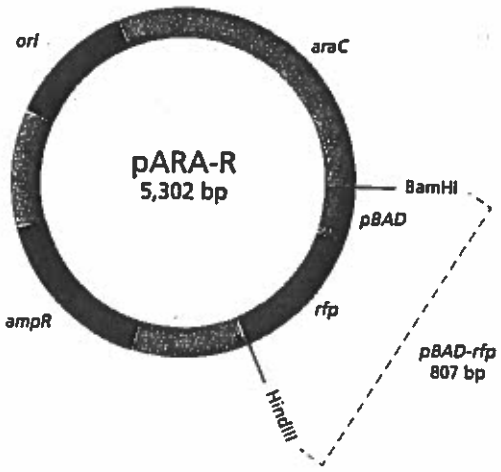
LABORATORY 2A: PREPARING TO VERIFY THE *RFP* GENE: DIGESTING THE pARA-R PLASMID

The purpose of this lab is to ensure that the recombinant plasmid, pARA-R, you have been given is the correct one for making the red fluorescent protein in bacteria. To do this you will use restriction enzymes to cut the plasmid (see **Figure 2A.3**), which will generate DNA fragments of lengths characteristic of the pARA-R plasmid. This procedure is called a *restriction digest* and the lengths of the fragments can be determined by gel electrophoresis (which you may do in Chapter 4A).

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

Figure 2A.3: The pARA-R plasmid



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

In addition to showing the relevant components, Figure 2A.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 recognition sites for these two restriction enzymes. (See Table 2A.1 on page C-7.) Figure P.4 in *What Is Genetic Engineering?* (on page A-12) 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 and in isolating the *rfp* gene. Using two different restriction enzymes has advantages: it allows the inserted gene to be oriented in the correct position for transcribing the "sense" strand of DNA (the strand that codes for the protein), and it prevents the plasmid from reforming a circle without the inserted gene. You'll learn more about this if you do Chapter 4A.

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 2A.3. If pARA-R 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 C-15 and C-16 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 pARA-R (pR)
  - 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 pipette tips
- 2 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 the marker to label two clean microfuge tubes "R+" and "R–." Include your group number and class period on each tube.
3. Review Table 2A.2, which summarizes the reagents that you will add in step 4.

Table 2A.2: Addition of reagents to the R+ and R– tubes

	R+ tube	R– tube
Step 4a: Restriction buffer (2.5xB)	4.0 µL	4.0 µL
Step 4b: pARA-R plasmid (pR)	4.0 µL	4.0 µL
Step 4c: Restriction enzymes (RE)	2.0 µL	
Step 4d: Distilled water (dH_2O)		2.0 µL

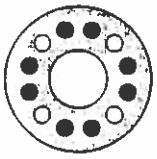
LAB TECHNIQUE: In step 4, be sure to use a new micropipette tip for each reagent to avoid contamination.

4. Add the following:
   a. 4.0 µL of 2.5xB to the R+ and R– tubes.
   b. 4.0 µL of pR to the R+ and R– tubes.
   c. 2.0 µL of RE to the R+ tube. 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 tube when done.
   d. 2.0 µL of dH_2O to the R– tube. Gently pump the solution in and out with the pipette to mix the reagents. Cap the tube when done.

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

5. Spin the two microfuge tubes (R+ and R–) in the microcentrifuge for four 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 both 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 60 minutes. After the incubation is complete, place both tubes in the freezer at –20°C.

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

CHAPTER 2A 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 an ampicillin-resistant gene and bacteria carrying a plasmid with a gene that provides resistance to another antibiotic (kanamycin) were accidentally mixed together. Design an experiment that will allow you to sort out the two kinds of bacteria. (Hint: Make sure that you do not kill off one of the kinds of bacteria you are trying to sort out!)

CHAPTER 2A 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: A biological process that occurs in all living organisms and copies their DNA. 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 template for the production of the complementary strand. Bases are then matched to synthesize the new partner strands.

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.

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 enzymes: Proteins that can cut DNA at very specific places, which are called *recognition sites.*

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.

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.

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

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

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