

Bio 3A Lab: DNA Isolation and the Polymerase Chain Reaction

Objectives

- Understand the process of DNA isolation
- Perform DNA isolation using cheek cells
- Use thermal cycler and *Taq* polymerase to perform DNA amplification
- Gain an understanding of the PCR process

Introduction

You are about to perform a procedure known as the polymerase chain reaction (PCR). This procedure will be used to amplify a specific sequence of your own DNA in a test tube. This particular piece of DNA 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 your genomic DNA carries this piece of DNA, or not.

The genome, composed of DNA, is our hereditary code. This is the so-called "hard-wiring", the blueprint that controls much of why we look like we do, why we act like we do, and how we do the things we do. Molecular biology is the study of genes and the molecular details that regulate the flow of genetic information from DNA, to RNA and 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 in order to get a complete picture. In the last 20 years, many advances in the areas of nucleic acid techniques have allowed researchers the means 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 diseases 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 at Cetus Corporation developed the molecular biology technique that has since revolutionized genetic research. This technique, termed 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 double-stranded DNA such as genomic DNA. A researcher can take trace amounts of genomic DNA from a drop of blood, a single hair follicle or cheek cell (in theory, only a single template strand is needed to copy and generate millions of new identical DNA molecules) and make enough to study. 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, is used in criminal investigations and courts of law to identify suspects on the molecular level, and has been a powerful tool in the sequencing of the human genome. Prior to PCR the use of molecular biology techniques for therapeutic, forensic, pharmaceutical, agricultural or medical diagnostic purposes was not practical nor cost effective. The development of PCR technology transformed molecular biology from a difficult science to one of the most accessible and widely used disciplines of biotechnology.

Procedure 1: DNA Extraction and Template Preparation

To obtain DNA for use in the polymerase chain reaction 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 be isolating DNA from epithelial cells that line the inside of your cheek. This is accomplished by using a sterile pipet tip to gently scrape the inside of both your cheeks about 10 times each to scoop up the cells lining the surface. 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 in the pipet tip are transferred into a micro test tube containing 200 μ l of InstaGene matrix. This particulate matrix is made up of negatively charged microscopic beads that "chelate", or grab metal ions out of solution. It acts to trap 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 within the cells cytoplasm that contain powerful enzymes, such as DNAases, which are used by cells to digest the DNA of invading viruses. When you rupture the cells, these DNAases can digest the released DNA of interest. 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 all enzyme degradation of the extracted DNA and results in a population of intact genomic DNA molecules that will be used as the template in your PCR reaction.

Your isolated cheek cells are first suspended in the InstaGene matrix and incubated at 56 °C for 10 minutes. This "pre-incubation" step helps to soften the plasma membranes and release clumps of cells from each other. The increased temperature also acts to inactivate enzymes such as DNAases, which will degrade the DNA template. After this 10 minute incubation period, the cells are then placed into a boiling (100 °C) water bath for 6 minutes. The boiling ruptures the cells and releases the DNA from the cell nucleus. Your extracted genomic DNA will then be used as the target template for PCR amplification.

Workstation Checklist

These materials and supplies should be present at your workstation prior to beginning this protocol:

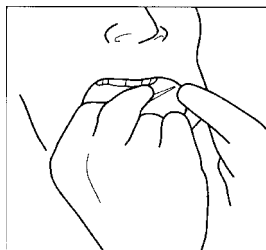
Student workstations	Number
Screw cap tubes with InstaGene™ matrix	8
Styrofoam microtube rack	2
P-20 micropipet	1
P-200 micropipet	1
Pipet tips (filter type) 20-200 µl	1 box
Lab marker	1
Waste container	1
Copy of Quick Guide or protocol	1

These materials, supplies, and equipment should be in a common location:

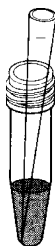
P-200 micropipet	1 - 8
Pipet tips (filter type) 20-200 µl	1 box
Water baths (56 and 100 °C)	2
Centrifuge	1

Protocol for DNA extraction

- Each member of your team should have two screw cap tubes, each containing 200 µl of InstaGene matrix. Label the tube on the cap and on the side with your initials. In addition, label the tubes as **"tube 1"** and **"tube 2"**. Each person should wash his or her hands before beginning step 2.
- Using a sterile 20-200 µl filter pipet tip, gently scrape the inside of both cheeks 10 times each with the tip. This is most easily done by pinching and extending the corner of your mouth with one hand, and scraping the cheek with the tip in the other hand. Use firm, but gentle pressure. The goal is to remove the surface layer of epithelial cells from your cheek lining. You should see a small volume of white cells in the pipet tip.



- Place the tip that contains your cheek cells into the screw cap tube labeled as **"tube 1"**.



- Using a second sterile 20-200 µl filter pipet tip, gently scrape the inside of both cheeks 10 times each with the tip. Place the tip that contains your cheek cells into your screw cap tube labeled as **"tube 2"**.

5. Place the tips on the end of a P-200 micropipet that is set on a 100 μ l setting. Pipet up and down 5 times into the InstaGene matrix - the action of pipetting up and down mixes and transfers your cheek cells into the InstaGene matrix.
6. Screw the lid tightly on your tubes and place in the foam microtube holder. When all members of your team have collected their samples, float the rack and tubes in a 56°C water bath for 10 min. At the halfway point (5 minutes), remix the contents of the tubes by shaking or vortexing several times and place back in the water bath for the remaining 5 minutes. We will use the flick and tap technique.
7. Remove the tubes from the water bath and remix by shaking the tubes several times. Now float the rack of tubes in a 100°C water bath for 6 minutes.
8. After 6 minutes, remove the tubes from the 100°C water bath and shake or vortex gently several times to resuspend the sample. Place the eight tubes in a balanced arrangement in the centrifuge. Pellet the matrix by spinning for 5 minutes at 6,000 x g (or 10 minutes at 2,000 x g) in the centrifuge.
9. Using a 200 μ l pipet tip, remove 170 μ l of the supernatant from one of your screw cap tubes and transfer the supernatant into the other. You will now be left with one screw cap tube that contains your isolated genomic DNA.
10. Store your screw cap tube in the refrigerator until the next laboratory period or proceed to Procedure 2 if you have enough time to set up and perform PCR reactions.

Procedure 2: PCR Amplification of DNA

It is estimated that there are 30,000-50,000 individual genes in the human genome. The true power of PCR is the ability to target and amplify a specific piece of DNA (or gene) out of a complete genome.

The recipe for a PCR amplification of DNA requires a simple mixture of ingredients. To replicate a given piece of DNA, the reaction mixture requires the following components.

1. Intact DNA template-containing the sequence of DNA to be amplified.
2. Individual deoxynucleotide bases (A, T, G and C-raw material of DNA.
3. DNA polymerase-an enzyme that assembles the nucleotides into a new DNA chain.
4. Magnesium ions -a cofactor (catalyst) required by DNA polymerase to create the DNA chain.
5. Oligonucleotide primers-pieces of DNA complementary to the template that tell DNA polymerase exactly where to start making copies.
6. Salt buffer-provides the optimum ionic environment and pH for the PCR reaction.

The template DNA in this exercise is genomic DNA that was extracted from your cheek cells. When all the other components are combined under the right conditions a copy of the original double stranded template DNA molecule is made-doubling the number of template strands. Each time this cycle is repeated, copies are made from copies and the number of template strands doubles-from 2 to 4 to 8 to 16 and so on-until after 20 cycles there are 1,048,554 exact copies of the target sequence.

PCR makes use of the same basic processes that cells use to duplicate their DNA:

1. Complementary DNA strand hybridization
2. DNA strand synthesis via DNA polymerase

The two DNA primers provided in this kit are designed to flank, or bracket, a DNA sequence within your genome and thus provide the exact start signal for the DNA polymerase to "zero-in on" and begin synthesizing (replicating) copies of that target DNA. Complementary strand hybridization takes place when the two different **primers** anneal, or hybridize, to each of their respective complementary base pair sequences on the template DNA molecule.

The primers are two short single-stranded DNA molecules (23 bases long); one that is complementary to a portion of the 5'-3' strand, and another that is complementary to the portion of the 3'-5' strand of your DNA. These primers hybridize to the separated template strands and serve as starting points for DNA amplification.

The enzyme, called *Taq* DNA polymerase, extends the annealed primers' sequence by "reading" the strand and synthesizing the complementary sequence. In this way, *Taq* replicates the template DNA strand(s). This polymerase has been isolated from a heat stable bacterium (*Thermus aquaticus*) which in nature lives within the steam vents in the Yellowstone National Park. For this reason the enzymes within these bacteria have evolved to withstand high temperatures (94°C) also used in each cycle of the PCR reaction.

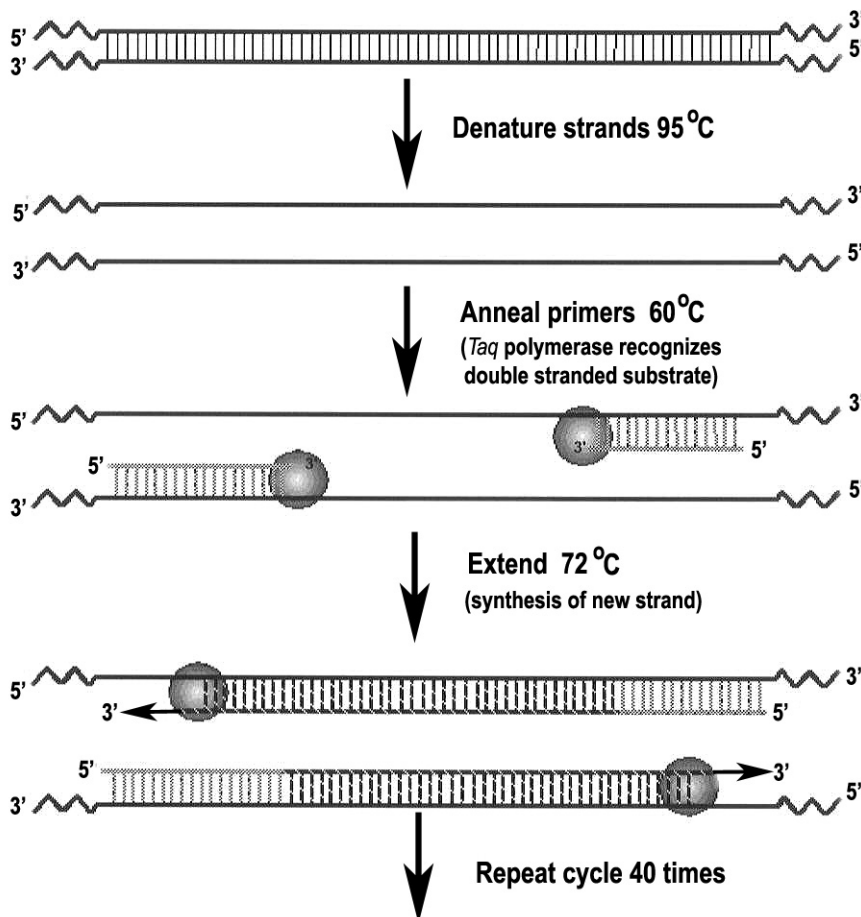


Figure One. Polymerase Chain Reaction (PCR), one cycle

The PCR Process

PCR amplification includes three main steps, **denaturation step**, an **annealing step** and an **extension step** (these steps are summarized in Figure 1). During denaturation, the reaction mixture is heated to 94°C for 1 minute, which results in the melting out or separation of the double-stranded DNA template into two single stranded molecules. In PCR amplification, DNA templates must be separated before the polymerase can generate a new copy. The harsh temperature required to melt the DNA strands normally would destroy the activity of most enzymes, but because *Taq* polymerase has been isolated from bacteria that thrive in the high temperatures of hot springs, it remains active.

During the annealing step, the oligonucleotide primers "anneal to" or find their complementary sequences on the two single-stranded template strands of DNA. In these annealed positions, they can act as "primers" for *Taq* DNA polymerase. They are called primers because they "prime" the single stranded DNA molecules by providing a short sequence of double stranded DNA for *Taq* polymerase to extend and build a new complementary strand. Binding of the primers to their template sequences is also highly dependent on temperature. In this lab exercise, a 60°C annealing temperature is optimum for primer binding.

During the extension step, the job of *Taq* DNA polymerase is to add nucleotides (A, T, G or C) one at a time to the primer to create a complementary copy of the DNA template. During polymerization, the reaction temperature is increased to 72°C, the temperature that produces optimal *Taq* polymerase activity. The three steps of denaturation, annealing, and extension, combine to form one "cycle" of PCR, and a complete PCR amplification undergoes 40 cycles.

The entire 40 cycle reaction is carried out in a test tube which is placed into the thermal cycler (also called a Gene Cycler). The Gene Cycler contains an aluminum block that holds the samples and can be rapidly heated and cooled across extreme temperature differences. The rapid heating and cooling of this thermal block is defined as "**temperature cycling**" or "**thermal cycling**".

Temperature Cycle =

Denaturation Step (94°C) + Annealing Step (60°C) + Extension Step (72°C)

Workstation Checklist

The following materials and supplies that should be present at your workstation prior to beginning this protocol:

The following materials and supplies that should be present at your workstation prior to beginning this protocol:	Number
PCR tubes	4
Microtubes, capless	4
Master mix	1
P-20 micropipet	1
Pipet tips (filter type), 2-20 µl	8 tips
Foam microtube rack	2
Lab marker	1
Ice bucket with ice	1
Waste container	1
Copy of Quick Guide or protocol	1
Instructor's (common) workstation	

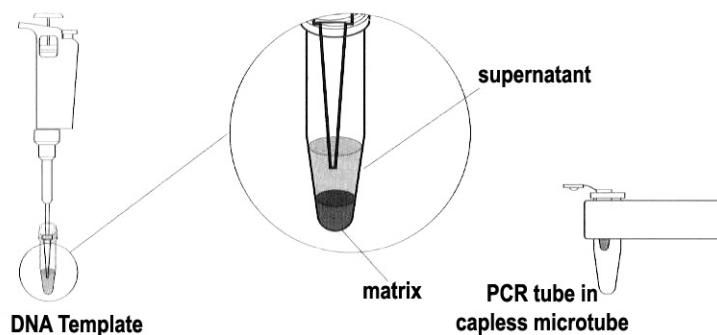
Centrifuge	1
Thermal cycler	1

Protocol for PCR amplification

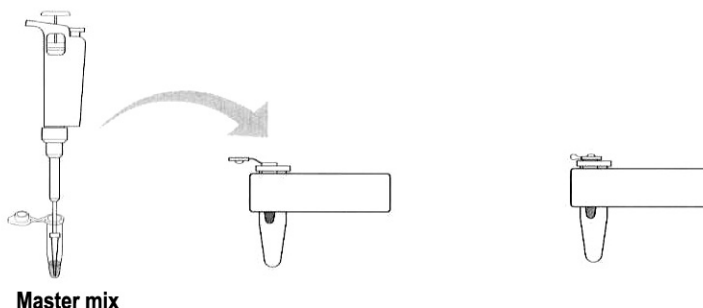
1. Obtain your screw cap tube that contains your genomic DNA template from the refrigerator and place on your lab bench. Centrifuge your tubes for 2 minutes at 6,000 x g or for 5 minutes at 2,000 x g in a centrifuge.
2. Each member of the team should obtain a PCR tube and capless microtube adaptor. Label each tube with your initials and place the PCR tube into the capless microtube (which acts as a convenient holder for the small PCR tube). Place the PCR tube in its holder in the foam rack.



3. Transfer 20 μ l of your DNA template from your screw cap tube into the bottom of the PCR tube. **Do not transfer any of your matrix beads into the PCR reaction-the reaction will be inhibited.**



4. Locate the tube of yellow PCR master mix (labeled "Master") in your ice bucket. Transfer 20 μ l of the master mix into your PCR tube. Mix by pipetting up and down 2-3 times.



5. Remove your PCR tube from its holder and place the tube in the thermal cycler.
6. When all of the PCR samples are in the thermal cycler, the teacher will begin the PCR reaction. The reaction will undergo 40 cycles of amplification, which will take approximately 3 hours.