Biotechnology Explorer™

Chromosome 16:
PV92 PCR Informatics Kit

Catalog #166-2100EDU

explorer.bio-rad.com

Note: Kit contains temperature-sensitive reagents. Open immediately upon arrival and store components at –20°C or at 4°C as indicated.

Duplication of any part of this document is permitted for classroom use only.

Welcome to Biotechnology Explorer

Technical advances over the past several decades have created a new branch of science, biotechnology, which has transformed and revolutionized life science research. Powerful methods to isolate, analyze and manipulate DNA, the basic building block of life, have already allowed many breakthroughs in understanding biological processes, human disease states and therapeutic methodologies. For these reasons, it is becoming increasingly important to expose students to these concepts. In the coming decades, when a routine visit to a family doctor might include a battery of DNA diagnostic tests — and DNA fingerprints will become the definitive form of personal identification — an understanding of these principles will be as important as learning about hygiene and nutrition.

In order to provide students at the high school, junior college and college levels with exposure to the technologies and applications of biotechnology, Bio-Rad has developed a series of easy-to-use instructional classroom kits supported by inquiry-based-curricula, equipment, and supplies. The Biotechnology Explorer Program has become the program of choice for both beginning and expert teachers seeking to bridge the gap between science in their classrooms and science in the real world.

Because of the increasing use of the polymerase chain reaction (PCR[1]) in modern medicine and science, and the potential impact on every member of society, it is important to provide students with an understanding of the basic principles and applications of PCR. In this kit, students perform PCR to amplify a segment of their own DNA. The segment of DNA they will amplify is present in the genes of many, but not all individuals. Analysis of the data generated in the laboratory will open the door to teaching basic principles of molecular biology, population genetics, and DNA fingerprinting and will illustrate how PCR is being used in many other areas of biology.

The Biotechnology Explorer program is totally unique and extremely innovative. Our laboratory-based activities capture the imagination while enhancing students' awareness and understanding of the applications of biotechnology which will increasingly influence their lives and affect their personal and community decisions.

Developed over five years in collaboration with the San Francisco Bay Area Biotechnology Educational Consortium, Rutgers University, Maxygen Inc., and the Stanford Human Genome Center Education Program, our curricula and kits were created by teachers and scientists working together. We strive to continually improve our curriculum and products. Your input is extremely important to us. We welcome your stories, comments and suggestions!

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Kit Inventory Checklist

This section lists the components provided in the PV92 PCR/informatics kit. It also lists required accessories. Each kit contains sufficient materials for 8 student workstations, with 4 students at each station. Please use this checklist to take inventory of your supplies before beginning this lab.

Note: If preparing genomic DNA using the hair follicle protocol (page 45), protease (catalog #166-2003EDU) must be ordered separately from the kit.

Kit Components	Quantity	(✔)
Store at –20°C (temperature-sensitive components)		
PV92 homozygous (+/+) control, 100 µl	1 vial	❒
PV92 homozygous (–/–) control, 100 µl	1 vial	❒
PV92 heterozygous (+/–) control, 100 µl	1 vial	❒
PCR master mix (dNTPs, *Taq* DNA polymerase, buffer), 2x, 1.2 ml	1 vial	❒
Forward and reverse primer mix, 50x, 25 µl	1 vial	❒
EZ Load™ molecular mass ruler (DNA standard), 100 µl	1 vial	❒
Store at 4°C		
50x TAE buffer, 100 ml	1	❒
Agarose powder, 5 g	1	❒
Fast Blast™ DNA stain, 500x, 100 ml	1 bottle	❒
InstaGene™ matrix, 20 ml	1 bottle	❒
PV92 XC loading dye, 5x, 1 ml	1 vial	❒
Store at room temperature		
PCR tubes	50	❒
Screwcap tubes, 1.5 ml	50	❒
Micro test tubes, capless, 1.5 ml	50	❒
Micro test tubes, with attached caps, 1.5 ml	60	❒
Foam micro test tube holders	16	❒
Gel staining trays	4	❒
Manual	1	❒

Refills available separately

PV92 PCR Kit TS refill: 166-2119EDU (includes PCR primers, positive controls, DNA molecular mass ruler, master mix containing dNTPs, buffer, DNA polymerase)

PV92 PCR kit RT refill: 166-2139EDU (includes InstaGene matrix, PV92 XC DNA loading dye, Fast Blast DNA stain, agarose, 50x TAE)

Required Accessories - Not Included in This Kit

Student workstation (4 students)	Quantity per Station	
P-20 micropipets, 2–20 µl (catalog #166-0506EDU)		
or 10 µl fixed-volume pipet	1	❐
and 20 µl fixed-volume pipet	1	❐
Xcluda pipet tips (filter type) 2–20 µl (catalog #211-2006EDU)	1 rack	❐
Mini-Sub® Cell GT electrophoresis chamber with 7 x 7 cm gel tray, 8-well comb (catalog #166-4400EDU)	1	❐
PowerPac™ Junior power supply (catalog #165-5048EDU), or	1	❐
PowerPac™ Basic power supply (catalog #164-5050EDU)	1	❐
Ice bucket with chipped or crushed ice	1	❐
Permanent markers	1	❐
Large containers for destaining (if applicable)	1–3 per 2 stations	❐
Cups with 10 ml 0.9% saline	4	❐
Copy of Quick Guide or protocol	1	❐
Tweezers or forceps (for hair follicle protocol)	1	❐
Scissors or razor blade (for hair follicle protocol)	1	❐

Teacher setup or lab equipment	Quantity per Kit	
P-20 micropipet, 2–20 µl (catalog #166-0506EDU)	1	❐
P-200 micropipet, 20–200 µl (catalog #166-0507EDU)	1	❐
P-1000 micropipet, 100–1,000 µl (catalog #166-0508EDU)	1	❐
Xcluda® pipet tips (PCR filter type)		
2–20 µl (catalog #211-2006EDU)	3 racks	❐
20–200 µl (catalog #211-2016EDU)	3 racks	❐
100–1,000 µl (catalog #211-2021EDU)	1 rack	❐
Gene Cycler™ thermal cycler (catalog #170-6700EDU) or	1	❐
MyCycler™ thermal cycler (catalog #170-9701EDU)	1	❐
Microwave oven	1	❐
Water bath (56 and 100°C) (catalog #166-0504EDU)	1 each	❐
Protease (catalog #166-2003EDU), for hair follicle protocol only	1.3 ml	❐
0.9% saline solution	500 ml	❐
Distilled or deionized water	500 ml	❐
1,000 ml Erlenmeyer flask for preparing agarose	1	❐
500 ml flask or beaker for DNA stain	1	❐
Lab tape (not Scotch tape)	1	❐
Microcentrifuge (catalog #166-0602EDU) or	1	❐
Mini centrifuge (catalog #166-0603EDU)	4	❐

Optional Accessories	Quantity per Class	
Gel support film for agarose (catalog #170-2984EDU)	1	❐
Rocking platform (catalog #166-0709EDU)	1	❐
Vortexer (catalog #166-0610EDU)	1	❐
Acetate sheets for tracing gels	8	❐

Storage and stability: Although the kit is shipped under ambient conditions, the components are guaranteed for 1 year from the date of purchase when stored under the appropriate conditions. The kit contains temperature sensitive components. Open the kit immediately and store the components at either –20°C, 4°C, or room temperature as indicated.

Background for Teachers

Introduction to PCR

In 1983, Kary Mullis[2] at Cetus Corporation developed the molecular biology technique that has since revolutionized genetic research, earning him the Nobel Prize in 1993. This technique, termed the **polymerase chain reaction (PCR)**, transformed molecular biology into a multidisciplinary research tool. Many molecular biology techniques used before PCR were labor intensive, time consuming and required a high level of technical expertise. Additionally, working with only trace amounts of DNA made it difficult for researchers in other biological fields (pathology, botany, zoology, pharmacy, etc.) to incorporate molecular biology into their research schemes.

PCR had an impact on four main areas of biotechnology: 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,[3] in criminal investigations and courts of law to identify suspects on a molecular level,[4] and in the sequencing of the human genome.[5] Prior to PCR the use of molecular biology techniques for therapeutic, forensic, pharmaceutical, or medical diagnostic purposes was not practical or cost-effective. The development of PCR technology changed these aspects of molecular biology from a difficult science to one of the most accessible and widely used tools in genetic and medical research.

PCR and Biotechnology — What Is It and Why Did It Revolutionize an Entire Research Community?

PCR produces exponentially large amounts of a specific piece of DNA from trace amounts of starting material (template). The template can be any form of double-stranded DNA, such as genomic DNA. A researcher can take trace amounts of DNA from a drop of blood, a single hair follicle, or a cheek cell and use PCR to generate millions of copies of a desired DNA fragment. In theory, only one single template strand is needed to generate millions of new DNA molecules. Prior to PCR, it would have been impossible to do forensic or genetic studies with this small amount of DNA. The ability to amplify the precise sequence of DNA that a researcher wishes to study or manipulate is the true power of PCR.

PCR amplification requires the presence of at least one DNA template strand. In this kit, human genomic DNA isolated from students' own cells will be the source of the template strands. One of the main reasons PCR is such a powerful tool is its simplicity and specificity. All that is required are inexpensive reaction buffers, four DNA subunits (deoxynucleotide triphosphates of adenine, guanine, thymine, and cytosine), a DNA polymerase, two DNA primers, and minute quantities of the template strand that one wants to amplify. Specificity comes from the ability to target and amplify one specific segment of DNA out of a complete genome.

PCR Makes Use of Two Basic Processes in Molecular Genetics

1. **Complementary DNA strand hybridization**
2. **DNA strand synthesis via DNA polymerase**

In the case of PCR, complementary strand hybridization takes place when two different **oligonucleotide primers** anneal to each of their respective complementary base pair sequences on the template. The two primers are designed and synthesized in the laboratory with a specific sequence of nucleotides such that they can anneal at the opposite ends and on the opposite strands of the stretch of double-stranded DNA (template strand) to be amplified.

Before a region of DNA can be amplified, one must identify and determine the sequence of a piece of DNA upstream and downstream of the region of interest. These areas are then used to determine the sequence of oligonucleotide primers that will be synthesized and used as starting points for DNA replication. Primers are complimentary to the up- and downstream regions of the sequence to be amplified, so they stick, or anneal, to those regions. Primers are needed because DNA polymerases can only add nucleotides to the end of a preexisting chain.

The DNA polymerase used in PCR must be a thermally stable polymerase because the polymerase chain reaction cycles between temperatures of 60°C and 94°C. The thermostable DNA polymerase (*Taq*) used in PCR was isolated from a thermophilic bacterium, *Thermus aquaticus*, which lives in high-temperature steam vents such as those found in Yellowstone National Park.[6]

Two new template strands are created from the original double-stranded template on each complete cycle of the strand synthesis reaction. This causes exponential growth of the number of template molecules, i.e., the number of DNA strands doubles at each cycle. Therefore, after 30 cycles there will be 2^{30}, or over 1 billion, times more copies than at the beginning. Once the template has been sufficiently amplified, it can be visualized. This allows researchers to determine the presence or absence of the desired PCR products and determine the similarities and differences between the DNA of individuals. Depending on the DNA sequence analyzed, differences among individuals can be as great as hundreds of base pairs or as small as a single base pair or single point mutation.

Genes and DNA

It is estimated that the 23 pairs of chromosomes (46 total chromosomes) of the human genome contain a total of 30,000–50,000 genes. Each gene holds the code for a particular protein. Interestingly, these 30,000–50,000 genes comprise only about 5% of chromosomal DNA. The other 95% is noncoding DNA. This noncoding DNA is found not only between, but within genes, splitting them into segments. In eukaryotes, these sequences within genes (called introns) are transcribed into RNA but in the end do not make a protein called **introns**. The sequences that do code for proteins are called **exons**. Both introns and exons are initially transcribed, then introns are spliced out of the RNA to create messenger RNA (mRNA).

In eukaryotes, genomic DNA is transcribed into RNA molecules containing both introns and exons for a particular gene. While the RNA is still in the nucleus (before being transported out of the nucleus), the introns (in = stay within the nucleus) must be removed from the RNA while the exons (ex = exit the nucleus) are spliced together to form the complete coding sequence for the protein (Figure 1). This process is called **RNA splicing**. Some genes may contain a few introns, others may contain dozens.

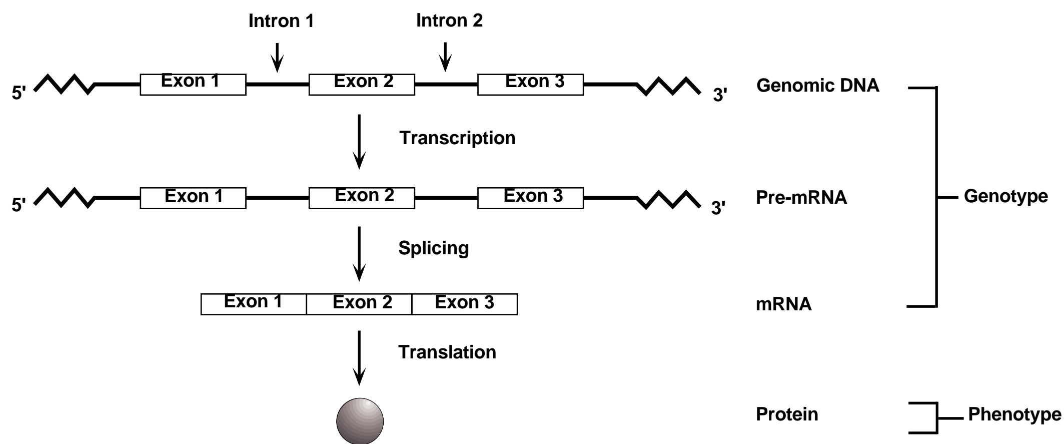


Fig. 1. Splicing of introns from genes.

As we have discussed, functional segments of genes (exons) code for proteins — molecules that carry out most cellular functions. Exon sequences are therefore similar among individuals. Introns, on the other hand, often vary in size and number among individuals. Intron sequences are thought to be the result of the differential accumulation of mutations throughout evolution that are silently passed to descendants through the hereditary code. It is this difference in intron sequences that allows us to determine human genetic diversity. The identification of these distinctive characteristics in the DNA represent the molecular basis for human identification and population genetics.

Throughout evolution, intron sequences have been the target of random insertions by short repetitive interspersed elements, also known as SINEs.[7] SINEs have become randomly inserted within our introns over millions of years. One such repetitive element is called the Alu sequence[7] (Figure 2). This is a DNA sequence about 300 base pairs long that is repeated, one copy at a time, almost 500,000 times within the human genome.[8] The origin and function of such randomly repeated sequences is not yet known. The Alu name comes from the *Alu* I **restriction enzyme** recognition site that is found in this sequence.

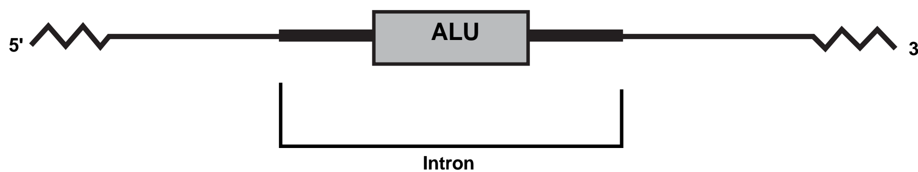


Fig. 2. Location of an Alu insertion within an intron.

Some of these Alu elements have characteristics that make them very useful to geneticists. If present within introns of genes associated with particular pathologies, they can thereby be associated with that disease. When present within the introns of genes, Alu repeats can also be used to estimate relatedness among individuals. In this activity, analysis of Alu repeats is used to estimate the frequency of an insert in a population and is a simple measure of molecular genetic variation — **with no reference to disease or relatedness among individuals**.

This kit provides a simple PCR-based screen for a single Alu sequence within the PV92 locus on chromosome 16. This particular Alu intron is dimorphic. That is, the element is present in some individuals but not others (Figure 3). Some people have the insert in the PV92 locus of one of their chromosome 16, others may have the insert in both homologous chromosomes (two alleles), and some do not have the insert in either chromosome. The presence or absence of this insert can be detected using the polymerase chain reaction followed by agarose gel electrophoresis.

In this activity, students will isolate their own genomic DNA from their cells. They will use primers that flank both the entire Alu insertion (300 base pairs in length) and 641 base pairs of the PV92 locus to amplify a 941 base pair fragment (if the Alu element is present) or a 641 base pair fragment (if the Alu element is absent). Agarose gel electrophoresis of the PCR products is sufficient to distinguish among homozygotes (+/+) for the presence of the Alu repeat (941 base pair product only), homozygotes (–/–) for the absence of the Alu repeat (641 base pair product only), and heterozygotes (+/–) having both the 641 and the 941 base pair products.

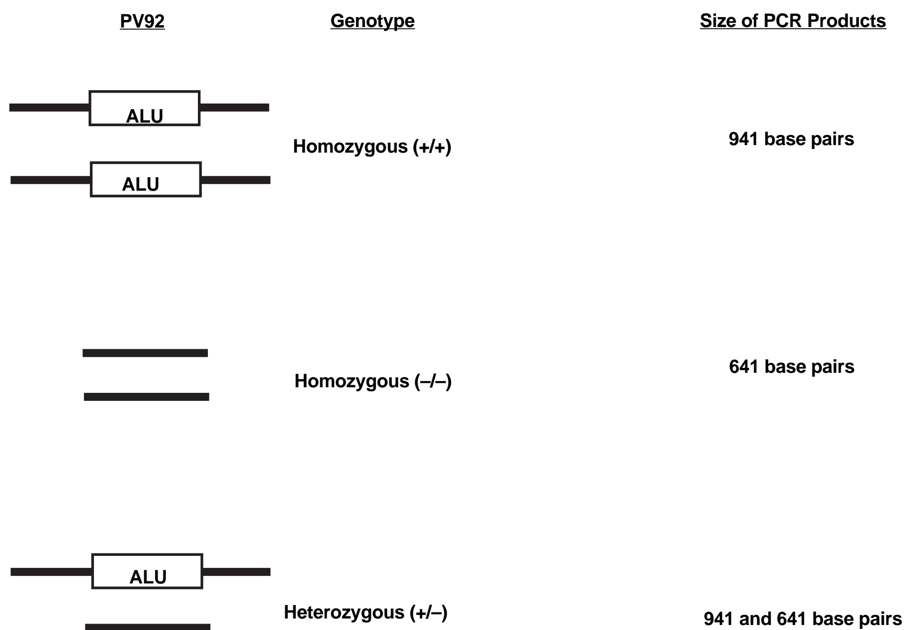


Fig. 3. The presence or absence of the Alu insert within the PV92 locus on chromosome 16.

Important Notes for the Instructor

Please note that since PV92 alleles are inherited from parents and can potentially reveal information about family relationships, we caution against generating genotypic data from multiple members of a family. If confidentiality is a concern, we suggest that the instructor mix up student samples to ensure anonymity. Student samples can be randomized at any point after cells are harvested.

Two protocols are provided for genomic DNA preparation. One involves the collection of oral epithelial cells using a saline mouthwash. The other isolates genomic DNA from hair follicles. Both methods are minimally invasive, and yield robust PCR products. Instructors may choose either protocol based on personal or student preference, or local restrictions.

PCR Step by Step

PCR involves a repetitive series of cycles, each of which consists of template denaturation, primer annealing, and extension of the annealed primer by *Taq* DNA polymerase. Before beginning DNA amplification, genomic DNA is prepared from students' cells.

Following sample preparation, the template DNA, oligonucleotide primers, thermostable DNA polymerase (*Taq*), the four deoxynucleotides (A, T, G, C), and reaction buffer are mixed in a single micro test tube. The tube is placed into the Gene Cycler™ or MyCycler™ thermal cycler. These thermal cyclers contain an aluminum block that holds the samples and can be rapidly heated and cooled across extreme temperature differences. The rapid heating and cooling of this thermal block is called **temperature cycling** or **thermal cycling**.

The first step of the PCR temperature cycling procedure involves heating the sample to 94°C. At this high temperature, the template strands separate (denature). This is called the **denaturation step**.

The thermal cycler then rapidly cools to 60°C to allow the primers to anneal to the separated template strands. This is called the **annealing step**. The two original template strands may reanneal to each other or compete with the primers for the primers complementary binding sites. However, the primers are added in excess such that the primers actually out-compete the original DNA strands for the primers' complementary binding sites.

Lastly, the thermal cycler heats the sample to 72°C for *Taq* DNA polymerase to extend the primers and make complete copies of each template DNA strand. This is called the **extension step**. *Taq* polymerase works most efficiently at this temperature. Two new copies of each complementary strand are created. There are now two sets of double-stranded DNA (dsDNA). These two sets of dsDNA can now be used for another cycle and subsequent strand synthesis.

At this stage, a complete temperature cycle (thermal cycle) has been completed (Figure 4).

Temperature cycle = denaturation step + annealing step + extension step

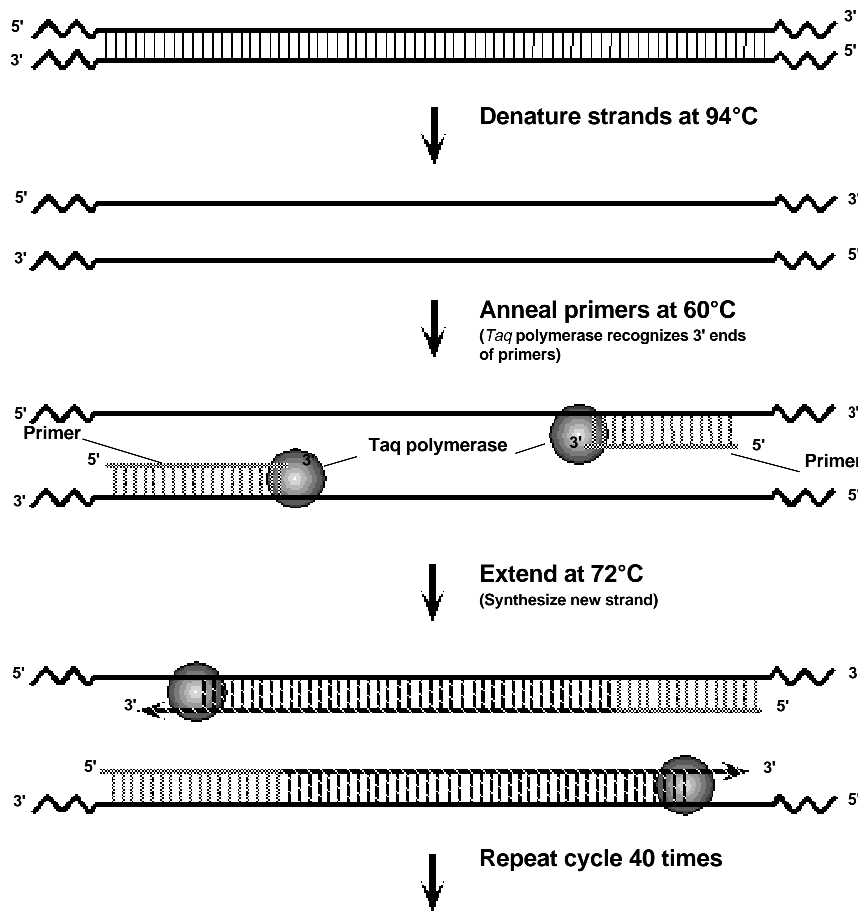


Fig. 4. A complete cycle of PCR.

Usually, thermal cycling continues for about 40 cycles. After each thermal cycle, the number of template strands doubles, resulting in an exponential increase in the number of template DNA strands. After 40 cycles there will be 1.1×10^{12} more copies of the original number of template DNA molecules.

PCR generates DNA of a precise length and sequence. On the first cycle, the two primers anneal to the original genomic template DNA strands at opposite ends and on opposite strands. After the first complete temperature cycle, two new strands are generated that are shorter than the original template strands but still longer than the length of the DNA that the researcher wants to amplify. It isn't until the third thermal cycle that fragments of the precise length are generated (Figure 5).

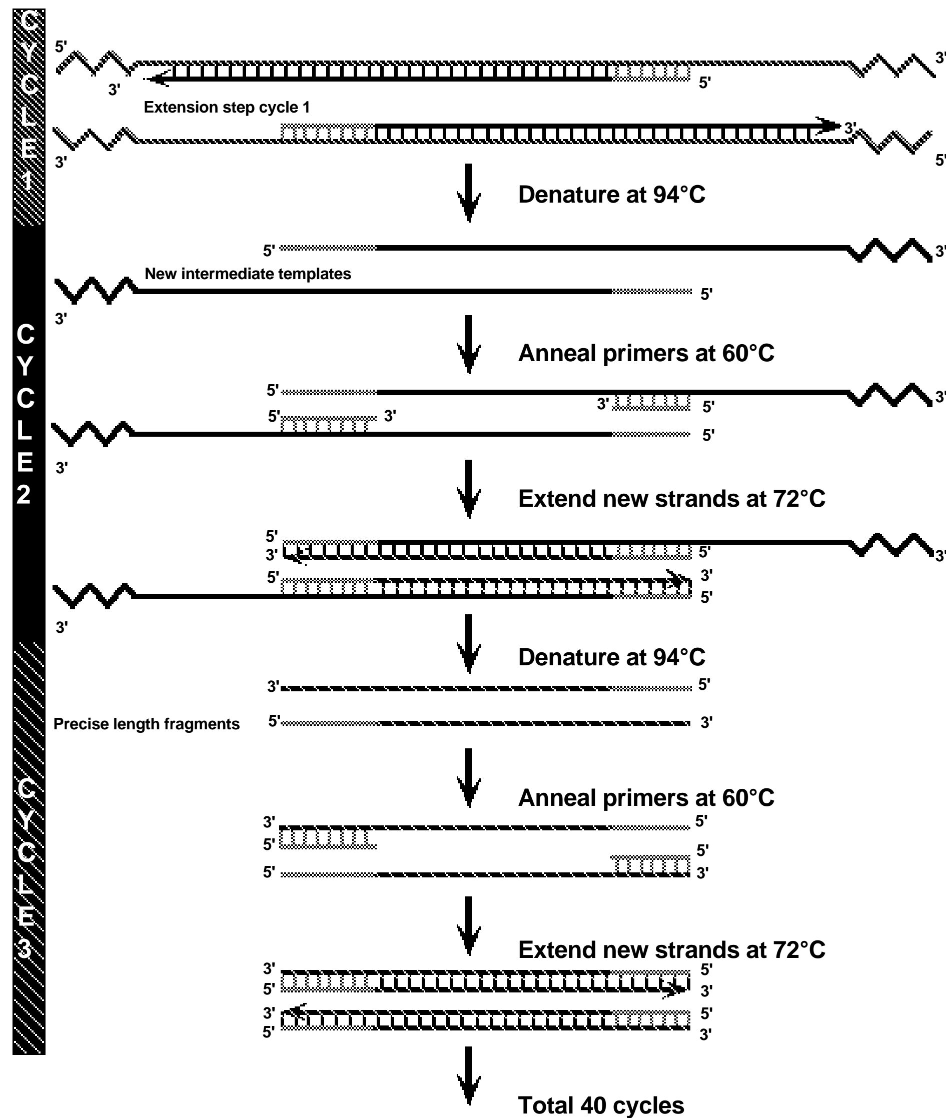


Fig. 5. Generation of precise-length fragments.

It is the template strands of the precise length that are amplified exponentially (X^n, where X = the number of original template strands and n = the number of cycles). There is always one set of original long-template DNA molecules which is never fully duplicated. After each thermal cycle, two intermediate-length strands are produced, but because they can only be generated from the original template strands, the intermediate strands are not exponentially amplified. It is the precise-length strands generated from the intermediate strands that amplify exponentially at each cycle. Therefore, if 20 thermal cycles were conducted from one double-stranded DNA molecule, there would be 1 set of original genomic template DNA strands, 20 sets of intermediate template strands, and 1,048,576 sets of precise-length template strands. After 40 cycles, there would be 1 set of original genomic template DNA strands, 40 sets of intermediate template strands, and 1.1×10^{12} sets of precise-length template strands (Figure 6).

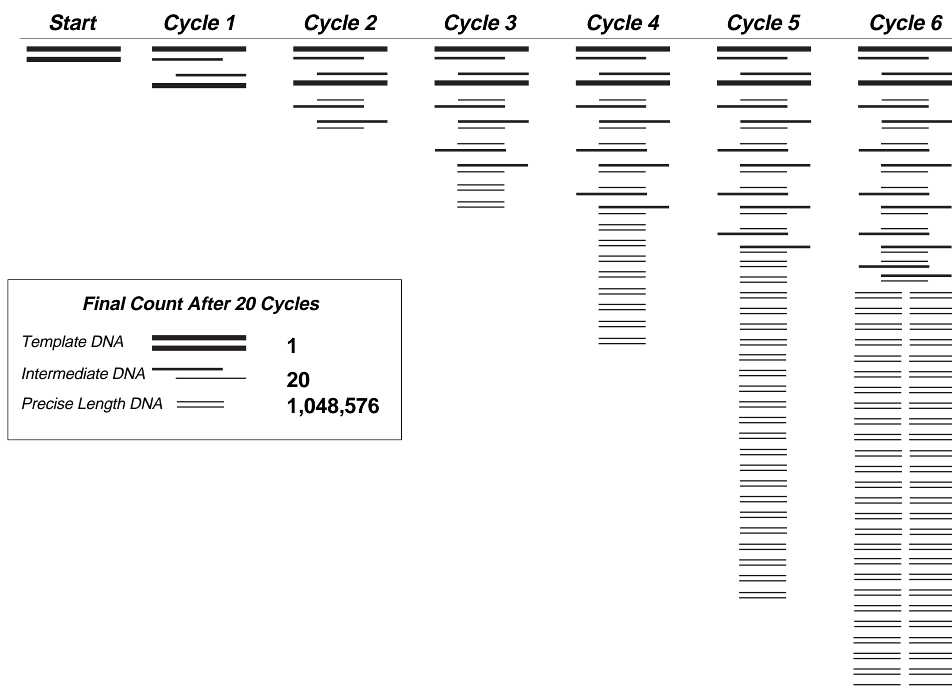


Fig. 6. Schematic of PCR amplification of DNA fragments.

The Relevance of Alu to Hardy-Weinberg Theory

The value of this Alu insert residing in a noncoding region of PV92 is that it does not code for any protein sequence and is not related to a particular disease. Because Alu repeats have inserted randomly in humans, the Alu insert in the PV92 locus can be very useful in studies of allele and genotype frequencies in the human population. In the lab exercise (Lesson 4), the principles of the Hardy-Weinberg theory can be applied to analyze genotypic and allelic frequencies of the Alu insert in populations. By determining the genotypic frequencies of the Alu genotype within your student population, the corresponding allelic frequencies can also be calculated. Additionally, the genotypic frequencies of your class population can be compared to published results of larger population sizes. This activity opens the door to teaching the principles of the Hardy-Weinberg theory. Use the student data generated in Tables 1 and 2 (pages 70–71) to discuss whether human populations are in Hardy-Weinberg equilibrium.

Analysis of Classroom Data Using Bioinformatics

Bioinformatics is a discipline that integrates mathematical, statistical, and computer tools to collect and process biological data. Bioinformatics has become an important tool in recent years for analyzing the extraordinarily large amount of biological information that is being generated by researchers around the world. In Lesson 5, students will perform a bioinformatics exercise to investigate the genotypic frequencies for the Alu polymorphism in their class population and compare them with the genotypic frequencies of other populations.

Following PCR amplification and electrophoresis of their samples, students will analyze their experimental data to determine their genotypes for the Alu insertion within the PV92 locus on chromosome 16. The classroom genotype data can then be entered into Allele Server of Cold Spring Harbor Laboratory's Dolan DNA Learning Center. Allele Server is a Web-based database that contains genotype data from populations around the world as well as other classrooms and teacher training workshops. It also provides a collection of statistical analysis tools to examine the Alu insertion polymorphism at the population level. Students can either analyze their classroom data as an individual population or compare their population with other populations in the database.

Once students enter classroom data into Allele Server, they can perform a Chi-square analysis to compare the Alu genotype frequencies within the class population with those predicted by the Hardy-Weinberg equation. The genotypic frequencies of the class population can also be compared with the genotypic frequencies of another population in the database. Using this database, students will determine if their class data are in agreement with the expected Hardy-Weinberg genotypic frequencies.

Suggested Lesson Flow

There are four student lessons in this PCR curriculum. All lessons are designed to be carried out in consecutive 50 minute periods. Lessons 1 and 2 have convenient stop points and two options. Teachers should choose cheek cell (page 41) or hair follicle (page 45) DNA preparation. Instructors may wish to offer either method as an option for students, or may elect to perform a particular protocol based on local restrictions. The samples can be stored for several days to accommodate weekends or labs that meet every other day.

Student Schedule

Lesson 1	**Cheek Cell DNA Template Preparation**
Activity	Isolate cheek cells Prepare genomic DNA from cheek cells (Stop point)
Lesson 1	**Hair Follicle DNA Template Preparation**
Activity	Isolate hairs Prepare genomic DNA from hair follicles (Stop point)
Lesson 2	**PCR Amplification**
Activity	Set up and perform PCR reactions Pour agarose gels (this may be performed by the instructor during the advance preparation) (Stop point)
Lesson 3	**Gel Electrophoresis of Amplified PCR Samples and Staining of Agarose Gels**
Activity	Load and run gels Stain gels (Note: If you are using the quick staining protocol, record the results and dry the gels)
Lesson 4	**Analysis and Interpretation of Results**
Activity	Record the results and dry the gels (if using the overnight staining protocol) Analyze and discuss results
Lesson 5	**Interpretation of Results: Bioinformatics**
Activity	Enter classroom data into PV92 Allele Server and analyze data

Instructor's Advance Preparation Overview

This section outlines the recommended schedule for advanced preparation on the part of the instructor. A detailed Advance Preparation Guide is provided on pages 16–24.

When	Activity	Time Required
Immediately	Read PCR manual	2 hours
Prior to Lesson 1	Aliquot InstaGene matrix Set up student workstations	30 min
Prior to Lesson 2	Prepare complete master mix and aliquot	1 hour
	Set up control PCR reactions	
	Prepare TAE buffer	
	Prepare molten agarose	
	Program Gene Cycler™ or MyCycler™	
	Set up student workstations	
Prior to Lesson 3	Prepare Fast Blast DNA stain	20 min
	Set up student workstations	
Prior to Lesson 4	Set up student workstations	10 min

Note: Choose **one** of the two options for DNA template preparation.

Workstation Daily Inventory Checklist

Student Workstations: Materials and supplies required at each student workstation prior to exercise are listed below. The components provided in this kit are sufficient for 8 student workstations with 4 students at each station.

Instructor's (Common) Workstation: Materials, supplies, and equipment required at a location that can be accessed by all students are also listed below. It is up to the teacher whether students will have access to common buffer solutions/equipment, or the teacher will aliquot the solutions and operate the equipment.

Lesson 1 DNA Template Preparation

Student workstations	Quantity per Station	(✔)
1.5 ml micro test tubes (for cheek cell protocol)	4	❐
Screwcap tubes with InstaGene™ matrix (for cheek cell protocol)*	4	❐
Screwcap tubes with InstaGene™ matrix and protease* (for hair follicle protocol)	4	❐
Foam micro test tube holders	2	❐
P-20 micropipet, for cheek cell protocol only	1	❐
Pipet tips (filter type), 2–20 µl, for cheek cell protocol only	4	❐
Permanent marker	1	❐
Waste container	1	❐
Copy of Quick Guide or protocol	1	❐
Cups with 10 ml 0.9% saline (for cheek cell protocol)	4	❐
Tweezers or forceps (for hair follicle protocol)	1	❐
Scissors or razor blade (for hair follicle protocol)	1	❐

*Choose one of the two methods.

Instructor's (common) workstation	Quantity per Class	
P-1000 micropipet, or P-200 micropipet	1	❒
Pipet tips (filter type), 20–200 µl,	1 box	❒
or Pipet tips (filter type), 100–1,000 µl	1 box	❒
Water baths (56 and 100°C)	1 each	❒
Microcentrifuge	1	❒
or mini centrifuge	4	❒
Vortexer (optional)	1	❒

Lesson 2 PCR Amplification

Student workstation	Quantity per Station	(✔)
PCR tubes	4	❒
Micro test tubes, capless	4	❒
Complete master mix (containing primers) on ice	1 tube	❒
P-20 micropipet	1	❒
or 10 µl and 20 µl fixed-volume pipets	1 of each	❒
Pipet tips (filter type), 2–20 µl	8	❒
Foam micro test tube holders	2	❒
Ice bucket with ice	1	❒
Permanent marker	1	❒
Copy of Quick Guide or protocol	1	❒
Waste container	1	❒

Instructor's (common) workstation	Quantity per Class	
Gel trays	1 per 2 stations	❒
Molten agarose (see advance prep)	40 ml per gel	❒
Lab tape for gel trays	1 per station	❒
Gene Cycler or MyCycler thermal cycler	1	❒
Microcentrifuge	1	❒
or mini centrifuge	4	❒

Lesson 3 Gel Electrophoresis of Amplified PCR Samples and Staining of Agarose Gels

Student workstations	Quantity per Station	(✔)
Agarose gel	1	❒
PCR samples	1 per student	❒
PV92 XC DNA loading dye	1 tube	❒
P-20 micropipet	1	❒
or 10 µl and 20 µl fixed-volume pipets	1 of each	❒
EZ Load™ molecular mass ruler (DNA standards)	1 tube	❒
Pipet tips (filter type), 2–20 µl	12	❒
Permanent marker	1	❒
Foam micro test tube holders	2	❒
Gel box and power supply	1	❒
Gel staining tray	1 per 2 stations	❒
Fast Blast™ DNA stain, 1x or 100x solution*	120 ml per 2 stations	❒
Gel support film (optional)	1	❒
Clear acetate sheets for tracing gels (optional)	1	❒
Warm tap water for destaining gels (if performing quick staining protocol)	1.5–2 L per 2 stations	❒
Large containers for destaining (if performing quick staining protocol)	1–3 per 2 stations	❒
Waste container	1	❒
Copy of Quick Guide or protocol	1	❒

Instructor's workstation	Quantity per Class	
1x TAE electrophoresis buffer	275 ml per gel box	❒
Amplified positive control samples (4 each)	12	❒
PV92 homozygous (+/+)		
PV92 homozygous (–/–)		
PV92 heterozygous (+/–)		
Rocking platform (optional)**	1	❒
Microcentrifuge	1	❒
or mini centrifuge	4	❒

Lesson 4 Analysis and Interpretation of Results

Student workstations	Quantity per Station	(✔)
Gel support film (optional)	1	❒
Clear acetate sheets for tracing gels (optional)	1	❒
Copy of Quick Guide or protocol	1	❒

Instructor's workstation

None required

* Depending on whether the quick staining or overnight staining protocol will be followed.

** Strongly recommended.

Instructor's Advance Preparation

This section describes the preparation to be performed by the instructor before each exercise. An estimate of preparation time is included in each section.

Lesson 1 DNA Template Preparation

Advance Preparation

Objectives	Aliquot InstaGene™ matrix Set up student and instructor workstations Set water baths to 56 and 100°C	
Time required	30 minutes	
Materials required	Screwcap tubes InstaGene™ matrix P-20 micropipet (2–20 µl) Filter tips (2–20 µl) P-200 micropipet (20–200 µl) Filter tips (20–200 µl) Vortexer	Micro test tubes Tweezers (for hair follicle prep) Scissors (for hair follicle prep) Cups (for cheek cell prep) Protease (for hair follicle prep)

Procedures

1. Aliquot InstaGene™ matrix (cheek cell preparation).*
   A. Thoroughly mix the InstaGene matrix by gently shaking or vortexing the bottle several times to resuspend the matrix. Be sure that the matrix is well mixed when you aliquot it. The beads settle out of solution quickly, so gently remix the bottle several times during aliquotting.
   B. Pipet 200 µl of InstaGene matrix into each screwcap tube. Distribute one tube to each student. Each student workstation should get 4 tubes of matrix for 4 students.
2. Prepare and aliquot InstaGene matrix with protease (hair follicle protocol).*
   A. Prepare a solution of InstaGene matrix containing 66 µg/ml protease. Protease (catalog #166-2003EDU) is supplied at a concentration of 20 mg/ml. To obtain 66 µg/ml, dilute stock protease 1:300 by volume into InstaGene matrix.
   For example, to make 5 ml of InstaGene matrix containing 66 µg/ml protease (enough for approximately 20 students):
   - Aliquot 5 ml InstaGene matrix to a new tube (be sure to mix the InstaGene well before aliquoting)
   - Add 17 µl of stock protease to the 5 ml of InstaGene matrix

   B. Pipet 200 µl of InstaGene matrix with protease into each screwcap tube. Distribute one tube to each student. Each student workstation should get 4 tubes of matrix for 4 students.
3. Prepare and aliquot saline solution (cheek cell protocol).
   A. Prepare a 0.9% saline solution. To a 500 ml bottle of drinking water, add 4.5 grams of noniodinated salt. Table salt is recommended. Invert the bottle until the salt goes into solution.
   B. For each student, place 10 ml saline into a separate cup. Each student workstation should have 4 cups of saline.

*Choose one of the two methods.

Lesson 2 PCR Amplification

Advance Preparation (for 8 student workstations)

Objectives

Prepare complete master mix by mixing in primers, and aliquot **(no more than 30 minutes before PCR cycling)**
Set up control PCR reactions
Program Gene Cycler or MyCycler thermal cycler
Pour agarose gels. **If you have your students pour their own gels during the lab, prepare the agarose ahead of time. Agarose, once molten, may be kept in a water bath set at 45–50°C until used by the students.**
Set up student and instructor workstations

Time required

1–1.5 hours (depending on how you choose to prepare agarose gels)

Prepare master mix and set up control samples towards the end of the advance preparation.

Materials required

Micro test tubes (with attached caps)
Screwcap tubes
Master mix
Primer mix
PV92 homozygous (+/+) control
PV92 homozygous (–/–) control
PV92 heterozygous (+/–) control
12 PCR tubes
Electrophoresis gel boxes, casting trays, and combs
Electrophoresis buffer (50x TAE)
Agarose powder
Micropipets (P-20 and P-200)
Filter tips (20–200 µl and 100–1,000 µl)

The Gene Cycler thermal cycler accommodates 24 samples, whereas the MyCycler thermal cycler holds 96 samples. If the Gene Cycler is being used, you will have to run the PCR machine more than once if there are more than 24 samples (including positive control samples). **For best results, prepare the student reactions and control samples no more than 30 minutes before PCR cycling.**

Procedures

Note: Before opening any of the reagent tubes, pulse-spin the contents (~3 seconds) in a centrifuge to bring contents to the bottom of the tubes. Contents often become lodged underneath the caps during shipping.

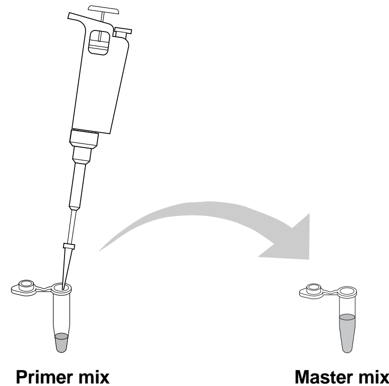
1. Prepare complete master mix by adding primers. For best results, the following steps should be performed **within 15–30 minutes of the PCR reaction**.

   A. Pipet 1,100 µl of master mix into a labeled micro test tube. If you choose to amplify 16 student samples or less, divide the master mix into two tubes with 550 µl each. One tube will be used immediately, and the remaining master mix can be refrozen for later use.

   B. For 32 students or 8 student workstations (halve for 16 students), label 8 micro test tubes “Master” and place the tubes on ice.

   C. Add 22 µl of the primer mix to the 1,100 µl of master mix. Vortex 10 seconds to mix. It is imperative that the master mix be evenly mixed after the addition of the primers. The solution should be yellow.

The primers are supplied as a concentrated yellow solution in a Tris buffer. Since the primers are much more stable in a concentrated form, add the primers to the master mix just prior to beginning the laboratory exercise — **not more than 15–30 minutes before** the PCR amplification.



D. Aliquot 95 µl of the complete master mix into the 8 micro test tubes labeled "Master", supplying one tube for each student workstation (1–8). Save the remaining complete master mix for the positive control reactions. Place these tubes on ice until they will be used.

2. Set up control PCR reactions.

   A. Label the control PCR tubes: +/+, –/–, and +/–. If you will be using the entire kit with a single lab period set up 4 of each control or 12 tubes total. If you will be splitting the kit between two lab periods, set up 2 of each control, or 6 tubes total. The unused control solutions should be stored in the freezer until used.
   - Pipet 20 µl of the +/+ template into each +/+ PCR tube.
   - Pipet 20 µl of the –/– template into each –/– PCR tube.
   - Pipet 20 µl of the +/– template into each +/– PCR tube.

   B. Pipet 20 µl of the complete master mix into each of the control tubes. **Use a fresh tip for each tube.**

   C. Place the tubes on ice until ready to load into the Gene Cycler or MyCycler. Amplify PCR control samples along with student samples during this lesson (see Quick Guide).

3. Prepare agarose gels. These procedures may be carried out 1 to 2 days ahead of time by the teacher or done during class by individual student teams.

   A. Prepare electrophoresis buffer. The electrophoresis buffer is provided as a 50x concentrated solution. 1x TAE buffer is needed to make the agarose gel and is also required for each electrophoresis chamber. Three liters of 1x TAE buffer will be sufficient to run 8 electrophoresis chambers and pour 8 agarose gels. To make 3 L of 1x TAE from a 50x TAE concentrate, add 60 ml of concentrate to 2.94 L of distilled water.

   B. Make the agarose solution. The recommend gel concentration for this classroom application is 1% agarose. This agarose concentration provides excellent resolution and minimizes run time required for electrophoretic separation of PCR fragments. To make a 1% solution, add 1 g of agarose to 100 ml of 1x TAE electrophoresis buffer. For 8 gels, you will need approximately 350 ml of molten agarose (3.5 g agarose per 350 ml 1x TAE buffer). The agarose must be made using electrophoresis buffer, **not** water.

Add the agarose powder to a suitable container (e.g., 1,000 ml Erlenmeyer flask, Wheaton bottle, etc.). Add the appropriate amount of 1x TAE electrophoresis buffer and swirl to suspend the agarose powder in the buffer. If using an Erlenmeyer flask, invert a 50 ml Erlenmeyer flask onto the open end of the 1,000 ml Erlenmeyer flask containing the agarose. The small flask acts as a reflux chamber, allowing boiling without much loss of buffer volume. The agarose can be melted for gel casting on a magnetic hot plate or in a microwave oven (see below). Heat the mixture to boiling using a microwave oven or hot water bath until the agarose powder has dissolved completely.

Careful observation is necessary to determine when the powder is completely dissolved. Hold the flask up to a light and look for small transparent bits of suspended agarose. These particles indicate that the agarose is not fully dissolved. Heating and swirling should be continued until no more of these transparent particles can be seen.

Caution: Use protective gloves, oven mitts, goggles, and lab coat as appropriate while preparing and casting agarose gels. Boiling molten agarose or the vessels containing hot agarose can cause severe burns.

Magnetic hot plate method. Add a stir bar to the flask containing agarose and buffer. Heat the mixture to boiling while stirring on a magnetic hot plate. Bubbles or foam should break before rising to the neck of the flask. Boil the solution until **all** of the small transparent agarose particles are dissolved. With the small flask still in place, set aside the agarose to cool to 60°C before pouring gels.

Microwave oven method. Place the agarose solution into the microwave. Loosen the bottle cap if present. Use a medium setting and set to 3 minutes. Stop the microwave oven every 30 seconds and swirl the flask to suspend any undissolved agarose. This technique is the fastest and safest way to dissolve agarose. Boil and swirl the solution until all of the small transparent agarose particles are dissolved. With the small flask still in place, set aside to cool to 60°C before pouring.

Convenient precast agarose gels (catalog #161-3057EDU) are available from Bio-Rad. These are 2 x 8-well, 1% TAE gels.

Procedure for Casting Gels

Using Bio-Rad's Mini-Sub® Cell GT system, gels can be cast directly in the gel box by using the casting gates with the gel tray. If casting gates are unavailable, use the taping method for casting gels, as outlined below. Other methods are detailed in the Bio-Rad Sub-Cell GT instruction manual.

Step 1. Seal the ends of the gel tray securely with strips of standard laboratory tape. Press the tape firmly to the edges of the gel tray to form a fluid-tight seal.

Step 2. Level the gel tray on a leveling table or workbench using the leveling bubble provided with the instrument.

Step 3. Prepare the desired concentration and amount of agarose in 1x TAE electrophoresis buffer. Boil the agarose until dissolved.

Step 4. Cool the agarose to at least 60°C before pouring.

Step 5. While the agarose is cooling to 60°C, place the comb into the appropriate slot of the gel tray. Gel combs should be placed within ~2 cm of the end of the gel casting tray (not in the middle of the gel).

Step 6. Allow the gel to solidify at room temperature for 10 to 20 minutes — it will be translucent when ready to use.

Step 7. Carefully remove the comb from the solidified gel. Remove the tape from the edges of the gel tray.

Step 8. Place the tray onto the leveled DNA electrophoresis cell so that the sample wells are at the cathode (black) end of the base. DNA samples will migrate towards the anode (red) end of the base during electrophoresis.

4. Program Gene Cycler or MyCycler thermal cycler.

The thermal cycler should be programmed for 3 steps in cycle 2, which will repeat 40 times. The final cycle 3 ensures that the final extension reaction goes to completion and all possible PCR products are made. The PCR reaction will take approximately 3.5 hours.

Cycle	Step	Function	Temperature	Time
1	Step 1	Pre-denaturation	94°C	2 minutes
	Repeat 1 time			
2	Step 1	Denature	94°C	1 minute
	Step 2	Anneal	60°C	1 minute
	Step 3	Extend	72°C	2 minutes
	Repeat 40 times			
3	Step 1	Final extension	72°C	10 minutes
	Repeat 1 time			

Refer to the Gene Cycler or MyCycler instruction manual for specific programming instructions or to the instructions in Appendix H.

Lesson 3 Electrophoresis of Amplified PCR Samples and Staining of Agarose Gels

Advance Preparation

Objectives	Prepare amplified positive control samples Aliquot PV92 XC loading dye Aliquot EZ Load molecular mass ruler (DNA standards) Set up student and instructor workstations Prepare either 1x or 100x Fast Blast DNA stain (depending on whether the quick staining or overnight staining protocol will be followed)
Time required	40 minutes
Materials required	8 micro test tubes with attached caps Micropipets (P-20, P-200, and P-1000) and filter tips 8 screwcap tubes Distilled or deionized water to prepare Fast Blast DNA stain Gel support film (if performing quick staining protocol) Acetate to trace gels (if performing quick staining protocol) 500 ml flask

Procedures

1. **Prepare positive control samples.** Add 10 µl of PV92 XC loading dye to each amplified positive control sample (+/+, –/–, +/–). Place the tubes at the teacher's workstation. Either you or a student group will load the positive and negative control samples on each gel, as indicated on page 28.

2. **Aliquot DNA size standards.** Aliquot 11 µl of the EZ Load molecular mass ruler into 8 microtubes and label "MMR". The sizes of the DNA standard bands are 1,000 bp, 700 bp, 500 bp, 200 bp, and 100 bp (see page 59).

3. **Aliquot PV92 XC loading dye.** Label 8 screwcap tubes "LD" for loading dye, and aliquot 50 µl into each tube. Distribute to student workstations.

4. **Prepare Fast Blast DNA stain.** Fast Blast DNA stain is provided as a 500x concentrate that must be diluted prior to use. The stain can be used as a quick stain when diluted to 100x to allow the visualization of DNA within 12–15 minutes, or it can be used as an overnight stain when diluted to 1x. When an agarose gel is immersed in Fast Blast DNA stain, the dye molecules attach to the DNA molecules trapped in the agarose gel. When the DNA bands are visible, your students can determine their genotypes for the Alu insert.

   Fast Blast DNA stain is a convenient, safe, and nontoxic alternative to ethidium bromide for the detection of DNA in agarose gels following electrophoresis. Fast Blast contains a cationic compound that belongs to the thiazin family of dyes. The positively charged dye molecules are attracted to and bind to the negatively charged phosphate groups on DNA. The proprietary dye formula stains DNA deep blue in agarose gels and provides vivid, consistent results.

WARNING

Although Fast Blast DNA stain is nontoxic and noncarcinogenic, latex or vinyl gloves should be worn while handling the stain or stained gels to keep hands from becoming stained blue. Lab coats or other protective clothing should be worn to avoid staining clothes. Dispose of the staining solutions according to protocols at your facility. Use either 10% bleach solution or 70% alcohol solution to remove Fast Blast from most surfaces. Verify that these solutions do not harm the surface prior to use.

1. To prepare 100x stain (for quick staining), dilute 100 ml of 500x Fast Blast with 400 ml of distilled or deionized water in an appropriately sized flask or bottle and mix. Cover the flask and store at room temperature until ready to use.
2. To prepare 1x stain (for overnight staining), dilute 1 ml of 500x Fast Blast with 499 ml of distilled or deionized water in an appropriately sized flask or bottle and mix. Cover the flask and store at room temperature until ready to use.

   (Detailed instructions on using Fast Blast are included in the student manual.)

Note:

- We recommend using 120 ml of diluted Fast Blast to stain two 7 x 7 cm or 7 x 10 cm agarose gels in individual staining trays provided in the kit (you may want to notch the gel corners for identification). If alternative staining trays are used, add a sufficient volume of staining solution to completely submerge the gels.
- Following electrophoresis, agarose gels must be removed from their gel trays before being placed in the staining solution. This is easily accomplished by holding the base of the gel tray in one hand and gently pushing out the gel with the thumb of the other hand.
- Because the gel is fragile, special attention must be given when handling it. We highly recommend using a large spatula or other supportive surface to transfer the gel from one container to another during the destaining steps involved with the quick staining protocol.
- Destaining (when performing the quick staining protocol) requires the use of at least one large-volume container, capable of holding at least 500 ml, at each student workstation. Each student team may utilize separate washing containers for each wash step, or simply use a single container that is emptied after each wash and refilled for the next wash.
- It is crucial that you shake gels gently and intermittently during the overnight staining in Fast Blast DNA stain; small DNA fragments tend to diffuse without shaking.
- 100x Fast Blast can be reused at least 7 times.
- No washing or destaining is required when using the overnight staining protocol.

Lesson 4 Analysis and Interpretation of Results

Advance Preparation

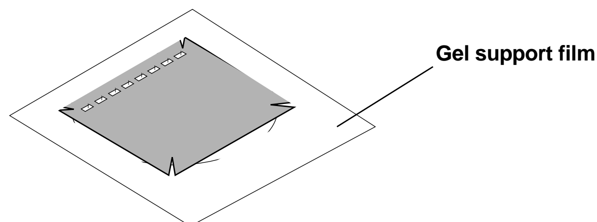
Objectives	No advance preparation required
Materials required	Gel support film (if performing the overnight staining protocol) Acetate to trace gels (if performing the overnight staining protocol)

Obtaining a Permanent Record of the Gel Before Drying

To obtain a permanent record of the gel before it is dried, either trace the gel outline (including wells and DNA bands) on a piece of paper or acetate, take a photograph using a standard camera and film (Bio-Rad's standard Polaroid gel documentation system, catalog #170-3742EDU), or photocopy the stained gel.

> **Note:** Drying agarose gels requires the use of Bio-Rad's specially formulated high-strength analytical grade agarose. Other gel media may not be appropriate for this purpose.

We recommend using Bio-Rad's exclusive gel support film (catalog #170-2984EDU) to dry agarose gels. Remove the stained agarose gel from its staining tray and trim away any unloaded lanes with a knife or razor blade. Place the gel directly upon the hydrophilic side of a piece of gel support film. (Water will form beads on the hydrophobic side but will spread out on the hydrophilic side of the film.) Center the gel on the film and remove bubbles that may form between the gel and film. Place the film on a paper towel and let the gel dry, making sure to avoid direct exposure to light. As the gel dries it will bond to the film but will not shrink. If left undisturbed on the support film, the gel will dry completely at room temperature after 2–3 days. The result will be a flat, transparent, and durable record of the experiment.



Note: Avoid extended exposure of dried gels to direct light to prevent band fading. However, DNA bands will reappear if the dried gels are stored in the dark for 2–3 weeks after fading.

Lesson 5   Analysis of Classroom Data Using Bioinformatics

Advance Preparation

Objectives	Become familiar with Allele Server on the Dolan DNA Learning Center web site Register to set up a free personal account
Time required	30–45 minutes
Materials required	None

Getting Started on Allele Server

Note: The Dolan DNA Learning Center web site is continually updated. Some of the following information may change.

1. On your Web browser, go to **vector.cshl.org**
2. Click on **Resources**
3. Click on **BioServers**
4. Under **Allele Server**, click on **Register**. Registration is free and allows you to set up a personal account. There is no need to register everytime you return to this site.

Using Allele Server

1. Log in to Allele Server using the username and password you registered.
2. Once you have logged in, follow instructions provided in the pop-up window for using Allele Server. You may also open a new window and go to **dnalc.org/help/sad/topic_3.html** to get more detailed instructions. Follow the detailed instructions on how to populate the workspace, analyze groups, compare groups, and query the database. Remeber that as a registered user, you may store any groups that you loaded in your personal Allele Server database and analyze them at your convenience.

Lesson Highlights — Frequently Asked Questions (FAQs)

This section describes steps in the experimental protocols that may be technically challenging or that are extremely important to the overall outcome and understanding of the experiments. Instructors should alert their students' attention to these points, and, when possible, demonstrate the technique before the students attempt the procedure.

The Student Manual and Quick Guide contain detailed descriptions and drawings of all laboratory steps and techniques employed in each of the Lessons. Refer to them for questions about the experimental protocols used in the labs.

Lesson 1 Sample Preparation

Processing Cheek Cell and Hair Follicle Samples to Obtain a Genomic DNA Template for PCR

A. InstaGene matrix: What function does it perform?

InstaGene matrix consists of a suspension of negatively charged Chelex® microscopic beads, which bind divalent cations like magnesium (Mg^{2+}). It is important to remove divalent cations from students' genomic DNA samples because the cations assist enzymes that degrade the DNA template. When cheek cells or hair follicles are lysed and boiled in the presence of InstaGene matrix, the divalent cations released from the cells bind to the beads, and the heat inactivates the DNA-degrading enzymes. The beads are then pelleted by centrifugation. The supernatant, which contains clean, intact genomic DNA, can be used as the template in the PCR reactions.

The beads in the InstaGene matrix quickly settle out of solution. It is extremely important that the vial of matrix be thoroughly mixed before pipetting aliquots for each student workstation, so that the aliquots contain equivalent amounts of beads.

Each student will prepare genomic DNA from cheek cells isolated using a saline mouthwash or from hair follicles. For students using the cheek cell protocol, 1 ml of cells collected using the mouthwash should provide sufficient material for DNA preparation. Some students may need to use 2 ml or more of the saline mouthwash to obtain sufficient cells to prepare DNA. Please note: it is **not** recommended to use more than 3 ml of the saline mouthwash to prepare DNA, (see 'Interpretation of Results and Troubleshooting Guide' on page 30). Once the cells have been spun in a centrifuge, a cell pellet about the size of a matchhead should yield enough cells for subsequent steps. Eating just prior to cell collection is not recommended, as food particles may make cell preparation more difficult. If a P-1000 micropipet is not available, students may carefully pour ~1 ml of their swished saline into a micro test tube. The gradations on the side of the micro test tube may be used to judge the amount of liquid in the tube.

If the DNA samples will not be amplified within 24 hours, they can be stored in the refrigerator in the InstaGene matrix for up to 1 week. For longer storage, place samples in the freezer to prevent DNA degradation. Before the samples are used in PCR, the beads should be re-pelleted by centrifugation just prior to making up the PCR reactions. However, processing the samples within 24 hours is recommended. See the next steps for processing tips.

B. Genomic DNA Preparation from cheek cells or hair follicles

For students using the cheek cell DNA protocol, cells are collected using a saline mouthwash. For students using the hair follicle DNA protocol, it is recommended that students collect two hairs for genomic DNA preparation.

C. Incubation: What are the functions of each incubation step?

The preincubation step is carried out at 56°C and performs two functions:

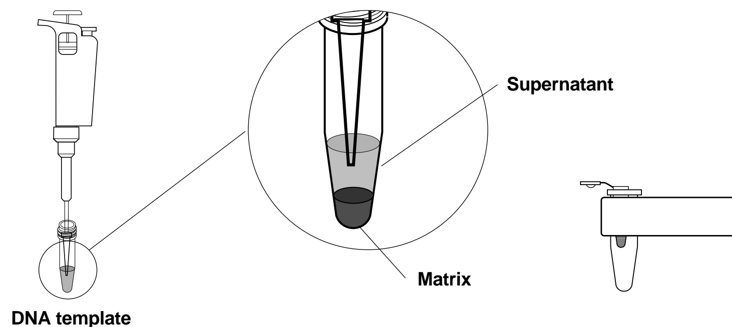
1. Heating the cell suspension aids in the breakup of connective tissue that holds the cells together. Breaking up the tissue makes the cells easier to lyse during the subsequent 100°C incubation step.
2. Preincubation at 56°C inactivates DNases, enzymes that are naturally present in the cell suspensions, and which could degrade the genomic DNA and inhibit the PCR reactions.

Heating the cell samples to 100°C ruptures the cell membranes, thereby releasing the cellular contents, which include genomic DNA. The genomic DNA serves as the template in the PCR reactions.

D. Do I have to remove the InstaGene matrix before PCR?

It is extremely important to pellet the InstaGene beads completely to the bottom of the tube before an aliquot of the lysate is removed for the PCR reaction. The beads bind to divalent cations such as Mg^{2+}, which are essential to the function of *Taq* DNA polymerase. Thus, if any beads are carried over into the PCR reaction, the reaction could be inhibited. The InstaGene matrix can be effectively pelleted by centrifugation (6,000 x g for 5 min).

The supernatant above the beads (which contains the genomic DNA) is taken for the PCR reaction. Carefully remove 20 µl of the supernatant, without disturbing the InstaGene matrix, and transfer it into a PCR tube that is placed within a capless adaptor.



Lesson 2 PCR Amplification of Genomic DNA Samples

Master mix: What is it?

The master mix contains a mixture of nucleotides, or dNTPs (dATP, dTTP, dCTP, and dGTP), buffer, and *Taq* DNA polymerase. Complete master mix is prepared by adding primers to the master mix just prior to the laboratory period. Thus, when a 20 µl aliquot of the cheek cell or hair follicle lysate (which provides the DNA template) is added to a 20 µl aliquot of complete master mix, all of the necessary components for a 40 µl PCR reaction are present. The 2x master mix contains 0.05 units/µl *Taq* DNA polymerase, 3 mM $MgCl_2$, 1.6 mM dNTPs, and 1 µM of each primer. The final 1x or working concentration of these components in the PCR tube after the primers, master mix, and template are combined will be one-half of the above values.

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

Why are the primers yellow?

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

PCR in a Thermal Cycler

The PCR amplification takes place in a thermal cycler that performs cycles of alternating heating and cooling steps. This lab utilizes a three-step cycle: the DNA undergoes denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 2 minutes. This cycle is repeated 40 times during the course of PCR amplification. During the denaturation, the two strands of the DNA template are melted apart to provide access for the PCR primers. During the annealing step, the PCR primers recognize and bind to the DNA template. Once the primers are bound, *Taq* DNA polymerase extends the primers to replicate the segment of DNA during the extension step. The PCR reaction will take approximately 3.5 hours to complete.

The PCR tubes are very small and require care when handling. It is important to carefully and completely cap the tubes before placing them into the thermal cycler. If the tubes are not closed completely, substantial evaporation can take place, and PCR amplification will be inhibited. For best results, if you have to run the thermal cycler more than once (that is, if you have more than 24 samples and you are using Bio-Rad's Gene Cycler), prepare each group of student reactions no more than 30 minutes before PCR cycling. Extended incubation of master mix and genomic DNA decreases amplification efficiency.

Bio-Rad's thermal cyclers were developed for oil-free operation. Oil is not needed in the thermal block wells or in the sample tubes. The sample wells are shaped to provide uniform contact with most standard 200 µl thin-wall PCR tubes. **Do not use 500 µl thin-wall micro test tubes with these thermal cyclers.** The heated sample block cover maintains a higher temperature than the sample block at all times during a thermal cycling program. This keeps water vapor from condensing under the cap of the sample tube, thereby reducing sample evaporation and eliminating the need for oil overlays in the tubes.

Manual PCR

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

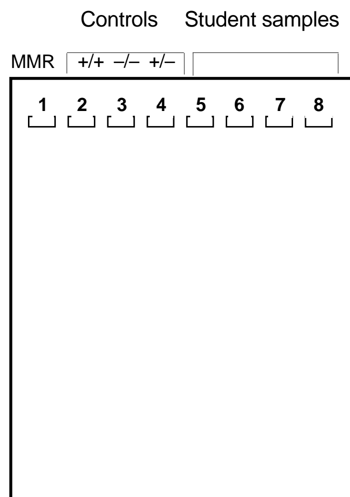
Lesson 3 Gel Electrophoresis of Amplified PCR Samples and Staining of Agarose Gels

Sample Preparation for Electrophoresis

Before the amplified samples are electrophoresed, students need to add 10 µl of 5x PV92 XC loading dye to each of their PCR tubes. The DNA loading dye contains glycerol, which increases the density of the sample and ensures that it sinks into the well of the agarose gel. In addition, the DNA loading dye contains a dye called Xylene Cyanole that comigrates at the same rate as a 4,000 bp DNA fragment toward the anode.

What volume of PCR samples is best loaded onto gels?

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. A gel template is provided below and the gel set up is also described (Figure 7).



Gel Setup (1 gel for 4 students; 8 gels for 32 students)

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)

Fig. 7. Gel template and suggested sample loading order.

Staining the Gels

Detailed instructions on using Fast Blast DNA stain are included in the student manual. **Because of the thickness of the gels, gentle rocking on a shaking table produces more efficient staining**.

The gels can also be stained and visualized with ethidium bromide (not included in the kit). If ethidium bromide is used as a stain, the gels should contain 0.05 µg/ml ethidium bromide in the agarose. This concentration of ethidium bromide produces maximum contrast of the amplified bands. Note: Fast Blast DNA stain quenches ethidium bromide staining, so visualize with ethidium bromide before Fast Blast stain.

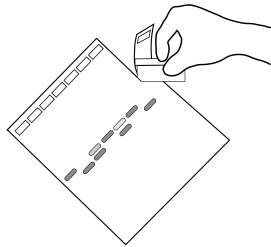
Lesson 4 Analysis and Interpretation of Results

Once the gel has been stained with Fast Blast DNA stain (using either the quick or overnight staining protocol), it will be analyzed. Because the bands fade slightly upon drying of the gel, it is best to analyze the stained gels before drying. The gels can be illuminated from below to enhance visualization of the bands.

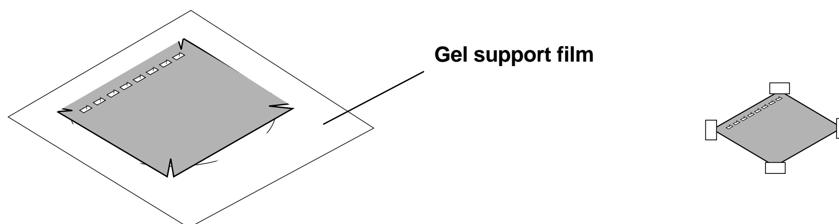
a. Place your gel on a light background and record your results by making a diagram as follows. Place a clear sheet of plastic or acetate over the gel. With a permanent marker, trace the wells and band patterns onto the plastic sheet to make a replica picture of your gel. Remove the plastic sheet for later analysis. Alternatively, gels can be photocopied on a yellow piece of transparent film for optimal contrast.

b. Dry the agarose gel as a permanent record of the experiment.

   i. Trim away any unloaded lanes with a knife or razor blade. Cut your gel from top to bottom to remove the lanes that you did not load samples into.



   ii. Place the gel directly upon the hydrophilic size of a piece of gel support film. (Water will form beads on the hydrophobic side of a piece of gel support film.) Center the gel on the film and remove bubbles that may form between the gel and film. Place the film on a paper towel and let the gel dry in a well-ventilated area, making sure to avoid direct exposure to light. As the gel dries it will bond to the film but will not shrink. If left undisturbed on the support film, the gel will dry completely at room temperature after 2–3 days. The result will be a flat, transparent, and durable record for the experiment.

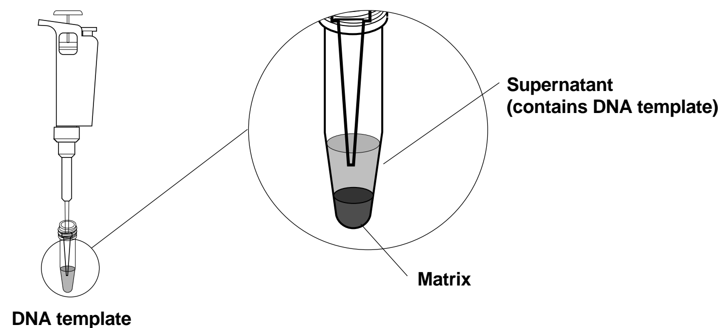


Interpretation of Results and Troubleshooting Guide

Explanations for "Empty Lanes" or Unamplified Samples

Multiple explanations can account for student samples not being amplified in the PCR reactions.

1. **Inadequate collection of cheek cells**. A visible cell pellet about the size of a match head should be obtained following centrifugation of the saline mouthwash. If no cell pellet is visible, or the pellet is too small, additional swished saline may be centrifuged until a pellet of the desired size is obtained. However, it is not recommended to collect more than 3 ml of cells (see point below).
2. **Excessive number of cells**. Just as too few cells will yield insufficient genomic DNA, an excessive number of cells will saturate the capacity of the InstaGene, resulting in little or no amplification.
3. **InstaGene matrix not transferred**. Each workstation is supplied with tubes of InstaGene matrix that were aliquotted by the instructor and placed on ice. These tubes of matrix must be mixed prior to each pipetting to bring the beads up into suspension. If no beads were transferred into the student's tube, the divalent cations will not be removed from the genomic DNA preparation, and the PCR reaction will be inhibited.
4. **Carryover of InstaGene into PCR reaction.** Although the beads in the InstaGene matrix are required for the DNA template preparation, it is critical that none of the InstaGene matrix be carried over into the PCR reaction. If beads are transferred into the PCR tube, the magnesium ions needed by the *Taq* polymerase will be removed, and the PCR reaction will be inhibited.



Interpretation of Heterozygous Samples

1. **Competition during amplification**. Amplification of heterozygous samples is more difficult than both homozygous amplifications because of competition between the reactions that produce the smaller (641 bp) and larger (941 bp) bands. Because the smaller 641 bp band is amplified more efficiently than the 941 bp band, heterozygous samples on agarose gels will show the smaller band being more intense than the larger band (see the band indicated by an asterisk in the gel below). For this reason, heterozygous samples can often be interpreted as homozygous (–/–) because of a faint upper band. **Careful examination of the gels is required to distinguish between heterozygous (+/–) and homozygous (–/–) individuals.** Alternatively, the use of ethidium bromide and photodocumentation equipment (the Bio-Rad gel documentation system) will increase the sensitivity and allow easier visualization of faint heterozygous samples.

2. **Larger band in (+/–) samples**. The heterozygous samples will often contain larger bands which migrate at ~1,100 bp and 1,700 bp in the gel (see the bands indicated by arrows in the gel below). These bands are heteroduplexes that form between the 641- and 941-nucleotide strands and contain secondary structure which results in the DNA bands migrating at a slower rate in the gel (Figure 8).

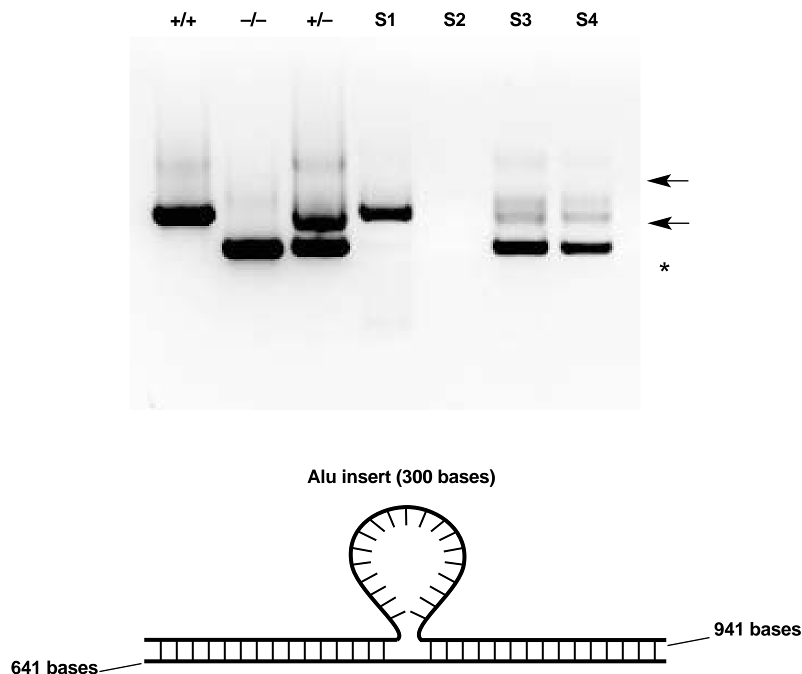
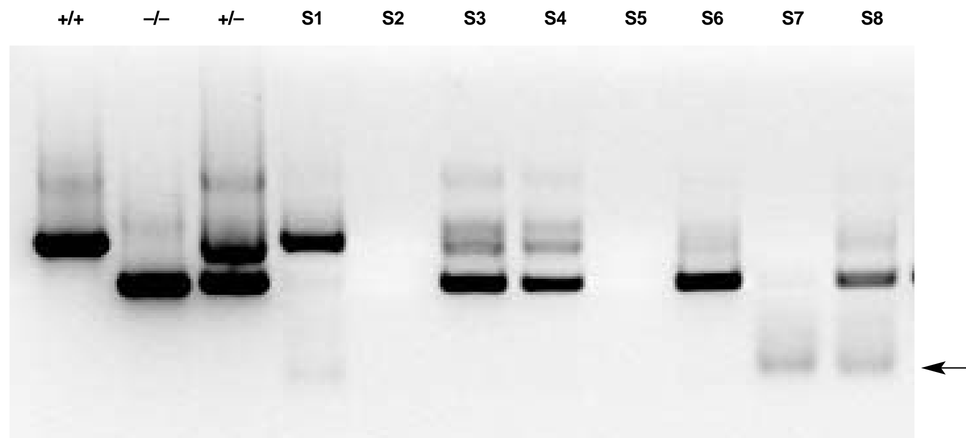


Fig. 8. Heteroduplex formed between 941- and 641-nucleotide strands.

3. **Primer-dimer formation**. Some PCR reactions may show primer-dimer formation. Primer dimers are bands that are seen at the bottom of the gels and which correspond to complexes of both primers. Primer-dimer formation is more intense in reactions that show little or no amplified product. Thus, primer-dimer formation is more likely to occur in reaction tubes with InstaGene contamination, little or no template, or in samples that were prepared well in advance of placing into the thermal cycler. The arrow in the figure below shows primer-dimers.

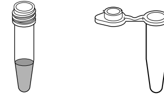


4. **Bands appear to be fading**. The blue dye in the Fast Blast DNA stain is subject to reversible bleaching when exposed to bright room lights. When the dried gels are examined 3–5 days after drying, the bands may appear faint. Placing the gels in a dark location (in a box or taped in a closed notebook) and examining several hours later will provide the most intense bands. It is most convenient to let the gels dry on the lab bench for 3–5 days, tape them into a lab notebook, and examine the gels the following day.

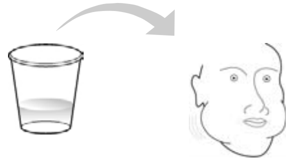
Quick Guide

Lesson 1 Cheek Cell DNA Template Preparation

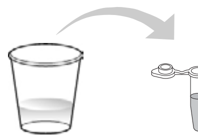
1. Label one 1.5 ml micro test tube with your initials. Label one screwcap tube containing 200 µl of InstaGene matrix with your initials.



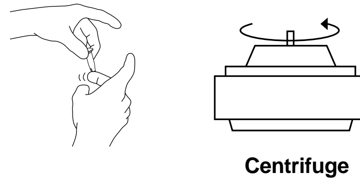
2. Obtain a cup containing saline solution from your instructor. Pour the saline into your mouth and rinse vigorously for 30 seconds. Expel the saline back into the cup.



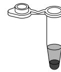
3. Transfer 1 ml of your saline rinse into the micro test tube (NOT the screwcap tube) with your initials. If a P-1000 micropipet is not available, **carefully** pour ~1 ml of your saline rinse into your micro test tube (use the graduations on the side of the micro test tube to estimate 1 ml).



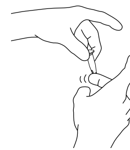
4. Spin your tube in a balanced centrifuge at full speed for 2 minutes. When the centrifuge has completely stopped, remove your tube. You should see a match-head sized pellet of whitish cells at the bottom of the tube. If you don't see a pellet of this size, decant the saline, refill your tube with more of your oral rinse, and repeat the spin.



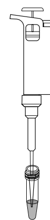
5. After pelleting your cells, pour off the saline. Being careful not to lose your pellet, blot your tube briefly on a paper towel or tissue. It's OK for a small amount of saline (< 50 µl, about the same size as your pellet) to remain in the bottom of the tube.



6. Resuspend the pellet by vortexing or flicking the tube so that no clumps of cells remain.

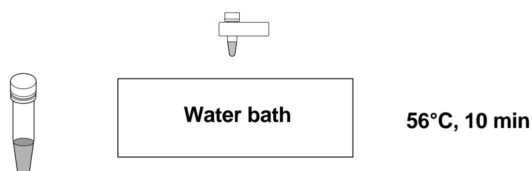


7. Using a 2–20 µl adjustable-volume micropipet set to 20 µl, transfer all of your resuspended cells to the screwcap tube containing InstaGene.

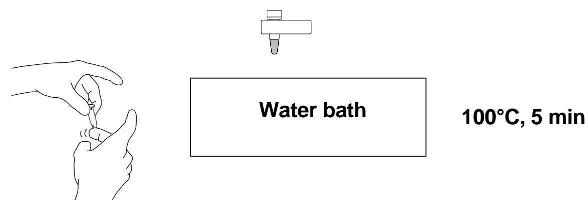


8. Screw the cap tightly on the tube. Shake or vortex to mix the tube contents.

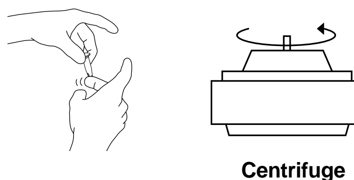
9. When all members of your team have collected their samples, place the tubes in the foam micro test-tube holder, and incubate at 56°C for 10 minutes in a water bath. At the halfway point (5 minutes), shake or vortex the tubes gently, then place back in the 56°C water bath for the remaining 5 minutes.



10. Remove the tubes, shake or vortex, and place the tubes in a boiling water bath (100°C). Incubate at 100°C for 5 minutes.



11. Remove the tubes from the boiling water bath and shake or vortex the contents to resuspend. Pellet the matrix by spinning at 6,000 x g for 5 minutes (or 2,000 x g for 10 minutes) in a centrifuge.



Centrifuge

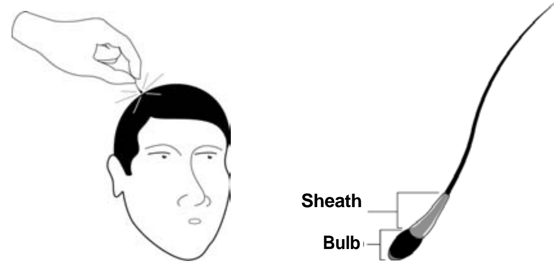
12. Store your screwcap tube in the refrigerator until the next laboratory period (or proceed to step 2 of Lesson 2).

Quick Guide

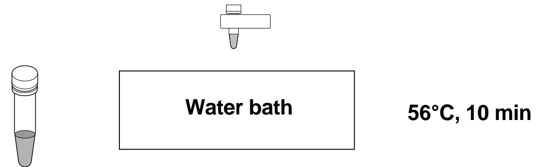
Lesson 1 Hair Follicle DNA Template Preparation

1. Label 1 screwcap tube containing 200 µl of InstaGene matrix plus protease with your initials.

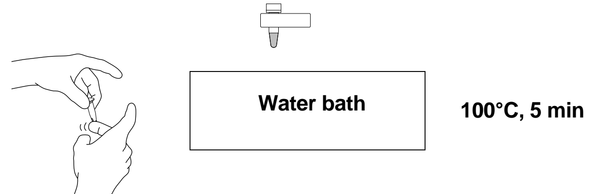
2. Collect 2 hairs from yourself. Select hairs that have either a noticeable sheath (a coating of epithelial cells near the base of the hair), or a good sized root (the bulb-shaped base of the hair). Trim the hair, leaving the last ~2 cm of the base of the hair. Place the trimmed hairs into the screwcap tube with your initials.



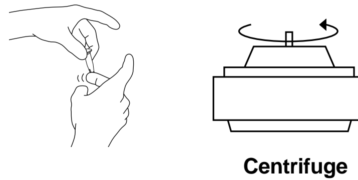
3. Place your tube in the foam micro test-tube holder and incubate at 56°C for 10 minutes in a water bath. At the halfway point (5 minutes), shake or vortex gently, then place it back in the 56°C water bath for the remaining 5 minutes.



4. Remove the tubes, gently shake or vortex, and place them in a boiling water bath (100°C). Incubate at 100°C for 5 minutes.



5. Remove the tubes from the boiling water bath and shake or vortex the contents to resuspend. Pellet the matrix by spinning at 6,000 x g for 5 minutes (or 2,000 x g for 10 minutes) in a centrifuge.



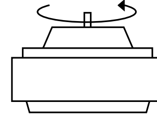
Centrifuge

6. Store your screwcap tube in the refrigerator until the next laboratory period (or proceed to step 2 of Lesson 2).

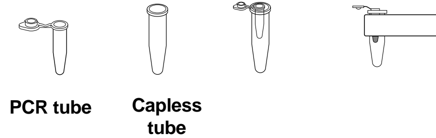
Quick Guide

Lesson 2 PCR Amplification

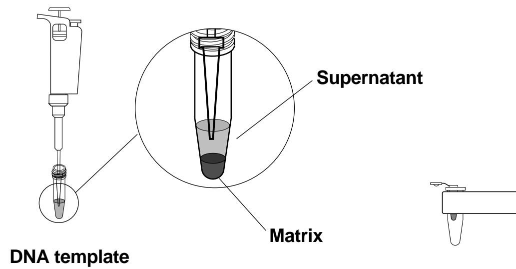
1. Obtain the tube with your DNA template from the refrigerator. Spin the screwcap tube for 2 minutes at 6,000 x g (5 minutes at 2,000 x g) in a centrifuge.



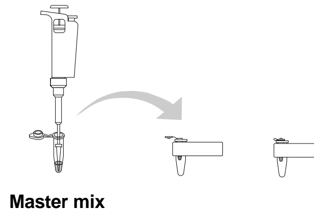
2. Label a PCR tube and a capless micro test tube with your initials, place the PCR tube in the capless tube as shown, and place both in the foam holder.



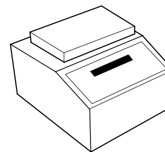
3. Transfer 20 µl of the DNA template (the supernatant) from the screwcap tube into the bottom of the PCR tube. Be very careful **not** to transfer any of the matrix beads into the PCR tube.



4. Locate the tube of yellow master mix on ice and transfer 20 µl of the master mix into the PCR tube. Mix by pipetting up and down 2–3 times. Cap the PCR tube tightly. The mixture should be yellow.

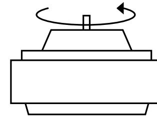


5. Place the PCR tube into the thermal cycler. Control reactions prepared by the instructor should also be placed into the PCR machine at this point. The reactions will undergo 40 cycles of PCR amplification.



Lesson 3 Electrophoresis of Amplified PCR Samples and Staining of Agarose Gels

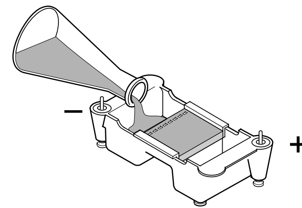
1. Obtain your PCR tube from the thermal cycler and place in the capless micro test tube. Pulse-spin the tube for ~3 seconds at 2,000 x g.



Centrifuge

2. Add 10 µl of PV92 XC loading dye into your PCR tube and mix gently.

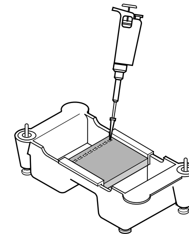
3. Place an agarose gel in the electrophoresis apparatus. Check that the wells of the agarose gels are near the black (–) electrode and the base of the gel is near the red (+) electrode.



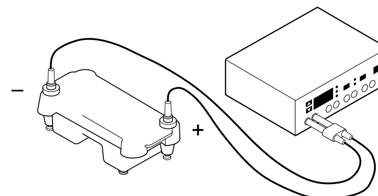
4. Fill the electrophoresis chamber and cover the gel with 1x TAE buffer. This will require ~275 ml of 1x buffer.

5. Using a clean tip for each sample, load the samples into 8 wells of the gel in the following order:

Lane	Sample	Load Volume
1	MMR (DNA standard)	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



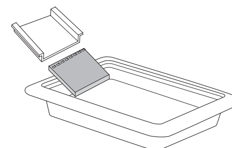
6. Secure the lid on the gel box. The lid will attach to the base in only one orientation: red to red and black to black. Connect the electrical leads to the power supply.



7. Turn on the power supply and electrophorese your samples at 100 V for 30 minutes.

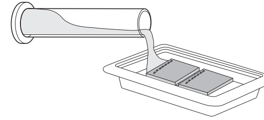
Staining of Agarose Gels

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

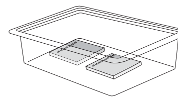


2. There are two protocols for staining your gel. Your instructor will inform you which one you will use.

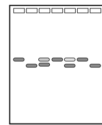
Protocol 1: Quick staining (requires 12–15 minutes)



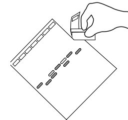
a. Add 120 ml of 100x Fast Blast stain into your staining tray (2 gels per tray).
b. Stain the gels for 2 minutes with gentle agitation. Save the used stain for future use. Stain can be reused at least 7 times.
c. Transfer the gels into a large washing container and rinse with warm (40–55°) tap water for approximately 10 seconds.
d. Destain by washing **twice** in warm tap water for 5 minutes each with gentle shaking for best results.



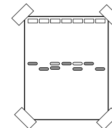
e. Place the gel on a light background and record your result. With a permanent marker, trace the wells and band patterns onto a clear sheet of plastic or acetate sheet.



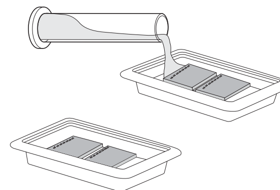
f. With the help of your instructor, determine whether you are homozygous or heterozygous for the Alu insertion.
g. Trim away any empty lanes of the gel with a knife or razor blade.



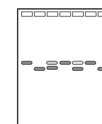
h. To obtain a permanent record, air-dry the gel on gel support film. Tape the dried gel onto your lab notebook. Avoid exposure of the stained gel to direct light, since it will cause the bands to fade.



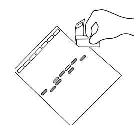
Protocol 2: Overnight staining



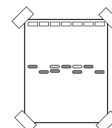
a. Add 120 ml of 1x Fast Blast DNA stain to your staining tray (2 gels per tray).
b. Let the gels stain overnight, with gentle shaking for best results. No destaining is required.
c. The next day, pour off the stain into a waste beaker.
d. Place the gel on a light background and record your result. With a permanent marker, trace the wells and band patterns onto a clear sheet of plastic or acetate sheet.



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



f. Trim away any empty lanes of the gel with a knife or razor blade.
g. To obtain a permanent record, air-dry the gel on gel support film. Tape the dried gel into your lab notebook. Avoid exposure of the stained gel to direct light, since it will cause the bands to fade.



Student Manual

Introduction to PCR — The Polymerase Chain Reaction

You are about to perform a procedure known as PCR[1] to amplify a specific sequence of your own DNA in a test tube. You will be looking for a particular piece of DNA that is present in the genes of many, but not all, people. Analysis of the data generated in this laboratory will enable you to determine whether or not you carry this specific DNA sequence.

The **genome**, composed of DNA, is our hereditary code. This is the so-called blueprint that controls much of our appearance, behavior, and tendencies. **Molecular biology** is the study of genes and the molecular details that regulate the flow of genetic information from DNA to RNA to proteins, from generation to generation. **Biotechnology** uses this knowledge to manipulate organisms' (microbes, plants, or animals) DNA to help solve human problems.

Within the molecular framework of biology, DNA, RNA, and proteins are closely tied to each other. Because proteins and enzymes ultimately play such a critical role in the life process, scientists have spent many lifetimes studying proteins in an attempt to understand how they work. With this understanding, it was believed we could cure, prevent, and overcome disease and physical handicaps as well as explain exactly how and why organisms exist, propagate, and die. However, the complete answer to how and why does not lie solely in the knowledge of how enzymes function; we must learn how they are made. If each enzyme is different, then what controls these differences and what is the blueprint for this difference? That answer lies within our genome, or genetic code.

Thus, you may realize why researchers today, in an attempt to understand the mechanisms behind the various biological processes, study nucleic acids as well as proteins to get a complete picture. In the last 20 years, many advances in nucleic acid techniques have allowed researchers to study the roles that nucleic acids play in biology. It took the imagination and hard work of many scientists to reveal the answers to one of the most mysterious puzzles of life — understanding the mechanisms that control how DNA is translated into proteins within living cells.

Before Beginning This Lab, See If You Can Answer the Following Questions

How is DNA faithfully passed on from generation to generation? What causes genetic disease in some people but not others? How do scientists obtain DNA to study? What secrets can DNA tell us about our origins? What human problems can an understanding of DNA help us solve? Should we unlock the secrets held in this most basic building block of life?

PCR Set the Stage for a Scientific Revolution

In 1983, Kary Mullis[2] at Cetus Corporation developed the molecular biology technique that has since revolutionized genetic research. This technique, called the **polymerase chain reaction** (PCR), transformed molecular biology into a multidisciplinary research field within 5 years of its invention. Before PCR, the molecular biology techniques used to study DNA required such a high level of expertise that relatively few scientists could use them.

The objective of PCR is to produce a large amount of DNA in a test tube (in vitro), starting from only a trace amount. Technically speaking, this means the controlled enzymatic amplification of a DNA sequence, or gene, of interest. The template strands can be any form of DNA, such as genomic DNA. A researcher can use tiny amounts of genomic DNA

from a drop of blood, a single hair follicle, or a cheek cell, and make enough DNA to study. In theory, only a single template strand is needed to copy and generate millions of new identical DNA molecules. Prior to PCR, this would have been impossible. It is the ability to amplify the precise sequence of DNA of interest that is the true power of PCR.

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;[3] in criminal investigations and courts of law to identify suspects,[4] and in the sequencing of the human genome.[5] 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 transformed molecular biology from a difficult science to one of the most accessible and widely used disciplines of **biotechnology**.

Two methods for DNA template preparation are provided in the manual. Your instructor will indicate which exercise to follow. Now, let's extract some of your own DNA.

Lesson 1 Cheek Cell DNA Template Preparation

To obtain DNA for use in the polymerase chain reaction (PCR) you will extract the DNA from your own living cells. It is interesting to note that DNA can be also extracted from mummies and fossilized dinosaur bones. In this lab activity, you will isolate DNA from epithelial cells that line the inside of your cheek. To do this, you will rinse your mouth with a saline (salt) solution, and collect the cells using a centrifuge. You will then boil the cells to rupture them and release the DNA they contain. To obtain pure DNA for PCR, you will use the following procedure:

The cheek cells are transferred to a micro test tube containing **InstaGene™ matrix**. This particulate matrix is made up of negatively charged, microscopic beads that chelate, or grab, metal ions out of solution. It traps metal ions, such as Mg^{2+}, which are required as catalysts or **cofactors** in enzymatic reactions. Your cheek cells will then be **lysed**, or ruptured, by heating to release all of their cellular constituents, including enzymes that were once contained in the cheek-cell lysosomes. Lysosomes are sacs in the cytoplasm that contain powerful enzymes, such as **DNases**, which are used by cells to digest the DNA of invading viruses. When you rupture the cells, these DNases can digest the released DNA. However, when the cells are lysed in the presence of the chelating beads, the cofactors are adsorbed and are not available to the enzymes. This virtually blocks enzymatic degradation of the extracted DNA so you can use it as the template in your PCR reaction.

You will first suspend your isolated cheek cells in the InstaGene matrix and incubate them at 56°C for 10 minutes. This preincubation step helps to soften plasma membranes and release clumps of cells from each other. The heat also inactivates enzymes, such as DNases, which can degrade the DNA template. After this 10 minute incubation period, place the cells in a boiling (100°C) water bath for 5 minutes. Boiling ruptures the cells and releases DNA from their nuclei. You will use the extracted genomic DNA as the target template for PCR amplification.

Lesson 1 Cheek Cell DNA Template Preparation (Lab Protocol)

Workstation Checklist

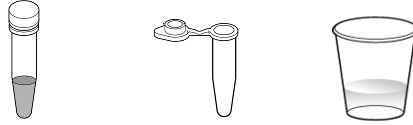
Materials and supplies required at the workstations prior to beginning this exercise are listed below.

Student Workstation	Quantity per Station	(✔)
1.5 ml micro test tubes	4	❒
Screwcap tubes with 200 µl InstaGene™ matrix	4	❒
Foam micro test-tube holder	2	❒
P-20 micropipet, 2–20 µl	1	❒
Pipet tips (filter type), 2–20 µl	4	❒
Permanent marker	1	❒
Copy of Quick Guide or protocol	1	❒
Waste container	1	❒
Cups with 10 ml 0.9% saline	4	❒

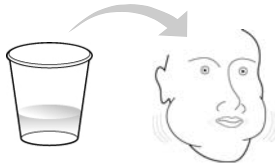
Instructor's Workstation	Quantity per Class	
P-20 micropipet, 2–20 µl	1	❒
P-200 micropipet, 20–200 µl	1	❒
Pipet tips (filter type), 2–20 µl	1 box	❒
Pipet tips (filter type), 20–200 µl	1 box	❒
Water baths (56 and 100°C)	1 each	❒
Microcentrifuge	1	❒
or mini centrifuge	4	❒
Vortexer (optional)	1	❒

Lesson 1   Cheek Cell DNA Template Preparation (Lab Protocol)

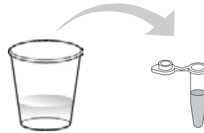
1. Each member of your team should have 1 screwcap tube containing 200 µl InstaGene™ matrix, 1.5 ml micro test tube, and a cup containing 10 ml of 0.9% saline solution. Label one of each tube and a cup with your initials.



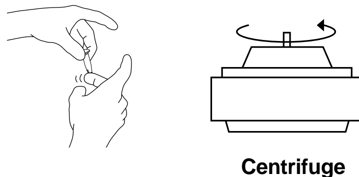
2. Do not throw away the saline after completing this step. Pour the saline from the cup into your mouth. Rinse vigorously for 30 seconds. Expel the saline back into the cup.



3. Set a P-1000 micropipet to 1,000 µl and transfer 1 ml of your oral rinse into the micro test tube with your initials. If no P-1000 is available, carefully pour ~1 ml of your swished saline into the micro test tube (use the markings on the side of the micro test tube 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.



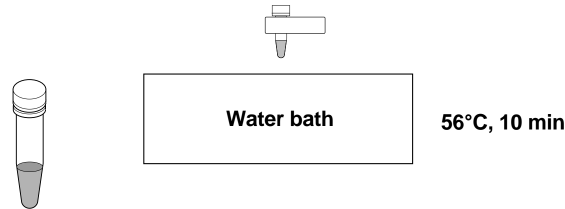
5. Pour off the supernatant and discard. Taking care not to lose your cell pellet, carefully blot your micro test tube on a tissue or paper towel. It's ok for a small amount of saline (~50 µl, about the same size as your pellet) to remain in the bottom of the tube.

6. Resuspend the pellet thoroughly by vortexing or flicking the tubes until no cell clumps remain.

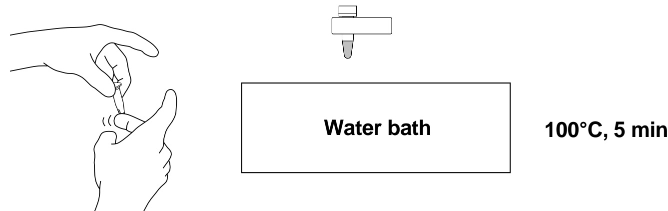
7. Using an adjustable volume micropipet set to 20 µl, transfer your resuspended cells into the screwcap tube containing the InstaGene with your initials. You may need to use the pipet a few times to transfer all of your cells.

8. Screw the caps tightly on the tubes. Shake or vortex to mix the contents.

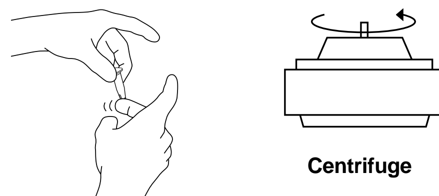
9. Place the tubes in the foam micro test-tube holder. When all members of your team have collected their samples, float the holder with tubes in a 56°C water bath for 10 minutes. At the halfway point (5 minutes), shake or vortex the tubes several times. Place the tubes back in the water bath for the remaining 5 minutes.



10. Remove the tubes from the water bath and shake them several times. Now float the holder with tubes in a 100°C water bath for 5 minutes.



11. Remove the tubes from the 100°C water bath and shake or vortex several times to resuspend the sample. Place the eight 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).



12. Store your screwcap tube in the refrigerator until the next laboratory period, or proceed to step 2 of Lesson 2 if your teacher instructs you to do so.

Lesson 1 Hair Follicle DNA Template Preparation

To obtain DNA for use in the polymerase chain reaction (PCR) you will extract the DNA from your own living cells. It is interesting to note that DNA can be also extracted from mummies and fossilized dinosaur bones. In this lab activity, you will isolate DNA from the epithelial cells that coat the base of your hair. To do this, you will collect two hairs from yourself. Good places to obtain hair are your head, arm, or leg. You will then boil the cells to **lyse**, or rupture them and release the DNA they contain. To obtain pure DNA for PCR you will use the following procedure:

You will trim 2 hairs with an obvious sheath (a coating of cells surrounding the base of the hair) or a large root (the bulb-shaped structure at the base of the hair) to about 2 cm then transfer them into a micro test tube containing **InstaGene™ matrix** and **protease**. This particulate matrix is made up of negatively charged microscopic beads that chelate, or grab, metal ions out of solution. InstaGene traps metal ions, such as Mg^{+2}, which are required as catalysts or **cofactors** in enzymatic reactions. Protease digests the connections between cells, allowing better lysis in the next step. Your epitheleal cells will then be lysed, or ruptured, by heating to release all of their cellular constituents, including enzymes that were once contained in the epitheleal cell lysosomes. Lysosomes are sacs within the cytoplasm that contain powerful enzymes, such as **DNases**, which are used by cells to digest the DNA of invading viruses. When you rupture the cells, these DNases can digest the released DNA. However, when the cells are lysed in the presence of the InstaGene beads, the cofactors are adsorbed and are not available to the enzymes. This virtually blocks enzymatic degradation of the extracted DNA so you can ust it as the template in your PCR reaction.

You first suspend isolated hair-follicle cells in the InstaGene matrix with protease and incubate them at 56°C for 10 minutes. This preincubation step helps to soften plasma membranes and release clumps of cells from each other and the hair. After this 10 minute incubation period, place the cells in a boiling (100°C) water bath for 5 minutes. Boiling ruptures the cells and releases DNA from cell nuclei. The heat also inactivates enzymes such as DNases, which can degrade the DNA template. You will use the extracted genomic DNA as the target template for PCR amplification.

Lesson 1 Hair Follicle DNA Template Preparation (Lab Protocol)

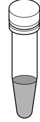
Workstation Checklist

Materials and supplies required at the workstation prior to beginning this exercise are listed below.

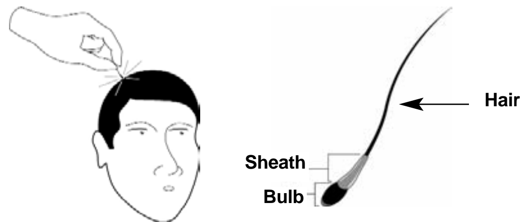
Student Workstations	Quantity per Station	(✔)
Screwcap tubes with InstaGene matrix plus protease	4	❒
Foam micro test tube holders	2	❒
Permanent marker	1	❒
Waste container	1	❒
Copy of Quick Guide or protocol	1	❒
Tweezers or forceps	1	❒
Scissors or razor blade	1	❒
Instructor's workstation	**Quantity per Class**	
P-1000 micropipet, 100–1000 µl	1	❒
Pipet tips (filter type), 100–1,000 µl	1 box	❒
Water baths (56 and 100°C)	1 of each	❒
Microcentrifuge	1	❒
or mini centrifuge	4	❒
Vortexer (optional)	1	❒

Lesson 1 Hair Follicle DNA Template Preparation (Lab Protocol)

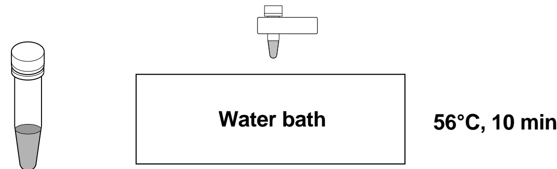
1. Each member of your team should have 1 screwcap tube containing 200 µl of InstaGene matrix plus protease. Label the tube on the cap and side with your initials.



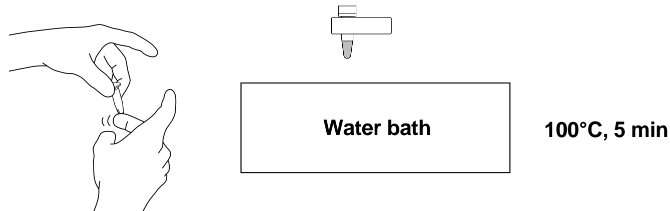
2. Collect 2 hairs from yourself. Choose hairs that leave noticeable sheaths (a coating of epitheleal cells around the base of the hair). Alternatively, choose hairs that have a large root. The root is the bulb-shaped base of the hair. Keeping the end of the hair with the sheath and bulb, trim the hair with scissors so it is ~2 cm long. Place your trimmed hairs into the screwcap tube with your initials. Screw the cap tightly on the tube.



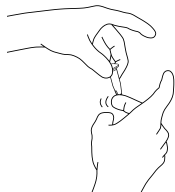
3. Place your tube in the foam micro test tube holder and incubate it at 56°C for 10 minutes in a water bath. At the halfway point (5 minutes), shake or vortex the tube gently, then place it back in the 56°C water bath for the remaining 5 minutes.

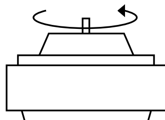


4. Remove your tube, gently shake or vortex it, then place it in a boiling water bath (100°C) 5 minutes.



5. Remove your tube from the boiling water bath and shake or vortex to resuspend the contents. Pellet the matrix by spinning at 6,000 x g for 5 minutes (or 2,000 x g for 10 minutes).





Centrifuge

6. Store your screwcap tube in the refrigerator until the next laboratory period (or proceed to step 2 of Lesson 2).

Lesson 1 DNA Template Preparation

Focus Questions

1. Why is it necessary to chelate the metal ions from solution during the boiling/lysis step at 100°C? What would happen if you did not use a chelating agent such as the InstaGene matrix?

2. What is needed from the cells for PCR?

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

4. Why do you think the DNA is stored cold with the InstaGene matrix after boiling the samples?

Lesson 2 PCR Amplification

It is estimated that there are 30,000–50,000 individual genes in the human genome. The true power of PCR is the ability to target and make millions of copies of (or **amplify**) a specific piece of DNA (or gene) out of a complete genome. In this activity, you will amplify a region within your chromosome 16.

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

1. DNA template — containing the intact sequence of DNA to be amplified
2. Individual deoxynucleotides (A, T, G, and C) — raw material of DNA
3. DNA polymerase — an enzyme that assembles the nucleotides into a new DNA chain
4. Magnesium ions — a cofactor (catalyst) required by DNA polymerase to create the DNA chain
5. Oligonucleotide primers — pieces of DNA complementary to the template that tell DNA polymerase exactly where to start making copies
6. Salt buffer — provides the optimum ionic environment and pH for the PCR reaction

The template DNA in this exercise is genomic DNA that was extracted from your cells. The complete master mix contains *Taq* DNA polymerase, deoxynucleotides, oligonucleotide primers, magnesium ions, and buffer. When all the other components are combined under the right conditions, a copy of the original double-stranded template DNA molecule is made — doubling the number of template strands. Each time this cycle is repeated, copies are made from copies and the number of template strands doubles — from 2 to 4 to 8 to 16 and so on — until after 20 cycles there are 1,048,576 exact copies of the target sequence.

PCR makes use of the same basic processes that cells use to duplicate their DNA.

1. **Complementary DNA strand hybridization**
2. **DNA strand synthesis via DNA polymerase**

The two DNA primers provided in this kit are designed to flank a DNA sequence within your genome and thus provide the exact start signal for the DNA polymerase to “zero in on” and begin synthesizing (replicating) copies of that target DNA. Complementary strand hybridization takes place when the two different **primers** anneal, or bind to each of their respective complementary base sequences on the template DNA.

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

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

PCR Step by Step

PCR amplification includes three main steps, a **denaturation step**, an **annealing step,** and an **extension step** (summarized in Figure 9). In denaturation, the reaction mixture is heated to 94°C for 1 minute, which results in the melting or separation of the double-stranded DNA template into two single stranded molecules. PCR amplification, DNA templates must be separated before the polymerase can generate a new copy. The high temperature required to melt the DNA strands normally would destroy the activity of most enzymes, but *Taq* polymerase is stable and active at high temperature.

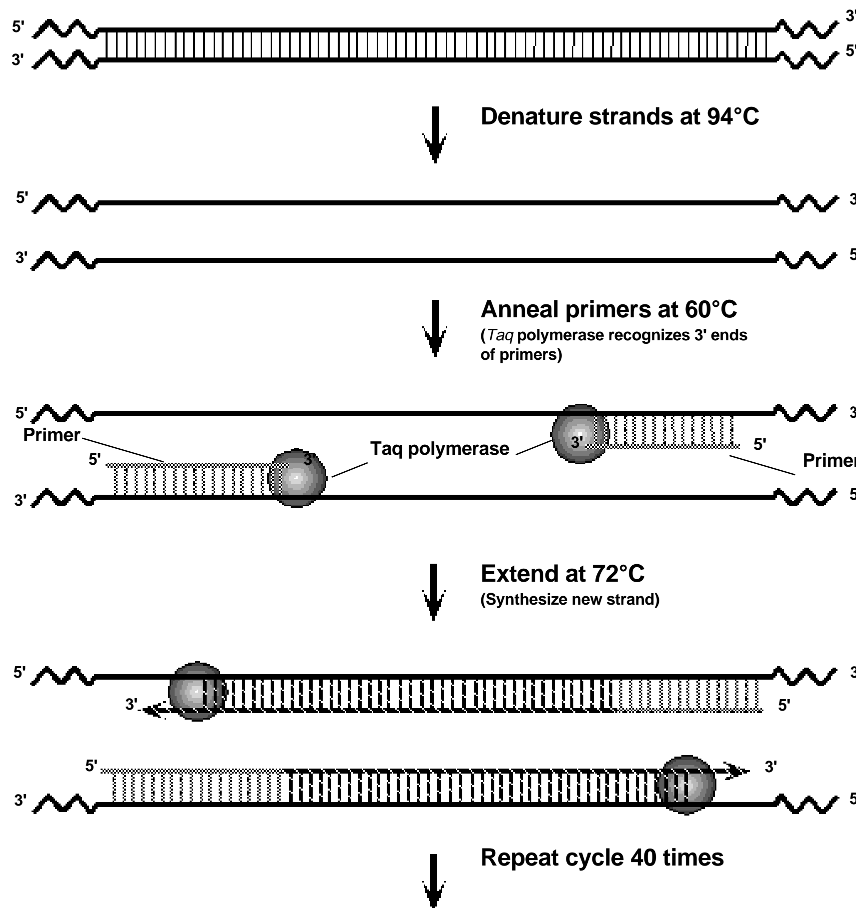


Fig. 9. A complete cycle of PCR.

During the annealing step, the oligonucleotide primers “anneal to” or find their complementary sequences on the two single-stranded template strands of DNA. In these annealed positions, they can act as primers for *Taq* DNA polymerase. They are called primers because they “prime” the synthesis of a new strand by providing a short sequence of double-stranded DNA for *Taq* polymerase to extend from and build a new complementary strand. Binding of the primers to their template sequences is also highly dependent on temperature. In this lab exercise, a 60°C annealing temperature is optimum for primer binding.

During the extension step, the job of *Taq* DNA polymerase is to add nucleotides (A, T, G, and C) one at a time to the primer to create a complementary copy of the DNA template. During

polymerization the reaction temperature is 72°C, the temperature that produces optimal *Taq* polymerase activity. The three steps of denaturation, annealing, and extension form one “cycle” of PCR. A complete PCR amplification undergoes 40 cycles.

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

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

Lesson 2 PCR Amplification (Lab Protocol)

Workstation Checklist

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

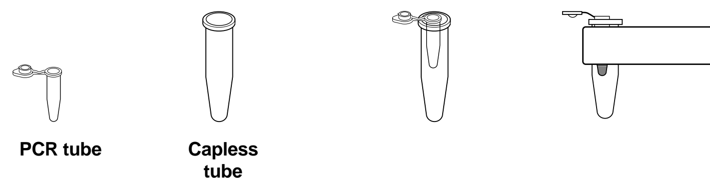
Student Workstation	Quantity per Station	(✔)
PCR tubes	4	❒
Micro test tubes, capless	4	❒
Complete master mix (with primers) on ice	1 tube	❒
P-20 micropipet	1	❒
Pipet tips (filter type), 2–20 µl	8	❒
Ice bucket with ice	1	❒
Foam micro test tube holders	2	❒
Permanent marker	1	❒
Waste containers	1	❒
Copy of Quick Guide or protocol	1	❒

Instructor's (common) workstation	Quantity per Class	
Gel trays	1 per 2 stations	❒
Molten agarose	40 ml per gel	❒
Lab tape for gel trays	1 per station	❒
Gene Cycler or MyCycler thermal cycler	1	❒
Microcentrifuge	1	❒
or mini centrifuge	4	❒

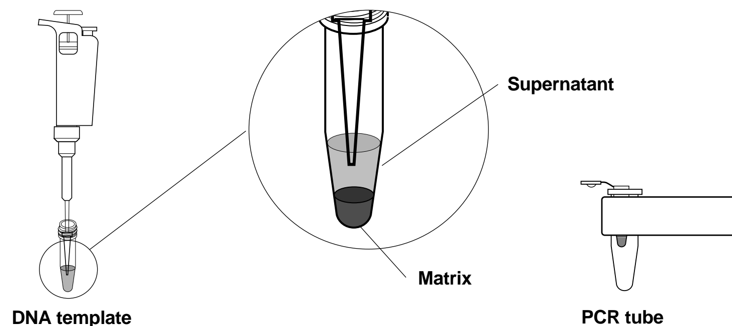
Lesson 2 PCR Amplification (Lab Protocol)

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.

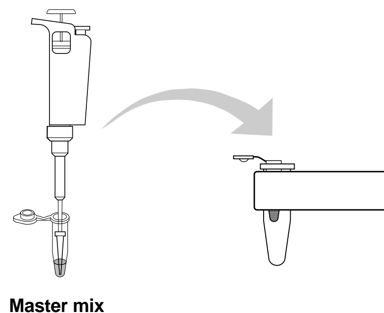
2. Each member of the team should obtain a PCR tube and capless micro test tube. Label each PCR tube on the side of the tube with your initials and place the PCR tube into the capless micro test tube as shown. Place the PCR tube in the foam micro test tube holder.



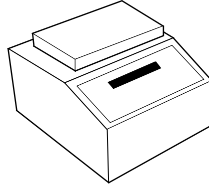
3. Transfer 20 µl of your DNA template from the supernatant in your screwcap tube into the bottom of the PCR tube. **Do not transfer any of the matrix beads into the PCR reaction because they will inhibit the PCR reaction.**



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.

7. If your teacher instructs you to do so, you will now pour your agarose gels (the gels may have been prepared ahead of time by the teacher).

Lesson 2 PCR Amplification

Focus Questions

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

2. How did *Taq* DNA polymerase acquire its name?

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.

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

Lesson 3 Gel Electrophoresis of Amplified PCR Samples and Staining of Agarose Gels

What Are You Looking At?

Before you analyze your PCR products, let's take a look at the target sequence being explored.

What Can Genes and DNA Tell Us?

It is estimated that the 23 pairs, or 46 **chromosomes**, of the human genome (23 chromosomes come from the mother and the other 23 come from the father) 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 30,000–50,000 genes only comprise 5% of the total chromosomal DNA. The other 95% is noncoding DNA. This noncoding DNA is found not only between, but within genes, splitting them into segments. The exact function of the noncoding DNA is not known, although it is thought that noncoding DNA allows for the accumulation of mutations and variations in genomes.

When RNA is first transcribed from DNA, it contains both coding and noncoding sequences. While the RNA is still in the nucleus, the noncodong **introns** (**in = stay within** the nucleus) are removed from the RNA while the **exons** (**ex = exit the nucleus**) are spliced together to form the complete messenger RNA coding sequence for the protein (Figure 10). This process is called **RNA splicing** and is carried out by specialized enzymes called **spliceosomes**.

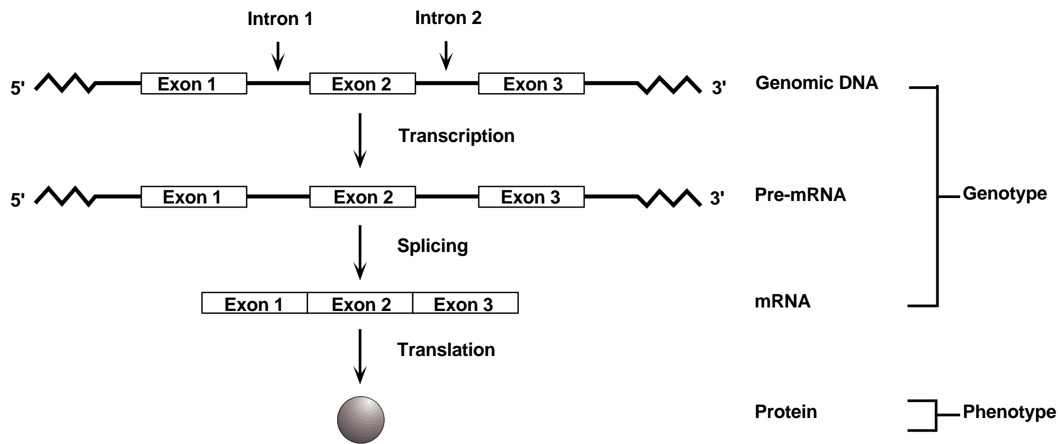


Fig. 10. Splicing of introns from genes.

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 noncoding 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 Target Sequence

The human genome contains small, repetitive DNA elements or sequences that have become randomly inserted into it over millions of years. One such repetitive element is called the "Alu sequence"[7] (Figure 11). This is a DNA sequence about 300 base pairs long that is repeated almost 500,000 times throughout the human genome.[8] The origin and function of these repeated sequences is not yet known.

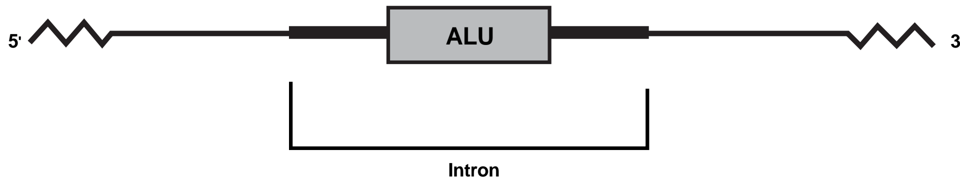


Fig. 11. Location of an Alu repetitive element within an intron.

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 its frequency in the population and as a simple measure of molecular genetic variation — **with no reference to disease or relatedness among individuals**.

In this laboratory activity you will look at an Alu element in the PV92 region of chromosome 16. This particular Alu element is **dimorphic**, meaning that the element is present in some individuals and not others. Some people have the insert in one copy of chromosome 16 (**one allele**), others may have the insert in both copies of chromosome 16 (**two alleles**), while some may not have the insert on either copy of the chromosome (Figure 12). The presence or absence of this insert can be detected using PCR followed by agarose gel electrophoresis.

Since you are amplifying a region of DNA contained within an intron, the region of DNA is never really used in your body. So if you don't have it, don't worry.

The primers in this kit are designed to bracket a sequence within the PV92 region that is 641 base pairs long if the intron does not contain the Alu insertion, or 941 base pairs long if Alu is present. This increase in size is due to the 300 base pair sequence contributed by the Alu insert.

When your PCR products are electrophoresed on an agarose gel, three distinct outcomes are possible. If both chromosomes contain Alu inserts, each amplified PCR product will be 941 base pairs long. On a gel they will migrate at the same speed so there will be one band that corresponds to 941 base pairs. If neither chromosome contains the insert, each amplified PCR product will be 641 base pairs and they will migrate as one band that corresponds to 641 base pairs. If there is an Alu insert on one chromosome but not the other, there will be one PCR product of 641 base pairs and one of 941 base pairs. The gel will reveal two bands for such a sample.

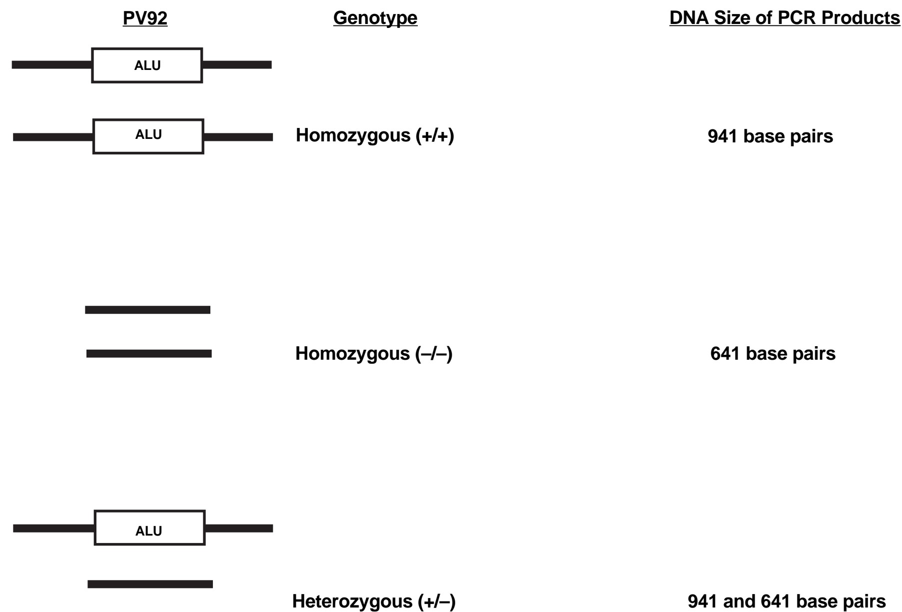


Fig. 12. The presence or absence of the Alu insert within the PV92 region of chromosome 16.

Electrophoresis separates DNA fragments according to their relative sizes. 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. In the sample gel below (Figure 13), PCR-amplified bands of 941 bp and 641 bp are separated based on their sizes.

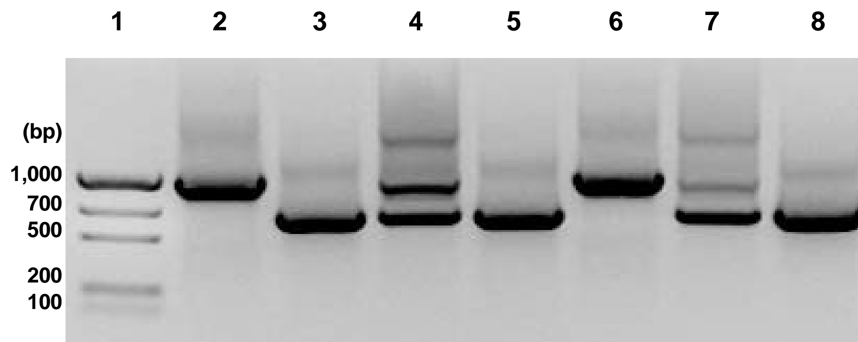


Fig. 13. Electrophoretic separation of DNA bands based on size. EZ Load DNA molecular mass ruler, which contains 1,000 bp, 700 bp, 500 bp, 200 bp, and 100 bp fragments (lane 1); two homozygous (+/+) individuals with 941 bp fragments (lanes 2, 6); three homozygous (–/–) individuals with 641 bp fragments (lanes 3, 5, and 8), and two heterozygous (+/–) individuals with 941 and 641 bp fragments (lanes 4 and 7).

Lesson 3 Gel Electrophoresis of Amplified PCR Samples and Staining of Agarose Gels (Lab Protocol)

Workstation Checklist

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

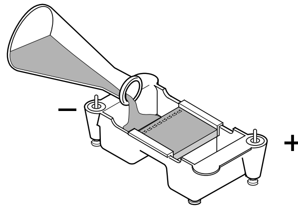
Student workstation	Quantity per Station	(✔)
Agarose gel	1	❒
Student PCR samples	1 per student	❒
MMR (DNA standard)	1 tube	❒
PV92 XC DNA loading dye	1 tube	❒
P-20 micropipet	1	❒
Pipet tips (filter type), 2–20 µl	12	❒
Permanent marker	1	❒
Foam micro test tube holder	1	❒
Gel box and power supply	1	❒
Fast Blast™ DNA stain, 1x or 100x solution	120 ml per 2 stations	❒
Gel support film (optional)	1	❒
Clear acetate sheets for tracing gels (optional)	1	❒
Warm tap water for destaining gels (if performing quick staining protocol)	1.5–2 L per 2 stations	❒
Large containers for destaining (if performing quick staining protocol)	1–3 per 2 stations	❒
Copy of Quick Guide or protocol	1	❒
Waste container	1	❒

Instructor's workstation	Quantity per Class	
1x TAE electrophoresis buffer	275 ml per gel box	❒
Amplified positive control samples (4 each)	12	❒
PV92 homozygous (+/+)		
PV92 homozygous (–/–)		
PV92 heterozygous (+/–)		
Shaking platform (optional)*	1	❒
Microcentrifuge	1	❒
or mini centrifuge	4	❒

*** Strongly recommended.**

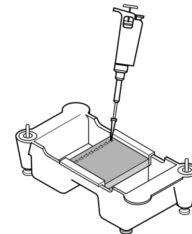
Lesson 3 Gel Electrophoresis of Amplified PCR Samples (Lab Protocol)

1. Remove your PCR samples from the thermal cycler and place in the micro test tube holder. If a centrifuge is available, place the PCR tubes in the capless micro test tubes and pulse-spin the tubes (~3 seconds at 2,000 x g) to bring the condensation that formed on the lids to the bottom of the tubes.
2. Add 10 µl of PV92 XC loading dye to each PCR tube and mix gently.
3. Obtain an agarose gel (either the one you poured or one pre-poured by your teacher). Place the casting tray with the solidified gel in it, onto the platform in the gel box. The wells should be at the cathode (–) end of the box, where the black lead is connected. Very carefully remove the comb from the gel by pulling it straight up, slowly.
4. Pour ~275 ml of electrophoresis buffer into the electrophoresis chamber, until it just covers the wells.

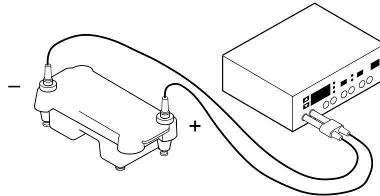


5. Using a clean tip for each sample, load the samples into the 8 wells of the gel in the following order:

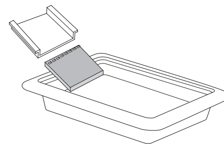
Lane	Sample	Load Volume
1	MMR (DNA standard)	10 µl
2	Homozygous (+/+) control	10 µl
3	Homozygous (–/–) control	10 µl
4	Heterozygous (+/–) control	10 µl
5	Student 1	20 µl
6	Student 2	20 µl
7	Student 3	20 µl
8	Student 4	20 µl



6. 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.
7. Turn on the power supply. Set it to 100 V and electrophorese the samples for 30 minutes.



8. When electrophoresis is complete, turn off the power and remove the lid from the gel box. Carefully remove the gel tray and the gel from the gel box. Be careful, the gel is very slippery. Nudge the gel off the gel tray with your thumb and carefully slide it into your plastic staining tray.



Staining of Agarose Gels

The moment of truth has arrived. What is your genotype? Are you a homozygote or a heterozygote? To find out, you will have to stain your agarose gel. Since DNA is naturally colorless, it is not immediately visible in the gel. Unaided visual examination of gel after electrophoresis indicates only the positions of the loading dyes and not the positions of the DNA fragments. DNA fragments are visualized by staining the gel with a blue dye called Fast Blast DNA stain. The blue dye molecules are positively charged and have a high affinity for the DNA. These blue dye molecules strongly bind to the DNA fragments and allow DNA to become visible. These visible bands of DNA may then be traced, photographed, sketched, or retained as a permanently dried gel for analysis.

Directions for Using Fast Blast DNA Stain

Below are two protocols for using Fast Blast DNA stain in the classroom. Use protocol 1 for quick staining of gels to visualize DNA bands in 12–15 minutes, and protocol 2 for overnight staining. Depending on the amount of time available, your teacher will decide which protocol to use. Two student teams will stain the gels per staining tray (you may want to notch gel corners for identification). Mark staining trays with initials and class period before beginning this activity.

WARNING
Although Fast Blast DNA stain is nontoxic and noncarcinogenic, latex or vinyl gloves should be worn while handling the stain or stained gels to keep hands from becoming stained blue. Lab coats or other protective clothing should be worn to avoid staining clothes.

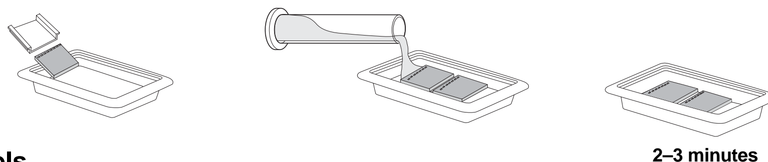
Protocol 1: Quick Staining of Agarose Gels in 100x Fast Blast DNA Stain

This protocol allows quick visualization of DNA bands in agarose gels within 15 minutes. For quick staining, Fast Blast DNA stain (500x) should be diluted to a 100x concentration. We recommend using 120 ml of 100x Fast Blast to stain two 7 x 7 cm or 7 x 10 cm agarose gels in individual staining trays provided in Bio-Rad's education kits. If alternative staining trays are used, add a sufficient volume of staining solution to completely submerge the gels.

Following electrophoresis, agarose gels must be removed from their gel trays before being placed in the staining solution. This is easily accomplished by holding the base of the gel tray in one hand and gently pushing out the gel with the thumb of the other hand. Because the gel is fragile, special attention must be given when handling it. We highly recommend using a large spatula or other supportive surface to transfer the gel from one container to another. Destaining requires the use of at least one large-volume container, capable of holding at least 500 ml, at each student workstation. Each student team may utilize separate washing containers for each wash step, or simply use a single container that is emptied after each wash and refilled for the next wash.

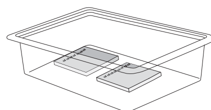
1. **Mark the staining tray with your initials and class period.** You will stain 2 gels per tray.

2. **Stain gels (2–3 minutes)**
Remove each gel from the gel tray and carefully slide it into the staining tray. Pour approximately 120 ml of 100x stain into the staining tray. If necessary, add more 100x stain to completely submerge the gels. Stain the gels for 2–3 minutes, but not for more than 3 minutes. Using a funnel, pour the 100x stain into a storage bottle and save it for future use. **The stain can be reused at least 7 times.**



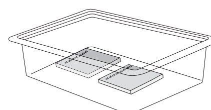
2–3 minutes

3. **Rinse gels**
Transfer the gels into a large container containing 500–700 ml of clean, warm (40–55°C) tap water. Gently shake the gels in the water for ~10 seconds to rinse.



10 seconds

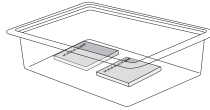
4. **Wash gels**
Transfer the gel into a large container with 500–700 ml of clean, warm tap water. Gently rock or shake the gels on a rocking platform for 5 minutes. If no rocking platform is available, move the gels gently in the water once every minute.



5 minutes

5. Wash gels

Perform a second wash as in step 4.



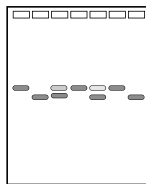
6. Record and analyze results

Examine the stained gels for expected DNA bands. The bands may appear fuzzy immediately after the second wash, but will begin to develop into sharper bands within 5–15 minutes after the second wash. This is due to Fast Blast dye molecules migrating into the gel and binding more tightly to the DNA molecules.

To obtain maximum contrast, additional washes in warm water may be necessary. Destain to the desired level, but do not wash the gels in water overnight. If you cannot complete the destaining in the allocated time, you may transfer the gel to 1x Fast Blast stain for overnight staining. **See Protocol 2.**

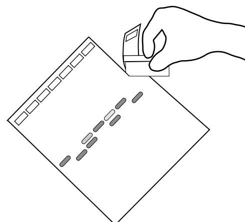
a. Place your gel on a light background and record your results by making a diagram as follows. Place a clear sheet of plastic sheet or acetate over the gel. With a permanent marker, trace the wells and band patterns onto the plastic sheet to make a replica picture of your gel. Remove the plastic sheet for later analysis. Alternatively, gels can be photocopied on a yellow piece of transparent film for optimal contrast.

b. With the help of your instructor, determine whether you are homozygous or heterozygous for the Alu insertion. First look at the control samples and note the migration patterns of the homozygous +/+, the homozygous –/–, and the heterozygous +/– samples (also refer to the example on page 59). You may notice that in the heterozygous sample the smaller 641 base pair band is more intense than the larger 941 bp band. This difference is due to the fact that the smaller fragment is amplified more efficiently than the larger fragment. Copies of the shorter fragment can be made at a faster rate than the bigger fragment, so more copies of the shorter fragment are created per cycle. Refer to pages 69–72 for more information on how to analyze your data.

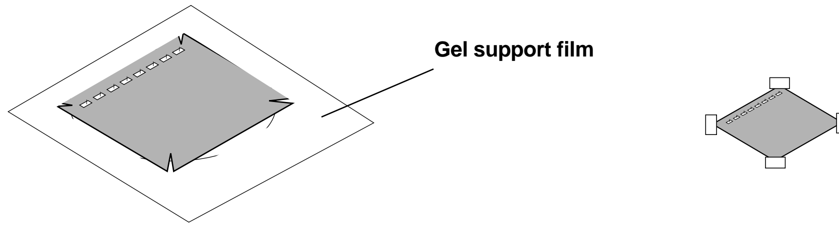


c. Dry the agarose gel as a permanent record of the experiment.

   i. Trim away any unloaded lanes with a knife or razor blade. Cut your gel from top to bottom to remove the lanes that you did not load samples into.



ii. Place the gel directly upon the hydrophilic size of a piece of gel support film. (Water will form beads on the hydrophobic side of a piece of gel support film.) Center the gel on the film and remove bubbles that may form between the gel and film. Place the film on a paper towel and let the gel dry in a well-ventilated area, making sure to avoid direct exposure to light. As the gel dries it will bond to the film but will not shrink. If left undisturbed on the support film, the gel will dry completely at room temperature after 2–3 days. The result will be a flat, transparent, and durable record for the experiment. Tape the dried gel into your laboratory notebook.



Note: Avoid extended exposure of dried gels to direct light to prevent band fading. However DNA bands will reappear if the dried gels are stored in the dark for 2–3 weeks after fading.

Protocol 2: Overnight Staining of Agarose Gels in 1x Fast Blast DNA Stain

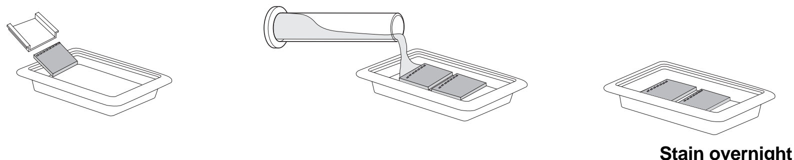
For overnight staining, Fast Blast DNA stain (500x) should be diluted to a 1x concentration. We recommend using 120 ml of 1x Fast Blast to stain two 7 x 7 cm or 7 x 10 cm agarose gels in individual staining trays provided in Bio-Rad's education kits. If alternative staining trays are used, add a sufficient volume of staining solution to completely submerge the gels.

Following DNA electrophoresis, agarose gels must be removed from their gel trays before being placed in the staining solution. This is easily accomplished by holding the base of the gel tray in one hand and gently pushing out the gel with the thumb of the other hand. Because the gel is fragile, special attention must be given when handling it.

1. Mark staining trays with your initials and class period. You will stain two gels per tray.

2. Stain gels (overnight)*

Pour 1x stain into a gel staining tray. Remove each gel from the gel tray and carefully slide it into the staining tray containing the stain. If necessary, add more 1x staining solution to completely submerge the gels. Place the staining tray on a rocking platform and agitate overnight. If no rocking platform is available, agitate the gels staining tray a few times during the staining period. You should begin to see DNA bands after 2 hours, but at least 8 hours of staining is recommended for complete visibility of stained bands.



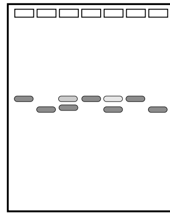
3. Analyze results

No destaining is required after staining with 1x Fast Blast. The gels can be analyzed immediately after staining.

a. Place your gel on a light background and record your results by making a diagram as follows. Place a clear sheet of plastic sheet or acetate over the gel. With a permanent marker, trace the wells and band patterns onto the plastic sheet to make a replica picture of your gel. Remove the plastic sheet for later analysis. Alternatively, gels can be photocopied on a yellow piece of transparent film for optimal contrast.

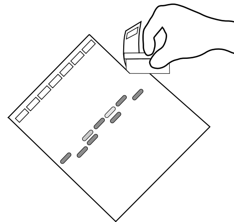
* Shake the gels gently and intermittently during overnight staining in 1x Fast Blast DNA stain; small DNA fragments tend to diffuse without shaking.

b. With the help of your instructor, determine whether you are homozygous or heterozygous for the Alu insertion. First look at the control samples and note the migration patterns of the homozygous +/+, the homozygous –/–, and the heterozygous +/– samples (also refer to the example on page 59). You may notice that in the heterozygous sample the smaller 641 base pair band is more intense than the larger 941 bp band. This difference is due to the fact that the smaller fragment is amplified more efficiently than the larger fragment. Copies of the shorter fragment can be made at a faster rate than the bigger fragment, so more copies of the shorter fragment are created per cycle. Refer to pages 69–72 for more information on how to analyze your data.

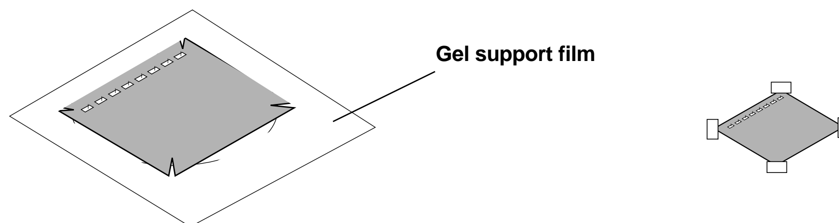


c. Dry the agarose gel as a permanent record of the experiment.

   i. Trim away any unloaded lanes with a knife or razor blade. Cut your gel from top to bottom to remove the lanes that you did not load samples into.



   ii. Place the gel directly upon the hydrophilic size of a piece of gel support film. (Water will form beads on the hydrophobic side of a piece of gel support film.) Center the gel on the film on a paper towel and let the gel dry in a well-ventilated area, making sure to avoid direct exposure to light. As the gel dries it will bond to the film but will not shrink. If left undisturbed on the support film, the gel will dry completely at room temperature after 2–3 days. The result will be a flat, transparent, and durable record for the experiment. Tape the dried gel into your laboratory notebook.



Note: Avoid extended exposure of dried gels to direct light to prevent band fading. However, DNA bands will reappear if the dried gels are stored in the dark for 2–3 weeks after fading.

Lesson 3 Gel Electrophoresis of Amplified PCR Samples

Focus Questions

1. Explain the difference between an intron and an exon.

2. Why do the two possible PCR products differ in size by 300 base pairs?

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

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

Lesson 4 Analysis and Interpretation of Results

If the overnight staining protocol was used to stain the gels, record your results and dry your gels as described earlier.

Analysis

Compare your sample lanes with the control lanes using the DNA size marker as a reference. Mark the location and size of your fragment or fragments. By comparing your DNA migration pattern to the controls, determine whether you are homozygous +/+, homozygous –/–, or heterozygous +/–. If your sample lane is blank, discuss the possible reasons with your classmates and teacher.

Remember that the interpretation of this gel allows you to determine your genetic makeup only at the locus (chromosomal location), being studied. There are three possible genotypes for the Alu insert at the location you have amplified. For a class, determine the number of individuals of each genotype: homozygous +/+, homozygous –/–, and heterozygous +/–. Tally the class results in the table on page 70.

A major factor affecting the reliability of DNA fingerprinting evidence in forensics is population genetics and genetic statistics. In humans, there are hundreds of loci, or DNA segments, like Alu, that can be selected and used for fingerprinting analysis. Depending on demographic factors such as ethnicity and geographic isolation, some populations show much less variation in particular DNA segments than others. A lower degree of variation will increase the odds of more than one individual having the same sequence. If 33% (1 out of three individuals) of a given population has the same fingerprinting pattern for a certain DNA segment, then little information will be obtained from using that segment alone to identify an individual. In such a case, a positive result would only identify a person with 33% accuracy.

In analyzing how incriminating the DNA evidence is, one needs to ask the question: Statistically, how many people in a population have the same DNA pattern as that taken from a crime scene: 1 in 1,000,000? 1 in 10,000? 1 in 10?

For a DNA fingerprint to identify a suspect in a criminal case or a father in a paternity suit, accurate identification required not a 1 out of 3 (1/3) chance of a match in a population, but closer to a 1 in 10 million ($1/10^7$) chance of a match. The frequency of a particular DNA pattern turning up in a population drastically decreases when multiple DNA segments are selected and amplified, rather than just one segment. For DNA fingerprinting to be admissible as evidence in court, it must analyze 30 to 40 different DNA segments on multiple chromosomes from the same person.

The Alu insert you have fingerprinted in this exercise has been used to study the migration patterns of human populations over time.[8] The data from these studies have been published, and your class samples can be compared to the data collected from much larger populations.

Lesson 4 Analysis and Interpretation of Results

Focus Questions

Remember that this Alu sequence is inserted into a noncoding region of the PV92 locus on chromosome 16 and is not related to a particular disease, nor does it code for any protein sequence. It is simply a sequence that can be used to study human genotypic frequencies.

Because Alu repeats appear in the general population at random, the Alu insert in chromosome 16 is very useful for the study of gene frequencies in localized human populations. Theoretically, in some small, geographically isolated populations, all individuals may be homozygous +/+. In others, the individuals may all be homozygous –/–. In a "melting-pot" population, the three genotypes (+/+, +/–, –/–) may exist in equilibrium.

The frequencies of genotypes and **alleles** are basic characteristics that population geneticists use to describe and analyze populations. The results you obtain in this exercise provide a real-life opportunity to calculate genotypic and allelic frequencies of the Alu insert in your class and to use the Hardy-Weinberg equation.

The results of the PCR reactions reveal your and your classmates' genotypes: +/+, +/–, and –/–. Knowing your genotypes, you can count up the alleles of your class "population" and determine their frequencies. You can then compare the allelic and genotypic frequencies of your class population to published reports of larger population sizes.[9]

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

2. What are the genotypic frequencies of +/+, +/–, 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

Allelic frequencies can be calculated from the numbers and frequencies of the genotypes in the population. Population geneticists use the terms p and q to represent the frequencies of the (+) and (–) alleles, respectively. Allele frequencies can be calculated from either the numbers or the frequencies of the genotypes (since they are related to each other).

$$p = \text{frequency of (+) allele} = \frac{\text{number of (+) alleles}}{\text{total number of alleles (both + and –)}}$$

$$= \frac{2(\text{\# of +/+ students}) + 1(\text{\# of +/– students})}{\text{total number of alleles (both + and –)}}$$

$$= \text{frequency of (+/+) students} + \tfrac{1}{2}\,(\text{frequency of (+/–) students})$$

$$q = \text{frequency of (–) allele} = \frac{\text{number of (–) alleles}}{\text{total number of alleles (both + and –)}}$$

$$= \frac{2\,(\text{\# of –/– students}) + 1(\text{\# of +/– students})}{\text{total number of alleles (both + and –)}}$$

$$= \text{frequency of (–/–) students} + \tfrac{1}{2}\,(\text{frequency of (+/–) students})$$

3. What is the frequency of each allele in your class sample? Fill in the table below with your class data. Remember, a class of 32 students (N) will have a total of 64 (2N) instances of each locus.

Table 2. Calculated Allelic Frequencies for the Class

Category	Number	Frequency
(+) alleles		p =
(–) alleles		q =
	Total alleles =	= 1.00

4. The following table presents data from a USA-wide random population study.

Table 3. Genotypic Frequencies for Alu in a USA Sample

Category	Number	Frequency
Homozygous (+/+)	2,422	0.24
Heterozygous (+/–)	5,528	0.55
Homozygous (–/–)	2,050	0.21
	Total = 10,000	= 1.00

Now, using the data above, calculate the allelic frequencies for the USA data as you did for your class population in Table 2.

Table 4. Calculated Allelic Frequencies for USA

Category	Number	Frequency
(+) alleles		p =
(–) alleles		q =
	Total alleles =	= 1.00

5. How do your actual class data for genotypic and allelic frequencies compare with those of the random sampling of the USA population? Would you expect them to match? What reasons can you think of to explain the differences or similarities?

The **Hardy-Weinberg equation**, $\mathbf{p^2 + 2pq + q^2 = 1}$, is one of the foundations of population genetics. It is the algebraic expansion of $\mathbf{(p + q)^2 = 1}$, where $\mathbf{p + q = 1}$. The equation describes the frequencies of genotypes in a population that is at "genetic equilibrium", meaning that the frequencies are stable from generation to generation. The Hardy-Weinberg theory states that, for a population to achieve this equilibrium, the population must be quite large, the members must mate randomly and produce offspring with equal success, and there must be no migration of individuals into or out of the population, or an excessive mutation converting one allele to another. Given these conditions, and the allelic frequencies p and q, the Hardy-Weinberg equation says that

p^2 = the expected frequency of the (+/+) genotype in the population
2pq = the expected frequency of the (+/–) genotype in the population
q^2 = the expected frequency of the (–/–) genotype in the population

It is important to understand that p^2, 2pq, and q^2 are expected, theoretical genotype frequencies of a population under Hardy-Weinberg equilibrium conditions, and they may not be realized in real-life population samples if one of the conditions is not met. These theoretical frequencies are calculated using the observed values for p and q; they may or may not be the same as the observed genotypic frequencies such as those shown in Table 1. If the observed and expected genotypic frequencies are the same, this indicates that the population is in Hardy-Weinberg genetic equilibrium.

6. Using the values for p and q that you calculated in Table 2 for your class population, calculate p^2, 2pq, and q^2. Do they come out to be the same as the genotype frequencies that you found in Table 1? If they do, your class resembles a Hardy-Weinberg genetic equilibrium. If your observed (actual) genotype frequencies are not the same as the expected values, what might be some of the reason(s) for the difference?
7. Using the values for p and q that you calculated in Table 4 for the USA population sample, calculate p^2, 2pq, and q^2. Do they come out to be the same as the genotype frequencies that you found in Table 3? Does this USA-wide sample suggest that the population of the USA is in Hardy-Weinberg equilibrium?

Lesson 5 Analysis of Classroom Data Using Bioinformatics

Bioinformatics is a discipline that integrates mathematical, statistical, and computer tools to collect and process biological data. Bioinformatics has become an important tool in recent years for analyzing the extraordinarily large amount of biological information that is being generated by researchers around the world. In Lesson 5, you will perform a bioinformatics exercise to investigate the genotypic frequencies for the Alu polymorphism in your class population and compare them with the genotypic frequencies of other populations.

Following PCR amplification and electrophoresis of your samples, you will analyze your experimental data to determine your genotypes for the Alu insertion within the PV92 locus on chromosome 16. The classroom genotype data can then be entered into the Allele Server database located at Cold Spring Harbor Laboratory's Dolan DNA Learning Center. Allele Server is a Web-based database that contains genotype data from a range of populations around the world as well as other classrooms and teacher training workshops. It also provides a collection of statistical analysis tools to examine the Alu insertion polymorphism at the population level. You can either analyze your classroom data as an individual population or compare your population with other populations in the database.

Once you enter classroom data into Allele Server, you can perform a Chi-square analysis to compare the Alu genotype frequencies within the class population with those predicted by the Hardy-Weinberg equation. The genotypic frequencies of the class population can also be compared with the genotypic frequencies of another population in the database, using the Chi-square analysis.

Using Allele Server

Note: The Dolan DNA Learning Center web site is continually updated. Some of the following information may change.

1. On your Web browser, go to **vector.cshl.org**
2. Log in to Allele Server using the username and password your instructor provides.
3. Once you have logged in, follow instructions provided in the pop-up window for using Allele Server. You may also open a new window and go to **dnalc.org/help/sad/topic_3.html** to get more detailed instructions. Follow the detailed instructions on how to populate the workspace, analyze groups, compare groups, and query the database.

Remember that as a registered user, you may store any groups that you loaded in your personal Allele Server database and analyze them at your convenience.

Appendix A
Review of Molecular Biology

This section provides an overview and concepts with which students should be familiar in order to get the most out of this lab. Please also refer to the Glossary Section (Appendix B) for definitions of molecular biology terms.

Any living organism functions based on the complicated interactions among nucleic acids, proteins, lipids (fat), and carbohydrates. In nearly all cases, certain proteins, termed enzymes, control the almost infinite number of interactions and life processes in living creatures. Think of enzymes and proteins as all the different people on earth. Each person performs a different role, function, or job on this planet, and although people are not the actual physical make-up of buildings, documents, food, and roads, it is the people that make these buildings and roads, and write the documents, and plant and nurture the crops. In the same way, enzymes and proteins do not comprise bones, lipids, sex hormones, and sugars, but enzymes control these structures, their interactions, and processes.

Because proteins and enzymes ultimately play such a critical role in the life process, scientists have spent many lifetimes studying proteins in an attempt to understand how they work and how they can be controlled. With a complete understanding, we could cure, prevent, and overcome many diseases and physical handicaps as well as explain exactly how and why organisms exist, propagate, and die. However, the complete answers do not lie solely in the knowledge of how enzymes function; we must learn how they are made. Before we can control enzymes, we must understand where they come from and what is the basis of the molecular information that encodes proteins. That answer lies within our **genetic code**.

Each living organism has its own blueprint for life. This blueprint defines how an organism will look and function (using enzymes as a means to form the appearance and control the functions). The blueprint codes for all the different enzymes. With amazing precision, this blueprint gets passed on from generation to generation of each species.

The transfer of this blueprint from generation to generation is called **heredity**. The blueprint for any organism is called its **genome**. The hereditary code is encrypted within the sequence of the DNA molecules that make up the genome. The molecule that constitutes the genome and thus the hereditary code is DNA (**deoxyribonucleic acid**).

The genome consists of very long DNA/protein complexes called **chromosomes**. Prokaryotes, organisms lacking a true nucleus, have only one chromosome. All other species, eukaryotes, have a defined cell nucleus that contains multiple chromosomes. The nucleus is a defined, membrane-enclosed region of the cell that contains the chromosomes. The number of chromosomes varies with the organism — from 2 or 3 in some yeasts to up to 100 or so in some fish. Humans have 46.

In most cases, chromosomes come in nearly identical pairs (one member of the chromosome pair from each parent). In general, the members of a pair differ in small details from each other, since they come from different parents, but are otherwise identical or **homologous**. Cells with homologous pairs of chromosomes are called diploid. Nearly all cells of an organism are diploid. Cells that have only one chromosome of each pair are called haploid. All sperm and ova are haploid.

The process of forming sperm and ova is called **meiosis**. **Meiosis** starts with a diploid cell that divides into two haploid cells. When a sperm fertilizes an ovum, the two nuclei fuse, and thus the new nucleus contains pairs of each chromosome, one partner from each parent. The result is called a diploid zygote.

All cells of diploid organisms duplicate chromosomal pairs when they divide (except when sperm and ova are formed), so that all body cells (called somatic cells) of an organism are diploid. The process of cell division in which the chromosomes are duplicated and each daughter cell gets pairs of chromosomes is called **mitosis**. It is through the processes of mitosis and meiosis that the hereditary code is passed from cell to cell and generation to generation. Now that we know where the code is and how that code is passed on, we need to know how the code produces the enzymes that control life. The actual DNA code for a protein is contained within a segment of a chromosome called a gene. In nearly all cases, diploid organisms will have the same gene on a specific chromosome pair. Each gene on a particular chromosome of a specific chromosome pair is also called an **allele**.

To clarify, a gene encodes a particular protein that performs a particular function. An allele is a specific version of a gene on a particular chromosome. Thus, there are genes for hair color and there is an allele for the hair color gene on each chromosome pair. The gene or allele's DNA code can also be called the genotype.

When the protein is made from this code and performs its function, the physical trait or result that is seen is called the **phenotype**. In many cases the two alleles on the specific chromosome pair coding for a protein differ slightly in their respective DNA code (genotype). Any slight difference in code between the two alleles can result in two different proteins, which, although intended to perform basically the same function, may carry out that function slightly differently, causing different results and thus different phenotypes.

Therefore, it is not only the various combinations of chromosomes a parent contributes to each offspring, but also the various combinations of alleles and how each of the enzymes coded from the alleles work together that decide how we look and allow us to function. The various combinations are nearly infinite and that is why we are all different. The study of genotypes and phenotypes is often referred to as **Mendelian genetics** (after Mendel, the individual who pioneered the study of heredity and genetics).

DNA: What Is It?

A DNA molecule is a long polymer consisting of four different components called **bases**. The four bases are also called **nucleotides**. It is the various combinations of these four bases or nucleotides that create a unique DNA code or sequence (also genotype, gene, and allele). Nucleotides are comprised of three different components:

- **Nitrogen base**
- **Deoxyribose sugar**
- **Phosphate group**

Each nucleotide contains the same ribose sugar and the phosphate group. What makes each nucleotide unique is its nitrogen base. There are four nitrogen bases:

Adenine (A)

Thymine (T)

Guanine (G)

Cytosine (C)

A DNA nucleotide chain is created by the connection of the phosphate group to the ribose sugar of the next nucleotide. This connection creates the “backbone” of the DNA molecule.

To designate the different ends of this single-stranded chain, we use some typical biochemistry terminology, in which the carbons on any sugar are numbered. The sugar of a nucleotide contains 5 carbons. The phosphate group (PO_4) of a given nucleotide is connected to the 5' carbon of the sugar. A hydroxyl group (OH) is attached to the 3' carbon of the sugar, and this 3' OH group connects to the phosphate group of the next nucleotide in the chain.

Thus, the end of a single-strand DNA molecule that has a free phosphate group (i.e., not attached to another nucleotide) is called the 5' end, and the end of the DNA molecule (with no subsequent nucleotide attached) is called the 3' end (see Figures 14 and 15).

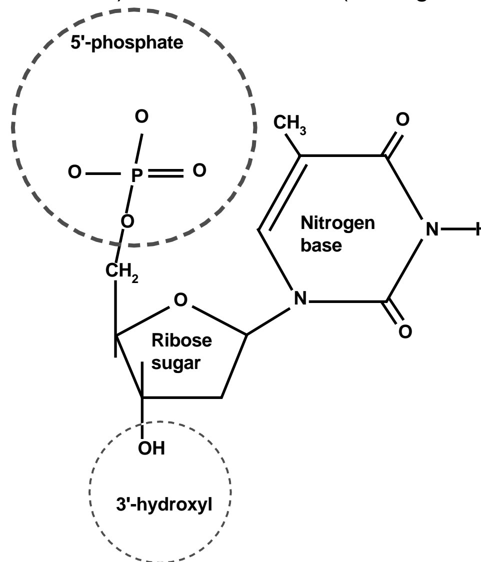


Fig. 14. Structure of one nucleotide of deoxyribonucleic acid.

It has become standard that a single-stranded DNA molecule is written with the 5' end on the left and the 3' end on the right. Therefore, a single-stranded DNA chain’s sequence is represented from left to right, starting on the left with the 5' nucleotide and moving to the right until the 3' nucleotide is last. Most DNA sequences are read 5' to 3'.

However, the long DNA molecules or chains that comprise the chromosomes are not single-stranded molecules. From X-ray crystallography patterns of DNA, and some imaginative molecular model building, Watson and Crick deduced that DNA is in fact a **double-stranded** molecule with the two single strands of DNA held together by **hydrogen bonds** between the nitrogen bases (A, T, G, and C). This double-stranded molecule is often

called a duplex (Figure 15). There are several important properties of double-stranded DNA molecules.

- Chromosomal (also called genomic) DNA is double-stranded.
- The overall structure is that of a helix with two strands intertwined.
- The structure can be viewed as a twisted ladder.
- The phosphate-deoxyribose backbones are the sides of the ladder.
- The nitrogen bases (A, T, G, and C) hydrogen bonded to each other are the rungs.
- Only the nitrogen bases A and T and C and G can form hydrogen bonds to each other. When A binds to T or C binds to G this is considered **base pairing**. Neither C and T, nor A and G form hydrogen bonds.
- The two strands are antiparallel; that is, the strands are oriented in opposite directions. This means that the ladder runs 5' to 3' in one direction for one strand and 5' to 3' in the opposite direction for the other strand.

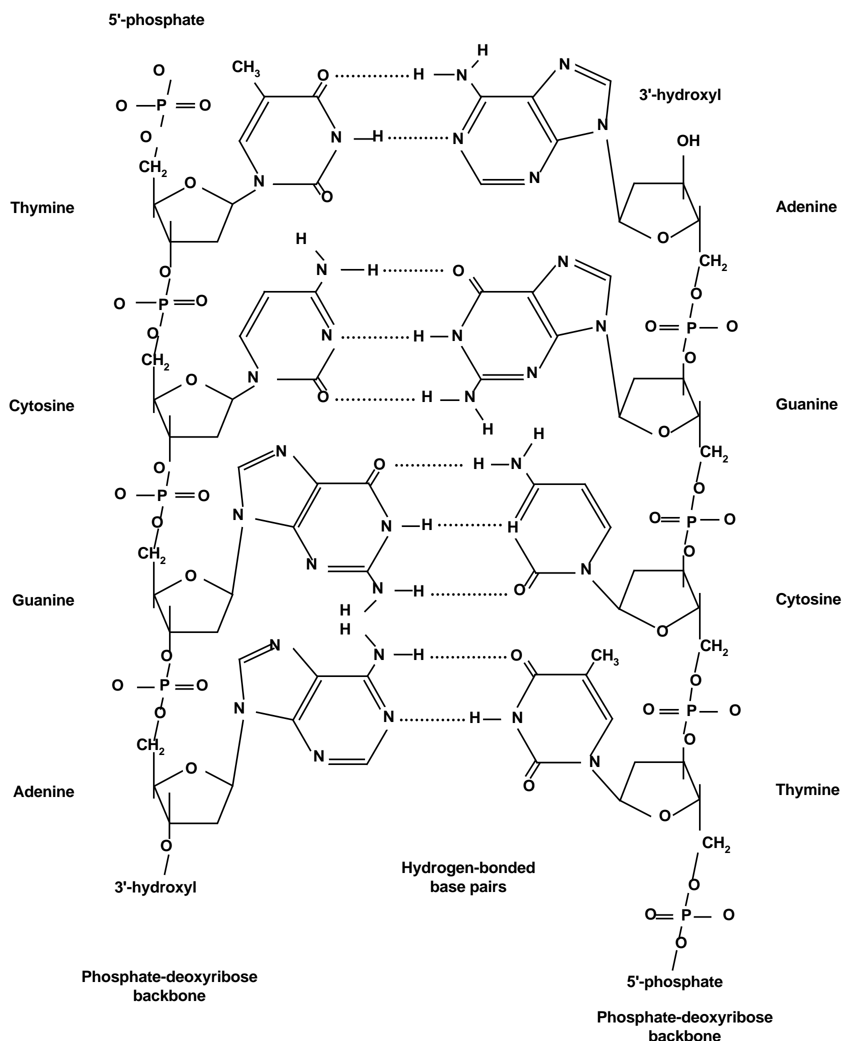


Fig. 15. Molecular structure of a portion of a double-stranded DNA molecule.

DNA Structure Conclusions

- Because A only binds to T, and G only binds to C, the two strands will have exactly the opposite, or complementary, sequence running in opposite directions (one strand 5' to 3', the other 3' to 5').
- These two complementary strands anneal or hybridize to each other through hydrogen bonds between the bases.
- A new strand of DNA can be synthesized using its complementary strand as the template for new synthesis.
- Each strand carries the potential to deliver and code for information.

The length of any double-stranded DNA molecule is given in terms of base pairs (bp). If a DNA strand contains over a thousand base pairs, the unit of measure is kilobases (1 kb = 1,000 bp). If there are over one million base pairs in a strand the unit of measure is megabases (1 Mb = 1,000 kb).

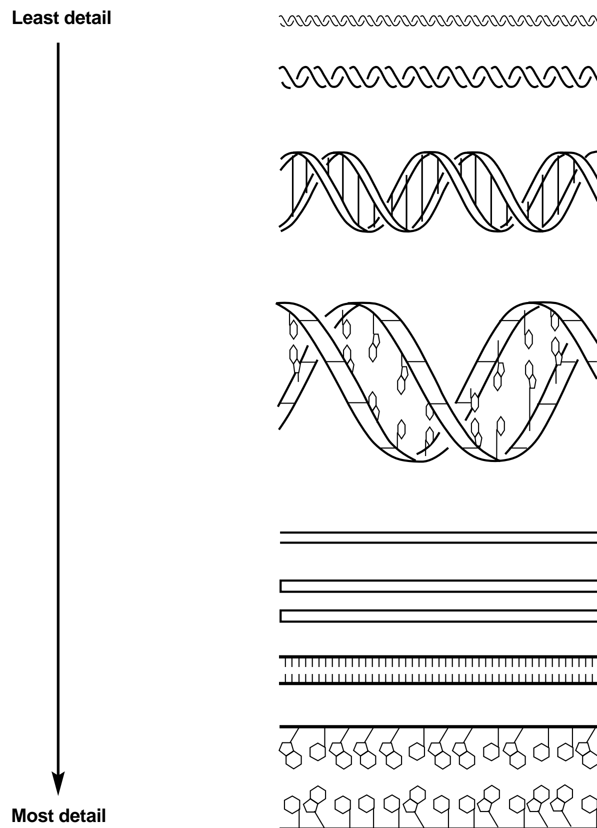


Fig. 16. DNA (deoxyribonucleic acid) — A long chainlike molecule that stores genetic information. DNA is graphically represented in a number of different ways, depending on the amount of detail desired.

DNA Replication — Strand Synthesis

New strands are synthesized by enzymes called **DNA polymerases**. New strands are always synthesized in the 5' to 3' direction. For a new single strand of DNA to be synthesized, another single strand is necessary. The single strand of DNA that will be used to synthesize its complementary strand is called the **template strand**.

However, in order for DNA polymerase to start synthesizing a new complementary strand, a short stretch of nucleotides (approximately 20 base pairs long) called an oligonucleotide primer must be present for the polymerase to start synthesis. This primer is a short stand of nucleotides complementary to the template where the researcher wants synthesis to begin. The primer must have a free 3' hydroxyl group (OH) for DNA polymerase to attach the 5' phosphate group of the next nucleotide.

The DNA polymerase grabs free (single) nucleotides from the surrounding environment and joins the 5' phosphate of the new nucleotide to the 3' hydroxyl group (OH) of the new complementary strand. This 5' to 3' joining process creates the backbone of the new DNA strand.

The newly synthesized strand maintains its complementarity with the template strand because the DNA polymerase only joins two nucleotides during new strand synthesis if the new nucleotide has its complement on the template strand. For example, the DNA polymerase will only join a G to the 3' end of the newly synthesized strand if there is the C counterpart on the template strand to form a hydrogen bond. Guanine will not be joined to the new strand if adenine, thymine, or guanine is the opposite nucleotide on the template strand.

DNA polymerase and strand synthesis allow DNA to replicate during mitosis. Both new DNA strands are synthesized simultaneously from the two original DNA template strands during mitotic DNA replication.

As you can see, DNA, RNA, and proteins are closely tied to each other. Thus, you can realize why researchers today, in an attempt to understand the mechanisms behind the various life processes, must study the nucleic acids as well as the proteins to get complete answers about the flow of information carried in the genetic code. In the last 20 years, many gains in the areas of nucleic acid techniques have finally allowed researchers the means to study the roles of nucleic acids in life processes.

Individual discoveries by many scientists have contributed the pieces that have begun to solve one of the most mysterious puzzles of life — understanding the hereditary code. In 1985, enough pieces of the puzzle were in place for a major breakthrough to occur. This understanding of how the necessary molecular components interact to faithfully replicate DNA within living cells led to the development of a technique for creating DNA in a test tube. This technique is called the **polymerase chain reaction**, or **PCR**.

Appendix B
Glossary of Terms

Aliquot — The division of a quantity of material into smaller, equal parts

Allele — A variation of a gene on a particular chromosome

Alu — A small piece of repetitive DNA that contains the *Alu*I restriction enzyme site, from which the sequence obtained its name

Annealing — Binding of oligonucleotide primers to complementary sequences on the template DNA strands

Biotechnology — The manipulation of organisms (microbes, plants or animals) DNA to help solve human problems

Chelate — To bind metal ions in solution. An example of a common chelating agent is EDTA or ethylenediamine tetraacetic acid

Cofactors — Ions or small molecules needed by an enzyme to function properly. For example, *Taq* DNA polymerase needs Mg^{2+} in order to function properly. Mg^{2+} would therefore be considered a cofactor

Denature — The process of melting apart two complementary DNA strands. In vivo denaturation is accomplished by enzymes; in the PCR reaction denaturation is accomplished by heat

DNases — Digestive enzymes that degrade DNA

dNTPs — Commonly used abbreviation for all four deoxynucleotide triphosphates (dATP, dTTP, dGTP, dCTP) used in synthesizing DNA

Ethidium bromide — A fluorescent dye molecule that intercalates between DNA base pairs and fluoresces when exposed to ultraviolet light

Eukaryotes — Organisms that are made up of cells containing a membrane-bound nucleus that contains the genetic material (DNA)

Exon — The region of a transcribed messenger RNA molecule that gets spliced together and leaves the nucleus for translation into protein sequence

Extension — This refers to the process of *Taq* polymerase adding dNTPs (deoxynucleotide triphosphates — dATP, dTTP, dCTP, or dGTP) onto the ends of the oligonucleotide primers. Extension follows the base pairing rule and proceeds in the 5' to 3' direction

Genome — The sequence of DNA molecules within the nucleus that codes for all proteins for a given species. Each segment of DNA that encodes a given protein is called a gene. The information contained in the genome constitutes the organism’s hereditary code

Genomic DNA — The sum total of the DNA that is found within the nucleus of a cell

Genotype — The combination of alleles carried by an individual

Hardy-Weinberg equilibrium	The conditions that enable a population to maintain its genetic frequencies; These conditions are: large population, random mating, no immigration or emigration, no mutations, and no natural selection
Homologous chromosomes	A pair of complementary chromosomes that contain the same genetic sequences, or genes, with one chromosome inherited from the mother and one chromosome inherited from the father
InstaGene matrix	Microscopic beads that bind divalent cations in solution; The binding or sequestering of divalent cations prevents their availability to enzymes that can degrade the DNA template
Intron	The region of a transcribed messenger RNA that is spliced out of the mRNA and is not translated into protein sequence
Lysis	The process of rupturing a cell to release its constituents. In this exercise, human cheek cells are lysed to release genomic DNA for PCR reactions
Master mix	The main solution of a PCR reaction which contains all of the necessary components (dNTPs, primer, buffer, salts, polymerase, magnesium) of the reaction except the template DNA
Messenger RNA	A type of RNA that is synthesized from the genetic material (DNA) and that attaches to ribosomes and is translated into protein
Molecular biology	The study of genes and the molecular details that regulate the flow of genetic information from DNA to RNA to proteins, and from generation to generation
Nucleotides	The fundamental unit of DNA or RNA; they consist of a sugar (deoxyribose or ribose), phosphate, and nitrogenous base (adenine, thymine, cytosine, or guanine, with uracil in place of thymine in RNA)
PCR	Polymerase chain reaction. The process of amplifying or synthesizing DNA within a test tube
Primer	A short sequence of nucleotides (usually 16–24 bases in length) that recognizes a particular sequence of nucleotides on the target DNA sequence; primers for the polymerase chain reaction are usually synthesized in a laboratory
Reagents	Materials needed to conduct an experiment; they are usually solutions or mixtures of various solutions
***Taq* DNA polymerase**	Thermostable DNA polymerase that was isolated from the thermophilic bacterium *Thermus aquaticus*. This DNA polymerase is commonly used in PCR reactions
Template	The strand of DNA that contains the target sequences of the oligonucleotide primers and that will be copied into its complementary strand

Appendix C
PCR Amplification and Sterile Technique

PCR is a powerful and sensitive technique that enables researchers to produce large quantities of DNA from very small amounts of starting material. Because of this sensitivity, contamination of PCR reactions with unwanted, extraneous DNA is always a possibility. Therefore, utmost care must be taken to prevent cross-contamination of samples. Steps to be taken to prevent contamination and failed experiments include:

1. **Filter-type pipet tips.** The end of the barrel of micropipets can easily become contaminated with DNA molecules that are aerosolized. Pipet tips that contain a filter at the end can prevent aerosol contamination from micropipets. DNA molecules within the micropipet can not pass through the filter and can not contaminate PCR reactions. Xcluda aerosol barrier pipet tips (catalog #211-2006EDU and 211-2016EDU) are ideal pipet tips to use in PCR reactions.
2. **Aliquot reagents.** Sharing of reagents and multiple pipettings into the same reagent tube will most likely introduce contaminants into your PCR reactions. When at all possible, aliquot reagents into small portions for each team, or if possible, for each student. If an aliquotted reagent tube does become contaminated, then only a minimal number of PCR reactions will become contaminated and fail.
3. **Change pipet tips.** Always change pipet tips when pipeting from a reagent for the first time. If a pipet tip is used repeatedly, contaminating DNA molecules on the tip will be passed into other solutions, resulting in contaminated PCR reactions. If you are at all unsure whether your pipet tip is clean, use the safe rule of thumb and discard the tip and get a new one. The price of a few extra tips is a lot smaller than the price of failed reactions.
4. **Use good sterile technique.** When opening, aliquotting, or pipetting reagents, leave the tube open for as little time as possible. Tubes that are open and exposed to the air can easily become contaminated by DNA molecules that are aerosolized or are present from your mouth, breath, etc. Pipet from reagent tubes efficiently, and close them when you are finished pipetting. Also, try not to pick tubes up by the rim or cap as you can easily introduce contaminating DNA molecules from your fingertips.

Appendix D
Teacher Answer Guide

Lesson 1 DNA Template Preparation

1. Why is it necessary to chelate the metal ions out of solution during the boiling/lysis step at 100°C? What would happen if you did not put in the InstaGene™ matrix?

   Metal ions, such as Mg^{2+}, act as cofactors for DNases which can degrade DNA. The InstaGene matrix removes the metal ions to inactivate the DNases and which keep the DNA template intact.

   If the InstaGene matrix was not present, DNases would remain active, and would degrade the DNA template, resulting in no PCR amplification.

2. What is needed from the cells to conduct the polymerase chain reaction (PCR)?

   The genomic DNA released from the nuclei of the cells is needed for the PCR reaction.

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

   The cell and nuclear membranes must be broken to release the template DNA into the solution.

4. After boiling the samples, why do you think the DNA is stored in the InstaGene matrix?

   If any residual DNases remain in solution, then keeping the DNA stored in the InstaGene matrix will keep the necessary metal cofactors (Mg^{2+}) bound, thus keeping the DNases inactive. Without the InstaGene matrix present, these residual DNases could degrade the DNA, resulting in loss of template DNA for the PCR reactions.

Lesson 2 PCR Amplification

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

   Two primers are needed so that both sides of the double helix are used as a template. The 5' to 3' primer allows the sense strand to be replicated, whereas the 3' to 5' primer allows the antisense strand to be replicated.

2. How did *Taq* DNA polymerase acquire its name?

   ***Taq* DNA polymerase was isolated from a thermophilic bacteriaum, *Thermus aquaticus*, which lives in steam vents. *Taq* DNA polymerase has been a valuable PCR tool because the enzyme can withstand the high temperature extremes of the denaturation cycle of PCR.**

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

   The four dNTPs are the nucleotides that are incorporated by *Taq* DNA polymerase into the complementary DNA strands.

   The other components are oligonucleotide primers, *Taq* DNA polymerase, salt buffer, and magnesium ions. The primers bind to the template strands and act as starting points of replication for *Taq* DNA polymerase. *Taq* DNA polymerase incorporates dNTPs into the complementary DNA strand. The salt buffer is required to provide the necessary concentration of salt ions and pH to provide optimal conditions for *Taq* DNA polymerase. Magnesium is a necessary cofactor for *Taq* DNA polymerase.

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

   The three main steps of PCR amplification are denaturation, annealing, and extension. During denaturation, the two strands of double stranded DNA are melted out to create two single-stranded templates. During annealing, the oligonucleotide primers find their complementary sequences on the template strands and anneal to the DNA template. During extension, *Taq* DNA polymerase incorporates dNTPs and extends the primers, resulting in the production of the complementary DNA strand.

5. Explain why the precise-length target DNA sequence doesn't get amplified until the third cycle. You may need to use additional paper and a drawing to explain your answer.

   In the first polymerization step, a new DNA strand is synthesized onto each primer. *Taq* DNA polymerase extends this new strand well beyond the target sequence, producing a new DNA strand that contains the downstream-upstream primers and is longer than the target.

   In the second cycle, the DNA is denatured and primers anneal to the original target DNA and to the new DNA strands produced in the first cycle. A mixture of small target sequences and larger hybrid DNA sequences are produced at the end of the second cycle.

   In the third cycle, the target DNA of defined length is reprimed with both upstream and downstream primers. *Taq* DNA polymerase then synthesizes two copies of target DNA of defined length. The number of these defined target sequences is doubled with each subsequent round of PCR, which thereby increase logarithmically. Thus, a 40 cycle PCR would amplify a given template 2^{40}, potentially producing 1.0×10^{12} DNA molecules.

Lesson 3 Gel Electrophoresis

1. Explain the difference between an intron and an exon.

   Introns, or intervening sequences, do not code for protein sequences and are spliced out of mRNA molecules before the mRNA leaves the nucleus. Exons code for the protein sequence and remain in the mRNA, are transported out of the nucleus, where they are finally translated into protein on ribosomes.

2. Why do the two possible PCR amplification fragments differ in size by 300 base pairs?

   The PCR primers amplify a 641 bp fragment within the PV92 region. Certain individuals contain a 300 bp Alu repeat within this region of chromosome 16, and amplification from these individuals produces a 941 bp fragment. Thus, the 300 bp difference in size is due to an insertion of a 300 bp Alu repeat.

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

   PCR fragments are separated in an electrophoretic field because DNA is a negatively charged molecule which moves when an electric field is applied to it. Since DNA is negatively charged it migrates toward the positive (red) electrode.

   The agarose acts as a sieve to separate the charged DNA molecules according to size. Large molecules of DNA move slowly through the agarose, while smaller molecules of DNA are less obstructed and move faster through the matrix of agarose.

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

   The controls that are run in this experiment are the homozygous +/+, homozygous –/–, and heterozygous +/– known samples. These bands have known base-pair lengths and can be used in comparison to unknown student samples.

Lesson 4 Analysis and Interpretation of Results

Focus Questions

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

   If there are two bands that match the +/– control the person is heterozygous +/–. If there is one band that matches the –/– control, then the person is homozygous –/–, and if there is one band that matches the +/+ control then the person is homozygous +/+.

2. What are the genotypic frequencies of +/+, +/–, and –/– in your class population? Fill in the table below with your class data.

Assuming a class size of 32 students, data may look something like this.

Table 1. Observed Genotypic Frequencies of the Class

Category	Number	Frequency (# of genotypes/Total)
Homozygous (+/+)	8	0.25
Heterozygous (+/–)	8	0.25
Homozygous (–/–)	16	0.50
	Total = 32	= 1.00

The frequencies are calculated by dividing the number of each genotype by the total number of samples taken.

Homozygous (+/+) = 8/32 = 0.25
Heterozygous (+/–) = 8/32 = 0.25
Homozygous (–/–) = 16/32 = 0.50

3. What is the frequency of each allele in your class sample? Fill in the table below with your class data. Remember, a class of 32 students (N) will have a total of 2(N) = 64 alleles.

Table 2. Calculated Allelic Frequencies for the Class

Category	Number	Frequency
(+) alleles	24	p = 0.375
(–) alleles	40	q = 0.625
	Total alleles = 64	= 1.00

4. The following table presents data from a USA-wide random population study.

Table 3. Genotypic Frequencies for Alu in a USA Sample

Category	Number	Frequency
Homozygous (+/+)	2,422	0.24
Heterozygous (+/–)	5,528	0.55
Homozygous (–/–)	2,050	0.21
	Total = 10,000	= 1.00

Now, using the data above, calculate the allelic frequencies for the USA data as you did for your class population in Table 2.

Table 4. Calculated Allelic Frequencies for the USA

Category	Number	Frequency
(+) alleles	10,372	p = 0.52
(–) alleles	9,628	q = 0.48
	Total alleles = 20,000	= 1.00

5. How do your actual class data for genotypic and allelic frequencies compare with those of the random sampling of the USA population? Would you expect them to match? What reasons can you think of to explain the differences or similarities?

   The student population in all probability will not match the data for the USA population. The data for the USA population come from a much larger sample size and give a better representation of frequencies expected for a large heterogeneous population. Because your class data represent a much smaller sample size, the allelic frequencies in all likelihood will be different. See Figure 17 for typical classroom results.

6. Using the values for p and q thqat you calculated in Table 2 for your class population, calculate p^2, 2pq, and q^2. Do they come out to be the same as the genotype frequencies that you found in Table 1? If they do, your class resembles a population in Hardy-Weinberg genetic equilibrium. If your observed (actual) genotype frequencies are not the same as the expected values, what might be the cause(s) of the discrepancy?

   It is most likely that the class population values for p^2, 2pq, and q^2 will not match the observed genotypic frequencies in Table 1, because a small class does not met the conditions for Hardy-Weinberg equilibration. A class too small a "population" to accurately represent the equilibrium that would occur with a larger population with the same values for p and q. Also, a class does not represent a population in which "random mating producing offspring with equal success" has been occurring for generations.

7. Using the values for p and q that you calculated in Table 4 for the USA population sample, calculate p^2, 2pq, and q^2. Do they come out to be the same as the genotype frequencies that you found in Table 3? Does this USA-wide sample suggest that the population of the USA is in Hardy-Weinberg equilibrium?

   In the USA population sample, p^2, 2pq, and q^2 are quite close to the observed genotypic frequencies. This sample, which is large enough to be fairly representative of the USA population at large, suggests that the USA population is in a state resembling genetic equilibrium. However, when you consider that Hardy-Weinberg equilibrium requires "random mating producing offspring with equal success" and "no migration of the individuals into or out of the population", it is apparent the population of the USA does not meet the conditions of Hardy-Weinberg equilibration. Keep in mind that the Hardy-Weinberg theory describes a theoretical, idealized situation which is never perfectly met in natural populations, but it is a useful way to evaulate and predict the genetic stability of sample natural populations.

Appendix E
Typical Classroom Results

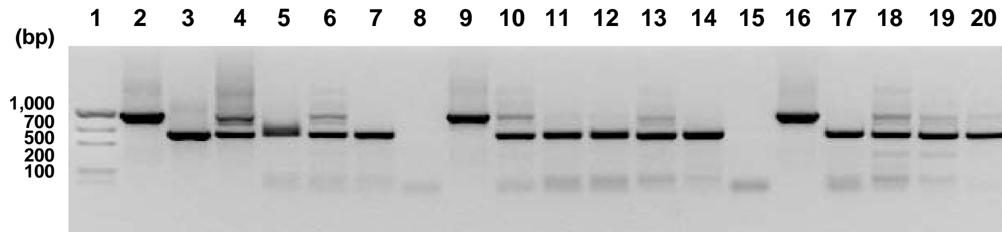


Fig. 17. Expected kit results. This gel shows typical results obtained from the PV92 PCR kit. PCR reactions were performed on the three positive controls (lanes 2, 3, and 4), and sixteen student samples (lanes 5–20). The DNA EZ Load molecular mass ruler (1,000 bp, 700 bp, 500 bp, 200 bp, and 100 bp fragments) is shown in lane 1. Note that it is typical to have some student samples that do not amplify (lanes 8 and 15; see possible explanations on page 30). Note that the amplification of heterozygous samples often results in a much more intense 641 bp fragment, and a much lighter 941 bp fragment (lanes 6, 10, 13, 18, 19, and 20; see interpretation notes on page 31).

Appendix F
References

1. Practice of the polymerase chain reaction (PCR) process requires a license. The Gene Cycler and MyCycler thermal cycler are Authorized Thermal Cyclers may be used with PCR licenses available from Applied Biosystems. Their use with Authorized Reagents also provides a limited PCR license in accordance with the label rights accompanying such reagents. Some applications may also require licenses from other third parties.
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Appendix G
Gel Loading Template

Use the template to enter students' names into the appropriate gel and well numbers.

	Controls			Student samples			
MMR	+/+	–/–	+/–	S1	S2	S3	S4
1	2	3	4	5	6	7	8

MMR = molecular mass ruler (DNA standard)

Appendix H
Programming Instructions for Thermal Cyclers

Abbreviated instructions for programming your Gene Cycler or MyCycler for the proper amplification cycles and temperatures used in this lab are provided below. Refer to the Gene Cycler or MyCycler instruction manual for more detailed instructions and troubleshooting.

Gene Cycler Thermal Cycler

Turn the machine on

Select “program”, then “enter”

Select “new”, then “enter”

Select “001” (or desired program number), then “enter”

Program Cycle 1: Pre-Denaturation

Select cycle repeats “001”, then “enter”

Select “edit” for step 1, then “enter”

Select “94 degrees, 2 min”, then enter

Skip step 2 by selecting the up arrow

Skip step 3 by selecting the up arrow

Skip step 4 by selecting the up arrow

Skip step 5 by selecting the up arrow

Select “no” for auto inc/dec

Select “yes” for the next cycle

Program Cycle 2: 40 PCR Cycles

Select cycle repeats “040”, then “enter”

Select “edit” for step 1, then “enter”

Select “94 degrees, 1 min”, then “enter”

Select “edit” for step 2, then “enter”

Select “60 degrees, 1 min”, then “enter”

Select “edit” for step 3, then “enter”

Select “72 degrees, 2 min”, then “enter”

Skip step 4 by selecting the up arrow

Skip step 5 by selecting the up arrow

Select “no” for auto inc/dec

Select “yes” for the next cycle

Program Cycle 3: Final Extension

Select cycle repeats “001”, then “enter”
Select “edit” for step 1, then “enter”
Select “72 degrees, 10 min”, then “enter”
Skip step 2 by selecting the up arrow
Skip step 3 by selecting the up arrow
Skip step 4 by selecting the up arrow
Skip step 5 by selecting the up arrow
Select “no” for auto inc/dec
Select “no” for the next cycle
Select “yes” for return to menu

To Ensure That You Programmed Your Gene Cycler Correctly

Select “program”, then “enter”
Select “edit”, then “enter”
Select “001” (or desired program number), then “enter”
Select enter three times to get through cycle 40
Scroll through steps in cycle 1 with up arrows
Select “no” on auto inc/dec, then “enter”
On next cycle, select “yes”, then “enter”
Select enter three times to get through cycle 001
Scroll through steps in cycle 2 with up arrows
Select “no” on auto inc/dec, then “enter”
On next cycle, select “no”, then “enter”
Select “yes” on return to menu

You Are Now Ready to Run Program 001

Select “run”, then “enter”
Select “program”, then “enter”
Select “001”, then “enter”
Select “040 µl”, then “enter”

The program will now flash “run”. You should monitor the first several cycles to ensure that the Gene Cycler is running the program according to your specifications.

MyCycler Thermal Cycler

Select “Standby” to turn the machine on
Select “Create”
Scroll down to “Standard-3”
Press “Enter”

Program the Initial Denaturation

Enter 94.0
Press the down arrow
Enter 2.00
Press the down arrow
Enter 1.00
Press the right arrow
Press the up arrow

Program the 40 PCR cycles

Enter 94.0
Press the down arrow
Enter 1.00
Press the right arrow
Press the up arrow
Enter 60.0
Press the down arrow
Enter 1.00
Press the right arrow
Press the up arrow
Enter 72.0
Press the down arrow
Enter 2.00
Press the down arrow
Enter 40X cycles
Press Enter

Program the Final Extension

Press the right arrow
Enter 72.0
Press the down arrow
Enter 10.00
Press the down arrow
Enter 1X cycle
Press the right arrow

Program the Final Chill Hold

Enter 1X cycle
Press “Done”

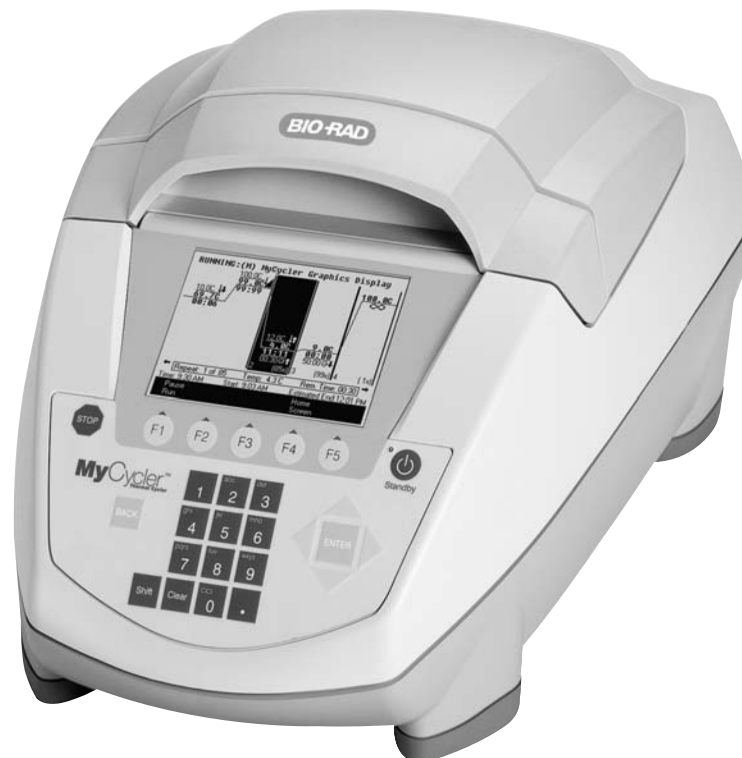
Save the Protocol

Press “Save Protocol As”
Press “Enter”
Enter PV92 using the alphanumeric keypad
Press “Enter”

Run the PV92 Program

Select “Protocol Library”
Select “PV92”
Press “Enter”
Press “Enter” to run protocol
Enter “Algorithmic Measurement”
Enter 40 µl volume
Select “No Hot Start”
Select “Begin Run”

The MyCycler should now begin running



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