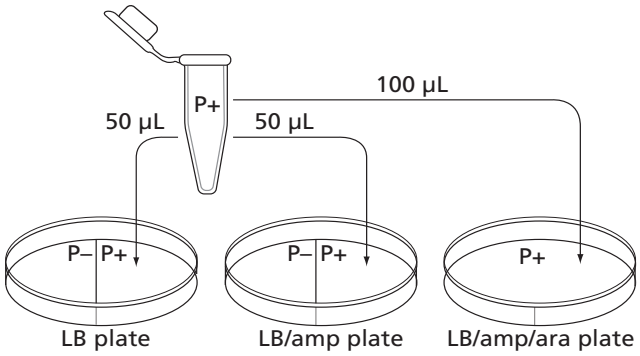
a. Hold the spreader only by the handle, and don't touch it to any other surfaces.

b. Open the lid of the LB plate like a clam shell, and gently spread the cells only across the P– side.

c. Repeat the process on the LB/amp plate using the same spreader.



16. Using a new micropipette tip for each plate, add 50 µL cells from the P+ tube to your LB and LB/amp plates, adding cells only to the P+ side of each plate. Be sure to gently mix the cells by pumping up and down before adding to plates.



17. Using the same technique, add 100 µL cells from the P+ tube to your LB/amp/ara plate. Be sure to gently mix the cells by pumping up and down before you add them, and distribute them across the plate as you dispense the solution.

18. Spread the cells from the P+ tube on your LB, LB/amp, and LB/amp/ara plates, being sure to spread cells only on the P+ side of the LB and LB/amp plates.

    a. Use the same spreader for all three plates

    b. For the LB/amp/ara plate, gently rotate the plate beneath the P+ spreader so that the cells can be spread evenly over the entire surface of this plate. Do not scrape the agar.

19. Allow all three plates to sit right side up for five minutes.

20. Place all microfuge tubes, micropipette tips, and cell spreaders in the biohazard container.

21. Stack and then tape all three plates together, and label the tape with your group identifier.

22. To prevent condensation on the agar, place the stack of plates in the 37°C incubator **upside down**. Wipe down your lab surface as directed by your teacher, and then wash hands.

23. Incubate the plates for 24–36 hours at 37°C.
24. After 24–36 hours, examine the plates and record the amount of growth in your notebook.
25. Discard the plates in the biohazard container when directed to do so.

STOP AND THINK

- **How is the P+ bacteria culture treated differently from the P– bacteria culture? (A culture is an isolated population of cells.)**
- **What is the purpose of the P– bacteria culture?**
- **Why do the cells need time to recover after the heat shock?**
- **Why are the cells incubated at 37°C?**
- **You used aseptic technique in this lab. Why is this important?**

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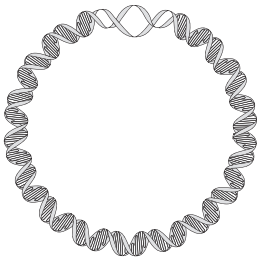


CHAPTER 2A

HOW DO YOU BEGIN TO CLONE A GENE?

INTRODUCTION

In the Program Introduction, you learned about the development of biopharmaceuticals and were introduced to the techniques used in developing these therapeutics. One of these techniques—bacterial transformation—allows human genes to be inserted into bacteria, enabling the bacteria to produce the human therapeutic proteins. Chapter 1 gave you a chance to work with two physical tools and techniques of genetic engineering that are used to clone a gene: the micropipette and gel electrophoresis. In this chapter you will work with two other important genetic engineering tools—plasmids and restriction enzymes. These "tools" are actually biomolecules found in many bacteria, and their discovery was crucial to genetic engineering. With these tools, scientists can modify microorganisms to make human proteins. You will now learn more about these tools and will then carry out the first steps in your quest to clone a gene.

CHAPTER 2A GOALS

By the end of this chapter, you will be able to do the following:

- Describe the characteristics of plasmids
- Explain how plasmids are used in cloning a gene
- Describe the function of restriction enzymes
- Explain how to use restriction enzymes to create a recombinant plasmid

WHAT DO YOU ALREADY KNOW?

Discuss the following questions with your partner and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these questions will help you think about what you already know about DNA, plasmids, and restriction enzymes.

1. What is the structure and function of DNA? Describe in words or a drawing the structure of a DNA molecule. Be as detailed as possible.

2. All living organisms contain DNA. In what ways is the DNA from different organisms the same, and in what ways does it vary?

3. Why it is possible for a bacterial cell to make a human protein from the instructions encoded in a human gene? Explain your answer, using your understanding of genes and how they are expressed.

4. As described in the Program Introduction, scientists use two biological tools to engineer organisms to make new proteins: plasmids and restriction enzymes. What do you remember about how these tools are used?

CLONE THAT GENE

You have learned about two biological tools for cloning a gene:

You now know about two biological tools for cloning a gene: plasmids and restriction enzymes.

1. Plasmids have several important features:
    - A sequence for the initiation of DNA replication, called the *ori* site, which allows the plasmid to replicate in the bacteria using the host DNA synthesis enzymes
    - A promoter for initiating transcription of the inserted gene
    - A gene encoding a protein for antibiotic resistance, which allows for identification of bacteria that have taken in the plasmid
2. Restriction enzymes digest both the plasmid and the human DNA containing the gene of interest (such as insulin) to be cloned.

How do scientists use these two tools to create a recombinant plasmid, which contains the insulin gene (or any other gene of interest) inserted into a bacterial plasmid? One important step is choosing a restriction enzyme or enzymes that cut the plasmid and the human DNA. The restriction enzyme(s) must do all of the following:

- Cut the plasmid at a site or sites that allow for the insertion of the new gene.
- Cut the plasmid at an appropriate site to ensure that no important genes or sequences are disrupted, including the *ori* site, the promoter, and at least one of the genes encoding antibiotic resistance.
- Cut the plasmid near the promoter so that the inserted gene can be expressed.
- Cut the human DNA as close as possible to both ends of the gene of interest so that it can be inserted into the appropriate site in the plasmid DNA, without cutting within the gene.

STOP AND THINK

Why is it important that the same enzyme or enzymes be used to cut both the plasmid and the insulin gene from the human DNA?

In this activity, you will make a paper model of a recombinant plasmid that contains an insulin gene. You have three tasks:

1. Cut the plasmid and the human DNA with the appropriate restriction enzyme.
2. Insert the insulin gene into the plasmid DNA.
3. Determine which antibiotic you would use to identify bacteria that have taken in the plasmid.

PROCEDURE

1. On the **Plasmid Diagram (RM 2)**, identify the plasmid. Then use scissors to cut out the plasmid sequence, and tape the ends together to make a paper model of the plasmid.
2. Choose the restriction enzyme that should be used to cut the plasmid. Verify that the restriction enzyme meets all the following criteria:
   - It leaves the *ori* site, the promoter, and at least one antibiotic-resistance gene intact.
   - It cuts the plasmid only once.
   - The cut is close to the promoter.
3. Review the **table below,** and use scissors to cut the plasmid at the recognition site exactly as the restriction enzyme would cut it. Write the sequences of the nucleotides that are left on each end of the plasmid.

Restriction enzymes used in this laboratory

Source	Restriction enzyme	Recognition site
Escherichia coli	*Eco*RI	5′ G↓AATTC 3′ 3′ CTTAA↑G 5′
Bacillus amyloliquefaciens	*Bam*HI	5′ G↓GATCC 3′ 3′ CCTAG↑G 5′
Haemophilus influenzae	*Hin*dIII	5′ A↓AGCTT 3′ 3′ TTCGA↑A 5′

The symbols ↑ and ↓ indicate where the DNA is cut.

4. On the **Human DNA Sequence (RM 3)**, scan the human DNA sequence, and determine where the three restriction enzymes, *Bam*HI, *Eco*RI, and *Hin*dIII, would cut the DNA.
5. Determine whether the restriction enzyme you chose in step 2 is a good choice for cutting out the insulin gene from the human DNA by verifying that it meets all the following criteria:
   - It does not cut within the insulin gene.
   - It cuts very close to the beginning and end of the gene.
   - It will allow the insulin gene to be inserted into the cut plasmid.
6. Review the **table above,** and use scissors to cut the human DNA at the recognition site exactly as the restriction enzyme would cut it. Write the sequences of the nucleotides that are left on each end of the insulin gene after it is cut from the human DNA.
7. Use tape to insert the insulin gene into the cut plasmid. Verify that the sticky ends will connect in the correct orientation. This is a paper model of a recombinant plasmid that contains an insulin gene. Once the plasmid replicates (copies) itself, the insulin gene is also copied, or cloned!

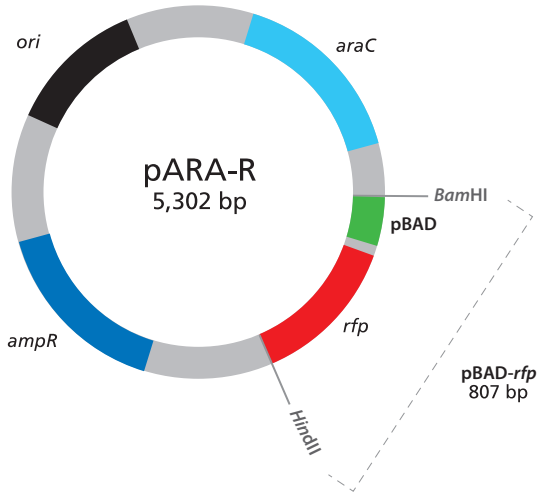
ACTIVITY QUESTIONS

1. Which restriction enzyme did you choose? Why did you choose that one?
2. Where would you insert the insulin gene and why?
3. Which antibiotic would you use to determine if the recombinant DNA was taken in?

LABORATORY 2A: PREPARING TO VERIFY THE *rfp* GENE: DIGESTING THE pARA-R PLASMID

To generate human therapeutic proteins, scientists need to first isolate a fragment of DNA that contains the human gene that codes for the desired protein and then insert that sequence into a plasmid. In this lab, you will do just that. You will ensure that the recombinant plasmid, pARA-R, you have been given is the correct one for making the RFP in bacteria. To do this you will use restriction enzymes to cut the plasmid (see **Figure 2A.5**), which will generate DNA fragments of lengths characteristic of the pARA-R plasmid. This procedure is called a restriction digest and the lengths of the fragments can be determined by gel electrophoresis (which you may do in Chapter 4A).

The recombinant DNA plasmid pARA-R contains the gene for ampicillin-resistance, the red fluorescent protein (*rfp*) gene, a promoter for initiating transcription, and the *ori* site for the initiation of DNA replication. The pARA-R plasmid also contains a DNA sequence that activates the promoter when the bacteria are grown in the presence of arabinose, a five-carbon sugar that naturally occurs in various plant and bacterial carbohydrates. This sequence is called the arabinose activator (*araC*). The activator controls the promoter. If arabinose is present in the bacteria, the promoter will bind RNA polymerase, and transcription will occur. In arabinose is not present, the promoter will not bind RNA polymerase, and transcription will not occur.



The pARA-R plasmid

The relevant components on the plasmid are the *rfp* gene, the promoter (pBAD), the ampicillin-resistance gene (*ampR*), and the arabinose activator (*araC*).

In addition to showing the relevant components, **the illlustration above** also shows the size of the plasmid (the number in the center, which indicates the number of base pairs [bp]) and the sequences where it can be cut by the restriction enzymes that will be used in the lab. The sites labeled "*Bam*HI" and

"*Hin*dIII" represent restriction sites for these two restriction enzymes. (See **the table on** on page SC-4.) In the cloning of the *rfp* gene, two restriction enzymes (*Bam*HI and *Hin*dIII) are used in cutting the plasmid and in isolating the *rfp* gene. Using two different restriction enzymes has advantages: It allows the inserted gene to be oriented in the correct position for transcribing the "sense" strand of DNA (the strand that codes for the protein), and it prevents the plasmid from reforming a circle without the inserted gene. You'll learn more about this if you do Chapter 4A.

BEFORE THE LAB

Respond to the following items with your group, and be prepared to share your responses with the class.

1. Review the **figure on the previous page**. If pARA-R is digested with *Bam*HI and *Hin*dIII, what fragments are produced? Record the nucleotide sequence of the sticky ends and the length of each fragment (bp), and indicate the genes and other important sequences present on each fragment.
2. To create a plasmid that can produce the red fluorescent protein in bacteria, what components are needed in the plasmid?
3. Bacteria can be killed by an antibiotic unless they carry a plasmid that has the gene for resistance to that antibiotic. These genes are known as selectable markers because only bacteria that carry the gene will survive an antibiotic. If the uptake of DNA by bacteria is inefficient, why is a selectable marker critical in cloning a gene in bacteria?
4. Read through the *Methods* section, and briefly outline the steps using words and a flowchart.

METHODS

1. With a marker, label the caps of two clean microfuge tubes with your group identifier and R+ and R–.
2. **To avoid contamination, use a new micropipette tip for each reagent,** and add the following:

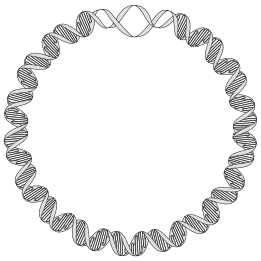
	R+ tube	R– tube
a. Restriction buffer (2.5xB)	4.0 µL	4.0 µL
b. pARA-R plasmid (RP)	4.0 µL	4.0 µL
c. Restriction enzymes (RE)	2.0 µL	
d. Distilled water (dH_2O)		2.0 µL

* For d and e: Add directly into the solution at the bottom of the microfuge tube, and gently pump the solution in and out to mix. Cap the tubes when done.

3. Distribute the two microfuge tubes (R+ and R–) evenly in the micro-centrifuge, and spin for several seconds to pool the reagents at the bottom of each tube.
4. Place both tubes into a floating microfuge rack. Place the rack in the 37°C water bath, and note your start time. Incubate for at least 5 minutes, but no longer than 15 minutes. After the incubation is complete, place both tubes in the freezer at –20°C. You will analyze the contents of the tubes in Laboratory 4A.

STOP AND THINK

Why might the enzymes work best at 37°C? (*Hint:* The normal human body temperature is approximately 37°C.) Why should the enzymes then be placed in the freezer?



CHAPTER 4A

MAKING SURE YOU'VE GOT A RECOMBINANT PLASMID

INTRODUCTION

When scientists clone a gene in order to produce a human therapeutic protein, they create a recombinant plasmid that includes the human gene of interest. To do so, they use restriction enzymes to create DNA fragments that contain the plasmid components (Chapter 2A) and then use DNA ligase to join those fragments together. As part of the gene cloning process, scientists have to verify (confirm) that they have created the recombinant plasmid they need—that is, the one with the gene of interest (which will make the therapeutic human protein) and all the necessary components for that protein to be made. In this chapter, you will continue to work with the tools of genetic engineering as you verify that you have the recombinant plasmid you need in order to produce RFP.

CHAPTER 4A GOALS

By the end of this chapter, you will be able to do the following:

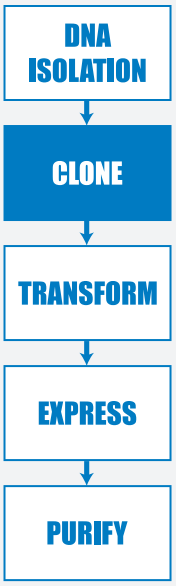
- Describe why it is important to verify products created in the genetic engineering process
- Predict the relative speed of DNA restriction fragments and plasmids through a gel during gel electrophoresis
- Separate and identify DNA restriction fragments and plasmids using gel electrophoresis

WHAT DO YOU ALREADY KNOW?

Discuss the following questions with your partner and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these questions will help you think about what you already know about gel electrophoresis and verification in the lab.

1. Why do DNA restriction fragments and plasmids separate when analyzed by gel electrophoresis?

2. Why is it important to identify and verify a recombinant plasmid?



LABORATORY 4A: VERIFICATION OF THE RECOMBINANT PLASMID USING GEL ELECTROPHORESIS

In this laboratory, you will use gel electrophoresis to examine the products from the restriction digest of the pARA-R plasmid (Laboratory 2A). It is important to verify work in the lab—there are many sources of potential error in any procedure, including the procedures used in cloning a gene. In gene cloning, there is also the problem that some procedures are not selective. For example, when a DNA ligase is used to ligate (bind together) DNA fragments, many different combinations result from the ligation process. Unless you verify your work, you do not know if you are using the recombinant plasmid that is needed.

You can determine the sizes of the DNA fragments by comparing them to a DNA ladder (a mixture of DNA fragments with known sizes). The DNA ladder is loaded adjacent to other DNA samples in order to make it easy to compare the bands in the samples with the bands in the ladder. Whereas short, linear pieces of DNA move as expected when run on gel electrophoresis, the movement of plasmids is not as straightforward. This is because a plasmid can exist in different configurations that move at different rates through the gel. There are three plasmid configurations—supercoiled, nicked, and multimer—so you may see several bands for the uncut plasmid where you would expect to see a single band.

The results from the gel electrophoresis will provide evidence that you are using the pARA-R recombinant plasmid that contains the *rfp* gene. The same procedure would be used to verify that a recombinant plasmid created in the lab contained the gene for a human therapeutic protein.

BEFORE THE LAB

Discuss the following items with your group, and be prepared to share your responses with the class:

1. The pARA-R plasmid you digested in Laboratory 2A was replicated in a bacterial cell. What configurations—supercoiled, nicked circle, and multimer—might the plasmid have before digestion?
2. You need to catalog all the products you might see, including the different plasmid configurations. Review your work in Laboratory 2A. What products might you expect to see in the R– and R+ tubes? Create a table that shows all the possible fragments and plasmids by tube. Include the length (bp size) of each possible fragment or plasmid, and arrange the products found in each microfuge tube by size, from smallest to largest. Include any possible plasmid configurations, and arrange them first by size and next by speed through the gel, from fastest to slowest.

3. Read through the *Methods* section, and briefly outline the steps using words and a flowchart.

METHODS

1. Obtain your samples, and add 2.0 µL of loading dye (LD) to the R– and R+ tubes.

STOP AND THINK

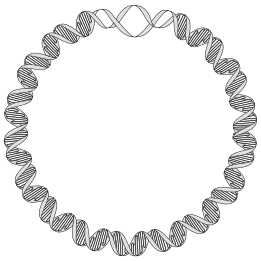
The DNA is not visible as it moves through the gel. The loading dye contains the three dyes that you separated in Laboratory 1.2. Why is it useful to use the loading dye in this lab?

2. Arrange the R– and R+ tubes evenly in the microcentrifuge and spin for several seconds to pool the reagents at the bottom of each tube.
3. Make sure that the wells in your gel electrophoresis unit are located near the negative (black) electrode.
4. Fill the box with 1x SB buffer to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more buffer.
5. Make a drawing in your notebook that shows the location of the wells in the electrophoresis box. The order of the samples in each well is shown **below**.

6. Using a new micropipette tip for each sample, dispense 10.0 µL of DNA marker/ladder (M), R–, and R+ into their designated wells.
7. When all the samples have been loaded, close the cover tightly over the electrophoresis box and turn on the power supply to the voltage indicated by your teacher.
8. After two or three minutes, check to see if the purple loading dye (bromophenol blue) is moving toward the positive (red) electrode. If it's moving in the other direction—toward the negative (black) electrode—check the electrical leads to see whether they are plugged into the power supply correctly.
9. Your teacher will explain what to do with your gel. You may not have sufficient time to complete the electrophoresis. The smallest loading dye molecules (yellow) will need to run just to the end of the gel, about 30–50 minutes (depending upon your machine). After the gel has finished running, it will need to be imaged to show the location of the DNA fragments and plasmids.

STOP AND THINK

- The DNA ladder serves as a standard because it contains a mixture of DNA molecules of known sizes. By running your samples and the DNA ladder side by side in your gel, you can estimate the actual size in base pairs of unknown molecules. The **DNA Ladder Diagram (RM 4A)** shows 10 DNA bands of known sizes. Using this information, can you predict the positions of DNA bands produced by the possible products found in the R– and R+ tubes by indicating their position on the **DNA Ladder Diagram**?
- The DNA samples and the DNA ladder are not visible on the gel. How might the DNA be made visible once the gel electrophoresis is complete?



CHAPTER 5A

GETTING RECOMBINANT PLASMIDS IN BACTERIA

INTRODUCTION

Once a recombinant plasmid that includes the gene of interest has been created, the next step is to replicate the plasmid and allow the bacteria to produce the protein. Both replication and protein expression (the way that proteins are synthesized, modified, and regulated in living organisms) can occur only inside a cell. Therefore, your next step in the gene cloning process is to put the recombinant plasmid into *E. coli* bacteria through a process called bacterial transformation, which changes the DNA content of the bacteria. In this chapter, you will carry out the transformation of *E. coli* bacteria using a recombinant plasmid that contains the *rfp* gene. If you were making a human therapeutic protein, the bacteria that you transform would contain the human gene and would be capable of producing the desired human therapeutic protein.

CHAPTER 5A GOALS

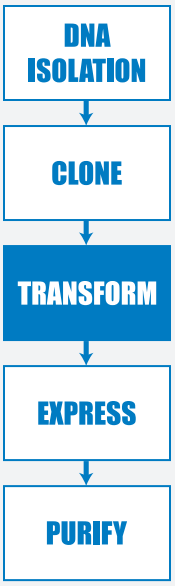
By the end of this chapter, you will be able to do the following:

- Describe the role of transformation in the gene cloning process
- Explain the purpose of each control in the transformation experiment
- Explain how the information encoded in a gene is expressed (the process of converting this information into messenger RNA and then to a protein) as a trait

WHAT DO YOU ALREADY KNOW?

Discuss the following questions with your partner, and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these questions will help you think about what you already know about plasmid uptake and gene expression in bacteria.

1. Do you think that bacterial uptake of a plasmid from the environment is a common event? Why or why not?
2. What are the steps involved in the transcription and translation (the process by which information encoded in messenger RNA is decoded and transformed into protein) of a gene?
3. What is the relationship among genes, proteins, and traits (or observable characteristics)?
4. What do bacteria and humans have in common that makes it possible for a human gene to be expressed in bacteria?



LABORATORY 5A: TRANSFORMING BACTERIA WITH THE pARA-R PLASMID

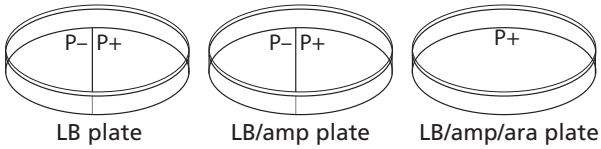
So far in your quest to clone a gene, you have prepared to verify the plasmid by performing a restriction digest (Laboratory 2A), then confirmed the identity of the provided plasmid using gel electrophoresis (Laboratory 4A). In this laboratory you will carry out another step of the gene cloning process: transforming *E. coli* bacteria with the provided plasmid. In the biopharma industry, this process would be used to create the human therapeutic protein. You will divide *E. coli* bacteria that have been pretreated with calcium chloride into two groups: a control group to which no plasmid will be added, and a treatment group to which you will add the pARA-R plasmid. After heat-shocking both groups of cells, you will grow them under several different conditions. All of the bacteria will be grown on three agar plates (Petri plates containing agar mixed with a medium or food source named Luria Broth [LB] that supports bacterial growth). One plate will just contain LB agar, a second will have the antibiotic ampicillin (amp) added to the LB, and a third will include LB, amp, and arabinose sugar (ara) to activate transcription of the protein of interest (RFP).

By examining the growth of bacteria under these conditions, you can verify that your transformation procedure worked, and you can identify the bacteria transformed with the pARA-R plasmid. How will you know if you are successful? The bacteria will have a new and highly visible trait: They will now produce RFP, which makes the cells red or bright pink! If you were making a human therapeutic protein, the bacteria would produce that protein, which would be invisible. However, the products created by the bacteria would be tested to ensure that they contained the desired protein.

BEFORE THE LAB

Discuss the following items with your group, and be prepared to share your responses with the class.

1. Ampicillin is an antibiotic that kills bacterial cells by disrupting the formation of cell walls. However, the pARA-R plasmid has the ampicillin resistance gene, which produces a protein that breaks down ampicillin. What is the purpose of growing bacteria that have been transformed in the presence of ampicillin?
2. What will happen when bacterial cells that contain the pARA-R plasmid are not given arabinose?
3. In the lab, you will add samples of the control group P– and the treatment group P+ to agar plates. The plates will be arranged as shown **below**.



LB plate LB/amp plate LB/amp/ara plate

Using the key on **Bacterial Growth Predictions (RM 5)**, show your predictions for the growth you would expect for each combination. Then fill in **Table 1** and **Table 2** in the handout by describing the conclusions that can be drawn if the predicted growth occurs or does not occur.

4. Read through the *Methods* section, and briefly outline the steps using words and a flowchart.

METHODS

SAFETY: Use caution when handling *E. coli* bacteria, and use aseptic technique as instructed by your teacher. Aseptic technique is a set of procedures that ensure protection of the lab worker and ensure your bacteria cultures are not contaminated with unwanted microbes, both of which are necessary for the experiment to be successful.

1. Obtain a tube of competent cells (CC) and keep it cold by picking it up by the upper rim and placing it immediately on ice. Label your CC tube with your group identifier.
2. Label two clean microfuge tubes with "P–" and "P+" and your group identifier.
3. Place the P– and P+ tubes on ice with the CC tube.
4. Obtain a P-200 micropipette. This will be used to add the competent cells from the CC tube to the P– and P+ tubes as directed below:
    a. Set the P-200 micropipette to 50 µL.
    b. Very carefully, resuspend the bacterial cells in the CC tube by gently pumping two times in the solution. Expel all of the cells back into the tube before moving on.
    c. Using a new micropipette tip, add 50 µL of CC to the chilled P– tube. Repeat for the P+ tube. Be sure to hold each tube at its rim to keep it cold, returning each tube quickly to the ice.
5. Obtain a P-20 micropipette to add RP to the tube labeled "P+" according to directions below:
    a. Set the P-20 micropipette to 10.0 µL.
    b. Hold the chilled P+ tube by the upper rim, and add 10.0 µL of RP. Mix the solutions by pumping two times in the liquids, and return the P+ tube to the ice.
6. Keep the P– and P+ tubes on ice for 15 minutes.

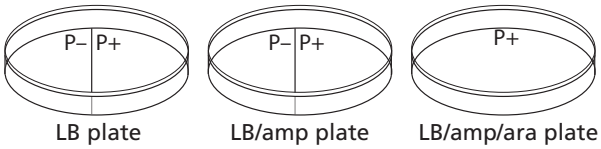
NOTE: During the 15-minute interval, share and discuss your answers to question 3 in Before the Lab and complete step 7.

7. While the cells are on ice, prepare your three agar Petri plates—one plate each of LB, LB/amp, and LB/amp/ara:

   a. Label the bottom of each plate (the part that contains the agar) with your group identifier.

   NOTE: Write small and on the edge of the plate.

   b. With the plates closed, draw a line on the LB plate and the LB/amp plate that divides each plate in the middle. Label half of each plate "P–" and the other half "P+." Label the LB/amp/ara plate "P+." The plates will be arranged as shown **below.**



8. Following the 15-minute incubation on ice, bring the P– and P+ tubes **on ice** to the water bath. Place the two tubes in the floating microfuge tube rack in the 42°C water bath **for exactly 45 seconds**.
9. After the 45-second heat shock, **immediately** place the tubes back on ice and leave them there for at least one minute.
10. Obtain a P-200 micropipette, and add LB to the P– and P+ tubes using the following steps:

    a. Set the P-200 micropipette to 150 µL.

    b. Add 150 µL of LB to the P– tube, and gently pump up and down two or three times to mix. Cap the tube.

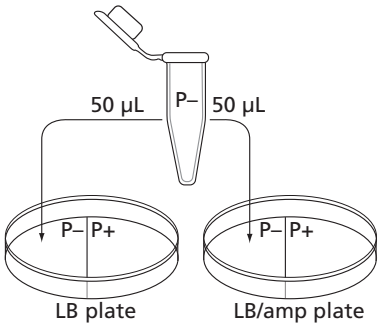
    c. Using a new micropipette tip, add 150 µL of LB to the P+ tube and gently pump up and down two or three times to mix. Cap the tube.

11. If time permits, allow the cells in the P– and P+ tubes to incubate at room temperature for 15 minutes.
12. Following the directions below, add cells from the P– tube onto your LB and LB/amp plates:

    a. Set the P-200 micropipette to 50 µL.

    b. Gently resuspend the cells in the P– tube, and load 50 µL of the P– cells.

    c. Open the lid of the LB plate like a clamshell, and add 50 µL of cells from the P– tube to the section marked "P–." Close the lid.



13. Using a new micropipette tip, repeat steps 12b and c, adding 50 µL of cells from the P– tube to the LB/amp plate.

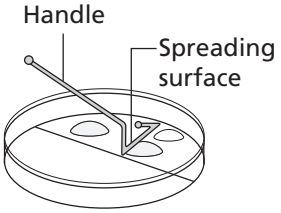
14. Spread the cells from the P– tube on your LB and LB/amp plates:

LAB TECHNIQUE: Hold the spreader by the handle, and do not allow the bent end to touch any surface, as this will contaminate the spreader. Place the used spreader in the biohazard bag.

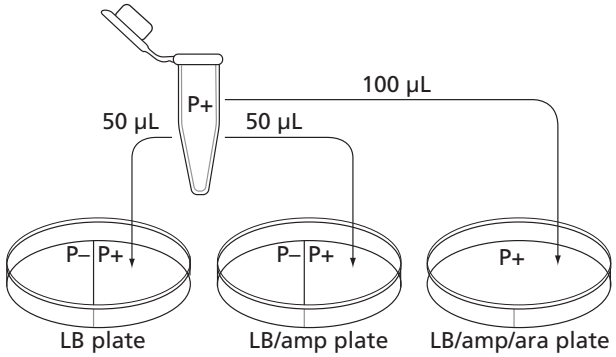
a. Hold the spreader only by the handle and don't touch it to any other surfaces.

b. Open the lid of the LB plate like a clam shell, and gently spread the cells only across the P– side.

c. Repeat the process on the LB/amp plate using the same spreader.



15. Using a new micropipette tip for each plate, add 50 µL cells from the P+ tube to your LB and LB/amp plates, adding cells only to the P+ side of each plate. Be sure to gently mix the cells by pumping up and down before adding to the plates.



16. Using the same technique, add 100 µL cells from the P+ tube to your LB/amp/ara plate. Be sure to gently mix the cells by pumping up and down before you add them, and distribute them across the plate as you dispense the solution.

17. Spread the cells from the P+ tube on your LB, LB/amp, and LB/amp/ara plates, being sure to spread cells only on the P+ side of the LB and LB/amp plates.

a. Use the same spreader for all three plates

b. For the LB/amp/ara plate, gently rotate the plate beneath the P+ spreader so that the cells can be spread evenly over the entire surface of this plate. Do not scrape the agar.

18. Allow all three plates to sit right side up for five minutes.

19. Place all microfuge tubes, micropipette tips, and cell spreaders in the biohazard container

20. Stack and then tape all three plates together, and label the tape with your group identifier.
21. To prevent condensation on the agar, place the stack of plates **upside down** in the 37°C incubator. Wipe down your lab surface as directed by your teacher, and then wash hands.
22. Incubate the plates for 24–36 hours at 37°C.
23. After 24–36 hours, examine the plates, and record the amount of growth in your notebook.
24. Discard the plates in the biohazard container when directed to do so.

STOP AND THINK

- **How is the P+ bacteria culture treated differently from the P– bacteria culture? (A culture is an isolated population of cells.) What is the purpose of the P– bacteria culture?**
- **Why do the cells need time to recover after the heat shock?**
- **Why are the cells incubated at 37°C?**
- **You used aseptic technique in this lab. Why is this important?**

SD

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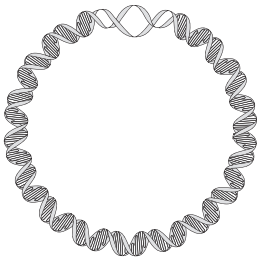


CHAPTER 5B

GETTING RECOMBINANT PLASMIDS IN BACTERIA

INTRODUCTION

Chapter 1 gave you a chance to work with two physical tools and techniques of genetic engineering that are used to clone a gene: the micropipette and gel electrophoresis. Two other important genetic engineering tools are plasmids and restriction enzymes—biomolecules found in many bacteria. These tools enable scientists to create a vector, a vehicle for carrying DNA sequences from one organism to another. This vector is a recombinant plasmid, a small piece of circular DNA that contains a gene. Once the plasmid has been taken up by *E. coli*—a common bacterium found in the gut of warm blooded animals—through a process called transformation, the plasmid can replicate (be copied) and its gene can be expressed (the protein can be made) using the bacterial cell's protein synthesizing machinery. In this chapter, you will model how a recombinant plasmid is made, and then practice the lab skills you learned in Chapter 1 as you carry out the transformation of *E. coli* bacteria using a recombinant plasmid that contains the *rfp* gene. If you were making human therapeutic protein, the bacteria that you transform would contain the human gene and would be capable of producing the desired human therapeutic protein.

CHAPTER 5B GOALS

By the end of this chapter, you will be able to do the following:

- Describe the characteristics of plasmids
- Explain how to use restriction enzymes to create a recombinant plasmid
- Describe the role of transformation in the gene cloning process
- Explain the purpose of each control in the transformation experiment
- Explain how the information encoded in a gene is expressed as a trait

WHAT DO YOU ALREADY KNOW?

Discuss the following questions with your partner, and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these questions will help you think about what you already know about DNA, plasmids, restriction enzymes, plasmid uptake, and gene expression in bacteria.

1. All living organisms contain DNA. In what ways is DNA from different organisms the same, and in what ways does it vary?
2. Scientists use two biological tools to engineer organisms to make new proteins: plasmids and restriction enzymes. How might each of these be useful in creating a new protein?
3. What is the relationship among genes, proteins, and traits (or observable characteristics)?
4. What do bacteria and humans have in common that makes it possible for a human gene to be expressed in bacteria?

CLONE THAT GENE

You now know about two biological tools for cloning a gene: plasmids and restriction enzymes.

1. Plasmids have several important features:
    - A sequence for the initiation of DNA replication, called the *ori* site, which allows the plasmid to replicate in the bacteria using the host DNA synthesis enzymes
    - A promoter for initiating transcription of the inserted gene
    - A gene encoding a protein for antibiotic resistance, which allows for identification of bacteria that have taken in the plasmid
2. Restriction enzymes digest both the plasmid and the human DNA containing the gene of interest (such as insulin) to be cloned.

How do scientists use these two tools to create a recombinant plasmid, which contains the insulin gene (or any other gene of interest) inserted into a bacterial plasmid? One important step is choosing a restriction enzyme or enzymes that cut the plasmid and the human DNA. The restriction enzyme(s) must do all of the following:

- Cut the plasmid at a site (or sites) that allows for the insertion of the new gene.
- Cut the plasmid at an appropriate site to ensure that no important genes or sequences are disrupted, including the *ori* site, the promoter, and at least one of the genes encoding antibiotic resistance.
- Cut the plasmid near the promoter so that the inserted gene can be expressed.
- Cut the human DNA as close as possible to both ends of the gene of interest so that it can be inserted into the appropriate site in the plasmid DNA, without cutting within the gene.

STOP AND THINK

Why is it important that the same enzyme or enzymes be used to cut both the plasmid and the insulin gene from the human DNA?

In this activity, you will make a paper model of a recombinant plasmid that contains an insulin gene. You have three tasks:

1. Cut the plasmid and the human DNA with the appropriate restriction enzyme.
2. Insert the insulin gene into the plasmid DNA.
3. Determine which antibiotic you would use to identify bacteria that have taken in the plasmid.

PROCEDURE

1. On the **Plasmid Diagram (RM 2)**:
    - Identify the plasmid. Then use scissors to cut out the plasmid sequence, and tape the ends together to make a paper model of the plasmid.
    - Locate the positions of the *ori* site, the promoter site, and the genes for antibiotic resistance.
    - Locate the positions of each restriction enzyme recognition site.
2. Examine the plasmid sequence, and choose the restriction enzyme that should be used to cut the plasmid. Verify that the restriction enzyme meets all the following criteria:
    - It leaves the *ori* site, the promoter, and at least one antibiotic-resistance gene intact.
    - It cuts the plasmid only once.
    - The cut is close to the promoter.
3. Review the **table below,** and use scissors to cut the plasmid at the recognition site exactly as the restriction enzyme would cut it. Write the sequences of the nucleotides that are left on each end of the plasmid.

Restriction enzymes used in this laboratory

Source	Restriction enzyme	Recognition site
Escherichia coli	*Eco*RI	5′ G↓AATTC 3′ 3′ CTTAA↑G 5′
Bacillus amyloliquefaciens	*Bam*HI	5′ G↓GATCC 3′ 3′ CCTAG↑G 5′
Haemophilus influenzae	*Hin*dIII	5′ A↓AGCTT 3′ 3′ TTCGA↑A 5′

The symbols ↑ and ↓ indicate where the DNA is cut.

4. On the **Human DNA Sequence (RM 3)**, scan the human DNA sequence and determine where the three restriction enzymes, *Bam*HI, *Eco*RI, and *Hin*dIII, would cut the DNA.
5. Determine whether the restriction enzyme you chose in step 2 is a good choice for cutting out the insulin gene from the human DNA by verifying that it meets all the following criteria:
    - It does not cut within the insulin gene.
    - It cuts very close to the beginning and end of the gene.
    - It will allow the insulin gene to be inserted into the cut plasmid.
6. Review the **table on the previous page,** and use scissors to cut the human DNA at the recognition site exactly as the restriction enzyme would cut

it. Write the sequences of the nucleotides that are left on each end of the insulin gene after it is cut from the human DNA.

7. Use tape to insert the insulin gene into the cut plasmid. Verify that the sticky ends will connect in the correct orientation. This is a paper model of a recombinant plasmid that contains an insulin gene. Once the plasmid replicates (copies) itself, the insulin gene is also copied, or cloned!

   The relevant components of this plasmid are the *rfp* gene, the promoter (pBAD), the ampicillin resistance gene (*ampR*), and the arabinose activator protein gene (*araC*). The *ampR* gene confers resistance to the antibiotic ampicillin. (Scientists call these genes "selectable markers" because only bacteria that carry the gene will survive an antibiotic.) The *araC* gene controls the promoter. If arabinose, a simple sugar, is present in the bacteria, the activator protein made by the *araC* gene turns on the promoter, which then binds RNA polymerase, and transcription of the *rfp* gene occurs. Activator proteins are used in some recombinant plasmids to control production of the protein of interest.

ACTIVITY QUESTIONS

1. Which restriction enzyme did you choose? Why did you choose that one?
2. Where would you insert the insulin gene and why?
3. Which antibiotic would you use to determine if the recombinant DNA was taken in?

LABORATORY 5B: TRANSFORMING BACTERIA WITH A RECOMBINANT PLASMID (pARA-R)

In this laboratory you will carry out an important step of the gene cloning process, which is to transform *E. coli* bacteria with the pARA-R plasmid. The pARA-R plasmid contains the *rfp* gene that can make red fluorescent protein (RFP). You will divide *E. coli* bacteria that have been pretreated with calcium chloride into two groups: a control group to which no plasmid will be added, and a treatment group to which you will add the pARA-R plasmid.

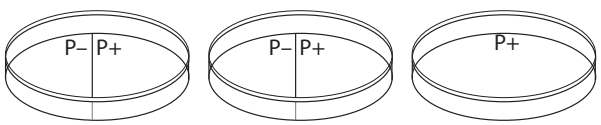
After heat-shocking both groups of cells, you will grow them under several different conditions. All of the bacteria will be grown on three agar plates (Petri plates containing agar mixed with a medium or food source named Luria Broth [LB] that supports bacterial growth). One plate will just contain LB agar, a second will have the antibiotic ampicillin (amp) added to the LB, and a third will include LB, amp, and arabinose sugar (ara) to activate transcription of the protein of interest (RFP).

By examining the growth of bacteria under these conditions, you can verify that your procedure worked, and you can identify the bacteria transformed with the pARA-R plasmid. How will you know if you are successful? The bacteria will have a new and highly visible trait: They will now produce RFP, which makes the cells red or bright pink!

BEFORE THE LAB

Discuss the following items with your group, and be prepared to share your responses with the class.

1. Ampicillin is an antibiotic that kills bacterial cells by disrupting the formation of cell walls. However, the pARA-R plasmid has the ampicillin resistance gene, which produces a protein that breaks down ampicillin. What is the purpose of growing bacteria that have been transformed in the presence of ampicillin?
2. What will happen when bacterial cells that contain the pARA-R plasmid are not given arabinose?
3. In the lab, you will add samples of the control group P– and the treatment group P+ to agar plates. The plates will be arranged as shown **below**.



LB plate LB/amp plate LB/amp/ara plate

Using the key on **Bacterial Growth Predictions (RM 5)**, show your predictions for the growth you would expect for each combination. Then fill in **Table 1** and **Table 2** in the handout by describing the conclusions that can be drawn if the predicted growth occurs or does not occur.

4. Due to a mishap in the lab, bacteria carrying a plasmid with an ampicillin-resistant gene and bacteria carrying a plasmid with a gene that provides resistance to another antibiotic (kanamycin) were accidentally mixed together. Design an experiment that will allow you to sort out the two kinds of bacteria. (*Hint*: Make sure that you do not kill off one of the kinds of bacteria you are trying to sort out!)
5. Read through the *Methods* section, and briefly outline the steps using words and a flowchart.

METHODS

SAFETY: Use caution when handling *E. coli* bacteria, and use aseptic technique as instructed by your teacher. Aseptic technique is a set of procedures that ensure protection of the lab worker and ensure your bacteria cultures are not contaminated with unwanted microbes, both of which are necessary for the experiment to be successful.

1. Obtain a tube of competent cells (CC), and keep it cold by picking it up by the upper rim and placing it immediately on ice. Label your CC tube with your group identifier.
2. Label two clean microfuge tubes with "P–" and "P+" and your group identifier.
3. Place the P– and P+ tubes on ice with the CC tube.
4. Obtain a P-200 micropipette. This will be used to add the competent cells from the CC tube to the P– and P+ tubes as directed below:
    a. Set the P-200 micropipette to 50 µL.
    b. Very carefully resuspend the bacterial cells in the CC tube by gently pumping two times in the solution. Expel all of the cells back into the tube before moving on.
    c. Using a new micropipette tube, add 50 µL of CC to the chilled P– tube. Repeat for the P+ tube. Be sure to hold each tube at its rim to keep it cold, returning each tube quickly to the ice.
5. Obtain a P-20 micropipette to add pARA-R plasmid (RP) to the tube labeled "P+" according to directions below:
    a. Set the P-20 micropipette to 10 µL.
    b. Hold the chilled P+ tube by the upper rim, and add 10 µL of LIG. Mix the solutions by pumping two times in the liquids, and return the P+ tube to the ice.

6. Keep the P– and P+ tubes on ice for 15 minutes.

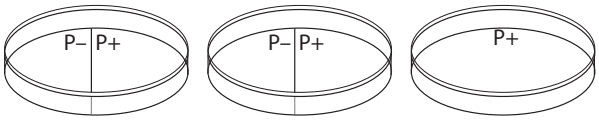
   NOTE: During the 15-minute interval, share and discuss your answers to question 3 in Before the Lab, and complete step 7.

7. While the cells are on ice, prepare your three agar plates—one plate each of LB, LB/amp, and LB/amp/ara:

   a. Label the bottom of each plate (the part that contains the agar) with your group identifier.

   NOTE: Write small and on the edge of the plate.

   b. With the plates closed, draw a line on the LB plate and the LB/amp plate that divides each plate in the middle. Label half of each plate "P–" and the other half "P+." Label the LB/amp/ara plate "P+." The plates will be arranged as shown in **below**.



8. Following the 15-minute incubation on ice, bring the P– and P+ tubes **on ice** to the water bath. Place the two tubes in the floating microfuge tube rack in the 42°C water bath **for exactly 45 seconds.**
9. After the 45-second heat shock, **immediately** place the tubes back on ice and leave them there for at least a minute.
10. Obtain a P-200 micropipette, and add LB to the P– and P+ tubes using the following steps:

    a. Set the P-200 micropipette to 150 µL.

    b. Add 150 µL of LB to the P– tube, and gently pump up and down two or three times to mix. Cap the tube.

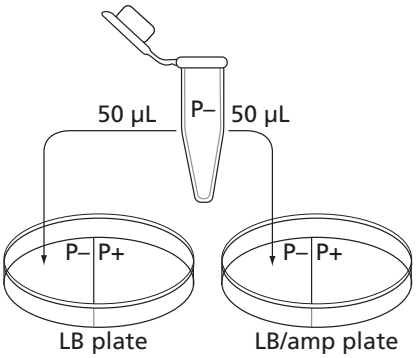
    c. Using a new micropipette tip, add 150 µL of LB to the P+ tube, and gently pump up and down two or three times to mix. Cap the tube.

11. If time permits, allow the cells in the P– and P+ tubes to incubate at room temperature for 15 minutes.
12. Following directions below, add cells from the P– tube onto your LB and LB/amp plates:

    a. Set the P-200 micropipette to 50 µL.

    b. Gently resuspend the cells in the P– tube, and load 50 µL of the P– cells.

    c. Open the lid of the LB plate like a clamshell, and add 50 µL of cells from the P– tube to the section marked "P–." Close the lid.



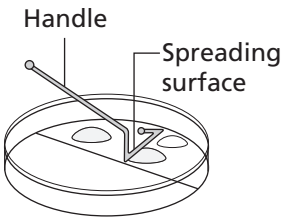
13. Using a new micropipette tip, repeat steps b and c, adding 50 µL of cells from the P– tube to the LB/amp plate.
14. Spread the cells from the P– tube on your LB and LB/amp plates:

    LAB TECHNIQUE: Hold the spreader by the handle, and do not allow the bent end to touch any surface, as this will contaminate the spreader. Place the used spreader in the biohazard bag.

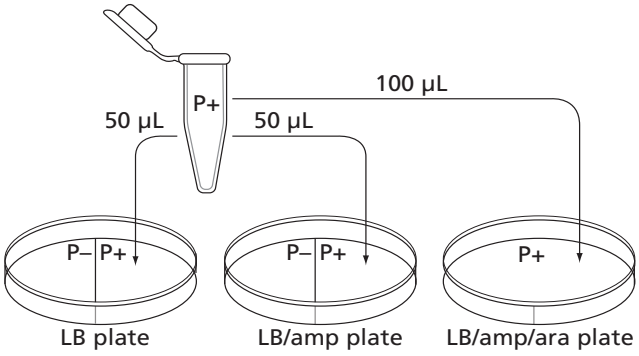
    a. Hold the spreader only by the handle and don't touch it to any other surfaces.

    b. Open the lid of the LB plate like a clam shell, and gently spread the cells only across the P– side.

    c. Repeat the process on the LB/amp plate using the same spreader.



15. Using a new micropipette tip for each plate, add 50 µL cells from the P+ tube to your LB and LB/amp plates. Add cells only to the P+ side of each plate. Be sure to gently mix the cells by pumping up and down before adding to the plates.



16. Using the same technique, add 100 µL cells from the P+ tube to your LB/amp/ara plate. Be sure to gently mix the cells by pumping up and down before you add them, and distribute them across the plate as you dispense the solution.
17. Spread the cells from the P+ tube on your LB, LB/amp, and LB/amp/ara plates, being sure to spread cells only on the P+ side of the LB and LB/amp plates.

    a. Use the same spreader for all three plates.

    b. For the LB/amp/ara plate, gently rotate the plate beneath the P+ spreader so that the cells can be spread evenly over the entire surface of this plate. Do not scrape the agar.

18. Allow all three plates to sit right side up for five minutes.
19. Place all microfuge tubes, micropipette tips, and cell spreaders in the biohazard container.

20. Stack and then tape all three plates together, and label the tape with your group identifier.
21. To prevent condensation on the agar, place the stack of plates **upside down** in the 37°C incubator. Wipe down your lab surface as directed by your teacher, and then wash hands.
22. Incubate the plates for 24–36 hours at 37°C.
23. After 24–36 hours, examine the plates and record the amount of growth in your notebook.
24. Discard the Petri plates in the biohazard container when directed to do so.

STOP AND THINK

- **How is the P+ bacteria culture treated differently from the P– bacteria culture? (A culture is an isolated population of cells.) What is the purpose of the P– bacteria culture?**
- **Why do the cells need time to recover after the heat shock?**
- **Why are the cells incubated at 37°C?**
- **You used aseptic technique in this lab. Why is this important?**

SE

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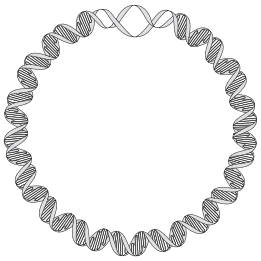


CHAPTER 6

GETTING WHAT YOU NEED

INTRODUCTION

Genetic engineering is used to produce therapeutic proteins. To provide a treatment for diabetes, for example, a recombinant plasmid is engineered to contain a cloned human insulin gene. Bacteria take up the recombinant plasmid and express the gene, producing insulin. To date, you have carried out all or some of these steps using the cloned *rfp* gene rather than a human gene that would produce a therapeutic protein.

The final step in the process is to obtain the protein. To do this, bacteria are treated with a reagent that lyses them (breaks open their cell walls), and the protein is separated from the cell contents by a method called column chromatography. (Chromatography is a method for separating similar substances by dissolving them and then flowing the solution over a material that binds the substances to different degrees. Column chromatography uses a column packed with beads coated with the binding material.)

In this chapter, you will complete this final step. You will lyse the bacteria you transformed in Chapter 5 and then use a column that separates proteins based on their solubility in water to obtain RFP made by the cloned *rfp* gene. This same process would be used to isolate a human therapeutic protein.

CHAPTER 6 GOALS

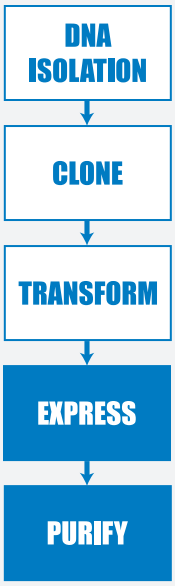
By the end of this chapter, you will be able to do the following:

- Describe the conditions that are favorable to bacterial growth
- Explain how a protein's conformation (three-dimensional shape) is related to its function
- Explain how protein folding (the physical process by which a protein folds into its characteristic three-dimensional structure, which is essential to its function) occurs
- Explain how column chromatography separates proteins

WHAT DO YOU ALREADY KNOW?

Discuss the following questions with your partner, and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these questions will help you think about what you already know about bacterial growth and proteins.

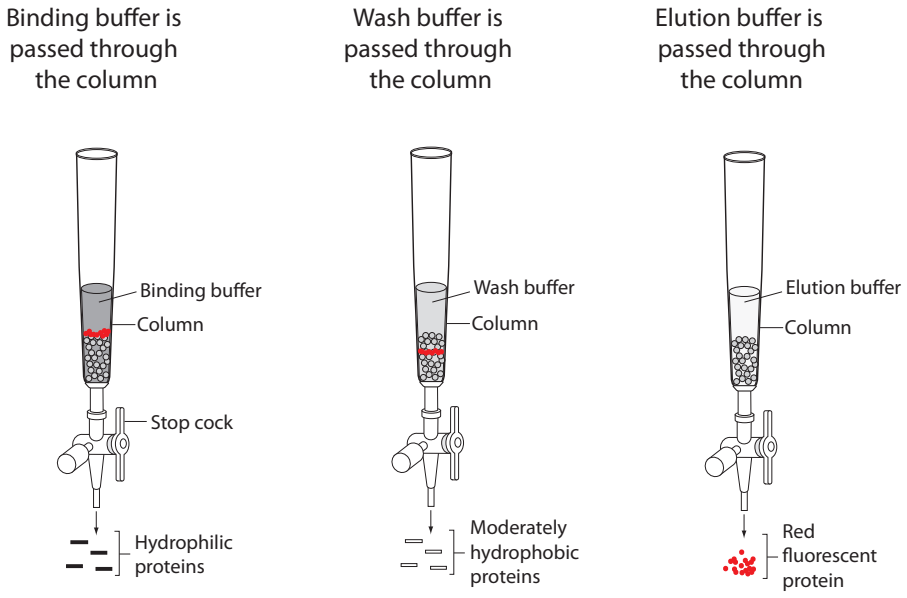
1. How do bacteria reproduce?
2. Why are proteins sometimes called workhorse molecules?
3. How might the conformation (shape or folding) of a protein be important for its function? Focus on one of the following protein functions: acting as an enzyme (speeding up reaction rates), transporting molecules, signaling, or forming structures.



LABORATORY 6: PURIFYING THE FLUORESCENT PROTEIN

In the previous laboratory, you transformed bacteria and then selected the bacteria that had the plasmid of interest by placing the cells on a plate that contained LB, ampicillin, and arabinose. One colony was then selected and grown in a shaker flask to provide a large population of identical cells that all contain one or more copies of the recombinant plasmid. The cells were given arabinose to turn on the *rfp* gene so that it would make red fluorescent protein.

In the first part of this laboratory, you will use a reagent called "lysis buffer" to lyse (break open) the cells. In the second part of this laboratory, you will use column chromatography to separate out the red fluorescent protein from your sample. The properties of the protein's amino acids allow us to separate it from the rest of the sample using column chromatography. Red fluorescent protein is composed of many hydrophobic (water-fearing) amino acids that fold into a cylindrical shape. In this experiment, you will use a column that is packed with small beads that are coated with a material (a resin) that attracts hydrophobic amino acids but not those that are hydrophilic (water-loving). When you pass your sample through the column, the hydrophobic red fluorescent protein will stick to the resin beads, while the hydrophilic amino acids will pass through. You will then wash out the remaining somewhat-hydrophobic proteins in your sample using a salt buffer solution. Finally, you will use another buffer solution to release the bound red fluorescent protein from the column and collect the product. This is the same process that would be used to isolate a human therapeutic protein.



Separation of red fluorescent protein by hydrophobicity using column chromatography

BEFORE THE LAB

Discuss the following items with your group, and be prepared to share your responses with the class.

1. How can solutions of different salt concentrations be used to help isolate the red fluorescent protein using column chromatography?
2. Read through the *Methods* sections for Part A and Part B, and briefly outline the steps using words and a flowchart.

SAFETY: Use caution when handling *E. coli* bacteria, and use aseptic technique as instructed by your teacher. Aseptic technique is a set of procedures that ensure protection of the lab worker and ensure your bacteria cultures are not contaminated with unwanted microbes, both of which are necessary for the experiment to be successful.

PART A: LYSE CELLS GROWN IN THE SHAKER

METHODS

1. Examine the *E. coli* (EC) tube, and record its color in your notebook.
2. Spin the EC tube in the microcentrifuge for five minutes to separate the cells from the media, and then carefully remove the EC tube from the microcentrifuge to avoid disturbing the solid pellet.

STOP AND THINK

How can you determine where the red fluorescent protein is in each separation step?

3. Using the micropipette, carefully remove as much of the liquid as possible without disturbing the pellet, and discard the liquid into the waste container.
4. Add another 1,000 µL of the LB/amp/ara culture of *E. coli* to your EC tube.
5. Repeat steps 2–4.

STOP AND THINK

What color is the supernatant? the pellet? What are the contents of each?

6. Using a new micropipette tip, add 150 µL of elution buffer (EB) to the cell pellet in the EC tube.
7. Close the cap of the EC tube tightly, and drag the tube vigorously across the surface of the microfuge tube rack to resuspend the cells until there are no visible clumps.

8. Using a new tip, add 150 µL of lysis buffer (LyB) to the EC tube and mix using the microfuge tube rack.
9. Label the EC tube with your group identifier. Your teacher will incubate the cells at room temperature overnight.
10. Place all microfuge tubes and micropipette tips in the biohazard bag.

PART B: SEPARATE THE RED FLUORESCENT PROTEIN WITH COLUMN CHROMATOGRAPHY

METHODS

Before completing steps 8–19, assign tasks in your group:

- Gather materials and equipment from your teacher, making sure it is ready (including step 3)
- Set up a chromatography column (steps 1–2)
- Spin lysed cells (steps 4–7).

1. Set up your chromatography column as directed by your teacher, being careful not to dislodge the stopcock attached to the lower portion of the tube. **Do not allow the column to run dry.**
2. Prepare the column:
    a. Set the liquid waste collection container under the stopcock.
    b. Carefully open the column by turning the stopcock valve, and allow the liquid to drain into the waste collection container.
    c. Close the valve once there is about 1–2 mm of liquid left above the resin bed.
    d. Make sure that the liquid is not draining from the column into the waste container.
3. Label two clean microfuge tubes with "SUPER" and "RFP" and your group identifier.
4. Spin the EC tube in the microcentrifuge for five minutes to create a pellet of the cell debris.
5. Examine the tube. You should see a supernatant and a solid pellet.
6. Carefully remove 200 µL of EC supernatant without disturbing the cell debris pellet, and dispense the supernatant into the SUPER tube. If you dislodge the pellet, you will have to centrifuge the tube again.
7. Using a new micropipette tip, add 200 µL of binding buffer (BB) to the SUPER tube, and mix by gently pumping the solution.
8. Using the same micropipette tip, slowly add 400 µL of the SUPER tube mixture **down the side** of the chromatography column.

9. Open the valve, and allow the solution in the column to drain into the waste collection container. Close the valve once there is about 1–2 mm of liquid left above the resin bed.
10. Examine the column, and locate the red fluorescent protein. Is it spread throughout the resin bed, or does it appear to be restricted to a single band? Record your observations.
11. Using a new micropipette tip, add 1,000 µL of wash buffer (WB) slowly down the side of the chromatography column.
12. Open the valve, and allow the solution in the column to drain into the waste collection container. Close the valve once there is about 1–2 mm of liquid left above the resin bed.
13. Examine the column, and locate the red fluorescent protein. Has the location of the red fluorescent protein changed in the resin bed?
14. Using a new micropipette tip, add 1,000 µL of EB **twice**, slowly, down the side of the chromatography column.
15. Set the RFP tube under the stopcock. Open the valve, and allow the red eluate to drain into the RFP tube. Once all of the red has flowed through, close the valve and cap the tube.
16. Set the waste collection container back under the stopcock. Open the valve, and allow the rest of the eluate to drain into the waste container. Close the valve once there is about 1–2 mm of liquid left above the resin bed.
17. Using a new micropipette tip, add 1,000 µL of column equilibration buffer (CEB) **twice** to the column to prepare it for the next class. Cap the column tightly.
18. Discard the contents of the waste collection container down the sink drain or as instructed.
19. Compare your RFP tube with RFP tubes from other groups. Is there a difference in intensity of color from sample to sample? Record your observations in your notebook.