Name _____ Per___

Ah, Lou! There really are differences between us!

Introduction

The human genome (the total sum of our genetic makeup) is made up of approximately 6 billion base pairs distributed on 46 chromosomes. All cells in your body, except red blood cells, sperm, and eggs, contain these 46 chromosomes (sperm and egg cells contain only 23 chromosomes). Only 3 to 10 percent of this enormous amount of DNA is used to directly code for the proteins required for supporting cellular metabolism, growth, and reproduction. The protein-encoding regions are scattered throughout the genome. Genes may be separated by several thousand bases. Furthermore, most genes in the human organism are themselves broken into smaller protein-encoding segments called exons, which, in many cases, have hundreds or thousands of base pairs intervening between them. These intervening regions are called introns and they make up between 90 to 97 percent of the entire genome. Since these non-coding areas such as introns have no defined role, they have been referred to as "Junk DNA". Whatever their function may entail in the genome, closer examination of these intervening DNA regions has revealed the presence of unique genetic elements that are found in a number of different locations. One of the first such repeating elements identified is *Alu*.

Alu repeats are approximately 300 base pairs in length. They got their name from the fact that most carry within them the base sequence AGCT; the recognition site for the *Alu* I restriction endonuclease, a type of enzyme that cuts DNA at a specific site. There are over 500,000 *Alu* repeats scattered throughout the human genome. On average, one can be found every 4,000 base pairs along a human DNA molecule. How they arose is still a matter of speculation but evidence suggests that the first one may have appeared in the genome of higher primates about 60 million years ago. Approximately every 100 years since then, a new *Alu* repeat has inserted itself in an additional location in the human genome. *Alu* repeats are inherited in a stable manner; they come intact in the DNA your mother and father contributed to your own genome at the time you were conceived. Some *Alu* repeats are fixed in a population, meaning all humans have that particular *Alu* repeat. Others *Alu* repeats are said to be dimorphic – some individuals have the repeat at a particular chromosomal location while others do not.

In the following laboratory exercise, you will use PCR (Polymerase Chain Reaction) to amplify a dimorphic *Alu* repeat (designated "Alu" PV92), which is present on chromosome 16. You will use your own DNA as template for this experiment. DNA is easily obtained from the human body. A simple saltwater mouth rinse will release cheek cells, from which you will extract the DNA. After you amplify the *Alu* repeat region, you will determine whether or not you carry this particular *Alu* sequence on one, both, or none of your number 16 chromosomes. This will be accomplished by separating the DNA in your PCR sample on an agarose gel via electrophoresis, a process that separates DNA by size.

Lesson 2 PCR Amplification

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 make millions of copies of (or **amplify**) a specific piece of DNA (or gene) out of a complete genome. In this activity, you will amplify a region within your chromosome 16.

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

1. DNA template — containing the intact sequence of DNA to be amplified
2. Individual deoxynucleotides (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 cells.
The complete master mix contains Taq DNA polymerase, deoxynucleotides, oligonucleotide primers, magnesium ions, and buffer. 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 1,048,576 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 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 bind to each of their respective complementary base sequences on the template DNA.

The primers are two short single-stranded DNA molecules (23 bases long), one that is complementary to a portion of one strand of the template, and another that is complementary to a portion of the opposite strand. These primers anneal to the separated template strands and serve as starting points for DNA Taq replication by DNA polymerase.

Taq DNA polymerase extends the annealed primers by “reading” the template strand and synthesizing the complementary sequence. In this way, Taq polymerase replicates the two template DNA strands. This polymerase was isolated from a heat-stable bacterium (Thermus aquaticus) which in nature lives within high temperature steam vents such as those found in Yellowstone National Park.[6] For this reason these enzymes have evolved to withstand high temperatures (94°C) and can be used in the PCR reaction.

PCR Step by Step

PCR amplification takes place in a thermal cycler that performs cycles of alternating heating -a **denaturation step**, cooling - an **annealing step,** and heating steps an **extension step** (summarized in Figure 9).

In denaturation, the reaction mixture is heated to 94°C for 1 minute, which results in the melting or separation of the double-stranded DNA template into two single stranded molecules. PCR amplification, DNA templates must be separated before the primers can access the DNA and polymerase can generate a new copy. The high temperature required to melt the DNA strands normally would destroy the activity of most enzymes, but Taq polymerase is stable and active at high temperature.

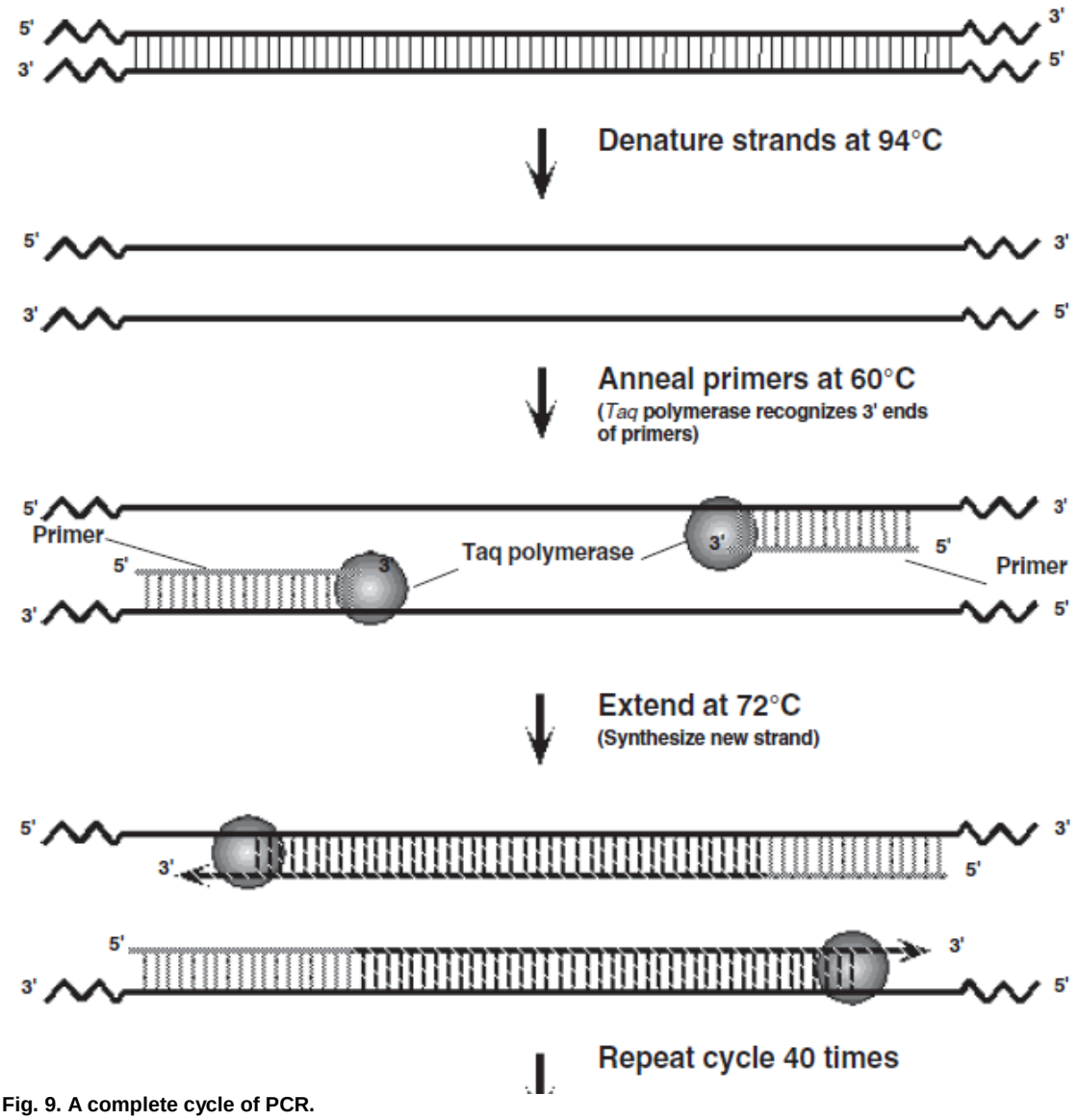


Fig. 9. A complete cycle of PCR.

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 synthesis of a new strand by providing a short sequence of double-stranded DNA for Taq polymerase to extend from 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 for 1 minute is optimum for primer binding.

During the extension step, the job of Taq DNA polymerase is to add nucleotides (A, T, G, and C) one at a time to the primer to create a complimentary copy of the DNA template. During polymerization the reaction temperature is 72°C for 2 minutes, which produces optimal Taq polymerase activity. The three steps of denaturation, annealing, and extension form one “cycle” of PCR. A complete PCR amplification undergoes 40 cycles.

The entire 40 cycle reaction takes approximately 3.5 hours and is carried out in a PCR tube that has been placed into a thermal cycler. The thermal cycler contains an aluminum block that holds the samples and can be rapidly heated and cooled across broad temperature differences. The rapid heating and cooling of this thermal block is known as **temperature cycling** or **thermal cycling**.

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

Ch 12 Alu Lab Part 2 PCR Amplification (Lab Protocol)

Workstation Checklist

Materials and supplies that should be present at the workstations prior to beginning this lab are listed below.

Student Workstation	**Quantity per Station**	**(✔)**
PCR tubes	4	❒
Complete master mix (with primers) on ice	1 tube (80 µl)	❒
P-20 micropipette	2	❒
Pipette tips	8	❒
Ice bucket with ice	1	❒
Permanent marker	4	❒
Waste containers with bleach solution	1	❒
Copy of Lab Procedure (Ch 12 Alu Lab Part 2)	4	❒

Class Equipment	**Quantity per Class**	
Thermal cycler	1	❒
Centrifuge (before class)	1	❒

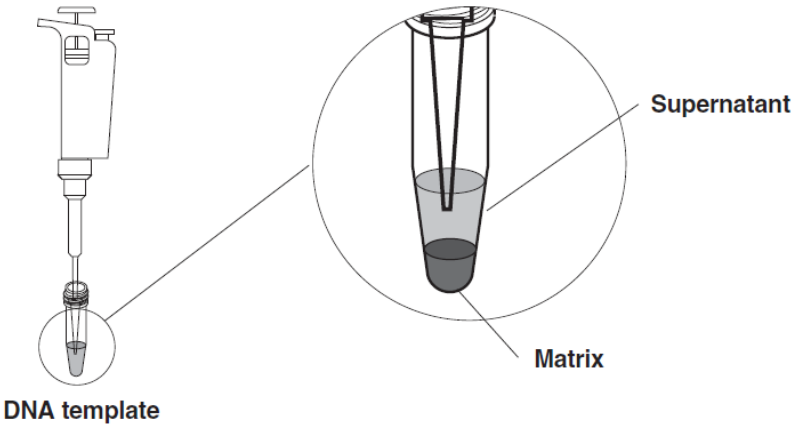
Lesson 2 PCR Amplification (Lab Protocol)

1. Obtain your screw cap tube that contains your genomic DNA template from the refrigerator. Centrifuge your tubes for 2 minutes at 6,000 x g or for 5 minutes at 2,000 x g in a centrifuge.

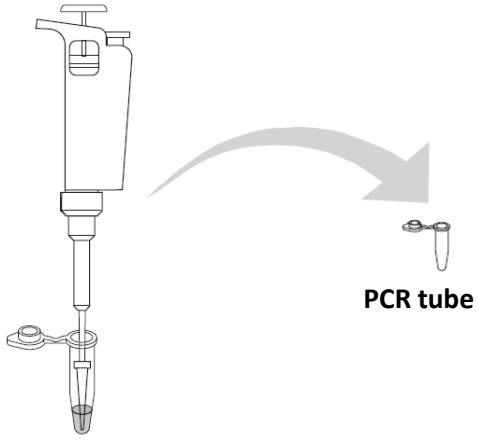
2. Each member of the team should obtain a PCR tube (PCR tubes are extremely small versions of reaction tubes). Label your PCR tube on the side of the tube with your 4 digit number and add colorful identifying marking.

PCR tube

3. Transfer 20 µl of your DNA template from the supernatant in your screw cap tube into the bottom of the PCR tube. **Do not transfer any of the matrix beads from the bottom of your screw cap tube into the PCR reaction because they will inhibit the PCR reaction.**



4. Locate the tube of yellow PCR master mix (labeled “Master”) in your ice bucket. Transfer 20 µl of the master mix into your PCR tube. Mix by pipetting up and down 2–3 times. Cap the PCR tube tightly and keep it on ice until instructed to proceed to the next step. Avoid bubbles, especially in the bottom of the tubes.



Master Mix: What is it?

The master mix contains a mixture of nucleotides or dNTPs (dATP, dTTP, dCTP , and dGTP –see p315 Figure 16.14 and the last paragraph on page 314 of your 8th edition Campbell Biology Book), buffer, *Taq* DNA polymerase, and $MgCl_2$ –as cofactor for Taq DNA polymerase. Complete master mix is prepared by adding primers to the master mix just prior to the laboratory period. Thus, when a 20 µl aliquot of the cheek cell lysate (which provides the DNA template) is added to a 20 µl of complete master mix, all of the necessary components for a 40 µl PCR reaction are present.

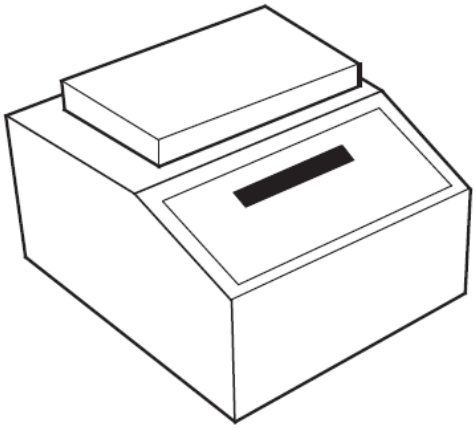
Note: The 2x master mix contains 0.05 units/µl Taq DNA polymerase, 3mM $MgCl_2$, 1.6 mM dNTPs, and 1 µl of each primer. The final 1x or working concentration of these components in the PCR tube after the primers, master mix, and template are combined will be one-half of the above values.

Note: Once the master mix and primers are mixed, they should be kept on ice and used within 15-30 minutes. These reagents are extremely sensitive.

Why is the master mix yellow?

The primer mix (which was added to the master mix) contains PCR-compatible dye called tartrazine, which comigrates with DNA of ~50bp. This yellow dye was added to allow students to easily visualize the sample.

5. Place your PCR tube in the thermal cycler. Record the location of your PCR tube in the PCR grid near the PCR machine. The PCR tubes are very small and require care when handling. It is important to carefully and completely cap the tubes before placing them into the thermal cycler. If the tubes are not closed completely, substantial evaporation can take place, and PCR amplification will be inhibited.



***Manual PCR**

It is possible to perform PCR manually without an automated thermal cycler, although the PCR will not be as efficient. For manual PCR amplification, reactions should be performed in screwcap tubes and topped off with a drop of mineral oil to prevent evaporation. The tubes are placed in a dry bath or water bath set at 95’C for 1 minute, then manually transferred to a dry bath or water bath set at 60’C for 1 minute, and finally transferred to a dry bath or water bath set at 72’C for 2 minutes. Forty cycles of manual PCR should take about 3 hours. It is tedious but it works.

Lab 2: Alu DNA Preparation Pre-Lab Questions

A. Introduction

1. What is Alu?

   How long is one Alu repeat?

   How many do we have?

   What is a 'fixed' allele?

   What chromosome is Alu found on?

   Why will we use gel electrophoresis?

After reading the Alu lab procedure answer the following questions.

B. IMPORTANT LAB PRACTICES

1. Why change pipette tips between each delivery?

C. Alu Lab Procedure

1. According to the lab, which micropipette will we be using?
   What is its range?
   What will the three window volume reading show for our measurements?

2. Why are we using PIN numbers?

3. Step 1: Why centrifuge the sample?

   How do you balance the centrifuge if you only need to spin your one single tube?

4. Why are we using bleach?

5. What is special about *Taq* DNA polymerase?

6. Step 3: What's in the matrix of your screwcap tube? What is in the supernatant?

7. Why can't we allow any of the heavy InstaGene beads into the PCR tube for the PCR reaction?

8. Why are there nucleotides (A, T, G, and C) in the master mix?
   What are the other components of the master mix, and what are their functions?

9. Why do you think we are using DNA primers? Not RNA primers?

10. Describe the three main steps of each cycle of PCR amplification and what reactions occur at each temperature.

11. How is this lab procedure able to amplify only one segment of your entire genome?

12. During PCR, explain why the precise length target DNA sequence doesn't get amplified until the third cycle. You may need to use your book, additional paper, and a drawing to explain your answer