Name:_____Period:_____Date:_____
Biotechnology Laboratory at William Floyd High School
Lab activity: ALU and you!

Background:

We are humans. We have two legs 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, or fish, is the molecule **deoxyribonucleic acid (DNA).** The basic building block of DNA is the nucleotide made of a deoxyribose sugar, a phosphate, and one of the four bases **A (adenine), C (cytosine), G (guanine), or T (thymine)**. 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 bound 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. 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.

The region of bases on DNA that holds the information 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 23,000 genes in human DNA. The human genome (the total sum of our genetic makeup) is made up of approximately 3 billion base pairs distributed on 23 chromosomes. All cells in your body, except red blood cells(which have no nucleus), sperm, and eggs, contain these 46 pairs of chromosomes (sperm and egg cells contain only 23 chromosomes). Only 15% of this enormous amount of DNA is used directly to 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 90–97% of the entire genome. Since these non-coding areas such as introns have no defined role, they were 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 was Alu. Alu repeats are approximately 300 base pairs in length. 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. No one is sure where they came from, 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 from your mother and father at the time you were conceived.

The polymerase chain reaction (PCR) is a method used by scientists to rapidly copy, in a test tube (in vitro), specific segments of DNA. By using 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. Deoxynucleoside triphosphates containing the bases A, C, G, and T (NTPs) 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 requires the metal ion magnesium (Mg^{+2}) 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 for the DNA polymerase reaction. PCR is accomplished by cycling a reaction through several temperature steps. Millions of copies of target 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 "Alu" PV92). If you have it, it will be found on your number 16 chromosome. You will use your own DNA as template for this experiment. After you amplify the Alu repeat region, you will determine whether or not you carry this particular Alu sequence on one or both or none of your number 16 chromosomes. This will be accomplished by separating the DNA in your PCR sample on an agarose gel using electrophoresis, a process that separates DNA by size. There is no medical significance to having or not having the insert.

(**AP Biology extension only:** 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.)

Objectives:

- Successfully isolate DNA from cheek cells
- Prepare a PCR reaction for amplification of an Alu insert

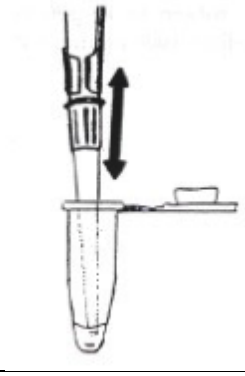
Pre-lab questions:

1. What is needed from the cells for PCR?

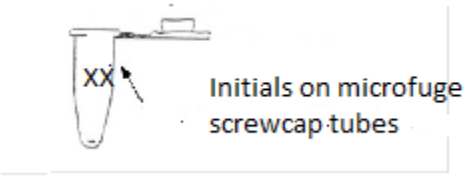
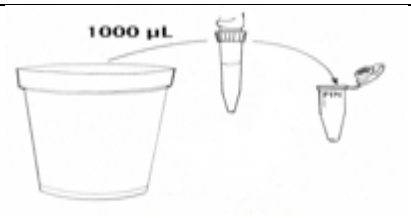
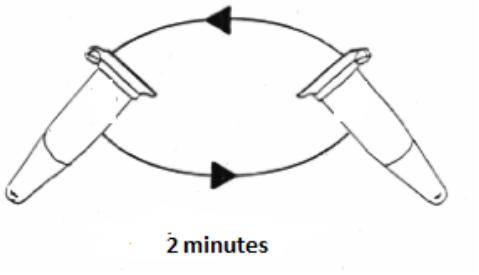
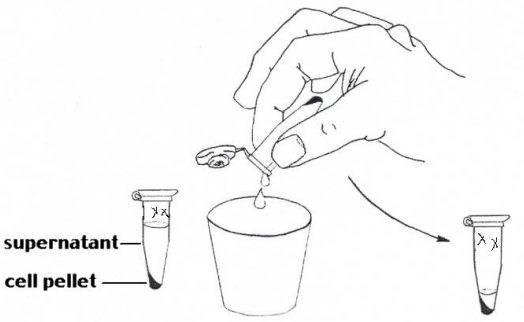
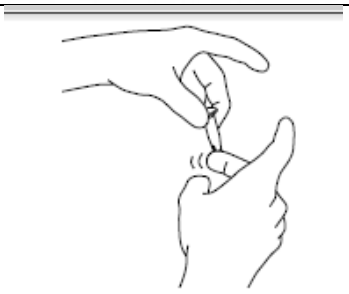
2. What structures must be broken to release the DNA from a cell?

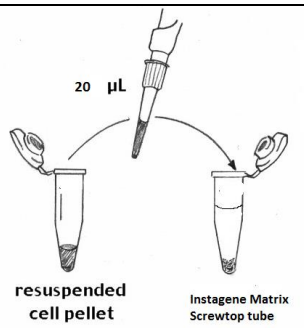
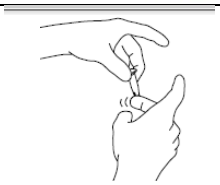
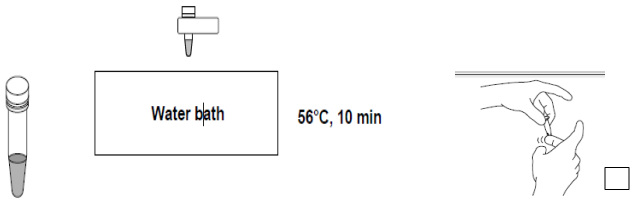
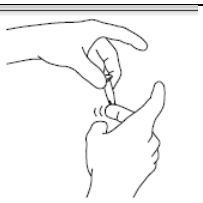
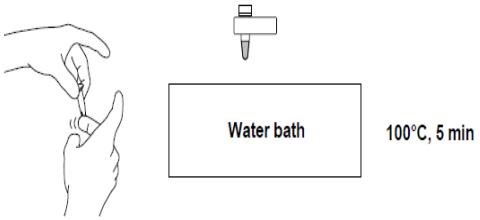
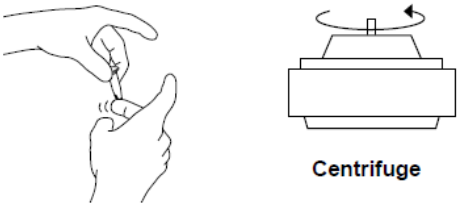
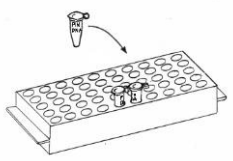
Procedure:

In the first step, you will rinse your mouth with a salt solution. This step typically dislodges hundreds of cells from the cheek epithelium. An aliquot of the mouthwash solution is centrifuged to collect the dislodged cells, which are then resuspended in a small volume of saline. The resuspended cells are then added to a solution of InstaGene™ matrix to remove any metal ions (such as magnesium) which might destroy your DNA. Magnesium (and other metal ions) can help enzymes that break down DNA present in saliva and the environment. The InstaGene™ matrix /cell sample is then boiled to break open the cells. Since the sample is heated at a high temperature, the DNA, following this step, will be in a single-stranded form. The sample is then centrifuged briefly to collect the InstaGene™ matrix and an aliquot of the liquid supernatant containing released DNA is used for PCR.

Important Laboratory Practices	
Add reagents to the bottom of the reaction tube, not to its side. b. Add each additional reagent directly into previously added reagent. c. Do not pipet up and down, as this introduces error. This should only be done only when resuspending the cell pellet and not to mix reagents. d. Make sure contents are all settled into the bottom of the tube and not on the side or cap of tube. A quick spin may be needed to bring contents down. 	Pipet slowly to prevent contaminating the pipette barrel. b. Change pipette tips between each delivery. c. Change the tip even if it is the same reagent being delivered between tubes. Change tip every time the pipette is used! 
Keep reagents on ice. 	Check the box next to each step as you complete it. 

Part I: DNA Preparation Using a Saline Mouthwash
Place a check mark in the box as you complete each step.

1. Your team of **two students** should have **1 screwcap tube** containing 200 µl InstaGene™ matrix, 1.5 ml **microcentrifuge tube**, and a cup containing 10 ml of **0.9% saline solution.** ***Label 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 (spit) the saline back into the cup**.	 ☐  ☐
3. Carefully use a disposable transfer pipet to place 1 ml of your swished saline into the microcentrifuge tube (use the markings on the side of the microcentrifuge tube and disposable transfer pipette 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.	 ☐
5. Pour off the supernatant (liquid) and discard. Taking care not to lose your cell pellet, carefully blot your microcentrifuge tube on a paper towel. It's OK for a small amount of saline (about 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. Incubate the tube for 10 min in the 56°C water bath. 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	 ☐
11. Incubate the tubes for 5 min at 100°C in the water bath (boiling) for 5 minutes.	 ☐
12. Remove the tubes from the 100°C water bath or dry bath and shake or vortex several times to resuspend the sample. Place the eight microcentrifuge 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).	 ☐
13. Store your screwcap tube in the refrigerator until the next laboratory period, or proceed to step 2 of Part II 2 if your teacher instructs you to do so.	 ☐

Part II: 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. Taq 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 the DNA that was extracted from your cheek 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.

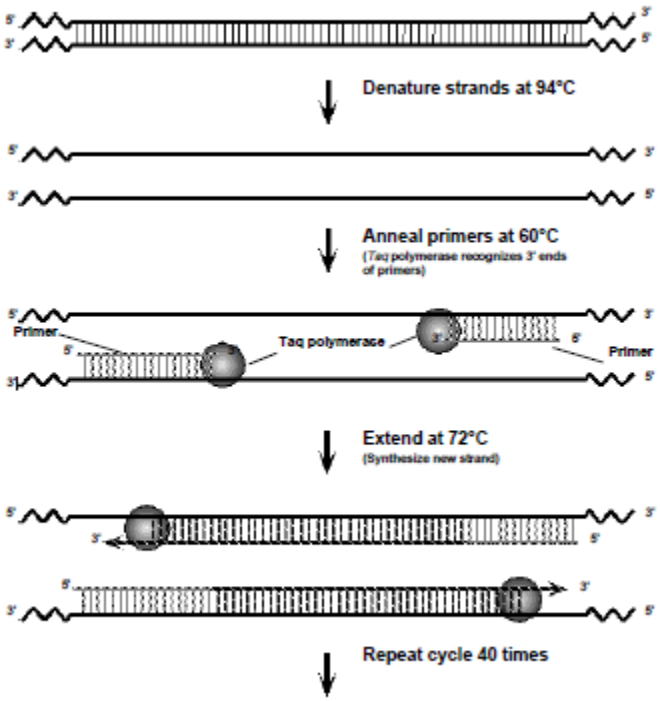
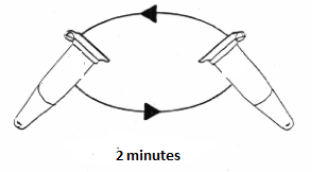
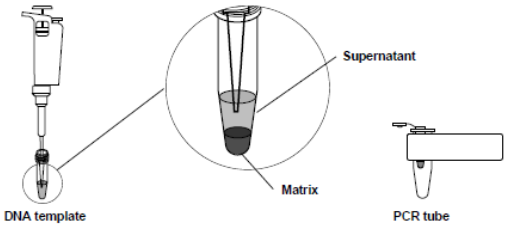
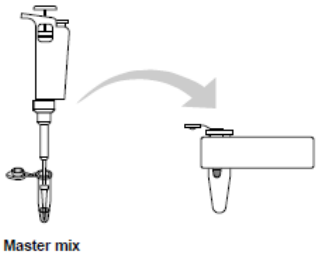
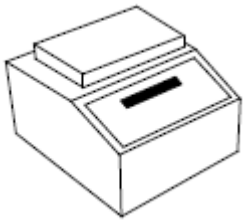


Fig. 9. A complete cycle of PCR.

Place a check mark in the box as you complete each step.

1. Obtain your screwcap 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 and capless micro test tube. Label each PCR tube on the side of the tube with your initials and place the PCR tube into the capless micro test tube as shown. Place the PCR tube in the foam micro test tube holder.	 ☐
3. Transfer 20 µl of your DNA template from the supernatant in your screwcap tube into the bottom of the PCR tube. **Do not transfer any of the matrix beads 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.	 ☐
5. Remove your PCR tube from the capless micro test tube and place the tube in the Gene Cycler or MyCycler 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.	

Analysis questions:

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

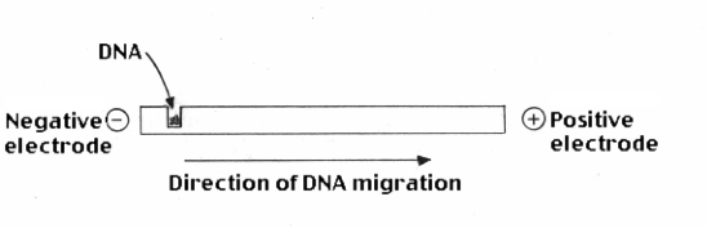
2. Why are there nucleotides (A, T, G, and C) in the master mix? What are the other components of the master mix, and what are their functions?

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

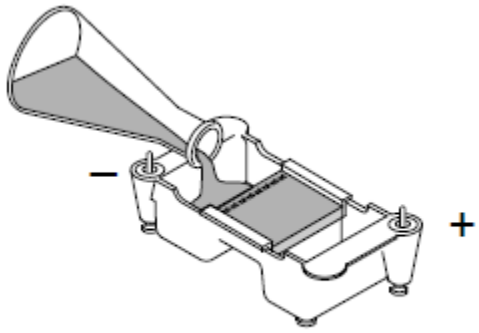
Part III: Gel Electrophoresis

Background: To determine whether or not you carry the *Alu* repeat, you will need to "see" the products of your amplification. This will be done using a process called **gel electrophoresis** in which electric current forces the DNA to move through a special gel material. Since DNA is negatively charged, it will migrate in an electric field towards the positive electrode (Figure 2). When moved through a gel, shorter fragments of DNA move at a faster rate than longer ones.

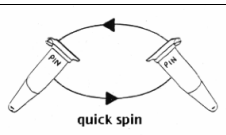
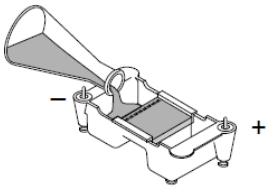
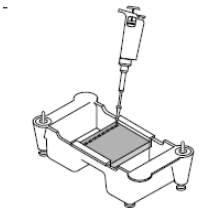
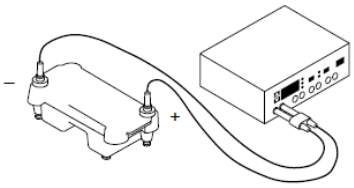
Figure 2. Side view of an agarose gel showing DNA loaded into a well and the direction of DNA fragment migration during electrophoresis.

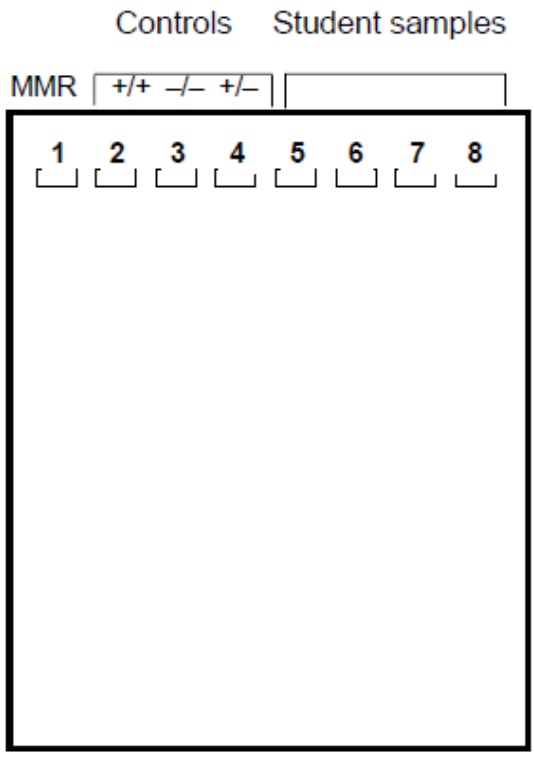


The gel material to be used for this experiment is called **agarose,** a Jell-O™ like substance made from red algae. The gel, in its casting tray, is placed in a buffer chamber connected to a power supply and running buffer is poured into the chamber until the gel is completely submerged. **Loading dye** is a colored, thick liquid containing dyes (making it easy to see) and sucrose or glycerol (making it dense). To a small volume of your total PCR reaction, you will add loading dye, mix and then put the mixture into one of the wells of your agarose gel. When all wells have been loaded with sample, you will switch on the power supply. The samples should be allowed to run until the dye front (either yellow or blue, depending on the dye used) is 1 to 2 cm from the bottom of the gel. The gel can then be moved, stained and photographed.



Place a check mark in the box as you complete each step.

Obtain your PCR tube from the thermal cycler and place in the capless micro test tube. Pulse-spin the tube for ~3 seconds at 2,000 x g.	 ☐
Add 10 µl of PV92 XC loading dye into your PCR tube and mix gently.	 ☐
Place an agarose gel in the electrophoresis apparatus. Check that the wells of the agarose gels are near the black (–) electrode and the base of the gel is near the red (+) electrode.	☐
Fill the electrophoresis chamber and cover the gel with 0.25x TAE buffer. This will require ~275 ml of 0.25x buffer.	 ☐
Using a clean tip for each sample, load the samples into 8 wells of the gel in the order on the next page	 ☐
Secure the lid on the gel box. The lid will attach to the base in only one orientation: red to red and black to black. Connect the electrical leads to the power supply.	 ☐
Turn on the power supply and electrophorese your samples at 200 V for 20 minutes.	



Lane	Sample	Load Volume
1	MMR	10 µl
2	Homozygous (+/+) control	10 µl
3	Homozygous (–/–) control	10 µl
4	Heterozygous (+/–) control	10 µl
5	Student 1	20 µl
6	Student 2	20 µl
7	Student 3	20 µl
8	Student 4	20 µl

* MMR = molecular mass ruler (DNA standard)

For optimum visibility, 10 µl of each control sample, 10 µl of the EZ Load molecular mass ruler (DNA standard), and 20 µl of amplified student samples should be loaded on each gel.

Staining of Agarose Gels

1. When electrophoresis is complete, turn off the power and remove the lid from the gel box. Using gloves, carefully remove the gel tray and the gel from the gel box. Be careful, **the gel is very slippery**. Push the gel off the gel tray with your thumb and carefully slide it into your plastic staining tray.

Protocol 1: Quick staining (requires 12–15minutes)

a. Add 120 ml of 100x Fast Blast stain into your staining tray (2 gels per tray).

b. Stain the gels for 2 minutes with gentle agitation. Save the used stain for future use. Stain can be reused at least 7 times.

c. Transfer the gels into a large washing container and rinse with warm (40–55°) tap water for approximately 10 seconds.

d. Destain by washing **twice** in warm tap water for 5 minutes each with gentle shaking for best results.

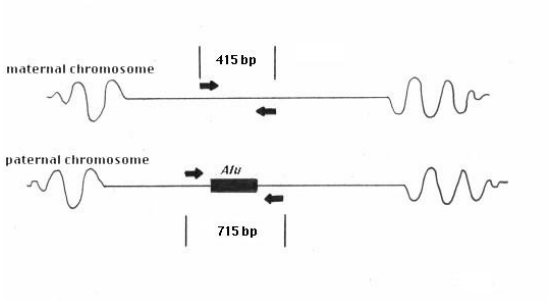
e. Place the gel on a light background and record your result. Draw the banding pattern on the gel template on the previous page.

f. With the help of your instructor, determine whether you are homozygous or heterozygous for the Alu insertion.

g. To obtain a permanent record, air-dry the gel on gel support film. Tape the dried gel onto your lab notebook. Avoid exposure of the stained gel to direct light, since it will cause the bands to fade.

Analysis:

By looking at 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 short 415 bp fragment if the repeat is not present. If the repeat is present, a long 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.



When you look at the gel, 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 long 715 bp band), we can say that they are positive for the insertion and write that allele with a "+" sign. If the *Alu* repeat is absent (a 415 bp short band is generated in the PCR), we write a "-" allele. If a student has a single band, whether it is a single short band or a single long 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 has a short band and a long 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**. See the table below for a quick summary of the allele designations.

Possible Bands	Allele Designation	Genotype	*Alu* Insert
1. One band at 415 bp	-/-	homozygous	No *Alu* insert
2. One band at 715 bp	+/+	homozygous	*Alu* insert present on both chromosomes
3. One band at 415 bp and a second band at 715 bp.	+/-	heterozygous	*Alu* insert on one of the chromosomes

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

2. How many people have +/+, +/–, and –/– in your class population?, Fill in the table below with your class data.

Table 1. Observed Genotypic Frequencies for the Class

Category	Number	Frequency (# of Genotypes/Total)
Homozygous (+/+)		
Heterozygous (+/–)		
Homozygous (–/–)		
	Total =	= 1.00

3. Explain how agarose electrophoresis separates DNA fragments. Why does a smaller DNA fragment move faster than a larger one?

4. What kinds of controls are run in this experiment? Why are they important? Could others be used?