CHAPTER 4

Out of Africa: Origin of Modern Humans

Introduction

The goal of this experiment is to analyze linkage disequilibrium between the *Alu* locus in the CD4 gene on chromosome 12 and the short tandem repeat (STR) locus located near by. We will calculate the extent of linkage disequilibrium between these genes for the whole class "population" and compare it with known linkage disequilibrium calculated for different non-African populations. Each student will determine his or her haplotypes using a PCR (polymerase chain reaction) and determine to which world populations they most probably belong.

During the course of this experiment, students will learn how to perform a PCR, use the thermal cycler, and analyze products using high-resolution agarose gel electrophoresis.

This experiment will take two laboratory periods. During the first laboratory period students will assemble and run a PCR. The second laboratory period will be dedicated to agarose gel electrophoresis and analysis of the results. A schematic outline of the experiment is shown in Fig. 4.1.

Background

Origin of humans

Humans arose from an ancestor common to contemporary primates that were present on earth for at least 35 million years. We diverged from the ancestor of our closest primate relatives, the chimpanzees, approximately 4–5 million years ago. These humanoids, which still retained an ape-like body shape, spread across all of Africa and probably the entire world. They are represented by ardipithecines and the more specialized australopithecienes.

The first evidence of animals that are more human-like also came from Africa and they date from approximately 2 million years ago (*Homo ergaster*).

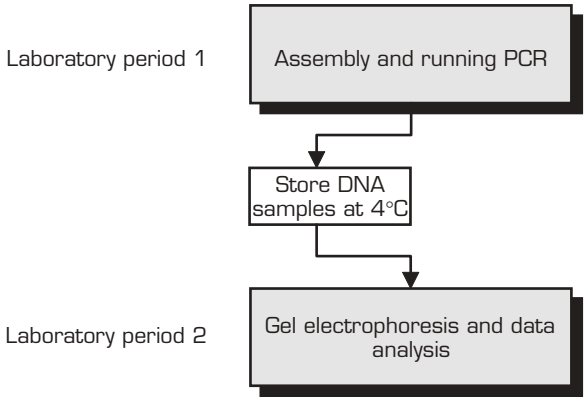

Figure 4.1 Schematic outline of experiment 4.

These animals or probably a variant of them, *Homo erectus*, emigrated from Africa approximately 1.7 million years ago as evidenced by finding *H. erectus* skeletons all over Eurasia (e.g. China and Java).

These findings led to a model of modern human evolution named the **multiple-origin model**. This theory states that major human races split one from another at the time of *H. erectus* dispersal from Africa. These groups evolved more or less independently (with some gene mixing) to modern humans (*Homo sapiens*). This version of our origin appears not to be true and has no serious advocates today.

The second hypothesis is known as the **out-of-Africa replacement (OAR)** hypothesis and is widely accepted today. In this model of human evolution, anatomically modern humans (*H. sapiens*) evolved first in Africa, probably from *H. erectus*, approximately 150,000–200,000 years ago. A small part of this group colonized Eurasia and the rest of the earth by migrating from Africa approximately 100,000 years ago driving local populations of *H. erectus*-like species to complete genetic extinction (**replacement**) without any or very little gene exchange. The migration from Africa may have occurred in a single wave or in multiple waves.

Evidence for the OAR hypothesis comes from three lines of reasoning. First is the observation of a large genetic diversity in African populations versus the rest of the world. This indicates that the African population is the oldest and has had more time to accumulate genetic variations. Large genetic variability in African populations was shown to exist for mitochondrial DNA, Y chromosome micro-satellites, and autosomal mini-satellites.

Second, phylogenetic analysis of nearly every genetically variant locus shows evolutionary trees the first branch of which separates African from non-African. This indicates that every polymorphic variant studied can be traced back to the African population. For example, well-known studies of maternally inherited mitochondrial DNA indicate that African sub-Sahara

populations have much higher genetic diversity than non-Africans. Moreover, each type of mitochondria of non-African populations can be traced back through the maternal lineage to one of African diversity, i.e. to ancestral females (Eve?) that existed in Africa between 100,000 and 300,000 years ago (Cann et al., 1987; Richards et al., 1996). The interpretation of the original "mitochondrial Eve" data has been hotly debated, but recent reinterpretation of these data and the emergence of some new data based on DNA sequencing confirms the original interpretation. Similar results were obtained for Y chromosome polymorphic sites. Since this chromosome is inherited thought the paternal line, the analysis also traces the origin of males (Y chromosome – Adam?) to the African population of humans (Semino et al., 1996; Casalotti, et al., 1999; Seielstad et al., 1999).

The third evidence comes from analysis of the age of the "genetic" ancestors of the non-African human population. This population is very young, being separated from the African population approximately 100,000 years ago (emigration from Africa).

In our experiment we will use one of the most ingenious experimental proofs existing for the OAR hypothesis introduced by Tishkoff et al. (1996). These authors used decay of linkage disequilibrium between two closely linked genes on chromosome 12 to prove the out-of-Africa hypothesis.

Linkage disequilibrium is defined as the non-random association between two polymorphic loci (e.g. A and B) on the same chromosome. The term "polymorphic" (meaning many forms) describes the state of a single genetic locus that exists in two or more forms at significant frequencies in the population (above 1 percent). When mutation of one of these loci occurs, e.g. of gene A, this mutation creates another polymorphic form of this gene. This mutation occurs on a particular chromosome on which a specific polymorphic variant of another gene, gene B, resides. Thus, these two specific polymorphic variants of both genes are now together on the same chromosome and are transferred from one generation to another more frequently together (no recombination) than separately (recombination). They are said to be in linkage disequilibrium. This association will decay with successive generations as recombination breaks the original association.

The rate of the breakage of this association (linkage disequilibrium disassociation) depends on the distance between both loci and the number of successive generations. Thus, it is possible to reconstruct recent evolutionary history using such closely linked markers.

In our experiment, you will use two linked markers located 9.8 kb apart. One of these markers is the *Alu* short interspersed nuclear element (SINE) and the other is an STR. *Alu* elements are approximately 300 bp in length and derive their name from a single recognition site for the endonuclease *Alu*I, which is located near the middle of it. The *Alu* element is the most abundant sequence in the human genome. The human genome contains approximately 1 million *Alu* repeats comprising an estimated 10 percent of

the genome. They are predominantly located in non-coding, intragenic locations, particularly in introns. The *Alu* element is thought to be derived from the 7 SL RNA gene that encodes the RNA component of a signal recognition particle that functions in protein synthesis.

The *Alu* element polymorphism used in our study resulted from the deletion of the *Alu* in locus CD4 located on chromosome 12. This element is present at this position in all primates and in approximately 82 percent of the chromosomes (haplotypes) in the African population. This indicates that the *Alu*(+) haplotype is the ancestral state and deletion of *Alu* (*Alu* polymorphism) occurred after divergence of humans from the great apes.

The second polymorphic marker that we will be using is an STR polymorphism consisting of a tandem repeated block of five nucleotides TTTTC. This locus has 12 alleles that differ by the number of repeats (four to 15). Only three of these alleles are seen in the human population outside of Africa at a frequency greater than 10 percent. These are STR 85 (five repeats), STR 90 (six repeats), and STR 110 (ten repeats) alleles. All of the great ape species have very limited polymorphism at this locus (three or six repeats), indicating that most of the STR polymorphic human alleles appeared after humans diverged from the great apes.

In this experiment oligonucleotide primers flanking the *Alu* insertion site will be used for amplifying a 661 bp fragment when *Alu* is present and a 438 bp fragment when it is deleted. Because humans are a diploid organism, containing two chromosome 12s, one from the father and the other from the mother, three genotypes are possible.

1. Homozygotic for the absence of *Alu*. In this case both chromosome 12s do not have the *Alu* locus. There will be only one PCR product, which is 438 bp long.

2. Homozygotic for the presence of *Alu*. In this case both chromosome 12s contain the insertion. There will be only one PCR product, which is 661 bp long.

3. Heterozygotic for the presence of *Alu*. There will be two PCR products, one which is 438 bp long and the other which is 661 bp long.

All three genotypes can be distinguished from each other following agarose electrophoresis of the PCR products.

The STR marker will be amplified using two primers (TT-F and TT-R) that are approximately 30 bp from the STR locus. Figure 4.2 shows the relative positions of the *Alu* and STR on chromosome 12.

Initially in our evolution, the *Alu*(–) allele was generated by the deletion of *Alu* on the chromosome that contained the "ancestral" STR 90 (six repeats). Thus, at this time there was total linkage disequilibrium between these alleles. In time, due to mutations and recombination, this association was lost in the ancestral African population. However, when some of the ancestral African population left Africa and populated the rest of the world most of them carried chromosome 12 containing the *Alu*(–) STR 90 haplotype. Thus, this combination of alleles (haplotype) shows almost

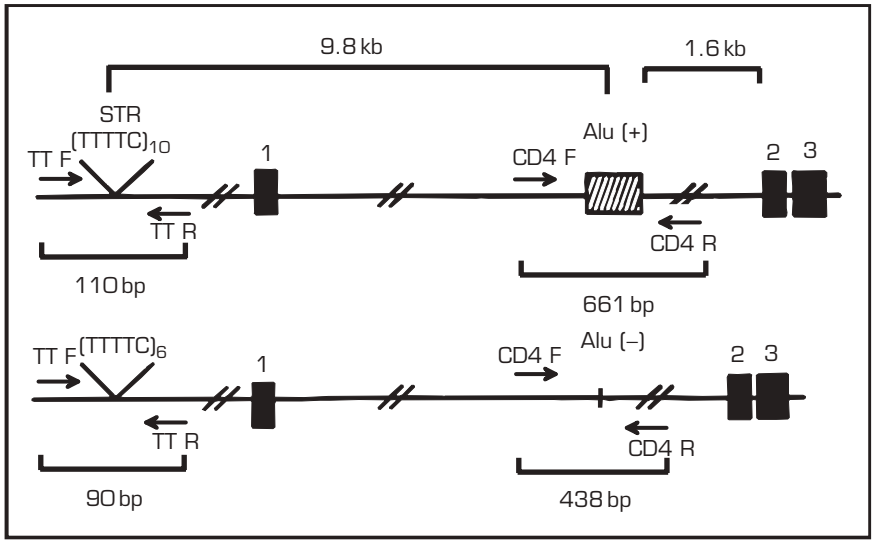

Figure 4.2 Positions of polymorphic markers at the CD4 locus on chromosome 12. Two haplotypes (chromosomes) are shown: *Alu*(+) STR with ten repeats (110 bp) and Alu(–) STR with six repeats (90 bp). Arrows indicate the positions of the *Alu* primers and STR primers. Solid blocks represent exons of the CD4 gene.

complete linkage disequilibrium in the non-African populations. Two other haplotypes, *Alu*(+) STR 85 and *Alu*(+) STR 110, were also present in a small population that migrated from Africa and are now distributed throughout the world.

The non-random association between the CD4 STR alleles and *Alu*(–) allele (linkage disequilibrium) can be calculated by estimating the excess of ancestral haplotype *Alu*(–) and any one of the STR alleles in non-African populations. This value is usually called P_{excess} or δ and is equal to

$$\delta_{-,x} = \frac{P_{-,x} - P_{+,x}}{1 - P_{+,x}} \tag{4.1}$$

where $P_{-,x}$ is the frequency of haplotype *Alu*(–) and any STR allele (e.g. STR 110) and $P_{+,x}$ is the frequency of haplotype *Alu*(+) and the same STR allele as above (e.g. STR 110). The δ values for world populations are presented in Table 4.1 and Table 4.2 presents the frequency estimates for Alu STR haplotypes for different world populations.

PCR

The PCR is a powerful method of *in vitro* DNA synthesis. Large amounts of a specific segment of DNA of defined length and sequence can be

Table 4.1 Values of δ for the three *Alu*(–) STRs in some world populations

Population	STR 85	STR 90	STR 115
Non-African			
Combined	–	0.98	–
Northeast Africa			
Egyptian	–	1.0	–
Ethiopian Jewish	–	0.61	–
Somalian	0.18	0.67	–
Sub-Sahara Africa			
Woloff	0.41	0.22	–
Mbuti	0.46	0.19	0.18
Bantu speakers	–	0.26	0.29
Kikuyu	–	0.11	0.64
Herero	0.01	0.38	0.13
Biaka	0.38	–	–
Nama	–	0.27	0.18

synthesized from a small amount of template. The technique has revolutionized molecular biology and is used in virtually every area of natural sciences and medicine.

The principal of a PCR is rather simple and involves enzymatic amplification of a DNA fragment flanked by two oligonucleotides (primers) hybridized to opposite strands of the template, the 3′-ends of which are facing each other. DNA polymerase synthesizes new DNA starting from the 3′-end of each primer. Repeated cycles of heat denaturation of the template, annealing of the primers, and extension of the annealed primers by DNA polymerase results in amplification of the DNA fragment. The extension product of each primer can serve as a template for the other primer, resulting in essentially doubling the amount of the DNA fragment in each cycle. The result is an exponential increase in the amount of a specific DNA fragment defined by the 5′-ends of the primers.

The products of a PCR will include, in addition to the starting DNA, an amount of a specific target sequence that can easily be visualized as a discrete band of a specific size by agarose gel electrophoresis. The practical consequence of the chain reaction is that one can start with nanogram amounts of DNA, carry out a PCR, and then run the sample on a gel in order to visualize a specific band corresponding precisely to the distance (including the length of the primers themselves) between the two primers used.

PCR kinetics

During the PCR reaction, products from one cycle serve as a template for DNA synthesis in the next cycle. Theoretically, the amount of product

Table 4.2 The percentage of *Alu* STR haplotypes for some world populations

All STR		STR 80		STR 85		STR 90		STR 100		STR 110		STR 115		STR 120		STR 125		STR 130	
Alu+	**Alu–**	**Alu+**	**Alu–**	**Alu+**	**Alu–**	**Alu+**	**Alu–**	**Alu+**	**Alu–**	**Alu+**	**Alu–**	**Alu+**	**Alu–**	**Alu+**	**Alu–**	**Alu+**	**Alu–**	**Alu+**	**Alu–**
New World																			
98	2.0	–	–	55.3	–	0.1	1.8	–	–	42.1	–	0.4	–	0.1	–	–	–	–	–
Pacific Island and Australo-Melanesian																			
98.7	1.3	–	–	72.7	–	1.3	0.7	–	–	22.7	–	1.3	–	–	–	–	–	–	–
Asian																			
95.1	4.9	–	–	60.3	0.1	1.1	3.7	0.3	–	31.0	–	2.3	–	0.2	–	–	–	–	–
European																			
72.4	27.6	–	–	36.1	0.1	1.0	27.6	–	–	28.9	0.1	3.7	–	–	–	–	–	–	–
Middle Eastern																			
68.0	32.0	–	–	33.5	0.5	1.5	30.5	–	0.5	24.0	–	4.5	–	2.0	–	2.5	–	–	–
Northeast African																			
79.2	20.8	–	–	27.3	2.0	1.0	16.0	–	0.4	28.0	–	11.3	–	6.3	–	0.6	–	2.7	–
Sub-Sahara African																			
82.2	17.8	0.2	–	18.7	4.6	2.3	4.2	7.4	0.7	17.2	–	15.6	5.4	10.7	0.7	2.4	0.5	2.1	0.1

doubles each cycle making the PCR process a true chain reaction, which is described by the equation

$$N = N_0 \times 2^n \tag{4.2}$$

where N is the number of amplified molecules, N_0 is the initial number of molecules, and n is the number of amplification cycles. This equation holds true if the efficiency of amplification (E), which is defined as the fraction of template molecules that take part in amplification during each cycle, is 1.0. An equation that describes the amplification process better, taking into account the efficiency of the process, is

$$N = N_0 \times (1 + E)^n \tag{4.3}$$

where E is the amplification efficiency.

Experimentally the accumulation of product during the course of the reaction is far from the case described by equation (4.3). This is primarily the result of changes in the amplification efficiency during the course of the reaction. At the beginning, the efficiency of amplification is close to 1.0 (0.8–0.97) and accumulation of the product proceeds exponentially. This constitutes the exponential phase of the reaction. During late PCR cycles accumulation of the product slows down and eventually stops. This effect is usually referred to as the "plateau effect" and occurs after accumulation of 0.3–1.5 pmol of amplification product (Innis et al., 1990; Sardelli, 1993).

The number of cycles it takes to reach a plateau depends mainly on the initial number of molecules present in the reaction (N_0). However, a plateau can be influenced by a number of conditions, such as the following.

1. Inhibition of polymerase by pyrophosphate.
2. Utilization of substrate.
3. The temperature of cycling.
4. The stability of enzyme and reagents.
5. The presence of impurities.
6. Reannealing of product at high concentration.
7. Incomplete strand separation at high concentrations of product.
8. Formation of primer dimers.
9. The GC content of the template.

Reaching a plateau should be avoided since it increases the probability of obtaining non-specific amplification products. Choosing the correct number of PCR cycles is one way of avoiding false initiation products. This can be achieved by correctly choosing the initial concentration of target DNA molecules. Since most standard amplification protocols use 30 amplification cycles, varying the concentration of target DNA is most frequently employed as a means of avoiding reaching a plateau.

It is important to emphasize that failure to obtain amplification products

commonly occurs due to too high a concentration of DNA in the reaction. This is because the plateau effect is for the most part a consequence of a high DNA concentration in the reaction. This can come about from *de novo* DNA synthesis or from too high a concentration of template (N_0 in equation (4.3)).

Rapid cycle DNA amplification

DNA amplification requires temperature cycling of the sample. This is usually performed using a thermal cycler instrument. Reactions of denaturation of DNA and annealing of primers are very fast kinetic reactions requiring only a few milliseconds to occur at the DNA and primer concentrations used in a PCR. Most of the commercial instruments utilize metal blocks for thermal equilibration and samples are contained in plastic microfuge tubes. In these instruments very fast temperature changes are not possible. A significant fraction of the cycle time in these machines is spent on heating and cooling of the blocks and tubes and the liquid contained in them. This extends amplification times to several hours, a much longer time than is kinetically necessary for carrying out all the steps of an amplification reaction.

Moreover, extended amplification times and long transition times make it difficult to determine the optimal temperature and times for each stage of the PCR reaction. This results in many false initiations by polymerase and, consequently, leads to poor product specificity. Rapid temperature transitions and small sample volumes improve both specificity and the time necessary for carrying out PCR reactions.

The instrument that makes it possible to carry out a PCR at speeds close to those theoretically required is an air thermal cycler (Wittwer and Garling, 1991; Wittwer et al., 1994). This rapid thermal cycler instrument uses heat transfer by hot air to samples contained in thin, glass capillary tubes. Because of the low heat capacity of air and the thin walls and increased surface area of capillary tubes, samples can be heated and cooled very quickly. The total amplification time in these instruments for 30 PCR cycles is 15–20 minutes and the volume of reaction is 10 μl. This makes it an ideal instrument for teaching applications. The specificity of amplification is also dramatically increased as compared to standard instruments and the cost of the instrument is low compared to standard thermal cyclers. We will be using this instrument for our amplification reactions.

FIRST LABORATORY PERIOD

In this laboratory period you will assemble and run two PCR reactions. The first reaction will amplify the region of chromosome 12 where the *Alu* SINE element is located. The second reaction will amplify the region of chromosome 12 where the polymorphic STR locus resides (see Fig. 4.2 for details).

Technical tips

The failure to amplify product or very weak amplification usually results from a failure of some component of the reaction. Most frequently this is due to primer degradation. Special care should be taken to prevent this degradation.

Using too high a concentration of DNA template is the second most frequent cause of reaction failure. To amplify human DNA sequences, the concentration of genomic DNA in the reaction should not exceed 100 ng. Careful determination of DNA concentration in the sample is paramount to successful amplification of DNA sequences.

The ability of a PCR for amplifying minute amounts of DNA template requires unusual care in order to avoid contamination with none-target DNA. Each pipette tip should be used only once and use of tips with barriers in order to avoid contaminating the pipettors, reagent stocks, and reaction is strongly recommended. In addition, to further avoid contaminating stock solutions, template DNA should be always added to the reaction mixture last.

In order to avoid DNase contamination of the reagents gloves should be worn all of the time and all buffer solutions should be autoclaved. Moreover, each experiment should always include a negative control reaction without template DNA.

Protocol

Running PCRs

1. Label 12 microfuge tubes (1.5 ml) as 1A, 2A, 3A, 4A, 5T, 6T, 7T, 8T, CA, CT, RSMA, and RSMT. Place the tubes on ice.

2. Dilute your DNA to a concentration of 50 ng μl^{-1}. For example, if the concentration of your DNA is 0.6 $\mu g\,\mu l^{-1}$ (i.e. 600 ng μl^{-1}) you will need to dilute your DNA 12 times (600 : 50 = 12). To prepare this dilution, add 50 µl of water to the microfuge tube. Prepare a wide-bore yellow tip by cutting off 2–3 mm from the end with a razor blade. Withdraw 4 µl (50 : 12 = 4.1) of

water from the tube and mark the level of the solution on the tip with a marking pen. Discard the water from the tip and withdraw DNA solution to the mark. Add the DNA to the tube with water and pipette up and down several times in order to remove the viscose DNA solution from the inside of the tip.

3. Prepare the reaction stock mixture for the PCR *Alu* reaction (RSMA). Add the reagents to the RSMA tube as described in Table 4.3. Start with the addition of water and buffer. Add enzyme last. Mix all of the ingredients by pipetting up and down. Centrifuge the tube for 30 seconds and place it on ice.

4. Prepare the reaction stock mixture for the PCR STR reaction (RSMT). Add the reagents to the RSMT tube as indicated in Table 4.4. Start with the addition of water and buffer. Add enzyme last. Mix all of the ingredients by

Table 4.3 Reaction stock mixture for *Alu* amplification (RSMA)

Ingredients concentration	Add for six reactions	Amount for one reaction	Final concentration
Buffer (ten times, low Mg^{2+})	6.0µl	1.0µl	one times
4dNTP (2.0mM)	6.0µl	1.0µl	200.0mM
Primer 1 *Alu* F (5.0µM)	6.0µl	1.0µl	0.5µM
Primer 2 *Alu* R (5.0µM)	6.0µl	1.0µl	0.5µM
Acetamide (50%)	3.0µl	0.5µl	2.5%
Enzyme (5 u μl^{-1})	1.2µl	0.2µl	1 u/10µl
Water	25.8µl	4.3µl	
Total	54.0µl	9.0µl	

Table 4.4 Reaction stock mixture for STR (RSMT)

Ingredient concentration	Add for six reactions	Amount for one reaction	Final concentration
Buffer (ten times, high Mg^{2+})	6.0µl	1.0µl	one times
4dNTP (2.0mM)	6.0µl	1.0µl	200.0mM
Primer 1 TT F (5.0µM)	6.0µl	1.0µl	0.5µM
Primer 2 TT R (5.0µM)	6.0µl	1.0µl	0.5µM
Acetamide (50%)	3.0µl	0.5µl	2.5%
Enzyme (5 u μl^{-1})	1.2µl	0.2µl	1 u 10µl^{-1}
Water	25.8µl	4.3µl	
Total	54.0µl	9.0µl	

Table 4.5 Running PCR

	Tube number									
	1A	**2A**	**3A**	**4A**	**CA**	**5T**	**6T**	**7T**	**8T**	**CT**
RSMA (*Aul* mixture)	9 µl	9 µl	9 µl	9 µl	9 µl	–	–	–	–	–
RSMT (TT mixture)	–	–	–	–	–	9 µl	9 µl	9 µl	9 µl	9 µl
Your DNA	1 µl	–	–	–	–	1 µl	–	–	–	–
Partner, DNA	–	1 µl	–	–	–	–	1 µl	–	–	–
Control 1 DNA	–	–	1 µl	–	–	–	–	1 µl	–	–
Control 2 DNA	–	–	–	1 µl	–	–	–	–	1 µl	–
Water	–	–	–	–	1 µl	–	–	–	–	1 µl

pipetting up and down. Centrifuge the tube for 30 seconds and place it on ice.

5. Prepare the reactions to run a PCR for *Alu* and TT amplifications. **Keep all tubes on ice.** Add 9 µl of RSMA to the first five tubes and RSMT to the remaining tubes as indicated in Table 4.5. Tubes 1, 2, 5, and 6 will contain your and your partner's DNA, respectively. Tubes 3, 4, 7, and 8 will have control DNA. Tubes CA and CT are controls without DNA.

6. Mix each tube briefly by pipetting up and down and centrifuge for 10 seconds. Place the tubes into an ice bucket.

7. Load the reactions into 10 µl capillary tubes. Insert the open end of the capillary tube into the white silicon end of the micro-dispenser approximately 5 mm deep and draw the reaction mixture into it by slowly turning the micro-dispenser knob counterclockwise. Position the liquid in the middle of the capillary and seal by flaming the end. Only a few seconds of heating the extreme tip of the capillary tube is necessary. Check the seal by gently turning the micro-dispenser knob back and forth. If the liquid does not move in the tube, the tube is sealed. Flame seal the other end of a capillary tube. Place the sealed tube into a microfuge tube **with the end sealed last on top**.

8. Set the cycling condition as follows: D (denaturation) = 94°C for 2 seconds, A (annealing) = 55°C for 2 seconds, and E (elongation) = 72°C for 30 seconds. Start with initial denaturation at 94°C for 2 minutes and finish the reaction with an extension step at 72°C for 5 minutes. Run the reactions for 30 cycles with the S setting on 9.

9. Place the capillary tubes into the DNA thermal cycler inserting the first sealed end into the holder. Start the machine and observe the end of the capillary tube extruding from the holder carefully. If the second end is improperly sealed, liquid will rise to the top of the capillary tube when the

temperature reaches approximately 90°C. If this happens, stop the cycler and reseal the open end. Restart the cycling again.

10. After cycling is complete, remove the capillary tubes and place them into appropriately labeled 1.5 ml centrifuge tubes. Store tubes in the refrigerator until the next laboratory period.

SECOND LABORATORY PERIOD

In this laboratory period, you will analyze the results of the PCR reactions prepared in the previous laboratory period. You will use agarose gel electrophoresis for this analysis. The products of STR TT reactions will be analyzed using high resolution MetPhor™ agarose gel electrophoresis. The products of the *Alu* element amplification will be analyzed on a regular agarose gel using TBE (Tris–borate EDTA) buffer. This buffer increases the resolving power of standard agarose gels.

Safety precautions

Ethidium bromide is a mutagen and suspected carcinogen. Contact with the skin should be avoided. Wear gloves when handling ethidium bromide solution and gels containing ethidium bromide.

For safety purposes, the electrophoresis apparatus should always be placed on the laboratory bench with the positive electrode (red) facing away from the investigator, that is away from the edge of the bench. To avoid electric shock always disconnect the red (positive) lead first.

Ultraviolet (UV) light can damage the retina of the eye and cause severe sunburn. Always use safety glasses and a protective face shield to view the gel. Work in gloves and wear a long-sleeved shirt or laboratory coat when operating UV illuminators.

Protocol

High resolution agarose electrophoresis: analysis of the results of TT amplification

This procedure uses MetaPhor™ agarose that has twice the resolution capabilities of standard agarose. DNA fragments that differ by only 4 bp can be resolved using this matrix in the size range between 40 and 800 bp. MetaPhor™ agarose has approximately the resolution power of standard polyacrylamide gels.

1. Use a mini-gel-casting tray when working with this agarose (e.g. gel size 7.5 cm × 7.5 cm × 0.4 cm). Preparing smaller gels limits the cost and does not affect the resolution of DNA bands. Seal the ends of the gel-casting tray with tape. Regular labeling tape or electrical insulation tape can be used. Check that the bottom of the comb is approximately 0.5 mm above the gel bottom. To adjust this height it is most convenient to place a plastic charge card (for example MasterCard) at the bottom of the tray and adjust the comb height to a position where it is easy to remove the card from under the comb.

2. Prepare 500 ml of one times TBE buffer by adding 50 ml of a ten times TBE buffer stock solution to 450 ml of deionized water.

3. Prepare a 3.5 percent MetaPhor™ agarose mini-gel in one times TBE buffer. Place 30 ml of the buffer into a 250 ml Erlenmeyer flask and add 1.0 g agarose. Allow the powder to swell in the buffer for at least 25 minutes. Melt the agarose by heating the solution in a microwave oven at full power for 20–30 seconds at a time until the agarose is fully dissolved. The MetaPhor™ agarose is more difficult to melt than regular agarose since it "boils over" very easily. If evaporation occurs during melting, adjust the volume to 30 ml with deionized water.

4. Cool the agarose solution to approximately 60°C and add 1 µl of ethidium bromide stock solution. Slowly pour the agarose into the casting tray. Remove any air bubbles by trapping them in a 10 ml pipette.

5. Position the sample comb at approximately 1.0 cm from the edge of the gel. Let the agarose solidify for approximately 15 minutes. In order to achieve maximum resolution, after the MetaPhor™ has solidified transfer the gel to a 4°C refrigerator for 25 minutes.

6. Remove the comb with a gentle back and forth motion, taking care not to tear the gel. Remove the tape from the ends of the gel-casting tray and place the tray on the central supporting platform of the buffer chamber.

7. Add electrophoresis buffer to the buffer chamber until it reaches a level approximately 0.5–1 cm above the surface of the gel.

8. First load 14 µl of a low molecular weight standard in the first well of the gel using a P20 Pipetman.

9. Next load TT series samples into following wells. Do not skip wells between samples. Holding the capillary end tightly with two fingers 5 mm from the end gently snap off the end. Insert the open end of the capillary tube into the white silicon tip of the micro-dispenser, approximately 5 mm deep. Holding the other end of the capillary with two fingers snap it off.

10. Remove the amplified sample from the capillary tube by slowly turning the micro-dispenser knob clockwise. Do not insert the end of the capillary tube into the well; keep the end above the well. Place the tip **under** the surface of the electrophoresis buffer and **above the sample well** opening. Deliver the sample slowly, allowing it to sink to the bottom of the well. During loading it is very important not to place the tip into the well or touch the edge of the well with it. This can damage the well resulting in uneven or smeared bands.

11. Place the lid on the gel box and connect the electrodes. DNA will travel towards the positive (red) electrode positioned away from the edge of the laboratory bench. Turn on the power supply and run gel electrophoresis for 20–30 minutes at 60–80 V.

12. Turn the power off and disconnect the leads from the power supply. To avoid electric shock always disconnect the red (positive) lead first.

13. Remove the gel tray from the electrophoresis chamber and place it on a UV illuminator. Photograph the gel using Polaroid film 667 and a Polaroid camera at speed 1 and F stop 8 or use a computer imaging system for recording the results.

High resolution agarose electrophoresis: analysis of the results of Alu *amplification*

A gel prepared in TBE buffer will be used for this analysis. Agarose gel prepared in TBE buffer has lower porosity than TAE (Tris–acetate EDTA) buffer-prepared gels. The DNA mobility in this buffer is approximately two times slower than in TAE buffer, but the DNA bands are sharper. This gel has the best resolving power for DNA fragments of less than 1,000 bp.

1. Prepare a 1.2 percent agarose mini-gel in one times TBE buffer. Use a mini-gel-casting tray (e.g. gel size 7.5 cm × 7.5 cm × 0.4 cm). Seal the ends of the gel-casting tray with tape. Regular labeling tape or electrical insulation tape can be used. Check that the bottom of the comb is approximately 0.5 mm above the gel bottom as described in step 1 of the previous procedure.

2. Use 30 ml of agarose solution. Weigh 360 mg of agarose and add it to 30 ml of one times TBE buffer. Melt the agarose in a microwave oven, adjust the volume to 30 ml with water, and add 1.0 µl of ethidium bromide. Pour immediately into the gel form. Let it solidify for 20–30 minutes.

3. First load 14 µl of a low molecular weight standard in the first well of the gel using a P20 Pipetman.

4. Next load *Alu* series samples into consecutive wells. Do not skip wells between samples. Holding the capillary end tightly with two fingers gently snap it off 5 mm from the end. Insert this end of the capillary tube into the white silicon tip of the micro-dispenser, approximately 5 mm deep. Holding the other end of the capillary with two fingers snap it off.

5. Remove the amplified sample from the capillary tube by slowly turning the micro-dispenser knob clockwise. Do not insert the end of capillary tube into the well and keep the end above the well. Place the tip **under** the surface of the electrophoresis buffer and **above the sample well** opening. Deliver the sample slowly, allowing it to sink to the bottom of the well. During loading it is very important not to place the tip into the well or touch the edge of the well with it. This can damage the well resulting in uneven or smeared bands.

6. Place the lid on the gel box and connect the electrodes. DNA will travel towards the positive (red) electrode positioned away from the edge of the laboratory bench. Turn on the power supply and run gel electrophoresis for 30 minutes at 110 V.

7. Turn the power off and disconnect the leads from the power supply. To avoid electric shock always disconnect the red (positive) lead first.

8. Remove the gel tray from the electrophoresis chamber and place it on a

UV illuminator. Photograph the gel using Polaroid film 667 at speed 1 and F stop 8 or use a computer imaging system to record the results.

Data analysis

Examine your *Alu* sample gel and determine to which *Alu* haplotype you belong. The presence of two bands indicates that you are heterozygote. A single band indicates you are a homozygote with or without the *Alu* insertion. Look for examples in Fig. 4.3.

Examine your TT sample gel. Determine your haplotype for STR elements. Consult Fig. 4.4 for examples of band locations and their sizes.

Using the data collected above, determine haplotype *Alu* and STR for both of your chromosomes. For example, individual 1 is heterozygotic for *Alu* insertion (well 1 in Fig. 4.3) and homozygotic for STR 85 (well 1 in Fig. 4.3). The haplotypes are as follows: *Alu*(+) STR 85 and *Alu*(–) STR 85. Inspection of Table 4.2 will indicate that this individual cannot belong to New World or Pacific Island populations.

In some instances exact determination of each haplotype will not be possible using the data collected. This situation occurs if you are heterozygotic for both loci. For example, individual 2 in Fig. 4.3 is heterozygotic for *Alu* insertion and also heterozygotic for STR 110 and STR 90 (Fig. 4.4). This person can have the following haplotypes.

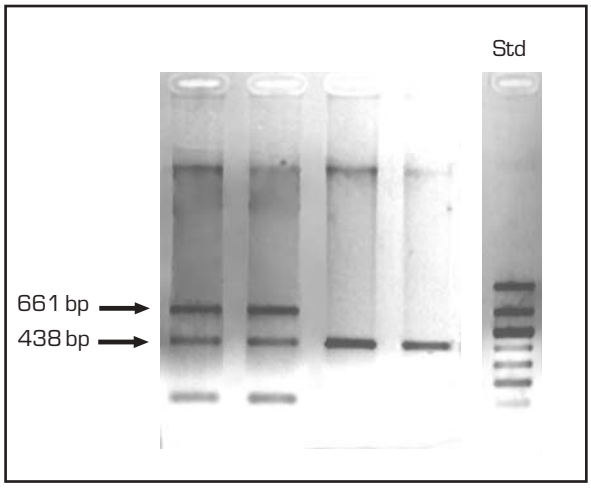

Figure 4.3 Agarose gel electrophoresis of amplification products using *Alu* primers. Wells 1 and 2 contain the DNA of heterozygotes containing chromosome with and without *Alu*. Wells 3 and 4 contain DNA from homozygotes with *Alu* deletion. Arrows indicate the position and size of haplotypes: *Alu* insertion (661 bp) and *Alu* deletion (438 bp), respectively.

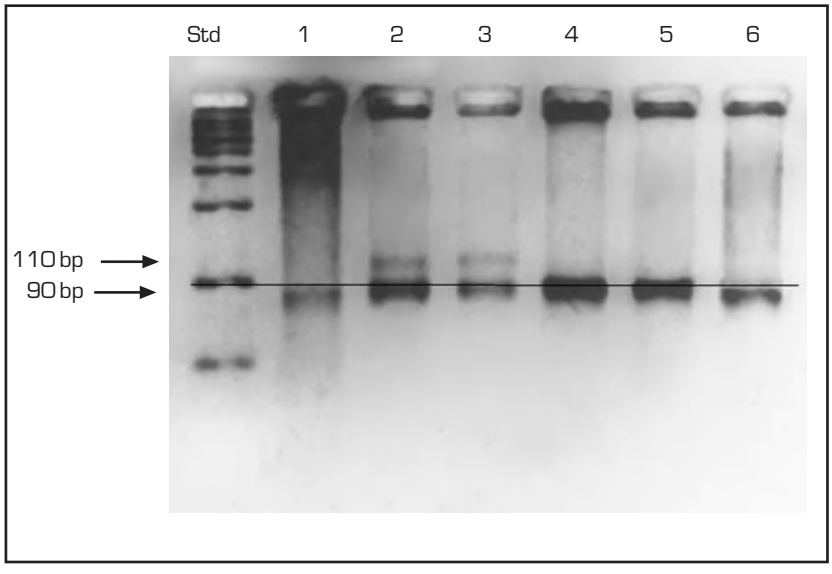

Figure 4.4 Amplification of human genomic DNA using STR primers. Wells 2 and 3 contain the DNA of heterozygotes with ten repeats (110 bp) and six repeats (90 bp). Wells 1 and 6 contain the DNA of homozygotes with five repeats (85 bp). Wells 4 and 5 contain the DNA of homozygotes with six repeats (90 bp). The line marks the position of a DNA standard fragment that is 100 bp long.

1. *Alu*(+) STR 90.
2. *Alu*(–) STR 90.
3. *Alu*(+) STR 110.
4. *Alu*(–) STR 110.

Inspection of Table 4.2 indicates that haplotype *Alu*(–) STR 110 is unlikely because it appears only in the European population. The most probable haplotypes for this individual are *Alu*(–) STR 90 and *Alu*(+) STR 110. This is because STR 90 allele is most frequently associated with *Alu*(–) and STR 110 allele is most frequently associated with *Alu*(+) in whole world populations, respectively.

Compile the data from the entire class and calculate the class δ value for the *Alu*(–) haplotype associated with STR 85, STR 90, and STR 115 ($\delta_{-,\text{STR }85}$, $\delta_{-,\text{STR }90}$, and $\delta_{-,\text{ STR }115}$) as described in the Introduction. Compare the calculated values to the δ values of world populations (Table 4.1).

Expected results

Figure 4.3 presents the amplification results of the *Alu* element polymorphism. All three haplotypes are clearly identified. Individuals 1 and 2 are

heterozygotes with one chromosome carrying an *Alu* insertion (661 bp) and the second having an *Alu* deletion (438 bp). Individuals 3 and 4 are homozygotic with respect to *Alu* deletion.

Figure 4.4 presents the amplification results for STRs. The three most common alleles can be identified with ease. The first and sixth individuals have only an 85 bp long repeat (five repeats) and the second and third individuals are heterozygotes with 90 bp (six repeats) and 110 bp (ten repeats) repeats, respectively. The fourth and the fifth individuals are homozygotes with a 90 bp long repeat. The other less common haplotypes 95 bp and 80 bp long cannot be clearly separated on these gels from haplotypes 90 bp and 85 bp long, respectively.

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