Student Manual

Introduction to PCR — The Polymerase Chain Reaction

You are about to perform a procedure known as PCR[1] to amplify a specific sequence of your own DNA in a test tube. You will be looking for a particular piece of DNA that is present in the genes of many, but not all, people. Analysis of the data generated in this laboratory will enable you to determine whether or not you carry this specific DNA sequence.

The **genome**, composed of DNA, is our hereditary code. This is the so-called blueprint that controls much of our appearance, behavior, and tendencies. **Molecular biology** is the study of genes and the molecular details that regulate the flow of genetic information from DNA to RNA to proteins, from generation to generation. **Biotechnology** uses this knowledge to manipulate organisms' (microbes, plants, or animals) DNA to help solve human problems.

Within the molecular framework of biology, DNA, RNA, and proteins are closely tied to each other. Because proteins and enzymes ultimately play such a critical role in the life process, scientists have spent many lifetimes studying proteins in an attempt to understand how they work. With this understanding, it was believed we could cure, prevent, and overcome disease and physical handicaps as well as explain exactly how and why organisms exist, propagate, and die. However, the complete answer to how and why does not lie solely in the knowledge of how enzymes function; we must learn how they are made. If each enzyme is different, then what controls these differences and what is the blueprint for this difference? That answer lies within our genome, or genetic code.

Thus, you may realize why researchers today, in an attempt to understand the mechanisms behind the various biological processes, study nucleic acids as well as proteins to get a complete picture. In the last 20 years, many advances in nucleic acid techniques have allowed researchers to study the roles that nucleic acids play in biology. It took the imagination and hard work of many scientists to reveal the answers to one of the most mysterious puzzles of life — understanding the mechanisms that control how DNA is translated into proteins within living cells.

Before Beginning This Lab, See If You Can Answer the Following Questions

How is DNA faithfully passed on from generation to generation? What causes genetic disease in some people but not others? How do scientists obtain DNA to study? What secrets can DNA tell us about our origins? What human problems can an understanding of DNA help us solve? Should we unlock the secrets held in this most basic building block of life?

PCR Set the Stage for a Scientific Revolution

In 1983, Kary Mullis[2] at Cetus Corporation developed the molecular biology technique that has since revolutionized genetic research. This technique, called the **polymerase chain reaction** (PCR), transformed molecular biology into a multidisciplinary research field within 5 years of its invention. Before PCR, the molecular biology techniques used to study DNA required such a high level of expertise that relatively few scientists could use them.

The objective of PCR is to produce a large amount of DNA in a test tube (in vitro), starting from only a trace amount. Technically speaking, this means the controlled enzymatic amplification of a DNA sequence, or gene, of interest. The template strands can be any form of DNA, such as genomic DNA. A researcher can use tiny amounts of genomic DNA

from a drop of blood, a single hair follicle, or a cheek cell, and make enough DNA to study. In theory, only a single template strand is needed to copy and generate millions of new identical DNA molecules. Prior to PCR, this would have been impossible. It is the ability to amplify the precise sequence of DNA of interest that is the true power of PCR.

PCR has made an impact on four main areas of genetic research: gene mapping; cloning; DNA sequencing; and gene detection. PCR is now used as a medical diagnostic tool to detect specific mutations that may cause genetic disease;[3] in criminal investigations and courts of law to identify suspects,[4] and in the sequencing of the human genome.[5] Prior to PCR, the use of molecular biology techniques for therapeutic, forensic, pharmaceutical, agricultural, or medical diagnostic purposes was neither practical nor cost-effective. The development of PCR transformed molecular biology from a difficult science to one of the most accessible and widely used disciplines of **biotechnology**.

Two methods for DNA template preparation are provided in the manual. Your instructor will indicate which exercise to follow. Now, let's extract some of your own DNA.

Lesson 1 Cheek Cell DNA Template Preparation

To obtain DNA for use in the polymerase chain reaction (PCR) you will extract the DNA from your own living cells. It is interesting to note that DNA can be also extracted from mummies and fossilized dinosaur bones. In this lab activity, you will isolate DNA from epithelial cells that line the inside of your cheek. To do this, you will rinse your mouth with a saline (salt) solution, and collect the cells using a centrifuge. You will then boil the cells to rupture them and release the DNA they contain. To obtain pure DNA for PCR, you will use the following procedure:

The cheek cells are transferred to a micro test tube containing **InstaGene™ matrix**. This particulate matrix is made up of negatively charged, microscopic beads that chelate, or grab, metal ions out of solution. It traps metal ions, such as Mg^{2+}, which are required as catalysts or **cofactors** in enzymatic reactions. Your cheek cells will then be **lysed**, or ruptured, by heating to release all of their cellular constituents, including enzymes that were once contained in the cheek-cell lysosomes. Lysosomes are sacs in the cytoplasm that contain powerful enzymes, such as **DNases**, which are used by cells to digest the DNA of invading viruses. When you rupture the cells, these DNases can digest the released DNA. However, when the cells are lysed in the presence of the chelating beads, the cofactors are adsorbed and are not available to the enzymes. This virtually blocks enzymatic degradation of the extracted DNA so you can use it as the template in your PCR reaction.

You will first suspend your isolated cheek cells in the InstaGene matrix and incubate them at 56°C for 10 minutes. This preincubation step helps to soften plasma membranes and release clumps of cells from each other. The heat also inactivates enzymes, such as DNases, which can degrade the DNA template. After this 10 minute incubation period, place the cells in a boiling (100°C) water bath for 5 minutes. Boiling ruptures the cells and releases DNA from their nuclei. You will use the extracted genomic DNA as the target template for PCR amplification.

Lesson 1 Cheek Cell DNA Template Preparation (Lab Protocol)

Workstation Checklist

Materials and supplies required at the workstations prior to beginning this exercise are listed below.

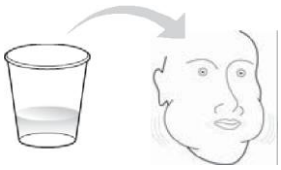
Student Workstation	Quantity per Station	(✓)
1.5 ml micro test tubes	4	❒
Screwcap tubes with 200 µl InstaGene matrix	4	❒
Foam micro test-tube holder	2	❒
P-20 micropipet, 2–20 µl	1	❒
Pipet tips (filter type), 2–20 µl	4	❒
Permanent marker	1	❒
Copy of Quick Guide or protocol	1	❒
Waste container	1	❒
Cups with 10 ml 0.9% saline	4	❒

Instructor's Workstation	Quantity per Class	
P-20 micropipet, 2–20 µl	1	❒
P-200 micropipet, 20–200 µl	1	❒
Pipet tips (filter type), 2–20 µl	1 box	❒
Pipet tips (filter type), 20–200 µl	1 box	❒
Water baths (56 and 100°C)	1 each	❒
Microcentrifuge	1	❒
or mini centrifuge	4	❒
Vortexer (optional)	1	❒

Lesson 1 Cheek Cell DNA Template Preparation (Lab Protocol)

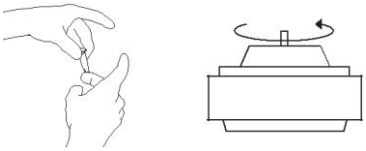
1. Each member of your team should have 1 screwcap tube containing 200 µl InstaGene™ matrix, 1.5 ml micro test tube, and a cup containing 10 ml of 0.9% saline solution. Label one of each tube and a cup with your initials.

2. Do not throw away the saline after completing this step. Pour the saline from the cup into your mouth. Rinse vigorously for 30 seconds. Expel the saline back into the cup.



3. Set a P-1000 micropipet to 1,000 µl and transfer 1 ml of your oral rinse into the micro test tube with your initials. If no P-1000 is available, carefully pour ~1 ml of your swished saline into the micro test tube (use the markings on the side of the micro test tube to estimate 1 ml).

4. Spin your tube in a balanced centrifuge for 2 minutes at full speed. When the centrifuge has completely stopped, remove your tube. You should be able to see a pellet of whitish cells at the bottom of the tube. Ideally, the pellet should be about the size of a match head. If you can't see your pellet, or your pellet is too small, pour off the saline supernatant, add more of your saline rinse, and spin again.



Centrifuge

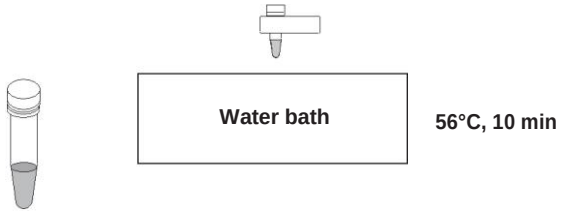
5. Pour off the supernatant and discard. Taking care not to lose your cell pellet, carefully blot your micro test tube on a tissue or paper towel. It's ok for a small amount of saline (~50 µl, about the same size as your pellet) to remain in the bottom of the tube.

6. Resuspend the pellet thoroughly by vortexing or flicking the tubes until no cell clumps remain.

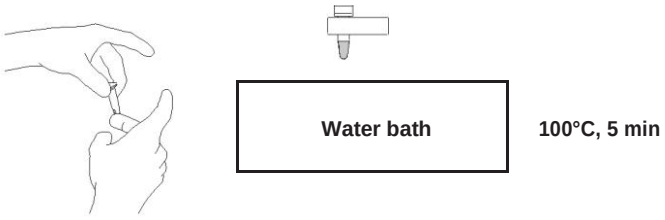
7. Using an adjustable volume micropipet set to 20 µl, transfer your resuspended cells into the screwcap tube containing the InstaGene with your initials. You may need to use the pipet a few times to transfer all of your cells.

8. Screw the caps tightly on the tubes. Shake or vortex to mix the contents.

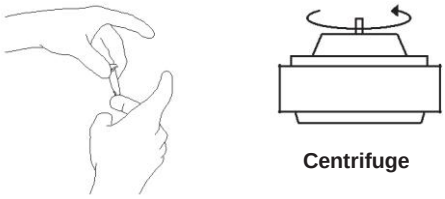
9. Place the tubes in the foam micro test-tube holder. When all members of your team have collected their samples, float the holder with tubes in a 56°C water bath for 10 minutes. At the halfway point (5 minutes), shake or vortex the tubes several times. Place the tubes back in the water bath for the remaining 5 minutes.



10. Remove the tubes from the water bath and shake them several times. Now float the holder with tubes in a 100°C water bath for 5 minutes.



11. Remove the tubes from the 100°C water bath and shake or vortex several times to resuspend the sample. Place the eight tubes in a balanced arrangement in a centrifuge. Pellet the matrix by spinning for 5 minutes at 6,000 x g (or 10 minutes at 2,000 x g).



12. Store your screwcap tube in the refrigerator until the next laboratory period, or proceed to step 2 of Lesson 2 if your teacher instructs you to do so.

Lesson 1 Sample Preparation

Processing Cheek Cell and Hair Follicle Samples to Obtain a Genomic DNA Template for PCR

A. InstaGene matrix: What function does it perform?

InstaGene matrix consists of a suspension of negatively charged Chelex® microscopic beads, which bind divalent cations like magnesium (Mg^{2+}). It is important to remove divalent cations from students' genomic DNA samples because the cations assist enzymes that degrade the DNA template. When cheek cells or hair follicles are lysed and boiled in the presence of InstaGene matrix, the divalent cations released from the cells bind to the beads, and the heat inactivates the DNA-degrading enzymes. The beads are then pelleted by centrifugation. The supernatant, which contains clean, intact genomic DNA, can be used as the template in the PCR reactions.

The beads in the InstaGene matrix quickly settle out of solution. It is extremely important that the vial of matrix be thoroughly mixed before pipetting aliquots for each student workstation, so that the aliquots contain equivalent amounts of beads.

Each student will prepare genomic DNA from cheek cells isolated using a saline mouthwash or from hair follicles. For students using the cheek cell protocol, 1 ml of cells collected using the mouthwash should provide sufficient material for DNA preparation. Some students may need to use 2 ml or more of the saline mouthwash to obtain sufficient cells to prepare DNA. Please note: it is **not** recommended to use more than 3 ml of the saline mouthwash to prepare DNA, (see 'Interpretation of Results and Troubleshooting Guide' on page 30). Once the cells have been spun in a centrifuge, a cell pellet about the size of a matchhead should yield enough cells for subsequent steps. Eating just prior to cell collection is not recommended, as food particles may make cell preparation more difficult.

Troubleshooting

Multiple explanations can account for poor student results

1. **Inadequate collection of cheek cells**. A visible cell pellet about the size of a match head should be obtained following centrifugation of the saline mouthwash. If no cell pellet is visible, or the pellet is too small, additional swished saline may be centrifuged until a pellet of the desired size is obtained. However, it is not recommended to collect more than 3 ml of cells (see point below).
2. **Excessive number of cells**. Just as too few cells will yield insufficient genomic DNA, an excessive number of cells will saturate the capacity of the InstaGene, resulting in little or no amplification.
3. **InstaGene matrix not transferred**. Each workstation is supplied with tubes of InstaGene matrix that were aliquotted by the instructor and placed on ice. These tubes of matrix must be mixed prior to each pipetting to bring the beads up into suspension. If no beads were transferred into the student's tube, the divalent cations will not be removed from the genomic DNA preparation, and the PCR reaction will be inhibited.
4. **Carryover of InstaGene into PCR reaction**. Although the beads in the InstaGene matrix are required for the DNA template preparation, it is critical that none of theInstaGene matrix be carried over into the PCR reaction. If beads are transferred intothe PCR tube, the magnesium ions needed by the Taq polymerase will be removed,and the PCR reaction will be inhibited.

If a P-1000 micropipet is not available, students may carefully pour ~1 ml of their swished saline into a micro test tube. The gradations on the side of the micro test tube may be used to judge the amount of liquid in the tube.

If the DNA samples will not be amplified within 24 hours, they can be stored in the refrigerator in the InstaGene matrix for up to 1 week. For longer storage, place samples in the freezer to prevent DNA degradation. Before the samples are used in PCR, the beads should be re-pelleted by centrifugation just prior to making up the PCR reactions. However, processing the samples within 24 hours is recommended. See the next steps for processing tips.

B. Genomic DNA Preparation from cheek cells or hair follicles

For students using the cheek cell DNA protocol, cells are collected using a saline mouthwash. For students using the hair follicle DNA protocol, it is recommended that students collect two hairs for genomic DNA preparation.

C. Incubation: What are the functions of each incubation step?

The preincubation step is carried out at 56°C and performs two functions:

1. Heating the cell suspension aids in the breakup of connective tissue that holds the cells together. Breaking up the tissue makes the cells easier to lyse during the subsequent 100°C incubation step.
2. Preincubation at 56°C inactivates DNases, enzymes that are naturally present in the cell suspensions, and which could degrade the genomic DNA and inhibit the PCR reactions.

Heating the cell samples to 100°C ruptures the cell membranes, thereby releasing the cellular contents, which include genomic DNA. The genomic DNA serves as the template in the PCR reactions.

Lesson 1 DNA Template Preparation

Focus Questions

1. How would you make 500mL of a 0.9% salt solution? Show your work
2. Why do you think we are using a salt solution for the mouthwash step?
3. Thinking back to your biotech experience in Biology, write down easy to follow instructions for how to use a micropipette. (Include how to withdraw a sample from a microfuge tube (reaction tube) taking it up into the micropipette, and how to expel the sample from the micropipette into a reaction tube.
4. According to the lab, which micropipettes will we be using in step 3 and 7? Wha is the range of each? From top down, which three digits will be displayed on the micropipette in step 3 and 7?
5. Why is it necessary to chelate the metal ions from solution during the boiling/lysis step at 100°C? What would happen if you did not use a chelating agent such as the InstaGene matrix?
6. What is needed from the cells for PCR?
7. What structures must be broken to release the DNA from a cell?
8. Why do you think the DNA is stored cold with the InstaGene matrix after boiling the samples?
9. Be sure you are able to describe why we are doing each step of the lab procedure.

Informed Consent Release

Date: _____

Dear Parent(s) or Guardian,

The __AP Biology_ class at __Lynbrook High__ school has the opportunity to participate in a class exercise in which an important technique in biotechnology will be used to analyze the students' DNA. The technique the students will be using is called the Polymerase

Chain Reaction (PCR). It is a method by which a particular piece of DNA can be amplified many million-fold. PCR has a number of applications in the scientific community, including uses in forensics, diagnostics, parentage testing, and evolutionary studies. It is used by forensic laboratories for the identification of possible suspects involved with a crime. It is used for the diagnosis of different genetic diseases. It is routinely used in most molecular biology laboratories for the cloning and characterization of specific genes.

In this laboratory protocol, students will be isolating DNA from their own cheek cells. They will then apply the PCR technique to amplify a particular segment of their DNA. This segment is not known to be associated with any genetic disease and variation between individuals in this region is in no way an indicator of health or genetic fitness. The results of this particular lab exercise are for teaching purposes only and will NOT be used for any diagnostic or identification purposes.
Your student's privacy will be protected. The student's name will not be linked to his/her DNA and the results of the lab exercise will remain anonymous.

Participation is voluntary. By signing this permission form, you are allowing your student to participate in this exciting learning experience. If you have any concerns or questions, please contact me at nicole_dellasantina@fuhsd.org.
Sincerely,

Ms. Della Santina

_____
Print Student's Name

_____ _____
Student's Signature Date

_____ _____
Parent's Signature Date