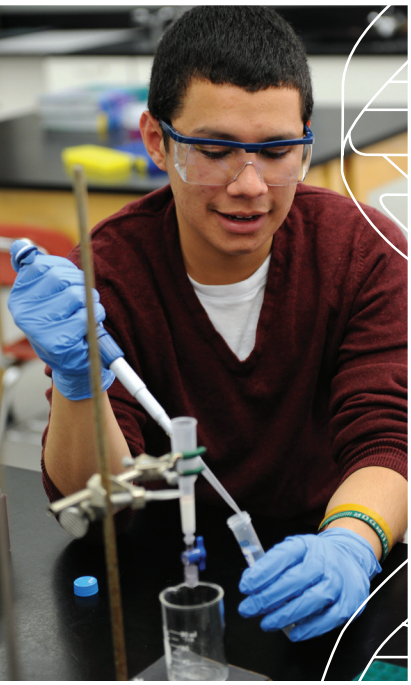
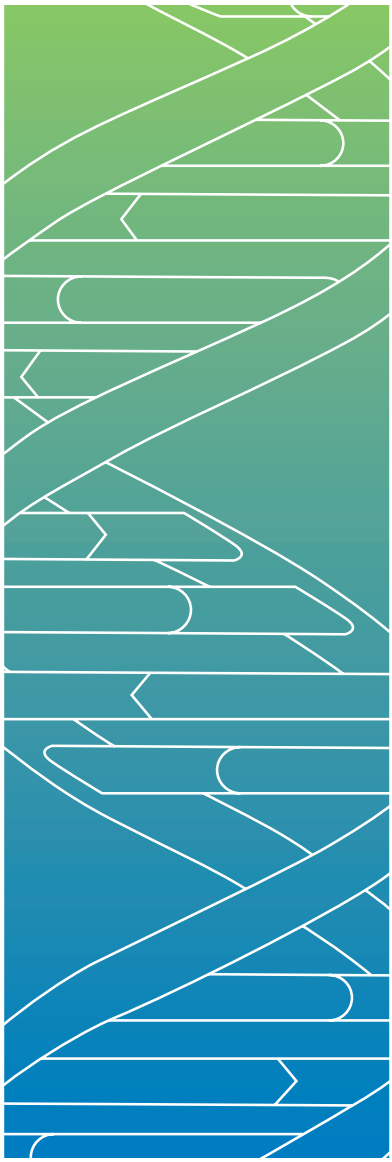
Amgen Biotech Experience

Scientific Discovery for the Classroom

Student Guide





COMPLETE GENETIC ENGINEERING SEQUENCE

www.amgenbiotechexperience.com

AMGEN®

TABLE OF CONTENTS

ABOUT THE AMGEN BIOTECH EXPERIENCE

Genetic engineering is a branch of biotechnology that uses special procedures and techniques to change an organism's *DNA*. This ability has had a huge impact on the field of medicine, as genetically modified bacteria can make human *insulin* (the hormone responsible for regulating *glucose* levels in the blood) and other life-saving products. It's rare for high school students to have the chance to learn about and actually practice the procedures and techniques that are the foundation of the biotechnology industry—but in this program, you will have just that opportunity. As you work in the laboratory and carry out the very experiments that led to breakthroughs in biotechnology, you will gain hands-on experience with producing genetically modified bacteria.

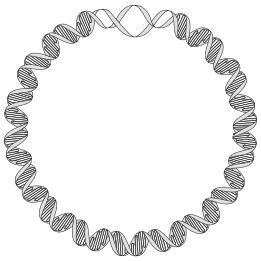
The procedures in this program were developed through a series of discoveries that led to important breakthroughs in biotechnology. Some of the pioneering scientists who made these discoveries received Nobel Prizes in Physiology or Medicine in 1978 and Chemistry in 1980 and 1993. (The Nobel Prize is the highest distinction awarded to scientists in these fields from around the world.) The work that you are about to do is based on this Nobel Prize-winning science—science that is significant and will continue to play an important role in the development of biotechnology and medicine. You will follow in the footsteps of the many scientists who have pushed and continue to push the boundaries of biotechnology. There are many advances still to be made—and students who decide to continue studying this field may contribute to those advances.

In science, the ability to keep track of what you are doing and communicate your work is extremely important. In order to demonstrate that you performed an experiment, either so that it can be duplicated and verified by others or if you want to apply for a patent—you need to have a very accurate record of what you've done. As you carry out this program, carefully record your notes, ideas, observations, results, and answers to questions in a science notebook, in pen. (For scientific purposes, it is important to keep a record—even of your mistakes.) If possible, use a separate bound composition notebook and organize the labs with a table of contents at the front. Since you will use a pen to write with, you'll need to cross out any mistakes you make—and it is good practice to simply "X" out the section you want to change (so that it can still be read) and to note

why you've done so. Following these best practices will make this program even better preparation for you!

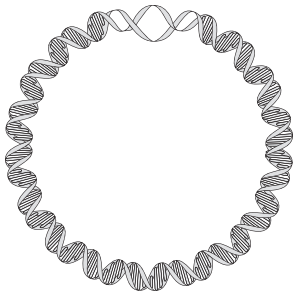
The Amgen Biotech Experience (formerly Amgen-Bruce Wallace Biotechnology Lab Program) had humble beginnings almost 25 years ago with visionary scientists and teachers who shared passion and energy for imparting their knowledge with students. Bruce Wallace, one of Amgen's first staff members, wanted all students to experience the joy of discovery and the excitement of having science at their fingertips. A desire for more robust science education at schools near Amgen's global headquarters led to involving area high school teachers and, later, a college professor, in developing curriculum and educator training in biotechnology. The program grew through word of mouth and teacher interest, and expanded over time to other states and countries.

Visit the ABE website at www.amgenbiotechexperience.com.



PROGRAM INTRODUCTION

AMGEN BIOTECH EXPERIENCE



WHAT IS GENETIC ENGINEERING?

CONSIDER: Preview the title, subtitles, and illustrations found on pages 7 through 14 and then list the topics that you think will be covered in this reading.

TREATING DISEASE WITH GENE CLONING

Until relatively recently, people with certain diseases had to rely on remedies that were expensive and sometimes difficult to obtain. Amazing as it might seem, many of these diseases are the result of the loss of a single protein function, either because the protein produced is defective or because it is not produced in normal amounts. (A *protein* is a large *biomolecule* that carries out essential functions in *cells*.) For example, individuals with *hemophilia*, a bleeding disorder in which blood fails to clot normally, make little or no clotting factor protein; a deficiency of human growth hormone can cause poor growth, delayed puberty, and muscle weakness in children, and fatigue, reduced muscle and bone mass, baldness, increased body fat, and memory loss in adults.

By providing the patient with a functional protein, the symptoms of these diseases can be alleviated. Before genetic engineering technology, these therapeutic proteins had to be extracted from natural sources, such as human blood or animal tissue, a process that was generally difficult, inefficient, and expensive. Pharmaceutical companies can use genetic engineering—or *gene cloning*, as it is often called—to make these proteins cost-effectively, in far greater quantities, without the impurities and viruses that can be transmitted from blood and tissue samples. Gene cloning involves inserting the human gene that encodes the protein into bacteria where the protein is made along with all the other bacterial proteins.

CONSIDER: What do you already know about cloning?

The ability to make enough of the proteins to treat diseases is the result of two key discoveries about bacteria made by scientists in the 1970s and '80s. The first discovery was that bacteria contain tiny circles of DNA, called *plasmids*, that sometimes contain genes that can make them resistant to antibiotics. The second discovery was that bacteria also contain proteins called *restriction enzymes* that can cut DNA at very specific places.

The findings made by basic research often lead to fundamental understandings about the nature of life. In some instances these findings can also lead to new technologies that can improve life. With the discovery of plasmids and restriction enzymes, a whole new era of genetic engineering was launched. Scientists now have the ability to generate products that can improve health in ways never before imagined.

One of the first pharmaceutical products produced using these tools was insulin, which is used to treat *diabetes*, a debilitating and sometimes fatal disease. To generate large quantities of human insulin, the sequences of DNA that contain the codes of human insulin are inserted into a plasmid that is introduced into the common intestinal bacterium *Escherichia coli* (*E. coli*), where the new protein is synthesized along with all the other bacterial proteins. The genetically modified bacteria are then grown in large batches, and the insulin is purified for use in the treatment of diabetes.

CONSIDER: Do you think that treating diabetes with insulin can be considered a cure? What is the difference between a treatment and a cure?

Why is the ability to produce large quantities of insulin so important, and how exactly is this done? In the following readings you will learn about diabetes: what it is and why insulin is in such demand. Then you will carry out some of the very same procedures that scientists use to produce human insulin in bacteria. But instead of producing insulin, you will engineer *E. coli* to produce a red fluorescent protein. This protein is made by a sea anemone gene that has undergone a mutation that makes the protein brighter in color. You will give *E. coli* a new protein and a trait it did not have before: *the ability to glow*.

DATELINE: AMERICA

MAY 2012

TEENAGE DIABETES ON THE RISE

The occurrence of type 2 diabetes in teenagers, once a disease found primarily in adults, has increased dramatically over the past 10 years. Nearly one in four teens between the ages of 12 and 19 is prediabetic (i.e., shows early signs of diabetes) or already has the disease. To make matters even worse, research suggests that the disease progresses more rapidly in children than in adults. In diabetes, the levels of glucose (a type of sugar) in the blood can become dangerously high, causing complications such as loss of vision, kidney failure, and nerve and blood vessel damage. The onset of diabetes early in life could mean serious health issues, such as heart disease, blindness, and amputation, for individuals in their 30s and 40s, far younger than such complications have been seen in the past.

Why this sudden rise in teenage diabetes? Although being overweight or obese can contribute significantly to developing diabetes, weight is not the only factor; 35 percent of teens of normal weight have glucose levels that are higher than normal, which is one indicator of being prediabetic. Factors such as lack of exercise in this age of increased computer and mobile device use may be part of the problem. While many prediabetics go on to develop full-blown type 2 diabetes, studies indicate that eating less fat and fewer calories and exercising a mere 20 minutes a day can reduce the risk of developing type 2 diabetes by 60 percent.

Diabetes can result from the body's inability to make sufficient insulin (type 1) or to effectively use the insulin that it does produce (type 2). Many patients with diabetes must take in insulin as an injection. More diabetes in the population will mean a greater demand for insulin.

What is it like for a teen to learn she has diabetes? Read the following story about one teenager's struggle with the disease.

JENNIFER'S STORY

Jennifer felt hungry all the time, but despite eating whatever she felt like whenever she wanted to, she was losing weight. She was also very thirsty and was constantly drinking water and then needing to pee. Initially, she just thought it was typical for a 15 year old; she was in a growth spurt and very active with soccer and track at school. Of course she was hungry and thirsty! She was also pleased by her weight loss, since she'd been a bit overweight for a while. Jennifer also felt unusually tired and draggy, especially in the afternoon. But again, who wouldn't be, since school started at the ungodly hour of 7:30? When she began to have trouble seeing the board in class, she thought, Drat! Do I really need glasses? But it was the cut on her leg that refused to heal and became infected that finally got her to talk to her parents and ultimately go to the doctor's office. There Jennifer was diagnosed with diabetes.

Jennifer had heard about a disease called diabetes but never gave it much thought. Now she really needed to pay attention. Diabetes is the result of too much of a sugar called glucose in the blood and not enough of it getting into cells, where it provides the energy to construct the biological molecules the body needs to survive. Jennifer learned that in order for glucose to get into cells, the body makes a hormone called insulin, which binds to the cells and enables glucose to enter them. For some reason, Jennifer's body no longer produced normal amounts of insulin, resulting in Jennifer having very high levels of glucose in her blood and not enough glucose getting into her cells. Jennifer hoped she could control her diabetes by eating more fruits and vegetables and getting more exercise. But although this change in habits helped some, it was not sufficient, and Jennifer had to begin injecting herself daily with insulin.

Jennifer now is very aware of what she eats, monitoring exactly how much sugar and other carbohydrates she ingests. She checks the level of her blood glucose several times a day by pricking her finger and testing her blood. She also injects her insulin faithfully. She knows that she can't cure her diabetes and that if the disease progresses further she could suffer very serious complications.

DIABETES TYPES 1 AND 2

DIABETES: TOO MUCH OF A GOOD THING

What is diabetes? Diabetes is the result of elevated levels of glucose in the blood. Glucose is a major source of energy and is used to construct biological molecules in the body. What you ate for breakfast or lunch today is rapidly being converted to glucose, which in turn will be used to generate energy, to synthesize new cells and tissues, and to carry out processes required to sustain life. The starch in your bread or potato is made up of long chains of glucose molecules (**Figure P.1a**). As food passes through your mouth, esophagus, and stomach (**Figure P.1b**), these chains are broken down to release glucose. The glucose is then absorbed through the intestinal wall and enters the bloodstream, where it is carried to all the cells in the body (**Figure P.1c**).

Figure P.1: How glucose gets to cells

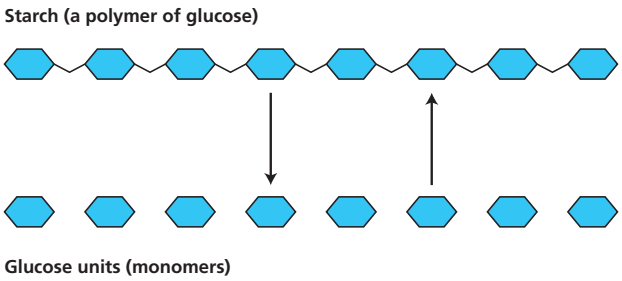


Figure P.1a: Starch is made up of subunits of glucose bonded together.

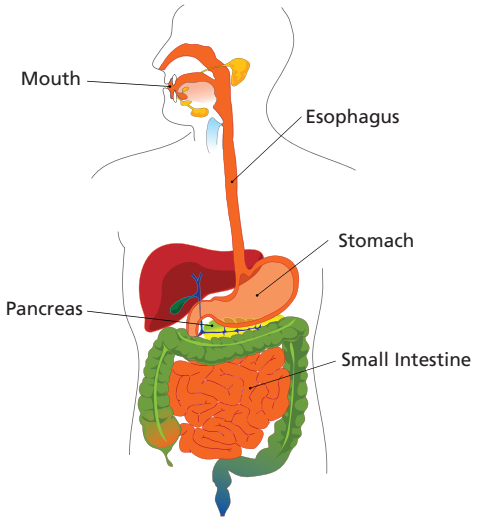


Figure P.1b: Nutrients such as starch are broken down into smaller molecules during digestion in the mouth, esophagus, and stomach.

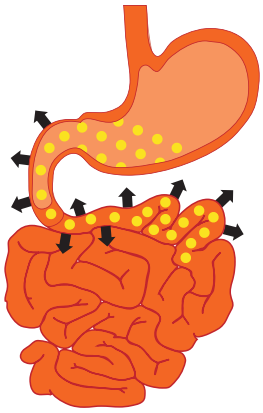
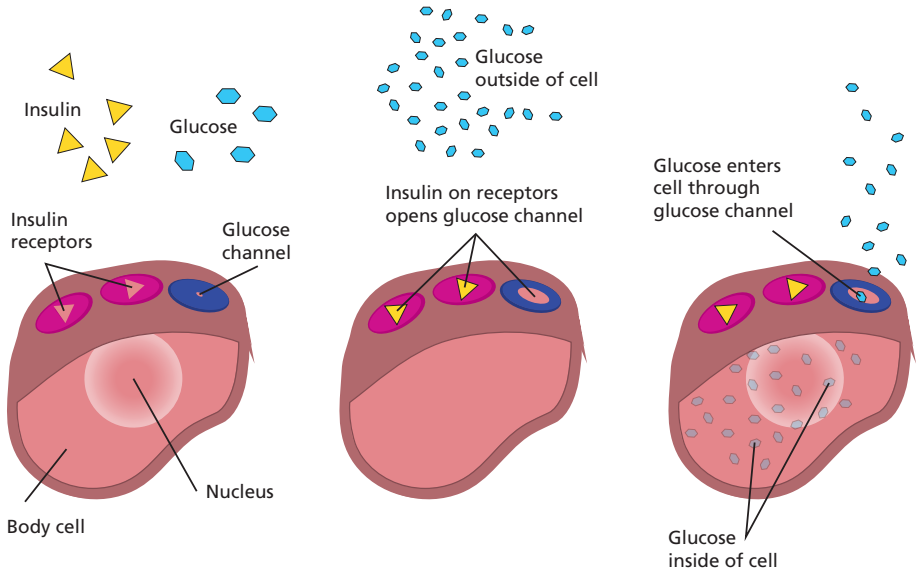


Figure P.1c: Glucose molecules pass through the small intestinal wall into the bloodstream, which delivers the glucose to cells in the body.

CROSSING THE CELLULAR DIVIDE

In order to get inside a cell, glucose must cross the cell membrane that separates the inside of the cell from its environment. Insulin, which is made by beta cells found in the pancreas (see **Figure P.1b**), binds to a special protein called a *receptor*, which causes an opening in the cell membrane and allows glucose to enter the cell (see **Figure P.2**). Without insulin, glucose cannot penetrate this cellular barrier.

Figure P.2: How glucose crosses the cell membrane



Insulin in the blood binds to specific receptors on the cell. This binding alters the conformation of the cell membrane, resulting in the formation of a glucose channel. Glucose in the blood can now enter the cell through these channels.

In both type 1 and type 2 diabetes, glucose is unable to enter the cells, resulting in elevated levels of glucose in the blood. In type 1 diabetes, the beta cells in the pancreas are unable to produce insulin. Without insulin to create glucose channels, the glucose remains in the blood (**Figure P.3a**). Type 2 diabetes is the result of a combination of two factors: (1) Cells become resistant to insulin, and the receptors can no longer bind the hormone (**Figure P.3b**). As the blood sugar levels rise, the beta cells pump out more and more insulin to no avail, since the cells cannot use it. (2) Eventually the beta cells are exhausted and can no longer produce insulin, and insulin levels in the blood drop while the sugar levels continue to increase.

Figure P.3: Reduced uptake of glucose by cells

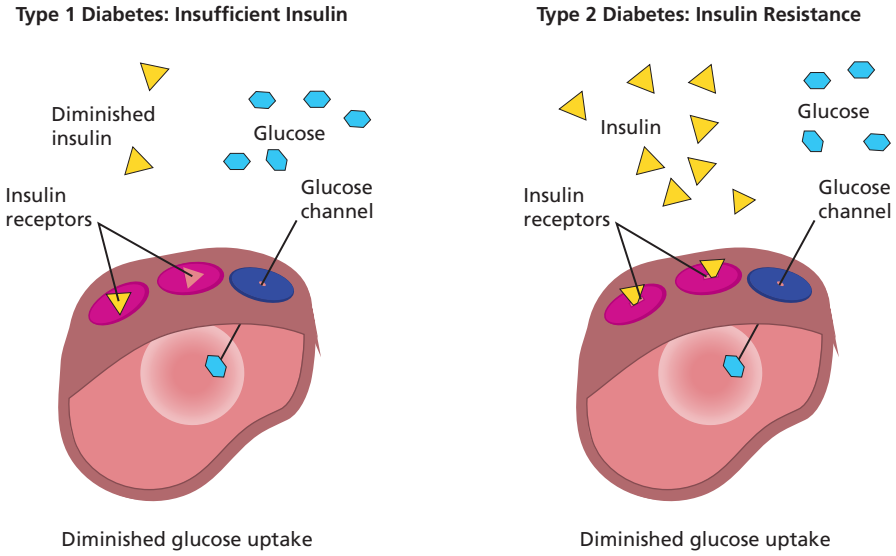


Figure P.3a: In type 1 diabetes, there is a lack of insulin in the body.

Figure P.3b: In type 2 diabetes, cells cannot bind the insulin.

THE PROBLEMS OF TOO LITTLE INTRACELLULAR GLUCOSE

When cells cannot get glucose, they cannot get the energy and biological molecules they need. The body responds by breaking down fats and proteins to obtain its needed energy. Loss of proteins and fats can cause serious damage to tissues and organs, leading to the symptoms of diabetes that patients like Jennifer experience, such as blindness and nerve damage (which can result in amputation).

TREATING DIABETES

Individuals with type 1 diabetes can regulate their sugar levels by monitoring their blood and injecting insulin as needed. Those with type 2 diabetes can sometimes regulate their blood sugar levels by changing their diet and increasing the amount that they exercise. However, in many cases, medications that reduce insulin resistance in cells and increase the levels of insulin in the blood are required to maintain normal blood sugar levels.

Currently, there is no cure for either type of diabetes.

E. COLI WITH A HUMAN GENE

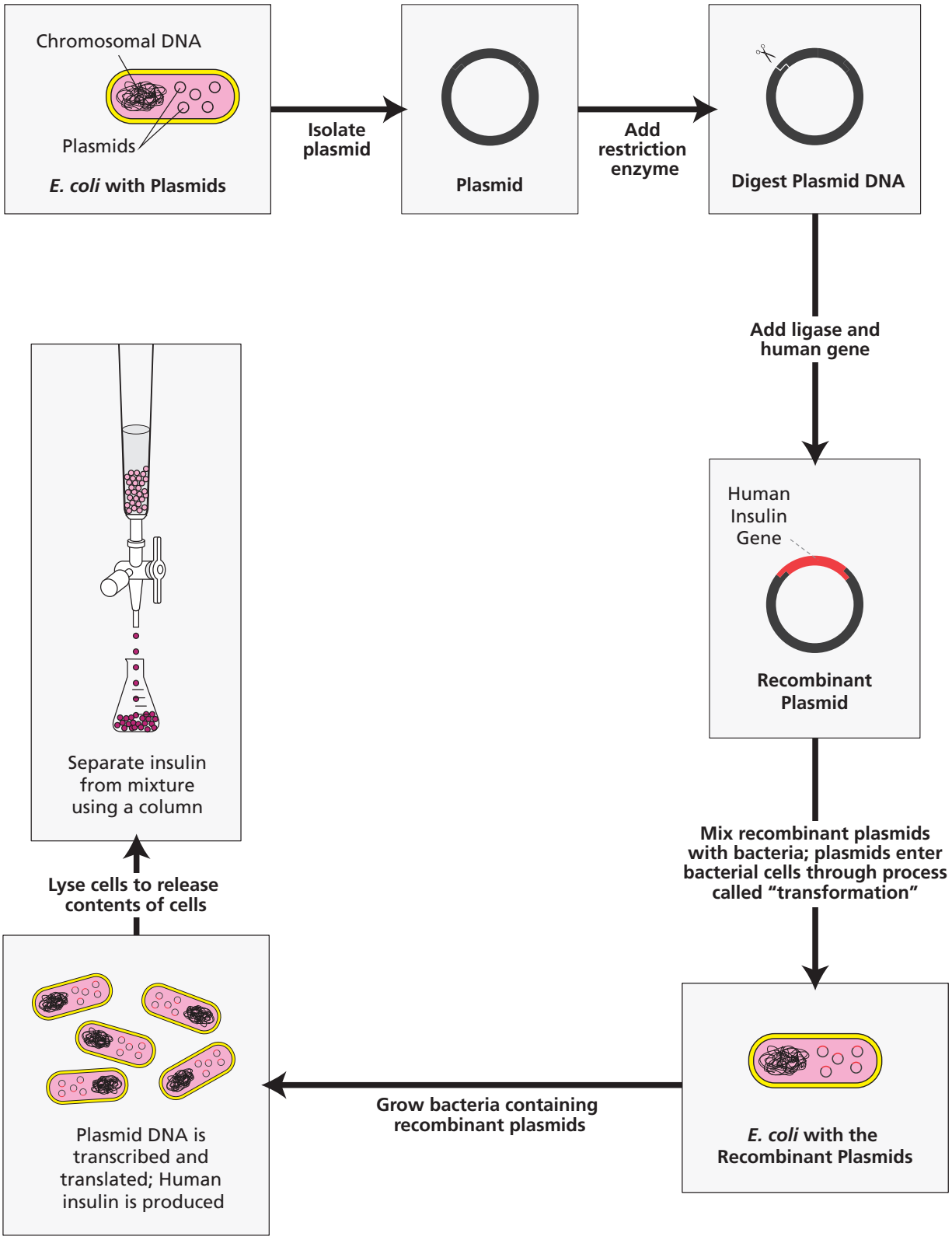
With the rise in diabetes in the population, the need for insulin for treatment is also on the rise. Originally isolated from the pancreases of pigs and cows, most of the insulin used today is genetically engineered human insulin, manufactured by bacteria. DNA sequences encoding human insulin in plasmids are taken up by bacteria, which make the hormone along with all of its bacterial proteins. Insulin is then isolated from the bacteria. In 1982, human insulin was the first commercially successful product made by recombinant DNA technology. (*Recombinant DNA* refers to DNA that contains sequences or genes from two or more sources.)

CONSIDER: **Why might diabetes be on the rise, especially in teenagers?**

MAKING NEW PROTEINS IN BACTERIA

Figure P.4 shows how a human protein—in this case, insulin—can be made in bacteria. The insulin is then purified so that it can be used by people with diabetes.

Figure P.4: Making insulin in bacteria



YOUR CHALLENGE

Your challenge in the Amgen Biotech Experience is to successfully carry out the steps of the genetic engineering process that is used to make insulin and other genetically engineered products. You will learn and practice the techniques and procedures that are part of this process. If you carry out all the steps in the program, you will create your own genetically modified bacteria.

Note: The number of steps will vary depending on how much time your class has available.

Instead of cloning insulin or another human gene, you will work with a gene from a sea anemone, a soft-bodied animal related to coral and jellyfish. (The gene is called *rfp* and the protein made by this gene is called red fluorescent protein.) How will you know if you are successful? The bacteria you create will have a new and highly visible trait: It will now produce red fluorescent protein!

DID YOU KNOW?

Red Fluorescent Protein in Sea Anemones

Red fluorescent protein is derived from a protein found in sea anemones. While sea anemones are sedentary, remaining attached to rocks, they are also predatory animals, using their stinging tentacles to catch their prey. The protein glows because it can absorb one color of light and then emit light of a different color—a process known as *fluorescence*. But why is it important for sea anemones to fluoresce? Our best guess is that fluorescent proteins somehow help sea anemones survive, but the role these proteins play is not yet well understood. Fluorescent molecules may serve as a sunblock, turning harmful UV light into light that is less damaging to the anemone's tissues. Another possibility is that while humans can't detect the fluorescence in bright sunlight, some animals may be able to, causing prey to be attracted to the glow.

PROGRAM INTRODUCTION GLOSSARY

Biomolecule: A molecule produced by living cells. Examples include proteins, carbohydrates, lipids, and nucleic acids.

Cells: The basic units of any living organism that carry on the biochemical processes of life.

Diabetes: A disease that occurs when the body doesn't produce or properly use insulin.

DNA (deoxyribonucleic acid): A double-stranded biomolecule that encodes genetic information.

***Escherichia coli* (*E. coli*):** *E. coli* is a common bacterium found in the gut of warm blooded animals. Most strains are harmless, including the strain used in these lab protocols.

Fluorescence: The production of light by a molecule (e.g., red fluorescent protein will release red light when exposed to ultraviolet light).

Gene cloning: Using genetic engineering techniques to create exact copies, or clones, of a gene or DNA sequence of interest.

Genetic engineering: A branch of biotechnology that uses specific procedures and techniques to change an organism's DNA.

Glucose: A sugar that is a major source of energy and biomolecules to sustain life processes. Glucose is absorbed through the intestine and travels in the blood to cells, where it is transported through the cell membrane to be used as energy, to synthesize cells and tissues, and to carry out other essential processes.

Hemophilia: A disease that occurs when the ability of blood to clot is reduced due to lack of one or more blood clotting factors.

Insulin: A hormone produced in the pancreas that controls the amount of glucose in the blood. Insulin is a protein.

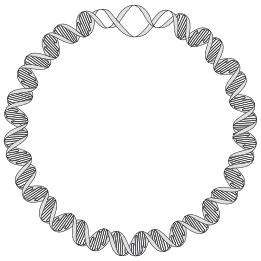
Plasmid: A circular molecule of DNA.

Protein: A large biomolecule. Proteins carry out essential functions in cells, from forming cellular structures to enabling chemical reactions to take place.

Receptor: A protein that receives signals from outside the cell. When a signal substance binds to a receptor, it directs the cell to do something, for example to allow biomolecules to enter the cell.

Recombinant DNA: DNA that contains sequences or genes from two or more sources.

Restriction enzyme: A protein that can cut DNA at a specific sequence.



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 induce bacterial *cells* to make human insulin by inserting human *DNA* into the cells. This new technology, termed *genetic engineering*, can be used to make products that treat the symptoms of certain genetic diseases.

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 carry out the subsequent labs and complete your challenge in this program.

CHAPTER 1 GOALS

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

- Use micropipettes and the technique of gel electrophoresis correctly
- 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 genetic disease and DNA.

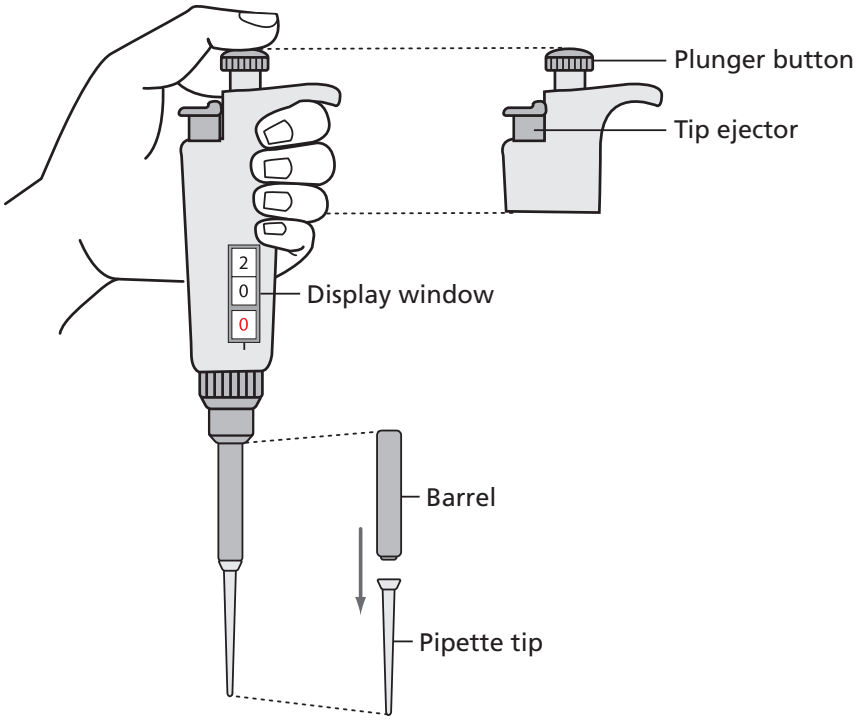
1. What does the term *genetic disease* mean? What examples of genetic diseases do you know about?

2. Adding human DNA to bacteria makes it possible to make human insulin. What do you already know about DNA? Be as detailed as possible and discuss the location of DNA in the cell, DNA structure, the replication of DNA, and the components of DNA.

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.

Figure 1.1: A P-20 micropipette



BEFORE THE LAB

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

1. Why do you think it is necessary to use very small and exact volumes of reagents in biotechnology?
2. Read through the Methods section on pages 21 through 23 and briefly outline the steps, using words and a flowchart.

MATERIALS

Reagents

- A plastic microfuge tube rack with a microfuge tube of red dye solution (RD)

Equipment and Supplies

- P-20 micropipette (measures 2.0–20.0 µL)
- Tip box of disposable pipette tips
- Laminated micropipette practice sheet
- Waste container for used tips and microfuge tubes (will be shared among groups)

SAFETY:

- **All appropriate safety precautions and attire required for a science laboratory should be used, including safety goggles. Please refer to your teacher's instructions.**
- **Wash your hands well with soap after completing the lab.**

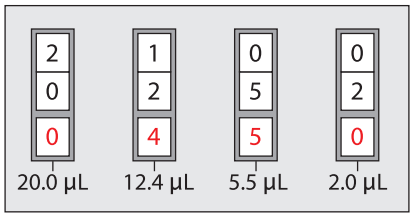
METHODS

1. Check your rack to make sure that you have the reagent listed.
2. Review the parts of the micropipette shown in (see **Figure 1.1** on page 20).
3. Find the display window on the handle of the micropipette.
4. Turn the plunger button on the top of the micropipette clockwise—to the right—to decrease the volume, or counterclockwise—to the left—to increase the volume.

5. **Figure 1.2** shows four micropipette volumes. Practice setting the micropipette to these volumes.

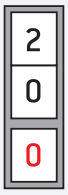
LAB TECHNIQUE: Never set the P-20 micropipette lower than 2.0 µL or higher than 20.0 µL or you could damage the equipment.

Figure 1.2: Four micropipette volumes



The display window of a micropipette shows how much fluid it will load and dispense. Four examples of displays and the corresponding amounts are shown.

6. 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.



7. Load the micropipette with 20.0 µL of RD by doing the following:

   a. Set the micropipette to 20.0 µL.
   b. Open the tip box. Lower the micropipette onto a tip and press down firmly (do not touch the tip with your fingers). Close the box when done.
   c. Bring the micropipette and the RD tube to eye level.
   d. Use your thumb to press the plunger to the first stop position, which is your first point of resistance.

LAB TECHNIQUE: When loading the micropipette, only press the plunger to the first stop or you will draw too much solution into the pipette tip.

   e. Put your pipette tip into the RD and slowly release the plunger to draw up the solution.

LAB TECHNIQUE: Do not lay down a micropipette with fluid in the tip or hold it with the tip pointed upward. If the disposable tip is not firmly seated onto the barrel, fluid could leak back into the pipette.

8. Dispense RD onto the laminated sheet by doing the following:

   a. Place the pipette tip over the 20.0 µL circle.

b. Use your thumb to press the plunger to the first stop position and then press down to the second stop.

LAB TECHNIQUE: When dispensing liquid from the micropipette, press the plunger to the first stop to dispense most of the liquid and then press the plunger to the second stop in order to dispense the last little bit.

c. With the plunger still depressed, pull the pipette out of the tube—this prevents you from accidentally pulling the liquid back into the tip.

9. Without setting down the micropipette, twist the plunger button to set it to 15.0 µL and repeat steps 7b–8c, dispensing over the 15.0 µL circle.

10. Without setting down the micropipette, twist the plunger button to set it to 10.0 µL and repeat steps 7b–8c, dispensing it over the 10.0 µL circle when dispensing the liquid.

11. Without setting down the micropipette, twist the plunger button to set it to 5.0 µL and repeat steps 7b–8c, dispensing it over the 5.0 µL circle.

12. Without setting down the micropipette, twist the plunger button to set it to 2.0 µL and repeat steps 7b–8c, dispensing it over the 2.0 µL circle.

13. Use the tip ejector to place your pipette tip into the waste container.

STOP AND THINK:

- **When loading or dispensing a solution, why is it important to actually see the solution enter or leave the pipette tip?**
- **You were instructed to avoid contact with the pipette tips—for example, you were asked to put the pipette tip on without using your hands, to avoid setting down the micropipette, 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?**

14. Using the micropipette practice sheet, each person in your group should have a chance to load and dispense the five drops of different volumes, with each person using a new pipette tip.

15. When everyone in your group has had a chance to dispense RD onto the micropipette practice sheet, draw the approximate sizes of each drop in your notebook (or take a photograph and tape it into your notebook) and label them with the amounts.

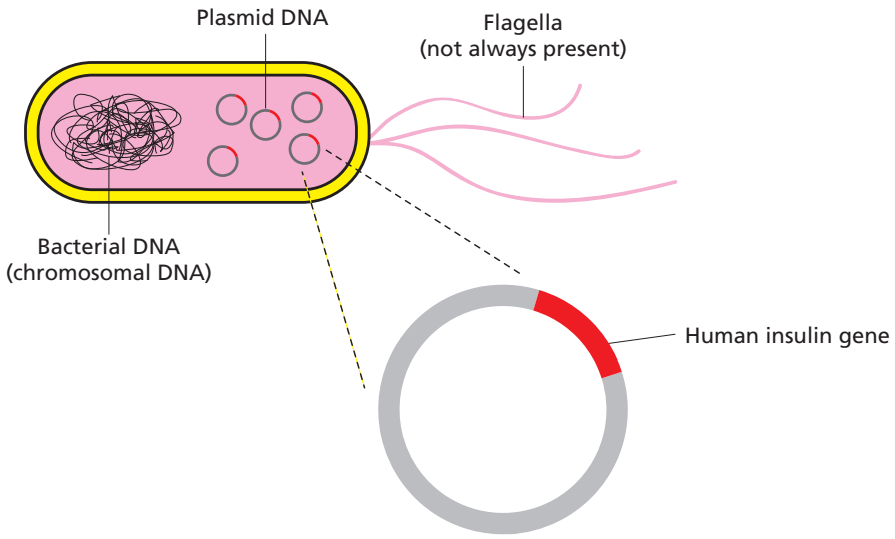
THE GENETIC ENGINEERING PROCESS

Do you know somebody who takes *insulin*, or a *blood clotting factor*, or *human growth hormone*? These substances are all *proteins* manufactured in certain human cells. If those cells fail to make these particular proteins, the diseases *diabetes*, *hemophilia*, and *growth deficiency* can result. A patient with one of these diseases must be treated with the missing protein.

CONSIDER: Prior to genetic engineering, how could people get missing proteins for a genetic disease?

Before the development of genetic engineering, it was difficult to obtain human proteins to treat people who needed them. Now, bacteria can make these proteins because scientists have figured out a way to change bacterial DNA by adding human DNA. (see **Figure 1.3**).

Figure 1.3: Bacterial cell with human DNA



What is the relationship between DNA and proteins? Both are biomolecules, large molecules made by living cells. When scientists investigated traits in organisms, they found that proteins were responsible for traits. For example, consider a plant that has the trait of red flowers. The flowers' red pigment is produced by the action of an enzyme (one kind of protein). The DNA in that plant contains instructions for making proteins, including that enzyme. The part of a DNA molecule that has the instructions for making a particular protein is called a *gene*.

In the genetic engineering process, a human gene is added to a *plasmid*, a small circular piece of DNA found in many bacteria. The plasmid is taken up by bacterial cells, and the cells make the human protein that is encoded by the human gene along with their own proteins. During this process, biotechnologists use a combination of tools, some human-made and some biological. Among the human-made tools are two that you'll work with in this chapter: micropipettes and gel electrophoresis.

DID YOU KNOW?

The DNA Code

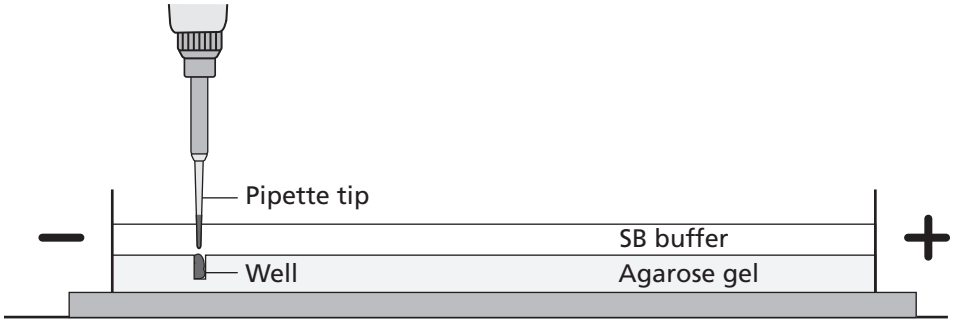
DNA information is encoded by the arrangement of *nucleotides*, small molecules that join together to form the DNA molecule. A DNA molecule has millions of nucleotides. There are four different kinds of nucleotides, and they are arranged in a specific *sequence* (order). A specific sequence of nucleotides in the DNA (i.e., a gene) is a code for how to make a specific protein. Think of a sequence of nucleotides as similar to a sequence of written musical notes—the code for how to play music. Just as different sequences of notes encode different songs, different sequences of nucleotides encode different proteins.

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 including DNA; the components of each mixture can then be identified by their location in the gel. Gel electrophoresis works based on the fact that biomolecules have a negative charge, which means that they will move in response to an electric charge. The biomolecules move through a gel, and their speed varies primarily according to their weight, although molecular 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.

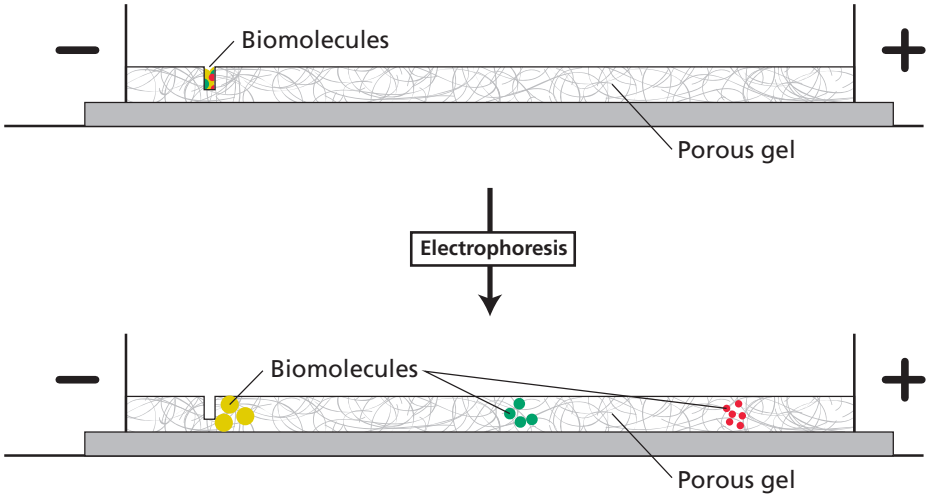
The electrophoresis setup consists of a box containing an agarose gel and two electrodes that create an electric field across the gel when the box is attached to a power supply. The negative electrode is black, and the positive electrode is red. Samples of biomolecules are pipetted into wells near the negative (black) electrode. The samples move through the gel toward the positive (red) electrode, as shown in **Figure 1.4**.

Figure 1.4: The gel electrophoresis unit



The gel that the biomolecules move through is 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 **Figure 1.5**.

Figure 1.5: How biomolecules, including DNA, move through the agarose gel matrix in gel electrophoresis



BEFORE THE LAB

Respond to the following questions with your group, and be prepared to share your answers 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 on pages 28 through 31 and briefly outline the steps for *Part A* and for *Part B*, using words and a flowchart.

MATERIALS

Reagents

- A plastic microfuge tube rack with the following:
  - Microfuge tube of red dye solution (RD)
  - Microfuge tube of dye solution 1 (S1)
  - Microfuge tube of dye solution 2 (S2)
  - Microfuge tube of dye solution 3 (S3)
- 50-mL flask containing 1x sodium borate buffer (1x SB) (shared with another group)

Equipment and Supplies

- P-20 micropipette (measures 2.0–20.0 µL)
- Tip box of disposable pipette tips

- 2 pipetting practice plates loaded with 0.8% agarose gel
- Electrophoresis box loaded with 0.8% agarose gel (will be shared among groups)
- Microcentrifuge (will be shared among all groups)
- Waste container for used tips and microfuge tubes (will be shared among groups)

SAFETY:

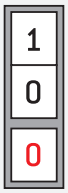
- **All appropriate safety precautions and attire required for a science laboratory should be used, including safety goggles. Please refer to your teacher's instructions.**
- **Wash your hands well with soap after completing the lab.**

METHODS

PART A: PIPETTING INTO WELLS

You will practice pipetting RD into preformed wells in an agarose gel.

1. Check your rack to make sure that you have the RD tube.
2. Fill the two pipetting practice plates with 1x SB to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more buffer.



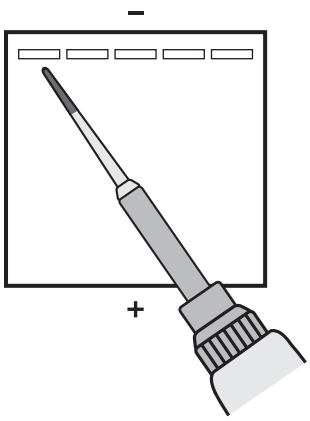
3. Set the P-20 micropipette to 10.0 µL and put on a pipette tip.
4. Load the RD into the pipette with 10.0 µL of RD.

LAB TECHNIQUE: **Do not lay down a micropipette with fluid in the tip or hold it with the tip pointed upward.**

5. Dispense RD into a well in one of the practice plates by doing the following:
   a. Place your elbow on the table to steady your pipette hand. If needed, also use your other hand to support your pipette hand.
   b. Lower the pipette tip until it is under the buffer but just above the well.

LAB TECHNIQUE: **Be careful not to place your pipette tip into the well or you might puncture the gel, which will make the well unusable.**

   c. Gently press the plunger to slowly dispense the sample. To avoid getting air into the buffer, do not go past the first stop. The sample will sink into the well.



6. Repeat steps 4 and 5 until all the practice plate wells have been filled. Everyone in your group should get an opportunity to practice pipetting into the wells.

7. Eject the pipette tip.

PART B: SEPARATING DYES WITH GEL ELECTROPHORESIS

Now you will use gel electrophoresis to separate different dyes. First you will add dyes into wells in the gel electrophoresis unit. You will then turn the unit on in order to move the negatively charged dyes through the gel. (You will share the electrophoresis boxes with one other group; your teacher will tell you which wells your group should use.)

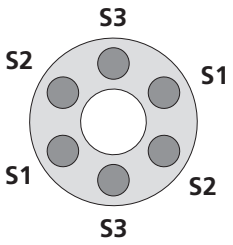
1. Check your rack to make sure that you have the three dye solutions (S1, S2, and S3).

2. Review **Figure 1.4** on page 26. Check to make sure that the wells in the gel are located near the negative (black) electrode.

3. Fill the box with 1x SB to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more buffer.

4. Centrifuge the S1, S2, and S3 tubes.

   LAB TECHNIQUE: **Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.**



5. Make a drawing in your notebook that shows the location of the wells in the electrophoresis box. Record which solution you will place in each well.

6. Set the P-20 micropipette to 10.0 µL and put on a pipette tip.

7. Load 10.0 µL of S1 into the pipette.

8. 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 pipette hand. If needed, also use your other hand to support your pipette hand.

   b. Lower the pipette tip until it is under the buffer but just above the well.

      LAB TECHNIQUE: Do not puncture the gel or it will become unusable.

   c. Gently press the plunger to slowly dispense the sample. To avoid getting air into the buffer, do not go past the first stop. The sample will sink into the well.

      LAB TECHNIQUE:

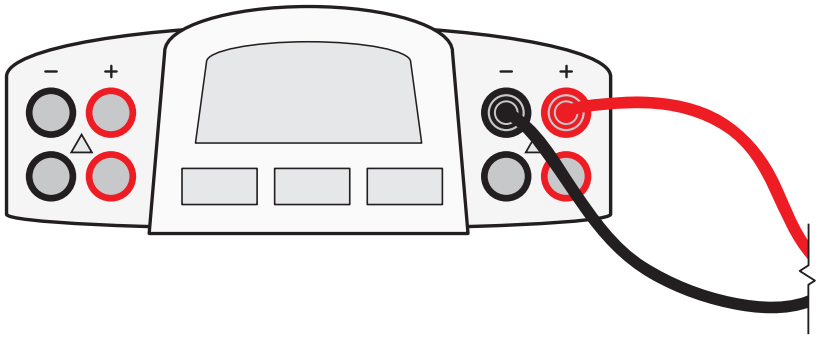
      - **While the plunger is still depressed, pull the tip out of the buffer so that you don't aspirate the solution or buffer.**
      - **Use a fresh pipette tip for each sample.**

9. Repeat steps 7 and 8 for S2 and S3, using a new pipette tip with each solution.

10. When all the samples have been loaded, close the cover tightly over the electrophoresis box. (Carefully close the cover in a horizontal motion, so that samples don't spill.)

11. Connect the electrical leads to the power supply. Connect both leads to the same channel, with cathode (–) to cathode (black to black) and anode (+) to anode (red to red). See **Figure 1.6**.

Figure 1.6: Leads from electrophoresis box connected to correct channel in power supply



12. Turn on the power supply and set the voltage to 130–135 V. (You will see bubbles form in the buffer at the red (+) end of the electrophoresis unit.)

13. After two or three minutes, check to see if the dyes are moving toward the positive (red) electrode. You should begin to see the purple dye (bromophenol blue) beginning to separate from the blue dye (xylene cyanole).

STOP AND THINK:

- **Study your gel electrophoresis results. Which solution sample contained a single dye: S1, S2, or S3? How do you know?**
- **What electrical charge do the dyes have? Explain your reasoning.**
- **The dyes that you are separating are orange G (yellow), bromophenol blue (purple), and xylene cyanole (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?**

14. In approximately 10 minutes, or when you can distinguish all three dyes, turn off the power switch and unplug the electrodes from the power supply. Do this by grasping the electrode at the plastic plug, NOT the cord.

15. Carefully remove the cover from the gel box and observe the dyes in the gel.

16. In your notebook, draw the relative location of the bands and their colors in each of the lanes containing your samples.

17. Leave the gels in the gel box.

CHAPTER 1 QUESTIONS

1. What is the importance of micropipettes and gel electrophoresis in genetic engineering?

2. How are recombinant plasmids used to treat genetic diseases?

DID YOU KNOW?

Gel Electrophoresis in DNA Fingerprinting

DNA fingerprinting uses gel electrophoresis to distinguish between samples of genetic material. In DNA fingerprinting, human DNA molecules are treated with enzymes that chop them at certain characteristic points, thereby reducing the DNA to a collection of smaller and more manageable pieces. The DNA fragments are loaded into a gel and placed in an electrical field, which electrophoretically sorts the DNA fragments into various bands. These bands can be colored with a radioactive dye to make them visible to imaging techniques. Methods of DNA identification have been applied to many branches of science and technology, including medicine (prenatal tests, genetic screening), conservation biology (guiding captive breeding programs for endangered species), and forensic science. In the latter discipline, analysis of the pattern of DNA fragments that results from the action of restriction enzymes enables us to discriminate between suspects accused of a crime, or potential fathers in a paternity suit.

CHAPTER 1 GLOSSARY

Agarose: A polymer made up of sugar molecules that is used as the matrix in gel electrophoresis procedures.

Biomolecule: A molecule produced by living cells. Most biomolecules are long polymers—made of repeating subunits called monomers—and include proteins, carbohydrates, lipids, and nucleic acids.

Blood clotting factor: A variety of proteins in blood plasma that participate in the clotting process.

Cell: The basic unit of any living organism that carries on the biochemical processes of life.

Diabetes: A disease that occurs when the body doesn't produce or properly use insulin.

DNA (deoxyribonucleic acid): A double-stranded molecule made up of nucleotide subunits that encodes genetic information.

Gel electrophoresis: The movement of charged molecules toward an electrode of the opposite charge; used to separate nucleic acids and proteins. When used to separate DNA fragments, electrophoresis will separate the fragments by size, with smaller fragments moving faster than larger fragments.

Gene: The fundamental physical and functional unit of heredity; an ordered sequence of nucleotides located in a specific place on the DNA that encode for a specific functional product.

Genetic engineering: The process of altering the genetic material of cells or organisms to enable them to make new substances or perform new functions.

Genetic disease: A disease caused by a change in DNA. Genetic diseases are often inherited from parents.

Growth deficiency: A disease that occurs when the body doesn't produce enough human growth hormone.

Hemophilia: A disease that occurs when the ability of blood to clot is reduced due to lack of one or more blood clotting factors.

Human growth hormone: A hormone secreted by the pituitary gland that stimulates growth. Human growth hormone is a protein.

Insulin: A hormone produced in the pancreas that controls the amount of glucose in the blood. Insulin is a protein.

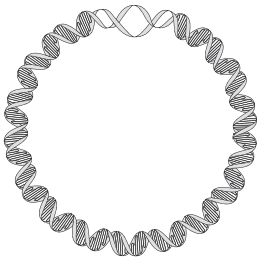
Micropipette: A laboratory instrument used to measure, dispense, and transfer very small amounts of liquid.

Nucleotide: A set of molecules that link together to form DNA or RNA. DNA and RNA each contain four types of nucleotides.

Plasmid: A circular molecule of DNA.

Protein: A large biomolecule. Proteins carry out essential functions in cells, from forming cellular structures to enabling chemical reactions to take place. Examples of proteins are enzymes, red fluorescent protein, cell receptors, and some hormones.

Sequence: A set of related events, movements, or items (such as nucleotides) that follow each other in a particular order.



CHAPTER 2

HOW DO YOU BEGIN TO CLONE A GENE?

INTRODUCTION

In the Program Introduction, you learned that the increase in diabetes in the United States has resulted in a great demand for its treatment, insulin. You also learned that the best way to meet this demand is to insert the human insulin gene into bacteria, enabling the bacteria to produce the insulin protein in quantities large enough to meet the demand. 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 insulin and other medicines. You will now learn more about these tools and 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 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 DNA from different organisms the same, and in what ways does it vary?

3. Using your understanding of genes and how they are expressed, explain why it is possible for a bacterial cell to make a human protein from the instructions encoded in a human gene.

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?

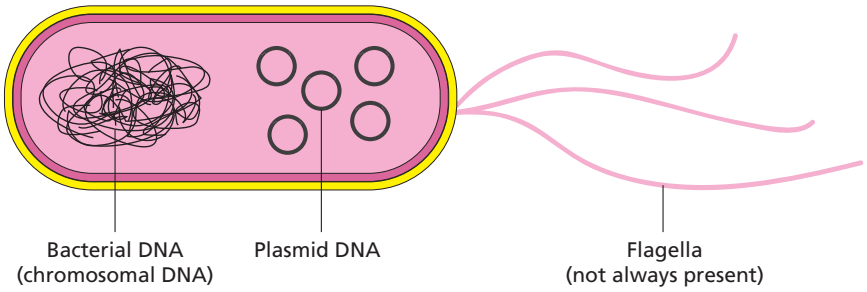
PLASMIDS AND RESTRICTION ENZYMES

The discovery of plasmids and restriction enzymes in bacteria is a classic example of how findings from basic research can revolutionize a field. Without the discovery of these biomolecules, major breakthroughs in understanding fundamental processes of life and in developing life-saving products might never have occurred.

PLASMIDS

Many different types of bacteria carry two forms of DNA: (1) a single chromosome made up of a large DNA molecule that contains all the information needed by the organism to survive and reproduce, and (2) plasmids, which are small circular DNA molecules, ranging in size from 1,000 to 200,000 *base pairs*—two nitrogenous bases joined to connect complementary strands of DNA—that are present in multiple copies separate from the chromosomal DNA (see **Figure 2.1**). Some bacteria carry as many as 500 plasmids in each cell.

Figure 2.1: DNA in bacterial cells



Several characteristics of plasmids make them ideal *vectors* (vehicles for carrying DNA sequences from one organism to another) for genetic engineering, for example:

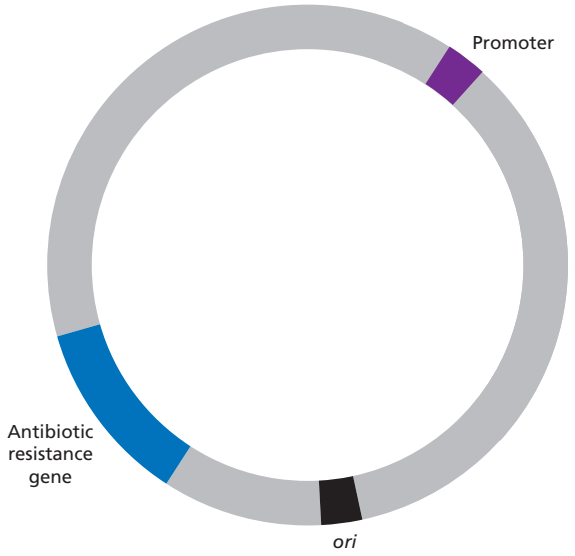
- The ability to replicate, that is, to make copies of itself independently of the bacterial chromosome. In order to do this, plasmids have a specific sequence where the host cell DNA synthesis enzymes bind and initiate *DNA replication* (a biological process that occurs in all living organisms to make copies of their DNA). This sequence is called the *ori* ("*origin of replication*") site.
- The ability to initiate *transcription* (the process by which information encoded in DNA is transferred to *messenger RNA* using the host cell *RNA polymerase*). This ability requires another specific sequence, called the *promoter* sequence. The promoter sequence binds RNA polymerase; this is where transcription is initiated. All genes have promoter sequences located

next to them in the DNA. In order for genes such as the insulin gene to be expressed in bacteria, they must be inserted in the plasmid next to the promoter sequence.

- A gene or genes that code for resistance to *antibiotics*, a class of compounds that kill or inhibit the growth of microorganisms. These genes code for proteins that inhibit the action of antibiotics secreted by microorganisms and can confer a selective advantage in nature to plasmid-containing bacteria in a microbial population in which bacteria compete for survival.

Figure 2.2 illustrates some of the characteristics of plasmids that make them ideal vectors for genetic engineering.

Figure 2.2: A plasmid vector



The basic components of a plasmid are the *ori* site for initiation of DNA replication, a promoter for the initiation of transcription, and a gene for *antibiotic resistance* (the state in which bacteria are no longer sensitive to an antibiotic and will continue to grow and divide in the presence of the antibiotic).

The plasmids you will work with in this and subsequent labs contain the genes for resistance to the antibiotics ampicillin and kanamycin. These genes produce proteins that inactivate the target antibiotic by chemically modifying its structure.

CONSIDER: Use what you know about natural selection and evolution to describe how plasmids might confer a selective advantage to their host bacteria.

A fourth feature of plasmids that is critical for genetic engineering is that they can be passed on from one bacterial strain to another in a process called *bacterial conjugation*, which enables bacteria to share and exchange genetic information. When a plasmid with a gene for antibiotic resistance is taken in by bacteria lacking that plasmid, the bacteria will then become resistant to that specific antibiotic. In nature, conjugation occurs with a very low efficiency; that is, only a small percentage of bacteria in a population can take in plasmid DNA at any point in time. The presence of an antibiotic resistance gene on the plasmid vector allows us to identify the small percentage of bacteria that took in the plasmid. Bacteria that did not take in the plasmid will be killed by the antibiotic. Those that have the plasmid with the gene of interest will survive and grow.

In developing techniques for cloning genes in bacteria, scientists had a powerful tool in plasmids—a vector that can be taken in by bacteria, that replicates in bacteria to produce many copies of itself, that has a promoter sequence for transcription of an inserted gene, and that carries a gene for antibiotic resistance. If you carry out the lab in Chapter 5, you will take advantage of these features of plasmids when you transfer your recombinant plasmid into bacteria.

Once scientists recognized the power of plasmids as a potential vector, the next challenge was to determine how to incorporate a foreign gene of interest, such as the insulin gene, into the plasmid DNA.

RESTRICTION ENZYMES

In the early 1950s, scientists observed that certain strains of *E. coli*, a common bacterium found in the human gut, were resistant to infection by *bacteriophage* (viruses that infect bacteria by injecting their DNA into the cell and commandeering the host cell's molecular processes to make more bacteriophage). Investigation of this primitive "immune system" led to the discovery of restriction enzymes, proteins that restricted the growth of bacteriophage by recognizing and destroying the phage DNA without damaging the host (bacterial) DNA. Subsequent studies demonstrated that restriction enzymes from different strains of bacteria cut DNA at specific sequences. These sequences are called *recognition sites*.

CONSIDER: How do bacteria that carry a restriction enzyme avoid cutting up their own DNA?

Table 2.1 provides examples of restriction enzymes isolated from different strains of bacteria and the DNA sequences they cut. In the examples shown, the enzymes cut asymmetrically on the strands of DNA, leaving single-stranded overhanging sequences at the site of the cut. For example, a cut (or *digestion*) with EcoRI will leave an AATT overhang (or "*sticky end*") on one strand and a TTAA sticky end on the other strand.

Table 2.1: Restriction enzymes used in this laboratory

Source	Restriction enzyme	Recognition site
Escherichia coli	EcoRI	5′ G↓AATTC 3′ 3′ CTTAA↑G 5′
Bacillus amyloliquefaciens	BamHI	5′ G↓GATCC 3′ 3′ CCTAG↑G 5′
Haemophilus influenzae	HindIII	5′ A↓AGCTT 3′ 3′ TTCGA↑A 5′

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

CONSIDER:

- **What is the sequence of the sticky end that results when DNA is cut with BamHI? With HindIII?**
- **Scientists can modify plasmids to have a single restriction enzyme site. Imagine that you have a plasmid with a single EcoRI site. Draw the structure of the plasmid after it has been cut with the enzyme, and show the nucleotide sequences left at the site of the cut. If you wanted to insert a gene from a plant at this site, what enzyme would you use to cut the plant DNA with? Explain your response.**

DID YOU KNOW?

The Rise of Antibiotic-Resistant Bacteria

Antibiotics and similar drugs have been used for the last 70 years to treat patients who have infectious diseases. When prescribed and taken correctly, antibiotics are enormously valuable in patient care. However, these drugs have been used so widely and for so long that the infectious organisms the antibiotics are designed to kill have adapted to them, making the drugs less effective. Antibiotic resistance occurs when some bacteria in a population are able to survive when exposed to one or more antibiotics. These species that have become resistant cause infections that cannot be treated with the usual antibiotic drugs at the usual dosages and concentrations. Some have developed resistance to multiple antibiotics and are dubbed multidrug-resistant bacteria or "superbugs."

Antibiotic resistance is a serious and growing phenomenon and has emerged as one of the major public health concerns of the 21st century. Drug-resistant organisms may have acquired resistance to first-line antibiotics, requiring the use of second-line agents. Typically, the first-line agent is selected on the basis of several advantages, including safety, availability, and cost; the second-line agent is usually broader in spectrum, may be less beneficial in relation to the associated risks, and may be more expensive or less widely available.

CLONE THAT GENE

You now know about two biological tools for cloning a gene:

1. A plasmid that has several important features:

    - A restriction enzyme site or sites that opens the plasmid circle and enables insertion of the gene of interest into the plasmid DNA
    - A sequence for the initiation of DNA replication, called the *ori* site, that allows the plasmid to replicate in the bacteria using the host DNA synthesis enzymes
    - A promoter sequence 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 for the digestion of 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 human insulin gene into the plasmid DNA
3. Determine which antibiotic you would use to identify bacteria that have taken in the plasmid

HANDOUTS

- **Plasmid Diagram (RM 2)**
- **Human DNA Sequence (RM 3)**

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 site, and the genes for antibiotic resistance.
    - Locate the positions of each restriction enzyme recognition site.
2. Choose the restriction enzyme that should be used to cut the plasmid. Verify that the restriction enzyme meets all the following criteria:
    - The *ori* site on the plasmid is intact
    - The promoter site is intact
    - At least one of the antibiotic resistance genes is intact
    - The enzyme cuts the plasmid only once
    - The cut is close to the promoter sequence
3. Review **Table 2.1** on page 41 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.
4. On the **Human DNA Sequence (RM 3)**, scan the human DNA sequence and determine where the three restriction enzymes, BamHI, EcoRI, and HindIII, 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 **Table 2.1** 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.) 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?

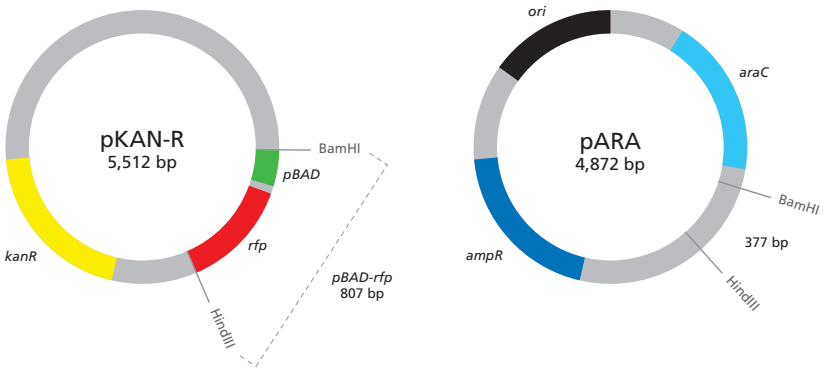
DNA ISOLATION
CLONE
TRANSFORM
EXPRESS
PURIFY

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

The purpose of this lab is to produce the DNA fragments that will be joined to make the recombinant plasmid, pARA-R, that can make the red fluorescent protein in bacteria. To do this you will use restriction enzymes to cut two plasmids, which will generate DNA fragments. 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 4).

So far, you've learned about using a single plasmid to clone the insulin gene. Under some circumstances scientists need to use plasmid DNA from different sources to generate a specific recombinant DNA. In order to clone the red fluorescent protein (*rfp*) gene, you will need DNA from two different plasmids. The plasmid pKAN-R (see **Figure 2.3**) carries the gene that makes bacteria resistant to the antibiotic kanamycin, the *rfp* gene, and a promoter sequence. The plasmid pARA (see **Figure 2.3**) contains the gene that makes bacteria resistant to the antibiotic ampicillin and 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*). If arabinose is present in the bacteria, the promoter will bind RNA polymerase, and transcription will occur. If arabinose is not present, the promoter will not bind RNA polymerase, and transcription will not occur. The plasmid pARA also contains the *ori* site for initiating DNA replication.

Figure 2.3: The pKAN-R and pARA plasmids



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

In addition to showing the relevant components, **Figure 2.3** 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 "BamHI" and "HindIII" represent the recognition sites for these two restriction enzymes. (See **Table 2.1** on page 41.) **Figure P.4** in *What Is Genetic Engineering?* (on page 14) shows the insulin gene being inserted in a single restriction enzyme site in the plasmid. In the cloning of the *rfp* gene, two restriction enzymes (BamHI and HindIII) are used in cutting the plasmid into which the *rfp* gene will be inserted and in isolating the *rfp* gene from the second plasmid. Using two different restriction enzymes has several 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 4.

STOP AND THINK: Why does using two different enzymes to cut the plasmid prevent the plasmid from reforming a circle without the inserted gene?

BEFORE THE LAB

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

1. Review **Figure 2.3**. If pKAN-R is digested with BamHI and HindIII, what fragments are produced? If pARA is digested with BamHI and HindIII, 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. In order 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. Biotechnologists call these genes *selectable markers* because only bacteria that carry the gene will survive an antibiotic. If the uptake of DNA by bacteria is inefficient (as discussed in the reading), why is a selectable marker critical in cloning a gene in bacteria?

4. Read through the Methods section on pages 49 and 50 and briefly outline the steps, using words and a flowchart.

MATERIALS

Reagents

- A rack with the following:
    - Microfuge tube of 2.5x restriction buffer (2.5xB)
    - Microfuge tube of pKAN-R plasmid (K)
    - Microfuge tube of pARA plasmid (A)
    - Microfuge tube of restriction enzymes BamHI and HindIII (RE)
    - Microfuge tube of distilled water (dH_2O)

Equipment and Supplies

- P-20 micropipette
- Tip box of disposable tips
- 4 1.5-mL microfuge tubes
- Permanent marker
- Microcentrifuge (will be shared among all groups)
- 37°C water bath with floating microfuge tube rack (will be shared among all groups)
- Waste container for used tips and microfuge tubes (will be shared among groups)

SAFETY:

- **All appropriate safety precautions and attire required for a science laboratory should be used, including safety goggles. Please refer to your teacher's instructions.**
- **Wash your hands well with soap after completing the lab.**

METHODS

1. Check your rack to make sure that you have all the reagents listed.
2. Use a marker to label four clean microfuge tubes as follows: K+, K–, A+, and A–. (Also include your group number and class period on each tube.)
3. Review **Table 2.2**, which summarizes the reagents that you will add in step 4.

Table 2.2: Addition of reagents to the K+, K–, A+, and A– tubes

	K+ tube	K– tube	A+ tube	A– tube
Step 4a: Restriction buffer (2.5xB)	4.0 µL	4.0 µL	4.0 µL	4.0 µL
Step 4b: pKAN-R plasmid (K)	4.0 µL	4.0 µL		
Step 4c: pARA plasmid (A)			4.0 µL	4.0 µL
Step 4d: BamHI and HindIII (RE)	2.0 µL		2.0 µL	
Step 4e: Distilled water (dH_2O)		2.0 µL		2.0 µL

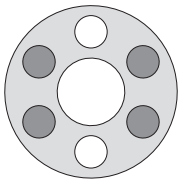
LAB TECHNIQUE: In step 4, be sure to use a new micropipette tip for each reagent in each tube to avoid contamination.

4. Add the following:
   a. 4.0 µL of 2.5xB to the K+, K–, A+, and A– tubes.
   b. 4.0 µL of K to the K+ and K– tubes.
   c. 4.0 µL of A to the A+ and A– tubes.
   d. 2.0 µL of RE to the K+ and A+ tubes. Add the enzymes directly into the solution at the bottom of the microfuge tube. Gently pump the solution in and out with the pipette to mix the reagents. Cap the tubes when done.
   e. 2.0 µL of dH_2O to the K– and A– tubes. Gently pump the solution in and out with the pipette to mix the reagents. Cap the tubes when done.

STOP AND THINK: In step 4, you are asked to set up two tubes without the restriction enzymes, BamHI and HindIII. What is the purpose of this step, and why is it important?

5. Spin the four microfuge tubes (K+, A+, K–, and A–) in the microcentrifuge for several seconds to pool the reagents at the bottom of each tube.

LAB TECHNIQUE: Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.



6. Place all four tubes into the 37°C water bath. (You will place your tubes in the floating microfuge tube rack; when the rack is full, your teacher will place it in the water bath.) Incubate for at least one hour but not longer than two hours. 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: Why might the enzymes work best at 37°C? Why should the enzymes then be placed in the freezer?

CHAPTER 2 QUESTIONS

Discuss the following questions with your partner and be prepared to share your answers with the class.

1. List in words or indicate in a drawing the important features of a plasmid vector that are required to clone a gene. Explain the purpose of each feature.

2. What role do restriction enzymes have in nature?

3. Using your understanding of evolution, why would bacteria retain a gene that gives them resistance to antibiotics? How is the existence of bacteria with antibiotic resistance affecting medicine today?

4. Bacteria, sea anemones, and humans seem, on the surface, to be very different organisms. Explain how a gene from humans or a sea anemone can be expressed in bacteria to make a product never before made in bacteria.

5. Due to a mishap in the lab, bacteria carrying a plasmid with a kanamycin-resistant gene and bacteria carrying a plasmid with an ampicillin-resistant gene 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!)

CHAPTER 2 GLOSSARY

Activator: A protein that regulates transcription of a gene by binding to a sequence near the promoter, thus enabling RNA polymerase to bind to the promoter and initiate transcription of the gene. The activator protein can also block the binding of RNA polymerase and thereby inhibit transcription of the gene.

Antibiotic: A class of compounds that kill or inhibit the growth of microorganisms.

Antibiotic resistance: The state in which bacteria are no longer sensitive to an antibiotic and will continue to grow and divide in the presence of the antibiotic.

Arabinose: A five-carbon sugar that naturally occurs in various plant and bacterial carbohydrates.

Bacterial conjugation: A process by which two bacterial cells join and transfer genetic material from one to another.

Bacteriophage: A virus that infects a bacterial cell and uses the cell machinery to replicate itself, eventually destroying the bacterial cell.

Base pair: Two complementary nitrogen-containing molecules paired together in double-stranded DNA by weak bonds.

Digestion: The cutting of DNA by a restriction enzyme.

DNA ligase: An enzyme that catalyzes the formation of covalent chemical bonds in the sugar-phosphate backbone, thereby binding fragments of DNA together.

DNA replication: The biological process of making an identical copy of DNA, which occurs each time a new cell is formed in living organisms. The double helix is unwound, and each strand of the original molecule serves as a template for the production of the complementary strand.

Enzyme: A protein that catalyzes a chemical reaction.

Messenger RNA: An RNA molecule transcribed from the DNA of a gene and used as the template for protein synthesis.

Origin of replication (*ori*): A sequence of DNA at which replication of the DNA is initiated.

Plasmid: A circular molecule of DNA.

Promoter: A specific DNA sequence that binds RNA polymerase and initiates transcription of the gene.

Recognition site: A specific DNA sequence that is cut by a restriction enzyme. Many restriction sites are palindromes, sequences that are the same when read forward or backward.

Recombinant DNA: DNA that contains sequences or genes from two or more sources.

Restriction digest: A technique in which naturally occurring enzymes are used to cleave DNA at specific sequences.

Restriction enzyme: A protein that can cut DNA at a specific sequence called a recognition site.

RNA (ribonucleic acid): A single-stranded biomolecule made up of a nitrogenous base, a ribose sugar, and a phosphate; RNA plays a critical role in protein synthesis, transmitting genetic information from DNA to the ribosome where proteins are then made.

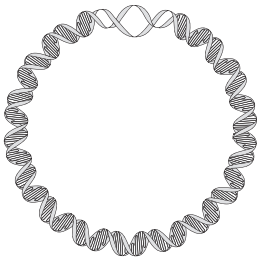
RNA polymerase: A protein that makes messenger RNA from DNA.

Selectable marker: A gene on a plasmid that is introduced into a cell along with a gene of interest that is being cloned. Selectable markers allow scientists to tell if the plasmid has been taken in by the cell because the marker can be seen or detected. A common selectable marker is an antibiotic resistance gene—only bacteria that have the gene will survive the antibiotic.

Sticky ends: Ends of a DNA molecule cut with certain restriction enzymes. These ends are asymmetrical in that one strand is longer than the other strand and therefore has unpaired bases. The sticky ends of two different pieces of DNA that have been cut with the same restriction enzyme(s) can be joined, as the unpaired bases on their ends are complementary.

Transcription: The process by which information encoded in DNA is transferred to messenger RNA, a single-stranded ribonucleic acid.

Vector: A vehicle for moving DNA sequences from one organism to another.



CHAPTER 3

BUILDING A RECOMBINANT PLASMID

INTRODUCTION

In Chapters 1 and 2, you learned about four important tools of genetic engineering that scientists use to clone the insulin gene: the micropipette, gel electrophoresis, plasmids, and restriction enzymes. In this chapter, you will use a fifth tool that is needed to clone genes—DNA ligase. *DNA ligase* is an *enzyme* that *catalyzes* the joining of DNA fragments; it is one of several enzymes involved in *DNA replication* in all cells. In Chapter 2, you prepared the DNA fragments required for cloning the *rfp* gene. The next step is to *ligate* (join together) these fragments to form the recombinant plasmid.

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 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 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. Describe what happens when two DNA fragments with complementary *sticky ends* join, and speculate how the activity of DNA ligase ensures that the join is permanent.

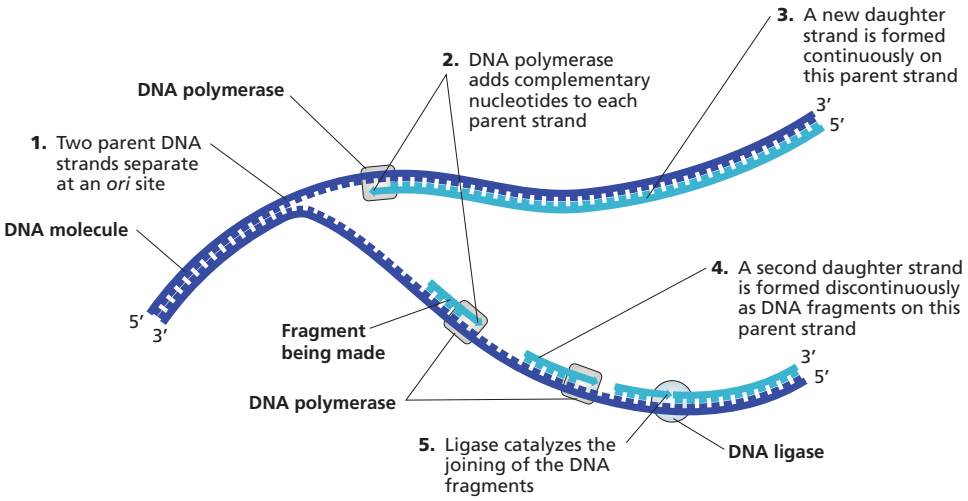
LIGASES

The discoveries of restriction enzymes, plasmids, and ligases in cells were important in the quest to understand how bacteria grow and reproduce themselves. But it was understanding how these biomolecules could be applied to manipulate DNA that launched a new era of genetic engineering, one in which humans could directly engineer organisms at the genetic level. Plasmids provided the vehicle to clone genes, and restriction enzymes provided the means to generate the fragments of DNA needed to form recombinant plasmids. The final essential ingredient was a way to "glue" the fragments together.

THE ROLE OF LIGASES IN DNA REPLICATION

In the early 1960s, scientists isolated enzymes that had this ability. These enzymes, called DNA ligases, were shown to be involved in DNA replication of chromosomes and plasmids. Replication occurs in a number of steps (as shown in **Figure 3.1**):

Figure 3.1: Summary of DNA replication



1. The two strands begin to separate at multiple locations on the DNA molecule. Each location is an origin of replication, or *ori* site (the same as you learned about with plasmids in Chapter 2). The two strands are called *parent strands*.

2. Once the parent strands are separated and the bases are exposed, an enzyme called *DNA polymerase* adds new nucleotides. Each new nucleotide base forms hydrogen bonds with existing nucleotide bases on the parent strand. *Complementary base pairs* are created in this step.

3. On one parent strand, DNA polymerase adds nucleotides continuously to form a new complementary strand, a *daughter strand*.

4. On the other parent strand, DNA polymerase adds nucleotides discontinuously, resulting in small fragments of DNA. (To learn about why one strand is replicated continuously and one discontinuously, refer to *Did You Know? DNA Directionality*.)

5. The DNA fragments must be joined by the enzyme DNA ligase in order to form the second daughter strand. The ligase joins the fragments by catalyzing the formation of a covalent bond between adjacent nucleotides.

Once the replication steps are complete, two new DNA molecules, each consisting of one parent strand and one daughter strand, have been made. Both molecules are exact copies, or *replicas*, of the original DNA molecule.

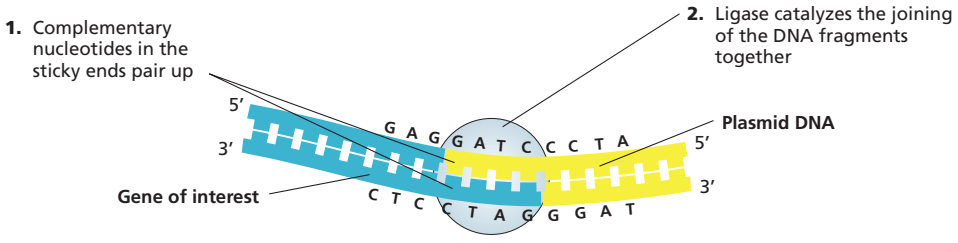
CONSIDER:

- **What are the roles of hydrogen bonds and covalent bonds in the structure of DNA?**
- **Based on what you just learned about DNA replication, what is the possible role of ligases in joining the sticky ends of DNA fragments?**

ROLE OF LIGASES IN GENE CLONING

The role of ligase in gene cloning is similar to its role in replication in that it binds DNA fragments together. In gene cloning, recombinant DNA is the result of ligation of fragments from a restriction digest (as shown in **Figure 3.2**). Any two fragments that have ends cut with the same restriction enzyme can be ligated together. First the unpaired bases at the sticky ends form hydrogen bonds to each other, and then the ligase catalyzes the formation of covalent bonds between adjacent nucleotides. If you have multiple fragments, the ligation procedure can lead to a number of possible products.

Figure 3.2: Ligation of DNA fragments in genetic cloning



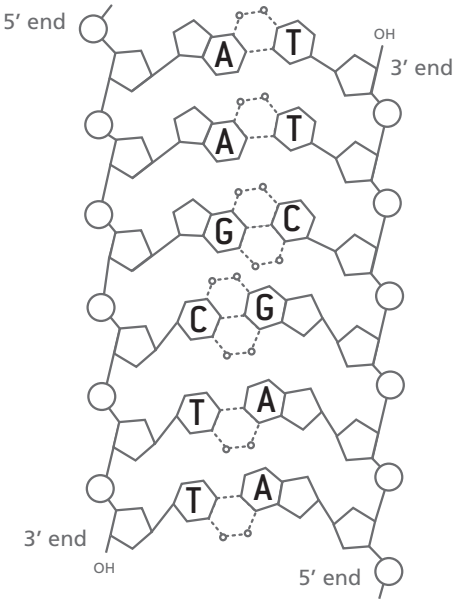
DID YOU KNOW?

DNA Directionality

DNA is a double helix of two intertwined strands that are hydrogen-bonded together through the bases of the nucleotides. Each strand has a different *directionality*, meaning that each strand has a chemical orientation based on the chemical group that it ends with. The *5′* (5 "prime") *end* of the DNA strand is the end that has the fifth carbon in the deoxyribose sugar ring. The *3′* (3 "prime") *end* of the DNA strand is the end that has the hydroxyl (OH) group on the third carbon in the deoxyribose sugar ring (see **Figure 3.3**).

This naming of the ends of the strands is useful in understanding how DNA polymerase works. DNA polymerase can only assemble new DNA strands in the 5′ to 3′ direction, adding new nucleotides to the 3′-hydroxyl group. Therefore, on one strand replication occurs continuously as polymerase adds nucleotides in the 5′ to 3′ direction to form the daughter strand. On the other strand, the DNA polymerase also adds nucleotides in the 5′ to 3′ direction, which results in short fragments of DNA that must be ligated together to form the other daughter strand.

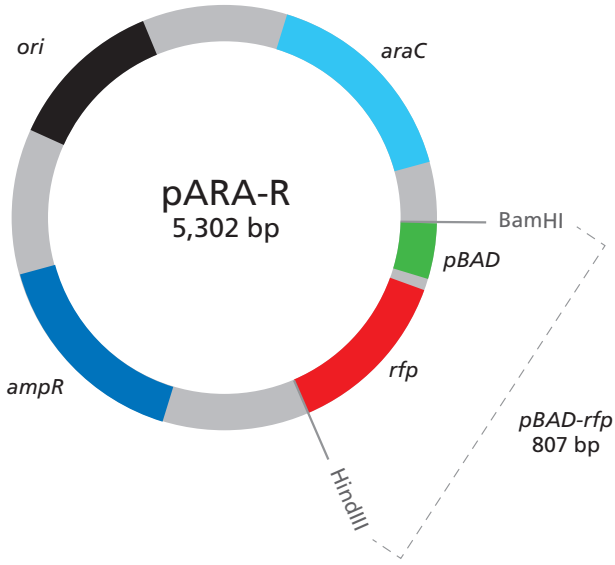
Figure 3.3: DNA structure



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 **Figure 3.4**).

Figure 3.4: The pARA-R plasmid



During the 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 questions with your group, record your answers, and be prepared to share your answers with the class.

1. Review your answer to question 1 in Before the Lab for *Laboratory 2* (page 47), in which you described the fragments that formed from the digestion of pKAN-R and pARA with BamHI and HindIII. Using this information, draw three possible recombinant plasmids resulting from the joining of two pARA and pKAN-R fragments. For each plasmid, identify the genes, other important sequences, and the number of base pairs each has.

2. Read through the Methods section on page 63 and briefly outline the steps, using words and a flowchart.

MATERIALS

Reagents

- A rack with the following:
    - Microfuge tube of digested pKAN-R from *Laboratory 2* (K+)
    - Microfuge tube of digested pARA from *Laboratory 2* (A+)
    - Microfuge tube of 5x ligation buffer (5xB)
    - Microfuge tube of DNA ligase (LIG)
    - Microfuge tube of distilled water (dH_2O)

Equipment and Supplies

- 70°C water bath with floating microfuge tube rack (will be shared among all groups)
- P-20 micropipette
- Tip box of disposable pipette tips
- Permanent marker
- Microcentrifuge (will be shared among all groups)
- Waste container for used tips and microfuge tubes (may be shared with another group)

SAFETY:

- **All appropriate safety precautions and attire required for a science laboratory should be used, including safety goggles. Please refer to your teacher's instructions.**

- **Wash your hands well with soap after completing the lab.**

METHODS

1. Check your rack to make sure that you have all the reagents listed.

2. Place K+ and A+ tubes from *Laboratory 2* in the 70°C water bath (you will place your tubes in the floating microfuge tube rack; once each group's samples are loaded, your teacher will place the rack in the water bath) for 30 minutes. This heat exposure will *denature* (inactivate) the restriction enzymes.

   NOTE: During the 30-minute incubation, share and discuss your answers to question 1 in Before the Lab. Also share and discuss your answer to the STOP AND THINK question that follows and begin answering the *Chapter 3 Questions* on page 64.

STOP AND THINK: Why is it important to inactivate the BamHI and HindIII restriction enzymes before ligating the fragments? What might happen if you did not perform this step?

3. Label the LIG tube with your group number and class period.

4. After 30 minutes, remove the K+ and A+ tubes from the water bath and place them in your rack.

5. Add the following solutions directly into the solution at the bottom of the LIG tube:

   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

   LAB TECHNIQUE: In steps 5a–d, be sure to use a new micropipette tip for each reagent to avoid contamination.

6. After adding the dH_2O, gently pump the solution in and out with the pipette to mix the reagents. Cap the tube when done.

7. Spin the LIG tube in the microcentrifuge for several seconds to pool the reagents at the bottom of the tube.

   LAB TECHNIQUE: Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

8. Place your LIG, A+, and K+ tubes in the microfuge racks designated by your teacher. (Your LIG tube will incubate at room temperature until the next class. Your A+ and K+ tubes will be returned to the –20°C freezer for use in *Laboratory 4*.)

CHAPTER 3 QUESTIONS

Discuss the following questions with your partner and be prepared to share your answers with the class:

1. What role do DNA ligases have in nature?
2. What role do DNA ligases have in gene cloning?
3. What properties of the DNA restriction fragments produced in *Laboratory 2* enable ligation of these fragments?
4. Could two *rfp* fragments join to form a plasmid during the ligation? If not, what would prevent that? If so, what would be the result?
5. During ligation, both hydrogen and covalent bonds form. Which bonds form first? Why do both types of bonds need to form?

DID YOU KNOW?

DNA Replication Errors and Evolution

Every time a cell divides and its DNA replicates, it copies and transmits the exact same sequence of nucleotides to its daughter cells. While DNA usually replicates with fairly high accuracy, mistakes do happen. Polymerase enzymes sometimes insert the wrong nucleotide or too many or too few nucleotides into a sequence. Incorrectly paired nucleotides that still remain following mismatch repair become permanent mutations after the next cell division. This is because once such mistakes are established, the cell no longer recognizes them as errors.

Mutations can be beneficial, harmful, or have no effect at all. If a mutation gives the organism a selective advantage, the trait may be passed to future generations as part of the evolutionary process. In fact, over the course of millions of years, mutations may build up in any given stretch of DNA at a reliable rate. This allows the gene to be used as a molecular clock, a tool for estimating how long ago two species split from each other. This general technique has been used to investigate several important questions, including the origin of modern humans and the date of the human-chimpanzee divergence.

CHAPTER 3 GLOSSARY

3′ end: The end of a DNA or RNA strand that has a hydroxyl group connected to the third carbon of the sugar molecule, which is deoxyribose or ribose. (The carbon number refers to the position of the carbon in the sugar molecule.)

5′ end: The end of a DNA or RNA strand that terminates at the fifth carbon of the sugar molecule, which is deoxyribose or ribose. (The carbon number refers to the position of the carbon in the sugar molecule.)

Catalyze: To increase the rate of a reaction.

Complementary base pair: Nitrogen-containing bases that are found opposite each other in a double-stranded DNA molecule. Complementarity is the result of the size and shape of the base and the number of hydrogen bonds between the adjacent bases in the pair (A and T form two hydrogen bonds, G and C form three). Adenine is complementary to thymine, and guanine is complementary to cytosine.

Daughter strand: In DNA replication, a new DNA strand that is created that is a complement to the original DNA strand.

Denature: To change the shape of a biomolecule and subsequently impact its function, which is often achieved by heating. For example, a protein folds into a complex three-dimensional shape that is directly related to its function. Any process that interferes with protein folding can change its three-dimensional shape and result in inactivation of the protein.

Directionality: In biochemistry, the orientation of a strand of nucleic acid.

DNA ligase: An enzyme that catalyzes the formation of covalent chemical bonds in the sugar-phosphate backbone, thereby permanently binding fragments of DNA together.

DNA polymerase: An enzyme used to replicate DNA molecules.

DNA replication: The biological process of making an identical copy of a section of DNA, which occurs each time a new cell is formed in living organisms. The process starts when one double-stranded DNA molecule produces two identical copies. The double helix is unwound, and each strand of the original molecule serves as a template for the production of the complementary strand.

Enzyme: A protein that catalyzes a chemical reaction.

Ligation: The reaction that chemically joins two fragments of DNA, resulting in a recombinant DNA molecule.

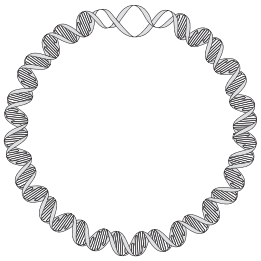
Ligate: To join together two DNA ends.

Parent strand: In DNA replication, the DNA strand that serves as a template for creating the new complementary strand.

Replica: An exact copy.

Restriction fragment: The piece of DNA that results from the cutting of the DNA molecule with a restriction enzyme. Fragments are often separated on a gel using electrophoresis.

Sticky ends: Ends of a DNA molecule cut with certain restriction enzymes. These ends are asymmetrical in that one strand is longer than the other strand and therefore has unpaired bases. The sticky ends of two different pieces of DNA that have been cut with the same restriction enzyme(s) can be joined, as the unpaired bases on their ends are complementary.



CHAPTER 4

MAKING SURE YOU'VE CREATED A RECOMBINANT PLASMID

INTRODUCTION

When biologists clone a gene in order to produce human insulin, they create a recombinant plasmid that has the insulin gene. To do so, they use restriction enzymes to create DNA fragments that contain the plasmid components and then use DNA ligase to join those fragments together. As part of the gene cloning process, biologists have to *verify* that they have created the recombinant plasmid they need—that is, the one with the gene of interest as well as all the necessary components for the protein of interest 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 to produce the red fluorescent protein.

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 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, 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?

VERIFICATION

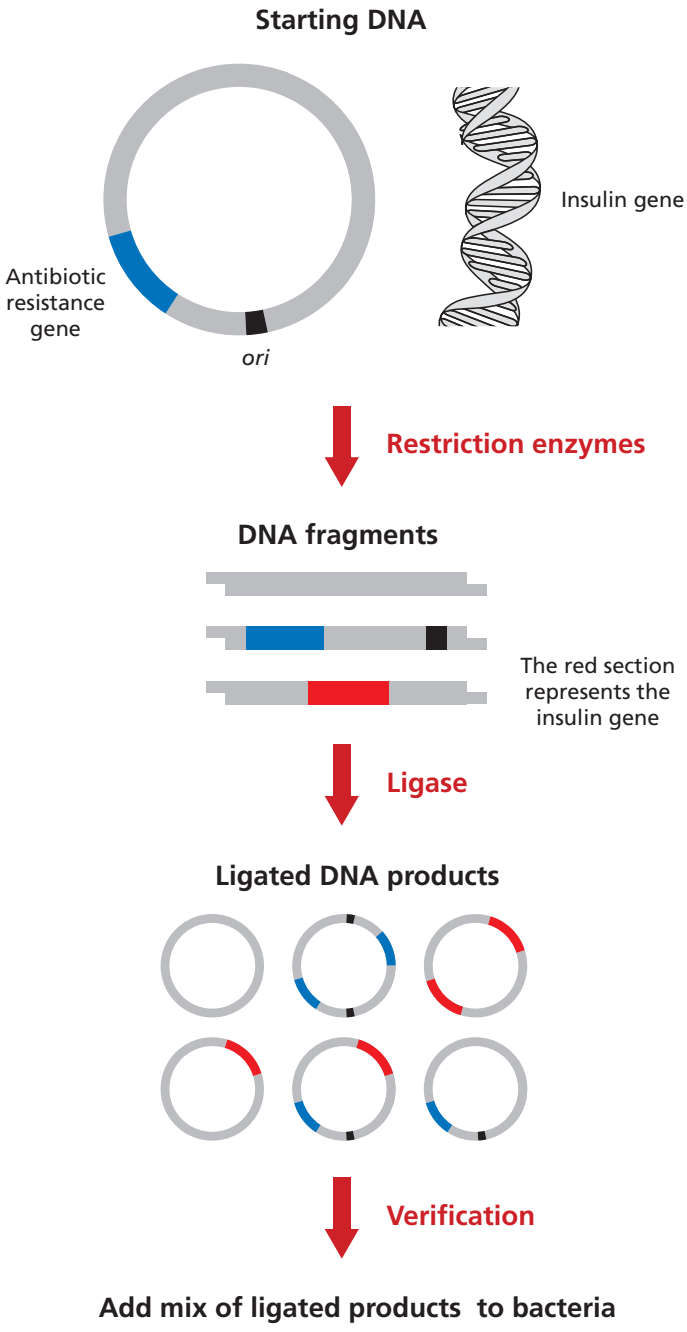
It is important to verify work in the lab—there are many sources of potential error in any procedure, including the procedures used in cloning a gene. In gene cloning, there is also the problem that some procedures are not selective. For example, when a DNA ligase is used to *ligate*—bind together—DNA fragments, many different combinations result from the ligation process. Unless you verify your work, you do not know if you have made the recombinant plasmid that is needed.

HOW TO VERIFY THE RECOMBINANT PLASMID

Figure 4.1 shows the method used for verifying your results when making a recombinant plasmid. You verify that the restriction digest and ligation procedures worked by comparing the products of both procedures with each other and with what you started with.

Figure 4.1: Verification method when making a recombinant plasmid

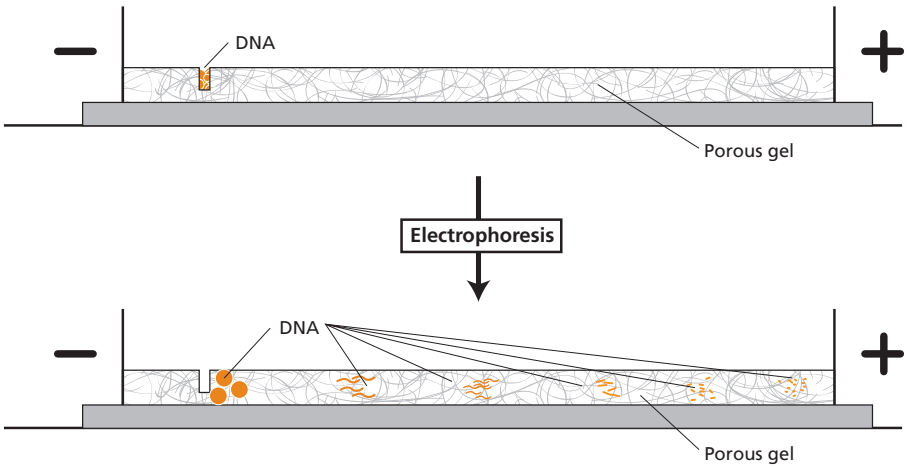
Verify that the restriction digest and ligation procedure was successful by comparing the products of the restriction digest, the products of the ligation procedure, and the starting materials



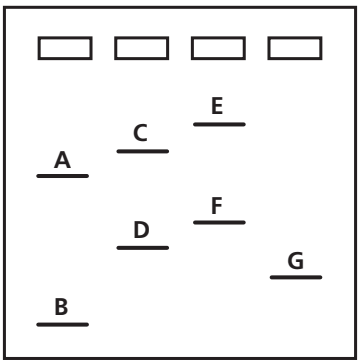
GEL ELECTROPHORESIS

Gel electrophoresis is used extensively in DNA verification and purification. In order to identify or purify DNA restriction fragments, it is necessary to separate the various-sized DNA molecules. Gel electrophoresis separates biomolecules primarily according to their molecular size, which for DNA is measured by the number of base pairs. The backbone of a DNA molecule, because of its phosphate groups, is negatively charged and therefore will move away from the negative (black) electrode and toward the positive (red) electrode. Because it is easier for small DNA molecules to move through the agarose matrix, they will migrate faster than larger DNA molecules. See **Figure 4.2**.

Figure 4.2: Separation of DNA by size using gel electrophoresis



CONSIDER: After different DNA fragments and plasmids have been separated by gel electrophoresis, the gel is stained to show bands that indicate the location of each kind of fragment and plasmid. The drawing of a stained gel below shows a series of bands that have been labeled with letters. The locations of the wells are also shown. What is the order of the fragments, from smallest to largest?



PLASMID CONFIGURATIONS

While 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:

- The most common plasmid configuration is *supercoiled*. You can visualize this configuration by thinking of a circular piece of plastic tubing that is twisted. This twisting or supercoiling results in a very compact molecule, one that will move through the gel very quickly for its size. This configuration is only seen in plasmids that have been replicated in bacteria because supercoiling of a plasmid requires an enzyme that is found in the bacterial cell. It is the default natural plasmid configuration found in bacteria.
- The second plasmid configuration is a *nicked circle*. You can visualize this configuration as a large floppy circle. This plasmid has a break in one of the covalent bonds located in its sugar-phosphate backbone along one of the two nucleotide strands. This circular plasmid configuration will not move through the agarose gel as easily as the supercoiled configuration. Although it is the same size, in terms of base pairs, it will be located closer to the well than the supercoiled form.
- The third plasmid configuration is a *multimer*. You can visualize this configuration by thinking of two or more plasmids that are connected like links in a chain. This configuration is only seen in plasmids that have been replicated in bacteria, because multimers form when plasmids are replicated so fast that they end up linked together. If two plasmids are linked, the multimer will be twice as large as a single plasmid and will migrate very slowly through the gel. In fact, it will move slower than the nicked circle.

The possible plasmid configurations are shown in **Figure 4.3**.

Figure 4.3: Plasmid configurations

Supercoiled Nicked circle Multimer

CONSIDER: **If you used gel electrophoresis to separate the same plasmid that has all three configurations, the supercoiled plasmid would move the fastest, while the multimer would move the slowest. Why do the different plasmid configurations move the way they do through the gel? Explain in words or a drawing.**

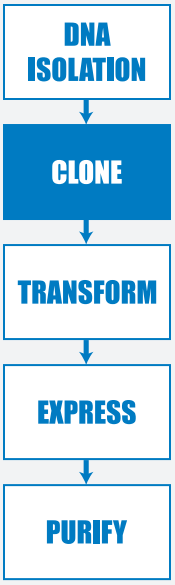
DID YOU KNOW?

History of Genetic Engineering

Genetic engineering is not a new phenomenon—it has been done for centuries in plant and animal breeding. Throughout history, humans have used selective breeding to produce organisms with desirable traits. The science of agriculture began with the selection of wild grasses and subsequent breeding to form the precursors of modern staples such as wheat, rice, and maize. In selective breeding, two members of the same species are paired as breeding partners in order to encourage desirable characteristics in the offspring. For example, cows that have been observed producing large volumes of milk may be bred to pass that trait on to future generations.

Selective breeding is one way for humans to nurture desirable traits in plants and animals, but it is much older and less predictable than genetic modification. Genetic modification is more precise than classical breeding, and, in many cases, it is much quicker. Our current understanding of genetics and heredity allows for the manipulation of genes and the development of new combinations of traits and new varieties of organisms. Actually going into a cell and changing its genome by inserting or removing DNA is a very new technology. But keep in mind that genetic modification isn't some "unnatural" process to create monsters! Genetic modification and traditional selective breeding are limited by the same constraints, and natural-occurring mutations may (but not always) have a negative outcome as well.

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 the products from the ligation (*Laboratory 3*). The sizes of the DNA fragments can be determined by comparing them to a *DNA ladder*—a mixture of DNA fragments with known sizes. (When the DNA ladder is run on gel electrophoresis and stained, the bands that show the fragments look like the rungs of a ladder.) 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. 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 questions with your group and be prepared to share your answers 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 catalog 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 on pages 77 through 79 and briefly outline the steps, using words and a flowchart.

MATERIALS

Reagents

- A rack with the following:
    - Microfuge tube of nondigested pKAN-R from *Laboratory 2* (K–)
    - Microfuge tube of digested pKAN-R from *Laboratory 2* (K+)
    - Microfuge tube of nondigested pARA from *Laboratory 2* (A–)
    - Microfuge tube of digested pARA from *Laboratory 2* (A+)
    - Microfuge tube of ligated plasmid from *Laboratory 3* (LIG)
    - Microfuge tube of *loading dye* (LD)
    - Microfuge tube of distilled water (dH_2O)
    - Microfuge tube of DNA ladder (M)
- 50-mL flask containing 1x sodium borate buffer (1x SB) (shared with another group)

Equipment and Supplies

- 5 1.5-mL microfuge tubes
- Permanent marker
- P-20 micropipette
- Tip box of disposable pipette tips
- Microcentrifuge (will be shared among all groups)
- Electrophoresis box loaded with 0.8% agarose gel (will be shared among groups)
- Waste container for used tips and microfuge tubes (will be shared among groups)
- **DNA Ladder Diagram (RM 4)**

SAFETY:

- **All appropriate safety precautions and attire required for a science laboratory should be used, including safety goggles. Please refer to your teacher's instructions.**
- **Wash your hands well with soap after completing the lab.**

METHODS

1. Check your rack to make sure that you have all the reagents listed.
2. Use the marker to label five clean microfuge tubes "geA–," "geA+," "geK–," "geK+," and "geLIG." Include your group number and class period on each tube.

   Note: The "ge" prefix signals that these tubes contain the gel electrophoresis samples.

3. Read **Table 4.1**, which summarizes the reagents you will add in steps 4–8 to verify the restriction and ligation procedures.

Table 4.1: Addition of reagents to the geK–, geK+, geA–, geA+, and geLIG tubes

Sequence	geK– tube	geK+ tube	geA– tube	geA+ tube	geLIG tube
Steps 4 and 5: Distilled water (dH_2O)	4.0 µL	4.0 µL	4.0 µL	4.0 µL	3.0 µL
Step 6: Loading dye (LD)	2.0 µL	2.0 µL	2.0 µL	2.0 µL	2.0 µL
Step 7: Nondigested pKAN-R (K–)	4.0 µL				
Step 7: Digested pKAN-R (K+)		4.0 µL			
Step 7: Nondigested pARA (A–)			4.0 µL		
Step 7: Digested pARA (A+)				4.0 µL	
Step 8: Ligated plasmid (LIG)					5.0 µL

LAB TECHNIQUE: In steps 4–8, be sure to use a new micropipette tip for each reagent to avoid contamination.

4. Add 4.0 µL of dH_2O to the geK–, geK+, geA–, and geA+ tubes.
5. Add 3.0 µL of dH_2O to the geLIG tube.
6. Add 2.0 µL of LD to the geK–, geK+, geA–, geA+, and geLIG 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?

7. Add the following (remember to use new micropipette tips):

    a. 4.0 µL of K– to the geK– tube.
    b. 4.0 µL of K+ to the geK+ tube.
    c. 4.0 µL of A– to the geA– tube.
    d. 4.0 µL of A+ to the geA+ tube.

8. Add 5.0 µL of LIG to the geLIG tube. Return the "LIG" tube to your teacher for use in the next lab.

9. Spin the five microfuge tubes (geK–, geK+, geA–, geA+, and geLIG) in the microcentrifuge for several seconds to pool the reagents at the bottom of each tube.

LAB TECHNIQUE: Distribute the tubes evenly in the microcentrifuge so that their weight is balanced, making sure that tubes of the same volume are directly opposite one another.

10. Make sure that the wells in your gel electrophoresis unit are located near the negative (black) electrode.

11. Fill the box with 1x SB to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more buffer.

LAB TECHNIQUE: If there are "dimples," add *very small* amounts of buffer to the electrophoresis box. While the gel needs to be completely under the buffer, you don't want too much buffer in the box, as this will allow the electrical current to run through the buffer and not the gel.

12. 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 as follows:

13. Using a fresh pipette tip for each sample, dispense 10.0 µL of the DNA ladder (M), geK–, geK+, geA–, geA+, and geLIG into their designated wells. For each sample, do the following:

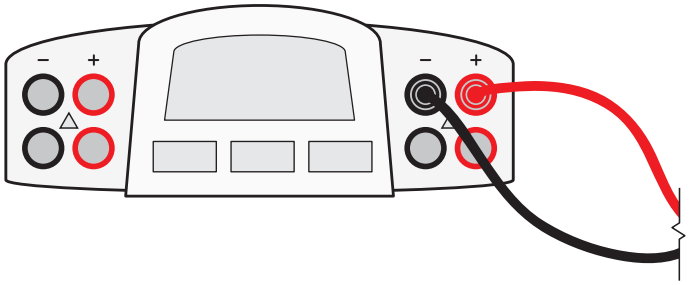
    a. Place your elbow on the table to steady your pipette hand. If needed, also use your other hand to support your pipette hand.
    b. Lower the pipette tip until it is under the buffer but just above the well.

LAB TECHNIQUE: Do not puncture the gel, or it will become unusable. Gently press the plunger to slowly dispense the sample. To avoid getting air into the buffer, do not go past the first stop. The sample will sink into the well.

14. When all the samples have been loaded, close the cover tightly over the electrophoresis box.

15. Connect the electrical leads to the power supply. Connect both leads to the same channel, with cathode (–) to cathode (black to black) and anode (+) to anode (red to red). See **Figure 4.4**.

Figure 4.4: Leads from electrophoresis box connected to correct channel in power supply



16. Turn on the power supply and set the voltage to 130–135 V.

17. 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 in to the power supply correctly.

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?**

18. Your teacher will explain what to do with your gel. You may not have sufficient time to complete the electrophoresis. The yellow loading dye will need to run just near the end of the gel, about 40–50 minutes. After the gel has finished running, it will need to be stained to show the location of the DNA fragments and plasmids and your teacher will provide you with a photograph of the stained gel to analyze.

CHAPTER 4 QUESTIONS

Analyze your gel photograph and discuss the following questions with your partner. Be prepared to share your answers with the class.

1. Why is it important to verify that you have the correct recombinant plasmid?
2. How did your actual gel results compare to your gel predictions?
3. Do you see any bands that are not expected? What could explain the origin of these unexpected bands?
4. Does the gel show that your restriction digest and ligation procedures were successful? Describe the evidence you used to make this assessment.
5. In the geK– and geA– lanes, do you see evidence of multiple configurations of plasmids? Explain your answer.
6. In the geK+ and geA+ lanes, do you see evidence of complete digestion? Explain your answer.
7. In which lane would you expect to find the *rfp* gene and the *ampR* gene in the gel photograph? Are you able to locate these two genes? Explain your answer.
8. Compare the lanes that have linear fragments with the lanes that have plasmids. Is there a difference in the shape of the bands between these two DNA forms?
9. In *Laboratory 3*, you described all the possible plasmids that you could make by ligating the digested fragments of the pKAN-R and the pARA plasmids. Two of the *rfp* gene fragments (807 bp each) may form a circularized fragment because each end of the fragments terminates in BamHI and HindIII sticky ends. Is there evidence of a circularized 1,614 bp fragment in the geLIG tube lane? Explain your answer.

CHAPTER 4 GLOSSARY

DNA ladder: A set of known DNA fragments with different sizes in base pairs (bp) or kilo bases (kb). These DNA fragments are separated and visualized as DNA bands on a gel. Together, the separated DNA bands look like a ladder on the gel. DNA ladders are used in gel electrophoresis to determine the size and quantity of DNA fragments.

Ligate: To join together two DNA ends.

Ligation: The reaction that chemically joins two or more fragments of DNA, resulting in a recombinant DNA molecule.

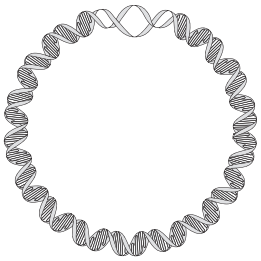
Loading dye: A set of dyes that are added to biomolecules such as DNA for gel electrophoresis. One dye moves farther than the sample, which indicates that it is time to stop running the gel.

Multimer: A plasmid configuration consisting of multiple plasmids that have interlocked during formation so they are like links in a chain.

Nicked circle: A plasmid configuration that consists of a single plasmid that has a break in one of its two strands. The shape of this plasmid is circular.

Supercoiled: A plasmid configuration consisting of a single plasmid that has been twisted. The shape of this plasmid is more compact (takes up less space) than the circular form.

Verify: Establish that something is true, accurate, or able to be defended.



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*, because it changes the DNA content of the bacteria. In this chapter, you will carry out the transformation of *E. coli* bacteria using the recombinant plasmid that contains the *rfp* gene.

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 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 transcription and *translation* 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?

TRANSFORMING BACTERIA WITH RECOMBINANT PLASMIDS

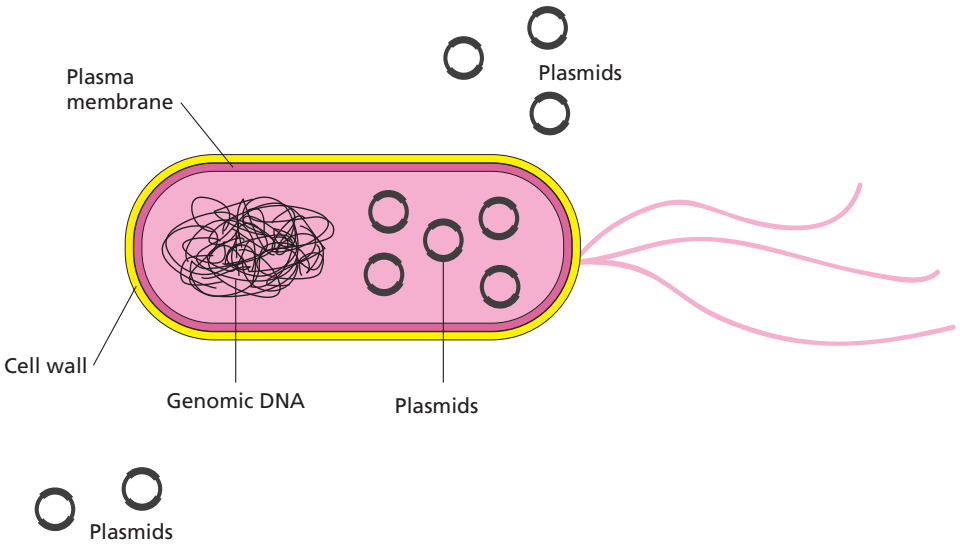
A plasmid is an ideal vector for carrying DNA sequences from one organism to another. The plasmid is equipped with (1) a *promoter* that enables gene transcription, (2) a sequence for the initiation of DNA replication, and (3) an antibiotic resistance gene. The plasmid can be taken up by bacteria where 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.

CONSIDER: Once a gene has been inserted into a vector, what do you think is required to make the product encoded by the inserted gene?

BACTERIA TRANSFORMATION

Once a recombinant plasmid is made that contains a gene of interest, such as insulin, the plasmid can enter bacterial cells by a process called transformation. **Figure 5.1** illustrates transformation.

Figure 5.1: Bacterial transformation



The uptake of DNA from the environment of a bacterial cell occurs with a very low efficiency in nature. *E. coli* bacteria have complex plasma membranes that separate the external environment from the internal environment of the cell and carefully regulate which substances can enter and exit the cell. In addition, the cell wall is negatively charged and repels negatively charged DNA molecules.

CONSIDER: Why is it important that the membranes of *E. coli* bacteria carefully regulate which substances can enter and exit the cell?

In order to increase the efficiency of DNA uptake, bacteria are treated in two ways. First, the *E. coli* bacteria are placed in a solution that contains positive calcium ions, which neutralize the negative charge on the cells' outer membranes, enabling DNA molecules to cross the plasma membranes and enter the cell. Next, the bacteria are subjected to a heat shock, a sudden increase in temperature, which causes the pressure outside the cell to increase. This pressure difference enables the plasmid DNA to enter the bacterial cell from the outside.

Cells treated with calcium and heat are considered *competent* to take up DNA more efficiently, but even with this treatment only about 1 in 10,000 bacterial cells takes up a plasmid in its environment. So how can the bacteria that have taken up the recombinant plasmid be identified? Recall that an important component of a recombinant plasmid is a gene for antibiotic resistance. If you place bacterial cells in the presence of the antibiotic, only those cells that have the recombinant plasmid will grow.

DID YOU KNOW?

Natural Uptake of Plasmids

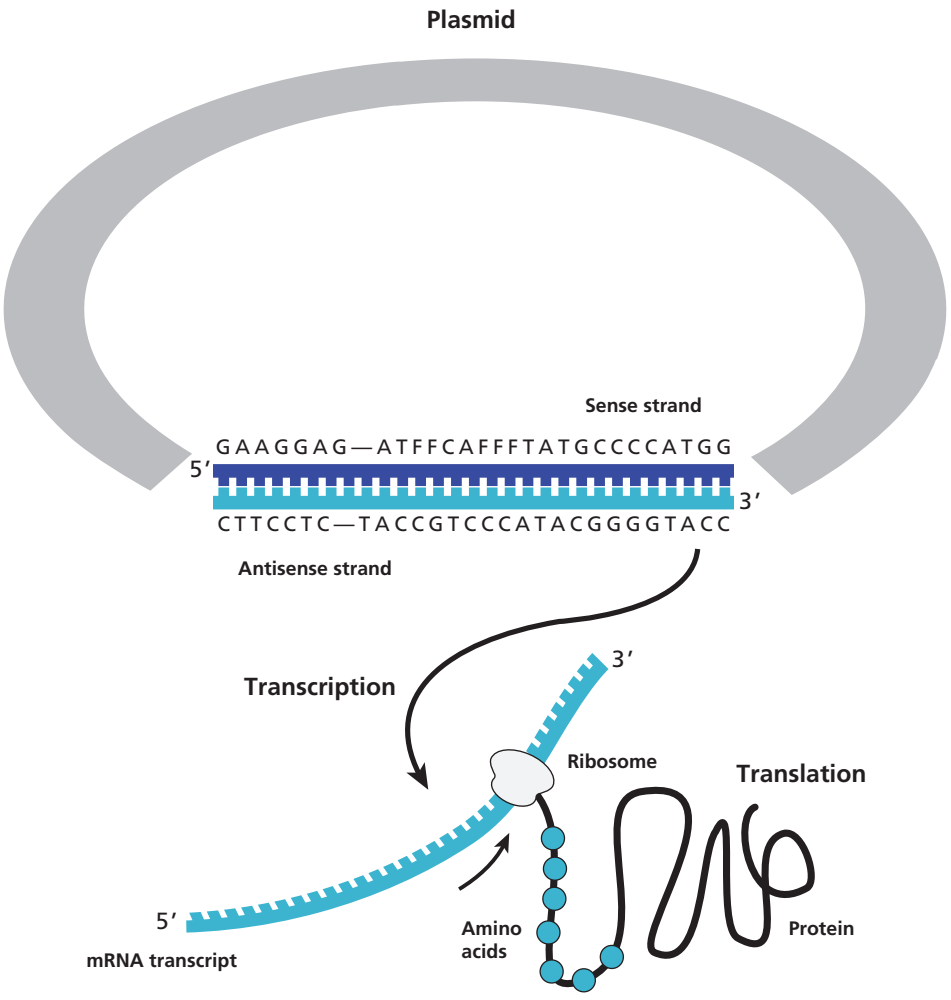
Some strains of bacteria naturally exchange plasmids, and those plasmids can provide a gene that gives a selective advantage, such as antibiotic resistance, to the cell. One mechanism for efficient transfer of DNA between bacterial species is *bacterial conjugation*, in which a plasmid is shared between two bacterial cells that are in contact. The other mechanism is transformation, in which bacteria take up DNA, including plasmids, directly from the external environment. In nature, the usual source of this DNA is cells that have died and released their contents into the environment.

While plasmids can be advantageous for bacteria cells, they are not always advantageous for people. For example, vancomycin was the only effective antibiotic against *Staphylococcus aureus* infections. Resistance to vancomycin is associated with the *VanA* gene, which is carried on a plasmid in other species of bacteria (such as the *Enterococcus faecium* [BM4147] strain). Exchange of this plasmid made vancomycin less effective against staph infections.

FROM PLASMID DNA TO PROTEIN

Once a recombinant plasmid has entered the bacterial cell, DNA polymerase initiates replication at the *ori* site, and the plasmid replicates using the bacterial DNA replication enzymes. These multiple copies of plasmids can now produce the protein of interest, such as insulin, in quantity. In this process, the information encoded in the human DNA is transferred from DNA to protein using the transcription and translation machinery of the cell (see **Figure 5.2**). The protein then alters the observable traits of the organism.

Figure 5.2: Gene expression from a plasmid in the bacterial cell



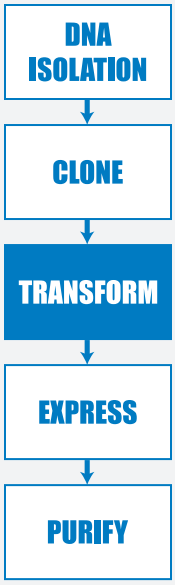
Genetic engineering is only possible because genes from different organisms can be expressed in bacteria. On Earth, all life is related, and the way that information is encoded in DNA is universal. As you may already know, proteins are made up of smaller subunits called *amino acids*, and a sequence of three nucleotides in DNA code for a single amino acid. These three-nucleotide sequences are called *codons*. For example, the codon TTG codes for the amino acid tryptophan, whereas the codon AAG codes for the amino acid lysine. In many cases, more than one codon can encode the same amino acid. For example, AAA is also a codon for lysine. In addition, there are informational codons, such as the *start codon* (ATG) and the *stop codon* (TTA), which show where in the DNA sequence the code for the protein begins and ends.

DID YOU KNOW?

Making DNA from RNA

Even though the DNA code is the same in all life forms, the transcription and translation of genes in *eukaryotes* and *prokaryotes* use different enzymes and structures. (Human cells are eukaryotes, and bacterial cells are prokaryotes.) One important difference between these two kinds of cells is that the genes in eukaryotes contain noncoding sequences called *introns*. The RNA polymerase transcribes the gene, producing a large precursor messenger RNA containing both introns and *exons*, which are the coding sequences. The precursor RNA is then *spliced*, which removes the introns and joins the exons into the mature messenger RNA.

Prokaryotes are unable to carry out the splicing of the introns. To solve this problem, scientists use an enzyme, *reverse transcriptase*, which can copy RNA into DNA, to make complementary DNA (cDNA) from the messenger RNA for a particular protein. The cDNA, which has only the exon sequences, is then inserted into the plasmid vector. The cloned human insulin gene used to make insulin is prepared in this way.



LABORATORY 5: TRANSFORMING BACTERIA WITH THE LIGATION PRODUCTS

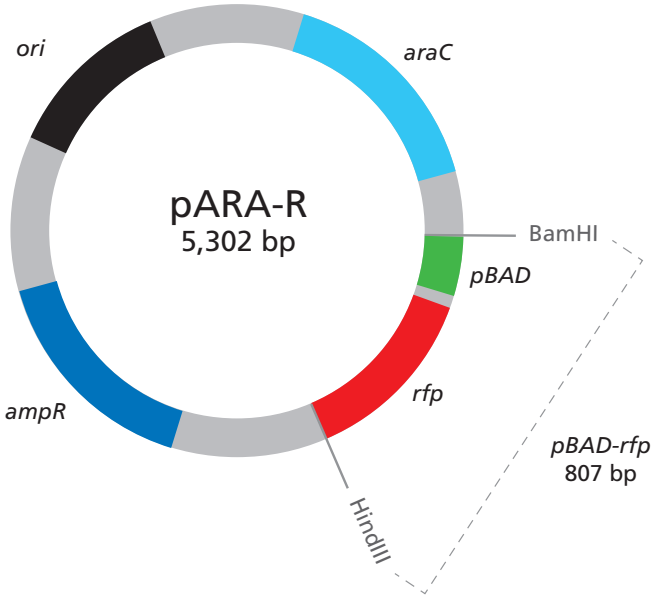
So far in your quest to clone a gene you have produced recombinant plasmids and verified that you made the pARA-R plasmid containing the *rfp* gene that can make the *red fluorescent protein*. In this laboratory you will carry out another step of the gene cloning process, which is to transform *E. coli* bacteria with this plasmid. Using *E. coli* bacteria that have been pretreated with calcium chloride, you will divide the bacteria into two groups: a control group to which no plasmid is added, and a treatment group to which you add the ligation products. After heat-shocking both groups of cells, you will grow them under several different conditions:

- The control group is grown in the presence of Luria Broth (a *medium* that supports bacterial growth).
- The control group is grown in the presence of Luria Broth and the antibiotic ampicillin.
- The treatment group is grown in the presence of Luria Broth.
- The treatment group is grown in the presence of Luria Broth and the antibiotic ampicillin.
- The treatment group is grown in the presence of Luria Broth, ampicillin, and the sugar arabinose.

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 that you created in *Laboratory 3*. How will you know if you are successful? The bacteria will have a new and highly visible trait: It will now produce red fluorescent protein, which makes the cells red or bright pink!

The pARA-R plasmid, which you reviewed in Chapter 3, is shown again in **Figure 5.3**.

Figure 5.3: The pARA-R plasmid



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. (Biotechnologists call these genes *selectable markers* because only bacteria having the gene will survive in the presence of 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.

HANDOUTS

- **Bacterial Growth Predictions (RM 5)**

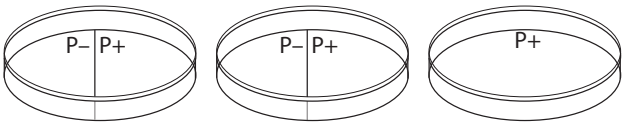
BEFORE THE LAB

Discuss the following questions with your group, and be prepared to share your answers 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 Luria Broth (LB), ampicillin, and the sugar arabinose. The plates will be arranged as follows:



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 on pages 93 through 97 and briefly outline the steps, using words and a flowchart.

SAFETY: All appropriate safety precautions and attire required for a science laboratory should be used. Please refer to your teacher's instructions.

SAFETY: Use caution when handling *E. coli* bacteria and use *aseptic technique*.

Aseptic technique is a set of procedures that ensure protection of the lab worker and protection of a bacterial sample, which is necessary for the experiment to be successful. Specifically:

- **Do not touch anything that has been or will be in contact with *E. coli* bacteria. Students handling equipment that comes into contact with bacteria should wear gloves.**
- **Try to avoid spills or contamination of surfaces with anything that has been in contact with *E. coli* bacteria. Immediately inform your teacher if a spill or contamination occurs.**
- **When you have finished using microfuge tubes, pipette tips, and cell spreaders, place them immediately into the biohazard bag or waste container, as directed by your teacher.**
- **When directed to do so, place your Petri plates back into the original sleeves and in the biohazard bag.**
- **Wash your hands well with soap after completing the lab.**

MATERIALS

Reagents

- A rack with the following:
  - Microfuge tube of ligated plasmid from *Laboratory 3* (LIG)
  - Microfuge tube of Luria Broth (LB)

- Microfuge tube of 100 µL of chilled competent *E. coli* cells (CC)

  Note: The CC tube must be kept on ice at all times.

- 3 Petri plates with agar:
  - 1 of LB
  - 1 of LB/amp
  - 1 of LB/amp/ara

Equipment and Supplies

- Styrofoam cup of crushed ice

  Note: Fill a cup with some of the crushed ice from the container holding the CC tubes before taking a CC tube. You'll need to keep the CC tube on ice at all times.

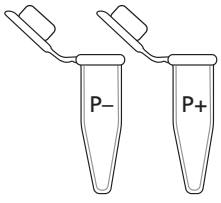
- 2 1.5-mL microfuge tubes
- Permanent marker
- Disposable gloves
- P-20 micropipette
- P-200 micropipette
- Tip box of disposable pipette tips
- Pack of cell spreaders (will be shared among groups)
- 42°C water bath with floating microfuge tube rack (will be shared among all groups)
- Timer or clock (will be shared among all groups)
- Tape (will be shared among all groups)
- 37°C incubator (will be shared among all groups)
- Biohazard bag for materials that come into contact with *E. coli* cells (will be shared among groups)
- Waste container (will be shared among groups)

METHODS

1. Check your rack to make sure that you have the reagents listed. Check that the LIG tube is labeled with your group number and class period.

2. Obtain a CC tube from the ice-filled container, placing it in a Styrofoam cup of ice.

   LAB TECHNIQUE: The competent cells in this lab must be kept cold—be sure to pick up microfuge tubes by the upper rim to avoid warming the cells.



3. Label two clean microfuge tubes "P–" and "P+."

4. Place the P– and P+ tubes in the Styrofoam cup of ice with the CC tube.

LAB TECHNIQUE: Bacterial transformation requires sterile techniques. It is essential that these directions be followed precisely.

5. Using the large P-200 micropipette, add the competent cells from the CC tube to the P– and P+ tubes:

   a. Set the P-200 micropipette to 50 µL.
   b. Very carefully, re-suspend the bacterial cells in the CC tube by gently pumping the pipette two times in the solution.
   c. Add 50 µL of CC to each of the empty chilled tubes (P– and P+), holding each tube at its rim to keep it cold, and return each tube quickly to the ice.

LAB TECHNIQUE: To avoid contamination, be sure to use a new micropipette tip for each addition.

6. Using the P-20 pipette, add LIG to the tube labeled "P+":

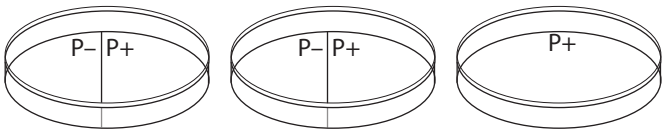
   a. Set the P-20 micropipette to 10.0 µL.
   b. Hold the chilled P+ tube by the upper rim and add 10.0 uL of LIG. Mix the solutions by pumping the pipette two times in the liquids, and return the P+ tube to the ice.

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

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

8. 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 number and class period. 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 follows:



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

9. Following the 15-minute incubation on ice, carry the P– and P+ tubes (in the cup of ice) to the 42°C water bath. Place the two tubes in the floating microfuge tube rack in the 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 a minute.

11. Using the large P-200 micropipette, add LB to the P– and P+ tubes:

    a. Set the P-200 micropipette to 150 µL.
    b. Add 150 µL of LB to the P– tube. Cap the tube and gently flick it two or three times to mix.

    LAB TECHNIQUE: To avoid contamination, be sure to use a new micropipette tip for each solution.

    c. Add 150 µL of LB to the P+ tube. Cap the tube and gently flick it two or three times to mix.

12. If time permits, allow the cells in the P– and P+ tubes to incubate at room temperature for 15 minutes.

STOP AND THINK:

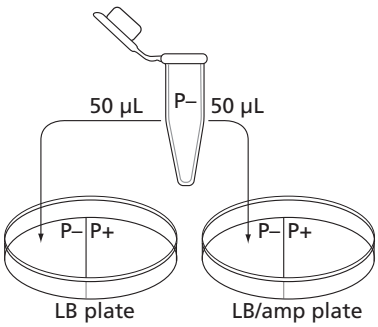
- **How is the P+ bacteria culture treated differently from the P– bacteria culture? (A *culture* is an isolated population of cells.) What is the purpose of the P– bacteria culture?**
- **Why do the cells need time to recover after the heat shock?**
- **Why are the cells incubated at 37°C?**
- **You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

13. Add cells from the P– tube onto your LB and LB/amp plates:

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

    LAB TECHNIQUE: To avoid contamination, be sure to use a new micropipette tip for each solution.

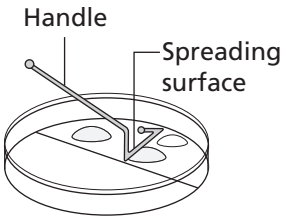
    b. Gently pump the pipette two or three times in the P– tube to suspend the cells, and load 50 uL of the P- cells.
    c. Open the lid of the LB plate, like a "clamshell," and add 50 µL of cells from the P– tube to the section marked "P–." Close the lid.
    d. Again, gently pump the pipette two or three times in the P– tube to suspend the cells, and load 50 uL of the P– cells.



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

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

a. Open the package of sterile cell spreaders at the end closest to the spreader handles. Remove only one spreader, and close the package to keep the others sterile.



b. Open the lid to the LB plate, like a clamshell, and spread the cells evenly across the entire P– side of the plate by gently moving the spreader across the agar surface. (Keep the cells on the P– side of the plate.) Close the lid.

c. Carefully spread the P– cells on the LB/amp plate, using the same spreader and technique.

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.

15. Add cells from the P+ tube to your LB, LB/amp, and LB/amp/ara plates:

a. Make sure that the P-200 micropipette is set to 50 µL.

LAB TECHNIQUE: To avoid contamination, be sure to use a new micropipette tip for each solution.

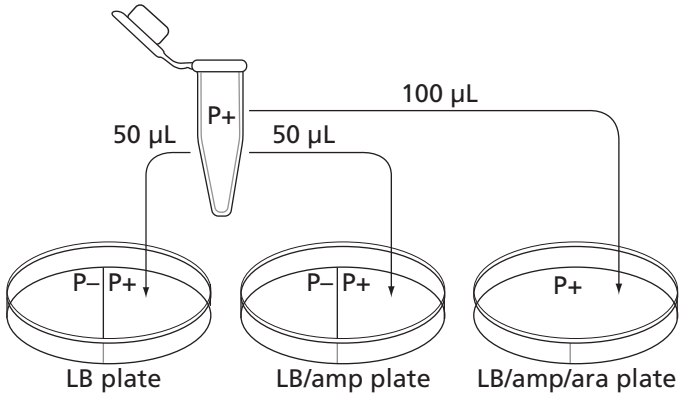
b. Gently pump the pipette two or three times in the P+ tube to suspend the cells, and load 50 uL of the P+ cells.

c. Open the lid of the LB plate, like a clamshell, and add 50.0 µL of cells from the P+ tube to the section marked "P+." Close the lid.

d. Again, gently pump the pipette two or three times in the P+ tube to suspend the cells, and load 50 uL of the P+ cells.

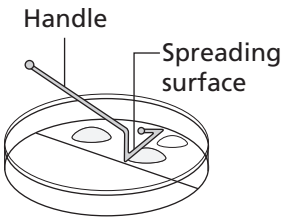
e. Open the lid of the LB/amp plate, like a clamshell, and add 50.0 µL of cells from the P+ tube to the section marked "P+." Close the lid.

f. Set the P-200 micropipette to 100 µL, gently pump the pipette two or three times in the P+ tube, and load 100 µL of the P+ cells.



g. Open the lid of the LB/amp/ara plate, like a clamshell, and add 100.0 µL of P+ cells to various areas across the surface—not just a single spot. Close the lid.

16. Spread the cells from the P+ tube on your LB, LB/amp, and LB/amp/ara plates:



a. Open the package of sterile cell spreaders at the end closest to the spreader handles. Remove only one spreader, and close the package to keep the others sterile.
b. Open the lid to the LB plate, like a clamshell, and evenly spread the cells on the P+ side of the plate (and only on this side) by gently moving the spreader across the agar surface. Close the lid.
c. Carefully spread the P+ cells on the LB/amp plate using the same spreader and technique.
d. Carefully spread the P+ cells on the LB/amp/ara plate using the same spreader. Then gently rotate the plate beneath the P+ spreader so that the cells can be spread over the entire surface of this plate. Close the lid.

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.

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

18. Using provided tape, tape all three plates together and label tape with your group number and class period.

19. Place the plates in the 37°C incubator upside down to prevent condensation from dripping onto the gels.

20. Place all microfuge tubes, pipette tips, and cell spreaders in the biohazard bag.

21. Incubate the plates for 24–36 hours at 37°C.

22. Examine the plates and in your notebook record the amount of growth on each half.

23. Discard the Petri plates in the biohazard bag when directed to do so.

CHAPTER 5 QUESTIONS

1. Look at the results of your transformation. Do your actual results match your predicted results? If not, what differences do you see, and what are some explanations for these differences?
2. How many red colonies were present on your LB/amp/ara plate?
3. Why did the red colonies only appear on the LB/amp/ara plate and not the LB/amp plate?
4. Recombinant plasmids are engineered so that they can replicate in the cell independently of the chromosome replication. Why is it important to have multiple copies of a recombinant plasmid within a cell?
5. How is the information encoded in the *rfp* gene expressed as a trait? Be sure to use what you have previously learned about gene expression and the relationship between DNA, RNA, protein, and traits.
6. Why is it possible for bacteria to make a human protein, such as insulin, or a sea anemone protein, such as the red fluorescent protein?
7. The only bacteria that could produce the red fluorescent protein in *Laboratory 5* were bacteria that were transformed with the pARA-R plasmid. Why?

DID YOU KNOW?

Making the Connection Between Genes and Proteins

How were scientists able to show that a gene codes for a protein? In 1941, George Beadle and Edward Tatum carried out an experiment in which they exposed bread mold to UV irradiation, a procedure known to cause *mutations* (changes) in genes. Beadle and Tatum created mutant strains of molds that had lost the ability to synthesize a necessary vitamin. By feeding the precursors of the vitamin one at a time to the mutants, Beadle and Tatum were able to determine that the mutants only lacked a single enzyme catalyzing one reaction.

Beadle and Tatum then investigated whether a single gene caused the loss of the single enzyme by genetic crosses between the mutants and a wild-type strain. After culturing the progeny, they found that half had the same defect as the parent mutant strain and half did not, confirming that a single gene had been mutated. From these results, Beadle and Tatum proposed that genes were responsible for coding the proteins of an organism and that a change in a gene could result in the production of a defective protein, which in turn could affect the traits of that organism. In 1958, Beadle and Tatum received the Nobel Prize for this work.

CHAPTER 5 GLOSSARY

Amino acid: The building block of proteins. There are 20 amino acids. Each amino acid is an organic substance that has two groups attached to it—an amino group (NH_2) and a carboxylic acid group (COOH).

Aseptic technique: A set of procedures and carefully controlled conditions to prevent contamination by pathogens.

Bacterial conjugation: A process by which two bacterial cells join and transfer genetic material from one to another.

Codon: A group of three mRNA bases that encode a single amino acid.

Competent: A cell that has the ability to be transformed genetically by taking up DNA from the environment.

Culture: An isolated population of cells that have been grown in a specially prepared nutrient medium.

Eukaryote: An organism that shelters its genes inside a nucleus and has several linear chromosomes.

Exon: The segment of a gene that codes for a protein. Exons are both transcribed and translated.

Expressed: When information encoded in a gene has been converted first into messenger RNA and then to a protein. This process is called expression.

Intron: The segment of a gene that does not code for a protein. Introns are transcribed into mRNA but are removed before the exons (the rest of the gene) are translated into a protein.

Medium: A solution that contains substances that support the growth of microorganisms. The medium may be solidified by the addition of agar. Luria Broth is an example of a medium.

Mutation: Change or damage occurring in a section of DNA that alters the products or processes associated with that section.

Prokaryote: Cell or organism with a single chromosome and no nuclear membrane; bacteria are prokaryotes.

Promoter: A specific DNA sequence that binds RNA polymerase and initiates transcription of the gene.

Red fluorescent protein: The protein encoded by the *rfp* gene. Mutant fluorescent protein is a molecule that is about 238 amino acids in size. When it is expressed in bacterial cells, the cells appear red or bright pink.

Reverse transcriptase: An enzyme that catalyzes the formation of DNA from an RNA template in reverse transcription.

Selectable marker: A gene on a plasmid that is introduced into a cell along with a gene of interest that is being cloned. Selectable markers allow scientists to tell if the plasmid has been taken in by the cell because the marker can be seen or detected. A common selectable marker is an antibiotic resistance gene—only bacteria that have the gene will survive the antibiotic.

Splice: To modify messenger RNA for translation by removing introns and joining exons.

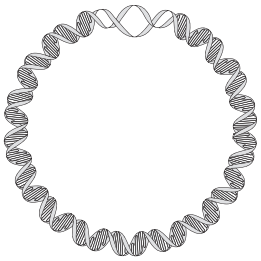
Start codon: The first codon of mRNA translated by a ribosome; typically AUG or GUG.

Stop codon: A nucleotide triplet within mRNA that signals a termination of translation.

Trait: A genetically determined characteristic. DNA codes for proteins, which determine traits.

Transformation: A process that places foreign DNA, such as a plasmid, into a cell.

Translation: The process by which information encoded in messenger RNA is decoded and transformed into protein.



CHAPTER 6

GETTING WHAT WE 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.

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 use a column that separates proteins based on their solubility in water to obtain the red fluorescent protein made by the cloned *rfp* gene.

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* 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.

4. A protein is a long linear molecule when it is made, but it immediately folds into a specific three-dimensional conformation. What properties of the *amino acids* in a protein control the folding process?

BACTERIA MULTIPLICATION AND PROTEIN PURIFICATION

Some bacteria can make you very sick. That's why in both the health care industry and food preparation, people try to prevent bacteria from growing. But when scientists use bacteria as hosts for recombinant plasmids, their goal is to encourage bacteria to grow and to produce the protein of interest.

BACTERIAL GROWTH

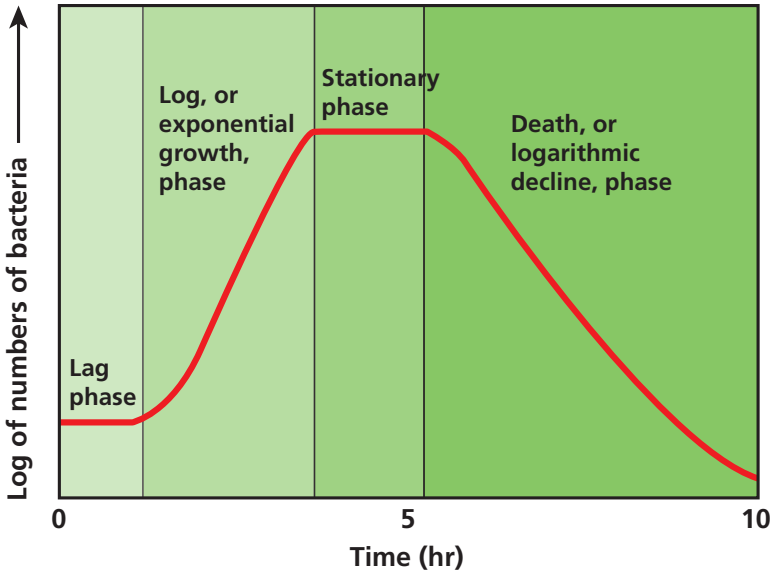
What factors affect bacterial growth? The last step in Chapter 5 was to place the bacteria that were transformed with the pARA-R plasmid in a *suspension culture* in a shaker flask. The cells were suspended in a nutrient broth, and the shaking mixed air into the suspension and prevented the bacteria from settling out of solution, providing conditions favorable to growth.

CONSIDER: Why might the shaker flask be better at supporting bacterial cell growth than a plate?

Under optimal conditions, such as those provided by the shaker flask, the growth of a bacterial population is predictable (see **Figure 6.1**). The growth occurs in four distinct phases:

- In the *lag phase*, there is a zero growth rate. There are no new cells and no cells dying. The cells adjust to the new conditions, grow larger, and prepare for cell division.
- In the *log phase*, there is a logarithmic growth rate. The number of new cells is greater than the number of cells dying. The cells undergo asexual cell division and double in number about every 20 minutes (which is the average doubling time for *E. coli*; other bacteria have different doubling times). This phase occurs as long as necessary resources such as food and oxygen are unlimited and there are no unusual factors that cause cell death.
- In the *stationary phase*, there is a zero growth rate. The number of new cells equals the number of cells dying. This phase occurs once resources such as food and oxygen become limited.
- In the *death phase*, there is a negative growth rate. The number of new cells is less than the number of cells dying. This phase occurs when resources are depleted and when toxic waste products build up.

Figure 6.1: Change in bacterial growth over time in the shaker flask



CONSIDER: If the gene of interest is controlled by an operon, such as the arabinose operon, when is the best time to turn on the gene? Keep in mind:

- **Production of the protein takes energy away from the processes of cell growth and cell division**
- **A greater number of cells will produce more protein**
- **Proteins can degrade over time**

PROTEIN PURIFICATION

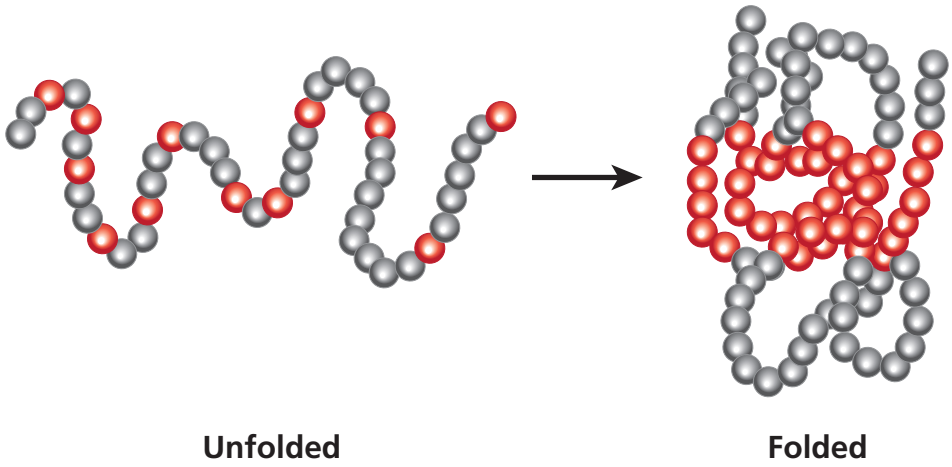
When the transformed bacteria are allowed to grow and multiply numerous times, they can produce enough therapeutic protein to meet the treatment needs of patients. However, the therapeutic protein must be purified, which requires separating it from the other contents of the cell, including other proteins. (A typical bacterium may contain more than 1,000 different kinds of protein.) Column chromatography is one method used to separate the proteins.

What physical characteristics of proteins enable them to be separated by column chromatography? Although all proteins are made up of amino acids, each kind of protein has a specific function and a specific conformation (shape). The conformation relates to function because the outside surface of a protein has specific sites that bind to other molecules. These *binding sites* allow proteins to attach to other molecules, which is how proteins can catalyze reactions, transport molecules, provide a signal, and form structures.

When a protein is first synthesized, it is a long flexible chain of amino acids, but it immediately attains its three-dimensional conformation by a process called protein folding (see **Figure 6.2**). Protein folding is dependent on the following properties of the amino acids in the protein:

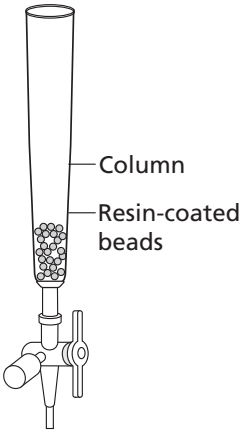
- Formation of weak noncovalent bonds between positively charged and negatively charged side chains of amino acids
- The tendency for *hydrophobic* (water-insoluble) amino acids to be buried on the inside of the protein away from water and for *hydrophilic* (water-soluble) amino acids to be found on the outside of the protein exposed to water
- Formation of covalent bonds, called disulfide bridges, that occur between sulfur-containing amino acids

Figure 6.2: Protein folding



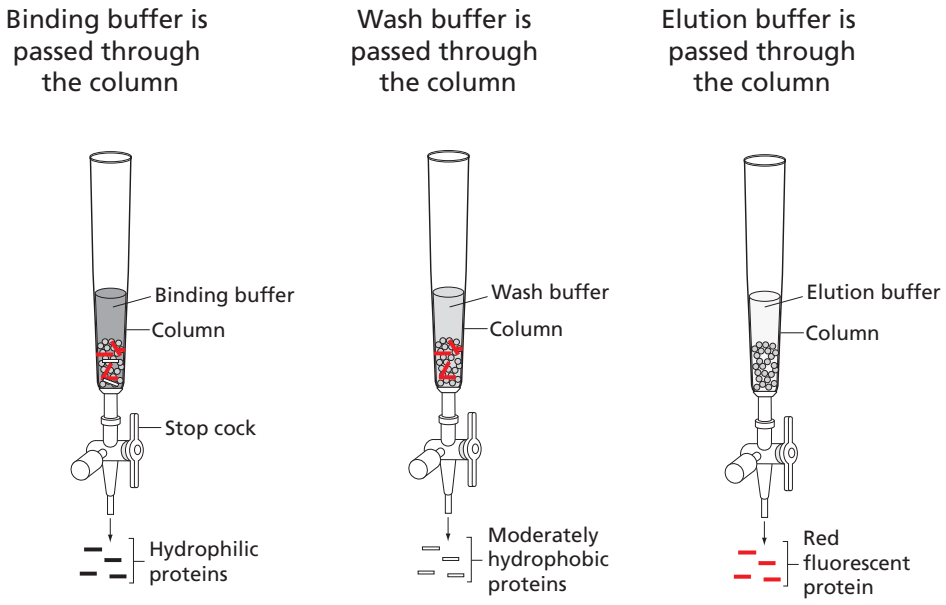
CONSIDER: If a mutation changes an amino acid, how might this change affect protein folding and protein function?

Specific proteins are either hydrophobic or hydrophilic, depending on the relative amount of hydrophobic and hydrophilic amino acids they contain. Hydrophobic proteins and hydrophilic proteins can be separated by column chromatography. In this method, a column is packed with small beads that are coated with a material (a *resin*) that attracts hydrophobic amino acids, and the mixture of proteins is dissolved and passed over the beads. In order for the hydrophobic amino acids to stick to the resin, the proteins must be unfolded to expose the hydrophobic amino acids, which tend to be found on the inside of the protein. Certain salt solutions called *buffers* will unfold proteins to varying degrees.



A series of buffers of different concentrations of salt can be used to separate many proteins from each other. **Figure 6.3** shows how three buffers are used to separate the highly hydrophobic protein red fluorescent protein in a column. A binding buffer is used to unfold all the proteins so that hydrophobic proteins stick to the resin and hydrophilic proteins pass through the column. A wash buffer is poured into the column to release moderately hydrophobic proteins from the resin, and then an *elution* buffer is poured into the column to release red fluorescent protein from the resin. Both the wash and the elution buffers have a lower salt concentration than the binding buffer and thus cause bound proteins to fold and begin to cover their hydrophobic amino acids. Depending on the extent of folding, the proteins are released from the beads.

Figure 6.3: Separation of red fluorescent protein by hydrophobicity using column chromatography



CONSIDER: If you were trying to use column chromatography to separate insulin from a mixture of proteins, would you use the same binding, wash, and elution buffers used for the red fluorescent protein, or would you use buffers with different salt concentrations? Explain the reasoning for your answer.

DID YOU KNOW?

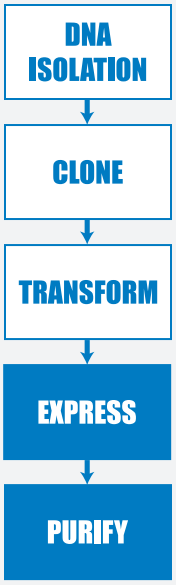
Recombinant Proteins

As you have learned, when recombinant proteins are produced for use as human therapeutics, host cells must be grown in large quantities so that enough recombinant protein is produced to meet treatment demand (the needs of patients). The recombinant protein is isolated, purified, and analyzed for activity and quality before it goes to market.

Producing a protein with the proper order of amino acids isn't always the whole story, however. Sometimes further processing or modification is required to yield an active or fully functioning protein. Many human proteins are glycosylated, meaning that they have a particular pattern of sugar molecules linked to them. If a protein is translated but not correctly glycosylated, it may not function properly. Another modification involves the addition of a phosphate group—a process known as phosphorylation. Phosphorylation of a protein can act as a kind of switch, allowing the protein to become more or less active by uncovering or covering its binding sites. In addition to saccharide (sugar) and phosphate groups, other chemical groups may be added to a protein in order to change its function.

Recombinant proteins for therapeutic use include *vaccines*, *hormones*, *monoclonal antibodies*, and *hematopoietic growth factors* for the treatment of diseases including cancer, AIDS, allergies, and asthma. The number of recombinant proteins has increased greatly in recent years as the technology used for their production and purification has advanced.

LABORATORY 6: PURIFYING THE FLUORESCENT PROTEIN



In the previous chapter 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. Near the end of the log phase of bacterial growth, 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, or break open, the cells. In the second part of this laboratory, you will use column chromatography to purify the protein.

BEFORE THE LAB

Discuss the following questions with your group and be prepared to share your answers with the class.

1. How can solutions of different salt concentrations, which will unfold proteins to varying degrees, be used to help purify red fluorescent protein using column chromatography?

2. Read through the Methods sections for *Part A* (on pages 110 and 111) and for *Part B* (on pages 112 through 114) and briefly outline the steps, using words and a flowchart.

SAFETY: All appropriate safety precautions and attire required for a science laboratory should be used. Please refer to your teacher's instructions.

SAFETY: Use caution when handling *E. coli* bacteria and use aseptic technique. Aseptic technique is a set of procedures that ensure protection of the lab worker and protection of the bacterial sample, which is necessary for the experiment to be successful. Specifically:

- **Do not touch anything that has been or will be in contact with *E. coli* bacteria. Hold microfuge tubes and Petri plates on the outside, hold only the handles of cell spreaders, and do not handle pipette tips at all.**
- **Try to avoid spills or contamination of surfaces with anything that has been in contact with *E. coli* bacteria. Immediately inform your teacher if a spill or contamination occurs.**

- **When you have finished using microfuge tubes, pipette tips, and cell spreaders, place them immediately into the biohazard bag.**
- **When directed to do so, place your Petri plates in the biohazard bag.**
- **Wash your hands well with soap after completing the lab.**

PART A: LYSE CELLS GROWN IN THE SHAKER

MATERIALS

Reagents

- Microfuge tube rack with the following:
  - Microfuge tube of the LB/amp/ara culture of *E. coli* (EC)
  - Microfuge tube of elution buffer (EB)
  - Microfuge tube of lysis buffer (LyB)
- Additional 1,000 µL (1 mL) of the LB/amp/ara culture of *E. coli* (obtain from your teacher in step 6)

Equipment and Supplies

- Microcentrifuge (will be shared among all groups)
- Liquid waste container
- P-200 micropipette
- Tip box of disposable pipette tips
- Permanent marker
- Waste container (will be shared among groups)
- Biohazard bag for materials that come into contact with *E. coli* cells (will be shared among groups)

METHODS

1. Check your rack to make sure that you have the reagents listed for *Part A*.
2. Examine the EC tube and record its color in your notebook.
3. Before you can lyse the cells, you will need to separate the cells from the growth *medium*. To do this, spin the EC tube in the microcentrifuge for five minutes.

   LAB TECHNIQUE: **Distribute the tubes evenly in the microcentrifuge so that their weight is balanced, making sure that two tubes of the same volume are directly opposite one another.**

STOP AND THINK: How can you determine where the red fluorescent protein is in each separation step?

4. Carefully remove the EC tube from the microcentrifuge to avoid disturbing the solid pellet that contains the bacterial cells.
5. Set the P-200 micropipette to 200 µL, place a new tip on the micropipette, and carefully remove the supernatant (liquid) from the EC tube without disturbing the cell pellet. (You can dispense the supernatant into the liquid waste container.)
6. Bring the EC tube to your teacher, who will add 1,000 µL (1 mL) of the LB/amp/ara culture of *E. coli* to your EC tube.
7. Repeat steps 3–5 (spin the tube for 5 minutes and remove the liquid).

STOP AND THINK: What color is the supernatant? The pellet? What are the contents of each?

8. Invert the EC tube containing the cell pellet, and use the micropipette to remove as much of the liquid as you can without touching the cell pellet.

LAB TECHNIQUE: Be sure to use a new micropipette tip for each reagent to avoid contamination.

9. Using the P-200 pipette, add 150 µL of EB to the cell pellet in the EC tube.
10. 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. Examine the EC tube carefully. If there are visible clumps of cells, again drag the tube vigorously across the surface of the microfuge tube rack.
11. Using the P-200 pipette, add 150 µL of LyB to the EC tube. Close the cap of the EC tube tightly and drag the tube vigorously across the surface of the microfuge tube rack two times to mix.
12. Label the EC tube with your group number and class period and give it to your teacher. Your teacher will incubate the cells at room temperature overnight.
13. Place all microfuge tubes and pipette tips in the biohazard bag.

PART B: SEPARATE THE RED FLUORESCENT PROTEIN WITH COLUMN CHROMATOGRAPHY

MATERIALS

Reagents

- Microfuge tube rack with the microfuge tube of lysed cells (EC, from *Part A* of this lab)
- Containers with the following:
    - Binding buffer (BB)
    - Wash buffer (WB)
    - Elution buffer (EB)
    - Column equilibration buffer (CEB)

Equipment and Supplies

- 2 1.5-mL microfuge tubes
- Liquid waste container
- P-1,000 micropipette
- Tip box of disposable pipette tips
- Chromatography column
- Microcentrifuge (will be shared among all groups)
- Waste container (will be shared among groups)

METHODS

1. Assign tasks in your group. Have one person make sure that your materials are ready (steps 2–3), another person set up the chromatography column (steps 4–5), and another person spin the lysed cells and remove the supernatant (step 6–7).
2. Check to make sure that you have all the reagents listed.
3. Label two clean microfuge tubes "SUPER" and "RFP."
4. 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.

LAB TECHNIQUE: Do not allow the column to run dry.

5. 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.

6. Spin the EC tube in the microcentrifuge for five minutes to create a pellet of the cell debris.

LAB TECHNIQUE: Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

STOP AND THINK: Three buffers you will use in this lab are the binding buffer (BB), the wash buffer (WB), and the elution buffer (EB). What is the function of each?

7. Examine the tube. You should see a supernatant and a solid pellet.

STOP AND THINK: What color is the supernatant? The pellet? What are the contents of each?

LAB TECHNIQUE: Be sure to use a new micropipette tip for each reagent to avoid contamination.

8. Using the P-1,000 pipette, 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 debris pellet, you will have to centrifuge the tube again.

9. Using a new tip, add 200 µL of BB to the SUPER tube. Mix by gently pumping the solution in and out.

10. Using the same tip but changing the volume, add 400 µL of the SUPER tube mixture to the chromatography column. Carefully dispense the solution down the side of the column to avoid disturbing the surface of the resin bed.

11. 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.

12. 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 in your notebook.

13. Using a new tip, add 1,000 µL (1 mL) of WB gently down the side of the chromatography column.

14. 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.

15. Examine the column and locate the red fluorescent protein. Has the location of the red fluorescent protein changed in the resin bed?

16. Using a new tip, add 1,000 µL of EB twice (2 mL total), gently, down the side of the chromatography column.

17. Set the RFP tube under the stopcock. Open the valve and allow the part of the eluate that is red to drain into the RFP tube. Close the valve and cap the tube when done.

18. 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.

19. Using a new tip, add 1,000 µL of CEB twice (2 mL total) to the chromatography column to prepare it for the next class. Cap the column tightly.

20. Pour the contents of the waste collection container down the sink drain.

21. 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.

CHAPTER 6 QUESTIONS

1. Why is a protein's conformation important for carrying out its function?
2. What properties of the amino acids in a protein relate to protein folding?
3. Does the eluate containing your red fluorescent protein appear less bright or brighter than it did in the cell lysate following centrifugation? If there is a noticeable difference in the intensity of the red color, what might account for that?
4. What characteristic of red fluorescent protein is used as the basis for separation by column chromatography?
5. How might the column chromatography procedure be adjusted or modified to increase the purity of the red fluorescent protein sample?

DID YOU KNOW?

Chimeric Proteins

When you determined which bacteria had taken up the recombinant plasmid that had the *rfp* gene, you were able to see the transformed colony because the bacteria fluoresced red when exposed to light. The ability of a fluorescent protein (FP) to "light up" is a powerful tool that is revolutionizing cell biology and biomedical research. Numerous FPs from different organisms have been isolated and even mutated to provide a toolbox of FP "tags." The FPs can be attached to other molecules to monitor processes that occur inside cells and in the body as a whole.

The function of a protein is directly related to its conformation, which is the result of protein folding. Other important pieces of information that shed light on a protein's role are its distribution, movement, interactions, and association with other proteins. To visualize these aspects of a protein, scientists create a molecule, known as a fusion protein or a chimeric protein, that contains both the protein of interest and an FP fused together. (A chimera is a mythical animal that has parts from different animals.) Scientists can measure *fluorescence* from a single FP, making fusion proteins powerful visualization tools. However, this procedure only works if the FP does not interfere with the protein's function, so tests are run to ensure that a particular protein acts the same with and without the FP tag.

CHAPTER 6 GLOSSARY

Amino acid: The building block of proteins. There are 20 amino acids. Each amino acid is an organic substance that has two groups attached to it—an amino group (NH_2) and a carboxylic acid group (COOH).

Binding site: The area of a biomolecule that binds to a specific substance or part of a substance.

Buffer: A solution that resists changes in pH, that contains either a weak acid and its salt or a weak base and its salt.

Colony: A group of the same kind of organisms living closely together, usually for mutual benefit. Within a bacterial colony, all organisms descend from a single ancestor and are genetically identical (except for mutations and contamination).

Column chromatography: A method of separating substances in which the substances are dissolved in a liquid that is allowed to flow through a glass column filled with small beads. The beads are coated with a material that binds the substances to different degrees.

Conformation: The three-dimensional shape or structure of something.

Death phase: The period of bacterial growth in culture when the bacteria run out of nutrients and die.

Eluate: The solution that washes out (e.g., solutions that drip from a chromatography column).

Elution: The process of extracting a substance that is bound to another by washing it with a solution.

Fluorescence: The production of light by a molecule (e.g., red fluorescent protein will release red light when exposed to ultraviolet light).

Hematopoietic growth factor: A group of proteins that promotes the growth, differentiation, and activity of blood cells.

Hormones: Substances that act as chemical messengers in the body.

Hydrophilic: Water loving; dissolves in water; polar. Some examples are sugar and salt.

Hydrophobic: Water fearing; does not dissolve in water; non-polar. Some examples are oil, wax, and red fluorescent protein.

Lag phase: The period of bacterial growth in culture when the bacteria adapt themselves to growth conditions. Individual bacteria are maturing and are not yet able to divide.

Log phase: The period of bacterial growth in culture when the number of bacterial cells doubles in a fixed period of time (also known as the logarithmic or exponential phase).

Lyse: To break open.

Medium: A solution that contains substances that support the growth of microorganisms. The medium may be solidified by the addition of agar. Luria Broth is an example of a medium.

Monoclonal antibody: A type of protein that binds to substances in the body and is made by clones of a cell formed in the laboratory.

Protein folding: The physical process by which a polypeptide folds into its characteristic three-dimensional structure, which is essential to the protein's function.

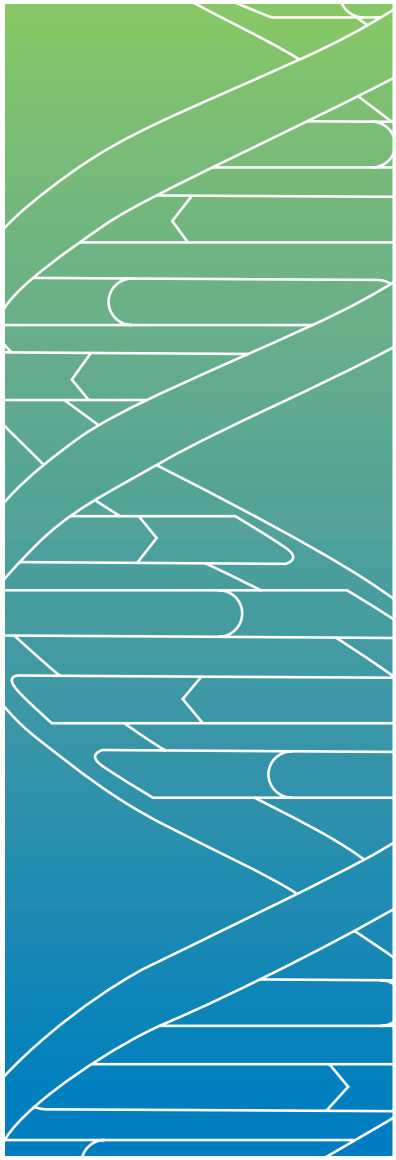
Resin: The material used in a chromatography column to coat the beads.

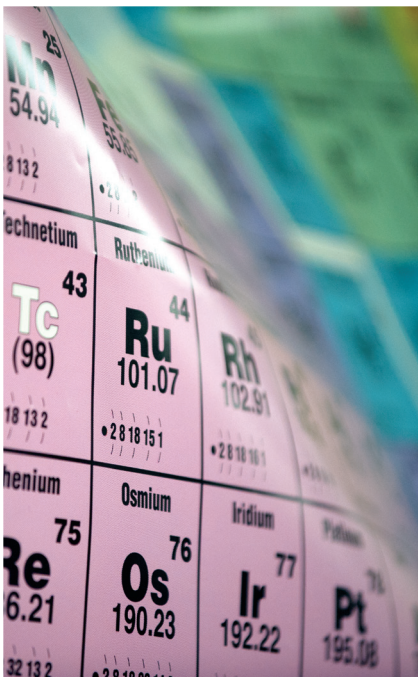
Stationary phase: The period of bacterial growth in culture when the population stays the same because rates of cell division and cell death are equal. This is often due to a growth-limiting factor, such as the depletion of an essential nutrient.

Supernatant: The clear liquid found on top of a solid precipitate after a mixture has been centrifuged.

Suspension culture: A method of growing cells in a liquid growth medium that is moved around by shaking or stirring. The movement mixes air into the suspension and prevents the bacteria from settling out of the solution.

Vaccine: A mixture that causes a body's immune system to make antibodies that will bind to a bacteria or a virus to fight a disease. The mixture contains killed or weakened bacteria or viruses.

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102.91
Osmium
76
Os
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77
Ir
192.22
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