

# Chapter 1

## Human DNA Fingerprinting by Polymerase Chain Reaction

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## Introduction

In this experiment, students use polymerase chain reaction (PCR) to amplify a short nucleotide sequence from chromosome 1 to create their own DNA fingerprint. The experiment is divided into three parts: sample isolation, amplification by PCR, and agarose gel electrophoresis. The first two parts have convenient stop points that allow the instructor to freeze student samples until ready to perform the next portion of the procedure. Since the objective is to illustrate variability found in the human genome, the experiment is performed by each student, rather than by lab groups. The experiment should be preceded by a lecture explaining polymerase chain reaction and its applications. The organization of the human genome is discussed with particular reference to repeated DNA sequences and their use in paternity/forensic applications.

Sample isolation uses a simple mouthwash procedure that may be completed in about 45 minutes. The isolation uses just two reagents, sodium chloride and the gel resin “Chelex.” Generally, students find the protocol easy to follow. The most common problem is students who mistakenly discard their cell pellet down the sink. Each time the experiment is carried out, instructors should verify that the Chelex solution has a pH of about 10.5. Use of Chelex at a more acidic pH will severely reduce the yield of PCR product. If desired, the DNA containing sample may be stored frozen until needed for amplification or in anticipation of further experimentation.

Setting up the PCR reaction consists of adding a small amount of DNA solution to a reaction tube containing the reagents needed for amplification. Usually, students are given an aliquot of a PCR mix that contains everything needed for amplification, except for magnesium chloride, which is added separately. The PCR set up can be completed in 15 minutes. The amplification is performed automatically using a thermal cycler and requires about 2.5 hours to finish. Students can perform other tasks during the amplification, or amplification can be started at the conclusion of the lab period. Following amplification, samples are retrieved from the thermal cycler and can be stored frozen until ready to carry out gel electrophoresis.

It is best to run gels that can accommodate 8–10 samples each, so that students will be able to compare their results with those from other classmates. Student alleles will range in size from 300 to 700 basepairs, so inclusion of a suitable molecular weight marker will aid gel interpretation. Ethidium bromide staining is recommended for this experiment.

## Materials

**Equipment required:** (per class of 25 students)

- Programmable DNA thermocycler
- Preparatory centrifuge (for 15 ml tubes)
- Microcentrifuge (for 1.5 ml tubes)

Hot plates and 600 ml beakers (2)  
 Adjustable micropipet  
 Electrophoresis chamber(s)  
 Power supply  
 UV transilluminator  
 Polaroid camera  
 Microwave oven (optional)  
 Test tube racks

**Supplies required:** (per class of 25 students)

Paper cups (25)  
 15 ml tubes (25)  
 1.5 ml tubes (100)  
 PCR tubes (25)  
 Disposable transfer pipets (100) (not needed if micropipets are available)  
 Large weigh boats (or other suitable containers) for staining gels  
 Permanent lab markers  
 Polaroid film (one exposure per student)

**Reagents required:** (per class of 25 students)

NaCl solution, 0.9% (300 ml)  
 Chelex (10%) from Carolina Biological (or BioRad) 100–200 mesh, sodium salt, pH 10.5 (20 ml)  
 PCR mix from Carolina Biological (1000  $\mu$ l in vial)  
 MgCl<sub>2</sub> solution from Carolina Biological (500  $\mu$ l in vial)  
 Distilled water  
 Mineral oil (5 ml in dropper vial)  
 Agarose, sufficient to analysis 25 student samples on 1.5% gels  
 Electrophoresis buffer  
 Loading dye  
 Ethidium bromide solution (1  $\mu$ g/ml)  
 0.05 M KMnO<sub>4</sub>, 0.25 N HCl, and 0.25 N NaOH are recommended for decontaminating ethidium bromide staining solution and stained gels.

**Notes for the Instructor**

**Pre-Laboratory Preparation**

**Solutions**

Saline and Chelex solutions may be prepared days in advance of the laboratory. Saline solution (0.9% NaCl) may be purchased sterile or prepared and autoclaved. It is critical that the pH of the 10% Chelex solution be sufficiently alkaline. Weigh out Chelex and add to a 50 mM Tris base (MW = 121.1) solution (at its natural pH). Shake the tube containing the Chelex solution to resuspend the resin and measure the pH. If less than 10.5, then add some concentrated NaOH to bring it within the 10.5–11.0 range.

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1. Aliquot 10 ml of saline solution into each of 25 15 ml culture tubes.
2. Aliquot 700  $\mu$ l of Chelex solution into each of 25 1.5 ml tubes. Be sure to shake Chelex supply tube to resuspend the resin beads *each time before pipetting*.
3. PCR mix and  $MgCl_2$  may collect in cap tops during shipping. *To have full volume available for student use, it may be necessary to pool these reagents* by spinning tubes briefly in a microcentrifuge or rapping tube sharply on lab bench. Store PCR mix and magnesium chloride solution in a beaker of cracked ice during the experiment.
4. Set up one boiling water bath per 12–15 students, composed of a hot plate and 600 ml beaker covered with aluminum foil. Poke holes in aluminum foil to receive 1.5 ml tubes.
5. Prepare student stations. The following should be available for each student:
  - Saline solution (10 ml in 15 ml tube)
  - Chelex (700  $\mu$ l in 1.5 ml tube)
  - 1.5 ml tubes (2)
  - Test tube rack
  - Lab marker
  - Paper cup
  - Micropipet (1000  $\mu$ l) or several disposable transfer pipets

#### Preparation for PCR Amplification

1. Samples should begin thermocycling within 30 minutes after the reaction components are added together. This helps to prevent mispriming and nonspecific amplification. After DNA samples have been isolated, prepare a class PCR master mix consisting of:
  - PCR mix (500  $\mu$ l)
  - $MgCl_2$  solution (250  $\mu$ l)
  - distilled water (375  $\mu$ l)
2. Aliquot 45  $\mu$ l of PCR master mix into each PCR reaction tube.
3. Program thermocycler for 30 cycles:
  - 94°C for 1 minute
  - 65°C for 1 minute
  - 72°C for 1 minute
4. Students will require approximately 1 hour for sample preparation and set up of PCR reactions. The PCR program will require about 135 minutes to complete the amplification. Amplified samples may be stored frozen for weeks.

#### Preparation for Electrophoresis

Prepare sufficient 1.5% agarose gels to accommodate the student samples. Gels should be poured thick enough so that 20  $\mu$ l of sample can be loaded into a well.

#### Sample Preparation

Human genomic DNA suitable for PCR analysis may be obtained from almost any type of cell. This experiment uses buccal (cheek) cells, because their collection is rapid, noninvasive, painless,

and bloodless. The cell lysate obtained by boiling cheek cells is extremely crude in biochemical terms — it contains the target DNA, as well as numerous molecules that can inhibit the activity of *Taq* DNA polymerase. Chelex, a negatively-charged resin, is employed to bind positively-charged metal ions present in the cell lysate, which are known inhibitors of *Taq* polymerase activity.

### Fine Points of the Lab Procedure

Be alert to the following cautions when performing the experiment. Where appropriate, discuss fine points with students, and have them make annotations on their Student Outlines.

*Effects of food particles:* Although it is not advisable for students to eat immediately prior to the experiment, food particles rinsed out with the mouthwash appear to have little effect on PCR amplification. However, fruit and vegetable particles (notably from apples) may clog pipet tips and make pipetting extremely difficult.

*Pelleting cheek cells:* A clinical centrifuge that develops 500–1,000 × g is sufficient pelleting cheek cells in Procedure A.5.

*Suspending chelex before use:* Make sure the resin beads are suspended before adding Chelex to cheek cells in Procedure A.7 and before removing Chelex/cell suspension in Procedure A.9.

*Storing cheek cell DNA samples:* Student DNA samples isolated in Procedure A should be held on ice until setting up PCR reactions. Samples may be stored frozen at -20°C for months without significant degradation.

*Setting up PCR reactions:* DNA thermal cycling should be started as soon as possible after preparing PCR reactions in Procedure B. Nonspecific priming may occur if PCR reagents sit too long without cycling.

*Electrophoresing:* The alleles typically amplified in this experiment range in size from 300–700 nucleotides. Larger alleles may not amplify as efficiently as smaller ones. Adequate separation of alleles is achieved when the bromophenol blue dye front has moved approximately 40 mm from the sample well.

*DNA Ladder:* A “DNA ladder,” composed of regularly-spaced fragments, is run along with student samples as an aid to calculate the base pair sizes of D1S80 alleles amplified by PCR. The ladder employed in this kit contains 1 to 34 repeats of a 123-base pair region of the rat prolactin gene. The larger alleles do not resolve one from another in the 1.5% agarose gel used in this experiment.

*Gel staining:* The signal obtained by PCR is rather weak — with typically 0.05–0.2 µg of DNA in each band (allele). Therefore, it is necessary to stain gels with a sensitive dye, such as ethidium bromide. Ethidium bromide, like many natural and man-made substances, is a mutagen by the Ames microsome assay and a suspected carcinogen. To avoid contamination of gel apparatuses and lab benches used by students, it is best to stain gels after electrophoresis — rather than incorporating ethidium in the gel and electrophoresis buffer. With responsible handling, the dilute staining solution (1 µg/ml) used in this kit poses minimal risk:

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1. Wear rubber gloves when staining gel, viewing gels, and cleaning up.
2. Confine all staining to sink area restricted from student use.
3. Flood gels with ethidium bromide solution, and allow to stain for 20 minutes. (Staining time depends on thickness of gel.)
4. Following staining, use funnel to decant as much ethidium bromide solution as possible from staining tray back into storage container. Stain may be reused to stain 15 or more gels. When staining time increases markedly, disable ethidium bromide solution as explained below.
5. Rinse gel and tray under running tap water to remove excess ethidium bromide solution.
6. Destain gel in tap or distilled water for 20–30 minutes to remove background ethidium bromide. Check periodically under UV light to ascertain when alleles are easily visible.
7. Wipe down camera, transilluminator, and staining area with paper towels and water.
8. After viewing and photographing, disable stained gels and used staining solution:
  - (a) Add 1 volume of 0.05 M  $\text{KMnO}_4$ , and *mix carefully*.
  - (b) Add 1 volume of 0.25 N HCl, and *mix carefully*.
  - (c) Let stand at room temperature for several hours.
  - (d) Add 1 volume of 0.25 N NaOH, and *mix carefully*.
  - (e) Discard disabled solution down sink drain. Drain disabled gels, and discard in regular trash.

*Viewing gels:* Transillumination, where light passes up through the gel, gives superior viewing of gels stained with ethidium bromide. A mid-wavelength ultraviolet lamp emits in the optimum range for illuminating ethidium bromide-stained gels (260–360 nm). Avoid shortwave lamps, whose radiation is dangerous to humans and damaging to DNA samples. Long-wavelength (“black light”) lamps, though safe, give less intense illumination. **Caution:** *Ultraviolet light can damage the retina of the eye. Never look at unshielded UV light source with naked eyes. Only view through filter or safety glasses that absorb harmful wavelengths.*

*Photographing gels:* A Polaroid “gun” camera, equipped with a close-up diopter lens, can be used to photograph gels on a UV or transilluminator. A plastic hood extending from the front of the camera forms a mini-darkroom and provides correct lens-to-subject distance. Alternately, a close-focusing 35 mm camera can be used.

## Student Outline

### Introduction

Although the DNA from different individuals is more alike than different, there are many regions of the human chromosomes that exhibit a great deal of diversity. Such variable sequences are termed “polymorphic” (meaning many forms) and provide the basis for genetic disease diagnosis and forensic/paternity testing. Many DNA polymorphisms are found within the estimated 90% of the human genome that does not code for protein. Most of this noncoding DNA lies between genes and has been called spacer or even “junk” DNA. A special type of polymorphism, called VNTR (variable number of tandem repeats), is composed of repeated copies of a DNA sequence that lie adjacent to one another on the chromosome. In this experiment, students amplify across a noncoding region of chromosome 1 containing a VNTR called D1S80, which has a repeat unit of 16 base pairs. At this locus, most individuals have alleles containing between 14 and 40 repeats, which are inherited in a Mendelian fashion on the maternal and paternal copies of chromosome 1.

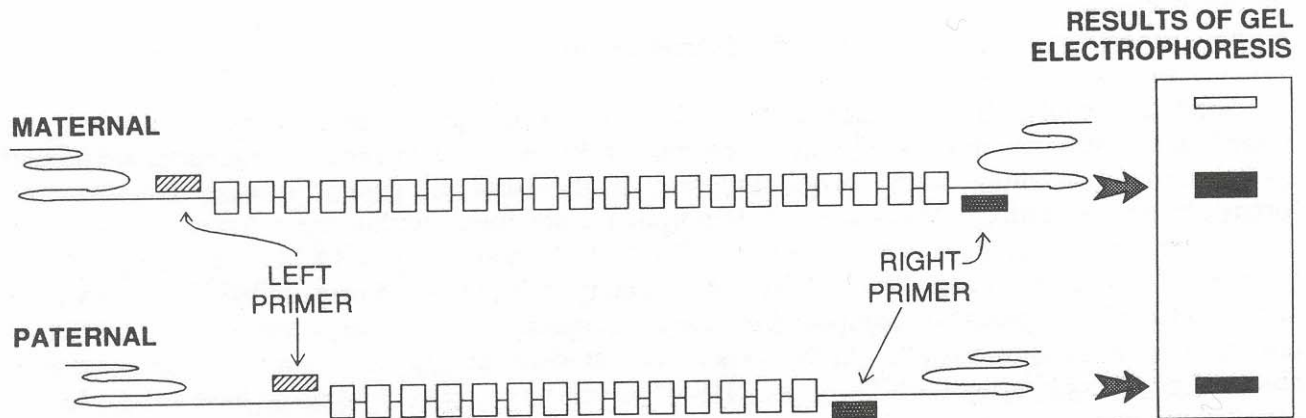
Students obtain a sample of their own cheek cells using a saline mouthwash (*bloodless and noninvasive*). The cells are collected by centrifugation and resuspended in a solution containing the resin “Chelex,” which binds metal ions that inhibit the PCR reaction. The cells are lysed by boiling and centrifuged to remove cell debris. A sample of the supernatant-containing chromosomal DNA is combined with a buffered solution of heat-stable *Taq* polymerase, short oligonucleotide primers, the four deoxynucleotide building blocks of DNA, and the cofactor  $MgCl_2$ . The PCR mixture is placed in a DNA thermal cycler and taken through 30 cycles consisting of:

1. 1 minute at 94°C, during which the chromosomal DNA is denatured into single strands;
2. 1 minute at 65°C, during which the primers hydrogen bond to their complementary sequences on either side of the D1S80 locus; and
3. 1 minute at 72°C, during which the *Taq* polymerase extends a complementary DNA strand from each primer.

The primers used in this experiment bracket the D1S80 locus and selectively amplify that region of chromosome 1 (Figure 1). Following PCR amplification, student alleles are separated according to size using agarose gel electrophoresis. After staining with ethidium bromide, one or two bands are visible in each student lane — indicating whether an individual is homozygous or heterozygous for the D1S80 locus. Different alleles appear as distinct bands, each composed of several billion copies of the amplified allele. A band's position on the gel indicates the size (and number of repeat units) of a D1S80 allele: smaller alleles move a longer distance from their origin, while larger alleles move a shorter distance.

### Procedure A: Isolate Cheek Cell DNA

1. Use a permanent marker to label your name on a test tube containing saline solution and on two clean 1.5 ml tubes.
2. Pour 10 ml of saline solution into your mouth and vigorously swish for 10 seconds. *Save test tube for later use.*
3. Expel saline solution into a paper cup. *Save paper cup for later use.*
4. Carefully pour saline solution from paper cup back into test tube, and close cap tightly.



**Figure 1.1** Map of D1S80 locus on chromosome 1.

5. Place your sample tube, together with other student samples, in a balanced configuration in a clinical centrifuge, and spin for 10 minutes.
6. Carefully pour off supernatant into a paper cup. *Take care not to disturb cell pellet at bottom.*
7. Set micropipet to 500  $\mu$ l. Draw Chelex solution in and out of pipet tip several times to suspend the resin beads. Then, before resin settles, rapidly transfer 500  $\mu$ l of Chelex to test tube containing your cell pellet.
8. Resuspend cells pipetting in and out several times. Examine against light to confirm that no visible clumps of cells remain.
9. Pipet several times to resuspend cells/resin, then transfer 500  $\mu$ l of your cell sample into a clean 1.5 ml tube.
10. Place your sample into boiling water bath for 10 minutes. Use forceps to remove your tube from boiling water bath, and allow to cool for 1 or 2 minutes. (Tube may be placed on ice.)
11. Place your sample tube in a balanced configuration in microcentrifuge, and spin for 30 seconds (or 1 minute in nanofuge).
12. Use fresh tip to transfer 200  $\mu$ l of the clear supernatant to a clean 1.5 ml tube. *Take care not to pick up Chelex/cell debris from bottom of tube.*
13. Store your sample on ice or in freezer until ready to begin Procedure B.
14. Pour supernatant from step 6 down sink and rinse with water.

**Procedure B: Set Up PCR Reaction**

1. Use a permanent marker to label the cap of a 0.5 ml PCR tube with your initials. Use the table below as a checklist while adding reagents to a 0.5 ml PCR reaction tube.

Water	Cheek DNA	PCR Mix	MgCl <sub>2</sub>
15 $\mu$ l	5 $\mu$ l	20 $\mu$ l	10 $\mu$ l

2. Add one drop of mineral oil from its dropper bottle to the PCR reaction tube. *Take care not to touch dropper to PCR reaction or tube.*
3. Close the tube top. Pool and mix reagents by pulsing in a microfuge or by sharply tapping tube bottom on the lab bench. (To spin in microcentrifuge, first place PCR tube inside an empty 1.5 ml tube.)
4. Store your sample on ice or in freezer until ready for amplification along with the other student samples.
5. **To be performed by instructor:** Program and start thermal cycler with a step file: 94°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute; repeat for 30 cycles.

**Procedure C: Cast 1.5% Agarose Gel**

1. Pour enough agarose solution into gel-casting tray to fill to depth of about 8 mm.
2. After agarose solidifies, place gel-casting tray into gel electrophoresis chamber, and pour in TBE electrophoresis buffer to level that just covers gel surface. (Close chamber top and submerged gel will remain in good condition for several days.)

**Procedure D: Load Gel and Electrophorese**

1. Use permanent marker to label a clean 1.5 ml tube with your name.
2. Transfer 20  $\mu$ l of your PCR sample to the clean 1.5 ml tube. *Take care not to transfer any of the mineral oil from the PCR tube. Submerge pipet tip through mineral oil layer to draw off sample, and use tissue to wipe off any mineral oil clinging to the outside of the pipet tip.*
3. Add 2  $\mu$ l of loading dye to the PCR sample. Close tube top, and mix by tapping tube bottom on lab bench, pipetting in and out, or pulsing in a microcentrifuge.
4. Add 20  $\mu$ l of PCR/loading dye sample into assigned well of a 1.5% agarose gel. Expel any air in tip before loading, and be careful not to punch tip of pipet through bottom of gel. (20  $\mu$ l of DNA size markers will be loaded into one lane of each group of student samples.)
5. Electrophorese at 100 volts for 45–60 minutes. Adequate separation will have occurred when the bromophenol dye front has moved approximately 40 mm from the wells.

- Turn off power supply, remove casting tray, and transfer gel to disposable staining tray. Take gel to controlled area for staining, viewing, and photographing.

### Results and Discussion

- Examine the photograph of the stained gel containing your experiment and those of other individuals. Orient the photograph with the wells at the top, and interpret results in each lane using the following key:
  - A diffuse (fuzzy) band, usually appearing at the same position in each lane toward the bottom of the gel. This so-called “primer dimer” is not amplified human DNA, but is an artifact of the PCR process that results from the primers amplifying themselves.
  - No bands visible. This usually results from an error during sample preparation, such as losing the cheek cell pellet or failing to resuspend Chelex beads prior to transferring solutions between test tubes.
  - One band visible. The simplest explanation is that the individual is homozygous at the D1S80 locus, having inherited the same allele on maternal and paternal chromosome 1. However, it is also possible that the individual is, in fact, heterozygous, but the two alleles are so similar in size that they cannot be resolved (separated) in this gel system. Another possibility is that a larger allele (with many repeats) has failed to amplify efficiently.
  - Two bands visible. The individual is heterozygous at the D1S80 locus. Often, the larger allele appears less intense than the smaller one.
  - Three or more bands visible. The two brightest bands are likely the true alleles. Additional bands may occur when the primers bind nonspecifically to chromosome loci other than D1S80 and give rise to additional amplification products.
- Population studies have identified 29 different alleles at the D1S80 allele, and estimate that 90% of individuals are heterozygous at this locus. Determine the number of different alleles represented among your classmates and the percent of heterozygous individuals. How does your class data compare with that of the general population? What reasons can you give for differences?
- Based on your results, do you think this protocol could be used to link a suspect with a crime or establish a paternity relationship? Why do you think so? How could you modify the experiment to improve its ability to positively identify individuals?
- The sizes of alleles can be estimated simply by comparing their positions with the ladder of size markers included in one lane of each gel. However, a closer determination of allele sizes can be obtained by graphing the function that determines the migration of linear DNA fragments in an electrophoretic field:

$$D = \frac{l}{\log^{10} MW}$$

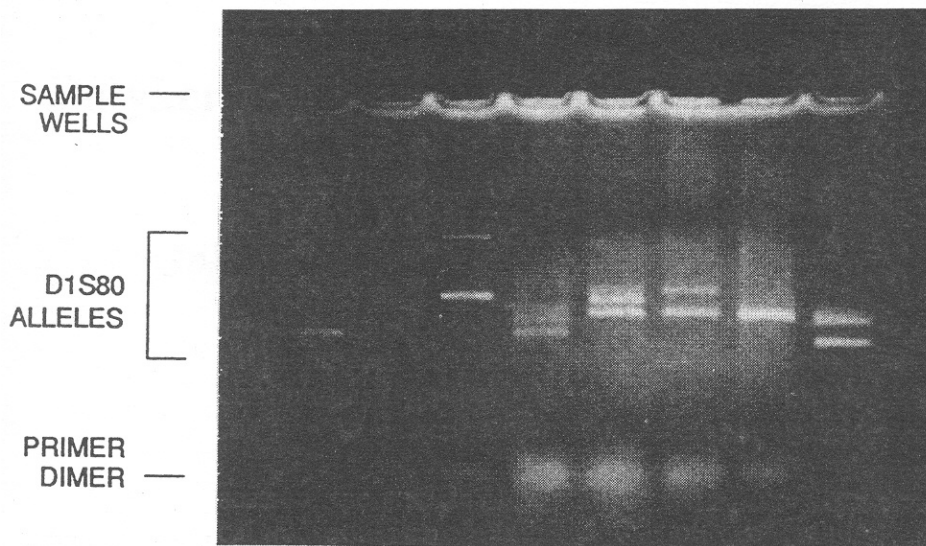
where  $D$  equals distance migrated and  $MW$  equals the molecular weight of the fragment. For simplicity's sake, biologists often substitute base pair length for molecular weight in this calculation.

- (a) The fragments in the marker ladder are multiples of a 123 base pair repeat. Orient your gel photo with the wells at the top and working from bottom to top, assign base-pair sizes to the first eight bands that appear in the ladder on your gel: 123, 246, 369, 492, 615, 738, 861, 984. Carefully measure the distance (in mm) each marker fragment migrated from the sample well. Measure from front edge of well to leading edge of each band.
- (b) Set up semilog graph paper with distance migrated as the  $x$  (arithmetic) axis and base pair length as the  $y$  (logarithmic) axis. Then, plot distance migrated versus base pair length for each marker fragment. Connect data points with a line.
- (c) Measure and record distances migrated by various alleles. To determine the base pair size of an allele, first locate the distance it migrated on the  $x$ -axis. Then, use a ruler to draw a vertical line from this point to its intersection with the marker data line. Now, extend a horizontal line from this point to the  $y$ -axis. The number on the  $y$ -axis is the calculated base pair size of the allele.
- (d) Compare the largest and smallest allele observed in your class with the known range of most D1S80 alleles: 300–700 base pairs.
- (e) Determine the size of the primer dimer.

APPENDIX A  
*Further Reading*

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APPENDIX B  
*Expected Results*



**Figure 1.2.** Photograph of an ideal gel.

Answers to questions in Discussion section:

2. Class results typically show six to eight different alleles and 50% heterozygosity. Reasons for the underrepresentation of alleles and heterozygosity in the class results include: inability of gel system to resolve similarly-sized alleles, degradation of DNA in the crude cell extract, small sample size, racial and ethnic make-up of the class, and inefficient amplification of large alleles.
3. No, there are not a large enough number of allele combinations to identify an individual within a population. Forensic scientists typically examine four or five different areas of the genome. The discriminating power of this experiment could be improved by amplifying across VNTR regions in addition to the D1S80 locus. Discrimination at the D1S80 locus itself could be improved by separating the amplification products in a gel system capable of resolving alleles that differ by only a single repeat.
4. Approximately 50 base pairs.