Part A. The Polymerase Chain Reaction

Biology 182
Arizona State University West

Introduction:
Today you will be performing a technique known as Polymerase Chain Reaction (PCR). The purpose of this reaction is to make a very small amount of DNA into a larger amount of DNA. PCR was developed by Kary Mullis at Cetus Corporation and is a very important tool in the study of DNA sequences because it can be used to make a large number of copies of a specific piece of DNA. This allows further analysis of that specific sample. In this experiment, you will amplify a segment of your DNA. By doing this you will determine whether you have a piece of DNA that is present in the genes of some people but not of others.

As we study DNA Replication, Transcription, and Translation you will come to understand the importance of DNA in the process of making proteins. Without DNA, you would not have proteins, so it is very important to understand that in order to make changes in proteins you must start with the DNA. The process of PCR has transformed the field of Molecular Biology by giving scientists an important tool in the study of DNA. Prior to its development, it was very difficult to get a large enough sample of DNA to study individual sequences carefully.

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. It is also used in criminal investigations and courts of law to identify suspects on the molecular level and has been a powerful tool in sequencing the human genome. 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 technology transformed molecular biology from a difficult science to one of the most accessible and widely used disciplines of biotechnology.

The PCR process specifically targets the DNA sequence you want to amplify if you get the right ingredients, which include: the DNA template (your sample), the deoxynucleotides (A,T,G,C), the enzyme *Taq* DNA Polymerase (which is a heat-stable form and comes from a bacterium that lives in the steam vents in Yellowstone National Park), magnesium ions (a cofactor for the DNA Polymerase), oligonucleotide primers (pieces of DNA that are complimentary to the sample DNA at either end of the sequence that you want to amplify. They tell the polymerase where to start making the DNA), and a salt buffer, which provides the right environment for the best function of the enzyme.

These ingredients combined cause the DNA to be doubled each time that the PCR cycle is completed. You have three main phases of DNA replication: denature the strands (94^{o}C), anneal the primers (temperature depends on primer composition and length; our primers anneal well at 60^{o}C), and extend the primers (72^{o}C). As this cycle is repeated you can see that the number of DNA templates of the segment will be doubled each time, so the growth is exponential and in only 40 cycles you can have enough DNA to be visible when it is properly stained and separated via electrophoresis.

Objectives: PCR Lab parts 1 and 2:

- Know what six kinds of ingredients are required in order to perform PCR and their importance to the process.
- Know what the temperatures in the PCR process are used for.
- Know the terms associated with RNA splicing.
- Know how the human genome is divided, and the type of DNA that the *Alu* element is located in.
- Know what the results of electrophoresis represent and what the bands tell you about the different DNA fragments.

Now let's extract and amplify some of your own DNA.

Procedure:

EduPrimer™ DNA Profiling Kit: Student Protocol

Do not eat or brush teeth one hour prior to cheek cell collection. Wear gloves and handle solutions carefully

Part A. Preparing Cheek Cell DNA for PCR.

1. Use the tube provided and add 200 µl of **Solution A** mark the tube with your initials

2. Collect cheek cells with the small swab located at your table. Thoroughly roll and swab inside your cheek for 10 seconds.

3. Place this swab into the tube you marked with your initials containing Solution A.

4. Cut the swab so that it will fit inside the tube. Make certain the cap will shut tightly.

5. Press the tube against the vortex machine to thoroughly mix the sample, do this for at least 10 seconds. *Solution A contains components which chemically disrupt cell membranes and begin to unravel proteins. Under these conditions, the cheek cells will begin to lyse or break open, spilling cell contents into the solution in the tube.*

6. Place the sample in the heat block containing water, or in a water bath whichever if provided, to incubate at 95°C for 5 minutes. Then immediately place tube in your ice bath until you are ready for next step,.*This process continues to destroy proteins, particularly, those that damage DNA.*

7. Load your sample into the microcentrifuge, taking care that there is another sample directly across from your sample to keep the centrifuge in balance as it spins. Close internal and external centrifuge lids.

8. Spin briefly (~10 seconds) using the "short spin" button on the mini spin to pool condensation that has collected on the cap.

9. Remove swab carefully with tweezers (then rinse tweezers with ethanol to prevent contamination).

10. Add 20 µl of **Solution B** (green label) to the sample tube. *Solution B neutralizes the harsh conditions needed for lysis, preparing the solution for DNA isolation and PCR to follow.*

11. Close the tube tightly and vortex to mix for at least 10 seconds

12. Load sample into microcentrifuge with the tube hinge pointing out, balance your sample tube with another and be sure you close the lids.

13. Spin sample for 1 minute at 12,000 rpm.

14. Look for a small clear round pellet near the bottom of the tube under the hinge. This pellet contains cellular debris. The aqueous solution (supernatant) that has not precipitated into the pellet contains cellular DNA (which is what you will use in the next step, **do not include any of the pellet.)**

Part B. Setting up your PCR Reaction Mixtures and Controls

***Wear gloves and handle solutions carefully**

Prepare and label a small PCR tube your name. Label both the top and side of the PCR tube to ensure clarity.

Label one additional PCR tube per group **(AS DESIGNATED BY INSTRUCTOR**), this will be used for a control. Depending on the number of groups in your lab this may vary.

3 Groups will do the + controls
3 Groups will do the – controls

You will share these next week when you run your gels.

For each student sample:

1. Add 10 µl of "2X PCR Master Mix" (see instructor), **(Use only the p10 pipettes for this WHITE TIPS)** and 10 µl of supernatant (avoid the pellet) from the 1.5 mL tube you prepared in part A. to the labeled PCR tube for at total of 20 µl Keep this solution on ice.

2. Mix the 20 µl PCR reaction mixture by pipetting it in and out with the pipette (only go to the first stop when doing this and use the white tips only), then close the lid tightly.

PCR reaction mixture table:

2x PCR Master Mix	10 µl
Your DNA Template	10 µl
Volume total	20 µl

3. Store the sample on ice until the thermal cycler is ready to run.

For each control sample:

1. Add 10 µl of "2X PCR Master Mix" (see instructor) and 10 µl of control DNA to a labeled (+ or -) depending on which you are assigned) PCR tube for at total of 20 µl. Keep this solution on ice.

2. Mix the 20 µl PCR reaction mixture by pipetting it in and out with the pipette (only go to the first stop when doing this and use the white tips), then close the lid tightly.

3. Store the sample on ice until the thermal cycler is ready to run.

PCR reaction mixture table

2x PCR Master Mix	10 µl
Control DNA Template	10 µl
Volume total	20 µl

4. When your samples as a class are all prepared your instructor will have you load the samples in the PCR machine and run it using the parameters listed in the document provided as background information on Blackboard.

5. These samples will be refrigerated until the next lab.

Questions 1-4:

1. What is needed from the cheek cells in order to conduct the polymerase chain reaction?

2. Why is it necessary to have a primer on each side of the DNA segment to be amplified?

3. 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?

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

Have you answered all four of the questions for this week?

Week two starts here.

Part B. Gel Electrophoresis of Amplified PCR Samples and Staining of Agarose Gels

What sequence are you looking at? What can genes and DNA tell us? It is estimated that the 23 pairs, or 46 **chromosomes**, of the human genome contain approximately 30,000 -50,000 genes. Each chromosome contains a series of specific genes. The larger chromosomes contain more DNA, and therefore more genes, compared to the smaller chromosomes. Each of the homologous chromosome pairs contains similar genes.

Each gene holds the code for a particular protein. Interestingly, the genes only comprise about 5% of the total chromosomal DNA. The other 95% is non-coding DNA. This non-coding DNA is interspersed in blocks between functional segments of genes and within genes, splitting them into segments. The exact function of the non-coding DNA is not known, although it is thought that non-coding DNA allows for the accumulation of mutations and variations in genomes.

When DNA is first transcribed from RNA, it contains both coding and non-coding sequences. While the RNA is still in the nucleus, the non-coding **introns** (in = stay with**in** the nucleus) are removed from the RNA, while the **exons** (ex = **ex**it the nucleus) are spliced together to form the complete messenger RNA coding sequence for the protein. This process is called **RNA splicing** and is carried out by specialized enzymes called **spliceosomes**.

Introns often vary in their size and sequence among individuals, while exons do not. This variation is thought to be the result of the differential accumulation of mutations in DNA throughout evolution. These mutations in our non-coding DNA are silently passed on to our descendants; we do not notice them because they do not affect our phenotypes. However, these differences in our DNA represent the molecular basis of DNA fingerprinting used in human identification and studies in population genetics.

The human genome contains small repetitive DNA elements that are located in various places within our genome. One such repetitive element is called the "*Alu* sequence". This is a DNA sequence, about 300 base pairs long, that is repeated almost 500,000 times throughout the human genome. The origin and function of these repeated sequences is not yet known.

Some of these *Alu* sequences have characteristics that make them very useful to geneticists. When present within introns of certain genes, they can either be associated with a disease or be used to estimate relatedness among individuals. In this exercise, analysis of a single *Alu* repeat is used to estimate relatedness among individuals and to estimate its frequency in the population. This particular DNA sequence is a simple measure of molecular genetic variation –

and has no reference to disease or relatedness among individuals. (this is confusing? It is used to estimate relatedness or it isn't?)

In this lab you will look at an *Alu* element in the PV92 region of chromosome 16. This particular *Alu* element is dimorphic, meaning the element is present in some individuals and not others. The presence or absence of this insert can be detected using PCR (what we just did), followed by electrophoresis (what we will now do).

The primers in this kit are designed to bracket a sequence within the PV92 region that is 415 base pairs long if the intron does not contain the *Alu* insertion, or 715 base pairs long if *Alu* is present. Three outcomes are possible: two 715 base pair sequences, two 415 base pair sequences, or one of each, 715 and 415 base pair sequences.

Electrophoresis separates DNA fragments according to their relative sizes (molecular weights). DNA fragments are loaded into an agarose gel slab, which is placed into a chamber filled with a conductive buffer solution. A direct current is passed between wire electrodes at each end of the chamber. DNA fragments are negatively charged and when placed in an electric field will be drawn toward the positive pole and repelled by the negative pole. The matrix of the agarose gel acts as a molecular sieve through which smaller DNA fragments can move more easily than larger ones. Over a period of time, smaller fragments will travel farther than larger ones. Fragments of the same size stay together and migrate in what appears as a single "band" of DNA in the gel.

Part C. Running your PCR samples on a gel.

Then you must cast a gel, you will use a very similar procedure to the one you used in the Separating organic Molecules lab.

Protocol for pouring an Agarose Gel.
If you are using the Carolina gel boxes.

1. Tape the ends of the gel tray with masking tape making sure to get a seal on all parts.

2. Insert the comb into the end of the gel tray.

3. Obtain 50mL of TAE or TBE buffer (whichever you are using) and place in a 125mL flask.

4. To this buffer add 0.5g of Agarose. And swirl making sure the agarose is evenly spread in the solution.

5. Boil this solution for ~1min swirl and make sure all flecks are dissolved your solution should appear clear. Do not overboil as this will cause the water to evaporate and your gel will be really thin.

6. Next add your **5 MICROLITERS** of GelRed. For this you will need to use the pipette with the white top and the smallest tips.

7. Swirl the solution so that the GelRed is evenly spread. Do not shake the solution since this will introduce a number of bubbles that will mess up your gel

8. Gently swirls the solution until the bottom of flask is easy to touch with your gloved hand.

9. Then pour this solution into the previously prepared gel tray, take a pipette tip and move any bubbles that form to the bottom edge away from the comb.

10. Wait until the gel is a uniformly cloudy color (no clear areas remaining)~15 min.

11. Then remove the gel tray from the gel box and reorient it so that the wells are on the end that will have the black electrodes (Run toward Red). (See top of gel tray to determine this.

12. After your gel is loaded this way you are ready to add TAE or TBE to your Tray whatever type of buffer you use make sure it is the same in the gel and in this step.

13. After your buffer is loaded then add your samples to the wells. Using the Table below for what goes in each well.

14. Hook up the power supply to the gel box Make sure black electrodes are plugged into black connections on your power supply and red are plugged into red connections on your power supply.

15. Then turn on the power supply, set the voltage and time (if available) , or if not available make sure you record the time. Use the voltage and time as described in your protocol. Usually 100-150V for 10-20 min.

Prepare your PCR samples for loading:

1. Spin tubes down as needed (5 sec), using a tube adapter (capless 1.5 mL tube).
2. Add 5 μL of loading dye to your sample and the control sample you ran in the PCR machine, and spin down again using a short 5 sec spin.

(Note positive and negative controls will need to be obtained from either the sample you did or from another group that did the opposite control from the one you did.)

Lane 1 Load 10 μL of DNA Ladder
Lane 2 Load 10μL of negative Control
Lane 3 Load 10μL of positive Control
Lane 4 Load up to 20μL of Student 1

Lane 5 Load up to 20μL of Student 2
Lane 6 Load up to 20μL of Student 3
Lane 7 Load up to 20μL of Student 4

16. **NOTE: THE BLUE BANDS DO NOT SHOW DNA!** You must view your gel on a Ultraviolet Light box. Detail your gel either by photographing it or drawing it and determine what your results were.
Drawing of gel and description of which student samples were positive and which were negative will need to be turned in.

Questions 5 - 8 :

5. *Explain how Agarose electrophoresis separates DNA fragments. Why does the smaller DNA fragment move faster than the larger one?*

6. *What is your genotype for the Alu insert in your PV92 region?*

7. *What are the observed genotypic frequencies of the +/+, +/-, or -/- in your class population? Fill in the table below with your class data.*

Table 1. Observed Class Genotypic Frequencies

Category	Number of genotypes	Frequency(# of genotypes/Total)
Homozygous (+/+)	p^2 =	
Heterozygous (+/-)	pq =	
Homozygous (-/-)	q^2 =	
	Total =	=

8. *Answer the following question after all data has been posted. What are the allele frequencies observed in your class? Remember, allele frequency is the total number of alleles of one type divided by the total number of alleles.*
For example: *If there are 60 students and five are heterozygous, and 10 are -/- the frequency of the – allele is 25/120 or 0.21.*