PCR 101: Amplification from the Lambda Phage Genome Student Worksheet

Group Discussion Questions

1. Many techniques in molecular biology require many copies of a specific DNA sequence to visualize a result or serve as a reagent for future experiments. For example, to see a band on a gel over a billion pieces of DNA, all of the same size, must be present! However, often the samples that scientists are starting with contain DNA from only a few cells. With your lab group, come up with two examples of situations where scientists would need to create many copies of DNA from a small number of starting copies.

2. With your lab group, brainstorm ways that scientists could produce many copies of a desired piece of DNA:

3. In the space below, sketch a model/diagram of DNA replication inside the cell during the cell cycle. Indicate how strands are separated, how replication is initiated, and any enzymes that are involved. Label all components of your diagram.

Introduction to PCR and the Lambda Phage Genome

This lab will introduce you to one of the most powerful concepts in modern molecular biology, a revolution that has transformed medical diagnostics, forensics, environmental science, and other life science disciplines over the past 30 years. By mastering Polymerase Chain Reaction (PCR) you will have the keys to developing your own molecular biology experiments and the necessary background for understanding new, increasingly complex technologies.

PCR technology addresses two major challenges in molecular biology; how to amplify DNA and how to target specific sequences for amplification. First, the DNA that scientists want to analyze is often collected in extremely low quantities (for example, a single drop of blood from a crime scene). To have usable quantities of DNA we must make many copies using the original DNA as a template. Second, among the whole genome we must find only the part we want to analyze, whether it is a gene that causes a disease or a sequence that can help identify a species. This is no small matter since the human genome is over 3 billion base pairs (bp) long and we are often interested in regions less than 300 bp long.

Stop and Think
How can we find a specific region of the genome among three billion base pairs? For inspiration, look back at your model of DNA replication.

The History of PCR

As with many ideas in biotechnology, nature provides us with most of the tools we need. Every time a cell divides it makes two replicates of its entire genome with the help of specialized enzymes. The phenomenon of complementary base pairing should give you a hint as to how a specific region can be targeted. In the late 1970s Frederick Sanger developed a method for copying DNA *in vitro* that used short pieces of DNA, called primers to initiate replication by a DNA polymerase, similar to the RNA primers in your model of cellular DNA replication. An artificial primer used in the Sanger method has a sequence that allows it to bind at only one location in the target DNA sequence.

Stop and Think
Do you think it's possible for a primer to bind to more than one location in the genome? Is this more likely for long primers or for short primers?

Using only one DNA primer, the Sanger method can only produce one copy of the target at a time. In 1983 Kary Mullis proposed a modification to this method where a second primer is used to initiate replication using the first copy as a template. Recall that DNA has an antiparallel structure where one strand runs in the opposite direction relative to the second strand. In order to use the first copy as a target for replication, the second primer that Mullis added needed to initiate replication in the opposite direction, thus one primer is called the forward primer and the other is called the reverse. With the original sequence and its copy serving as templates, two copies are produced instead of one. Further, these two copies can each be used as templates in a second round of copying, which produces four copies. After each round, or cycle, the number of copies has doubled. Over multiple cycles, billions of copies of the DNA sequence between the two primers are generated. This method is called Polymerase Chain Reaction (PCR) – polymerase because of the enzyme that is used to copy DNA and chain reaction because the products of one cycle serve as templates for the

next round of copying. This rapid and efficient technology for generating usable quantities of DNA won Mullis the Nobel Prize in Chemistry. The first application of PCR was a test for sickle cell anemia.

> **Stop and Think**
> **Starting with a single copy, how many copies are produced after two cycles of PCR? Three? Write out the number of copies produced over the first ten cycles. Can you think of a mathematical function that describes the increase in copy number with cycle number? Why is PCR often called DNA amplification?**

How PCR Works

Instead of trying to recreate the cell's intricate biochemical machinery in a test tube, scientists rely on heat to control the various steps of the PCR reaction. In order for DNA to be copied the nucleotide bases must be exposed, which means double stranded DNA must be separated into single strands. Recall that heat applied to an ice cube weakens the hydrogen bonds between water molecules and causes a phase transition to liquid water. Similarly, heat applied to double-stranded DNA weakens hydrogen bonds between bases causing separation of the strands into single-stranded DNA. As with ice, this is sometimes called melting, but is commonly referred to as denaturation. In a PCR cycle **denaturation** is performed at 90-98°C for 5-30 seconds. This temperature is just below the boiling point of water.

Once the template DNA has been separated into single strands, the temperature is lowered to encourage primers to bind to their target sequences. Just as in the water analogy, lower temperature favors the formation of hydrogen bonds between molecules, in this case the primer and the template DNA. This step, called **annealing,** is typically between 45 and 65°C for 5-30 seconds. The ideal temperature and duration of the annealing step depends of the sequences of the primers being used. It must be low enough that hydrogen bonds are able to form between the primer and specific complementary sequence, but not so low that non-specific, or random, binding between primer and template occurs.

> **Stop and Think**
> **Will long primers or short primers require a lower annealing temperature to bind to their complementary sequences? Can both forward and reverse primers anneal to the same strand of single-stranded DNA?**

Just like in your model of cellular DNA replication the DNA polymerase used in PCR binds to the primer-template complex and begins to add nucleotides (dNTPs) onto the 3' end of the primer. This step, called **extension,** results in a new copy attached to the template as double-stranded DNA. The duration of the extension step depends on the length of segment of DNA that is being copied and can be anywhere from 5 seconds to 5 minutes.

At this point you may have noticed a problem – DNA polymerase, which the entire PCR process relies on, is a protein enzyme that needs a very specific three-dimensional structure to copy DNA. Before getting to the extension step, the entire PCR mixture, including the polymerase, has already been through the denaturation step where the reaction was heated almost to boiling. Anyone who has cracked an egg into a frying pan will know what high heat does to proteins!

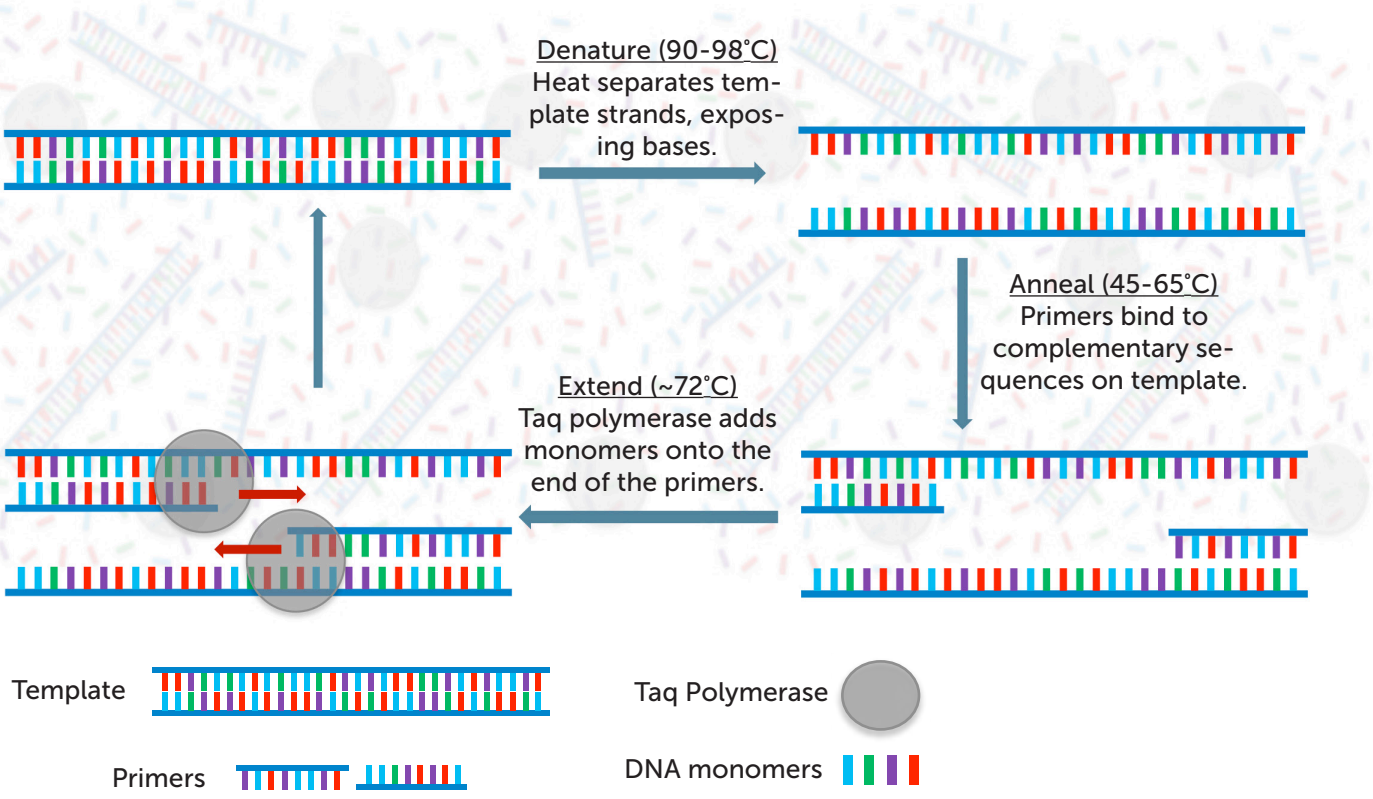
In the early days of PCR, fresh polymerase was added to the reaction tube every cycle to replace the polymerase that had been destroyed by the denaturation step. Having to open the tube and add new enzyme for up to 30 cycles was expensive, inefficient, and increased the chances of contaminating the reaction. The innovation that would remove these obstacles came from an unusual source – Yellowstone National Park.

In the 1970s scientists had isolated a species of bacteria called *Thermus aquaticus* from a hot spring in Yellowstone. *T. aquaticus* thrives at 75-80˚C and can survive much higher temperatures. Its enzymes are similarly heat-tolerant. *E. coli*, the original source for the polymerase used in PCR, thrives at 37.5˚C, the same temperature as the gut of mammals. Since the biochemistry of DNA is similar across the tree of life, polymerase from *T. aquaticus* (called Taq Polymerase) can replace *E. coli* polymerase in PCR, with the modification that the extension step is performed at 70-75˚C.

This innovation led to PCR becoming the ubiquitous, inexpensive, and efficient tool that it is today. Reactions can be set up once, sealed inside a tube, and placed in an automated thermal cycler. The thermal cycler (or PCR machine) is a specialized instrument that accurately and rapidly changes the temperature of the tube between denaturation, annealing, and extension. After 20-40 cycles of these three temperatures, trillions of copies of the desired DNA product can be produced. The amplified DNA can be analyzed with gel electrophoresis at the end of the experiment, or detected as they are being formed using more advanced equipment.

Stop and Think
Can PCR use the same DNA nucleotides that the cell uses?

Figure 1. The mechanism of PCR



Summary – Becoming a PCR Pro

Designing an efficient and specific PCR reaction is a skill that researchers develop with experience. The temperatures and durations of each step in the PCR protocol must also be carefully chosen. Some are standard for all reactions and some must be optimized for each reaction.

Several factors contribute to the success or failure of a PCR reaction, primer design being the most important. The primers must be carefully designed to be specific to the target sequence. If sequences complementary to the primers exist in other parts of the genome you can end up with multiple products, or no product. The annealing temperature must be carefully selected to favor specific binding of the primer to the target. pH and Mg^{2+} concentration must be carefully controlled for maximum activity of the DNA polymerase. Finally, even small concentrations of contaminants can interfere with PCR resulting in complete failure of the reaction.

In this lab we will examine how different primers are used to amplify different segments of a single genome. You will be supplied with three different primer sets that target different sequences of the lambda phage genome. From the primer sequences and the genome sequence of the phage you can predict the sizes of the segments that will be amplified. You will test your prediction by performing PCR with the different primer sets and analyzing the results with agarose gel electrophoresis.

Take a moment to look back over the Introduction and use your new understanding of PCR to fill in the missing details on the two summary tables:

Table 1. Reagents Required for PCR

1	
2	
3	
4	
5	
6	Mg2+ (required for polymerase function)
7	Buffer (to maintain a consistent pH)

Table 2. PCR Cycling Protocol- Typical Values

# Cycles	Step Name	Temperature	Time
_____	Denaturation		
	Annealing		
	Extension		

The Lambda Phage

Bacteriophage lambda, or lambda phage, is a virus that infects *Escherichia coli* (*E. coli*), a species of bacteria common in the mammalian gut. The phage attaches itself to the outside of the bacterial cell and injects its genome into the cytoplasm. The phage can then enter the lytic or the lysogenic cycle. In the lytic cycle the genome is replicated inside the host cell and the genes are expressed. Gene products then assemble into phage particles that are released when the host cell lyses. In the lysogenic cycle the phage genome recombines with the host genome integrating itself into the bacterial chromosome. In this stage it is called a prophage and it is replicated every time the host genome replicates. Under certain environmental conditions the prophage is cut out of the host genome and can enter the lytic cycle.

Since its discovery in 1950 the lambda phage has been intensively studied as a model for basic molecular genetics and for its applications in biotechnology. Experiments with the phage have led to important discoveries in fields such as protein folding, genetic regulation, and enzyme biochemistry. The ability of the phage to inject its DNA into bacterial cells and recombine with the host genome has made it a valuable tool for editing the genomes of bacteria.

The genome of lambda phage is 48,502 base pairs of linear double-stranded DNA, coding for over a thousand proteins. The relative simplicity and accessibility of this genome make it an excellent tool for exploring basic biochemical techniques, including PCR.

Pre-Lab Questions and Exercise

1. Using what you have just learned about PCR, modify your diagram of DNA replication in the cell to show the mechanism of DNA replication during a PCR reaction.

2. PCR targets a specific region of the genome for copying. What reagent in the PCR reaction makes it specific for one region rather than another? How is this specificity achieved?

3. After the PCR reactions are complete, you will run the products on a gel to determine the size of the fragment you have copied. Aside from the band corresponding to this fragment, what other bands might you see on the gel? Justify your reasoning.

4. Sometimes after running a PCR reaction no bands are seen on the gel. Name three reasons why this might happen. Justify your reasoning.

5. What would you expect to happen if primers were left out of your PCR reaction?

6. Why are simple organisms like the lambda phage and its host useful in molecular biology?

7. On the next page you will find a DNA sequence corresponding to a region of the lambda phage genome as well as the sequences of the three primer pairs we will use. Using this information and your knowledge of PCR, record the sizes of the fragments that will be amplified with each primer pair on Table 3.

8. The copying rate of FastTaq polymerase you will use in this lab is 100 bp/sec. Using the fragment lengths you determined in Question 7, calculate the minimum extension time required for each cycle. (Hint: perform this calculation for the longest fragment you found in Question 7.)

Lambda Phage Genome - Partial

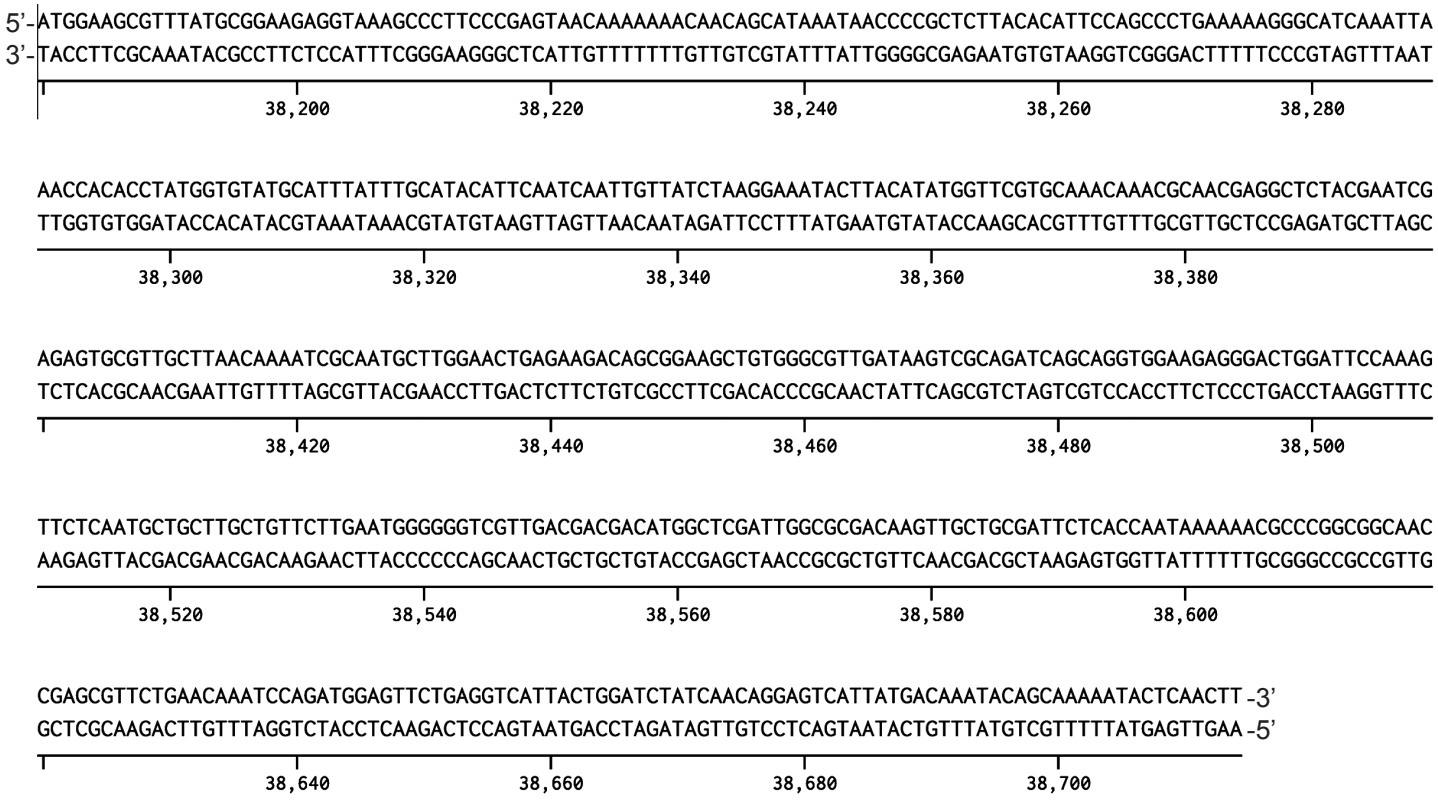


Table 3. Forward and reverse primer sequences

	Forward Primer	Reverse Primer	Fragment Length (bp)
Primer Set 1	5'-GAAGCGTTTATGCGGAAGAG-3'	5'-CGTTGCGTTTGTTTGCAC-3'	
Primer Set 2	5'-GAAGCGTTTATGCGGAAGAG-3'	5'-ACCTGCTGATCTGCGACTTA-3'	
Primer Set 3	5'-GAAGCGTTTATGCGGAAGAG-3'	5'-TGACTCCTGTTGATAGATCCAGT-3'	

Experimental Procedures

Today you will set up PCR reactions with three sets of primers targeting three regions of the lambda phage genome along with one control reaction. In addition to the primer sets and lambda phage DNA, you will be supplied with a FastTaq MasterMix, which contains a special fast-copying Taq DNA polymerase, the PCR buffer, Mg2+ ions, and dNTPs (DNA monomers). Follow the protocol below to prepare your samples and set up the thermal cycler.

1. Label the tops and sides of four thin-walled PCR tubes with your initials and P1, P2, P3, and -C, for the three primer sets and a negative control (no primers). These primer sets are the same as the primer sets you used for your calculations in Question 7 of the Pre-Lab.

2. Add reagents to each of the tubes according to Table 4. Pipette the reagents directly into the bottom of the PCR tubes and try to avoid creating bubbles.

Table 4. PCR Setup

	Tube			
	P1	P2	P3	-C
FastTaq 2x MasterMix (µL)	10	10	10	10
Lambda DNA (µL)	5	5	5	5
Primer Mix (µL)	5 (PS1)	5 (PS2)	5 (PS3)	5 (H_2O)
Total Volume (µL)	20	20	20	20

3. If there are some reagents stuck to the sides of the tubes briefly spin down with a centrifuge to collect all liquid at the bottom of the tube. If a centrifuge is not available, tap the bottom of the tube on the bench. Gently flick the tube with your finger to make sure the reagents are well mixed and there are no bubbles trapped at the bottom of the tube.

4. Turn on your MiniOne PCR thermal cycler and place your tubes in the wells on the aluminum plate. Follow the instructions in your Getting Started Guide to program the cycling parameters (Table 5) on the tablet app and start the PCR protocol.

Table 5. Cycling protocol for lambda phage genome PCR

# Cycles	Step Name	Temperature	Time
20	Denaturation	94°C	5 sec
	Annealing	54°C	5 sec
	Extension	72°C	5 sec

5. Monitor the progress of the PCR protocol with the real-time graph on the tablet app.
6. When the PCR protocol is complete, remove your tubes from the thermal cycler. Samples can be used immediately for gel electrophoresis or stored in the refrigerator overnight.
7. Prepare your samples for gel electrophoresis by adding 5 µL of 5x Loading Dye to each sample (Table 6). Flick with your finger to mix. If necessary, centrifuge or tap on the benchtop to bring all liquid to the bottom of the tube.

Table 6. Prepare the samples for gel analysis

Sample	Add 5x Loading Dye	Load on Gel
MiniOne Marker	-----	10 µL
Tube P1	5 µL	10 µL
Tube P2	5 µL	10 µL
Tube P3	5 µL	10 µL
Tube -C	5 µL	10 µL

8. Cast the 2% agarose GreenGel using the MiniOne Casting Stand. Use the 6 well side of the comb.
9. After the gel has solidified (~10 minutes) carefully remove the comb and follow the directions to set up the MiniOne Electrophoresis chamber.
10. Add 135 mL TBE running buffer to the tank and load 10 µL of each sample and the MiniOne Marker into the wells, keeping track of the placement using Table 7.

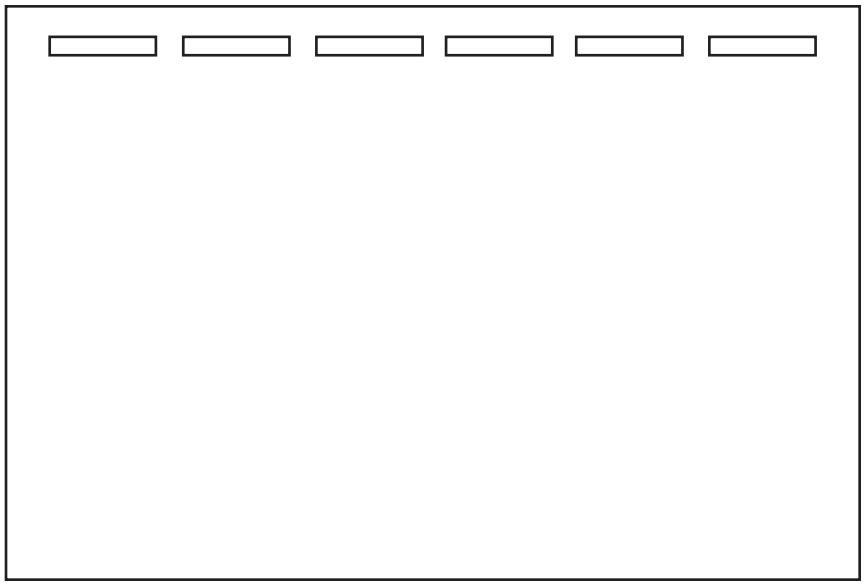
Table 7. Record the sample loaded into each lane

Well	1	2	3	4	5	6
Sample						

11. Run your gel for 20 minutes or until the bands have clearly separated. Document your results with your cell phone or camera and paste an image of your gel in your lab notebook.

Data Collection Worksheet

After completing the gel electrophoresis analysis of your PCR products, sketch your final results on the diagram below, or paste an image of your gel:



Post-Lab Questions and Analysis

1. The sizes of the bands in the MiniOne Marker are: 100, 300, 500, 1000, 2000 bp. Estimate the sizes of the bands in your PCR lanes by comparing to the MiniOne Marker lane. Are your estimates consistent with your sizes you predicted in the pre-lab exercise? If not, what do you think accounts for the difference?

2. Did you observe any other bands on the gel besides the expected PCR products?

3. Tube C was a negative control- we deliberately left out the primers to demonstrate one of the requirements for a successful PCR reaction. Another common negative control in PCR reactions is a 'no template control' where water is added in place of the template DNA. What is the purpose of a "no template" negative control?

4. What differences would you expect to see in your gel if you programmed the thermal cycler for 30 cycles instead of 20?

5. This protocol uses a high-speed polymerase that can copy DNA at 100 bp/sec. What would you expect to see on your gel if you programmed 2 seconds extension time instead of 5 seconds?

6. Bonus: A multiplex reaction is when multiple primer pairs are combined in the same tube to amplify multiple genomic regions at once. Do you think the primer pairs used in this lab could be used to amplify all three fragments in the same tube? Why or why not?

Appendix A: Recommended Resources

The DNA Learning Center at Cold Spring Harbor Laboratory has a number of excellent resources on DNA and Biotechnology that are helpful in understanding the background for this lab:

- Animation of the Sanger method of copying DNA:
  https://www.dnalc.org/view/15479-Sanger-method-of-DNA-sequencing-3D-animation-with-narration.html

- Interactive animation and graph of exponential DNA amplification by PCR:
  https://www.dnalc.org/view/15924-Making-many-copies-of-DNA.html

- A video illustrating the mechanism of PCR using 3D molecular models:
  https://www.dnalc.org/view/15475-The-cycles-of-the-polymerase-chain-reaction-PCR-3D-animation.html

Scitable by Nature Education is also a useful resource for a variety of topics in molecular biology:

- Basic introduction to cellular DNA replication:
  https://www.nature.com/scitable/topicpage/cells-can-replicate-their-dna-precisely-6524830

- A more detailed explanation of the molecular mechanism:
  https://www.nature.com/scitable/topicpage/major-molecular-events-of-dna-replication-413

You can learn more about the lambda phage and its history in molecular biology here:

- Murray, N. E., Gann, A. (2007) What has phage lambda ever done for us? *Current Biology*, 17 (9), R305-R312.

Appendix B: PCR Glossary

Term	Definition
Annealing	As the temperature of a PCR reaction is lowered, short pieces of DNA, called primers, bind to specific sequences within the genome targeting this region to be copied. Annealing temperature is specific to the primers used in your reaction- typical settings are 45-65˚C for 5-30 seconds.
Buffer	A salt added to aqueous solution that helps maintain a consistent pH. Buffers are essential in PCR because the DNA polymerase's function is sensitive to pH changes.
Cycle	A cycle refers to one round of denaturation, annealing, and extension steps of the PCR reaction. The number of cycles needed for a particular reaction will depend on how much DNA you are starting out with and how much DNA you are trying to produce. With high starting concentration, 20-25 cycles is sufficient to produce enough DNA to visualize on a gel. Where the starting concentration is low or large quantities of product are needed, 35-40 cycles can be used.
Denaturation	Denaturation uses high temperature to break the bonds between bases on opposing strands. Double-stranded DNA is split into single-stranded DNA exposing the bases so they can be copied. Typical settings are 90-98˚C for 5-30 seconds.
dNTPs	Nucleotides, the molecular building blocks of DNA.
Enzyme	An enzyme is a biological catalyst that speeds up a chemical reaction without changing the products or being consumed by the reaction. Most enzymes are proteins and they control a wide range of reactions in cells, from copying DNA to extracting energy from food.
Extension	At around 70˚C the polymerase gets to work and starts adding nucleotides (dNTPs) to the 3' end of the annealed primers, copying the complementary strand. Typical settings are 72˚C for 5 seconds – 5 minutes.
Final Extension	In some protocols an additional extension step is used. This ensures that the polymerase can add the final base pairs onto the end of the strands, which is necessary in some applications. The typical duration is 2-10 minutes.
Initial denaturation	When copying a piece of genomic DNA, an initial denaturation step is often used to make sure the long strands of DNA are fully separated and freed from bound proteins before thermal cycling begins. Typical settings are 90-96 C for 30 seconds - 10 minutes.
Monomer	A molecule that can be bonded with other similar molecules to form a polymer.
Polymer	A molecule that consists of many similar units bonded together.
Primers	Short pieces of DNA with sequences complementary to the sequences flanking the region to be copied. Primers are designed specifically for every PCR reaction taking many variables into account, including length, nucleotide content, and structural features. Many computer tools are available to assist in primer design.
Template	DNA containing the sequence that will be copied in a PCR reaction. Can be a short fragment or a whole genome.
Thermal Cycler	Also called a PCR machine, a thermal cycler is an instrument that automatically changes the temperature of the PCR reaction according to a program set up by the user. It heats and cools the reaction between denaturation, annealing, and extension temperatures over a specified number of cycles.

Appendix C - Gel Electrophoresis

Gel electrophoresis is a technique used in many areas of science to analyze the components of complex chemical mixtures. Mixtures of DNA, RNA, proteins, or dyes can be separated into their individual components based on molecular size and electrical charge using a separation matrix within an electric field.

The gel used in gel electrophoresis is a tangle of polymers forming a three-dimensional matrix with water-filled pores through which molecules migrate. A higher density of polymers creates smaller pores. Like the holes in a sieve or colander, the size of the pores has to be the appropriate size for the molecules being separated. Gels can be made from different substances depending on the application. One of the most commonly used and effective materials is agarose, a polymer extracted from seaweed. Agarose gels are formed (or cast) by pouring molten (melted) agarose into a tray where it solidifies into the desired shape as it cools. A comb is placed while the agarose is molten and then removed after it solidifies to create wells where the samples are loaded.

After the gel solidifies it is placed in an electrically conductive buffer between parallel positive (⊕ anode) and negative (⊖ cathode) electrodes. A voltage is applied between the electrodes, creating a uniform electric field within the gel. Molecules in the wells begin to move under the influence of the electric field: positively charged molecules migrate toward the ⊖ cathode and negatively charged molecules migrate toward the ⊕ anode.

The speed of a molecule's movement in an electric field is determined by the strength of its electric charge relative to its molecular weight. This is quantified as the charge to mass ratio. Speed of movement within a gel is also influenced by the size of the molecule relative to the pores in the gel. The polymers in the gel are like an obstacle course: smaller molecules maneuver easily through the pores, traveling faster and farther than large, bulky molecules. However, a large molecule can move faster through a gel than a smaller molecule when the strength of its charge relative to its mass is significantly higher. Shape can also affect how a molecule moves through the gel. Long spaghetti-like molecules will move slower than compact molecules, which slip easily through the pores. Molecules of the same size, shape, and charge will move together and form a distinct band. If multiple types of molecules are present in the sample, they will separate from each other and each will form a distinct band.