Chapter 1

Use PCR and a Single Hair to Produce a "DNA Fingerprint"

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Contents

Introduction 2
Student Time Table 3
Materials 3
  Student Equipment 3
  Student Consumable Supplies 3
Notes for the Instructor 4
  Overview 4
  PCR 4
  VNTR 5
  Electrophoresis 6
  Safety Precautions 6
  Teaching Applications 7
  Overview of Protocols 7
  Detailed Protocols for Two Loci 10
Student Handouts 13
  First Lab Meeting 14
  Second Lab Meeting 16
Acknowledgements 16
Literature Cited 16
  Appendices
  A.Recipes for Reagents and Proper Storage Conditions 18
  B.Sources for Reagents and Equipment 19
  C.Suggested Retailers for Major Equipment 20
  D.Potential Problems and Trouble Shooting 21
  E.Pouring and Running an Aragose Gel 23
  E.Photographing Gels 25
  F.Calculating Molecular Weights of DNA Brands 26
  H.Photographs of Gels Obtained from Students Labs 29
  I.Molecular Weight Markers 30
  J.Checklist for Equipment and Reagents 31

Introduction

As biology teachers, we are aware of the ever growing amount of information and new methods that we would like to incorporate into our classes. One area of particular growth has been in the field of DNA manipulations, or molecular biology. All of us want to share with our students these new and exciting techniques that are driving the revolution in biotechnology and altering our understanding of population genetics, DNA structure, replication, and mutations.

We have developed a laboratory procedure that draws upon all of these topics and is appropriate for introductory level college biology courses (for majors and/or non-majors). For upper level students, we have had them design potential modifications to improve the protocol. Using the protocol described in this article, in two lab periods of two hours each, every student extracts his or her own DNA from a single hair follicle, uses polymerase chain reaction (PCR[1]) to amplify a polymorphic locus, electrophoreses the PCR products on an agarose gel, and visualizes the alleles to generate a "DNA fingerprint". These single-locus fingerprints can be used to generate population genetics data or to solve a fabricated crime. Setup time for the instructor will vary due to available equipment but for six sections of 32 students each, it takes us from 8 to 10 hours to pour all the gels, make all the stock solutions, and aliquot/distribute all reagents.

Student Time Table

Day #1

pluck hairs (5 - 10 minutes)
add hair to DNA extraction buffer (5 minutes)
incubate hair at 55° C (1 hour)
incubate hair at 95° C (10 minutes)
set up PCR mixtures in clean tubes (10 minutes)
allow PCR to automatically cycle 30 times (less than 3 hours)

Day #2

mix PCR sample with loading dye (5 minutes)
load gel (10 minutes)
electrophorese fragments (1-2 hours)
photograph gel (5-10 minutes)
discuss results

Materials

There are two ways to do PCR, manually and automatically. Manual PCR has been described extensively by Garrison and dePamphilis (1994) and is cheaper since the cost of a temperature cycler begins at about $3,000. However, we have found automated PCR to be much more reproducible, convenient, and faster, so the equipment listed in this article will be that needed for automated PCR. The only difference between the two methods is the temperature cycler itself, and once the temperature cycler is purchased, the cost of a given experiment is identical between manual and automated PCR. If you buy a thermocycler, we recommend you get one with a heated lid which enables you to perform PCR on small volumes without having to use an oil overlay. We have found that students have a difficult time loading their samples when oil was used in the PCR. Although we have noticed that reactions conducted with oil overlays produced fewer extraneous bands and given "cleaner" results, students find the samples too difficult to pipette.

Student Equipment

One temperature cycler (a programmable heating block) - one for all students in lab(s)
Micropipetters (students need to pipet 100 µl, 17.5 µl, 15 µl, 10 µl) - one per group of 4 students
Microcentrifuge (optional, but convenient)
Power supply for electrophoresis - one per two electrophoresis chambers
Electrophoresis chamber - one per gel
Uv light box for detection of ethidium bromide stained dna - one for all students in lab(s)
Polaroid camera (not required but highly recommended) - one for all students in lab(s)
Tweezers for hair plucking - two for all students in lab(s)
Ice bucket - one per group of from four to six students
Scissors - one per group of from four to six students

Student Consumable Supplies

Marking pen - one per group of from four to six students
Microcentrifuge tubes (500 microliter size) - two per student

Agarose gel - number required varies, depending on number of lanes in gel
Micropipette tips - about 5 per student
Gloves, disposable (reusable dishwashing gloves are ok) - one pair for anyone handling gel
Ice water bath or crushed ice - one per group of from four to six students
Distilled water (grocery store quality is acceptable)
Toothpicks - one per student
Dna extraction buffer - 100 µl per student
Pcr reagents (see details below) - less than 20 µl per student
Molecular weight marker - 5 µl per gel

Notes for the Instructor

Overview

There are a few fundamental concepts of biology that are critical for students to understand in order to appreciate this laboratory exercise. We use “DNA fingerprinting” as a motivational tool to make relatively abstract concepts seem more tangible, exciting, and easier to learn. In this article, we describe the basics of PCR, variable number of tandem repeats (VNTRs), and electrophoresis which are integral to this laboratory procedure. We conduct this experiment with our Introductory Biology students towards the end of the semester when they have had experience with pipets and covered the necessary background information.

PCR

Dr. Kary Mullis developed the Nobel Prize winning technique of PCR which has been described previously (Garrison and dePamphilis, 1994; Mullis, 1990). PCR allows you to start with one molecule of double-stranded DNA and replicate over a billion times within 3 hours a selected portion of that DNA. The portion that is replicated is defined by two primers, short stretches of single-stranded DNA, which are used to prime the DNA polymerase. The sequences of the two primers are complementary to opposite strands of the double helix and have their 3’ ends facing towards each other (figure 1). The power behind PCR is the chain reaction component; replication is expanded exponentially because after each replication, the resulting DNA is unzipped (denatured), by raising the temperature to 95° C. After a newly polymerized segment of DNA is denatured, it becomes the template for the next round of replication. The DNA polymerase used in this process can withstand such extreme temperatures because the enzyme was isolated from a thermophillic bacterium that lives in hot springs. Since the cycle is repeated thirty times, the original copy of DNA will be replicated over one billion (2^{30}) times, which is enough DNA for visualization on an agarose gel.

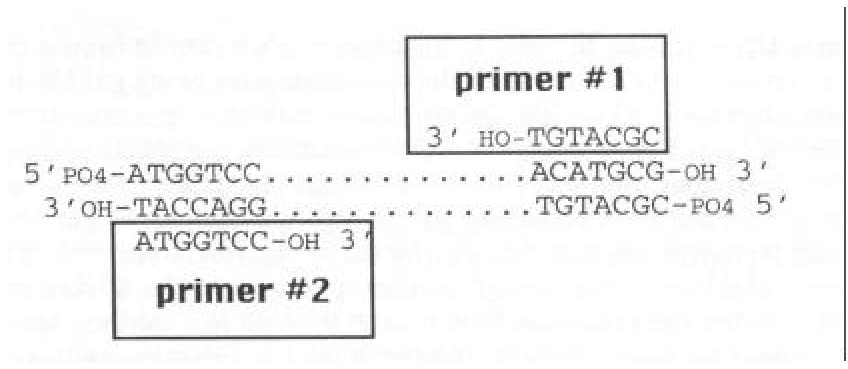

Figure 1.1 Diagram of two PCR primers annealing to their complementary strands. A segment of double-stranded DNA is shown with an undefined length of interviening sequence, as shown by the dots. In a real PCR mixture, the primers would be longer.

VNTR

The evolutionary principle of variation within a population is a cornerstone in biology. This variation results from subtle differences in the DNA sequence in individuals of a given species, and the DNA of *Homo sapiens* is no exception. Variation commonly originates by the mistaken duplication of a small sequence of nucleotides when only one copy was present before replication. This results in a tandem repeat of the original sequence. If this mistake occurs again in another round of replication, then three copies of a sequence will be in tandem (figure 2). These tandem repeats are part of our chromosomes and as such, they will be inherited according to Mendelian genetics. Over the centuries, the number of tandem repeat units has increased, therefore each of us has inherited a variable number of tandem repeats (VNTRs) at many loci scattered throughout our genomes. A VNTR can be thought of as a locus with each particular number of repeated units being analogous to different alleles. Therefore, each human (except for identical twins) carries a unique combination of VNTRs and these alleles can be used in population studies or to identify a particular individual.

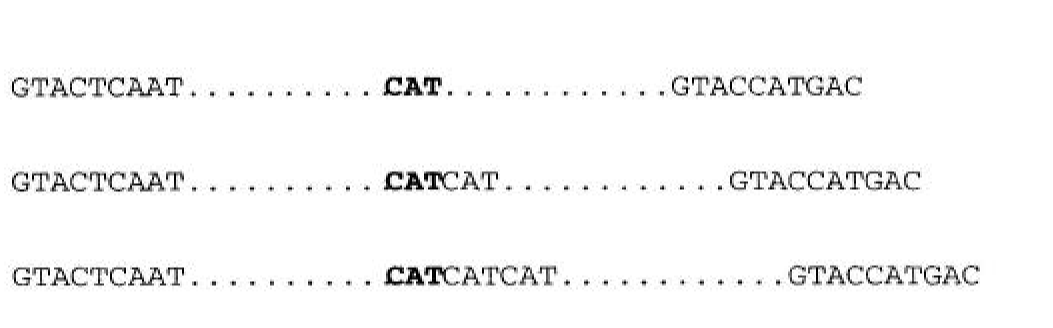

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Figure 1.2. Illustration of variable number of tandom repeats (VNTRs). Single strands of DNA from the same locus and three different individuals are shown. Within this region, the trinucleotide repeat CAT is present once, twice, or three times which results in alleles of three different lengths.

Electrophoresis

PCR can be used to amplify portions of human DNA that are known to contain VNTRs. By the end of the first lab period, each student will have generated PCR products that contain his or her own VNTR alleles. Next, the students need to be able to see their DNA "fingerprint", more appropriately referred to as a DNA profile. In order to visualize a DNA profile from a given locus, the DNA fragments need to be separated according to their variable sizes using gel electrophoresis. The gel is a matrix of agarose that looks like white gelatin and is analogous to a microscopic thicket of bushes and trees with lots of branches and twigs. An electric current is applied to the gel and all the negatively charged DNA molecules (negative because of all the phosphate groups) race towards the positive pole. But this is not a fair race. Returning to our analogy, the race is between children and adults of different sizes trying to run through the thicket of agarose trees and branches. Of course, it is easiest for small children to run through narrow passages in the thicket and so they move the fastest, the oldest children and the smallest adults run through at a medium speed, and the largest adults come in last place. Like people running through a thicket, DNA molecules of different sizes migrate through the gel at different speeds, depending on their size. By this process, students can separate their different sized VNTR alleles with the smallest alleles migrating the fastest and the largest alleles migrating the slowest. Once the DNA is stained in the gel, it can be visualized to reveal the DNA profile of each student.

Safety Precautions

The major concerns with this laboratory are ethidium bromide and the electrical current. Since most electrophoresis equipment is designed to be "idiot proof", the risk of electrical shocks should be minimal. However, one should always remember that the current used in agarose gel electrophoresis is powerful enough to be fatal. Ethidium bromide (EtBr) is a known mutagen and should be handled with extreme caution. Gloves should be worn by anyone handling EtBr. Of course the greatest risk comes when handling the concentrated stock solution but we do not let students do this. We make the gels and all buffers which contain EtBr. When the lab is complete, EtBr must be disposed of properly, according to your institution's guidelines. Schleicher and Schuell makes the Extractor, an EtBr waste reduction system which is a filtering device that extracts EtBr from aqueous solutions. If the Extractor is used, then only the gels and the Extractor require special disposal. Some people do not like to have EtBr in the gel buffer as, described below. The reason we include EtBr in our gel buffer is so the progress of the electrophoresis can be monitored at any time and to eliminate an extra staining and destaining procedure. However, either method will give satisfactory results.

There are two alternatives to ethidium bromide for staining DNA. Molecular Probes makes a fluorescent dye (SYBR Green I) that is reported to be more sensitive and less mutagenic, though it requires a different filter for photography than the orange one used with ethidium bromide. Alternatively, you can use methylene blue which is non-toxic and can be seen with visible light but is less sensitive. Kits for DNA detection and photography can be purchased from many suppliers including Fisher, Carolina Biological, and Fotodyne.

Dimethyl sulfoxide (DMSO) is used for the D1S80 locus protocol described in this article DMSO is not a major concern by itself but since it can be absorbed through the skin and is a universal solvent, it can be a means of absorbing other more toxic substances. Finally, there is a certain amount of superstition about working with DNA that circulates in laboratories. One is that every piece of plastic that comes into contact with DNA must be autoclaved. We do not wear gloves when working with DNA nor do we autoclave the pipet tips or the microfuge tubes and we have no problems with DNAses degrading the DNA.

Teaching Applications

The results from this experiment can be integrated into a biology curriculum at several points throughout the semester, plus the experiment exposes students to several techniques used in molecular biology. Below we have compiled a list of some areas of the curriculum that can be enhanced by this laboratory experiment. Since PCR is based on DNA replication, you may want to discuss: the mechanism of the DNA polymerase activity; the semiconservative nature of replication; the need for primers to initiate a DNA polymerase; and the 5' and 3' orientations of DNA strands.

By their very existence, VNTRs are a good example of mutations that occur naturally in our DNA. From this starting point, you could discuss other mutations which might have physiological or evolutionary repercussions.

Mendelian genetics can be illustrated if students can bring in hair follicles of family members. They can observe the genotypes of their parents and determine which alleles they have inherited. However, there is the potential for a student to learn that his or her biological father is not the man they thought.

If all the class data are compiled, they can be used to discuss specific areas of population genetics, such as the frequencies of different alleles, pedigree analysis, and the use of the Hardy-Weinberg equation.

Biotechnology can be introduced by using this experiment as a launching pad to discuss forensic uses of DNA, bioengineering, Jurassic Park, etc. With the incessant example of the O. J. Simpson trial, the use of DNA profiles in criminal cases is an obvious application. An amazing example can be found in Hochmeister *et al.*, (1991) where the analysis was conducted on DNA isolated from skin cells deposited on cigarette butts. The possibilities for discussion continue to increase and are often quoted, or misquoted, in daily newspapers.

We have our students calculate the molecular weights of all the alleles using the molecular weight marker as standards. The D1S80 PCR product with zero repeat units is 142 base pairs long, so every repeat unit will add 16 base pairs to the VNTR. A student can use the estimated molecular weights of each band to determine how many repeat units are in each allele.

In conjunction with a quantitative analysis, a fun approach is to fabricate a crime where the criminal has left behind a single hair follicle. The students are mentally prepared to find a guilty party and in our experience, they always do, even if the hair did not come from someone in the class. This is a good opportunity to discuss objective evaluation of data, the handling of evidence, the number of loci necessary to identify the source of the DNA, and statistical methods to calculate the odds of this DNA profile belonging to more than one person.

A related area ripe for discussion is the statistical basis for saying whether or not a given DNA sample is likely to belong to only one person. There is a general agreement that, in principle, DNA could be used to identify any given person if enough DNA is examined. However, how much is enough? Examining the VNTRs at a single locus is not sufficient to distinguish individuals within a large population since the number of possible allele combinations is small compared to the number of people. The number of VNTR loci examined is a minor problem compared to the statistical analysis of a given set of data. There are several different methods to analyze the data and these methods are eloquently discussed in the National Research Council's DNA Technology in Forensic Science (1992).

Overview of Protocols

Collecting the Hair Sample

The most critical step is plucking a substantial hair follicle. We do this with our fingers and have found that most people can harvest good follicles if they pull between 3 and 20 hairs out at a time. For those with fragile hair, we use tweezers to grip the hair shaft at its base, pull quickly, and harvest the follicle. A "good"

follicle is comprised of many cells and is easily identified because it has a black bulbous base that is sticky and surrounding the lowest portion of the hair shaft that is covered with a shiny layer of cells. Many follicle cells means more template DNA, which means better student results, since the number of DNA molecules replicated equals the initial number of template molecules raised to the thirtieth power. We cut off most of the hair shaft and use a toothpick to transfer one follicle to a labeled microfuge tube since a good follicle will stick to the toothpick. If a student cannot get a "good" follicle, we have used as many as 10 "bad" follicles in one tube to extract as much DNA as possible.

APOC2 Locus

We have used two different loci; APOC2 and D1S80. We have found the APOC2 locus to be easier to execute successfully because it is technically easier to amplify than the other locus. Therefore, you might want to attempt PCR amplification with the APOC2 locus first, though the D1S80 locus gives much better results. APOC2 (figure 3; Weber and May, 1989) is a locus that encodes apolipoprotein C2 (which is involved in cholesterol transportation in the blood).

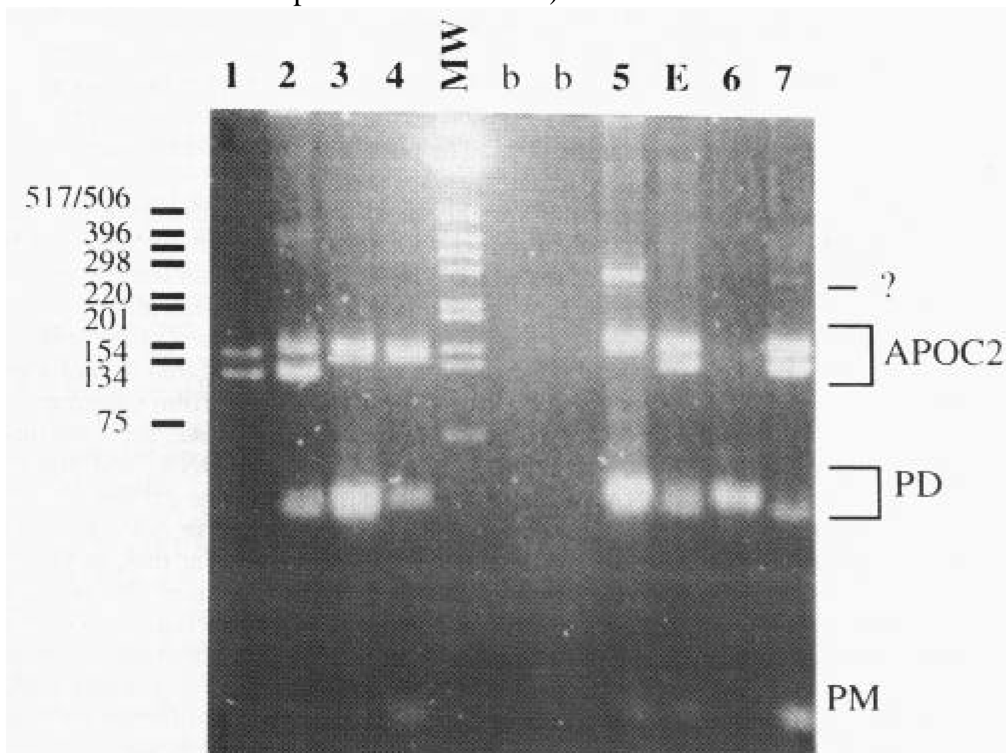

Figure 1.3. Photograph of a 3% agarose gel, stained with ethidium bromide, showing students' results for the APOC2 locus. Lanes: 1 - 7 are from seven different students' PCR products; MW is the one kb molecular weight marker lane and the sizes of the major bands are indicated in base pairs on the left side of the photograph; b indicates blank lanes, E is the evidence DNA we used for a fabricated crime. The sample from lane 7 evaporated when the lid popped open during the PCR and an extra tube of evidence DNA was substituted for the student's sample (see Appendix C., Potential Problem #6). Lane 6 produced very faint bands of the expected size and is an indication of a pipetting error or a "bad" hair follicle. The labels on the right side of the photograph, beginning at the bottom, note the positions of: PM, primer monomers; PD, primer dimers; APOC2, the APOC2 alleles; ?, the bands found in lanes 5 and 7 that are unidentified. The white spots scattered across the gel are dust and powder from our gloves.

D1S80 Locus

D1S80 (Figure 4; Budowle *et al.*, 1991; Nakamura *et al.*, 1988; Skowasch *et al.*, 1992) is located on the distal portion of the short arm of chromosome one, is not a part of any gene, and it is used in several countries for forensic analysis of DNA samples (Kloosterman *et al.*, 1993; Sajantila *et al.*, 1992). D1S80 requires hotstart PCR which means that the Taq DNA polymerase is not added to the PCR mixture until the mixture has been heated to 95° C. The hotstart is necessary because the D1S80 primers have a tendency to anneal to each other rather than the template while the mixture is heating up for the first time, which allows the DNA polymerase to generate "primer dimers" (figure 5). If addition of the DNA polymerase is delayed, then inappropriately annealing primers are denatured as the kinetic energy increases, so no replication occurs until the temperature is lowered later in the procedure, allowing the primers to anneal to the proper portion of the template DNA. DMSO has been included in the reaction mixture to enhance the specificity of the primers (Filikin and Gelvin, 1992).

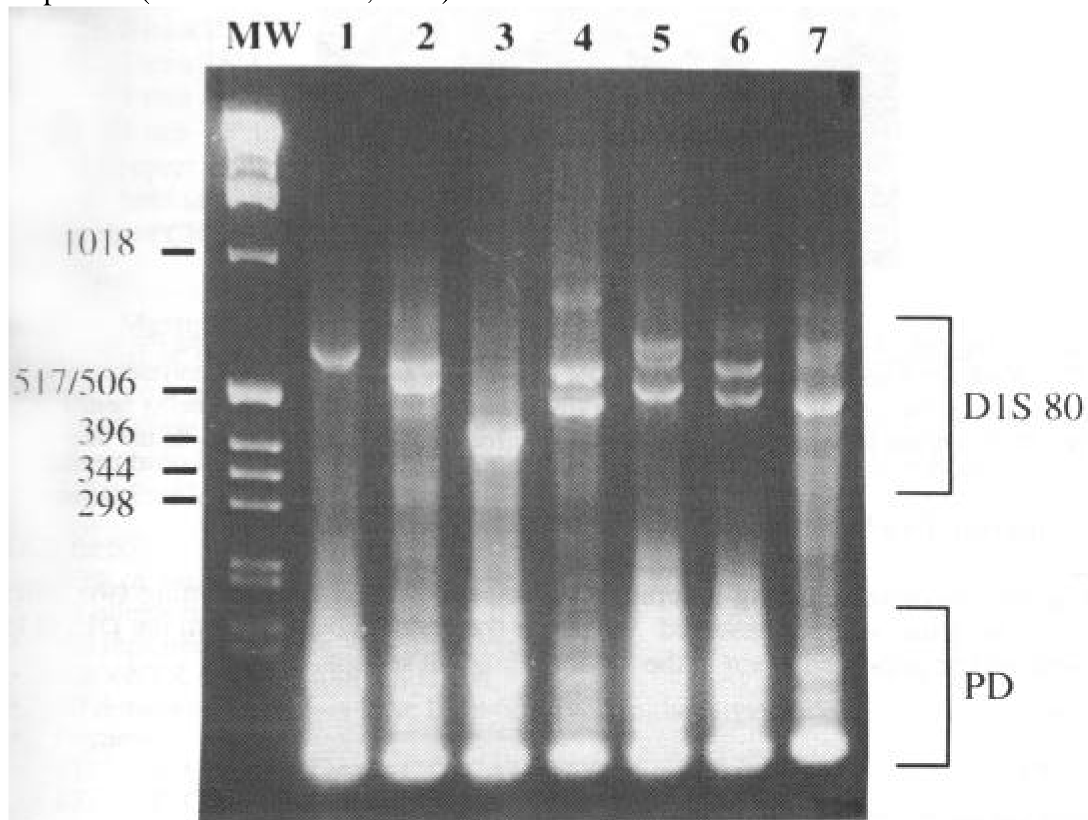

Figure 1.4. Photograph of a 1.5% agarose gel, stained with ethidium bromide, showing the results for the D1S80 locus using seven different sources of DNA (lanes one through seven). Examples were chosen to illustrate the wide range of alleles found in a class of 17 students, with a bias to highlight apparently homozygous individuals. The DNA used in lane five was extracted from five "bad" follicles (see Potential Problems #4). Labels: MW, the one kb molecular weight markers with the size in base pairs of relevant bands indicated to the left of the photograph; PD, primer dimers; D1S80, the D1S80 alleles.

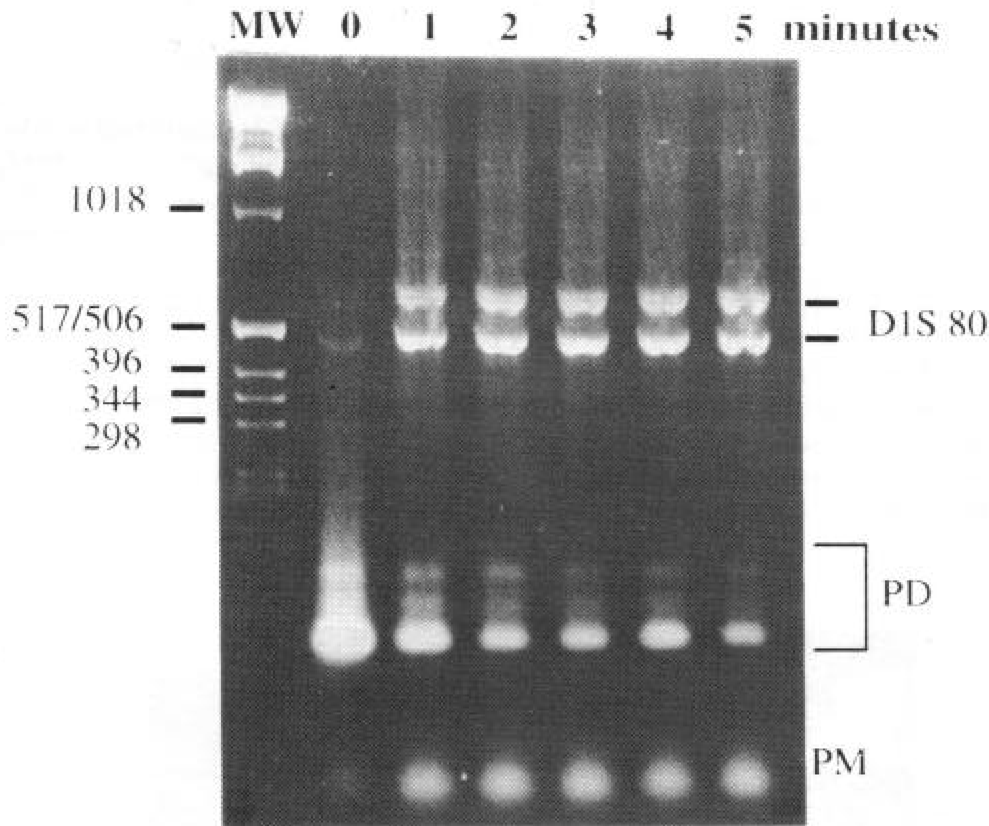

Figure 1.5. Photograph of a 1.5% agarose gel, stained with ethidium bromide, showing the need for hotstart PCR. One DNA sample was used as template for all lanes and the Taq DNA polymerase was added to the appropriate tube at the indicated time (from zero to five minutes) after being heated to 95° C. The labels are the same as in figure 4.

Detailed Protocols for Two Loci

Since we have developed teaching laboratory exercises for PCR[1] fingerprinting two different loci, protocols for both loci will be presented. We prefer the results obtained from the D1S80 locus and that protocol will be presented again in the Student Handout section.

APOC2 Locus

DNA extraction (adapted from Erlich, 1992)

1. Pluck a hair so that a follicle is removed from your head.
2. Cut off most of the hair shaft but keep the follicle (~5 mm). Be careful, sometimes the follicle jumps away when you cut the hair.
3. With the heated lid disabled, incubate the follicle in 100 µl extraction buffer (which contains 6 µg of proteinase K) for 1 hour at 55° C, 10 minutes at 95° C, then cool the samples to room temperature.
4. When the DNA extraction cools, set up a new 500 µl microfuge tube by adding the following:

Reagent	Volume	Final Concentration
extracted DNA	7.5 µl	~ 50 ng of DNA
reaction mixture	17.5 µl	see below**

**The reaction mixture contains the following cocktail:

Reagent	Volume	Final Concentration
H_2O	11.55 µl	
10X PCR buffer (without Mg)	2.50 µl	0.75 mM $MgCl_2$
20X dNTPs	1.25 µl	200 µM each
#1 primer	1.00 µl	100 ng primer
#2 primer	1.00 µ	100 ng primer
Taq DNA polymerase	0.20 µl	1 unit
Total Volume	17.50 µl	

PCR

5. Start the following PCR program with the heated lid enabled.
   - Step 1 5 min 95° C
   - Step 2 1 min 95° C
   - Step 3 1 min 55° C
   - Step 4 1 min 72° C
   - Step 5 repeat steps two through four 29 more times
   - Step 6 hold at 20° C
6. When the PCR is completed, the tubes are removed and stored at 4° C until the second lab meeting.

Second Lab Meeting

1. Add 2.5 µl of 10X loading dye to your PCR products and load up to 25 µl of each sample into a separate well.
2. Electrophorese the 25 µl of the reactions + loading dye on a 3.0% (w/v) agarose gel in 0.5X TBE + ethidium bromide (200 ng/ ml final concentration) at 90 volts for 1.75 hours (see Appendix D., Potential Problem #7).

APOC2 facts:

80% of American population is heterozygous
11 alleles have been published
the repeated sequence is two nucleotides long
the VNTR occurs within an intron
30 dinucleotide repeats have been observed in the largest allele
Primers:
#1 5' CATAGCGAGACTCCATCTCC 3'
#2 5' GGGAGAGGGCAAAGATCGAT 3'

D1S80 Locus

First Lab Meeting

DNA extraction

Follow the same procedure given above (steps one through three) for DNA extraction in the first laboratory meeting under APOC2 Locus.

4. When the DNA extraction cools, set up a new 500 µl microfuge tube by adding the following:

Reagent		**Volume**	**Final Concentration**
extracted DNA	15.0 µl	~ 100 ng of DNA	
reaction mixture	10.0 µl	see below **	

**The reaction mixture contains the following cocktail:

Reagent	**Volume**	**Final Concentration**
H_2O	4.00 µl	
10X PCR buffer (without Mg)	2.50 µl	1.5 mM MgCl2 (from extraction buffer)
DMSO	1.25 µl	5% v/v
20X dNTP's	1.25 µl	200 µM each
#1 primer	0.50 µl	100 ng primer
#2 primer	0.50 µl	100 ng primer
Total Volume		10.00 µl

PCR

5. To initiate hot start PCR, denature the DNA by incubating the tubes for four to five minutes at 95° C (Step 1), maintain the tubes at 95° C while you add 0.5 µl Taq DNA polymerase to each tube. Do not allow the tubes to cool and do not take time to mix the reaction mixture after adding the Taq polymerase.
6. .Resume the following PCR program with the heated lid enabled:
   - Step 2 1 min 95° C
   - Step 3 1 min 65° C
   - Step 4 1 min 72° C
   - Step 5 repeat steps two through four 29 more times
   - Step 6 hold at 20° C
7. When the PCR is completed, the tubes are removed and stored at 4° C until next lab meeting.

Second Lab Meeting

Add 2.5 µl of the 10X loading dye to each tube, load up to 25 µl of the PCR product into a well, and electrophorese the DNA on a 1.5% agarose gel using 0.5X TBE and ethidium bromide (200 ng/ml final concentration). We usually run these gels at 90 volts for 1 - 1.5 hours. The exact time and voltage will depend on the gel box configuration and appropriate conditions can be refined accordingly (see Appendix D., Potential Problem #7).

D1S80 facts:

>80% of all populations tested are heterozygous
28 alleles have been published
repeat unit is 16 nucleotides long
D1S80 PCR product with zero repeat units is 142 base pairs long, so every repeat unit will add 16 base pairs to the VNTR.
PCR products range from 430 to 814 base pairs long
41 repeated units have been observed in the largest allele
Primers:
#1 5' GAAACTGGCCTCCAAACACTGCCCGCCG 3'
#2 5' GTCTTGTTGGAGATGCACGTGCCCCTTGC 3'

Student Handouts

What is your genotype? A PCR Answer

Introduction

Unless you have spent the last couple of years in a cave, you have heard about the increasing use of "DNA fingerprinting" in court cases. The technology available is so sensitive that unbelievable sources of DNA have been used to convict criminals. In Minnesota for example, DNA was extracted from the back of a postage stamp since some epidermal cells from the suspect's tongue had been deposited on the glue when the stamp was licked. My prediction is that the pivotal point in future court cases will be the collection and handling of the evidence. For example, what if some DNA from the crime scene is shown to be the accused? The defense attorney could suggest that the police collected some epidermal cells from the sidewalk (from a visit the day before) at the same time as the blood drops. What do you think of this possibility as a defense? If you want to read more about this area, the library has several books on this topic.

There are two standard methods for "DNA fingerprinting": 1) Southern blots and, 2) PCR. We have discussed Southern blots *ad nauseam* in class but have not covered PCR in much detail. Dr. Kary Mullis, the inventor of PCR, was awarded a Nobel Prize in 1993 for his revolutionary innovation. As you know, PCR allows you to amplify a single copy of DNA into millions of copies, provided the DNA has been sequenced because you have to supply DNA polymerase with primers that will specifically hybridize to the target gene and no other DNA. Over the next two weeks, we will use PCR to determine the genotype of every student taking this class. We are using a hair root as our source of genomic DNA and are looking at a locus called D1S80. D1S80 contains a Variable Number of Tandem Repeat sequence (VNTR). As the term implies, there is a section of DNA that is repeated to varying degrees in each person. As a simplified example, the repeat unit is the two nucleotides (CG). So if we were to sequence this portion of 4 different D1S80 alleles, we might see the following:

1) ATGCCGTATTA*CGCGCGCGCGCGCG*CCTATTAGGTATTAG
2) ATGCCGTATTA*CGCGCGCGCGCGCGCGCGCGCGCG*CCTATTAGGTATTAG
3) ATGCCGTATTA*CGCGCGCG*CCTATTAGGTATTAG
4) ATGCCGTATTA*CGCGCGCGCGCGCGCGCG*CCTATTAGGTATTAG

In this example, there are 4 copies of the VNTR with 4 different lengths. If we electrophoresed these 4 segments of DNA on a gel, we would find 4 different bands of different sizes (2>4>1>3). In a criminal case, we might have 4 suspects and one DNA sample from the crime scene. The resulting gel might look like this:

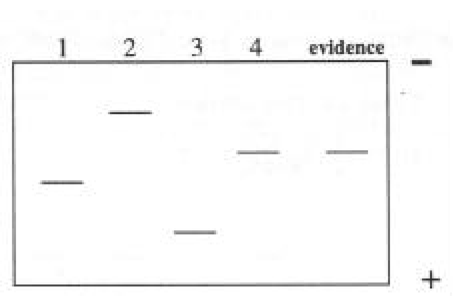

Figure 1.6. Diagram of fictitious gel using the data depicted above. Lanes one through four contain suspect DNA.

Questions:

1) So, “who done it”?
2) What is wrong with the above gel? Why is this too easy?

First Lab Meeting

Now it is time for us to determine our genotypes. You should be forewarned, this is a delicate procedure that does not always work for everyone. In order to process this kind of evidence for a criminal case, a technician usually has a master’s degree in Forensic Science, and a few years of “on-the-job training”. Nevertheless, even these experts sometimes make mistakes. So do not be discouraged if your sample does not “work”, but try to avoid this situation by observing these guide lines:

1. Follow the protocol as carefully as possible.
2. Do not contaminate your hair or DNA with that of others (remember one cell is enough to be amplified).
3. When the DNA extraction is just finished, visually check to verify that you have extracted DNA by gently removing the tube from the thermocyler and flicking the tube while holding it up to a light and looking very carefully. You should see a more dense portion of the solution as it mixes with the less dense water.
4. The most common mistakes are pipetting errors. Be sure to visually check to see that you are transferring about the right volumes and always use clean tips; when in doubt get a new one.
5. Be very careful loading the gel. We will have time to practice this week so that when you are loading your real sample next week, you will be a pro.

Protocol

DNA extraction[1]

1. Pluck a hair so that some root is removed from your head (yikes!) The root is white/translucent in appearance. Check to make sure you got some root and not all shaft.
2. Cut off most of the hair but keep the root (~5mm). Be careful, sometimes the root will jump away when you cut the hair.
3. Incubate the root in 100 µl digestion buffer (which contains 6 µg of proteinase K) for 1 hour at 55° C, then 10 minutes at 95° C (what is the purpose of this step?). Use thermocycler program HAIR 1 - lid disabled.

PCR Reaction Mixtures

1. When the DNA extraction cools to 20° C, set up a new 500 µl microfuge tube by adding the following:

Reagent	**Volume**	**Final Concentration**
extracted DNA	15.0 µl	~ 100 ng of DNA
reaction mixture	10.0 µl	see below **

[1] (Adapted from: *PCR Technology* by Henry A. Erlich, W. H. Freeman and Co., NY, 1992, pp. 35-37.)

**the reaction mixture already contains the following cocktail and has been aliquoted for you:

Reagent	**Volume**	**Final Concentration**
H_2O	4.00 µl	
10X PCR buffer (without Mg)	2.50 µl	1.5 mM MgCl2 (from extraction buffer)
DMSO		1.25 µl 5% v/v
20X dNTP's	1.25 µl	200 µM each
#1 primer	0.50 µl	100 ng primer
#2 primer	0.50 µl	100 ng primer
Total Volume		10.00 µl

PCR

This locus requires hotstart PCR which means that the Taq DNA polymerase is not added to the PCR mixture until it has been heated to 95° C. The hotstart is necessary because the D1S80 primers have a tendency to anneal to each other rather than the template while the mixture is heating up for the first time, which allows the DNA polymerase to generate "primer dimers". If addition of the DNA polymerase is delayed, then inappropriately annealing primers are denatured as the kinetic energy increases, so no replication occurs until the temperature is lowered later in the procedure, allowing the primers to anneal to the proper portion of the template DNA. DMSO has been included in the reaction mixture to enhance the specificity of the primers.

5. To initiate hot start PCR, denature the DNA by incubating the tubes for 5 minutes at 95° C (Step 1), maintain the tubes at 95° C while the instructor adds 0.5 µl Taq DNA polymerase to each tube. Do not allow the tubes to cool and do not take time to mix the reaction mixture after adding the Taq polymerase.
6. Resume the following PCR program with the heated lid enabled:

   Step 2 1 min 95° C
   Step 3 1 min 65° C
   Step 4 1 min 72° C
   Step 5 repeat steps two through four 29 more times
   Step 6 hold at 20° C
7. When the PCR is completed, the tubes are removed and stored at 4° C until next lab meeting.

D1S80 facts:

>80% of all populations tested are heterozygous
28 alleles have been published
repeat unit is 16 nucleotides long
if there were zero repeat units, the PCR product would be 142 bp long
PCR products range from 430 to 814 base pairs long
41 repeated units have been observed in the largest allele
Primers[2]:
#1 5' GAAACTGGCCTCCAAACACTGCCCGCCG 3'
#2 5' GTCTTGTTGGAGATGCACGTGCCCCTTGC 3'

[2] Budowle, B., Chakraborty, R., Giusti, A. M., Eisenberg, A. J., and Allen, R. C. (1991) Analysis of the VNTR locus D1S80 by the PCR followed by high-resolution PAGE. *American Journal of Human Genetics* 48: 137 - 144.

Second Lab Meeting

The PCR Results

Add 2.5 µl of the 10X loading dye to each tube and electrophorese the DNA on a 1.5% agarose gel using 0.5X TBE and 200 µg/ml ethidium bromide. We usually run these gels at 90 volts from 1 to 1.5 hours. The exact time and voltage will depend on the gel box configuration and appropriate conditions can be refined accordingly.

While these gels are running, we will purify a lot of genomic DNA from our friend, *Chlamydomonas*. The protocol can be found on the next page.

Recipe for 0.5X TBE

45 mM Tris-borate pH 8.0
1 mM EDTA

Acknowledgements

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APPENDIX A
Recipes for reagents

0.5X TBE — store at room temp.

45 mM Tris-borate pH 8.0
1 mM EDTA

Hair DNA Extraction Buffer — store at +4° C

Amount	*Reagent*	*Final Concentration*
1.86 g	KCl	50 mM KCl
0.61 g	Tris	10 mM Tris
0.25 g	$MgCl_2 \cdot 6H_2O$	2.5 mM $MgCl_2$,
0.05 g	gelatin	0.1 mg/ml gelatin
2.25 ml	NP40	0.45% NP40
2.25 ml	Tween 20	0.45% Tween 20

pH to 8.3 at room temperature.
water to 500 ml and autoclave (will look white until it cools)

20X dNTPs — store at -20° C

final concentration of this stock is 4 mM of each nucleotide

Primers — store at -20° C

dilute each primer to 200 ng/ µl

Taq DNA polymerase — store at -20° C

do not alter concentration, use as supplied by manufacturer
very temperature sensitive, do not leave out except to pipet

Proteinase K — store at -20° C

dissolve in 1 ml of sterile water (10 mg/ml final concentration)
aliquot in 20 µl volumes; do not thaw and refreeze
add 0.6 µl for every 100 µl of extraction buffer

Molecular Weight Markers — store at -20° C

make a stock solution of the 1 kb ladder (0.1 ug/ µl)
that contains a 1X concentration of loading dye
use 5 µl of the marker per lane

10 X loading dye — store at +4° C

35% glycerol
0.25% bromophenol blue
0.25% xylene cyanol FF

ethidium bromide KNOWN MUTAGEN — store at +4° C

10 mg/ml stock solution
use 1 µl for every 50 ml of buffer or gel
200 ng/ml final concentration in gel and buffer

APPENDIX B

Sources for Reagents and Equipment

This is a list of the reagents and equipment we use but substitutes can be purchased from wide variety of sources such as: Amresco, Fisher, Fotodyne, New England Biolabs, Pharmacia, Promega, and Boehringer Mannheim.

Item	**Company**	**Phone Number**
thermocycler	MJ Research	1-800 -729-2165
Taq DNA polymerase	Promega #M1862 (in storage buffer A)	1-800 -356-9526
10X reaction buffer	free from Promega	
deoxynucleotides (dNTPs)	Promega #U1240 (40 umoles each)	
1 kb ladder	Gibco BRL 1#15615-016 250 µg	1-800 -828-6686
nonidet P-40	Sigma # N-6507	1-800 -325-3010
Tween 20	Sigma # P-1379	
proteinase K	Sigma # P2308 (10 mg amount)	
ethidium bromide	Sigma # E-8751 KNOWN MUTAGEN	
agarose (low EEO)	Fisher # BP160-100	1-800 -766-7000
gelatin	Sigma # G-9382	
Extractor	Scleicher and Schuell #448030	1-800 -245-4024
Primers	see list below	

There must be over 100 sources of oligonucleotides that can be used for PCR primers. We have used:

Retrogen	1-619-586-7918
National Biosciences	1-800-369-5118
Genset	1-619-551-6551

You want to have the oligos desalted and you should get a minimum of 5 OD units worth for each primer. Currently, we use Geneset because the primers arrive in solution, the concentration of each primer is written on the vial, and they charge about $1.80 per nucleotide and no set-up charges. A new and inexpensive source that we have not tried yet is Universal DNA which charges $1.00 per nucleotide for desalted oligos, no set-up charge, and 5 ODs [(800) 768-8744].

APPENDIX C

Suggestions for Equipment Sources

We have found some places that sell particularly good equipment that can be recommended. Of course there are plenty of other sources for equipment that are comparable, but if you are going to buy something new, you might want to try these vendors.

1. *Electrophoresis chambers* - Jordan Scientific's Gel-o-Submarine Electrophoresis Systems. They have a very nice casting system that eliminates the need for taping the gel molds. They come in different sizes, depending on your needs. (800) 222-2092
2. *PCR machine* - MJ Research's PTC-100 cycler with heated lid (you do not need the temperature probe). This unit is nice because it can go from 100° C to 0° C but does not use a compressor or oils which can create some problems. It has a very small footprint and the new generation of heated lids can be adjusted to ensure that microfuge tube lids cannot pop open. (800) 729-2165
3. *Photo-documentation* - Fotodyne has a wide range of systems that range from handheld models to photostands, to video documentation systems that store images electronically and print on thermal paper. Fotodyne is very good at supplying kits that contain every component you need but they also sell individual components (e.g. UV light boxes, safety equipment, Polaroid cameras, filters, and films) (800) 362-3686

   We have just learned about Kodak's new EDAS system which is a digital camera attached to a hood that fits over the UV light box. The images are then moved to either a Mac or PC and Kodak has software that can analyze the data. To get hard copies, you can print the pictures either on a normal laser jet printer, or Kodak sells printers for this as well. (800) 225-5352
4. *Micropipets* - Rainin brand "Pipetman". This is still the industry standard and with two micropipetrs (the P-20 and P-200), you can deliver volumes from 0.2 to 200 µl. The Pipetman, despite its name, is very user-friendly and durable. Rainin also sells pipetrs (P-2) that specialize in very small volumes that are good for delivering the 0.5 µl volume required for the hotstart PCR. However, this is not a requirement, and since only one is needed for the instructor, it is a nice option that will not add significantly to your budget. (617) 935-3050

There are a number of other manufacturers of micropipets which are represented by Fisher, VWR, Baxter, etc.

APPENDIX D.
Potential Problems and Trouble Shooting

In the process of designing and trouble shooting this protocol, we have learned a few tips to facilitate good results.

Make sure students are comfortable working with the micropipets. In our introductory labs, we have the students pipet only three solutions: 100 µl of the extraction buffer, 15 µl of extracted DNA, and 10 µl of the reaction buffer. We add the 0.5 µl of Taq DNA polymerase to the tubes, and make the common stock solutions of extraction buffer with proteinase K, and the reaction mixture. If pipetting is a trouble spot, the teacher should do all the pipetting.

Make sure students use fresh tips for every volume they pipet. Students want to conserve tips, but this results in contaminated reagents.

Make sure the students do not add the reaction mixture to their entire 100 µl of extracted DNA; usually happens about once per lab day.

Make sure students get the best possible hair follicles; this is the most common mistake. We offer to pluck hairs from those with brittle hair. We use the tweezers and grip a few hairs very close to the scalp, near the crown. A "good" follicle should be obvious from several feet away. African American hair follicles are more difficult to distinguish visually since the hair and its follicle are equally pigmented. However, all "good" follicles are always sticky. If a student cannot get a good follicle, we have use as many as 10 "bad" follicles in one tube to extract as much DNA as possible. When the one hour extraction is complete, make sure the students mix the contents of their tubes since the DNA tends to collect at the bottom of the tube (you can see it go into solution). It is critical that the tube with the extracted DNA be shaken vigorously to ensure the DNA is mixed into solution; it tends to accumulate at the bottom of the tube during the extraction process.

Care should be taken by working close (about 5 cm) to the bench top when cutting off the hair shaft, since follicles tend to jump when cut free. We find it helpful to cut dark hair over white paper and light colored hair over dark surfaces.

With the APOC2 locus, make sure the lids of the microfuge tubes do not pop open during the first 3 cycles. This is especially a problem if any liquid was dispensed on the lip of the tube before it was closed. We periodically check the microfuge tubes during the first three cycles by quickly lifting the heated lid of the temperature cycler. We recommend that you set up a few extra PCR tubes for two reasons. First, it ensures that every student will have some DNA to load even if their lid pops open. Second, if you are working on a fabricated crime, use the evidence DNA in your extra tubes and exchange one of the extra tubes with a student's tube. This guarantees that at least one student will have identical bands to the evidence (figure 2).

Run the gels at a higher voltage for less time, only if time is limited since the resolution and clarity of the bands are reduced compared to gels run at lower voltage for longer times. You will get more distinct bands if the gels are run at 90 volts rather than higher voltages (e.g. 120 volts maximum).

During the first lab, we have a practice gel into which students load water mixed with 10X loading dye. This allows them to make mistakes when there is no harm; for the next lab, they get only one chance to load their PCR samples.

WARNING: ethidium bromide is a mutagen and should be handled carefully while wearing gloves. Use the same concentration of ethidium bromide in the gel and in the running buffer. Differences in ethidium bromide concentration will result in a gel that is half light and half dark, since the ethidium will electrophorese through the gel in the opposite direction of the DNA.

If you do use oil, we recommend freezing the samples and removing the oil before adding the 10X loading dye to the DNA solution.

1. Answers to questions in Student Handout:

The figure is too easy because there is only a single band in each lane. Humans are diploids and therefore should carry two alleles and it is unlikely that everyone involved would be homozygous.

The 95° C incubation during the DNA extraction is required to inactivate the proteinase K. Without this step, the Taq DNA polymerase would be degraded during the PCR.

APPENDIX E
Pouring and Running an Agarose Gel

One of the basic properties of DNA is that it is negatively charged, a consequence of ionization of its many phosphate groups and the reason that the substance is an acid. Because they are acidic, molecules of DNA in an aqueous solution will migrate towards the positive pole when exposed to an electric field. Agarose gels are highly porous, with pore size varying inversely with concentration of agarose. Small molecules of DNA can migrate through the pores relatively easily, while larger molecules migrate through pores with some difficulty and are retarded in their movement. Thus agarose gel electrophoresis allows us to separate molecules of DNA according to molecular size, usually represented as base pairs (bp) or kilobase pairs (kb).

Once the samples have 2.5 µl of 10X loading dye added to them, they are ready to be loaded onto the gel. It is important that a fresh tip be used for each sample to avoid contamination. The pipet tip must be positioned over the well in the gel, the pipet tip dipped into the buffer just over the appropriate well and the plunger very carefully depressed. Do not stick the pipet tip deep into the well because the tip's opening may get clogged with agarose or a hole may be created in the well. Glycerol in the loading dye will cause the sample to sink to the bottom of the well. If there is air in the pipet tip, in front of the sample, it can be expelled by carefully depressing the plunger to push the sample to the end of the tip before submerging the tip in the buffer. (Air bubbles over the well may deflect the sample so that it does not fall into the well.) The gel may be checked to determine if the bands of DNA have separated adequately by turning off the power supply and removing the gel to the UV light box for observation. If the bands of DNA are not well separated, the gel may be put back into the gel box for additional separation. Good separation will usually have occurred when the indicator dye (bromophenol blue) has moved several centimeters from the wells. The bromophenol blue should never be allowed to run off the end of the gel.

How to Pour an Agarose Gel

1. Seal off the ends of the gel mold with lab tape or other appropriate means. Use your fingernails to make sure it is a good seal.
2. Put the comb into place and confirm that it is set for the proper depth (1-2 mm clearance).
3. Dilute the 5X stock of TBE so that you wind up with the desired volume of 0.5X TBE. For example:
   50 ml of 5X TBE
   450 ml water
   500 ml total of 0.5 X TBE
4. Weigh the appropriate amount of agarose. For example, if you want a 50 ml gel of 1.5%, you would weigh out 0.75 g of agarose (0.75 ÷ 50 = 1.5%).
5. Pour the agarose into a large flask (typically 2-5 times larger than the gel volume) along with the 50 ml of buffer, swirl gently, and cover with saran wrap or a beaker.
6. Microwave the mixture for about 1 minute and 20 seconds.
7. Using insulated gloves, remove flask and swirl. Look to see if the agarose has begun to melt but is still solid and translucent. Continue to microwave for 20 seconds and swirl until *all* the agarose is melted.
8. Now add in 1 µl of EtBr (10 mg/ml stock) for every 50 ml of agarose solution. For example, add 1.0 µl for a 50 ml gel.
9. For these high percentage gels, you do not usually need to worry about leakage. Simply pour the molten agarose and make sure the gel comb is in place.
10. Allow this to cool completely; it will turn white as it gels.

11. Slowly and smoothly, remove the comb from the gel with one side (e.g. the left side) coming out slightly before the other (the right side). Remove the tape from the mold and put the gel and the mold into gel box.
12. With the remaining 0.5X TBE buffer, pour in enough buffer to just cover the gel and the wells. Now add in 1 µl of EtBr (10 mg/ml stock) for every 50 ml of buffer in the gel box.
13. To load DNA onto a gel, set your pipettor for the appropriate volume.
14. Slowly remove the blue DNA mixture (assuming you have already added the required DNA loading dye). Avoid getting bubbles in the pipet tip.
15. Slowly and smoothly pipet the blue mixture into the well (see figures below). Put the pipet tip into the well but do not jab it into the agarose. The DNA loading dye will cause the mixture to sink.
16. Close the gel box and turn on the current (typically 90 volts). Verify direction and current. (An easy way to remember which way the DNA will migrate is 'DNA Runs Red"; it migrates to the red electrode.)

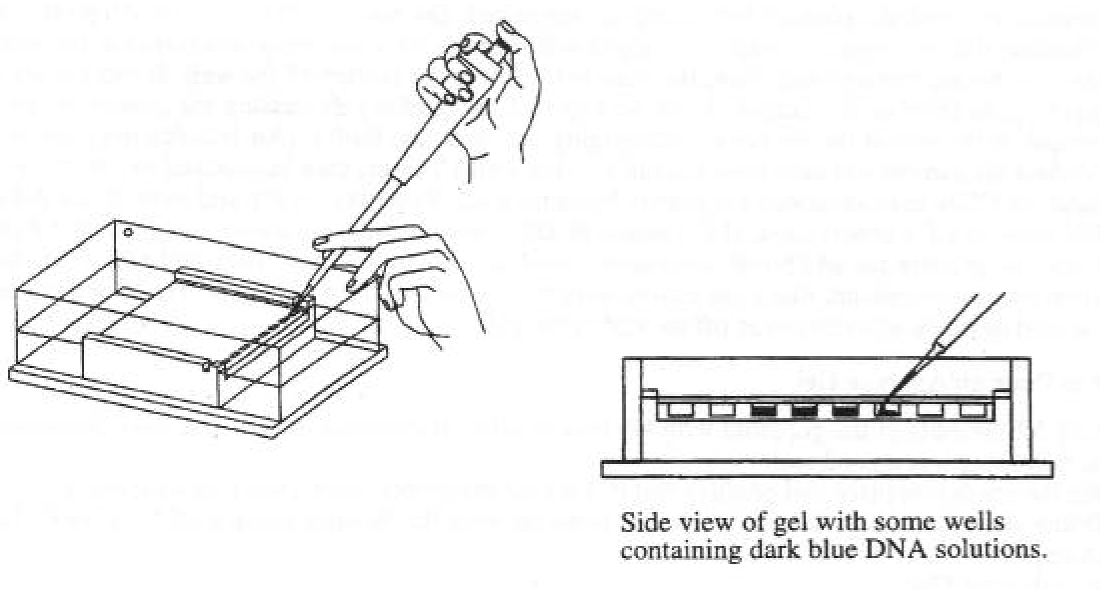

Figure 1.7. Diagrams depict the proper way to load DNA samples into agarose gels. Reprinted with permission from Cold Springs Harbor Laboratory Press. *From*: DNA Science: A first course in recombinant DNS technology. By David A. Micklos and Greg A. Freyer, 1990, Cold Springs Harbor Laboratory Press, USA, pages 263-264/

APPENDIX E
Photographing Gels

Given that we can separate DNA molecules in an agarose gel, we need to visualize these molecules to determine how far they have moved through the gel. This is accomplished by “staining” the molecules of DNA with some compound having an affinity for DNA. We routinely stain DNA with ethidium bromide, a highly planar molecule that intercalates between base pairs in DNA. When subjected to UV irradiation, ethidium fluoresces orange-red, allowing us to visualize DNA in the gel. We then make a copy of the gel using a Polaroid camera and use a photocopier with enlargement capabilities to make a print for each student. Our students collect their data directly from this photocopy.

When we have determined that our DNA samples have separated adequately, we photograph our gel. We obtain excellent photographs using the versatile Polaroid M4 photographic system. However, very good photographs can be obtained using a hand-held Polaroid camera. The latter system has only one focal length, but is less expensive to purchase. Since the gels and the running buffer contain ethidium bromide, caution must be taken to wear gloves when handling gels.

The gel is removed from the gel box and placed on the UV transilluminator. While 1.5% agarose gels are firm enough to remove from the tray and to place back on the tray after visualization, they are sippery and need to be handled carefully. Type 667 (ASA 3000) film is used to make a photographic copy of the gel. Excellent photographs are obtained using the orange filter that is part of the camera package with the aperture set at f/8 and the shutter speed set at 1/4 second. You may adjust these setting to meet your individual preferences. Once all the photographs have been taken for a laboratory section, they can be enlarged *en masse* so each student can have a copy of all the data.

APPENDIX F
Calculating Molecular Weights of DNA bands

We have found photocopies of gels to be convenient when measuring migration distances of DNA in agarose gels. We measure from the front of the well to the front of each band of DNA. With a little practice, one can quickly learn to estimate fractions of millimeters. It is important to remember that each gel must contain at least one lane of molecular weight marker DNA and that molecular weight marker DNA on one gel may not be used with data from a second gel. In other words, each gel must contain its own molecular weight marker.

Calculating the Molecular Weights of the PCR Products

We use three sequential steps when teaching our students to analyze gels (see the three graphs below for examples of student data). First, we have students plot their data using normal graph paper. They plot the size of the molecular weight marker DNA fragments in base pairs, versus migration distances of those fragments. Next we have them use semi-log graph paper to graph the same data. After mastering semi-log plots, we use standard graph paper again but plot sizes of fragments as $\log_{10}$ base pairs versus migration distances. Of course, these second two graphs are not different from the first graph, but some students need time to recognize the relationship between these three ways of managing the same data. It is important to remember that the migration distance of a molecule moving through a gel is proportional to the $\log_{10}$ of its molecular weight. Threfore, the last two graphs should provide the students with a straight line. Lastly, we have students use the regression program in their calculators to do these chores (see Table I for the regression program for a TI-35X calculator). We find that students need to draw and visualize graphs several times before using calculators effectively. They need to "know" what their machine is really doing.

When our students first perform these experiments, they invariably ask "Why are my data wrong?" or "Why didn't I get the right answer?" This becomes an ideal situation for teaching students by letting them answer their own questions. We suggest to our students that they determine just how "bad" their estimations really are. They will determine that their estimates usually vary less than ten per cent from the actual size of the fragments. If necessary, we point out that 90 per cent accuracy on a test or exam might be very acceptable. This leads our analysis to a discussion of sources of experimental error and precision of techniques.

Practice Data Used to Generate Regression Analysis and 3 Standard Curves

Base Pairs	Log10	Distance Migrated (mm)
6557	3.8167	20
4361	3.6396	23.8
2322	3.3659	28.3
2027	3.3069	30.8
564	2.7513	44

Table I: Regression analysis for model TI-35X calculator to determine sizes of DNA fragments separated by agarose gel electrophoresis.

Procedure	View
1. Press: 3rd, Stat 2	0
2. Press: 2nd, CSR	0
3. Press: 3.8167, X<-->Y, 20, +	1
4. Press 3.6396, X<-->Y, 23.8, +	2
5. Press 3.3659, X<-->Y, 28.3, +	3
6. Press 3.3069, X<-->Y, 30.8, +	4
7. Press 2.7513, X<-->Y, 44, +	5
8. Press 3rd, COR	-0.996339584

To determine the molecular weights of bands (in bp) using the standard curve from above, one would need the distances traveled by the different DNA bands. Below is a series of calculations for several different sized bands (data not shown). The distances each band migrated is the number which follows the word “Press”.

Procedure	*View*
Lane 2. Press 28 (distance in mm), 2nd, X’, 2nd, 10x	2737 (bp)
Lane 3. Press 23, 2nd, X’, 2nd, 10x	4567 (bp)
Lane 4. Press 23, 2nd, X’, 2nd, 10x	4567 (bp)
Lane 5. Press 23, 2nd, X’, 2nd, 10x	4567 (bp)
Lane 6. Press 24.4, 2nd, X’, 2nd, 10x	3957 (bp)
Press 39.5, 2nd, X’, 2nd, 10x	844 (bp)
Lane 7. Press 27.5, 2nd, X’, 2nd, 10x	2882 (bp)
Press 30.3, 2nd, X’, 2nd, 10x	2164 (bp)
Lane 8. Press 25.2, 2nd, X’, 2nd, 10x	3646 (bp)
Press 34.7, 2nd, X’, 2nd, 10x	3679 (bp)
Lane 9. Press 28, 2nd, X’, 2nd, 10x	2738 (bp)
Press 35, 2nd, X’, 2nd, 10x	1338 (bp)
Press 39, 2nd, X’, 2nd, 10x	888 (bp)

Key to symbols and abbreviations*:*

CSR = Clearing Statistical Registers

COR = CORrelation

X’ = 1st deduced value for X in log MW

X⇔Y = exchange key for dependent and independent variables

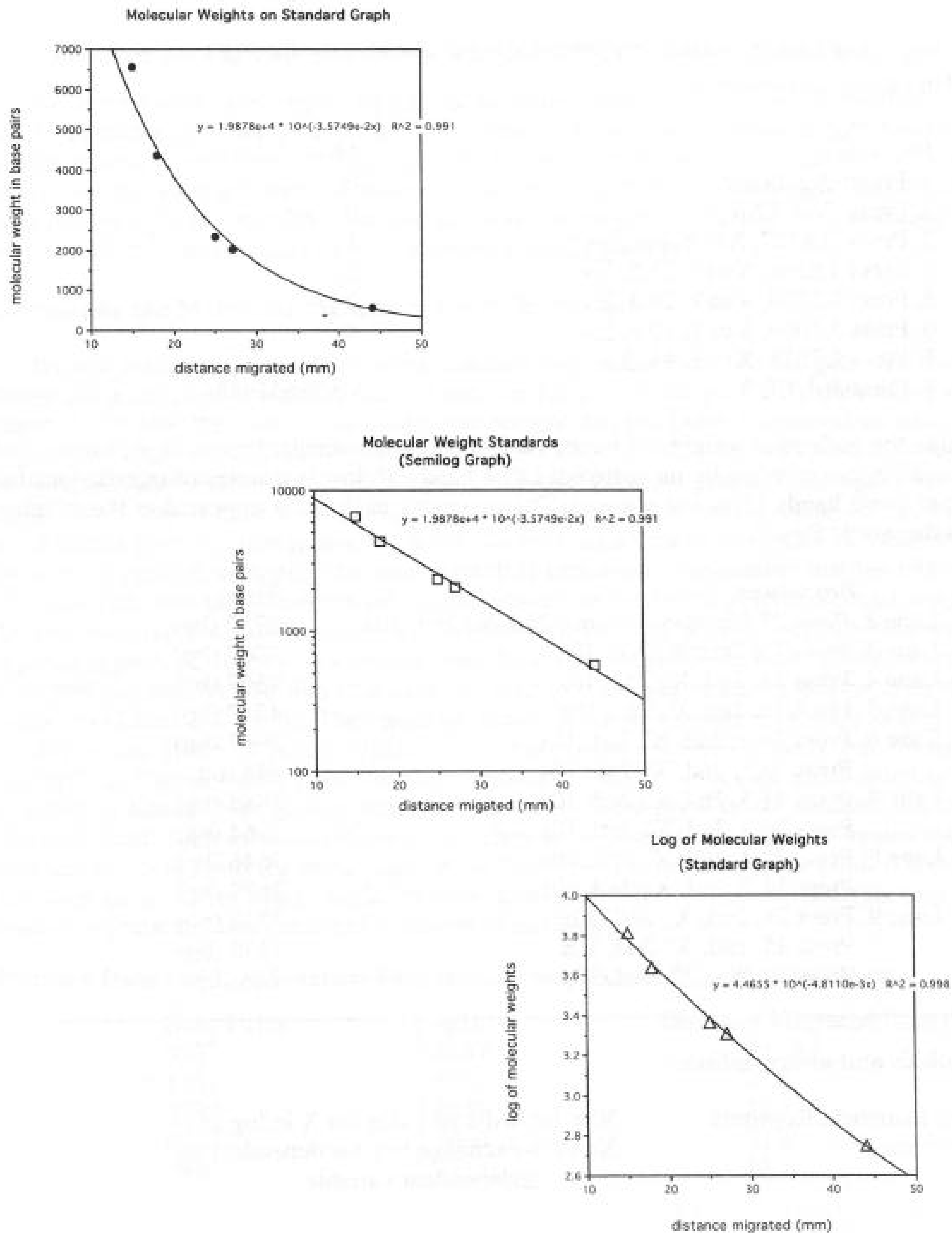

Figure 1..8. Three graphs, generated with a graphing program, depicting the same data (see text above) plotted three different ways. The top graph uses regular graph paper to plot the distance each band migrated (x-axis) as a function of its molecular weight (y-axis). The middle graph uses semi-log paper to generate a straight line with a correlation of 0.991, which is identical to the correlation in the top graph. The bottom graph uses regular graph paper again and plots the distance migrated as a function of the $\log_{10}$ of the molecular weight with a correlation of 0.998. Students can generate similar plots by hand on the appropriate graph paper.

APPENDIX H
Photographs of Gels Obtained from Student Labs

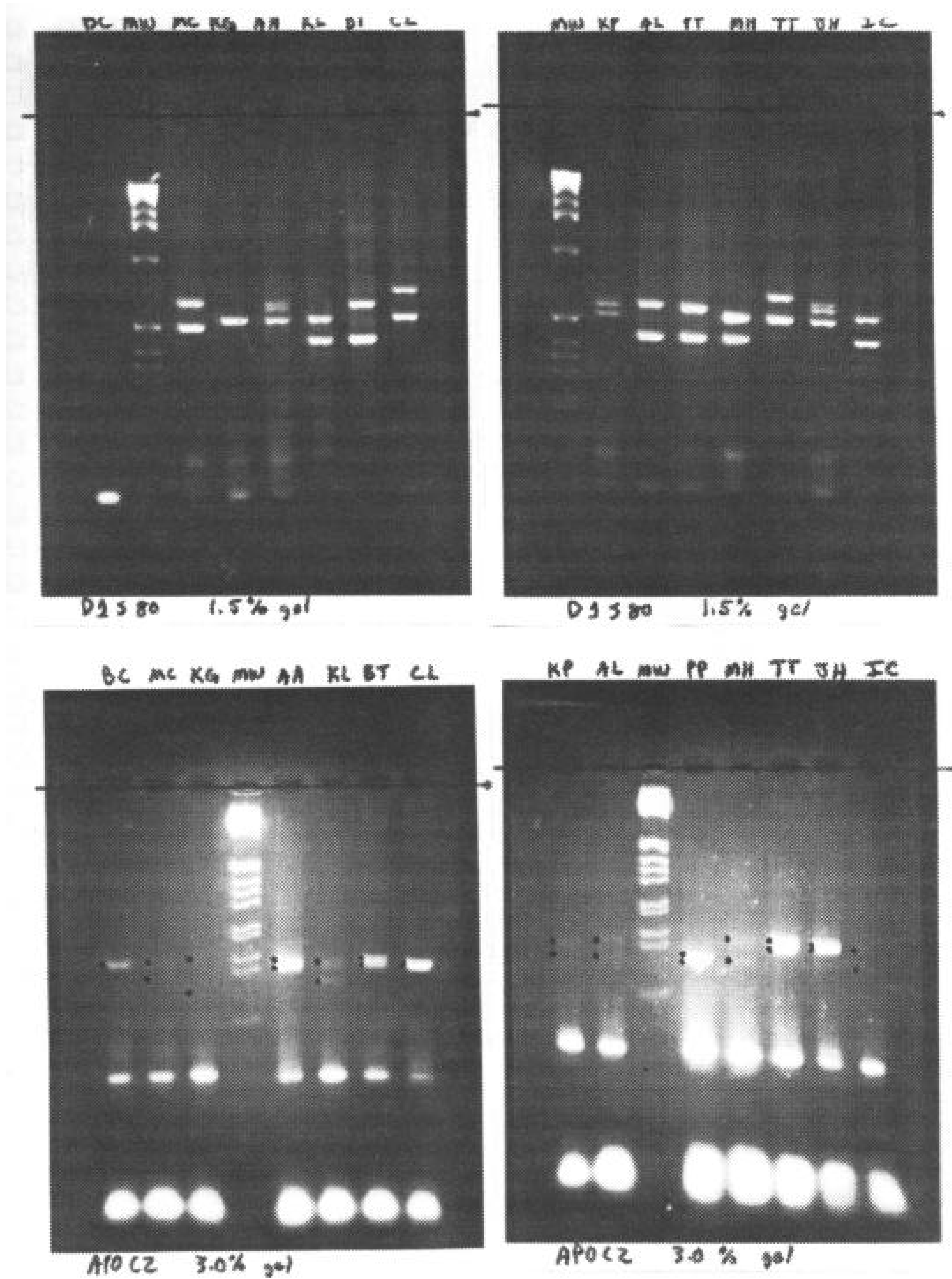

Figure 1.9. The top two gels are 14 different sources of DNA templates analyzed at the D1S80 locus. The bottom two gels are the same 14 different samples analyzed at the APOC2 locus.

APPENDIX I

Molecular Weight Markers (one KB Ladder on 1.5% Gel)

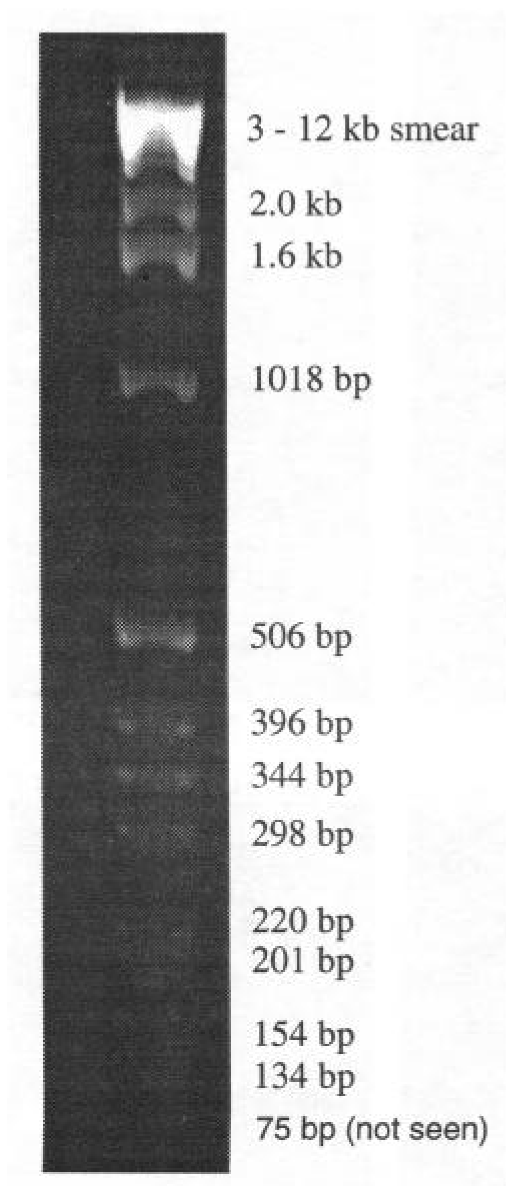

Figure 1.10. Photograph of one KB molecular weight markers that were electrophoresed on a 1.5% agarose gel. The molecular weights (in kilobases or base pairs) are listed next to the appropriate bands.

APPENDIX J

Checklist For Equipment and Reagents

agarose: (low EEO) Fisher #BP160-100 or equivalent
proteinase K Sigma # P2308 10 mg
5X TBE:
micropipets (P200 and P20): x4 or more for each size
yellow tips
parafin oil: grocery store quality (only needed if PCRmachine does not have heated lid)
parafilm: 4" roll
toothpicks: grocery store qualitye
tweezers: drugstore quality
microfuge tubes: (500 ul) (1.5 ml) for preparation of stock solutions
microfuge tubes: (1.5 ml) for preparation of stock solutions
photo setup and UV box: Polaroid is good
microwave: standard, should hold 250 ml flasks
250 ml flasks
saran wrap: 5 ft long or more
hot gloves
ethidium bromide solution: (10mg/ml stock) - 1 ml
balance: (0.01 - 10 gram range minimum)
weigh paper
small spatulas
distilled water
graduated cylinders: 1 liter and 100 ml
lab tape: Fisher # 11-880-20F
PCR machine that holds 500 µl microfuge tubes
VWR lab markers: (black; extra fine tips) #52877-150
microfuge tube racks
gel molds, boxes and power supplies
crushed ice and ice buckets
microfuge (nice to have but optional)
-20° C freezer (preferably frost free) and +4° C refrigerator