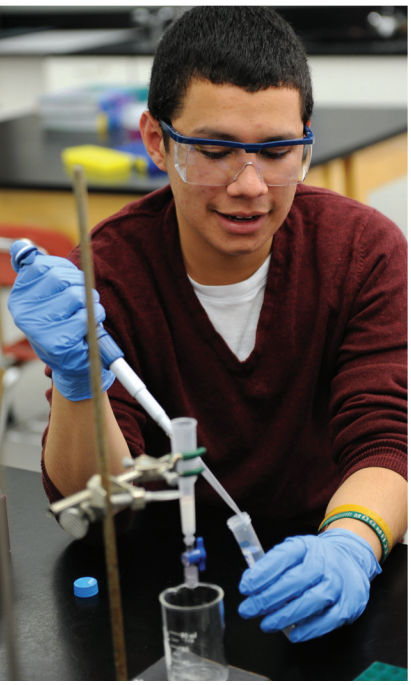
AMGEN® Biotech Experience

Scientific Discovery for the Classroom

Student Guide



Colony PCR

www.amgenbiotechexperience.com

AMGEN® Foundation

TABLE OF CONTENTS

INTRODUCTION

One of the most widely used techniques in biotechnology is ***polymerase chain reaction (PCR)***. PCR is like a molecular copy machine—it is used to make many (often several billion!) copies of genetic material. PCR was first developed in 1983 by Kary Mullis, an American biochemist who later received the Nobel Prize for his work. PCR has had a profound impact on biotechnology and is now used in many areas of research and applied biotechnology, including genetic engineering, ***forensics*** (the use of scientific tests or techniques in crime investigation), and medicine.

In this chapter, you will learn about the multiple uses of PCR and then use PCR to determine if the transformed bacterial colonies carry the gene of interest, *rfp*. This type of PCR is referred to as a colony PCR.

GOALS

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

- Use PCR to detect the presence of a gene of interest
- Describe some of the applications of PCR
- Explain the role of enzymes and DNA primers in PCR

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 gene cloning and PCR, and what questions you may have.

1. What might be some reasons to make many copies of a gene?
2. Under what circumstances might it be important to copy DNA quickly using PCR?
3. In what situations might you want to know if an organism carries a particular gene or DNA sequence?

WHAT IS PCR?

Gene cloning can be carried out ***in vivo*** (inside a living organism) by adding genes to recombinant plasmids and ensuring that they are replicated inside bacterial cells. This is what happens during bacterial transformation: a plasmid with a gene of interest is added to a bacterial cell, and the bacteria begin replicating that gene of interest. Gene cloning can also be carried out ***in vitro*** (outside of a living organism—for example, in a test tube) by using PCR.

In vitro and *in vivo* gene cloning are currently used for very different reasons. *In vivo* gene cloning allows the production of the ***gene product***—the protein that results from the expression of a gene, such as red fluorescent protein or a human therapeutic protein such as insulin. On the other hand, *in vitro* gene cloning produces many copies of a DNA fragment. These fragments are just pieces of genetic material; they will not produce a protein unless they are introduced into a living cell. Recently, scientists have begun developing methods that allow cost-effective cell-free expression of proteins. These methods are called ***IVT*** (in vitro transcription and translation reactions).

PCR copies a specific region of DNA from a sample, then very rapidly produces billions of copies of that specific region of DNA. Scientists sometimes call PCR ***DNA amplification*** because it allows a small amount of DNA to be "heard." Essentially, PCR will duplicate a desired segment of DNA so that there are many copies of it. Those copies will vastly outnumber any other DNA in a sample, making the sequence far easier to detect and analyze. Prior to the development of PCR, the only way to make multiple copies of a specific sequence of DNA was through biological amplification in bacteria, which was very expensive and took a long time. PCR is much less expensive and can be done very quickly.

HOW DOES PCR WORK?

Like other biotechnology methods, PCR is based on basic scientific discoveries. PCR relies on discoveries related to DNA replication. It involves multiple rounds of DNA replication, which leads to the production of over 1 billion copies of a specific portion of DNA.

There are five ingredients required to set up a PCR reaction:

1. DNA, from which you will make copies.
2. ***Primers***, which are short stretches of DNA designed to match the beginning and end of the section of DNA that you want to copy.
3. DNA nucleotide bases (dNTPs) that you will use to build the new copies of DNA.
4. **Taq *polymerase*** *enzyme*, which comes from *Thermus aquaticus*—a type of heat-loving bacteria. *Taq* polymerase is very stable at high temperatures

(unlike other polymerases) and can withstand the heat used to denature the DNA. This enzyme catalyzes the reaction that will build the new DNA strands.

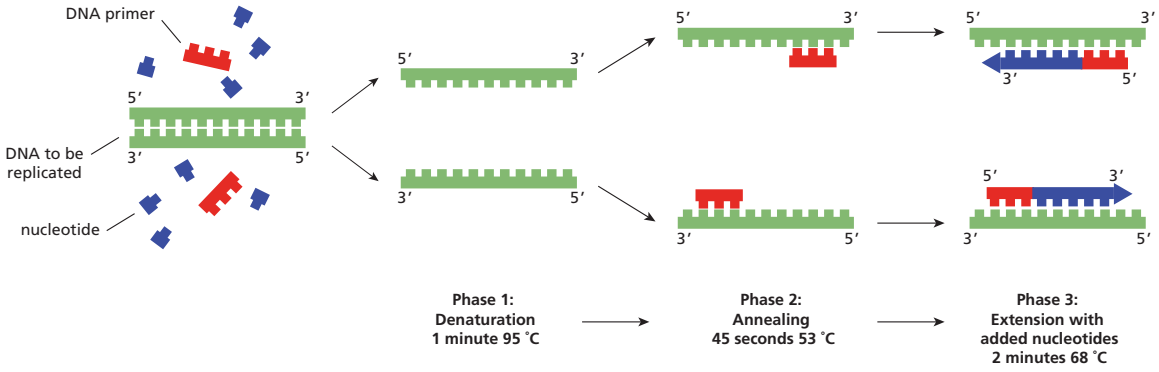
5. A buffer that creates optimal conditions for the reaction.

There are three phases in PCR:

1. *Denaturation phase*: DNA becomes single-stranded at high temperatures because the hydrogen bonds between the bases in two strands of DNA break, allowing the strands to separate. The ***melting temperature of DNA*** (the temperature at which 50% of the DNA in a sample is single-stranded and 50% is double-stranded) depends on its physical properties, but generally temperatures above 70°C cause DNA to melt. In PCR, the mixture is heated to 94–95°C long enough to ensure that the DNA strands have separated completely.
2. *Annealing phase*: In this phase, the mixture is cooled, allowing the primers to ***anneal*** (attach) to the denatured single-stranded DNA. The annealing temperature is calculated based on the melting temperature of the primers being used in the PCR reaction.
3. *Extension phase*: In this phase, the temperature is raised. *Taq* polymerase replicates the region of interest by adding dNTPs to the 3′ end of the primers.

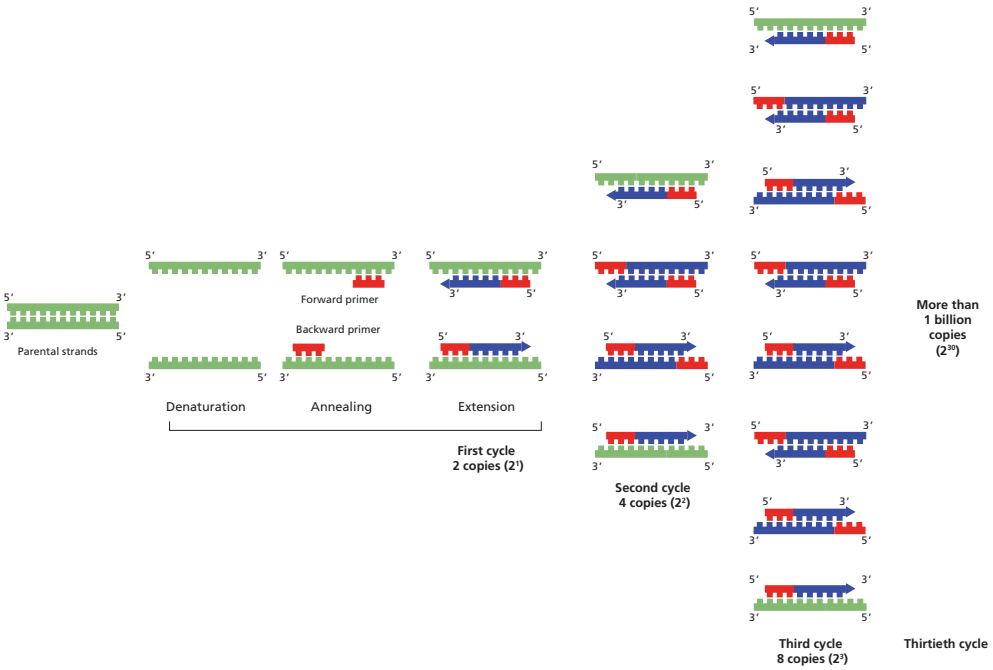
These three phases make up one cycle of PCR. An example of a single cycle of a PCR reaction is shown below.

Figure 5-EXT.1: Three Phases of the PCR Reaction



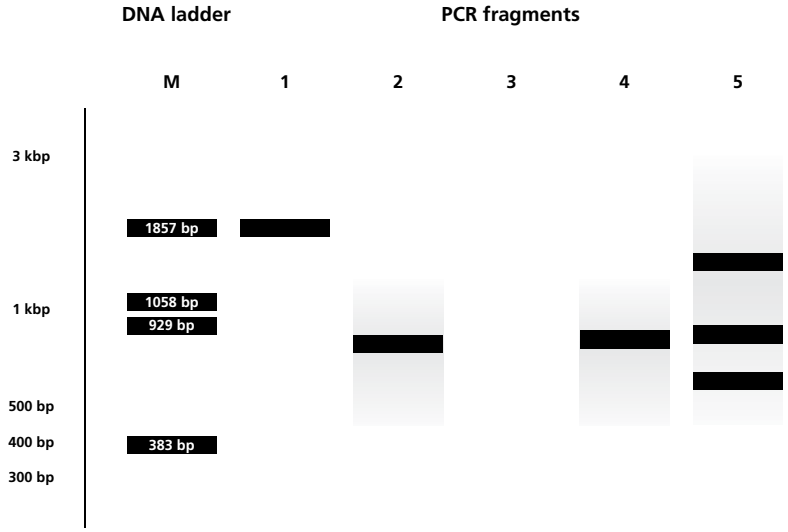
PCR is carried out in an instrument called a ***thermocycler***, which controls the temperature and length of time for each phase of the reaction. During the cycle, the number of copies of DNA from the region of interest doubles. In the example reaction above, one cycle would take approximately four minutes. This cycle is then repeated to make more copies. A reaction that runs for 30 cycles can result in more than 1 billion copies. The **Figure 5-EXT.2** shows the results after just three cycles.

Figure 5-EXT.2: DNA Amplification in PCR



The success of the PCR is then determined by using gel electrophoresis to analyze the products. By comparing the products of the PCR to standard-sized pieces of DNA in a DNA ladder, it is possible to determine whether the reaction has been successful. The gel will show if the expected product has been made, if it is the expected length, and if only one type of product has been synthesized. In the sample gel shown in **Figure 5-EXT.3**, lane 1 shows a PCR product of about 1,850 base pairs, lanes 2 and 4 represent a PCR product of about 800 base pairs, and lanes 3 and 5 show PCR failures—in lane 3, no product was formed, and in lane 5, multiple bands show that something went wrong in the reaction.

Figure 5-EXT.3: Verification of PCR Reaction Product



PCR is used in diagnostics, genetic testing, forensics, and basic research. **Table 5-EXT.1** gives examples of how PCR is currently used.

Table 5-EXT.1: PCR Applications and Examples

Genetic Testing	Example
Genetic testing (pre- and post-natal)	• Detecting mutations that lead to genetic disease (e.g., ***sickle cell disease***, cystic fibrosis) • Detecting chromosomal aberrations (e.g., duplications or deletions)
Tissue typing	• Determining tissue matches prior to organ transplantation to avoid immune rejection
Diagnostics	**Example**
Cancer detection and therapy	• Diagnosing cancers (e.g., breast and pancreatic) • Determining the origins of cancer during metastasis • Predicting response to, resistance to, or toxicity of therapeutic drugs
Detection and identification of pathogenic organisms	• Diagnosing viruses (e.g., HIV, HPV, and Ebola), bacteria (e.g., those causing tuberculosis or strep throat), and parasites (e.g., those causing malaria or trichinosis) • Determining drug sensitivities of infectious agents • Mapping the spread of infectious diseases for epidemiological studies
Forensics	**Example**
Identifying remains	• Identifying victims of crimes and natural disasters (e.g., earthquakes)
Identifying suspects	• Identifying DNA from blood, semen, saliva on cigarette butts, or other evidence left at crime scenes
Testing family relationships	• Identifying family relationships (e.g., paternity testing)
Determining origins	• Determining family lineages
Basic Research	**Example**
Drug discovery	• Examining the effectiveness of a trial drug by measuring production of enzymes in the body that facilitate distribution or disposal of the drug
Molecular anthropology, archaeology, and evolution	• Investigating evolutionary links between ancient and modern humans • Identifying common ancestry among organisms
Patterns of gene expression	• Investigating mechanisms and regulation of embryogenesis, cell differentiation, and initiation of cancer • Investigating molecular responses to environmental factors
Genetic mapping	• Determining the physical position of genes within chromosomes

LABORATORY 5-EXT: USING PCR TO AMPLIFY THE *rfp* GENE

The transformation lab (Laboratory 5/5A/5B) produced both red and white bacterial colonies. All colonies that grow on the ampicillin plate will contain the gene for antibiotic resistance, but only the colonies of bacteria that have been correctly transformed are red, while all other colonies are white. This expression of color makes it very easy to determine if a colony has taken up the correct plasmid. However, the production of other proteins, including human therapeutic proteins, cannot be confirmed visually—scientists need another method to verify that a colony has been transformed with the correct plasmid. This confirmation can be done with PCR.

COLONY PCR

In this laboratory, you will use PCR and gel electrophoresis to examine the DNA from the colonies produced in the transformation lab (Laboratory 5/5A/5B) and confirm whether or not the bacterial cells on your plates have been transformed with the plasmid carrying the *rfp* gene, pARA-R, by using PCR to make copies of the DNA and then analyzing your results, using gel electrophoresis.

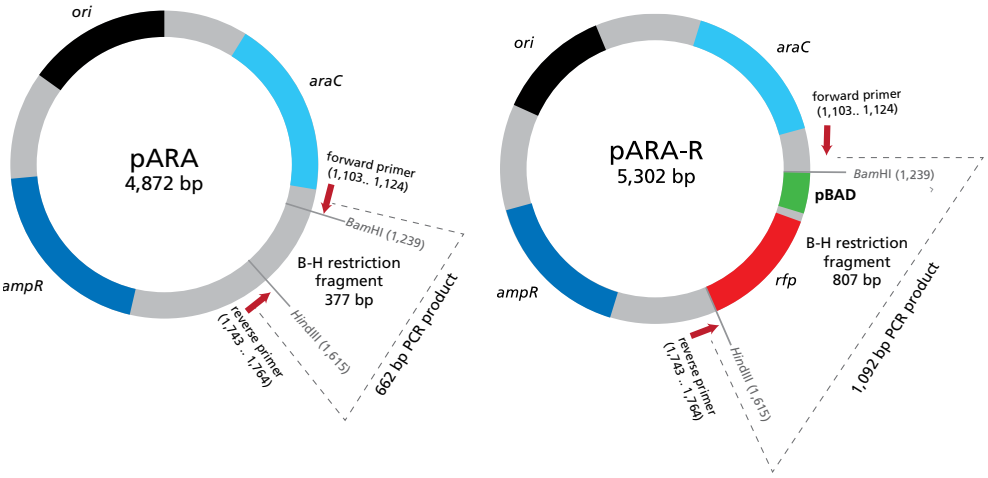
To obtain plasmid samples, you will use a pipette tip to transfer cells from a red colony to a PCR microfuge tube. The microfuge tube will contain a mixture of reagents, called a master mix, which contains all the reagents necessary for DNA replication, including dNTPs, *Taq* polymerase, and the appropriate DNA primers. The sequences of the two primers used in this lab are shown in **Table 5-EXT.2.** The sequence of each primer is unique to the large restriction fragment of pARA and is not found anywhere else in the plasmid or genome of *E. coli.*

Table 5-EXT.2: Primers for ABE PCR

Forward primer	5′-TGTAACAAAGCGGGACCAAAGC-3′
Reverse primer	5′-GCGTTTCACTTCTGAGTTCGGC-3′

You will also sample cells from a white colony not producing red fluorescent protein and will set up control tubes carrying the two plasmids: pARA and pARA-R. The figure on the following page shows the important components on the pARA and pARA-R plasmids, including the two sites where the DNA primers will bind. PCR replicates the DNA sequence between those two sites.

Figure 5-EXT.4: pARA and pARA-R Plasmid Components



BEFORE THE LAB

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

1. In this lab, we use PCR and gel electrophoresis to confirm whether the bacteria have been transformed with the correct plasmid. Why is it necessary to perform PCR before gel electrophoresis? What might happen if you attempted to perform gel electrophoresis on the sample from the agar plate?
2. You read about the importance of using specific primers to frame the target sequence for PCR. How might it impact the products of PCR if a scientist added very short primers to the DNA?
3. Read through the *Methods* sections for Part A (on pages 10–12) and for Part B (on pages 13), and briefly outline the steps for Part A and Part B, using words and a flowchart.

PART A: PIPETTING INTO WELLS

MATERIALS

FOR EACH GROUP OF STUDENTS

Reagents Kept on Ice

- PCR master mix (PCR)
- 0.025 ng/µL pARA-R (+)
- 0.025 ng/µL pARA (–)

Other Equipment and Supplies

- Cup with wet ice
- 4 empty 0.25-mL PCR tubes and caps
- Fine-tip permanent marker
- Empty tip box to use as PCR tube rack
- P-20 micropipette
- Tip box of disposable pipette tips
- Waste container
- 2 disposable gloves
- 1 LB/amp/ara plate with transformed colonies (plate can be shared by 3–4 groups)

FOR THE CLASS

- Microcentrifuge with PCR tube adaptor
- Thermocycler

SAFETY: This procedure involves opening agar plates containing genetically modified bacteria. It is not good practice to open agar plates that contain unknown microbes after incubation, so only uncontaminated agar plates (containing only the expected red and white *E. coli* colonies) should be used. Plates and all other disposable materials used in this laboratory must be sterilized prior to disposal. Follow your teacher's instructions to dispose of all waste from this laboratory. Wear disposable gloves during the procedure and wash your hands well with soap and warm water after completing this laboratory.

METHODS

1. Obtain a tube of PCR master mix (PCR) and two tubes of control plasmids (pARA-R [+] and pARA [–]) in a cup of wet ice. The reagents in this lab must be kept cold—be sure to pick up tubes only by the upper rim to avoid warming the contents with your hand.
2. Label the four empty PCR tubes 1 through 4, and initial them. Label the PCR tubes on both the side and the top, because the ink can come off the top of the tube in the thermocycler. Place the tubes in the empty tip rack.
3. You will set up four reactions: one using cells from a red colony, one using cells from a white colony, and two with plasmid controls. Review the table below, which summarizes the reagents you will add to the PCR tubes in steps 4–8. Assume that each cell sample is approximately 2 µL.

Table 5-EXT.3: Addition of Reagents to the PCR Tubes

	1	2	3	4
Step 4: PCR master mix (PCR)	23 µL	23 µL	23 µL	23 µL
Step 5: Red colony	2 µL			
Step 6: White colony		2 µL		
Step 7: pARA-R (+)			2 µL	
Step 8: pARA (–)				2 µL
Total volume	**25 µL**	**25 µL**	**25 µL**	**25 µL**

4. Set the P-20 pipette to 11.5 µL, and carefully dispense 23 µL (2 × 11.5 µL) of master mix into each of the four tubes. Place each tube on ice as soon as you add the master mix.
5. Decide who will pick the red colony. This student should put a glove on their dominant hand and, using their gloved hand, carefully pull out a pipette tip from the box. The procedure for sampling cells is as follows:
    a. Locate a red colony that is isolated from other colonies.
    b. Open the Petri dish like a clamshell, and use the pipette tip to lightly touch the red colony. **Do not** pick up any agar. Examine the pipette tip: You should be able to see cells from the colony. Lower it into the PCR tube pre-labeled for red cells (Tube 1).
    c. Transfer the bacteria into the PCR mixture by gently twirling the pipette tip in the PCR mixture without creating bubbles. Place the tip in the waste container. Return the PCR tube to the ice. Throw the used glove into the waste container.
6. Choose a different student to transfer the cells from a white colony. Have this student put a glove on their dominant hand. Using a new pipette tip, repeat steps 5a–c, but this time transfer cells from an isolated white colony into the second PCR tube (Tube 2).
7. Set up the ***positive control*** (an experimental sample with a known response that is compared to experimental samples with unknown responses) by carefully pipetting 2 µL of pARA-R into your labeled PCR tube (Tube 3). Pipette in and out several times to mix. Avoid splashing the reagents or creating bubbles. Return the PCR tube to the ice.
8. Using a new pipette tip, repeat step 7 to set up the pARA control plasmid (Tube 4). Return the PCR tube to the ice.
9. If there are any bubbles or reagents splashed on the sides of the tubes, gently tap the bottom of the PCR tube on a tabletop. If large bubbles are present, have your teacher centrifuge your PCR tube. Return the PCR tube to the ice.
10. Take the ice cup with your PCR tubes to your teacher. Once your teacher has collected all the tubes, they will be placed in a thermocycler that has been pre-programmed for this reaction. The PCR program you will use is

shown in the table below. The amplification will take approximately two hours to complete. When it is finished, the thermocycler will hold the temperature of the PCR tubes at 4°C until your samples can be transferred to the freezer, where they will be stored until you can perform the agarose gel electrophoresis.

Table 5-EXT.4: Thermocycler Program for Colony PCR

		Temperature (°C)	Time (sec)
Initial denaturation		95	270
30 cycles [	Denaturation	95	30
	Annealing	53	30
	Extension	68	60
Final extension		68	300
Hold		4	Indefinite

PART B: SEPARATE PCR PRODUCTS USING GEL ELECTROPHORESIS

MATERIALS

Equipment and Supplies

- 4 PCR tubes with amplification products (from Part A) in rack
- Microfuge tube of DNA ladder (marked "M")
- Plastic microfuge tube rack
- P-20 micropipette
- Tip box of disposable pipette tips
- Electrophoresis box loaded with 0.8% agarose gel (will be shared among groups)
- 1x SB buffer
- Waste container for used tips and microfuge tubes
- Copies of **DNA Ladder Diagram (RM 5-EXT.1)** (1 for each group member)

SAFETY:

- **Use all appropriate safety precautions and attire required for a science labratory, including safety goggles. Please refer to your teacher's instructions.**
- **Wash your hands well with soap and warm water after completing the lab.**

METHODS

1. Obtain your PCR tubes from Part A, and check your rack to make sure that you have the reagents listed.
2. Set up your gel electrophoresis box as directed by your teacher.
3. 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 should be as follows:
   a. DNA ladder (M)
   b. Red colony (Tube 1)
   c. White colony (Tube 2)
   d. pARA-R (Tube 3)
   e. pARA (Tube 4)
4. Using a fresh pipette tip for each sample, dispense 10 µL of each prepared sample and of the DNA ladder into their designated wells.
5. When you have loaded all the samples, close the cover tightly over the electrophoresis box.
6. Turn on the power supply and set the voltage as directed by your teacher.
7. While you are waiting for your gel to run, complete the **DNA Ladder Diagram**. The **DNA Ladder Diagram** shows 12 DNA bands of known sizes. Using this information, predict the positions of DNA bands produced by the PCR procedure by drawing their position on the diagram.
8. Let your gel run until the dark blue-purple dye is just past halfway down the gel. Your teacher will explain what to do with your gel once it is finished running.

DID YOU KNOW?

Some Like It Hot

PCR uses an enzyme—DNA polymerase—that is used to replicate DNA in living cells. Most living organisms survive at temperatures between 25°C and 37°C. Normal enzymes and DNA are stable at these temperatures; at higher temperatures, most proteins will denature, and DNA will unzip, causing the two strands to separate. However, in the 1960s, a new kind of microorganism called thermophiles was discovered. Thermophiles live at much higher temperatures, ranging from 55°C to a sizzling 121°C (much higher than the temperature of boiling water). Strains of these thermophiles can be found anywhere it is warm, from compost piles to thermal vents in the ocean floor to the boiling hot springs in Yellowstone National Park. Originally, all thermophiles were considered bacteria, but some earned recognition as a new domain of life, the Archaea, because they are so different from bacteria in their genetic and biochemical makeup.

How do thermophiles stay alive at temperatures that would kill most other organisms? The proteins found in thermophiles contain certain amino acids that can form stabilizing bonds, and these bonds cause the protein to fold in ways that are more resistant to denaturation by heat. The DNA in some thermophiles is supercoiled, a form that is more heat-stable. In addition, high levels of potassium and magnesium in the cells prevent breakdown of the phosphodiester backbone of the DNA. The proteins and DNA of thermophiles not only survive but actually function best at higher temperatures.

Taq polymerase, a type of DNA polymerase, was discovered in thermophile bacteria. *Taq* polymerase can function at higher temperatures, a key prerequisite for PCR. PCR requires high temperatures to denature the DNA and make the nucleotides accessible for the primers to anneal to them. The annealing temperature ensures that only primers with the exact sequence will anneal and allow replication at the desired site in the DNA sequence. Without *Taq* polymerase, PCR would not be possible.

CHAPTER 5-EXT QUESTIONS

PART A

1. Why are multiple cycles of denaturation, annealing, and extension required in PCR?
2. The recombinant plasmids used to transform the colonies in this lab were made in the gene cloning lab (Lab 2/2A) and the building a recombinant plasmid lab (Lab 3). The initial plasmid restriction digest resulted in the formation of four different fragments, each with a *Bam*HI and a *Hin*dIII sticky end.
   a. How many different two-fragment recombinant plasmids would you expect to have formed in Lab 3 (the ligation lab)?
   b. Of the two-fragment recombinant plasmids that could form while building the recombinant plasmid, which would you expect could be carried by the cells growing on the LB/amp/ara plates used in this lab?

      HINT: The large pARA and large pKAN-R fragment combination is unlikely because of its large size and multiple origins of replication.
   c. What single restriction fragment would each plasmid need to be present in all the cells that grew on the LB/amp/ara plates?
   d. Of the most likely plasmids, what would be the size of the amplification product from a red colony? _____*bp* From a white colony? _____*bp*

      HINT: **Figure 5-EXT.4** should help you determine the sizes.

PART B

1. Why is important to examine the PCR products?
2. How did your gel results from this laboratory compare to your gel predictions? Did you see any bands that were not expected? What could explain the origin of these unexpected bands?
3. Does the gel photograph show that your PCR procedure was successful? Describe the evidence you used to make this conclusion.
4. In this laboratory, you used two controls. Can you think of any additional controls this laboratory might have included? Explain.
5. Why does DNA have to be denatured to carry out PCR?
6. What are the roles of DNA polymerase and DNA primers in the PCR method?

CHAPTER 5-EXT GLOSSARY

Anneal: In molecular biology, the binding of two complementary DNA sequences by hydrogen bonding; usually one sequence is a short DNA sequence, such as a primer. In order for annealing to occur, the two sequences must be complementary.

DNA amplification: The production of multiple copies of a sequence of DNA.

Forensics: The use of scientific tests or techniques in crime investigation.

Gene product: The RNA or protein that results from the expression of a gene.

***In vivo*:** Taking place inside a living organism.

***In vitro*:** Taking place outside a living organism, such as in a test tube.

Melting temperature of DNA: Temperature at which 50% of the DNA in a sample is single-stranded and 50% is double-stranded.

Polymerase chain reaction (PCR): A technique for amplifying DNA sequences—it can amplify a specific sequence of DNA by as many as 1 billion times.

Positive control: An experimental sample with a known response that is compared to experimental samples with unknown responses.

Primers: Short stretches of DNA that bind to a complementary DNA sequence during the annealing step, allowing DNA polymerase to extend a specific region of the DNA.

Sickle cell disease: A genetic blood disorder that is characterized by abnormally shaped red blood cells.

***Taq* polymerase:** A DNA polymerase used in PCR. It is found in the thermophilic bacterium *Thermus aquaticus*, and it functions at high temperatures.

Thermocycler: A laboratory device that can change temperature rapidly and accurately and is used in the PCR method to carry out successive cycles of DNA amplification.