# PCR 101: Amplification from the Lambda Phage Genome

## Teacher's Guide

## Cat# M6001 & M6002

Version 081617

In this hands-on MiniLab students explore the basics of Polymerase Chain Reaction (PCR) using the genome of Bacteriophage Lambda, a classical model system for molecular genetics. Students will use PCR to amplify three segments of the Lambda Phage genome using three different sets of primers and use the supplied genome sequences and primer sequences to predict the size of the fragments. Students will test their predictions by running the products of the PCR reactions on an agarose gel.

**This MiniLab contains enough supplies for**

**10 groups**

**to perform experiments**

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# Laboratory Safety

1. Exercise caution when heating or melting reagents.
2. Exercise caution when working with electrical equipment.
3. Gloves and eye protection should be used whenever needed as part of good laboratory practice.
4. Always wash hands thoroughly after handling biological materials or reagents.
5. Follow all safety precautions described on the MSDS for the reagents.
6. Elements of the PCR thermal cycler, including the PCR plate and heated lid, can be extremely hot. Exercise caution when adding or removing tubes from the machine.

# MiniLab Components

## Supplies Included in included in Cat# M6001 & M6002

| | |
|---|---|
| FastTaq DNA Polymerase 2x MasterMix | 475 µL |
| Primer Set 1, Primer Set 2, Primer Set 3, $dH_2O$ | 80 µL each |
| Lambda Genomic DNA | 275 µL |
| 5x Gel Loading Dye | 275 µL |
| MiniOne Molecular Weight Marker | 130 µL |
| 0.65 mL Microcentrifuge Tubes | 88 tubes |
| 0.2 mL thin-wall PCR tubes | 45 tubes |

## MiniOne Electrophoresis Supplies included in Cat# M6001

| | |
|---|---|
| 2% Agarose GreenGel in-a-cup | 10 cups |
| Tris-Borate EDTA (TBE) Concentrate | 100 mL |

# Additional Materials Required

| MiniOne™ Systems | Quantity Needed |
|---|---|
| **MiniOne™ Electrophoresis System:** Carriage Base, Running Tank, Photohood, 42V Power Supply | 1 each per group |
| **MiniOne™ PCR System:** Thermal Cycler, 12V Desktop Power Supply | 1 for every 4 groups |
| **MiniOne™ Casting System:** Casting Stand, Gel Tray, Gel Comb | 1 each per group |

| Other Equipment | Quantity Needed |
|---|---|
| Micropipette (1-10 µL or 2-20 µL) | 1 per group |
| dH2O for diluting running buffer | 1900 mL |
| Tablet or iPad for running PCR software | 1 per PCR System |
| Digital camera or cell phone camera | 1 per group |
| Microwave oven | 1 per class |
| Benchtop microcentrifuge (optional) | 1-3 per class |
| PCR tube rack (optional) | 1 per group |

# NGSS Alignment

| | |
|---|---|
| **Phenomenon** | How do scientists use enzymes and heat to copy DNA in a test tube? |
| **Grade Level** | This lab, which is appropriate for grades 9-12, introduces students to the theory and methods of polymerase chain reaction (PCR), one of the most powerful and groundbreaking methods in molecular biology. Completing student worksheets requires a basic understanding of the structure and function of DNA and the mechanism of DNA replication during the cell cycle, as well as algebra and exponential math. Experience with gel electrophoresis and pipetting small volumes is highly recommended. Students should be familiar with basic physics and chemistry and units of measure commonly used in molecular biology. |
| **Science and Engineering Practices (SEPs)** | **Using Mathematics and Computational Thinking**<br>Use mathematical and/or computational representations of phenomena or design solutions to support explanations. (HS-LS2-1)<br><br>**Developing and Using Models**<br>Develop a model based on evidence to illustrate the relationships between systems or components of a system. (HS-LS2-5)<br><br>**Analyzing and Interpreting Data**<br>Analyze data using tools, technologies, and/or models (e.g., computational, mathematical) to make valid and reliable scientific claims or determine an optimal design solution. |
| **Cross-cutting Concepts (CCs)** | **Scale, Proportion, and Quantity**<br>The significance of a phenomenon is dependent on the scale, proportion, and quantity at which it occurs. (HS-LS2-1)<br><br>**Systems and System Models**<br>Models (e.g., physical, mathematical, computer models) can be used to simulate systems and interactions—including energy, matter, and information flows- within and between systems at different scales. (HS-LS1-2), (HS-LS1-4)<br><br>**Energy and Matter**<br>Energy drives the cycling of matter within and between systems. (HS-LS2-3)<br><br>**Science is a Human Endeavor**<br>Technological advances have influenced the progress of science and science has influenced advances in technology. (HS-LS3-3) |
| **Disciplinary Core Ideas (DCIs)** | **LS1.A Structure and Function**<br>All cells contain genetic information in the form of DNA molecules.<br>Multicellular organisms have a hierarchical structural organization, in which any one system is made up of numerous parts and is itself a component of the next level. (HS-LS1-2)<br><br>**LS3.A Inheritance of Traits**<br>Each chromosome consists of a single very long DNA molecule, and each gene on the chromosome is a particular segment of that DNA. The instructions for forming species' characteristics are carried in DNA.<br><br>**Growth and Development of Organisms**<br>In multicellular organisms individual cells grow and divide via a process called mitosis [...]. The organism begins as a single cell that divides successively to produce many cells, with each parent cell passing identical genetic material to both daughter cells. |

# Learning Objectives

## Intellectual Objectives

1. Understand the molecular mechanisms of Polymerase Chain Reaction (PCR) and how gel electrophoresis is used to analyze PCR products
2. Appreciate the importance of model systems in molecular biology
3. Make predictions about PCR reactions based on their background knowledge and test their predictions using experimental data
4. Calculate the size of PCR fragments from a genomic DNA sequence and primer sequences
5. Understand the purpose of each step in a PCR protocol and interpret a graph of temperature changes over time
6. Use gel electrophoresis to estimate the size of a PCR fragment by comparison to a molecular weight standard

## Technical Objectives

1. Correctly handle and use an adjustable volume micropipette
2. Combine reagents in the correct proportions to set up a PCR reaction
3. Program and monitor the PCR thermal cycler
4. Cast, load, and run an agarose gel to analyze results of a PCR reaction
5. Follow appropriate lab safety protocols

# Required Student Background

1. Students should have some exposure to the theory and applications of gel electrophoresis before beginning this lab.
2. Hands-on experience with pipetting and gel electrophoresis is strongly encouraged.
3. Students must understand the basic structure of DNA and the mechanism of DNA replication during the cell cycle.
4. Students should be familiar with units of measure commonly used in molecular biology (µL, mL, ng).
5. A basic understanding of molecules, chemical bonding, monomers and polymers, phase transitions, and electricity is helpful for understanding the mechanism of PCR and electrophoresis.
6. If students do not have experience using a micropipette or pipetting small volumes of liquid, we recommend the MiniOne Practice Pipetting and Gel Loading MiniLab (Cat# M3002) as preparation.
7. If students do not have experience with DNA gel electrophoresis we recommend any of the MiniOne Electrophoresis MiniLabs including DNA Fingerpringting (Cat# M3004), CSI Forensics (Cat# M3005), and Electrophoresis 101 (Cat# M3001).

# Group Discussion Questions

*The purpose of these questions is to access students' prior knowledge and inspire discussion about how technology advances in response to scientific discoveries and how scientific applications follow technological progress. There are many possible answers to these questions. Students can discuss these questions in their lab groups then share their answers in a class discussion.*

1. Many techniques in molecular biology require numerous copies of a specific DNA sequence to visualize a result or to 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.

   *Students may suggest a police lab analyzing traces of biological material found at a crime scene, public health investigators detecting disease organisms present at low levels in food, or researchers identifying a species based on a small sample.*

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

   *Suggestions may refer to the cellular mechanism of DNA replication which uses the existing copy as a template through complementary base pairing. They might also suggest synthesizing it from scratch or introducing it into an organism that could replicate it with it's cellular machinery.*

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.

   *Responses will vary depending on students' level of preparation. This question assumes that students have learned about DNA replication in class and can access information from their notes or a textbook. If DNA replication has not been covered yet, consider letting students do background research on the topic using one of our recommended resources (Appendix A).*
   *Students should include double stranded DNA being separated into single strands by helicase, binding of primers by complementary base pairing, DNA polymerase binding and copying the exposed bases at a minimum. Subsequent questions will reference this model, so all students should have the basics sketched out before moving on.*

# 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 between 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?**

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 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!

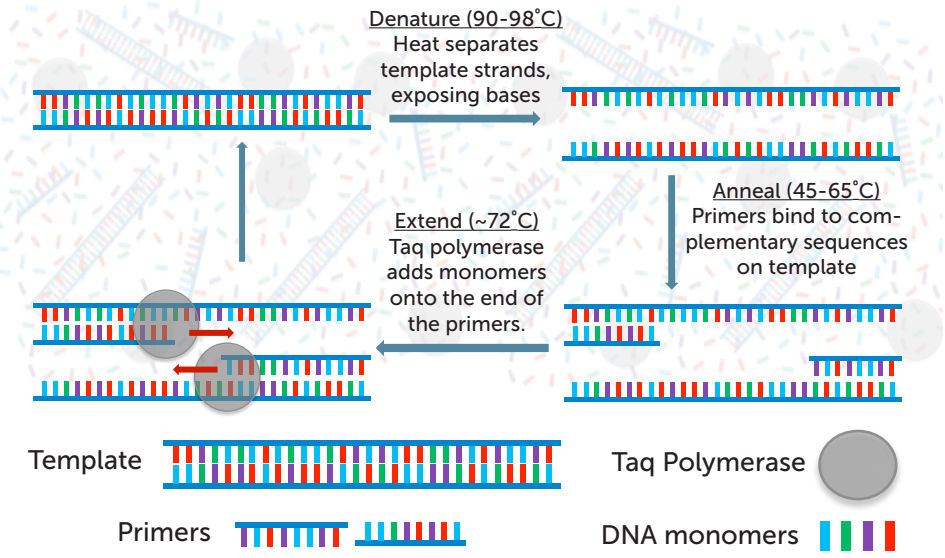
In the early days of PCR, fresh polymerase was added to the reaction tube every cycle to replace the polymerase that had been destroyed by the denaturation step. Having to open the tube and add new enzyme for up to 30 cycles was expensive, inefficient, and increased the chances of contaminating the reaction. 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 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 amplificed 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 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. 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 Mg2+ 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 | *Taq Polymerase* |
| 2 | *DNA template* |
| 3 | *Forward primer* |
| 4 | *Reverse primer* |
| 5 | *Nucleotides (or dNTPs)* |
| 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 |
|---|---|---|---|
| *_20-40_* | Denaturation | *94-98˚C* | *5-30 sec* |
| | Annealing | *45-65˚C* | *5-30 sec* |
| | Extension | *70-75˚C* | *5 sec- 5 min* |

## 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

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.

   *Students should recognize that the mechanism of PCR is similar to the mechanism of DNA replication in cells except that heat is used as the energy input for denaturation and annealing rather than enzymes. They should add the temperature of each step to the diagram, and indicate that temperatures are applied in a cycle to produce many copies. Depending on their knowledge of DNA replication, they may also indicate that the cell uses RNA primers while DNA primers are used in PCR.*

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?

   *Students should know that primers target a specific region of DNA to be copied through complementary base pairing between primer and target sequence.*

3. After the PCR cycles 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?

   *Unused primers or template may be seen on the gel. Knowing that the specificity of PCR is highly dependent on the reaction conditions, some students might point out that other parts of the genome besides the specific target could also be copied.*

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

   *Some possible causes of reaction failure: an essential ingredient was left out of the reaction, the wrong primers were used, the wrong temperatures were used, the reagents were contaminated, the pH of the buffer was wrong, there was an error in programming the PCR machine.*

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

   *Students should understand that no PCR product would be produced if there were no primers because they are essential for targeting a region to be copied.*

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

   *Simple and accessible model organisms such as the lambda phage have been instrumental in exploring basic molecular mechanisms, including DNA replication, that are common to all organisms. Useful model organisms often present fewer experimental complications than more complex organisms. When one type of organism is used in many studies and many laboratories the accumulated knowledge of its biology makes it increasingly useful in experiments.*

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 in Table 3.

   *Students should be able to find the binding sites of the primers and calculate the lengths of the fragments as 202 bp, 304 bp, and 501 bp (see Table 3 and sequence on Page 14). Some might correctly identify the binding sites of the primers but mistakenly think that the primer sequence itself is not part of the resulting fragment.*

8. The copying rate of the 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.)

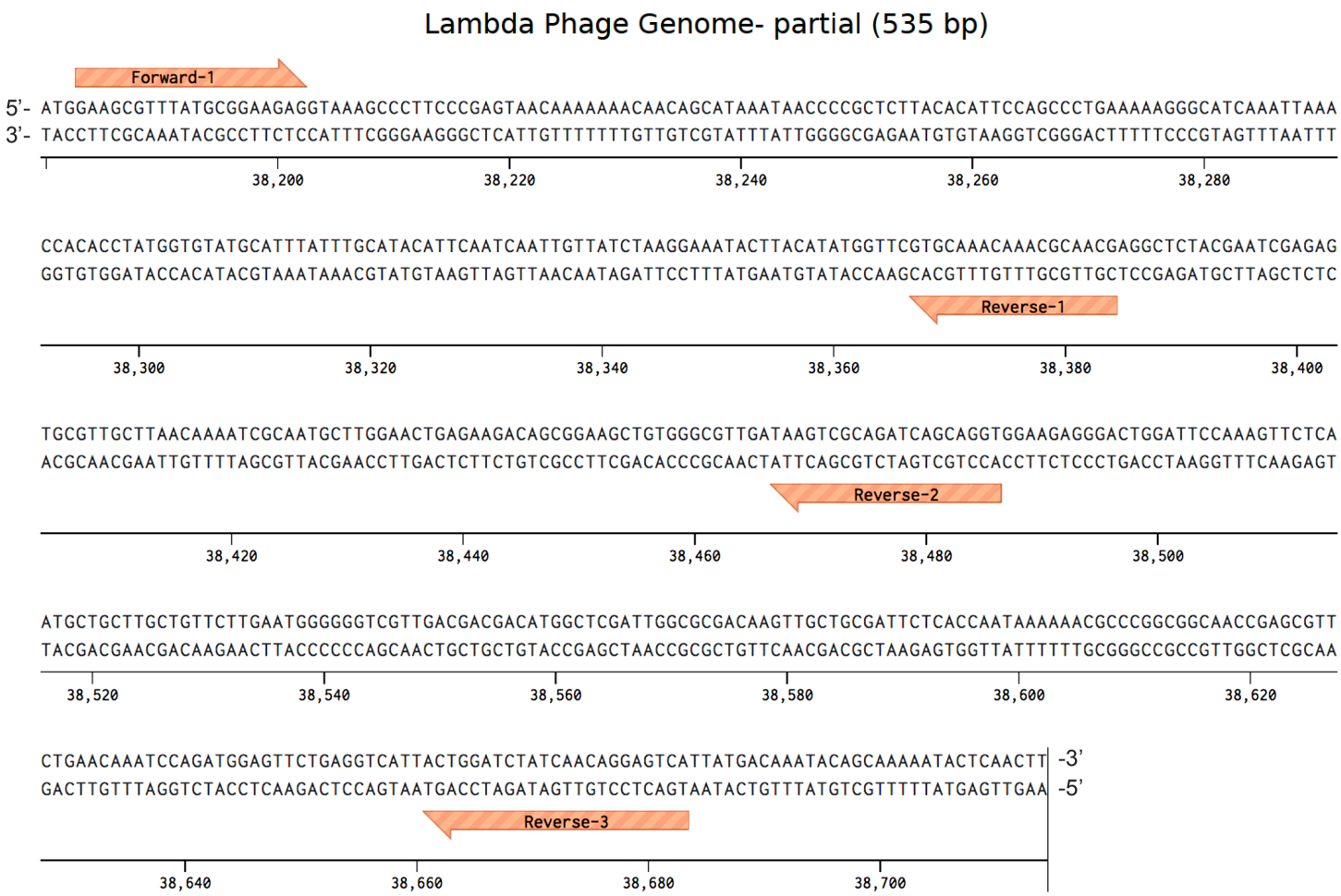
   *Speed of TaqFast polymerase: 100 bp/sec*

   *Length of longest fragment: 501 bp*

   *Extension time = 501 bp/(100 bp/sec) = 5.02 sec*

## Table 3. Forward and reverse primer sequences

| | Forward Primer | Reverse Primer | Fragment Length (bp) |
|---|---|---|---|
| Primer Set 1 | 5'-GAAGCGTTTATGCGGAAGAG-3' | 5'-CGTTGCGTTTGTTTGCAC-3' | *202* |
| Primer Set 2 | 5'-GAAGCGTTTATGCGGAAGAG-3' | 5'-ACCTGCTGATCTGCGACTTA-3' | *304* |
| Primer Set 3 | 5'-GAAGCGTTTATGCGGAAGAG-3' | 5'-TGACTCCTGTTGATAGATCCAGT-3' | *501* |


Lambda Phage Genome- partial (535 bp)
Forward-1
5'- ATGGAAGCGTTTATGCGGAAGAGGTAAAGCCCTTCCCGAGTAACAAAAAAACAACAGCATAAATAACCCCGCTCTTACACATTCCAGCCCTGAAAAAGGGCATCAAATTAAA
3'- TACCTTCGCAAATACGCCTTCTCCATTTCGGGAAGGGCTCATTGTTTTTTTGTTGTCGTATTTATTGGGGCGAGAATGTGTAAGGTCGGGACTTTTTCCCGTAGTTTAATTT
38,200
38,220
38,240
38,260
38,280
CCACACCTATGGTGTATGCATTTATTTGCATACATTCAATCAATTGTTATCTAAGGAAATACTTACATATGGTTCGTGCAAACAAACGCAACGAGGCTCTACGAATCGAGAG
GGTGTGGATACCACATACGTAAATAAACGTATGTAAGTTAGTTAACAATAGATTCCTTTATGAATGTATACCAAGCACGTTTGTTTGCGTTGCTCCGAGATGCTTAGCTCTC
Reverse-1
38,300
38,320
38,340
38,360
38,380
38,400
TGCGTTGCTTAACAAAATCGCAATGCTTGGAACTGAGAAGACAGCGGAAGCTGTGGGCGTTGATAAGTCGCAGATCAGCAGGTGGAAGAGGGACTGGATTCCAAAGTTCTCA
ACGCAACGAATTGTTTTAGCGTTACGAACCTTGACTCTTCTGTCGCCTTCGACACCCGCAACTATTCAGCGTCTAGTCGTCCACCTTCTCCCTGACCTAAGGTTTCAAGAGT
Reverse-2
38,420
38,440
38,460
38,480
38,500
ATGCTGCTTGCTGTTCTTGAATGGGGGGTCGTTGACGACGACATGGCTCGATTGGCGCGACAAGTTGCTGCGATTCTCACCAATAAAAAACGCCCGGCGGCAACCGAGCGTT
TACGACGAACGACAAGAACTTACCCCCCAGCAACTGCTGCTGTACCGAGCTAACCGCGCTGTTCAACGACGCTAAGAGTGGTTATTTTTTGCGGGCCGCCGTTGGCTCGCAA
38,520
38,540
38,560
38,580
38,600
38,620
CTGAACAAATCCAGATGGAGTTCTGAGGTCATTACTGGATCTATCAACAGGAGTCATTATGACAAATACAGCAAAAATACTCAACTT -3'
GACTTGTTTAGGTCTACCTCAAGACTCCAGTAATGACCTAGATAGTTGTCCTCAGTAATACTGTTTATGTCGTTTTTATGAGTTGAA -5'
Reverse-3
38,640
38,660
38,680
38,700

# Instructor's Preparation Guide

## Experimental Timing

From start to finish this lab takes around 90 minutes. Background material and pre-lab questions can be covered during a separate class period or assigned as homework.

- Pre-lab discussion and review of PCR principles and experimental protocol (20 minutes). *Students can work on calculating fragment sizes as homework, as an in-class exercise, or while the PCR protocol is running.*
- Set up and PCR reactions (10 minutes)
- Program, run, and monitor the MiniOne PCR System thermal cycler (25 minutes).
- *After the PCR protocol has completed, products can be used immediately or stored in the refrigerator until you are ready to run the gel electrophoresis.*
- Prepare TBE running buffer and agarose gel Using the MiniOne Casting System (15 minutes). *To save time students can cast their gels while the PCR protocol is running.*
- Gel electrophoresis of PCR products on the MiniOne Electrophoresis System (20 minutes).
- Documentation and interpretation of gels (10 minutes).

## Before the Lab

**This MiniLab provides enough materials to run 10 experiments. The following suggested preparation steps assume that you are going to run all 10 experiments.**

## Prepare Running Buffer

1. The kit provides 100 mL of Tris-Borate EDTA (TBE) Concentrate. We recommend diluting buffer in batches for accuracy.
   **Dilute 1 volume of the TBE Concentrate with 19 volumes of DI water** for TBE running buffer. Estimate that each experiment will need 135 mL of running buffer.

   **Sample calculation: (enough for 10 runs plus extra)**

   Final volume: 2000 mL
   TBE Concentrate needed: 2000 mL / 20 =100 mL
   DI water needed: 2000 mL – 100 mL = 1900 mL

2. Following the calculation, add 100 mL of TBE Concentrate to 1900 mL of DI water to get 2 L of TBE for running buffer.

3. Optional: Aliquot 135 mL of TBE running buffer for each group of students before class starts.

## Prepare Sample Aliquots

Aliquot the following reagents for each student group using the provided micro-centrifuge tubes:

- 45 µL FastTaq 2x Master Mix
- 7 µL Primer Set 1
- 7 µL Primer Set 2
- 7 µL Primer Set 3
- 7 µL $dH_2O$
- 25 µL Lambda DNA
- 12 µL MiniOne Marker
- 25 µL 5x Loading Dye

## PCR Materials For Each Group

- Aliquots of primers, $dH_2O$, FastTaq MasterMix, and Lambda DNA
- Four 0.2 mL PCR tubes
- 1 micropipette (2-20 µL) & 15 pipette tips
- PCR tube rack
- Fine point permanent marker

## Electrophoresis Materials For Each Group

- 1 MiniOne Casting System
- 1 MiniOne Electrophoresis System
- 1 micropipette (2-20 µL) & 10 pipette tips
- 1 Agarose GreenGel in-a-Cup
- 135 mL of TBE running buffer
- Loading Dye
- MiniOne Marker

## Common Workstation

- MiniOne PCR Systems
- Tablets with MiniOne PCR App
- Microwave
- Benchtop microcentrifuge

# 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 PCR machine.

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.

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 chamber and load 10 µL of each sample and the MiniOne Marker into the wells, keeping track of the placement using Table 7.

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.

### Table 6. Prepare the samples for gel analysis

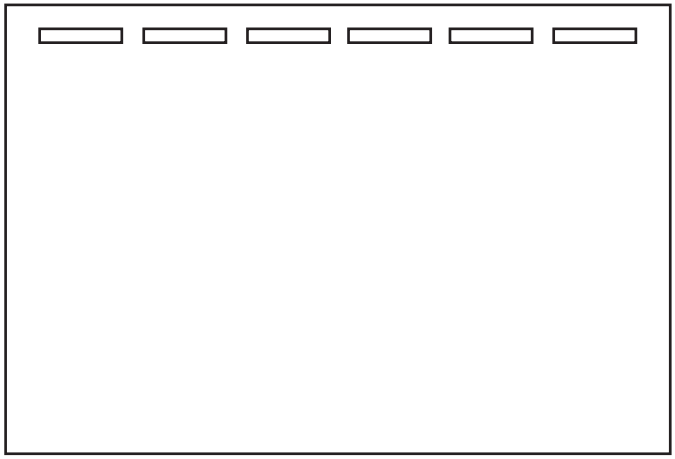
| Sample | Add 5x Loading Dye | Load on Gel |
|---|---|---|
| MiniOne Marker | ----- | 10 µL |
| Tube P1 | 5 µL | 10 µL |
| Tube P2 | 5 µL | 10 µL |
| Tube P3 | 5 µL | 10 µL |
| Tube -C | 5 µL | 10 µL |

# Data Collection Worksheet

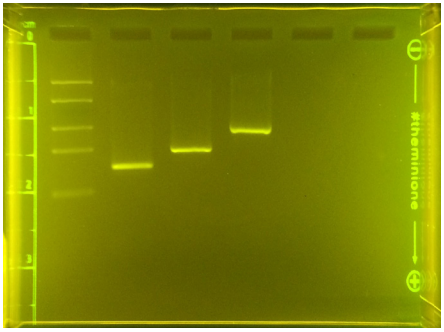
## Table 7. Record the sample loaded into each lane

| **Well** | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **Sample** | marker | PS1 | PS2 | PS3 | -C | |

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:



## Example Gel Image:



# 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?

   *Students should see bands corresponding to the 202, 304 and 501 bp fragments. If they see no band or bands of the wrong size this probably means they made a mistake in mixing the reagents or programming the machine. If they see a band in the negative control lane this means they probably mixed up the reagents between the tubes.*

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

   *If there were mistakes in preparing the reaction or programming the machine students might see multiple bands in the lane corresponding to non-specific PCR products or low molecular weight bands corresponding to primer dimer. They are unlikely to see the template DNA as a band because we are adding it at a very low concentration.*

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?

   *The purpose of the no template control is to make sure there is no contamination of the PCR reagents by DNA fragments or organisms that could also contain DNA that is amplified by your primers. This is especially important where the reaction is designed to detect very low copy numbers of the template, such as forensics or environmental sampling.*

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

   *Higher number of cycles means more DNA is being produced. You would see a brighter band at the same position on the gel. You would not see a band at a different molecular weight.*

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?

   *You would probably not see the 304 and 501 bp bands because the polymerase would not have time to copy these fragments during the elongation step.*

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?

   *One possible answer: probably not, because the three primer sets are targeting regions of the genome that overlap they would be competing with each other to bind and amplify this region. Since the lowest molecular weight fragment can be amplified from the higher molecular weight fragments but not the other way around, the 202 bp fragment would probably amplify at the expense of the others. Other answers are possible – this is just one way to get the students thinking about the mechanism of PCR.*

# 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 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 being used in your reaction. Typical settings 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 chemical building blocks of DNA. |
| Enzyme | An enzyme is a biological catalyst that speeds up a chemical 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 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.

# Additional MiniOne Products

## MiniOne Equipment

| Catalog # | Description | List Price |
|---|---|---|
| M1000 | MiniOne Electrophoresis Unit | $279.00 |
| M1010 | MiniOne Electrophoresis Classroom Package of 10 | $2490.00 |
| M2031 | MiniOne Microcentrifuge | $350.00 |
| M4000 | MiniOne PCR System | $799.00 |

## MiniLabs

| Catalog # | Description | Shelf Life | List Price |
|---|---|---|---|
| M3001 | Electrophoresis 101 - The "fun"-damentals of electrophoresis (10 groups) | 6 months | $39.00 |
| M3002 | Pipette Practicing Kit - Master the skills to load a gel (20 groups) | 6 months | $49.00 |
| M3003 | PTC Genetics - Mendelian inheritance and taste blindness (10 groups) | 6 months | $69.00 |
| M3004 | DNA Fingerprinting - Help a whale calf find her father (10 groups) | 6 months | $69.00 |
| M3005 | CSI Forensics - Solve the crime using DNA & other evidence (10 groups) | 6 months | $69.00 |
| M3006 | Forensic Science - Foodborne Outbreak Investigation AP Bio (10 groups) | 6 months | $120.00 |
| M6001 | PCR 101- Master PCR basics by amplifying DNA from a viral genome | 6 months | $99.00 |

## Consumables

| Catalog # | Description | Shelf Life | List Price |
|---|---|---|---|
| M3101 TBE | Tris-Borate EDTA (TBE) Concentrate, 500 mL | 12 months | $18.00 |
| M3102 TBE | 1% GreenGel-in-a-Cup with 100 mL TBE Concentrate, 10 cups | 6 months | $21.00 |
| M3103 TBE | 2% GreenGel-in-a-Cup with 100 mL TBE Concentrate, 10 cups | 6 months | $21.00 |
| M3104 | MiniOne DNA Marker, 500 µL/ 1 vial, 100, 300, 500, 1000 and 2000 bp | 12 months | $35.00 |
| M3105 | Electrophoresis Grade Agarose, 5 g | 24 months | $15.00 |
| M3106 | Electrophoresis Grade Agarose, 25 g | 24 months | $45.00 |
| M3108 | 0.6 mL Microcentrifuge Tubes, Assorted Colors, 200/pk | N/A | $10.00 |
| M3110 | 1.7 mL Microcentrifuge Tubes, Assorted Colors, 200/pk | N/A | $10.00 |
| M3111 | 1 – 200 µL Universal Fit Micropipette Tips, 250/pk | N/A | $9.50 |
| M3112 | 1 – 10 µL Micropipette Tips, 250/pk | N/A | $9.50 |
| M3113 | GelGreen DNA Stain, 10,000X stock, 50 µL | 12 months | $20.00 |
| M3114 | GelGreen DNA Stain, 10,000X stock, 500 µL | 12 months | $100.00 |
| M3115 | 5X Sample Loading Dye w/ Orange G and Xylene Cyanol, 10 mL | 12 months | $10.00 |
| M3116 | 1 Kb DNA Ladder, 1000 µL | 12 months | $65.00 |
| M3117 | 100 bp Ladder, 1000 µL | 12 months | $65.00 |

## Pipettes

| Catalog # | Description | U.S. List Price | 10+ Units |
|---|---|---|---|
| M2008 | Variable Volume, 2–20 µL | $89.00 | $79.00 |
| M2010 | Variable Volume, 20–200 µL | $89.00 | $79.00 |
| M2011 | Variable Volume, 100–1000 µL | $89.00 | $79.00 |
| M2012 | Variable Volume, 1–10 µL | $89.00 | $79.00 |

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