Name _____

Ah, Lou! There really are differences between us!

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BABEC

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We gratefully acknowledge David Micklos of the DNA Learning Center at Cold Spring Harbor Laboratory for his generous help. Some materials for this exercise were adapted, by permission, from the *Genomic Biology: Advanced Instructional Technology College Biology Faculty* laboratory manual, Cold Spring Harbor Laboratory, copyright 1999.

Introduction

We are humans. We are bipedal and stand upright. We have hands, feet, fingers, and toes. You can look at the student next to you and easily recognize that person to be human too. What makes us look similar to each other while different from frogs, fish, or fuchsias is the molecule **deoxyribonucleic acid** (**DNA**).

The basic building block of DNA is the **nucleotide** comprising a deoxyribose sugar, a phosphate, and one of the four bases A (adenine), C (cytosine), G (guanine), or T (thymine). In the DNA molecule, nucleotides are linked together in a chain. DNA is a **double helix**; two chains of nucleotides are wound around each other to form a spiral structure. Interactions (hydrogen bonds) between the bases on the opposing strands hold the double helix together. The A's on one strand hydrogen bond with the T's on the other strand. The G's on one strand interact with the C's on the other. Therefore, A's and T's are said to be **complementary** as are G's and C's. Complementary bases, when hydrogen bonding in the double helix, are called **base pairs** (**bp**). It is the order of the bases along the strands of the DNA molecule that makes each species unique.

Our bodies are caldrons for thousands of chemical reactions carried out to support the process of life. We ingest food for energy and for the raw materials needed to build the structures of the cell. We breathe oxygen; it assists in the moving of electrons from one molecule to another. We manufacture protein molecules called **enzymes** needed for the building or breakdown of still other molecules. We all look like humans because we all share the same cellular makeup.

The information for the construction of all the enzymes in the cell and all the proteins giving the cell its shape and function is stored within DNA's sequence of bases. One particular base sequence may carry the information for the assembly of hemoglobin, a protein that carries oxygen to your cells. Another sequence of bases may direct the manufacture of an actin molecule, a protein found in muscle. The region of bases on DNA that holds the information directly needed for the construction of a particular protein is called a **gene**. The average gene is approximately 10,000 base pairs long. There are approximately 140,000 genes in human DNA.

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, however, are actually 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 many thousands of base pairs. Furthermore, most genes in the human organism are themselves broken into smaller protein-encoding segments, called **exons**, with, in many cases, 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 introns have an ill-defined or possibly even non-essential role, they have been referred to by many as "Junk DNA". Whatever their function, examination of these intervening DNA regions has revealed the presence of unique genetic elements that can be found in a number of different locations within the genome. 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 are said to be **dimorphic**; different individuals may or may not carry a particular *Alu* sequence at a particular chromosomal location.

The Polymerase Chain Reaction

Objectives:
You should be able to list and explain the importance of each component of PCR.
You should be able to associate the temperature changes with the cycling steps of PCR.

The polymerase chain reaction (PCR) is a method used by scientists to rapidly copy, in a test tube, specific segments of DNA. By mimicking some of the DNA replication strategies employed by living cells, PCR has the capacity for churning out millions of copies of a particular DNA region. It has found use in forensic science, in the diagnosis of genetic disease, and in the cloning of rare genes. One of the reasons PCR has become such a popular technique is that it doesn't require much starting material. It can be used to amplify DNA recovered from a plucked hair, from a small spot of blood, or from the back of a licked postage stamp.

There are some essential reaction components and conditions needed to amplify DNA by PCR. First and foremost, it is necessary to have a sample of DNA containing the segment you wish to amplify. This DNA is called the **template** because it provides the pattern of base sequence to be duplicated during the PCR process. Along with template DNA, PCR requires two short single-stranded pieces of DNA called **primers**. These are usually

about 20 bases in length and are complementary to opposite strands of the template at the ends of the target DNA segment being amplified. Primers attach (**anneal**) to their complementary sites on the template and are used as initiation sites for synthesis of new DNA strands. **Deoxynucleotides** containing the bases A, C, G, and T are also added to the reaction. The enzyme **DNA polymerase** binds to one end of each annealed primer and strings the deoxynucleotides together to form new DNA chains complementary to the template. The DNA polymerase enzyme absolutely requires the metal ion magnesium ($\mathbf{Mg^{++}}$) for its activity. It is supplied to the reaction in the form of $MgCl_2$ salt. A **buffer** is used to maintain an optimal pH level.

PCR is accomplished by cycling a reaction through several temperature steps. In the first step, the two strands of the template DNA molecule are separated, or **denatured**, by exposure to a high temperature (usually 94° to 96°C). Once in a single-stranded form, the bases of the template DNA are exposed and are free to interact with the primers. In the second step of PCR, called **annealing**, the reaction is brought down to a temperature usually between 37˚C to 55 ˚C. At this lower temperature, stable hydrogen bonds can form between the complementary bases of the primers and template. Although human genomic DNA is billions of base pairs in length, the primers require only seconds to locate and anneal to their complementary sites. In the third step of PCR, called **extension**, the reaction temperature is raised to an intermediate level (65˚C to 72˚C). During this step, the DNA polymerase starts adding nucleotides to the ends of the annealed primers. These three phases are repeated over and over again, doubling the number of DNA molecules with each cycle. After 25 to 40 cycles, millions of copies of DNA are produced. The PCR process taken through four cycles is illustrated on the following page (Figure 1).

In the following laboratory exercise, you will use PCR to amplify a dimorphic *Alu* repeat (designated PV92) found on your number 16 chromosome. You will use your own DNA as template for this experiment. DNA is easily obtained from the human body. A simple saltwater mouthwash 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 or both of your number 16 chromosomes. This will be accomplished by electrophoresing your PCR sample on an agarose gel. Finally, using a program developed by the DNA Learning Center at Cold Spring Harbor Laboratory, you will determine how rare this *Alu* sequence is in the human population and make some assessment as to when and where it arose.

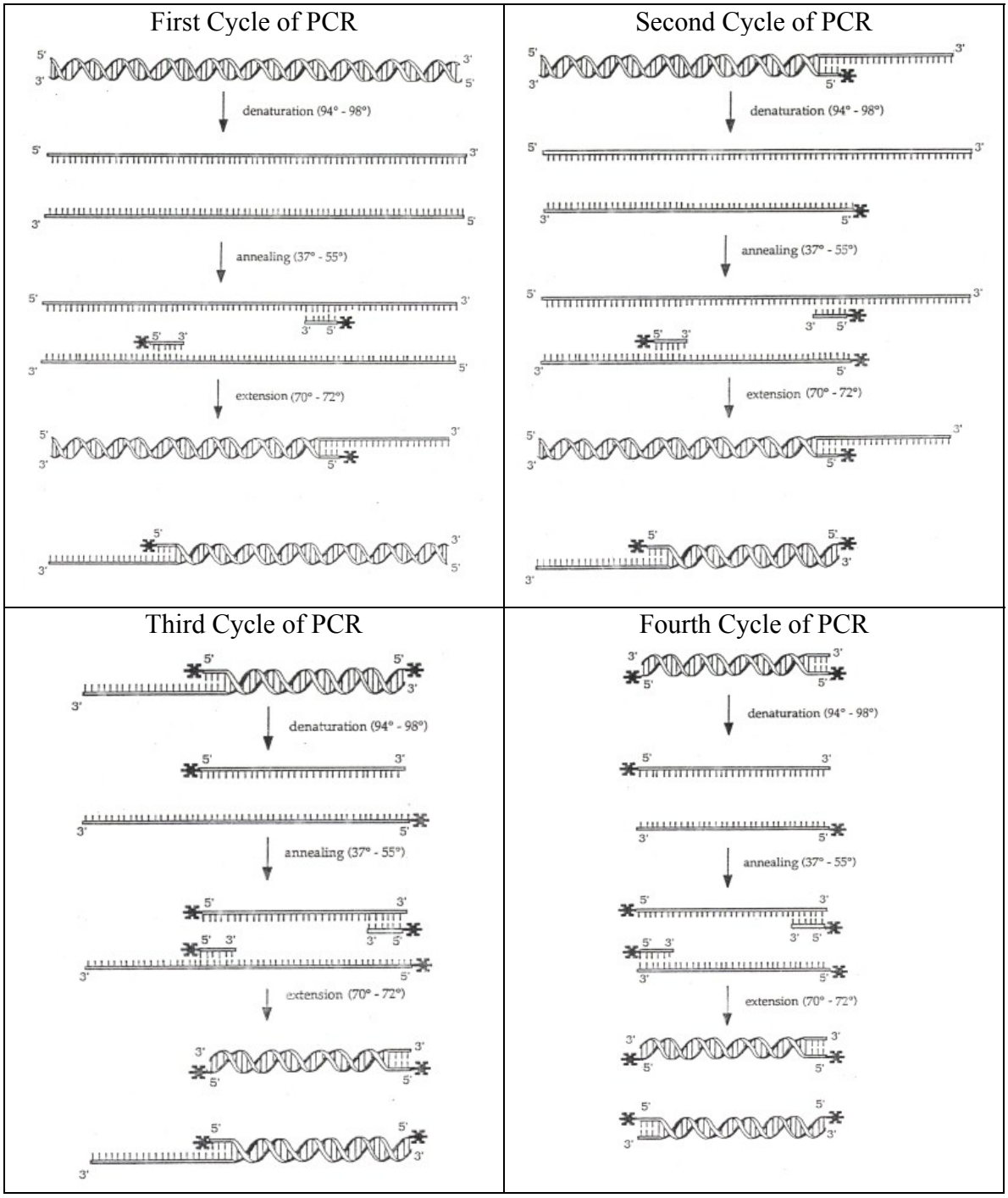


Figure 1. The first four cycles of the polymerase chain reaction.

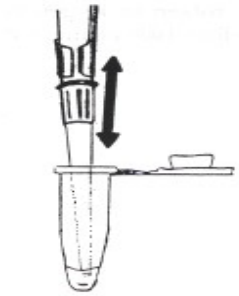
An excellent animated tutorial showing the steps of PCR is available at the Cold Spring Harbor web site. **http://vector.cshl.org/shockwave/pcranwhole.html**

Laboratory Exercise: Week 4 Tuesday

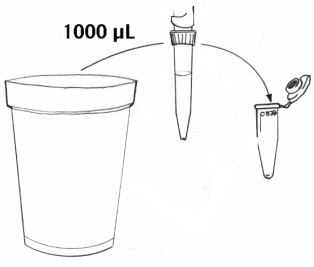
Objectives:

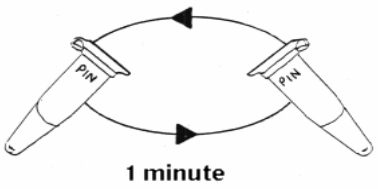
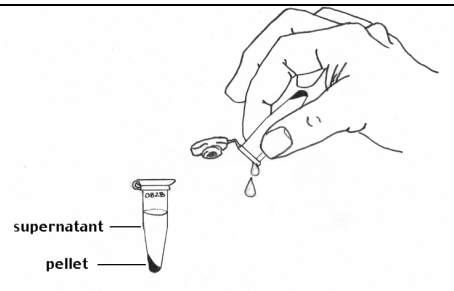
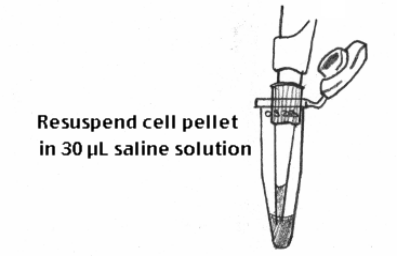
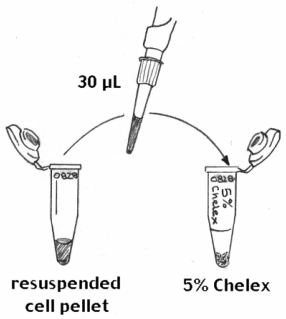
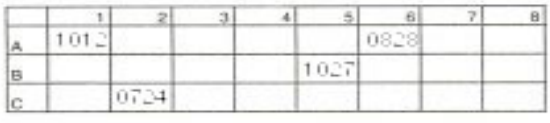
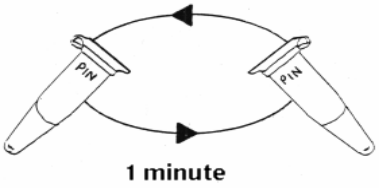
You should be able to successfully isolate DNA from cheek cells.

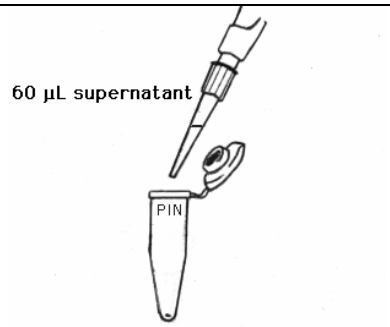
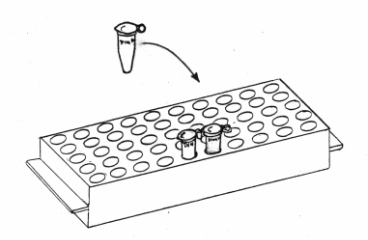
You should be able to prepare a reaction for PCR amplification of an *Alu* insert.

IMPORTANT LABORATORY PRACTICES	
Add reagents to the bottom of the reaction tube, not to its side. You should add each additional reagent directly into previously-added reagent and pipet the combined liquid up and down several times to ensure proper mixing. Pipet slowly to prevent contaminating the pipette barrel. 	**Change pipette tips between each delivery. You should change the tip even if it is the same reagent being delivered between tubes.** 

Place a check mark in the box of each step as it is completed.

DNA Preparation Using a Saline Mouthwash	
1. Swirl 10 mL of 0.9% saline in your mouth for 30 seconds.	
2. Expel saline into a cup and swirl to mix the cells.	
3. Transfer 1000 µL of the liquid into a 1.5 mL microfuge tube, labeled with your initials.	

4. In a balanced centrifuge, spin sample for 1 minute.	
5. Observe your cell pellet at the bottom of the tube. Pour off the supernatant, being careful not to lose your cell pellet. **Note**: It is okay if some supernatant is left in the tube.	
6. Resuspend your cell pellet in 30 µL of saline. Make sure the entire cell pellet is thoroughly mixed by vortexing, pipeting up and down several times, or "racking" your tube. **Note**: To "rack" your sample, be sure the top of the tube is closed, hold tube firmly at the top, and pull it across a microfuge rack 2-3 times.	
7. Withdraw 30 µL of the cell suspension and add it to a 0.2 mL tube containing 200 µL of 5% Chelex. **Note**: Do not pipet up and down at this step or else you will clog the tip with Chelex beads.	
8. Place your 0.2 mL tube with 200 µL of Chelex and 30 µL of cell suspension in the 99˚C thermal cycler for 10 minutes. **Note:** Remember to record the location of your tube in the thermal cycler.	
9. Shake your tube well or briefly vortex it and then place it in a balanced centrifuge. Spin for 1 minute.	

10. Withdraw 60 µL of supernatant (no Chelex beads) to a clean tube, labeled with your PIN. **Note**: This stored sample is your "DNA" tube.	
11. Place your DNA tube in an ice bucket while you prepare your PCR amplification.	

Polymerase Chain Reaction

1. Label a 200 µL PCR tube with your initials.

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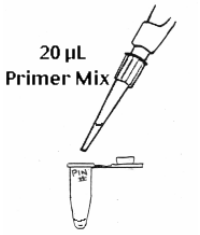
2. Change your tip and dispense **20 µL of Master Mix** into your PCR tube.

❒

3. Change your pipet tip and add **20 µL of Primer Mix** into your PCR tube.



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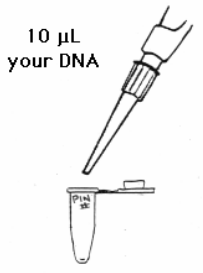
4. With a new pipet tip, add **10 µL of your purified DNA** into your PCR tube.

Note: Slowly pipet up and down several times to mix all the reagents in your reaction tube.



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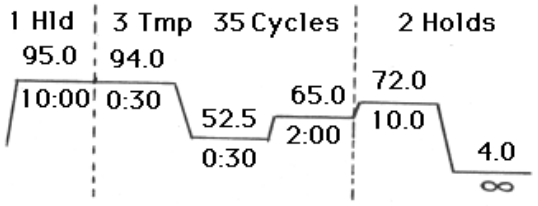
5. Place your reaction into the thermal cycler and record the location of your tube on the grid provided.

	1	2	3	4	5	6	7	8
A	1012					0828		
B					1027			
C		0724						

❒

6. The cycling protocol for amplification of this Alu region is:
   95˚C, 10 minutes;
   94˚C, 30 seconds;
   52.5˚C, 30 seconds; X 35 cycles
   65˚C, 2 minutes;
   72˚C, 10 minutes;
   4˚C, hold



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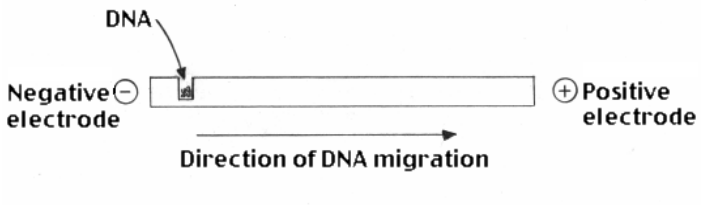
Agarose Gel Electrophoresis: Week 4 Thursday

To determine whether or not you carry the *Alu* repeat, you will need to visualize the products of your amplification. This will be done using a process called **electrophoresis** in which electric current forces the migration of DNA fragments through a special gel material. Since DNA is negatively charged, it will migrate in an electric field towards the positive electrode (Figure 2). When electrophoresed through a gel, shorter fragments of DNA move at a faster rate than longer ones. The *Alu* repeat adds 300 base pairs of length to a DNA fragment and thus will slow its migration during electrophoresis.

Figure 2. Side view of an agarose gel showing DNA loaded into a well and the direction of DNA fragment migration during electrophoresis.



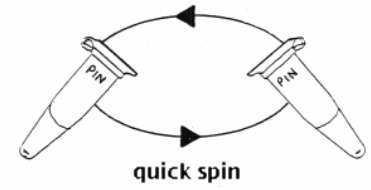
The gel material to be used for this experiment is called **agarose.** When agarose granules are placed in a buffer solution and heated to boiling, they dissolve and the solution becomes clear. A casting tray is set up with a comb to provide a mold for the gel. The agarose is allowed to cool slightly and is then poured into the casting tray. Within about 15 minutes, the agarose solidifies into an opaque gel having the look and feel of coconut Jell-O. The gel, in its casting tray, is placed in a buffer chamber connected to a power supply and buffer is poured into the chamber until the gel is completely submerged. The comb can then be pulled out to form the wells into which your PCR sample will be loaded.

Loading dye is a colored, viscous liquid containing dyes (making it easy to see) and sucrose, ficoll, or glycerol (making it dense). You will add loading dye to your amplification reaction and then pipet an aliquot of the mixture into one of the wells of your agarose gel. When all wells have been loaded with sample, your instructor will switch on the power supply. The samples should be allowed to electrophorese until the blue loading dye is 1 to 2 cm from the bottom. The gel can then be stained with ethidium bromide and photographed.

You will use a 2% agarose gel for electrophoresis of your PCR products.
Nancy will demonstrate how to cast a gel.

Electrophoresis of Amplified DNA

1. Retrieve your PCR tube and spin it briefly to bring the liquid to the bottom of the reaction tube. **Make sure the centrifuge is balanced before you begin spinning your sample!**



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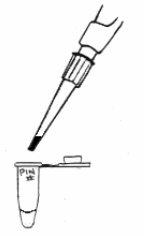
2. Add 10 **µL** of loading dye to your PCR tube. Slowly pipet the mixture up and down until the contents in the tube are uniformly colored.



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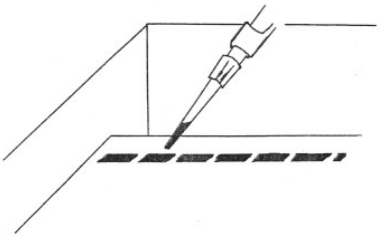
3. Carefully load **20 µl** of your reaction into a well in your gel. Avoid poking the pipette tip through the bottom of the gel or spilling sample over the sides of the well. Use a new tip for each sample.



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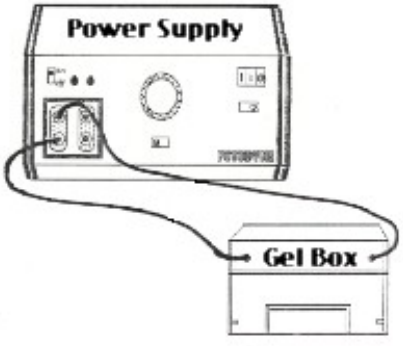
4. Load **10 µl** of the 1Kbp ladder (molecular weight marker) into one of the wells of each gel.

❒

5. When all samples are loaded, attach the electrodes from the gel box to the power supply. Have Nancy check your connections and then electrophorese your samples at **100 Volts** for **30-45 minutes**.



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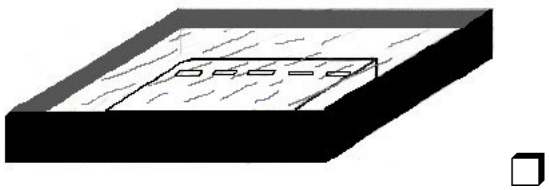
	

Staining and Photographing Agarose Gels

You will stain your agarose gel and take a photograph so that you may analyze your *Alu* results. Gel staining is done as follows.

1. Place the agarose gel in a staining tray.
2. Wait 15 minutes.

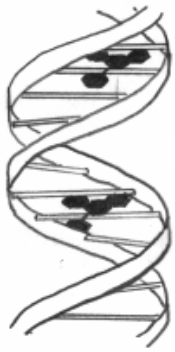
CAUTION: Ethidium bromide is a carcinogen. Always wear gloves and safety glasses when handling.

3. Transfer the gel to the destain tray and allow to sit for 5 minutes
4. Remove gel from staining tray onto a piece of Saran wrap. Carefull transfer to UV light box.

CAUTION: Ultraviolet light can damage your eyes and skin. Always wear protective clothing and UV safety glasses when using a UV light box.

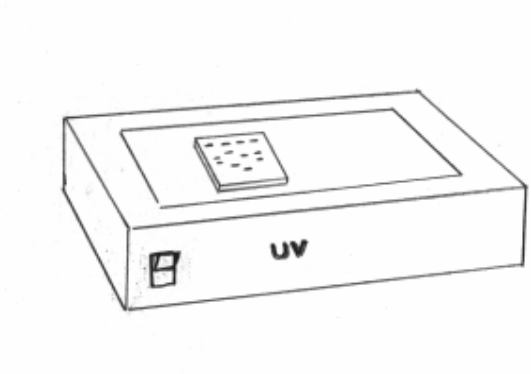
5. Place the camera over the gel and take a photograph.

Figure 3. Ethidium bromide molecules stacked between DNA base pairs.



The PCR products run on your agarose gel are invisible to the naked eye. If you look at your gel in normal room light, you will not be able to see the amplified products of your reaction. In order to "see" them, we must stain the gel with a fluorescent dye called **ethidium bromide**. Molecules of ethidium bromide are flat and can nestle between adjacent base pairs of double stranded DNA (Figure 3). When this interaction occurs, they take on a more ordered and regular configuration causing them to fluoresce under ultraviolet light (UV). Exposing the gel to UV light after staining, allows you to see bright, pinkish-orange bands where there is DNA(Figure 4).

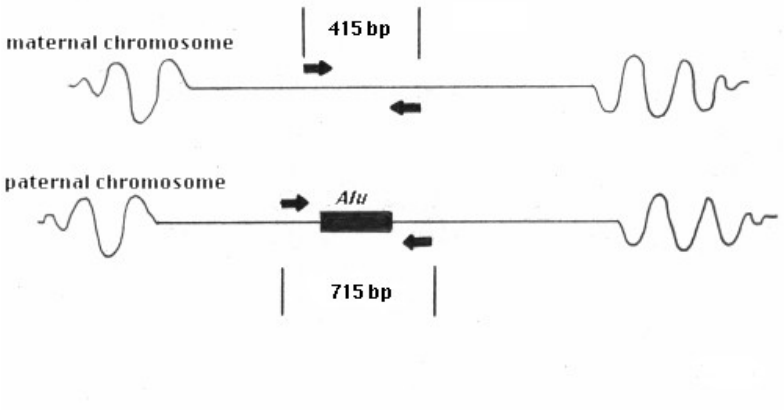
Figure 4. After staining an agarose gel with ethidium bromide, DNA bands are visible upon exposure to UV light.



Results

By examining the photograph of your agarose gel, you will determine whether or not you carry the *Alu* repeat on one, both, or neither of your number 16 chromosomes. PCR amplification of this *Alu* site will generate a 415 bp fragment if the repeat is not present. If the repeat is present, an 715 bp fragment will be made. Figure 5 shows the structure of an individual's two number 16 chromosomes in a case where one carries the *Alu* repeat and the other does not.

Figure 5. The chromosomes you inherit from your parents may or may not carry the *Alu* repeat on Chromosome 16.



When you examine the photograph of your gel, it should be readily apparent that there are differences between people at the level of their DNA. Even though you amplified only one site, a site that every one has in their DNA, you will notice that not all students have the same pattern of bands. Some students will have only one band, while others will have two.

We use the term **allele** to describe different forms of a gene or genetic site. For those who have the *Alu* repeat (they have at least one 715 bp band), we can say that they are positive for the insertion and denote that allele configuration with a "+" sign. If the *Alu* repeat is absent (a 415 bp band is generated in the PCR), we assign a "-" allele designation. If a student has a single band, whether it is a single 415 bp band or a single 715 bp band, then both their number 16 chromosomes must be the same in regards to the *Alu* insertion. They are said to be **homozygous** and can be designated with the symbols "-/-" or "+/+", respectively. If a student's DNA generates a 415 bp band and an 715 bp band during PCR, the student is said to be **heterozygous** at this site and the designation "+/-" is assigned. A person's particular combination of alleles is called their **genotype**.

Figure 6 on the following page shows a representation of a possible experimental outcome in which all possible allele combinations have been generated.

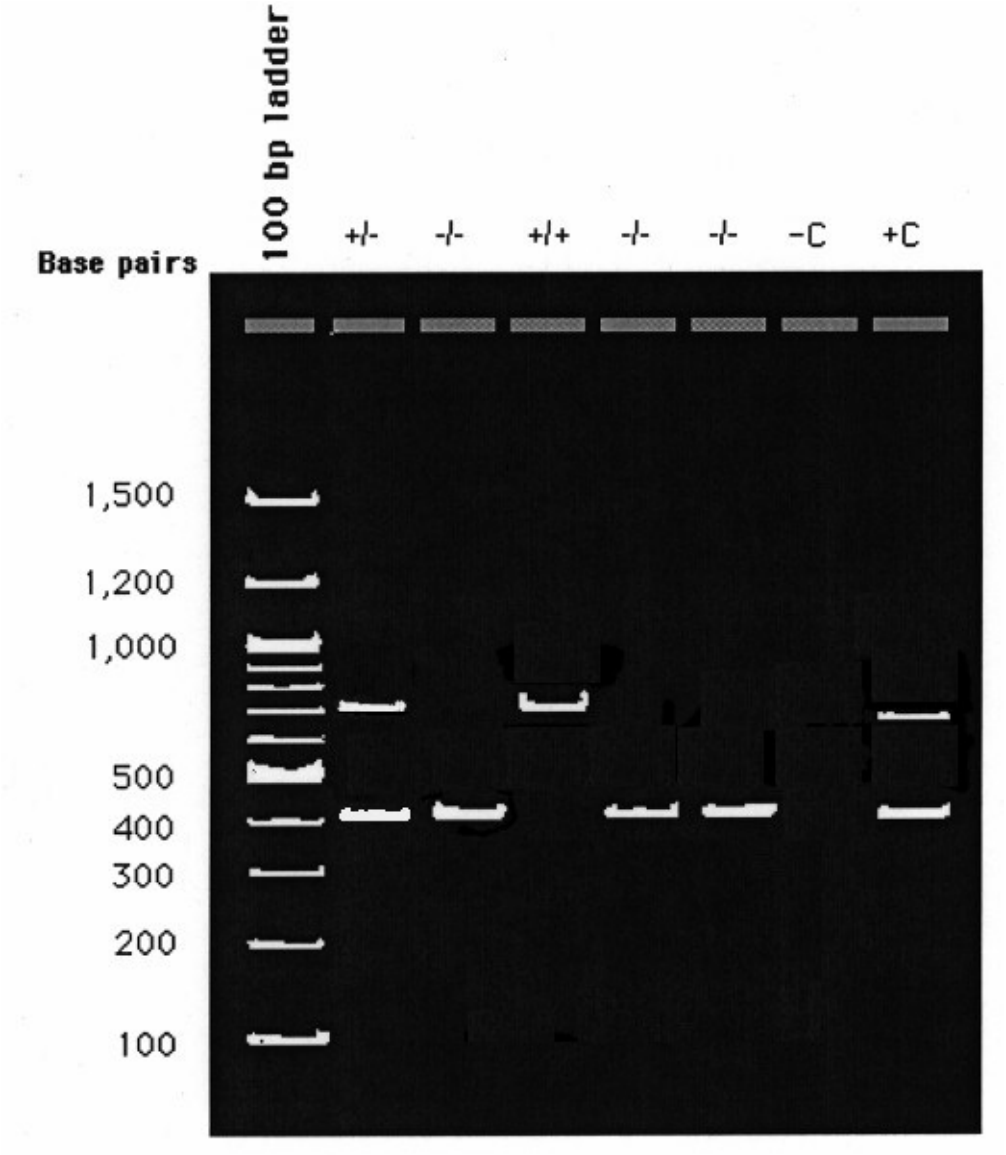


Figure 6. Agarose gel of homozygous and heterozygous individuals for the PV92 *Alu* insertion. A 100 base pair ladder is loaded in the first lane and is used as a size marker. The bands differ by 100 bp in length. The 500 bp band and the 1,000 bp band are more intense when stained with ethidium bromide than re the other bands of the ladder. The next 5 lanes contain the results of homozygous and heterozygous individuals. A negative control (-C) does not contain any template DNA and should therefore contain no bands. The positive control (+C) is heterozygous for the *Alu* insertion; it contains a 415 bp band and an 715 bp band.