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DETECTION OF HETEROPLASMY IN HUMAN HEAD HAIR AND BLOODSTAINS USING A MITOCHONDRIAL DNA LINEAR ARRAY ASSAY

by

KATHERINE A. ROBERTS

A dissertation submitted to the Graduate Faculty in Criminal Justice in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2002

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20 June 2002
Date

Chair of Examining Committee

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Date

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THE CITY UNIVERSITY OF NEW YORK

Abstract

DETECTION OF HETEROPLASMY IN HUMAN HEAD HAIR AND BLOODSTAINS USING A MITOCHONDRIAL DNA LINEAR ARRAY ASSAY

by

Katherine A. Roberts

Adviser: Professor Peter De Forest

This study examines mitochondrial DNA polymorphisms in human head hair and bloodstains with respect to their potential for forensic application. Mitochondrial DNA was isolated and polymorphisms were detected by applying sequence-specific oligonucleotide probe analysis, a technique that provides objective, timely and cost-effective results. The particular focus was to further the understanding of the factors that influence the condition and detection of heteroplasmy in human head hair.

131 bloodstains and 2551 head hairs from 132 individuals from four population groups were amplified. Amplification success was assessed as a function of several independent variables. The findings indicate that the overall success rate is independent of hair growth phase, donor age, use of cosmetic hair treatments, medulla structure, or whether the donor is living or deceased. However, the race of the donor, the length of the hair sample and the pigmentation of the hair shaft all affect the success rate.

Samples that successfully amplified were typed using a mitochondrial DNA linear array assay. The genetic diversity value for each population group was analyzed and the frequency of each mtDNA haplotype was determined. The results of this study demonstrate differences in heteroplasmic expression between tissues. Of the 132 individuals typed, 5 exhibit heteroplasmy in their blood samples and 38 show evidence of heteroplasmy in at least one hair sample. Each of the 5 individuals with heteroplasmic blood also exhibits heteroplasmy in at least one hair. For the remaining 33 individuals, the heteroplasmic condition is found exclusively in the hair tissue.

The findings show that the frequency of heteroplasmy differs across racial population groups and is higher in the Caucasian population. A χ^2 test for independence indicates that this is statistically significant at $P > 0.005$. The data also reflect that the frequency of heteroplasmy does not differ significantly with age, sex, medulla morphology, region of the scalp, hair growth or, when comparing living and deceased donors.

Acknowledgements

I would like to thank all of the criminalists at the Los Angeles County Coroner's Office who assisted in the collection of samples for this study. To my "hair team" – Angela, Jamie and Ramona – I express my enduring gratitude for the commitment that you gave. Special thanks to my Dissertation Committee: Robert Shaler, for your kind words of encouragement; Lorah Perlee, for sharing your vast knowledge of the field of molecular biology; and Peter De Forest, for being a mentor who continues to inspire me to this day.

This research was made possible due to the support of several sources: the generous donation of reagents by Roche Molecular Diagnostics (thank you Sandy and Rebecca); the California Association of Criminalists (E. Reed & Virginia McLaughlin Endowment); and, Barry Fisher, to whom I extend my deep gratitude.

Finally, I would like to thank those that I hold closest to my heart, my family and friends. To Ira Sommers, for your guidance in making statistical sense of my data, to Debbie Baskin, for your unwavering belief in me from the day I was hired, and to Christine for so many reasons I have lost count – this research is as much yours as it is mine. Thanks for your encouragement, love and support. You are an angel. To my mother, "Cymru am Byth!"

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CHAPTER 1

Introduction

Background

DNA technology has evolved rapidly in the past fifteen years to the extent that, at this time, the use of certain DNA-based tests can conclusively match biological evidence with a particular individual. Until recently, forensic scientists have focused exclusively on nuclear DNA (nuDNA), and although the analysis of this DNA presents a number of advantages, it also has certain shortcomings. For example, scientists have reported difficulty analyzing nuclear DNA extracted from aged specimens, from samples that have been degraded and from material that contains limited quantities of nucleated cells, such as hair shafts, dental enamel and bone tissue. Consequently, scientists have devoted time evaluating a second, distinct DNA genome, located within the mitochondrion.

All human eukaryotic cells contain mitochondria within the cellular cytoplasm (figure 1). The mitochondria are relatively large organelles that are surrounded by a double membrane. The inner membrane is highly folded, forming chamber-like cristae. These present a large surface area for many of the enzymes and electron carriers involved in cellular respiration. Since mitochondria are primarily responsible for generating energy, it follows that the higher the energy requirements of a cell, the more mitochondria will be present. This has implications in the present study because human mitochondrial DNA (mtDNA) is also located within the mitochondria. The fact that a high copy number of

mitochondrial DNA is present within each cell is important in the context of forensic hair analysis, since the amount of nuclear DNA recovered from telogen (inactive) hairs has been shown to be low. For example, it has been shown that a human hair root undergoes rapid self-proliferation, which, coupled with recovery or the recycling of DNA, produce an abundance of mitochondria surviving in the hair shaft.

Table 1. Comparison of Nuclear and Mitochondrial DNA

[illegible]	[illegible]	[illegible]
Genetics	Diploid	Haploid
Genome Size	Three billion base pairs	16,569 base pairs
Structural Conformation	Linear Duplex	Circular Duplex
Inheritance	Paternal & Maternal	Maternal
Number of Copies/Cell	2	10 to >1000
Mutation Rate	Slow	X 5 to 10 Faster
DNA Recombination	Yes	No
Discrimination Power	High	Low

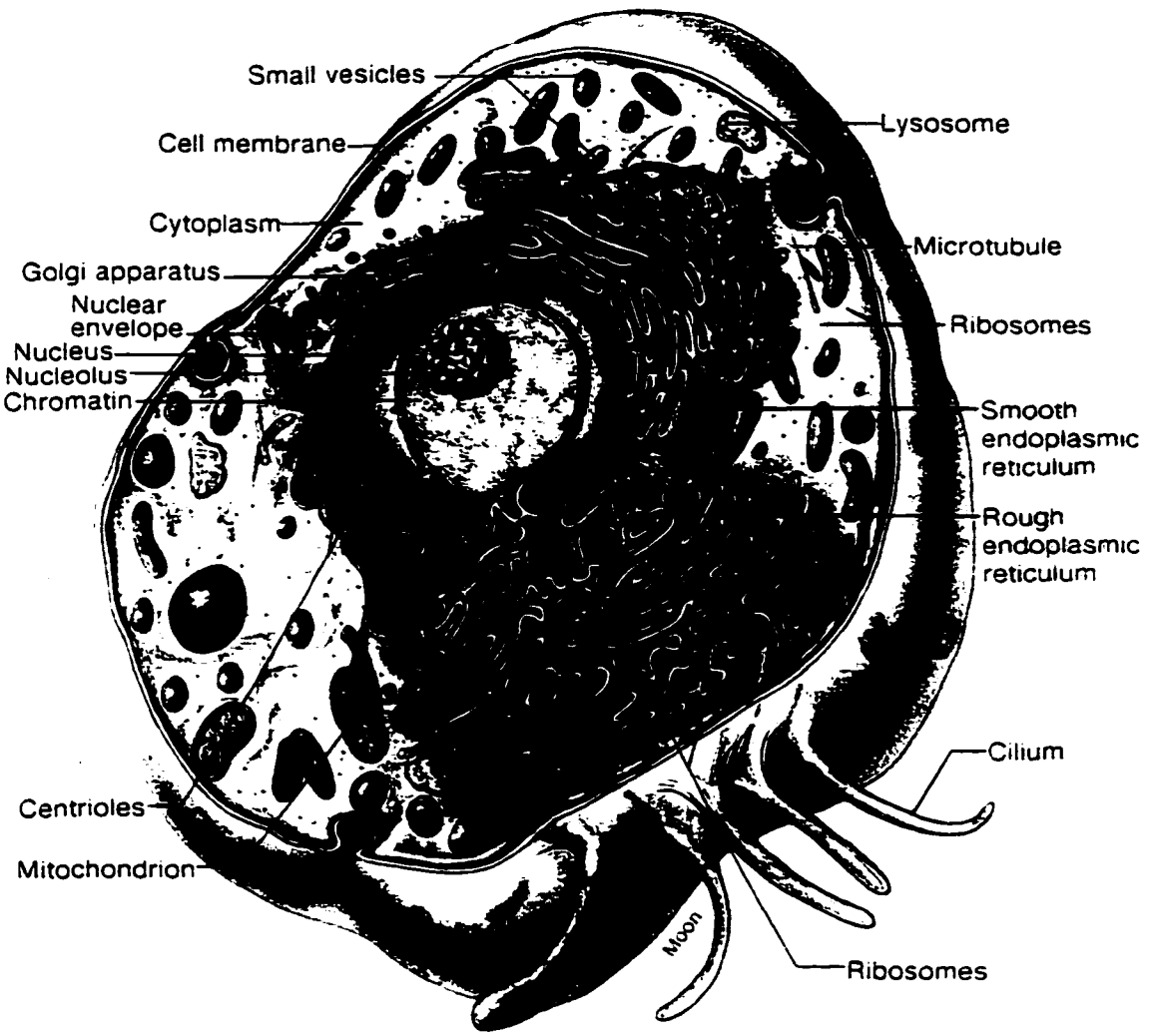


Figure 1. Somatic Cell Illustrating Location of Nuclear and Mitochondrial Genome

[Adapted from Tortora & Anagostakos

Principles of Anatomy and Physiology, Harper & Row, Publishers, Inc. 1990]

While both nuclear and mitochondrial DNA constitute duplex molecules, the nuclear genome is diploid and follows classical Mendelian inheritance. The mitochondrial genome is haploid because it is derived exclusively from the maternal parent (figure 2). This is due to the fact that during meiosis only the cytoplasm from the ovum is incorporated into the zygote. Since there is no cytoplasm from the spermatozoa contributed, all of the mitochondria originate from the maternal lineage. Consequently, Mendelian genetics cannot be applied to mitochondrial DNA inheritance. The conformational structures of both genomes also differ; mitochondrial DNA forms a closed, circular molecule whereas nuclear DNA is linear. The mitochondrial genome is also much smaller than its nuclear counterpart, and includes a control region that constitutes approximately 1100 nucleotide base pairs (table 1). The control region is essentially non-coding and accounts for most of the sequence polymorphism manifested by mitochondrial DNA. Haploid inheritance and the size of the genome both contribute to the lower discrimination power of mitochondrial DNA compared to nuclear DNA. The fact that mtDNA does not undergo genetic recombination means that any polymorphism is attributed exclusively to mutation. The control region is the only portion of the mtDNA genome that is analyzed for mutations in forensic casework, so the degree of variation in the population is also limited by the size of the region studied. Finally, nuclear DNA is present in multiple chromosomes, each of which contains loci that are considered independent markers. Therefore, unlike mtDNA (a single locus marker) the product rule can be applied to nuclear DNA to generate statistical calculations that give rise to the high discrimination power. The limited discrimination power of

mtDNA is revealed in figure 2, which illustrates how fourteen individuals in a family pedigree all fall into one of five haplotype groups.

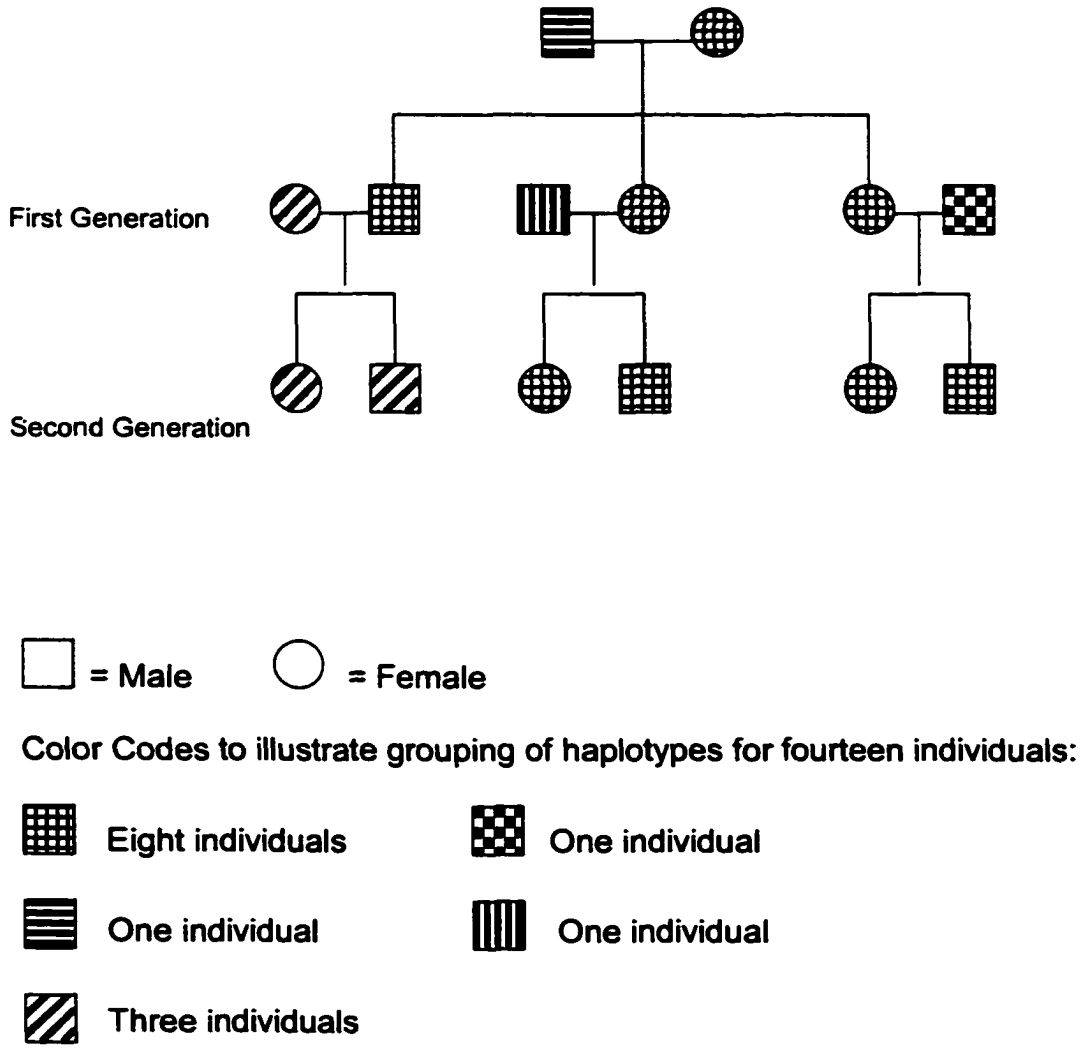


Figure 2. Illustration of Maternal Inheritance of MtDNA Through Two Generations of a Family Pedigree

Heteroplasmy

Like nuclear DNA, mitochondrial DNA is susceptible to genetic mutation. The mutations may be somatic (mitotic) or may originate in the female germ-line (meiotic). Evidence suggests that the mitochondrial genome mutates at a rate that is an order of magnitude greater than that of nuclear DNA.[24,27-28] This is generally attributable to the fact that mtDNA replication occurs more frequently than nuclear DNA and is more susceptible to error. In addition, mutations can be initiated by a number of environmental mutagens, including cosmic rays, UV light, or chemical exposure. Generally, only a single mitochondrial DNA type is detected within an individual, a condition referred to as homoplasmy. However, when a mutation is introduced, but only reflected in some copies of an individual's mtDNA, it results in a combination of normal (wild) and mutant types. Ordinarily, the normal type can be considered the major component and the mutant type the minor component, although, during the process of replicative segregation, there may be preferential selection of the mutant type such that it becomes the major component. This mixture of haplotypes is referred to heteroplasmy.

Heteroplasmy can impact a forensic identification in a number of ways. By convention, the consensus sequence is defined as that reported by Anderson[11] (light strand). In direct sequencing, the mtDNA under investigation is compared base by base to the Anderson Sequence and mutations are noted as polymorphisms in a given position. For example, a mutation from G to A at Position 233 is recorded as 233A, whereas a heteroplasmic case at the same

position is recorded as 233A/G. Therefore, if the condition is detected in both the evidence and reference sample, it can serve to provide additional discriminatory power. If the condition is identified in either the evidence or the reference sample, but not both, certain precautions need to be taken in order to avoid a false exclusion. Finally, heteroplasmy may be detected in both samples, but the levels of the major and minor component observed within the two samples may not be the same. This illustrates the precautions that need to be taken when evaluating samples containing heteroplasmy since differences in the levels of each variant may not necessarily provide exclusionary information.

When evaluating heteroplasmy, the tissue source must be considered, since inter-tissue variation has been observed within an individual. Studies have indicated that higher levels of mutated mtDNA are found in skeletal muscle and brain tissue, compared to lower levels present in blood[73]. Since different detection methodologies vary in their sensitivity, this may lead to difficulties when comparing two different tissue types, particularly if the level of the minor component falls below the detection limit of the technique. This is important in instances when evidential bloodstains are compared to reference samples other than blood, for example, postmortem tissues. Another factor to consider is the potential for an intra-tissue variation in heteroplasmy. In this case, a sufficient number of samples may need to be analyzed in order to gain a representative sampling. This is particularly true in the case of hair analysis, where each hair may reflect varying levels of the heteroplasmic condition given that they grow independently of each other within a hair follicle. Finally, if the condition is

identified in either the evidence sample or the reference sample obtained from a maternal relative of the suspect but not both, precautions are necessary to avoid a false exclusion. Given the mechanism of germ-line mutations, a homoplasmic mother may give birth to heteroplasmic offspring. Conversely a heteroplasmic mother may produce homoplasmic progeny. One study[45] using Holstein cattle found that heteroplasmy resolved to homoplasmy in two to three generations, while a follow up study[46] observed a genotype shift in a single generation. This issue is particularly important in cases involving the identification of human remains.

Forensic Hair Analysis

Hair analysis is an integral part of many forensic investigations, mainly as associative evidence. Hair is a proteinaceous outgrowth of follicles present in the skin of all mammals. The hair follicles begin to form during the first eight to twelve weeks of embryonic development. Discrete clusters of cells are organized into both the ectodermal and mesodermal layers of the primitive skin epithelium, ultimately forming the hair bulb and dermal papilla, respectively (figure 3). Once formed, the dermal papilla stimulates mitotic growth of the matrix cells within the hair bulb by releasing growth factors, which result in the growth of the hair shaft within the follicle. The follicles develop independently and once formed, represent the complement that individuals will retain during their lifetime.

The human scalp contains an estimated 100,000-150,000 hair follicles in three stages of growth (figure 4). In the anagen phase, the hair is actively

growing and can last from two to seven years, growing an average of 30cm uncut. During this phase, the germinal cells within the bulb matrix undergo rapid self-proliferation, requiring high energy. It is not surprising, therefore, that this area particularly in the region proximal to the follicles has an abundance of mitochondria. It is estimated that each germinal cell within the bulb matrix contains approximately one thousand mtDNA molecules, a number that remains constant for the life of the cell. Once these cells leave the bulb matrix they no longer divide and are differentiated into cuticular, medullary and cortical cells (figure 5). Plucked hairs are mostly in the anagen phase of development. They generally have follicular material adhering to their surface that is usually ectodermal in origin, although mesodermal tissue may possibly be attached. Both the hair root bulb and the follicular tissue provide good sources of nuclear DNA. In addition, the hair bulb contains melanocytes, the cells responsible for imparting color to the hair shaft. Melanocytes themselves remain within the root bulb, although, as they mature, they form dendritic arms that contain mitochondria and melanosomes (pigment granules). The pre-cortical cells are believed to pick up the mitochondria and melanosomes as they pass from the root bulb to the hair shaft, undergoing differentiation to cortical cells in the process. These cortical cells begin the synthesis of keratin shortly afterward, which requires mitochondria. Thus, cortical cells in the hair shaft may contain two sources of mtDNA, one from the germinal bulb matrix cells and one from the melanocytes. Linch *et al.*[137] report that this may potentially provide an explanation for the existence of heteroplasmy within the hair shaft. In addition,

the authors reason that when cortical cells become keratinized, they generate little if any additional mitochondria, whereas melanocytes are continually producing mitochondria. This may be one explanation for the differing levels of the major and minor variant observed in a heteroplasmic mixture. This finding has implications in the present study because, potentially, different hairs *and* different portions of each hair shaft may exhibit different levels of variants, possibly even shifting from heteroplasmy to homoplasmy.

Once the hair enters the catagen phase, the hair growth activity gradually ceases with the termination of mitosis in the hair bulb matrix cells. This process can last several weeks before making a transition to the dormant telogen phase. The melanocytes also become dormant prior to the catagen phase, which explains why telogen hairs generally lack pigmentation in the region immediately proximal to the root. In addition, the root itself shrinks in length and retracts upwards, producing a club-like morphology, a phase which generally lasts approximately three months. Quarmy *et al.*[135] have reported that approximately eighty percent of human head hairs are in the anagen phase, compared to ten to twenty percent in the telogen phase, with catagen hairs representing approximately two percent.

It is believed that individuals lose an average of about one hundred head hairs daily, suggesting that investigators can potentially uncover hair evidence from the victim and, where applicable, the perpetrator. Primarily, hair analysis compares microscopic characteristics of a representative sample of a known source of hairs with the questioned hair(s). With the aid of a compound microscope and

transmitted light, hair examiners rely on the microscopical appearance of the cuticle, cortex and medulla. In particular, the following characteristics are recorded: the color, size, distribution and pattern of the pigment granules within the cortical cells; appearance of the medulla; hair shaft diameter; indications of chemical treatments; and, the physical condition of the shaft. This examination is generally preceded by a macroscopical examination using a stereomicroscope and reflected light to examine the three-dimensional configuration of the hair, overall length, texture, and the existence of trace material adhering to the hair shaft.

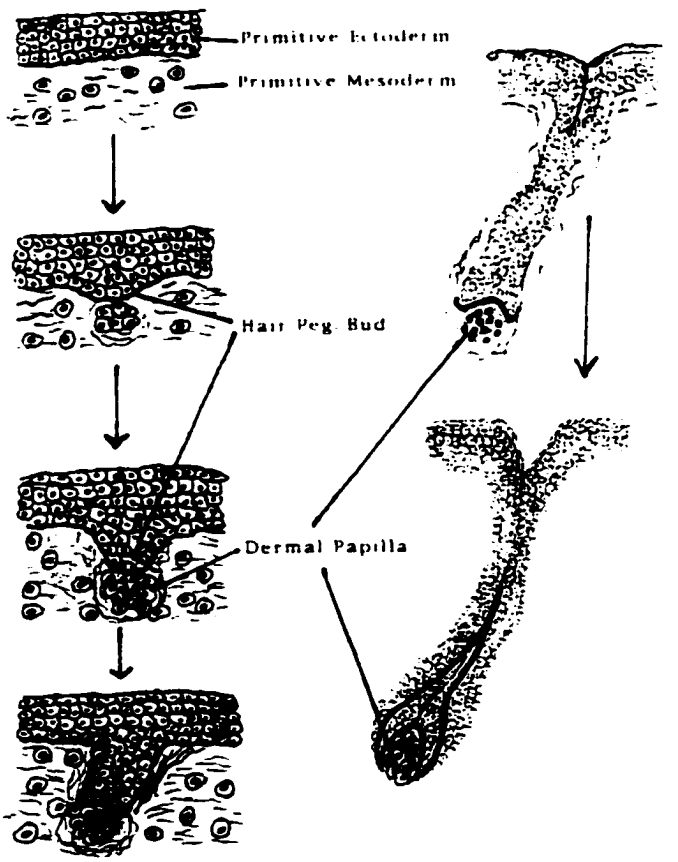


Figure 3. Embryonic Hair Development Showing Cellular Differentiation to Form the Hair Follicle and Hair Growth
[Adapted from Linch *et al.* J Forensic Sci 2001;46(4) p846]

This type of morphological evaluation is not amenable to the "one-to-one" comparisons typically performed with fingerprint evidence, bullet striations, and footwear, due to the intra-individual variation exhibited by head hairs. Intra-sample variation makes it more difficult for the hair examiner to assign a match between known and unknown hairs, and, as a result, the approach is criticized because of the inherent subjectivity involved. While it is generally accepted that inter-individual variation of microscopic characteristics greatly exceeds that of intra-individual variation, it is possible that hairs from two different individuals may be morphologically indistinguishable. Compounding this problem is the fact that hair examiners are unable to predict the expected frequency of this coincidental match. Further, it could be argued that the greater the intra-individual variation, the greater the potential for a coincidental match. Conversely, an individual's hair may change between samplings, which may lead to a false exclusion based on a morphological hair examination.

The introduction of PCR-based DNA technology has provided a more objective method for making hair comparisons on hairs that have a root sheath or a partial root sheath attached (anagen), as they can be subjected to nuclear DNA analysis. However, hair fragments, or hairs that lack a root sheath cannot be analyzed in this manner due to the absence or low quantity of nuclear DNA recovered from within the hair shaft. Hairs that lack a root sheath are generally, although not exclusively, telogen hairs.

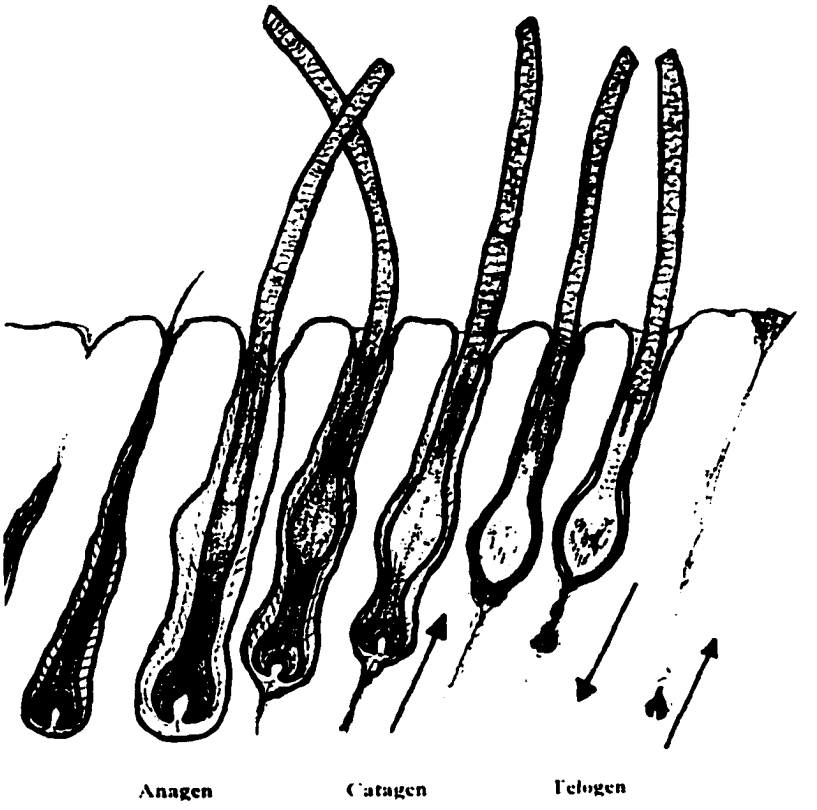


Figure 4. Human Hair Growth Phases
[Adapted from Linch *et al.* J Forensic Sci 2001;46(4) p848]

Thus, there has been a continued reliance on microscopy as the primary means of analyzing telogen hairs. Within the last few years, research conducted by the FBI[1] and others has provided alternative methods that can now be applied to hair analysis. Using mitochondrial DNA, present in large quantities within the hair shaft, PCR-based technology can now be applied to both telogen *and* anagen hairs.

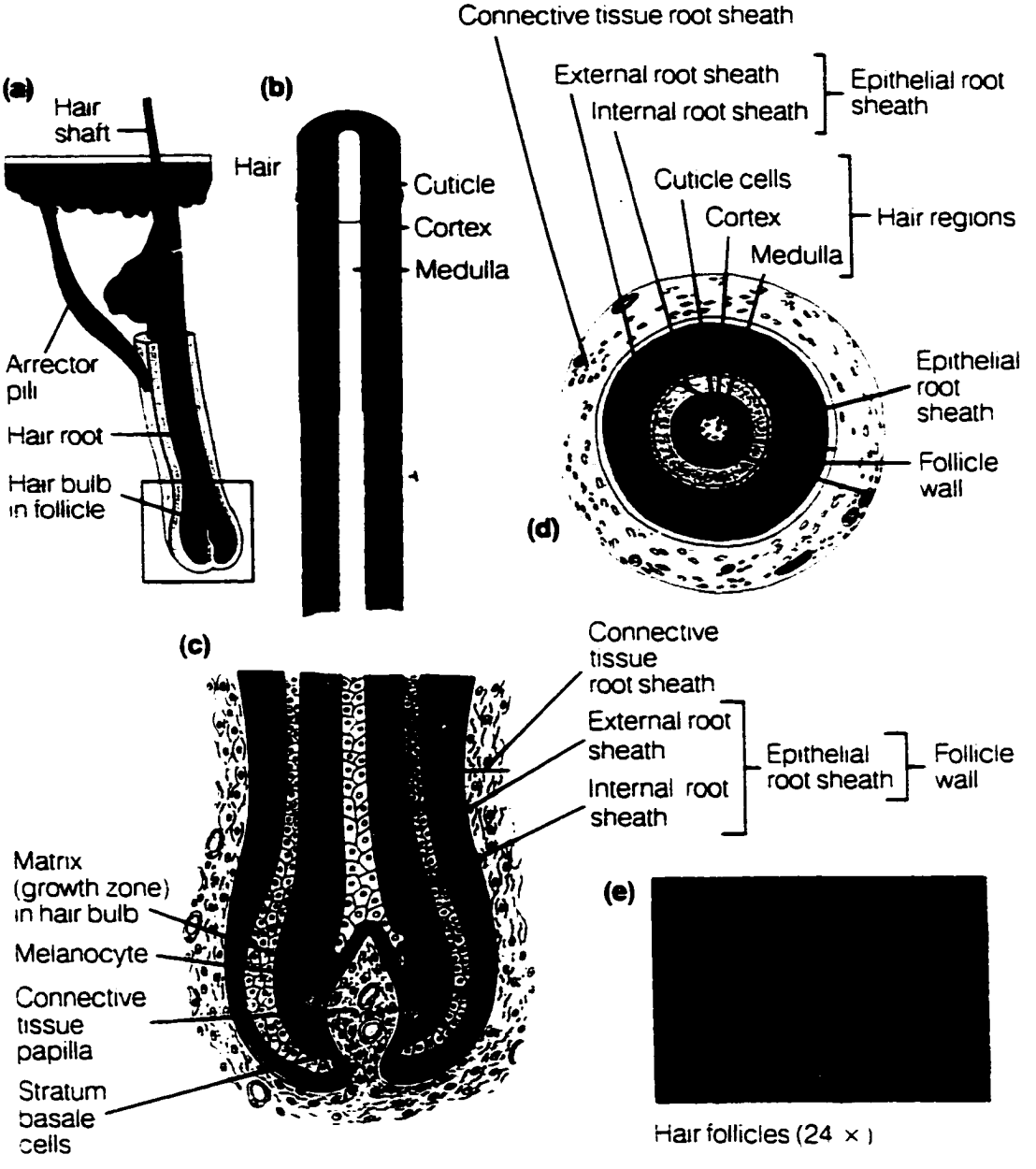


Figure 5. Structure of Human Hair and Hair Follicle

[Adapted from Tortora & Anagostakos

Principles of Anatomy and Physiology, Harper & Row, Publishers, Inc. 1990]

Forensic Mitochondrial DNA Analysis

Mitochondrial DNA can be analyzed in several different ways. One approach has been sequencing, which suffers the drawbacks of being both labor-intensive and expensive. Researchers have also reported problems with sequence analysis due to the presence of a string of repeated cytosine bases in the control region.[32] Alternatively, less expensive screening methods have been developed in order to identify the most probative samples for sequence analysis. These include mini-sequencing, single-strand conformational polymorphism (SSCP), restriction fragment length polymorphism (RFLP), denaturing gradient-gel electrophoresis (DGGE) and dot-blot analysis using sequence specific oligonucleotide probes (SSOP). The last method is the most cost-effective but can be tedious in a forensic setting where only a few samples need to be compared. A modification of this screening approach is the reverse dot-blot procedure, which is amenable to forensic laboratories, as it allows for rapid screening of samples in a cost-effective format.

Negative Impact of DNA Analysis

The use of DNA in the criminal justice system has been criticized since its inception. Early commentators voiced concern over the lack of validation studies to assess the reliability of the technology[131]. These have now been addressed, along with the implementation of accreditation and certification programs[132]. The focus of the controversy, somewhat ironically, has now shifted to the probative value of DNA evidence. There is a minority within the criminal justice system that argues that DNA evidence is becoming so compelling, that there exists the

possibility that juries will disregard other forms of evidence. It has also been suggested that defense attorneys will often challenge the selection of a juror who possess a scientific background, particularly in the field of human genetics. Another controversial debate revolves around the FBI's national CODIS DNA program, which is comprised of three indices: DNA profiles obtained from certain convicted felons, a population index, and a missing persons database. The concern here is access to genetic information in the database.

Research Statement

This study examines mitochondrial DNA polymorphisms in human head hair and bloodstains with respect to their potential for forensic application. Mitochondrial DNA was isolated and polymorphisms were detected by applying sequence-specific oligonucleotide probe analysis, a technique that provides objective, timely and cost-effective results. The particular focus was to further the understanding of the factors that influence both the condition and detection of heteroplasmy in human head hair.

The research project was initiated by conducting a sensitivity study to determine the amount of hair sample required to produce a typable result. The next series of experiments were designed to determine if exposing a hair to different chemical treatments affects the ability to obtain a typable result. Indirectly, this addresses whether or not chemical treatments degrade or otherwise reduce the quantity of mitochondrial DNA in the shaft. Another avenue pursued in this research project was an attempt to determine if discrete tissues

from the same individual could manifest different mitochondrial DNA haplotypes. The next evaluation focused on whether or not individual head hairs obtained from the same donor exhibit different haplotypes. In another experiment, an investigation was directed at determining whether hairs exhibiting certain morphological characteristics render either different haplotypes or different levels of the heteroplasmic condition. The study was continued by considering whether or not the age of an individual is a strong basis for predicting a heteroplasmic condition. Next, an experiment was designed to assess whether certain races are more genetically predisposed to heteroplasmy. The next study examined differences in the detection of heteroplasmy observed in living versus deceased individuals. It was also important to examine if the heteroplasmic condition represents a mosaicism or a random distribution across the scalp. Finally, the relationship between the growth phase of a hair and the ability to detect heteroplasmy was investigated.

For this study, the sex of the donor of each hair sample was noted, although, for pragmatic reasons it was not believed to be correlated to heteroplasmy. It is known that nuclear DNA mutations related to sex are sex-linked in the sense that they are transmitted via chromosome twenty-one (XY locus). Mutations located on the "Y" locus are therefore exclusive to males. Since mtDNA is only inherited maternally, it also provides a sex-linked mutation. However, since male and female offspring inherit the mtDNA, logically, there is no reason to believe that either sex may preferentially inherit the heteroplasmic condition. There may, however, be sex related issues that may prohibit a typable result: for example,

the length of the hair (shorter in males) or exposure to cosmetic treatments (more common in females).

CHAPTER 2

Literature Review

Mitochondrial DNA Genetics

According to classical Mendelian theory, a genotype, and therefore a phenotype, is based on genetic information that is transmitted biparentally. However, the existence of the mitochondrial genome adds a nontraditional element to mammalian origin because it is derived solely from the maternal parent[2-4]. Experimental research indicates that during fertilization with the ovary (figure 6), only components of the spermatozoon head contribute to the zygote and the tailpiece, where the mitochondrial sheath is located, is disassembled in the process (figure 7). Sutovsky[5] has studied the *in* vitro fertilization of cattle microscopically and suggests that although the mitochondrial sheath is initially incorporated into the oocyte, it is no longer visible by the third mitotic division. Allen[6] has postulated a theoretical basis for maternal inheritance. The hypothesis states that since a high degree of energy is expended by the male gamete during motility, this exposes the male mtDNA to free-radical by-products that can cause mutations. The author proposes that an evolutionary division of labor occurs in order to maintain the fidelity of the mitochondrial DNA whereby the father does not contribute to the compliment of the fertilized zygote. This fact has been confirmed by genetic analysis of family trees. Recent evidence suggests that certain microorganisms inherit mitochondrial DNA biparentally[7], although this appears to be limited to a few species. For example, Gyllensten[8]

detected low levels of paternally inherited mtDNA in mice at a frequency of 10^{-4} relative to maternal contributions; Saville[9] reported mtDNA recombination in a natural population of fungus, while Kondo[10] observed similar mtDNA recombination in *Drosophila*.

However, no documented examples have been found to support mitochondrial genetic recombination in the human population. Theoretically, mtDNA recombination may be the result of three situations. First, a crossing over event is possible between different mtDNA molecules in heteroplasmic oocytes. A second option is that mtDNA recombination may occur with nuclear DNA. Since it has been established that mtDNA sequences - pseudogenes - occur in the nuclear genome, it is possible that the mtDNA might enter the nucleus and undergo recombination with the pseudogenes. Finally, as discussed above, recombination might occur between maternal and paternal mtDNA during fertilization. Potential biparental transmission impacts clinical medicine because if males do contribute mtDNA, then they have the potential to transmit mtDNA diseases. This possibility also has important implications in the present study because "leakage" of parental mtDNA could provide a plausible explanation for the existence of a genetic mixture or "heteroplasmy".

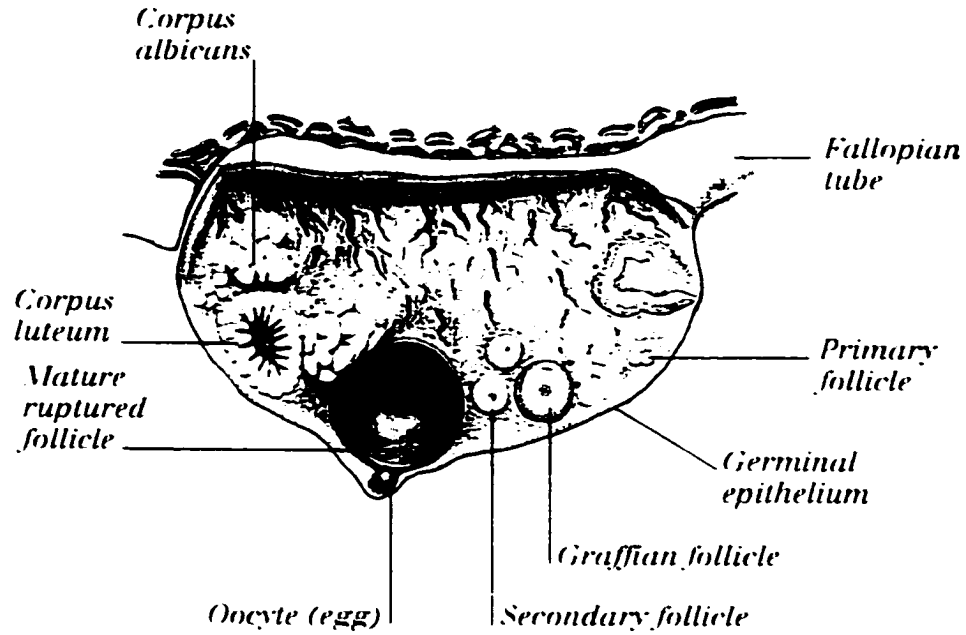


Figure 6. External Structure of a Human Ovary

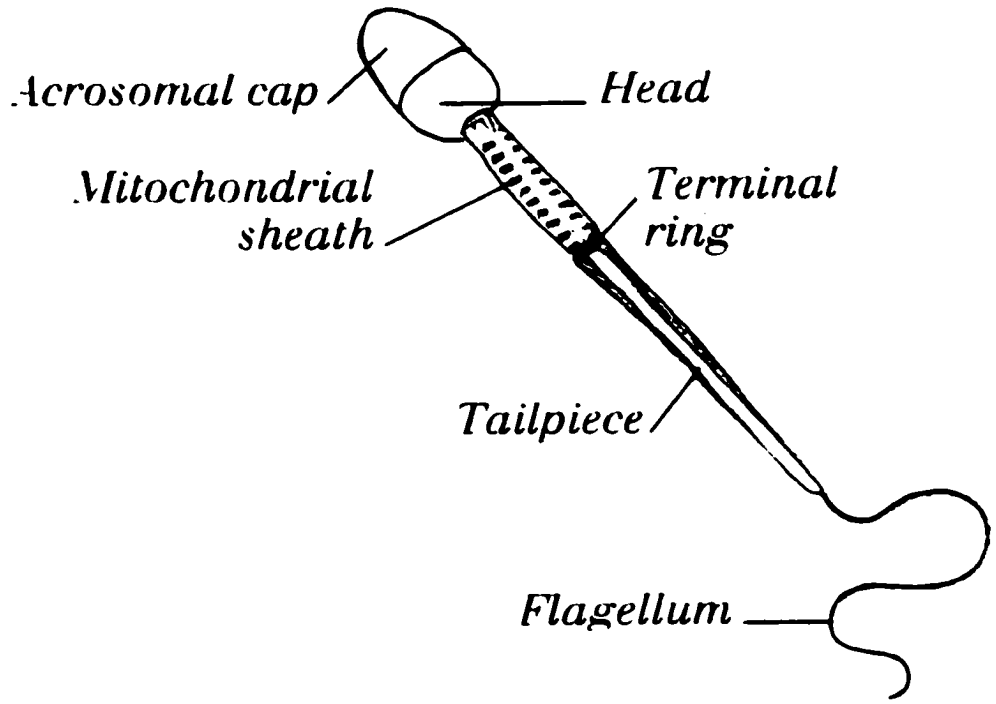


Figure 7. External Structure of a Human Spermatozoon

Mitochondrial DNA is extra-nuclear, located exclusively within organelles referred to as mitochondria. It is generally believed that these structures selectively evolved from bacteria that developed a symbiotic relationship with eukaryotic cells in early ancestral development[33]. This is inferred from both the size of the mitochondrion, approximately one micron in diameter, and the coding sequences in the mitochondrial genome. The mitochondrial DNA molecule is a circular duplex comprised of 16,569 nucleotide base pairs. Anderson[11] has determined the entire sequence of the genome, which encodes thirty-seven genes: a small (12S) and large (16S) ribosomal RNA, twenty-two tRNAs, and thirteen polypeptides. All of these mtDNA-encoded polypeptides are sub-units of the mitochondrial energy-generating pathway, oxidative phosphorylation (OXPHOS). This is the primary energy-generating pathway for cellular respiration. It consists of five enzyme complexes that form an array within the inner membrane of the mitochondrion (figure 8). Briefly, energy is generated by mitochondria as follows: complex I removes electrons from NADH and complex II collects electrons from succinate. Both enzymes transport these electrons via coenzyme Q to complex III, transferring them to cytochrome c, and finally complex IV. The end products of this electron transfer chain at complex IV are oxygen and water, with the concomitant release of energy. The released energy is used to pump protons out through the mitochondrial inner membrane, forming an electrochemical gradient between the inner and outer membrane of the mitochondrion. Ultimately, complex V utilizes this gradient as a source of energy to generate adenosine triphosphate (ATP) from adenosine diphosphate

(ADP) and phosphate. The outer strand of the mitochondrial DNA duplex is referred to as the heavy strand (H-strand) because of its rich guanine content, and comprises twenty-eight of the thirty-seven genes. The complementary inner strand is known as the light strand (L-strand) because of its rich cytosine content and constitutes the nine remaining genes.

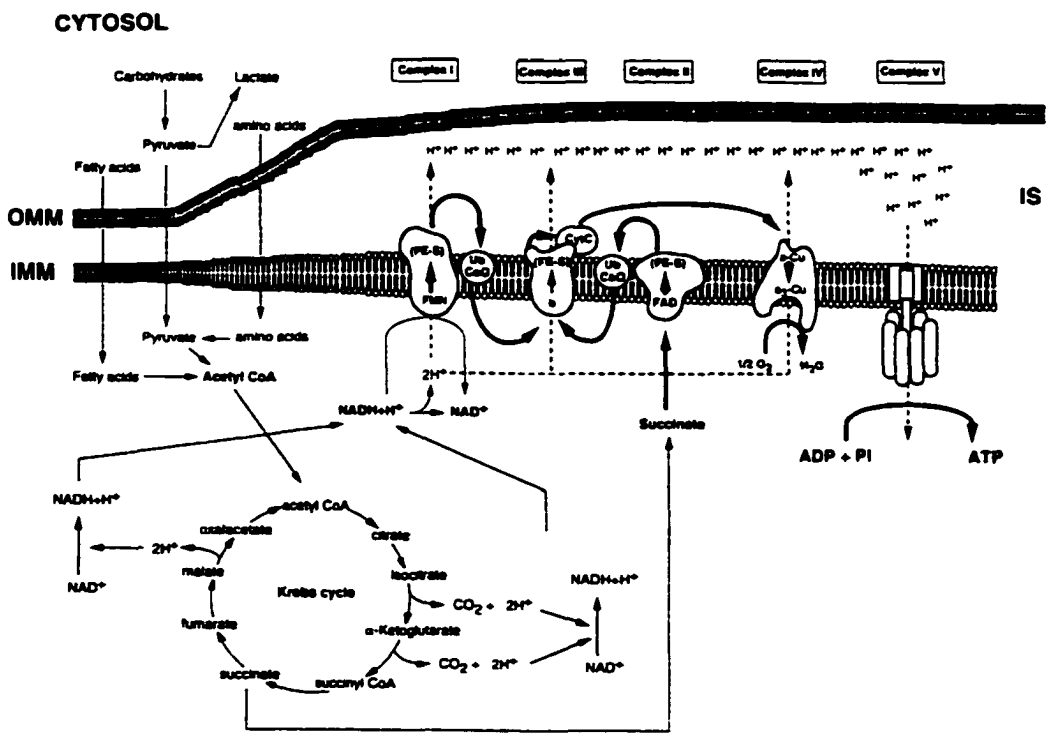


Figure 8. The Mitochondrial DNA Respiratory Chain:
Schematic Representation of the Mitochondrial OXPHOS System
[Adapted from Fernandez-Morenet *et al.* Molecular Genetics and Metabolism 2000; 71: p482]

Approximately ninety-three percent of the mitochondrial genome is comprised of coding genes, which are either contiguous, or, separated by less than one or two bases. The remaining seven percent of the total mtDNA length make up the non-coding sequences. These sequences are concentrated in the control region, a 1,122bp sequence located between proline and phenylalanine tRNA. This region includes the displacement loop (D-loop), which is a triple-stranded region generated by the synthesis of a short piece of H-strand DNA (7S DNA). Included in the control region are the transcription promoters for the H and L-strands, PH1 and PL respectively. Mammalian mtDNA transcription has been characterized both *in* vitro and *in* vivo.[12-15] Transcription of the heavy strand runs clockwise around the circle and transcribes all genes into one large transcript, while the light strand promoter causes transcription to run in the opposite direction. The mechanism of mammalian mtDNA replication has also been characterized[15-16]. The origin of replication of the mtDNA H-strand is also located within the control region. H-strand replication starts at the 7S DNA and proceeds around the L-strand, displacing the single-stranded template H-strand. After traversing two-thirds of the genome, the L-strand is exposed. L-strand replication is then initiated and proceeds back along the displaced H-strand template.

Replication of mtDNA is semi-conservative and uses DNA γ-polymerase, which is specific to the mitochondrion. Thus, when undergoing a mitotic division, the mitochondria independently replicate and then distribute randomly into daughter cells. MtDNA replication occurs throughout the cell cycle, depending on the energy requirements of the cell. This is supported by the fact that, in general, the energy demands of a specific tissue correlate with an elevated expression of

genes encoding components of the oxidative phosphorylation system.[17] Most of the mitochondrial proteins involved in the maintenance, replication and expression of the mitochondrial genome are encoded in the nucleus. Therefore, the process of oxidative phosphorylation is controlled by both the nuclear and mitochondrial genomes.[18]

Mitochondrial DNA Mutations

Similar to nuclear DNA, mtDNA sequences are susceptible to mutation. It is estimated that mtDNA mutates at a rate that is five to ten times greater than that of nuclear DNA. It has also been stated that non-coding control region sequences appear to diverge ten times faster than mitochondrial coding sequences.[19-21] Recombinant DNA techniques have been used to quantitate the degree of nucleotide sequence divergence within coding regions. A study conducted by Monnat[22] indicates a high degree of mitochondrial nucleotide sequence homogeneity in human somatic cells from normal individuals (subjects with no indication of disease symptoms). Estimated mutation rates can be obtained directly and indirectly. Indirect measurements are obtained by evolutionary studies and applying phylogenetic or coalescent methods to infer the mutation rate based on pattern variation in human mtDNA sequences. Recent advances in sequence analysis have permitted a direct estimation of mtDNA mutation rates by counting the number of mutational events that occur in pedigrees. A number of pedigree-based studies have been conducted: Howell[23] suggests a mutation occurs in the control region approximately every 25

generations (a generation is defined as 20 years) while Parsons[24] reports a mutation rate for the control region that is approximately twenty times higher than the estimates obtained from phylogenetic analysis at 2.5/site/1 million years. To date, the most extensive study has been conducted by Siguroardottir et al[25], who report an estimated mutation rate of 0.32/site/1 million years. All of the studies concur that the higher mutation rate in the control region appears to be concentrated in two distinct segments, referred to as hypervariable region I and II (figure 9). Each of these regions is comprised of 444bp and 415bp sequences respectively, and are separated by a central conserved sequence[26]. Nucleotide position 1 of the Anderson sequence[11] falls within this conserved sequence. It has also been demonstrated that hypervariable region I exhibits more than twice the variability observed in hypervariable region II.[27] The regions that are more susceptible to mutations are referred to as "hot spots".[24,28-29]

Given that the mitochondrial DNA genome is transmitted maternally, and therefore, not subject to recombination, the only means of altering the mitochondrial DNA sequence is by accumulation of the mutations. These mutations account for the polymorphism that exists in the population and is used as a basis for distinguishing individuals. Specifically, there are two types of mtDNA polymorphisms, point mutations and length polymorphisms. Point mutations involve single base substitutions, resulting in sequence variation between individuals[30]. Two common hotspots have been identified at positions 16093 and 16129, as T/C and A/G respectively. Length polymorphisms have also been observed in the non-coding region and can result from large insertions or

deletions of oligonucleotide sequences. This type of mutation gives rise to a variation in the length of the mtDNA fragment that is most commonly observed in the HVII region at nucleotide base pair 303[31]. It is generally associated with a thymine to cytosine transition due to a long stretch of ten to thirteen cytosine residues. This region is commonly referred to as the polycytosine tract or "C-stretches" and is difficult to sequence.

There are a number of reasons why mtDNA is more predisposed to mutation when compared to nuclear DNA. Mitochondria are present in the cytoplasm, which means that they are not protected by the nuclear membrane. Hence, their location close to the respiratory chain exposes them to the effects of the oxygen radicals that are generated by oxidative phosphorylation. Secondly, they replicate more frequently than nuclear DNA, increasing the opportunity for a mismatch. In addition, the mitochondrial genome lacks protective proteins, such as histones and has a poorly evolved DNA repair system.[7] Mitochondrial DNA mutations can arise either in somatic tissues or in the female germ-line. Somatic mutations accumulate randomly in tissues and may have a deleterious effect on the individual, but essentially the fatal mutations terminate with the individual. The mutations that arise in the female germ-line however, are inherited and are therefore transmitted to the next generation as new polymorphisms.

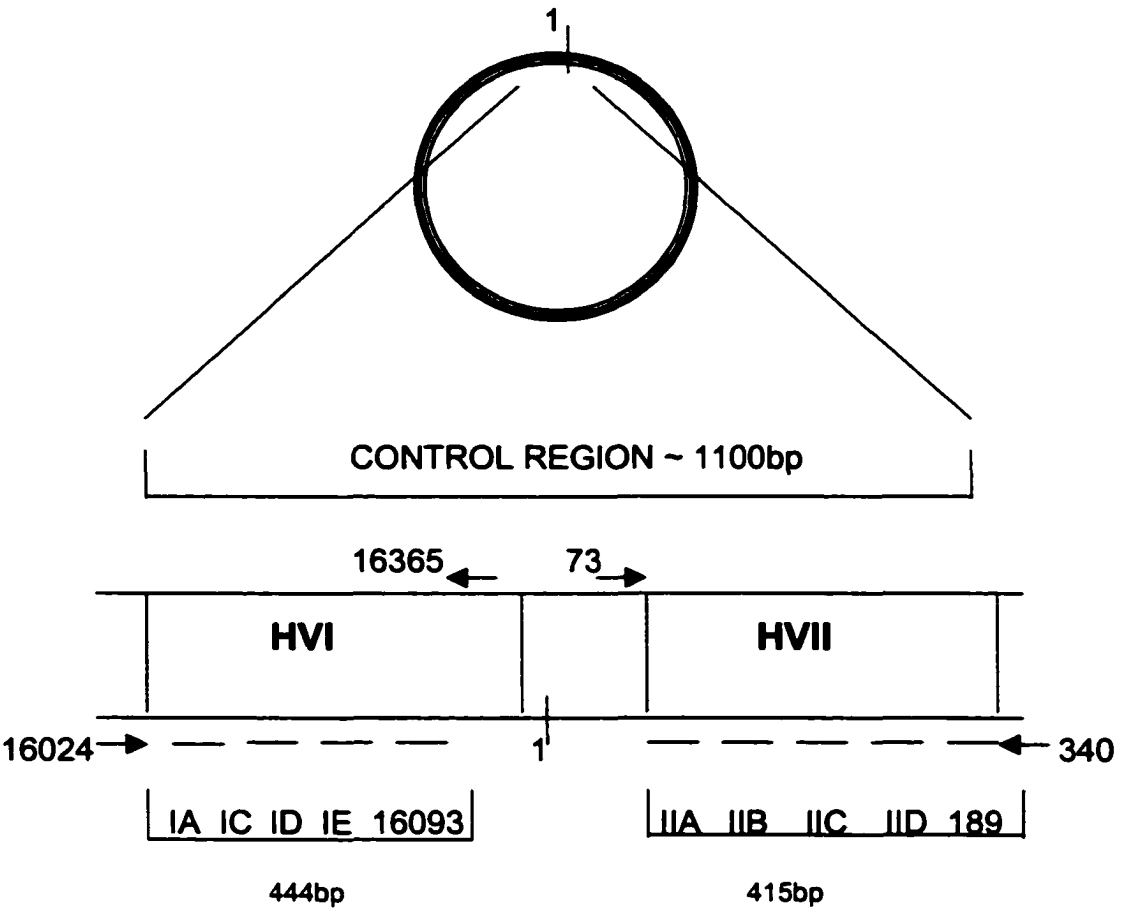


Figure 9. Mitochondrial DNA Control Region: HVI and HVII

Heteroplasmy Studies

Mitochondrial DNA is present in high copy number in a cell, ranging from 1000 to 10,000 molecules, depending on the energy requirements of the specific tissue.[33] The number of molecules within a mitochondrion also varies, typically from five to ten per mitochondrion. As a result, mitochondrial DNA is said to be polyplasmic, and estimated to comprise one percent of the total amount of DNA in a human cell[34]. In normal individuals, nearly all of the mtDNA is thought to be identical, in what is referred to as a homoplasmic state. Homoplasmic, by definition, means 99.9% sequence concordance.[35] However, when a mutation arises, it creates an intracellular mixture of mutant and wild-type molecules called heteroplasmy. Given that the human body contains trillions of cells, each of which can retain over a thousand copies of the mtDNA genome, it is not surprising that such a mixture can exist. Generally, two types of heteroplasmy are observed. Sequence heteroplasmy[50] is attributed to point mutations in mtDNA, whereas length heteroplasmy[51] is associated with mutations involving nucleotide insertions or deletions. Bendall and Sykes[32] have observed length heteroplasmy in HVI between sites 16184 and 16193 with C-stretch variation ranging from eight to fourteen residues. The authors propose that the mechanism responsible for generating length heteroplasmy in HVI involves replication slippage after a thymine to cytosine transition has occurred. Greenberg *et al.*[21] reported that similar insertions occur in HVII at nucleotide positions 309, where an eight base C-stretch is observed preceding a thymine residue and at 315, where a six base C-stretch follows a thymine residue. Again, it is important to

emphasize that both sequence and length heteroplasmy can arise from somatic and/or germ-line mutations. If samples from the mother are available, they can be typed and compared with the progeny's "heteroplasmic" type in order to determine the origin of the mutation. Although the incidence of heteroplasmy was originally thought to be quite low, recent studies indicate otherwise. This may be due partly to the fact that heteroplasmy can now be more readily detected given the improved sensitivity of currently available sequencing techniques.

The heteroplasmic condition was initially identified in a forensic context in 1994 when Gill[38] found a single heteroplasmic polymorphism in the control region (T/C mixture at Position 16,169) of the seventy-year old skeletal remains of Tsar Nicholas II. Further, Ivanov[39] reported that the same heteroplasmy was also present in the Tsar's brother, but had resolved to homoplasmy in a maternally related descendant four generations later. This information, coupled with parentage information obtained from short-tandem-repeat analysis enabled a 98.5% certainty that the remains were those of the Romanovs. Based on similar information, forensic analysis was able to refute the claim of Anna Anderson Manahan that she was the missing Royal Duchess, Anastasia.[40]

If the mutation causing heteroplasmy is somatic in origin, the mutant and normal mtDNAs are randomly distributed into the daughter cells during mitotic division[36]. As a result, the percentage of mutant and wild-type molecules drifts within the cell, toward either pure mutant or pure wild type, approaching homoplasmy. This process, referred to as replicative segregation, causes the

phenotype to be largely influenced by the proportion of mtDNA molecules harboring the mutation in the different tissues. If the mutation is germ-line, random distribution of wild and mutant-types into the daughter cells occurs during meiotic division and can also drift toward either pure mutant or pure wild-type. If the mixture does persist in the oocyte, it is transmitted to the progeny. Given the polyplasmic nature of the mitochondrial genome, mitotic replicative segregation usually requires multiple cell divisions to approach homoplasmy, but meiotic replicative segregation can be quite rapid. For example, one longitudinal study of the somatic cell-line conducted over one to two decades suggests that the level of heteroplasmy in somatic cells remains stable over an individual's lifetime. This indicates that the heteroplasmic condition within the control region of mtDNA is inherited rather than a somatic age-related accumulation[37]. In comparison, germ-line studies have indicated that mutant molecules have changed from predominantly wild-type to mutant-type in one generation. Therefore, it is conceivable that progeny could exhibit a different mtDNA sequence from both the mother and the siblings.

The rapid germ-line segregation described above has been attributed to the rapid sorting of mitochondrial genomes in oocytes during early embryogenesis[41-43]. At fertilization, the oocyte contains approximately 200,000 mtDNAs, one per mitochondrion. Once fertilized, the nuclear DNA replicates and the oocyte cleaves, but the mtDNA does not replicate until after the blastocysts are formed. These are the primordial female germ-line cells that randomly segregate a small number of the oocyte's mtDNAs during a process referred to

as bottlenecking (figure 10). In most cases, only wild-type mtDNA are isolated. However, occasionally a mixture of mutant and wild-type mtDNA is acquired, resulting in a large increase in the proportion of mutant mtDNAs in the oocytes of the next generation. Since each oocyte can vary in the ratios of the mutant and wild-type components, subsequent replication may result in multiple offspring manifesting varying levels of heteroplasmy. An additional reduction in mtDNA copy number occurs between a mature oocyte and an oogonium (a descendent of a primordial cell that gives rise to an oocyte) in female offspring, which may also constitute a bottleneck. Another way to think about this is to consider the wild-type as the major component and the mutant-type the minor component. Depending on the selection process during replicative segregation, there may be a genetic shift where the mutant-type becomes the major component. In this case, due to the inability of a technique to detect the minor component, an apparent homoplasmic mother will exhibit a different haplotype that her apparent homoplasmic offspring. Once the mutant mtDNA predominates in the germ-line, the next generation's mtDNA genotype can shift to pure mutant. This provides one possible explanation of how mitochondrial mutations become 'fixed' within a population.

A few studies of mammalian species have investigated segregation of mitochondrial genotypes within heteroplasmic lineages. Upholt[44] studied sheep and goats, and estimated that if a mammalian germ line comprised one hundred mtDNA molecules, assuming random segregation during cellular division of the

developing oocyte, homoplasmy of a mixed mtDNA population would be achieved in approximately twenty generations. A study of Holstein cattle found that heteroplasmy resolved to homoplasmy in two to three generations, concluding that a bottleneck may comprise from twenty to one hundred segregating units[45]. Koehler *et al.*[46] conducted a follow up study on cattle and observed a genotype shift in a single generation, suggesting the possibility of a bottleneck comprised of a single segregating unit. Jenuth *et al.*[41] constructed a heteroplasmic model using a mouse to investigate mtDNA distribution in oocytes. Their results indicated that random segregation and a bottleneck of ~200 mtDNA molecules in mouse progenitor oocytes could explain the heteroplasmic mtDNA distribution in mice.

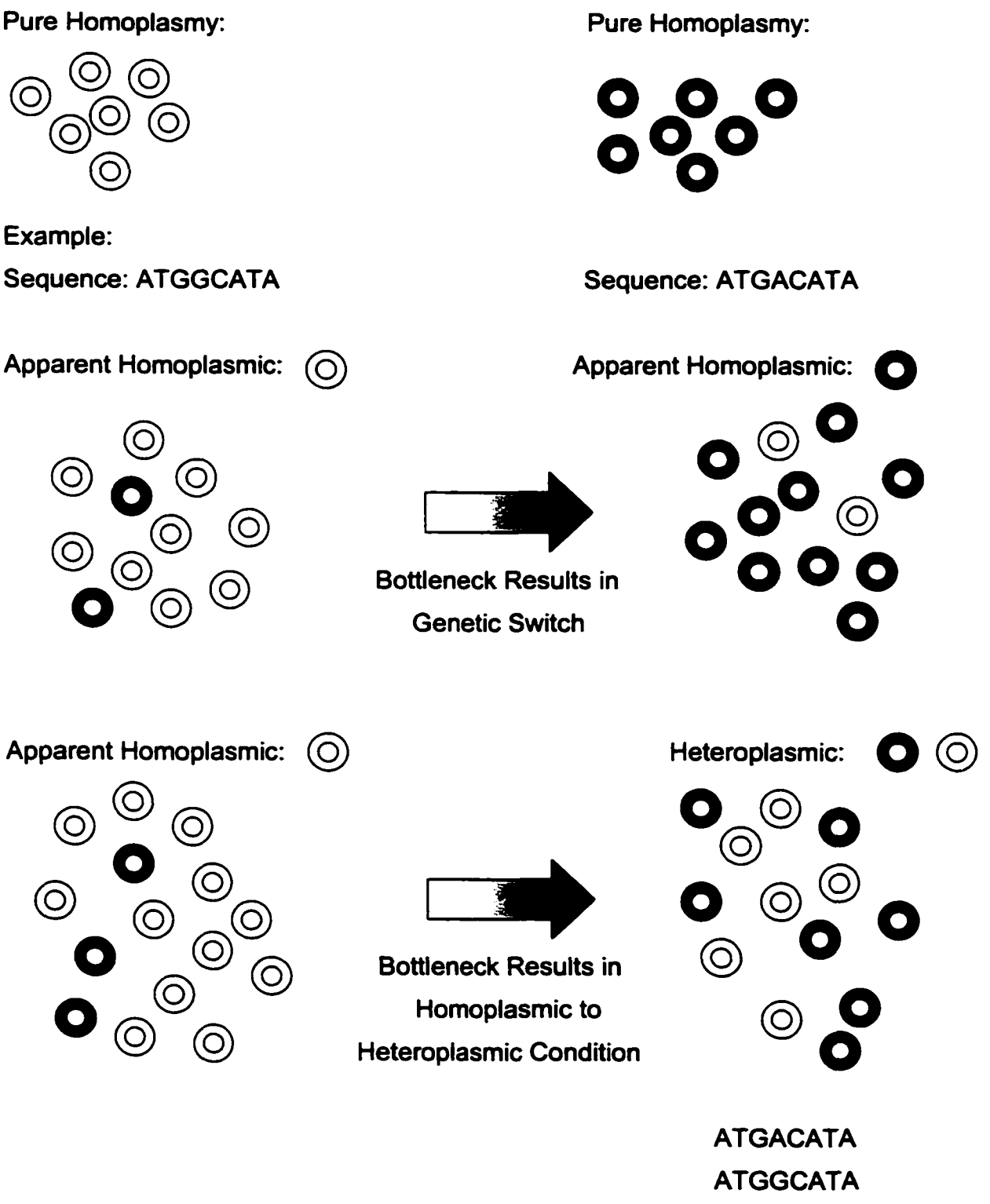


Figure 10.

Illustration of the Bottleneck Theory of Mitochondrial DNA Segregation

Variations in segregation rate have also been found in human population studies. Howell *et al.*[47] found slow segregation in one study but, in a follow-up analysis, found both slow and fast segregation within the same pedigree.[23] Parsons, however, reported segregation within a single generation.[24] Bendall *et al.*[50] analyzed two pedigrees and calculated that a bottleneck comprises three to twenty segregating units. Johns[48] first demonstrated the same mtDNA deletion in two different heteroplasmic tissues from a living patient, indicating that the mutation occurred before divergence of the cell lines, either early in embryonic development or in the maternal germ-line. Research conducted by Blok *et al.*[49] suggests that additional factors influence the distribution of wild and mutant mtDNA. This study analyzed seven oocytes from a female with heteroplasmic expression at base pair position 8993 (T→G). The results indicated a skewed expression, with one oocyte exhibiting no mutation and the remaining oocytes exhibiting high levels (>95%) of mutated genomes. They suggest that during amplification of mtDNA in the developing oocyte, mtDNA from one mitochondrion is preferentially, although not exclusively amplified (rather than random segregation and replication). Thus, subsequent mature oocytes may contain predominantly wild-type or mutant–type mitochondrial genomes. This hypothesis would support rapid germ-line segregation and also suggests a mechanism to minimize inheritance of deletion mutations.

These studies, amongst others, have increased the understanding of mitochondrial genetics, along with the implications of the faster mutation rate and degree of variation exhibited within the Mitochondrial DNA genome. As a result, it

generated interest in at least three different, although not unrelated disciplines: the medical profession, anthropology/paleontology, and forensic science. Much of what we have learned in the last few years has been gleaned from the sequence analysis of mtDNA, in an effort to study mitochondrial diseases.

Mitochondrial Diseases

Mitochondrial dysfunctions that hinder the cellular respiration process may be attributed to mutations that are encoded in both the nuclear and genetic genomes.[52-56] These disorders can show different patterns of inheritance, can affect any tissue that relies on oxidative metabolism, and may present symptoms anytime from early infancy to old age. Studies indicate that the most common degenerative mitochondrial diseases are associated with mutations in the coding regions of mtDNA,[57] although the number of reported nuclear defects have increased in recent years. Mitochondrial DNA defects can be classified into two types, mtDNA rearrangements (deletions or duplications) and mtDNA point mutations. These disorders generally affect muscle and nervous tissue. Examples of phenotypes associated with mtDNA rearrangements include progressive external ophthalmoplegia (PEO); Kearns-Sayre[58] syndrome (KSS), which results in paralysis of external eye muscles; and Pearson syndrome.[59] Although these mutations are typically small-scale deletions or duplications, Schon *et al*[60] have identified a large-scale (5kb) deletion associated with KSS and PEO. This study suggests that deletions of this size appear adjacent to

"hotspots" of direct repeat sequences. The distribution of normal and mutant DNA influences the severity of the clinical symptoms in individuals with mtDNA deletions. However, even significant mutations may not be fatal, producing only mild clinical symptoms. Similarly, a number of phenotypes have been associated with point mutations in mtDNA including Leber's Hereditary Optic Neuropathy (LHON),[61] which causes bilateral visual loss and optic atrophy; neuropathy, ataxia and retinitis pigmentosa (NARP);[62] and, Leigh disease.[63] Interestingly, both NARP and Leigh disease, whilst manifesting distinct symptoms, are both caused by a T-to-G mutation at base pair position 8993. Leigh disease is typically observed in cases where the percentage of mutant mtDNA is particularly high. The two most common phenotypes associated with point mutations in tRNA-coding genes within the mtDNA genome are myoclonic epilepsy with ragged-red fibers (MERRF);[64] and mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS).[65] Again, the distribution of normal and mutant DNA influences the severity of the clinical symptoms in individuals with mtDNA point mutations.

An interesting feature of mitochondrial diseases associated with mtDNA point mutations is the potential relationship between the defects and their symptoms. In some cases, there is a frequent association between a symptom and a specific mutation, such as the 8344A>G mutation in the $tRNA^{Lys}$ gene and MERRF. However, this correlation is not seen in the majority of the mutation cases observed. Compounding this issue is the fact that mutations in the same tRNA

can cause different syndromes or mutations in different genes can cause the same syndrome.

Ordinarily, mtDNA diseases resulting from base substitutions are maternally inherited. They can alter polypeptide genes, cause mis-sense mutations, or affect structural RNAs. Insertions and deletions are typically spontaneous somatic mutations, which are rarely inherited maternally, although they may be inherited along Mendelian lines in the case of nuclear-encoded mitochondrial genes. For example, it is known that MERRF is caused by a point mutation in a gene coding for a protein essential for electron transport chain function. Consequently, in this disorder the mitochondria do not produce ATP through oxidative phosphorylation. With other disorders, it is believed that individuals contain heteroplasmic mixtures of both the wild and mutant-type mtDNA but eventually segregate back to normal-type homoplasmy. It also appears as if mutant mtDNAs must reach a critical threshold level within a particular tissue before a phenotypic expression is observed. If the proportion of mutant molecules is low, the wild-type molecules may override the manifestation of the mutation. Therefore, most people with sub-threshold levels are probably asymptomatic for the disease. In contrast, if the percentage of mutated molecules exceeds the threshold, the wild-type mtDNA molecules are not able to complement the mutation, producing a dysfunction in the oxidative metabolism that manifests itself phenotypically as a diseased condition.[66] Further, the phenotype may vary in different tissues within individuals. It follows that tissues with higher oxidative requirements contain a higher absolute number of mtDNA molecules, which means they may potentially

have a higher percentage of mutant mtDNA expressed. Therefore, they would be more sensitive to the effects of pathogenic mtDNA mutations. For example, the brain, heart, or bone tissues are more sensitive to a defect in energy production than are blood or skin tissue. Thus, a blood sample may indicate a homoplasmic type ("A" identified at a given position) whereas brain tissue sample may generate heteroplasmy (mixture of "A" and "G" at this same position).

Mitochondrial DNA and Aging

It has also been postulated that mutations in mtDNA are associated with the aging process.[67] Evidence seems to indicate that with increasing age there is an increase in mtDNA deletions, resulting in a decrease in energy production and a decline in mitochondrial activity in post-mitotic cells and tissues. While these effects may be compatible with a normal adult life, they can manifest as devastating symptoms later in life.[68] A number of studies have focused on the 4977bp deletion,[69-72] as it is believed to be the most common age-related deletion. Another study reports that the frequency of heteroplasmy in the HV II region increases with age.[73]

Evolutionary Studies

The control region of the mitochondrial genome has been analyzed for human population studies.[129] There are two reasons why mtDNA is employed in evolutionary studies. First, it is maternally inherited, and therefore, trees relating mtDNA types reflect the maternal history of a particular species.[91] Second, since it evolves quickly, many differences arise even among mtDNAs from closely

related populations. In theory, mutations accumulate at a constant rate and therefore, by comparing differences in the nucleotide sequence, a determination can be made as to when two population groups diverged. Some studies have focused on comparing the variation in human mtDNA to the prediction of neutral equilibrium models.[78-79] These models are based on the assumption that the human population is at equilibrium with respect to variation that is gained compared to the loss due to genetic drift effects. Research conducted on mtDNA sequences suggests that a correlation exists between sequence and ethnic background.[74-76] These studies appear to show important differences, both among racial groups and within the same ethnic group. This implies the possibility of predicting ethnicity based upon observed sequences, which may be useful in situations where there is no suspect. Connor and Stoneking[77] have developed a logistic regression model to predict ethnic groups from mtDNA sequences. Extensive analyses of cleavage sites and sequences of mtDNA[80] have demonstrated the highest variation in the African ethnic group. This finding supports an earlier suggestion by Brown[81] that the last common ancestor of contemporary humans existed about 200,000 years ago. However, this notion is strongly disputed by proponents of the multi-region hypothesis[82] who claim that man originated simultaneously in various geographic regions and that the age of the last common ancestor exceeds 200,000 years.

The main controversy regarding the use of mitochondrial DNA for evolutionary studies has centered on the estimated age and reliability of the mtDNA

"molecular clock" to gauge rates of evolution (a measure of nucleotidesubstitution rates). Reliance on the "molecular clock" has been criticized because varying mutation rates have been identified for different DNA sequences within the mtDNA genome. To resolve close relationships among humans, recent studies[74,83-85] have focused on sequence analysis in the displacement loop of mtDNA. Another study by Hasegawa *et al.*[86] emphasizes the importance of correcting for rate heterogeneity (variation in substitution rates) among sites when analyzing non-coding regions of mtDNA in order to estimate the last common ancestor.

The most widely used mutation rate for non-coding human mtDNA relies on estimates of when humans and chimpanzees shared a common ancestor, and is taken to be five million years ago. This date is based on counting the mtDNA and protein differences between primates and timing their divergence dates using fossil records. In humans, this yields a rate of about one mutation in three hundred to six hundred generations, or one every six to twelve thousand years. Horai *et al.*[87-88] compared homonoid mtDNA by analyzing complete mtDNA sequences from human and non-human hominoids in discussing the origins of modern humans.

Population Statistics

A mtDNA population database, referred to as the Scientific Working Group on DNA Analysis Methods (SWGDAM) database, has been compiled by the FBI, AFDIL and other cooperating laboratories to estimate the prevalence of a mtDNA

haplotype. It contains the mtDNA sequences of both HVI and HVII from four racial groups: Asians, Hispanics, African Americans and Caucasians. The groups are further divided into geographic areas, or ethnic origin. In April 2001, the database contained approximately 4,360 human mtDNA sequences. Since the mitochondrial genome is inherited as a single unit, it must be considered as one entire genetic locus. Therefore, although an individual can exhibit different sequences across the various regions, unlike nuclear DNA, these regions cannot be considered discrete alleles because they are not independent of each other. Consequently, the counting method relies on population frequency calculations to predict how common the mtDNA profile is in the population. This is achieved by comparing the evidentiary sample with the reference database to determine how frequently it occurs in the database. A confidence level of the estimated frequency can also be calculated based on the size of the database and is established with an upper bound that benefits the defendant. Further, although the majority (60%) of the sequences in the SWGDAM database are "unique" in that they occur only once, the total number of different mtDNA sequences and the frequency of these sequences in the human population is unknown. As a result, until a comprehensive mtDNA database is compiled, it will not be possible to obtain reliable frequency estimates for most mtDNA sequences. An international standard forensic database of the human mtDNA control region has been also compiled.[130] As of April 2001, the database comprises a total of 10,828 mtDNA sequences, drawn from SWGDAM and other nationally and internationally published data. The database includes information on the

continent/country of origin and ethnicity, and incorporates the geographical region, the number of populations, the number of mtDNA types per population and the sequence range.

Forensic-Related Studies

MtDNA typing is also valuable in a forensic context in the identification of human remains.[92-93] Given the high copy number per cell, the mitochondrial genome is particularly suited to the genetic analysis of samples containing limited amounts of biological material. Insufficient sample may be attributed to small sample size, DNA degradation, or due to the absence of a nuclear genome. For example, mtDNA sequence information has been obtained from the ancient human remains of a 7000-year-old brain[94] and a 5000-year-old mummified body,[95] as well as teeth,[96-99] skeletal fragments,[100-101] dried tissue[102] and human feces.[103] Several studies of mtDNA from plucked human hair have also been performed.[105] The aforementioned have all proven difficult samples when attempting to yield nuclear DNA markers. In the study conducted by Paabo *et al.*[94] it was demonstrated that mtDNA from ancient sources is highly fragmented, such that PCR amplicons greater than approximately 150bp lengths could not be recovered from aged tissues. While this does not apply to most mtDNA analyses of forensic samples, since amplicons approximately 250bp in length are typically obtained, samples exposed to extreme conditions of heat, humidity and chemical action may not be successfully amplified. A study by Gabriel *et al.*[104] suggests

that the use of a "mini primer set" (MPS) that generates small amplicon products (126 – 170 bp) can be more successful when compared to primers generating larger amplicons, particularly when analyzing degraded samples. Since head hairs are frequently encountered as physical evidence, mitochondrial DNA analysis is particularly applicable to the analysis of telogen head hairs, which generally lack a nuclear genome.[92] A method has been reported for the analysis of mtDNA from human hair shafts[106] and recently, Allen described a sensitive technique for analyzing shed hairs, saliva, nail scrapings and small bloodstains.[107] In 1996, Sullivan reported the first heteroplasmic point mutation in hair shafts from an individual whose blood was homoplasmic.[108] Huhne *et al.*[109] recently compared the mtDNA sequences of head hair shafts of individuals with blood and saliva samples and found no evidence of heteroplasmy. Pfeiffer *et al.*[110] conducted a similar study of mtDNA sequence comparisons from human axillary, pubic and head hair shafts, again, reporting no incidence of heteroplasmy. Bendall *et al.*[111] found highly variable levels of heteroplasmy in single hair roots within an individual despite the fact that there was consistent levels of heteroplasmy in samples obtained from peripheral blood and buccal cells. In another study, Grzybowski[112] reports finding several cases of multiple heteroplasmy (more than one mutant type is detected in the mixture) in single hair roots from the same individual. Also, Stewart *et al.*[113] demonstrated that different hairs from the same individual exhibit length polymorphism in the HVII region. This intra-individual variation results in a heteroplasmic condition that can

manifest itself in both a length variation and different levels of expression of the mixture from hair to hair.

It has also been demonstrated that domestic dog hairs are amenable to mtDNA analysis.[114] This illustrates the potential of animal hair and other sources of trace evidence in providing investigative leads, particularly when combined with a comprehensive database. One such database is described by Savolainen *et al.*[115] and comprises one hundred and two domestic dogs spanning fifty-two different breeds. Within the database, nineteen mitochondrial variants are identified, with frequencies that vary from 1 - 21%.

Methods of Analysis

A number of approaches have been used to type the mitochondrial genome. Connor[77] used PCR and subsequent dot-blot analysis with sequence-specific probes to detect variation in the hypervariable parts of the control region. Wong[116] employed a similar technique with allele-specific probes to screen for multiple point mutations in mtDNA, while Schollen[117] developed a reverse dot-blot format for screening mtDNA mutations associated with LHON. Other methods that have been explored combine PCR with single-strand conformation polymorphism analysis (SSCP)[118] and restriction fragment length polymorphism (RFLP) variation using restriction enzymes.[73,75,119] Tully's group developed a multiplex mini-sequencing approach to detect mtDNA variation.[120] This technique, referred to as "multiplex solid-phase fluorescent mini-sequencing", detects and types ten substitution polymorphisms and two length polymorphisms.

The procedure involves multiplex PCR amplification of several mtDNA loci, which are subsequently immobilized on magnetic beads prior to the addition of mini-sequencing primers for all of the loci. The primers are then extended using DNA polymerase and dideoxynucleotides, each labeled with a different fluorescent dye. Separation of each loci is achieved based on their differing mobility by polyacrylamide gel electrophoresis. Each separated locus is then analyzed using an automated sequencer. Hanekamp *et al.* have developed another means for screening mtDNA polymorphisms.[121] This method involved denaturing gradient gel electrophoresis (DGGE) and allows DNA strands that exhibit minimal sequence differences to be separated electrophoretically. This technique is based on the fact that sequence-specific melting domains exist along the double-stranded DNA. By exposing the melting domains to increasing concentrations of a denaturant, the melting temperature of the domain is attained, and the strand is partly or totally denatured. This, in turn, affects the mobility of the strand, which can be detected in an electrophoresis gel. Given that the melting temperature of a melting domain is sequence-specific, a mutation in the sequence can alter this temperature and affect its migration in a gel. Thus, a sequence variant will appear as a polymorphic band on the DGGE.

The most common method of mtDNA analysis is accomplished by analyzing the PCR amplification product with direct sequencing, performed manually[81,90,98-99] or using an automated sequencer.[100-105,122-128] In either case, the sequence of the mtDNA genome under analysis is compared to a consensus sequence. By

convention, the consensus sequence is defined as that reported by Anderson[11] (light strand).

Legal Issues

In 1996 a Tennessee court admitted the results of mitochondrial DNA typing for the first time in *State of Tennessee v. Paul William Ware*. The case involved a comparison of the mtDNA typing results of two hairs (one recovered from a bed sheet at the crime scene and another removed from the pharynx of the victim) with a saliva sample obtained from the suspect. The results indicated a "match" in the mtDNA profile; however, the prosecution failed to address certain issues relating to heteroplasmy. Properly interpreted, heteroplasmy may discriminate among individuals in the same maternal lineage. However, an incorrect interpretation has the potential to falsely exclude a suspect or, less commonly, falsely include a potential suspect. MtDNA analysis has subsequently been reviewed in other jurisdictions. These include Ohio (US *v.* Turns), Michigan (People *v.* Holtzer), South Carolina (State *v.* Council), North Carolina (State *v.* Underwood), Maryland (State *v.* Williams), Pennsylvania (Commonwealth *v.* Dillon), California (People *v.* Torres), New York (People *v.* Klinger), Connecticut (State *v.* Pappas), Florida (Crow *v.* State), Washington (State *v.* Smith) and Indiana (Anderson *v.* State).

CHAPTER 3

Methods and Procedures

Subjects and Design of the Study

Announcements were distributed to crime laboratory personnel in Southern California (LAPD, Scientific Investigation Division; LA County Sheriff, Scientific Services Bureau; and Orange County Sheriff Laboratory) and to the faculty, staff and students affiliated with California State University at Los Angeles. These announcements requested participation in the study and the need for hair and blood samples. Participants were asked to self-collect sixty head hairs in order to ensure a sample that represented the natural variations that can exist within individuals.

Individuals who agreed to participate were issued a sample collection packet. Each packet was labeled with a unique donor # and contained a clean, unused comb and ten small envelopes. Each envelope was pre-labeled with the region of the scalp that the hair was to be removed from and the means used to obtain the sample (for example, "Head hair sample: front/plucked", "Head hair sample: front/combed", etc.). The collection packet included an instruction sheet on hair collection and packaging procedures. Specifically, each donor was asked to collect ten hairs from each of five regions on the scalp designated: front, center, rear, left, and right side. Of these ten hairs, five were plucked and the other five were obtained by combing the respective areas. Participants were reminded to

seal each envelope once collection was complete, but not to initial the seal. Each participant was also supplied with a sealed, sterile lancet and a sealed, sterile alcohol pad in order to obtain a blood sample using a finger puncture technique. The participant was asked to transfer 0.2 milliliters of blood (~4 drops) directly onto the bloodstain cards supplied, allowing the bloodstain card to air dry prior to folding it and inserting it into the small manila envelope provided. Again, each was reminded to seal the envelope, but not to sign or mark the seal in any way. Participants were also asked to answer three demographic questions related to their age, sex and race and one relating to chemical treatment of their hair. The study required that all of the subjects were eighteen years or older, and excluded individuals who were pregnant.

In addition to these living subjects, permission was granted to collect postmortem hair samples from decedents discharged to the Los Angeles County Coroner's Office. In the case of the decedents, blood samples were collected by venipuncture of the femoral vein and the demographic information was obtained from official Los Angeles County Coroner autopsy records. Although information relating to chemical treatments was not available for the decedents, morphological observations of the hair using light microscopy provided a limited amount of information with regard to certain potential treatments.

A total of seven thousand nine hundred and twenty head hairs (132 individuals x 60 hairs each) and one hundred and thirty-one blood samples were collected over a period of approximately eight months. Initially, subjects were accepted one at a time and assigned to a subgroup base on age range, sex

and race. In order to draw statistical inference from the data, a target number of at least five individuals across all three variables was obtained. Once the target for each subgroup was accomplished, specific individuals were selected based on the need to balance the number of subjects across all of the subgroups. In all, 64 males and 68 females were assigned. The age of the subjects ranged from 18 to 88 years, each falling into one of four age ranges: 54 individuals in the 18-29 years category; 46 in the 30-49 years category; 23 in the 50-69 years category; and 9 in the 70 years and above category. The distribution of population groups collected for this study can be summarized as follows: 44 Caucasian, 35 African American, 25 Asian and 28 Hispanic individuals.

The described method of collection represents a non-probability sampling procedure. However, by using a purposive/convenience sample which describes the characteristics of the target population (in terms of age, race, and sex) weighted appropriately to their proportion in the general population, the overall data provides a reasonable representation of the demographic characteristics of the United States population (tables 2 & 3). This type of sampling is considered reasonable given the exploratory nature of the research.

A labeling code was assigned to each hair to give it a unique identifier. For example, A33(1)1F represents the initial portion of the proximal end of first anagen hair, selected from the front region of donor 33.

Table 2. Sub-Groups of Sample Demographics

	C	C	B	B	A	A	H/OTHER	H/OTHER	
AGE	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE	TOTAL
18-29	5	10	5	5	9	6	6	8	54
30-49	7	5	5	8	5	5	6	5	46
50-69	7	5	3	5			3		23
70+	2	3	1	3					9
TOTAL	21	23	14	21	14	11	15	13	

Table 3. Demographics of Total Sample

SAMPLE #	SEX	RACE	18-29	30-49	50-69	70+
01	M	A	26			
02	M	A		49		
03	F	A	25			
04	M	A	26			
05	M	A	26			
06	M	H	26			
07	M	C		34		
08	M	A	28			
09	M	A	25			
10	F	C	27			
11	M	C	26			
12	F	C		32		
13	M	A	27			
14	M	A	28			
15	F	A	28			
16	M	A	26			
17	F	H	19			

SAMPLE #	SEX	RACE	18-29	30-49	50-69	70+
18	F	H	22			
19	F	H	26			
20	F	A	25			
21	F	H	21			
22	M	C	27			
23	F	A	27			
24	F	H	27			
25	F	H		39		
26	F	B	24			
27	M	H	26			
28	M	A	23			
29	F	C	29			
30	F	C	28			
31	F	C	24			
32	F	C	24			
33	F	C		33		
34	F	C				80
35	F	H		38		
36	M	C			56	
37	F	B		41		
38	M	H		45		
39	F	C				83
40	F	C		30		
41	M	C			50	
42	F	B		39		
43	M	H		41		
44	F	C	23			
45	F	H	22			
46	M	C		44		
47	M	B	26			
48	F	C		49		
49	M	C		38		
50	M	C			59	
51	M	C		47		
52	M	B/OTHER		41		
53	F	A	29			
54	M	C		46		
55	M	C		45		
56	M	H			62	
57	F	H	28			
58	M	C		41		

SAMPLE #	SEX	RACE	18-29	30-49	50-69	70+
59	M	H			65	
60	F	C			66	
61	F	C	23			
62	F	C				79
63	F	H		36		
64	M	B			63	
65	M	B			63	
66	F	A		44		
67	M	H		49		
68	F	B		44		
69	F	H	26			
70	M	C				74
71	F	C			59	
72	M	C			60	
73	F	C			50	
74	M	C			62	
75	F	C			61	
76	F	B		47		
77	F	C	24			
78	M	C			66	
79	F	C			54	
80	F	B	18			
81	F	B	22			
82	F	C	23			
83	F	A	23			
84	M	H			53	
85	F	H		37		
86	F	C		35		
87	F	C	25			
88	M	H		40		
89	F	B				88
90	M	C			67	
91	M	H		38		
92	M	C				77
93	M	B		42		
94	F	A		48		
95	M	H	20			
96	M	B			62	
97	F	H		38		
98	M	B		36		
99	M	A		48		
100	F	A		43		

SAMPLE #	SEX	RACE	18-29	30-49	50-69	70+
101	F	B	21			
102	M	A		33		
103	M	B	19			
104	M	A		31		
105	F	A		31		
106	F	B			55	
107	M	B		32		
108	F	H		34		
109	F	A		34		
110	F	B			62	
111	F	B	27			
112	F	B			59	
113	F	B		30		
114	F	B				82
115	F	B		45		
116	M	H	29			
117	M	B				71
118	F	B		47		
119	M	B	23			
120	M	A		33		
121	M	B	24			
122	M	B	23			
123	M	C	29			
124	F	B		48		
125	F	B			57	
126	F	B			66	
127	F	B				73
128	M	H	19			
129	M	C	23			
130	M	H	21			
131	M	C	27			
132	M	B		44		

Experimental Procedures for Examining the Research Questions

In order to better understand the factors that contribute to heteroplasmy in human head hair, this study examined six primary research questions. In addressing each research question, the total subject pool was partitioned into comparison groups. The procedures used to study these questions are outlined below. During each experiment, an attempt was made to control for the following independent variables: morphological characteristics; donor age and sex; growth phase; scalp origin; portion of the hair shaft; cosmetic treatment; and living/deceased donor.

1. How much hair sample is required for successful polymerase chain reaction (PCR) amplification? What other variables influence the success rate?

This was a sensitivity study to determine the amount of extracted mitochondrial DNA that can be reliably typed based on the length and diameter of the hair shaft. The experiments were designed to determine the amount of template hair that should be processed to yield the optimal amount of mitochondrial DNA that is added into an amplification reaction. The study also assesses the additional experimental factors that may influence the amplification success rate. Sample size is important for several reasons. Presumably, the greater the sample size, the greater the potential to detect the minor component in a heteroplasmic mixture. However, there are counteracting effects when dealing with larger samplings. First, there is a greater likelihood of introducing a

contaminant when sampling over a larger area, which will be amplified along with the target template. A contamination can be thought of as any non-relevant cellular material or mitochondrial DNA that was introduced before or after the material was deposited. Secondly, the larger the DNA input, the greater the possibility of introducing inhibitors into the amplification reaction. The presence of a sufficient level of PCR inhibitor can reduce the activity of the *Taq* polymerase, which may decrease product yield. Inhibitors may be indigenous to the sample or may derive from extraneous sources. Examples of the former that relate to this study are hemoglobin (from erythrocytes) and melanin (hair pigment), whereas extraneous sources include dyes present on sample substrates or possibly substances adhering to a hair shaft. Finally, increasing the amount of template DNA does not guarantee successful amplification, since it may not be the limiting reactant in the polymerase chain reaction. To demonstrate the relationship between sampling, successful amplification and subsequent typing, hair samples measuring up to two centimeters in length were extracted and amplified. Those hairs that were successfully amplified were subsequently typed. The shaft diameter and the racial origin of each hair were also recorded, as these variables may influence total DNA yield. For example, the length and diameter of the hair shaft, coupled with the degree of pigmentation may be a factor affecting DNA extraction. Blood samples measuring 3 millimeters square were also analyzed to generate a reference sample. In addition, since each hair was recorded photomicrographically, this permitted an assessment of whether hair morphology can be used as a reliable predictor of amplification success. This

information is invaluable in terms of selection of hairs for subsequent mtDNA typing.

2. What is the effect of heteroplasmy on the interpretation of forensic samples obtained from the hair shafts and blood of the same individual?

To demonstrate differences in heteroplasmic expression between tissues, parallel experiments were conducted to compare the mitochondrial DNA type of the peripheral blood with hair samples from the same donor. This study is important because initially, the reference typing was obtained from a sample of peripheral blood that represented the donor's dominant haplotype. Thus, it defined the homoplasmic type in order to assess whether a heteroplasmic mixture existed. The study also assesses whether there are differences in mitochondrial DNA type between tissues within the same individual, and if so, the nature and frequency of occurrence. This is important because genetic differences attributed to heteroplasmy may prevent the use a blood sample as a "universal" reference source for mtDNA comparison purposes. It has long been established that discrete tissues have different energy demands, which is reflected in the number of mitochondria within the cellular cytoplasm. A comparison of the tissues permits both the identification and extent of sequence mosaicism that existed between different tissues in the same individual. For example, different patterns may be detected. This might include blood homoplasmy/hair heteroplasmy; blood heteroplasmy/hair homoplasmy; blood homoplasmy/hair homoplasmy with a genetic difference observed between tissues; or blood heteroplasmy/hair heteroplasmy expressed at different levels.

3. Is the mitochondrial DNA yield from the shaft affected by exposure to various chemical hair treatments?

This study involved collecting and comparing hairs that had been subjected to different cosmetic treatments (dyed, permanent chemical waving, semi-permanent waving, highlighting, straightening, etc.). The question here is whether or not chemical hair treatments degrade or otherwise reduce the quantity of typable mitochondrial DNA in the shaft. Although it is fairly well established that the mitochondrial DNA type does not change with various cosmetic treatments, it is not known how much mitochondrial DNA survives treatment. This may impact the sample size needed to obtain a typable result, or in a heteroplasmic case, the ability to detect the minor component. In addition, the chemicals may have an inhibitory effect, which may also affect the ability to identify the minor sequence. If a reduction in yield is observed, it may be necessary "spike" the extract with a positive control in order to distinguish inhibition from degradation. A comparative quantitative analysis was conducted of the mitochondrial DNA yield extracted from each hair (based on examination of the ethidium bromide intensity signal of the PCR product when run in a product gel). This permitted an estimate of the amount of mtDNA extracted.

4. What is the frequency of the heteroplasmy observed using the mtDNA linear array assay method?

This experiment addressed the issue of whether heteroplasmy is a common condition identified using the linear assay method. The current literature states that a heteroplasmic state exists at a higher rate than earlier suggestions indicated, with one study conducted by the Armed Forces DNA Identification Laboratory (AFDIL) estimating an occurrence exceeding 50% of the population (n=21, discrimination power = 31.0-73.8 at the 95% confidence level). [133]
The purpose of this experiment is to quantify the incidence of heteroplasmy for the blood and hair samples collected in this study.

5. Do different hairs from the same individual exhibit different haplotypes?

In this experiment, hairs were selected from the same individual in order to determine if they exhibit different haplotypes. It has been established that hairs grow independently of each other in the hair follicle, which means that they may accumulate different mutations. In addition, discrete hairs may reflect different levels of heteroplasmy. This may manifest itself in different ways: for example, two hairs may be homoplasmic but reflect different haplotypes due to a genetic difference; or hairs from the same individual may reflect a heteroplasmic mixture of both haplotypes. The levels of the major and minor components may also differ from hair to hair. This study is important because it will determine the number of hairs that will be necessary to sample in order to obtain a representative haplotype of an individual, and determine if minor differences in mitochondrial DNA type constitute exclusionary data.

6. Does detection of the heteroplasmic condition in a given individual correlate with the microscopic morphology of distinctly different hair types in the scalps of individuals?

In this experiment, hairs were selected from a representative sample obtained from a given individual. This study focused on whether a microscopic examination may be a useful predictor of the heteroplasmic condition. The study determined whether the heteroplasmic condition correlates to those hairs exhibiting distinct morphologies. The basis for selection was that the hairs exhibited distinct morphological features. These features were macroscopic (color, texture, thickness) and/or microscopic (color, medulla structure, cortex structure) in nature. For example, hairs exhibiting a medulla generally contain less cellular debris compared to hairs that lack a medulla. The greater number of cells in the later may reflect an increase in mtDNA copy number. In addition, heavily pigmented hairs comprise a larger number of melanosomes. Since it has been established that melanosomes have their own source of mtDNA, hairs that are more pigmented may also reflect an increase in mtDNA copy number. Further, since there are two potential sources of mtDNA within the hair follicle, a mutation in one source may provide an explanation for the existence of a heteroplasmic mixture. The study evaluated what constituted a representative sample for morphological characteristics and mtDNA typing. Since each hair was photographed prior to extraction and analysis, it allowed for an assessment of the morphological appearance of a hair as being a reliable predictor of successful amplification.

In addition to the six primary issues listed above, this study addressed five secondary issues, two of which are related to demographic factors:

7. Is the heteroplasmic condition correlated with age?

Studies have indicated that somatic deletion mutations accrue with age. This is based primarily on research in clinical medicine on mitochondrial DNA diseases in the coding region of the genome. It appears that certain diseased states manifest when the mutated gene represents a certain threshold level within the heteroplasmic mixture, which, it seems, becomes more pronounced with age. This is important from a forensic standpoint because variation in individuals that takes place over time implies that different haplotypes may be obtained when comparing contemporaneous and historical samples from the same individual. Likewise, when comparing contemporaneous samples with a reference sample from a maternal relative. In order to investigate if expression of the mutant form of the genome is more discernible with age, blood and hair samples were collected from individuals in the age range of 18-88 years.

8. Is the heteroplasmic condition correlated to race?

Human population studies on mitochondrial DNA sequences appear to show important differences both among racial groups and within the same ethnic group. Since two population groups diverge as a result of an accumulation of mutations in the genome and because a mixture of mutant and wild-type genomes manifests itself as a heteroplasmic condition, it seems reasonable to suppose that different races and/or ethic groups may be more susceptible to

heteroplasmy. This is true of mutations associated with nuclear DNA. For example, a number of conventional protein markers exhibit various levels of polymorphisms within different racial groups in the population due to the inheritance of mutated genes. This variation within racial groups has also been recognized when analyzing DNA directly and different population databases have been compiled as a result. This study is important because it will inform analysts of the need to be particularly cognizant of interpretation problems with certain racial groups. In order to investigate whether the incidence of heteroplasmy correlates with a particular racial group, blood and hair samples were collected from individuals within four racial groups: Asian, African American, Hispanic, and Caucasian. These racial groups were either self-reported (living donors) or from medical examiner autopsy reports (decedent donors).

9. Are there differences in the level of detection of heteroplasmy when comparing living with deceased individuals?

Here, the issue is whether postmortem changes in the hair shaft or root impact the recovery of mitochondrial DNA. Postmortem tissues undergo decompositional changes due to microbial and enzyme action. This activity may degrade the mitochondrial DNA and impact its recovery. Research indicates that various postmortem morphological changes occur within a hair shaft. These include root banding, formation of a hard keratin point, or the development of brush-like roots. Postmortem root banding is attributed to the formation of air pockets and typically occurs in the region where the sebaceous duct enters the

follicle. The presence of air pockets could impact the success rate of the amplification because fewer DNA templates may be available. In order to assess whether postmortem changes have an effect on the recovery of mitochondrial DNA, blood and hair samples were collected from living subjects and decedents.

10. Does the heteroplasmic condition represent a mosaicism or a random distribution across the scalp of an individual?

As stated earlier, each scalp hair grows independently of any other hair. The issue here is whether or not mutations can accumulate in discrete areas of the scalp, or are distributed randomly. This has important ramifications because it impacts hair collection procedures in attempting to obtain a representative sample intended for mtDNA analysis. In order to investigate spatial mosaicism, hair samples were collected from five regions of the scalp: front, back, left-side, right-side, and center.

11. Is heteroplasmy correlated to the growth stage of the hair?

This study investigated the relationship between the growth phase of a hair and the ability to detect heteroplasmy. Research has indicated that an anagen root bulb matrix contains a higher proportion of mitochondrial DNA relative to telogen hairs. Therefore, even in the absence of follicular tissue, it is reasonable to believe that these hairs are more likely to amplify successfully, permitting detection of the minor component in a heteroplasmic mixture. This is important because it may be the source of observation of different haplotypes for telogen

and anagen hairs from the same individual. In order to investigate whether the growth phase of a hair impacts the successful recovery of mitochondrial DNA, combed and plucked hair samples were collected from individuals.

Experimental Methods of Analysis

Photomicrography

Representative hairs from each donor were initially mounted on acetate sheets, labeled with their unique identifier, and observed microscopically. This was performed in order to confirm that the hairs were correctly designated "telogen" and "anagen" (as indicated by the root morphology) in the collection packets. Hairs that exceeded two centimeters in length were preferentially selected since this was considered to be the minimal length required to obtain sufficient mtDNA. For each telogen hair selected, a 2 cm portion was cut from the proximal end. In the case of anagen hairs, the root tissue was initially removed (labeled appropriately and retained for future analysis) prior to cutting a 2 cm portion from the proximal end. Each hair was subsequently placed on a microscope slide, and mounted in a temporary medium (Cargille mineral oil N_D@23°C = 1.5150±0.0002) with a cover slip. The hairs were examined microscopically at x100 using Kohler illumination (Olympus Model POS). Photomicrographs were taken of each hair to record the root end in addition to representative regions along the shaft (Honeywell Pentax 35mm SLR SP 500

with Asahi Pentax microscope adapter II, shutter speed exposure @1/8", illuminator voltage 6). Several 35mm color reversal films were selected to record the photomicrographs: Kodak 64T 135-36 EPY; Kodak select series Elitechrome 100 ASA, with 80B filter; Fujichrome Sensia II 100 ASA, with 80B filter; and Fujichrome Provia 100F RDP III 135, with 80B filter. The Fujichrome Provia 100F provided the best combination of resolution and economy.

MtDNA Chelex Extraction of Blood

The blood samples used in this study were prepared as stains on FTA™ Cards (Life Technologies/GIBCO BRL Product). Mitochondrial DNA was extracted by incubating a $3mm^2$ portion of the bloodstain with 1mL of sterile distilled water in a microcentrifuge tube at room temperature for fifteen to thirty minutes, mixing occasionally by inversion or gentle vortexing. The extract was transferred to a microcentrifuge for three minutes at 10-15K x g. The supernatant was discarded (all but 20-30 μL) leaving the FTA™ substrate in the tube with the pellet. A 5% Chelex solution was added to the microcentrifuge tube to a final volume of 200 μL. The samples were incubated at 56°C for 15-30 minutes, vortexed at 10-15K x g for ten seconds and placed in boiling water bath for eight minutes. The samples were then vortexed vigorously for an additional ten seconds and spun for three minutes at 10-15K x g in a microcentrifuge tube.

MtDNA Chelex Extraction of Hair

In order to isolate the mitochondrial DNA from the hair shaft, each hair was removed from the temporary mounting medium and washed, initially with 95% ethanol, followed by DI water. This was performed in order to remove material adhering to the surface of the hair. (Potential sources of exogenous DNA include blood, shed scalp cells and, in the case of deceased individuals, brain tissue. In addition, since physiological fluids and tissues may transfer on contact, the adhering material may or may match the mitochondrial DNA type obtained from the hair shaft). The hair was then placed into a pre-labeled microcentrifuge tube containing 500µl 2% SDS, and sonicated in an ultrasonic water bath for ten minutes. Each hair was removed from the SDS solution, rinsed in distilled and deionized water and placed in a micro-tissue grinder (Kontes Glass Company) containing 150µL TE buffer. Each hair was homogenized by grinding in a circular fashion using a matching pestle until no hair was visible. The homogenate was transferred to a color-coded, pre-labeled microcentrifuge tube containing 50µL 20% Chelex. (Color coding was designated to indicate the region on the scalp that the hair was removed from: red – front, orange - center, yellow - back, green – right-side, blue – left-side). 10µL of proteinase K was added to each tube and the samples were incubated in a water bath at 56°C from 6-8 hours to overnight. Each tube was then vortexed vigorously for ten seconds and placed in the microcentrifuge for ten seconds at 10-15K x g. Each sample was then boiled in a water bath for eight minutes. The samples were then vortexed vigorously for an

additional ten seconds and centrifuged for three minutes at 10-15K x g in a microcentrifuge tube.

Procedure for Amplification of mtDNA

a. PCR Amplification Reactions

The polymerase chain reaction (PCR) was employed to amplify segments of the highly polymorphic control region of mitochondrial DNA (figure 11). The reactions were performed in either 50 or 60µL volumes as follows: 20µL template mtDNA or diluted template; 10µL or 20µL biotinylated HVI/HVII primers; 20µL mtDNA Reaction Mix. The amount of template mtDNA added to the reaction mixture was dependent on the tissue source and on the success of prior amplifications of the sample(s): 10-20µL of hair extract and 5µL of bloodstain extract (diluted to 20µL with DI water) was used. In this study, two different approaches were used to add the primer component to the amplification reaction mix. The first approach involved addition of a duplex primer set. For each amplification reaction, the duplex comprises two biotinylated primer sets: one set that specifically hybridizes to the heavy and light chain of HVI and a second set that is specific to the heavy and light chain of HVII. The second approach involved the addition of each singleplex primer set separately. This required the addition of each primer set (HVI and HVII) in a separate amplification reaction. The composition of the mtDNA Reaction Mix is as follows: 1X Ampli*Taq* DNA Polymerase Buffer [(12m*M* Tris-HCl, pH 8.3, 60m*M* KCl) 2.4 m*M*/L $MgCl_2$ final concentration] (Applied Biosystems, Foster City, CA), 200µM each dinucleotide

triphosphate (dNTP), and 250 U/mL Ampli*Taq* Gold® DNA Polymerase (Applied Biosystems). The 0.2mL PCR tubes were also color coded to duplicate the corresponding microcentrifuge tubes: red – front, orange – center, yellow - back, green – right-side, blue – left-side.

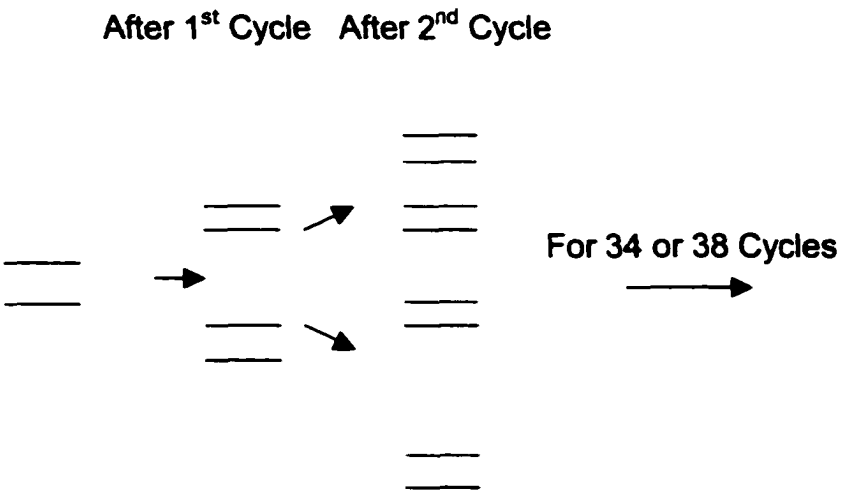


Figure 11. Theoretical Illustration of the Polymerase Chain Reaction

Table 4.
Sequences for the Biotinylated HVI and HVII Primers
Used to Amplify mtDNA Products[139]

Singleplex Primer Pair		Primer Sequence (5'→3')
HVI (16024-16365)	F15975	5'-CTCCACCATTAGCACCCAA-3'
	R16418	5'-ATTTCACGGAGGATGGTG-3'
HVII (73-340)	F15	5'-CACCCTATTAACCACTCACG-3'
	R429	5'-CTGTTAAAAGTGCATACCGCCA-3'

b. PCR Amplification Protocol

The amplification reaction was conducted using a Perkin-Elmer 2400 DNA Thermal Cycler, which was programmed with the PCR profile times and temperatures provided in the table below (table 5). Either thirty-four or thirty-eight cycles were performed for each PCR reaction, depending on the yield. A pre-PCR negative control (no template DNA) and a PCR negative-control (DI water) were run in the Thermal Cycler along with the research samples.

Table 5. Perkin-Elmer GeneAmp® 2400 PCR Profile Time and Temperature Parameters for Amplification

Steps	Temperature	Intervals	Cycle
Activation	92°C	12 minutes	1
Denaturation	92°C	30 seconds	34 - 38
Annealing	60°C	30 seconds	34 - 38
Extension	72°C	30 seconds	34 - 38
Final Extension	72°C	10 minutes	1
Hold	4°C	∞	

c. PCR Product Analysis

The quality and size of the amplified mitochondrial DNA product was analyzed by agarose gel electrophoresis on either a 2% product gel (2.0g NuSieve GTG agarose in 100mL 1 X TBE Gel Running Buffer, run at 100V for 30 minutes; stained with 0.5µg/mL ethidium bromide) or a 3%/1% product gel (3.0g NuSieve GTG agarose and 1g SeaKem GTG agarose in 1 X TAE Gel Running Buffer, run

at 140 V for 60 minutes; stained with 0.5µg/mL ethidium bromide). Gel Loading Buffer (6X) was added to the wells of a 96-well microtiter plate corresponding to the number of samples and controls to be analyzed.

For analysis using a 2% product gel, 5µL of the amplified DNA sample or control was withdrawn from each reaction tube and added to 1µL of Gel Loading Buffer (6X) in the appropriately labeled microplate well. The amplified DNA samples/controls plus loading buffer solutions were mixed and then dispensed via pipette into the wells of the gel. A pre-PCR negative control (reagent blank with no template DNA) and a PCR negative control (amplification blank/distilled water) were run in parallel with the research samples. In addition, 5µL of a DNA Mass Ladder (Apex™ DNA Quantladder; 100-1,000bp) mixed with 1µL of loading buffer designated for the molecular weight marker and included on the gel for estimation of the concentration of PCR product in the research samples. Under optimal conditions using a 3%/1% product gel, the analysis was repeated using 4µL volumes of each sample/control.

The mtDNA was observed using transmitted ultraviolet light (Sigma Chemical Company, Model T1202) and recorded photographically (35mm Kodak black and white film; macro-lens with a 590DF100 filter attached).

Quantitation of PCR Product

The addition of too little or too much product can lead to ambiguous typing results. To ensure that an optimal amount of product was added during the hybridization step, it was necessary to quantitate the PCR product. This was

achieved by comparing the PCR product from the samples to a DNA Mass Ladder with bands of known quantity. Since samples are likely to have variable yields and because both high and low product yields may be encountered within a single gel, it was useful to run two concentrations of DNA Mass Ladder for comparison. Electrophoresis of 4µL of the DNA Mass Ladder neat and at a 1:2 dilution on a 3%/1% product gel, results in bands containing 5-200ng DNA (table 6). The mass of the PCR product was estimated by comparing the intensity of the 444bp HVI and the 415bp HVII to the bands of the DNA Mass Ladders. The less intense of the two bands was compared if unequal amplification of products occurred. The concentration of the PCR product was determined for each sample using the following calibration:

$$\text{Concentration PCR product (ng/µL)} = \frac{\text{Estimated amount of DNA (ng)}}{\text{Vol. PCR product loaded on gel (4µL)}}$$

In cases where the concentration of the PCR product was below the low range of the target quantity of 45ng (<3ng/µL), one or more of the following procedures was followed:

1. If the concentration of the PCR product was less than ~3ng/µL and the DNA sample was limited, a new amplification was performed for a total number of thirty-eight cycles (table 5).

2. If the concentration of the PCR product was less than ~3ng/µL and the DNA sample was not limited, the volume of DNA may be increased up to

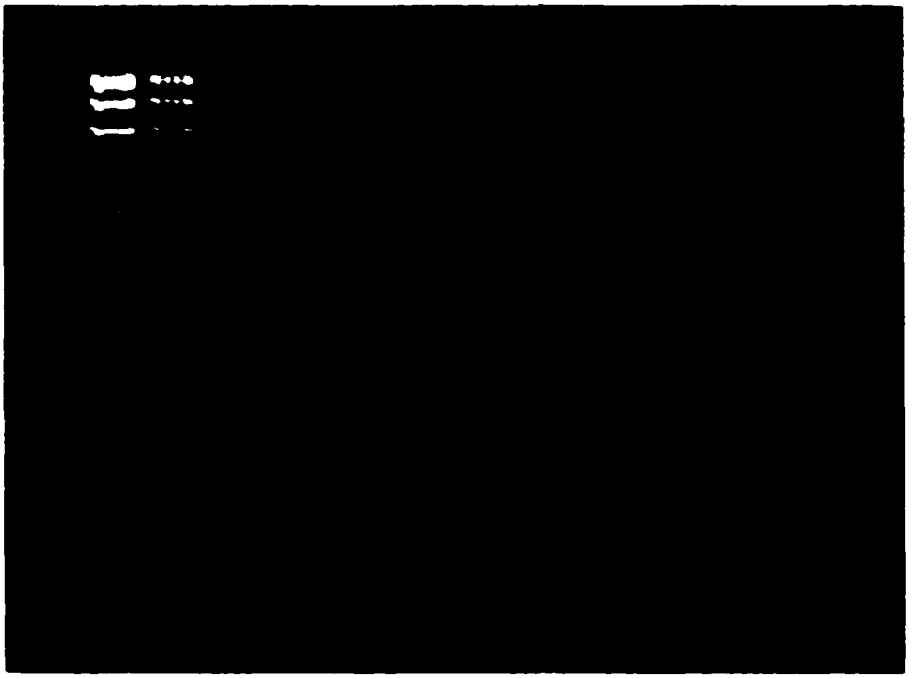
20µL. Depending on the yield of the initial reaction, the number of cycles for PCR may also be increased up to thirty-eight cycles.

3. If the concentration of the PCR product was less than ~3ng/µL and the DNA sample was not limited, and 20µL of DNA was amplified in the initial reaction, the number of cycles for PCR may be increased up to thirty-eight cycles.

4. If the concentration of the PCR product was less than ~3ng/µLand none of the above were successful or if the DNA did not appear to amplify, PCR inhibition was considered to be a possibility. This is discussed below under validation studies.

Table 6. Amount of DNA (ng) in DNA Mass Ladder

Fragment Size	Low DNA Mass Ladder (neat)	Low DNA Mass Ladder (Diluted 1:2)
2000 bp	200 ng	100 ng
1200 bp	120 ng	60 ng
800 bp	80 ng	40 ng
400 bp	40 ng	20 ng
200 bp	20 ng	10 ng
100 bp	10 ng	5 ng



Lane #	Sample	Estimated Product Yield (ng)	Concentration of PCR Product (ng/μL)
1	Low DNA Mass Ladder 1	-	-
2	Low DNA Mass Ladder 2 (1:2)	-	-
3	Product 1A-01	~20	~5.0
4	Product 1A-02	~15	~3.75
5	Product 1A-03	~20	~5.0
6	Product 1A-04	~18	~4.5
7	Product 1A-05	~18	~4.5
8	Product 1A-06	~15	~3.75
9	Product 1A-07	~15	~3.75
10	Product 1A-08	~15	~3.75
11	Product 1A-09	~16	~4.0
12	Product 1A-10	~15	~3.75
13	Product 1A-11	~15	~3.75

Figure 12. Illustration of DNA Mass Ladder Quantitation with 3%/1% Product Gel
[Adapted from Study 1A: Roche Molecular Diagnostics Beta Site Testing of the HVI/HVII mtDNA Linear Array Assay]

Mitochondrial DNA Linear Array Assay Typing System

This research adopts the reverse dot-blot format that was developed in 1989 by Saiki *et al.*[136] in order to type mitochondrial DNA. The technique involves the immobilization of sequence specific oligonucleotide probes to a nylon membrane and the addition of labeled amplified DNA (biotin labeled primers are incorporated into PCR product during the amplification reaction) to the membranes. Consequently, denatured PCR products hybridize to the immobilized single stranded probes bearing a complementary sequence. This hybridization product is detected colormetrically: the horseradish peroxidase-strepdavidin enzyme conjugate binds to the biotin-labeled PCR product, which oxidizes TMB to form a colored product. The pattern of colored bands produced determines the individual's mitochondrial DNA type. Roche Molecular Systems (RMS) have developed a mitochondrial DNA typing format whereby the conventional immobilized probe "dots" have been replaced by probe "lines", thereby accommodating more probes on a given piece of membrane (See Appendix F for protocol).

a. Mitochondrial DNA Linear Array Assay

The "RMS mitoTYPE" linear array strips are made up of thirty-one probes that reveal the haplotypes of an individual within the control region of the mitochondrial DNA genome. Within the control region, two hypervariable regions have been defined based on the higher mutation rates (higher polymorphism) observed in these regions. These have been designated HVI and HVII. Four

regions have been identified within each: for HVI these are A, C, D, and E; for HVII these have been designated A, B, C, D. Each of the eight regions detects a number of polymorphisms at 1-3 nucleotide positions that differ from the Anderson Consensus Sequence. These are summarized as follows: HVIA has three probes and can detect three polymorphic variants; HVIC has four; HVID has two; HVIE has three; HVIIA has two; HVIIB has seven; HVIIC has four; and HVIID has two. Those probes designated by a "1" correspond to sequence motifs found in the Anderson Sequence. Two other regions within the control region have also been identified as being highly polymorphic: basepair 189 (within HVII) and 16093 (within HVI), each of which can exist in two variant forms. These are also represented on the SSOP linear arrays. One of four types of probe signals is detected: a single probe is positive; a single probe gives a weak signal; no probe signals are visible; and, two probes are visible (either with equal or uneven intensity). If two probes within the same region hybridize to the PCR product, this is an indication that the sample may be heteroplasmic. The sequences for the SSOP probes immobilized on the linear arrays are unavailable. This information is considered proprietary, as per a confidentiality agreement. The sequences that are distinguished by the probes in the HVI and HVII regions are indicated in table 7 and 8 respectively.

Sample #	HVI REGION					HVII REGION				
	A	C	D	E	16093	A	B	C	D	189
1A-01	1	1	1	2	1	W2	7	4	1	0
1A-02	1	1	1	1	1	2	6	5	1	2
1A-03	2	2	1	1	1	2	3	1	1	1
1A-04	1	1	2	1	2	2	1	0	1	0
1A-05	1	w2/w3	1	1	1	2	2	0	0	0
1A-06	3	4	1	2	1	2	4	2	1	W1
1A-07	3	3	1	1	W1/2	1	1	0	2	0
1A-08	1	1	1	3	1	2	5	1	1	1
1A-09	1	3	1	1	1	2	1/W3	1	1	1
1A-10	0	1	2	1	2	2	6	W1	1	2
1A-11	1	0	1	1	1	2	5	4	1	0

Figure 13. "RMS mitoTYPE" Linear Arrays

[Adapted from Roche Molecular Diagnostics Beta Site Testing of the HVI/HVII Linear Array Assay, Study IA]

Table 7.
SSOP Sequence Variation in the HVI Region

PROBE DESIGNATION	POSITION THAT SEQUENCE VARIATION IS DETECTED
	16126 16129
A1	T G ***T*** A C ***G*** G T
A2	T G ***C*** A C ***G*** G T
A3	T G ***T*** A C ***A*** G T
	16304 16309 16311
C1	A G ***T*** A C A T ***A*** G ***T*** A C
C2	A G ***C*** A C A T ***A*** G ***T*** A C
C3	A G ***T*** A C A T ***A*** G ***C*** A C
C4	A G ***T*** A C A T ***G*** G ***T*** A C
	16362
D1	C G ***T*** C C
D2	C G ***C*** C C
	16270 16278
E1	C A ***C*** T A G G A T A ***C*** C A
E2	C A ***C*** T A G G A T A ***T*** C A
E3	C A ***T*** T A G G A T A ***C*** C A

Table 8.
SSOP Sequence Variation in the HVII Region

PROBE DESIGNATION	POSITION THAT SEQUENCE VARIATION IS DETECTED
	73
A1	G T ***A*** T G
A2	G T ***G*** T G
	146 150 152
B1	C C ***T*** C A T ***C*** C ***T*** A T
B2	C C ***C*** C A T ***C*** C ***T*** A T
B3	C C ***T*** C A T ***C*** C ***C*** A T
B4	C C ***C*** C A T ***C*** C ***C*** A T
B5	C C ***T*** C A T ***T*** C ***T*** A T
B6	C C ***T*** C A T ***T*** C ***C*** A T
B7	C C ***C*** C A T ***T*** C ***C*** A T
	189 195 198 200
C1	G A ***A*** C A T A C ***T*** T A ***C*** T ***A*** A A
C2	G A ***A*** C A T A C ***C*** T A ***C*** T ***A*** A A
C4	G A ***A*** C A T A C ***C*** T A ***T*** T ***A*** A A
C5	G A ***G*** C A T A C ***T*** T A ***C*** T ***G*** A A
	247
D1	T T ***G*** A A
D2	T T ***A*** A A
	189
189A	G A ***A*** C A
189G	G A ***G*** C A
	16093
16093T	A T ***T*** T C
16093C	A T ***C*** T C

b. Sample Preparation

For optimal hybridization, 15μL of ~3-6ng/μL PCR product (~45-90ng of PCR product) should be added during the hybridization step. The actual volume added was adjusted based on the intensity of the product with respect to the DNA Mass Ladders. In cases where the concentration of the PCR product exceeds 6ng/μL, it is necessary to dilute the concentration to between ~3-6ng/μL to obtain optimal typing results using the following dilution factor:

$$\text{Dilution Factor for PCR product} = \frac{\text{Estimated Concentration of DNA (ng/μL)}}{\text{Target Concentration (ng/μL)}}$$

15μL of Denaturation Solution (1.6% 0.5M sodium hydroxide (w/w), Amplicor®, Roche Diagnostics) was added to each well of a microtiter plate in order to denature the PCR product. The appropriate volume of the quantitated PCR product was aliquoted into the corresponding well containing the Denaturation Solution and mixed to ensure homogeneity.

c. Mitochondrial DNA Typing Procedure

Two water baths are required in order to perform mtDNA linear array typing. A rotating water bath, heated at 55°C (±1°C), is necessary for the hybridization and wash steps. In order to maintain the temperature accurately, a calibrated total immersion thermometer was installed in the water bath. While performing the linear array typing, the covered rotating water bath was maintained at a

rotation of 50-70 rpm with a water level of approximately ¼ - ½ inch above the shaker platform. A stationary water bath, heated at 56°C, is required to maintain the temperature of the Wash Buffer (2 x SSPE, 0.5% SDS) prior to typing.

The packets containing the linear array "strips" were removed from their light-tight location (stored at 2-8°C) just prior to their use, and, using forceps, the required number of strips was removed. Each array was labeled with a hair identifier number in the designated location on the right-side of the strip using the pen included with the AmpliType™ DNA Typing Trays. One mtDNA probe strip was placed into each of twenty-four wells of the AmpliType™ DNA Typing Tray and the tray was subsequently covered with the lid supplied. As illustrated in figure 14, the typing tray was tilted slightly prior to adding 3mL of pre-warmed Wash Buffer to each well at the reference end of each strip.

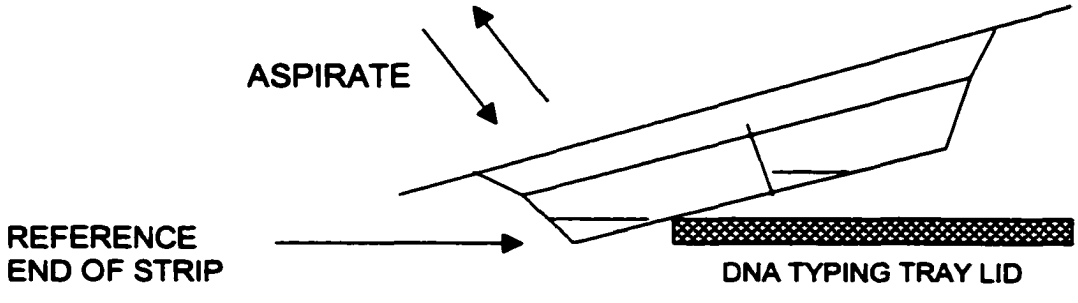


Figure14. DNA Typing Tray Arrangement
[Adapted from Perkin Elmer Amplitype® PM + DQA1 PCR Amplification and Typing Kit Procedure]

The amplified mtDNA/Denaturation Solution was added to the Wash Buffer in the well containing the appropriately labeled mtDNA probe strip. The tray was covered with the lid supplied and placed in the rotating water bath (a weight was placed on top of the tray to secure it down during the rotation). The amplified samples were hybridized to the mtDNA probe arrays by incubating at 55°C for 15 minutes. Prior to the end of the hybridization step, the Enzyme Conjugate Solution (3.3 mL Wash Buffer, 8μL SA-HRP Conjugate per array) was prepared, and mixed to ensure homogeneity, as indicated below:

Arrays X 3.3mL Wash Buffer = Volume Wash Buffer
Arrays X 8μL Enzyme Conjugate: SA-HRP = Volume Enzyme Conjugate: HRP-SA

The contents of each well were aspirated from the reference end of the strip to remove unbound PCR product (figure 14) and 3mL of pre-warmed (56°C) Wash Buffer was added to each well to ensure removal of excess amplified DNA. The tray was gently agitated for several seconds, aspirated, and 3mL of Enzyme Conjugate Solution was dispensed into each well and incubated at 55°C for 5 minutes. Following incubation, the tray was removed and the content of each well was aspirated. 3mL of pre-warmed Wash Buffer was dispensed into each well, the tray was covered, and the arrays were rinsed for several seconds by gently agitating the tray. The Wash Buffer was aspirated, and an additional 3mL of Wash Buffer was dispensed into each well. The tray was covered

and incubated at 55°C for 12 minutes (stringent wash step). Subsequently, the contents were aspirated, subjected to a final wash step in which 3mL Wash Buffer was added to each well, agitated for several seconds and aspirated as previously described. The three successive wash steps outlined above were performed to remove any residual Enzyme Conjugate Solution remaining in the wells.

d. Color Development

In order to prepare the linear arrays for color development, 3mL Citrate Buffer (0.1M, pH 5.0) was dispensed into each well; the tray was covered and placed on an orbital shaker set at 50 rpm at room temperature (15 – 30°C) for 5 minutes. At this time, the Color Development Solution was prepared by adding the following reagents in the order indicated, protecting the final solution from exposure to light:

Arrays X 3.3mL Citrate Buffer = Volume of Citrate Buffer
Arrays X 4µL 3% Hydrogen Peroxide = Volume of Hydrogen Peroxide
Arrays X 150µL Chromogen: TMB = Volume of Chromogen: TMB

The tray was removed from the orbital shaker, the contents aspirated, and 3mL of the freshly prepared Color Development Solution (3.3mL 0.1M Citrate Buffer, pH 5.0, 4µL 3% H_2O_2, 150µL Chromogen Solution per array) was dispensed into each well. The tray was covered with the lid provided to protect

the linear arrays from exposure to light and the arrays were allowed to develop at room temperature for 5 – 35 minutes on an orbital shaker (50 - 100rpm). Following development, the Color Development Solution was poured out, 3mL of sterile DI water was added to each well, and the arrays were rinsed by rocking back and forth at room temperature for 20 - 30 seconds. The sterile DI water was removed following the initial rinse and the wash step was repeated a minimum of two times.

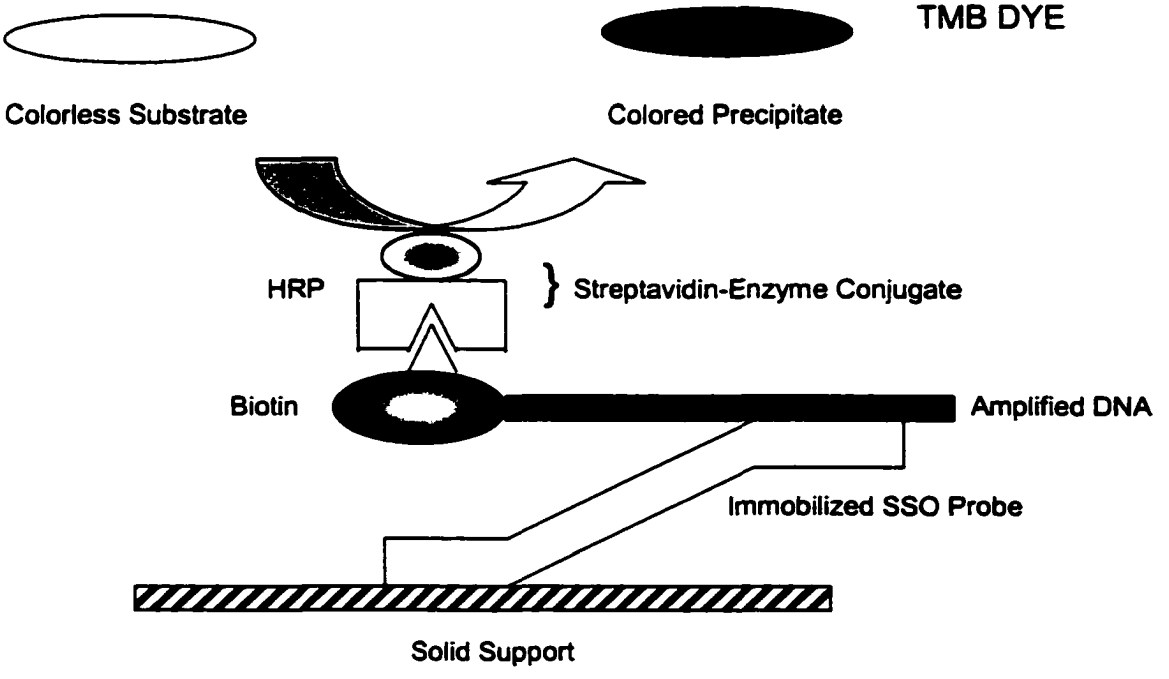


Figure 15. Enzyme Conjugation with Immobilized SSO Probes
[Adapted from Perkin Elmer Amplitype® PM + DQA1 PCR Amplification and Typing Kit Procedure]

e. Interpretation of Results

In order to interpret the results, the wet arrays were placed on a mtDNA linear array template. As illustrated in figure 16, the template is designed to accommodate a total of twenty arrays using the reference line on each strip to align them correctly. The pattern of blue lines was recorded on a mtDNA Interpretation Sheet (see Appendix F). As indicated earlier, the "RMS mitoTYPE" SSOP linear arrays are comprised of thirty-one probes that distinguish polymorphisms within the control region of the mitochondrial DNA genome. A total of ten regions have been associated with HVI and HVII. Each of the ten regions reflects varying degrees of polymorphism. A region that is represented by more than one polymorphic form is tentatively identified as a heteroplasmic case. However, the existence of multiple lines in more than one region within a strip suggests the possibility of either a cross-hybridization reaction or a DNA mixture. The intensity of the probe signal within an array reflects the amount of DNA that hybridizes to the probe. Therefore, when heteroplasmy is observed, it is possible to estimate the relative amounts of each sequence in the mixture. However, it is not possible to compare the intensity of the probes between arrays since the amount of DNA added may differ.

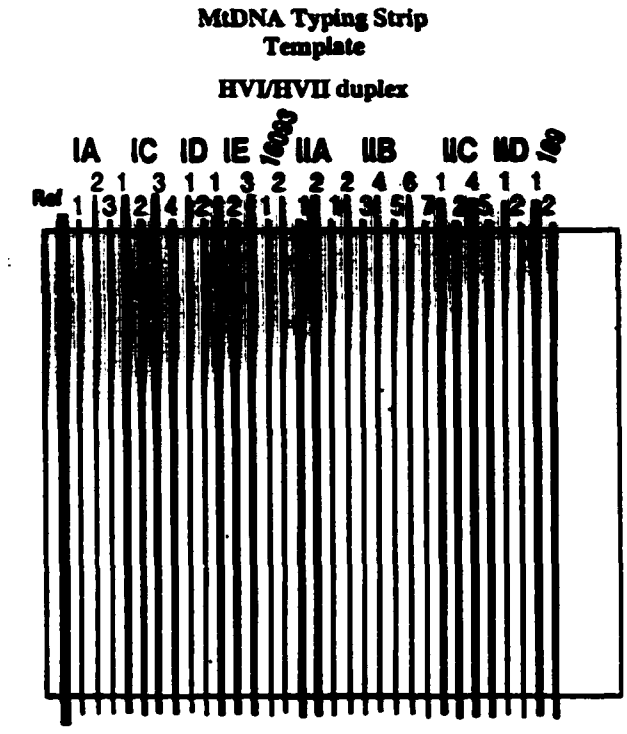


Figure 16. Mitochondrial DNA Linear Array Template

f. Photography

A permanent record of the typing results was obtained by photographing the linear arrays against the interpretation template. Kodak 35mm black and white print film was selected as the photographic medium (ASA 400 F/5.6, shutter speed 1/30 - 1/60"). A SLR camera was used (Pentax Honeywell SP 500/Super-Takumar 50mm lens/Marumi 49mm +3 macro lens) on a copy stand using tungsten floodlights (even illumination @ 45°).

Validation Studies of Mitochondrial DNA Linear Array Assay Typing

In order to optimize the results obtained with the mitochondrial DNA linear array assay typing method, a number of validation studies were performed. These are summarized below:

a. Sample Extraction Procedures

A preliminary study was performed to compare the efficiency of the Chelex extraction method with and without the inclusion of an incubation step with Proteinase K. It was found that the addition of 10μL Proteinase K to the homogenate followed by an incubation step at 56°C for at least 6 - 8 hours or overnight increased the mtDNA yield from the hair shaft.

b. Sample Amplification

A study of the amplification parameters was also performed. As indicated above, modifications of the cycle parameters (thirty-four v. thirty-eight cycles), primer sets (duplex v. singleplex) and the amount of primer incorporated (0.1μM v. 0.2μM) can be made based on the results obtained. In addition, research is ongoing to compare the amplification success rates obtained with an optimized protocol (see Discussion under *Future Studies).*

c. Linear Array Typing

In order to optimize the linear array assay typing for interpretation, a study was conducted to determine both the optimal input of mtDNA for hybridization and the development time for the assay. The study indicated

that 45 - 90ng of mtDNA should be targeted and developed with the chromogenic reagent for 15 minutes. This combination gave average intensity bands with no cross-hybridization.

d. Amplification Inhibition

In order to study the possibility that inhibitors were contributing to the failed amplification attempts, a sub-set of hair sample extracts was spiked with known blood (5μL blood + 15μL hair extract). In each case, the blood samples failed to amplify. The blood samples alone successfully amplified, which suggests that components in the hair extract have an inhibiting effect. A number of ongoing studies are addressing this problem. These include the use of bovine serum albumin to remove inhibitors, reducing the template DNA input for amplification (to reduce inhibitor input) and a comparison of other extraction methods (e.g. organic extractions or PCR purification using centricon tubes).

Statistical Analysis

The data collected for each research question were subjected to three types of statistical analyses: descriptive, bivariate and multivariate. The data obtained for each question was described by indicating how many times a particular event or value of a variable occurs in the assembled data. Counting and reporting the raw number of responses falling into each response category for a given variable allowed for a determination of the frequency of occurrence (descriptive statistics). For example, the first statistical analysis involved determining the distribution of mitochondrial DNA blood haplotypes for the samples in the study.

With respect to the first question "How much hair sample is required for successful polymerase chain reaction (PCR) amplification?" and "What other variables influence the amplification success rate?" The success rate of the amplification reaction was measured by estimating the amount of DNA extracted as a function of the length of hair shaft isolated. Analysis of the product gel allowed for an assessment of the estimated quantitation of mtDNA required (minimum threshold) in order to obtain a typable result for the hair samples. Chi-square analysis was also applied to examine the relationships among several variables in this experiment, hair length, hair shaft diameter and hair pigmentation.

For the question "What is the effect of heteroplasmy on the interpretation of forensic samples obtained from the hair shafts and blood of the same individual?" a two-tier statistical analysis was applied. Initially, the frequency of occurrence of

heteroplasmy in each tissue (blood and hair) was assessed, followed by a bivariate comparison of both tissues.

The question "Is the mitochondrial DNA yield from the hair shaft affected by exposure to various chemical hair treatments?" involved quantifying the amount of DNA within each region of the hair shaft. Initially, frequency data were collected related to the amount of DNA isolated from treated and untreated hairs. This data was then subjected to a t-test paired comparison, followed by a bivariate analysis.

The next research question was "What is the frequency of the heteroplasmy observed using the linear array assay method? For this question, a direct assessment of the frequency of occurrence of heteroplasmy in blood and hair samples was examined.

The next question, "Do different hairs from the same individual exhibit different haplotypes?" was addressed using descriptive statistics. This involved a direct count and reporting of the pooled haplotypes exhibited across an individual's scalp.

In addressing the question "Does detection of the heteroplasmic condition in a given individual correlate with the microscopic morphology of distinctly different hair types in the scalps of individuals", a numerical value was assigned to each characteristic. For example, in describing the medulla as continuous, discontinuous, fragmented and absent, a code of 0, 1, 2 and 3 was assigned respectively. A determination of the frequency of occurrence of heteroplasmy for hairs that had a distinct morphology was obtained. A t-test paired comparison

was then performed on these frequencies, followed by a multivariate analysis that took into consideration the other known variables.

A chi-squared test for independence was used to determine whether the frequency of heteroplasmy differed significantly with age, race, sex; scalp origin; growth phase of the hair; or, for living compared to deceased subjects.

CHAPTER 4

Results

PCR Amplification Success Rates

The data pertaining to the amplification success rate was collected and organized into three groups according to: PCR amplification conditions, hair morphology characteristics and demographic factors.

PCR Conditions

The results show that telogen hairs have a higher success rate (75%) when compared to anagen hairs (53%) for the first duplex amplification (table 9A). However, this difference is compensated for by subsequent amplification attempts. Hairs that initially did not amplify were successful with the second duplex and gave a combined duplex success rate of 77% (958/1248) and 64% (840/1303) for the telogen and anagen hairs respectively. All of the hairs that were unsuccessful with the duplex primer set were amplified with both singleplex primer sets (i.e. in separate reactions). Factoring in the success rate for the singleplex primer set for both hair growth phases, the combined amplification success rate is 79% (989/1249) and 79% (1014/1303) for telogen and anagen hairs respectively. Therefore, the total amplification success rate for all hairs in this study is 79% (2003/2552). Further analysis of the data reveals that of the two singleplex primer sets, the HVI success rate greatly exceeds that of HVII (table 9B). This is true for telogen hairs ($HVI_{Success\ Rate}$ = 16% and $HVII_{Success\ Rate}$ = 2%) and

anagen hairs ($HVI_{Success\ Rate}$ = 41% and $HVII_{Success\ Rate}$ = 13%). Combining both growth phases, a success rate of 33% (HVI) and 9% (HVII) is obtained (table 9C).

Table 9A. Duplex Hair Amplification Success Rate Summary

	First Duplex (T)	Second Duplex (T)	First Duplex (A)	Second Duplex (A)
Amplified	936	22	683	157
Not Amplified	312	264	619	72
Total Number Hairs Attempted	1248	286	1302	229
% Success	75%	8%	53%	69%

Table 9B. Singleplex Hair Amplification Success Rate Summary
(Includes only those hairs that were unsuccessful with the duplex)

	HVI ONLY (T)	HVII ONLY (T)	HVI & HVII (T)	HVI ONLY (A)	HVII ONLY (A)	HVI & HVII (A)
Amplified	28	0	3	117	6	47
Not Amplified	164	192	189	287	398	357
Total Number Hairs Attempted	192	192	192	404	404	404
% Success	15%	0%	2%	29%	2%	12%

Table 9C. Total Hair Amplification Success Rate Summary

	First Duplex	Second Duplex	Singleplex[A]	Total Amplified	Total Not Amplified	Total Success %
Amplified Telogen	936	22	31	989	260	79%
Amplified Anagen	683	157	174	1014	288	79%
Total	1630	179	205	2003	548	79%

Hair Morphology Characteristics

Closer analysis of the amplification success rate by hair growth phase reveals that a high success rate (91-100%) is obtained for telogen hairs from 74 individuals; only two hairs failed to amplify for these individuals combined (0.12%). An intermediate success rate (50-90%) is obtained for telogen hairs from 31 individuals, with seventy hairs (27%) failing to amplify. A low success rate (1-50%) is attributed to twenty-three individuals, who, combined, account for one hundred and fifty one of the telogen hairs that did not amplify (58.1%). In addition, all of the hairs failed to amplify for four individuals, which accounts for the remaining thirty-seven telogen hairs (14.2%).

A high success rate (91-100%) is obtained for anagen hairs from 61 individuals; no failed amplifications were observed for these 61 individuals combined. An intermediate success rate (50-90%) is obtained for anagen hairs from 45 individuals, with one hundred and three hairs (36%) failing to amplify. A low success rate (1-50%) is attributed to eighteen individuals, who, combined, account for one hundred and thirteen of the anagen hairs that did not amplify (39%). In addition, in the case of eight individuals, none of the anagen hairs successfully amplified, which represent the remaining seventy-two hairs (25%). This data is summarized in table 10 and figure 17. In general, the data reflect similar patterns for both anagen and telogen hairs in terms of the number of cases falling within a particular success range. However, the higher number of anagen hairs that did not amplify appears to be attributed to individuals/hairs that failed to show any amplification success. In the case of the telogen hairs,

approximately the same number of unsuccessful amplifications is more evenly distributed over a lower success rate.

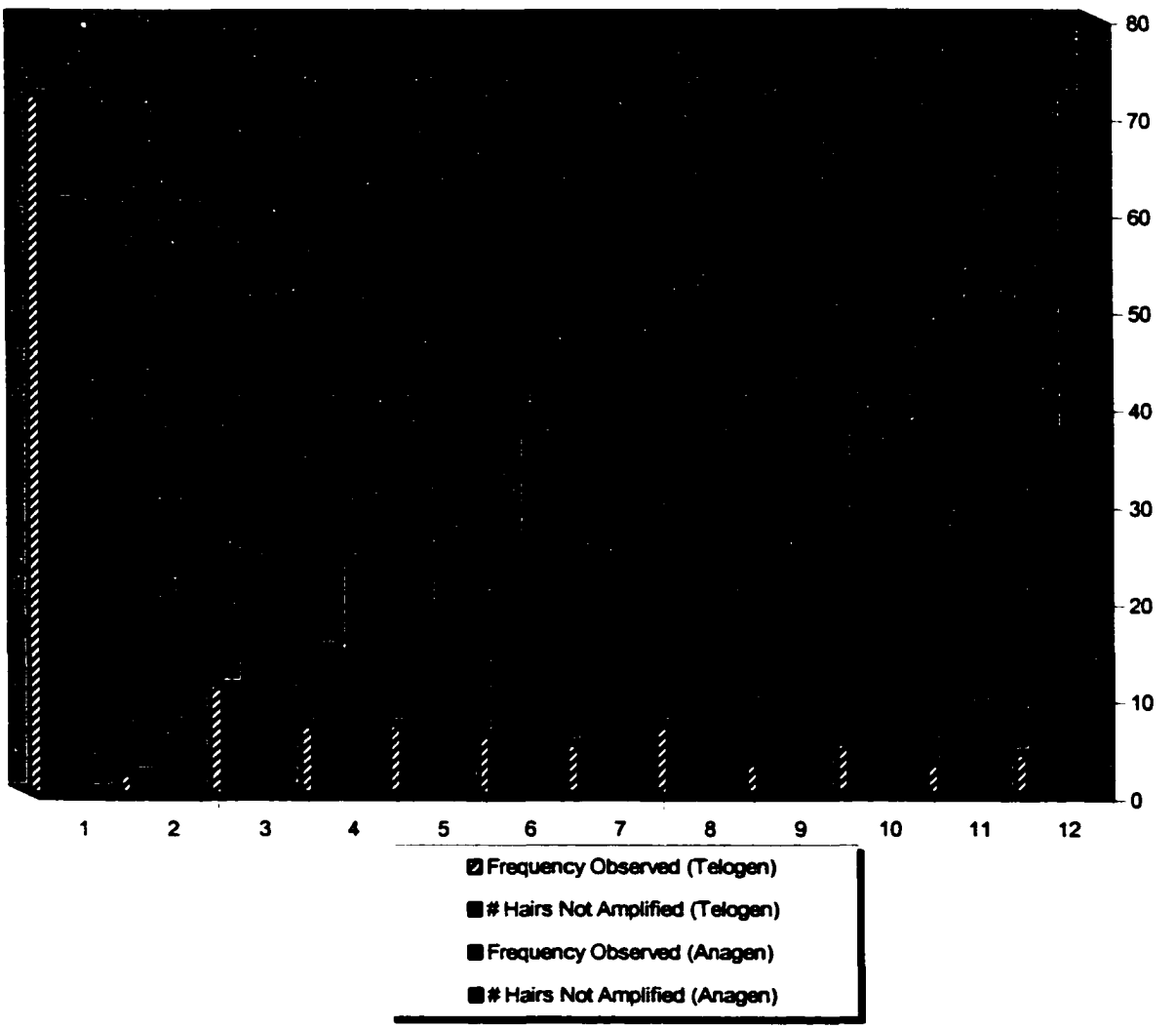


Figure 17. Distribution of Amplification Success Rates by Growth Phase (Refers to Data in Table 10)

Table 10. Frequency of Amplification For Each Individual Case by Hair Growth Phase

Group Distribution (Graph X Axis)	% Hairs Amplified Per Individual	Frequency Observed (Telogen)	Total # Hairs Not Amplified	Frequency Observed (Anagen)	Total # Hairs Not Amplified
1	100	72	0	61	0
2	91-99	2	2	0	0
3	81-90	11	11	15	15
4	71-80	7	15	12	24
5	61-70	7	20	9	27
6	51-60	6	24	9	37
7	41-50	5	25	5	25
8	31-40	7	39	6	36
9	21-30	3	21	4	27
10	11-20	5	39	2	16
11	1-10	3	27	1	9
12	0	4	37	8	72
	Total	132	260	132	288

In order to determine whether the amplification success rate is correlated to other hair characteristics, the data were analyzed to test for statistical significance using the chi-square distribution. The overall data for all success ranges (table 11) indicate that the use of cosmetic treatments does not influence the amplification success rate (not significant at the 95% confidence level). However, of the thirty-four individuals who exhibited a 100% success rate, twenty-five (74%) had no reported hair treatment whereas nine (26%) describe using one of several types of treatments. Similarly, the statistical test for significance of amplification success rate when comparing living and deceased individuals showed that there is no correlation for either test group (95% confidence level). This result is consistent across all success rate categories

(table 12). In terms of hair morphology, the data for the telogen growth phase indicate that there is no statistically significant correlation (95% confidence level) between amplification success rate and either the medulla structure (table 13) or the diameter of the hair shaft (table 15). However, hair length does appear to correlate, with shorter hairs showing significantly lower success rates. A chi-square analysis comparing the success rate of hairs that measured 2cm with those that were less than 2cm showed that the results were significant at the 99% confidence level (table 14). In addition, the pigmentation within the hair shaft also affects the amplification success rate. A chi-square analysis indicated that this was significant at the 95% confidence level (table 16). Closer analysis of the pigmentation data revealed that the significance of the result is attributable to the lower than expected amplification rate of the "brown" hairs.

Table 11. Total Hair Amplification Success Rate by Cosmetic Treatment

Treatment	Count	# Individuals (100% Amplified)	# Individuals (50-99% Amplified)	# Individuals (0-49% Amplified)	Total[a]
No	Observed	25	30	10	65
	Expected	20	34	11	65
Yes	Observed	9	26	8	43
	Expected	14	22	7	43
Total	Observed	34	56	18	108
	Expected	34	56	18	108

Table 12. Total Hair Amplification Success Rate For Living/Decedent Cases

Living	Count	# Individuals (100% Amplified)	# Individuals (50-99% Amplified)	# Individuals (0-49% Amplified)	Total
No	Observed	8	16	0	24
	Expected	8	13	3	24
Yes	Observed	34	56	18	108
	Expected	34	59	15	108
Total	Observed	42	72	18	132
	Expected	42	72	18	132

Table 13. Telogen Hair Amplification Success Rate by Medulla Structure

# Hairs	Count	Absent Medulla	Fragmented Medulla	Discontinuous Medulla	Continuous Medulla	Total[c]
Amplified	Observed	703	169	50	36	958
	Expected	715	160	49	34	958
Did Not Amp	Observed	203	34	12	7	256
	Expected	191	43	13	9	256
Total	Observed	906	203	62	43	1214
	Expected	906	203	62	43	1214

Table 14. Telogen Hair Amplification Success Rate by Length of Hair Shaft

Length of Hair Shaft (cm)	Count	Amplified	Did Not Amplify	Total
2	Observed	942	232	1174
	Expected	930	244	1174
< 2cm	Observed	47	28	75
	Expected	59	16	75
Total	Observed	989	260	1249
	Expected	989	260	1249

Table 15. Telogen Hair Amplification Success by Diameter Range of Hair Shaft

Diameter of Hair Shaft (μm)	Count	Amplified	Did Not Amplify	Total
10 - 30	Observed	63	26	89
	Expected	71	18	89
31 - 60	Observed	392	109	501
	Expected	398	103	501
61 - 90	Observed	465	111	576
	Expected	457	119	576
91 - 140	Observed	45	5	50
	Expected	40	10	50
Total	Observed	965	251	1216
	Expected	965	251	1216

Table 16. Telogen Hair Amplification Success by Pigmentation Range of Hair Shaft

Pigmentation of Hair Shaft	Count	Amplified	Did Not Amplify	Total
Blond Pale Yellow – Pale Brown	Observed	55	7	62
	Expected	52	10	62
Light – Medium Brown	Observed	233	44	277
	Expected	230	47	277
Brown	Observed	192	62	254
	Expected	211	43	254
Dark – Very Dark Brown	Observed	138	18	156
	Expected	130	26	156
Grey Brown	Observed	3	0	3
	Expected	3	0	3
No Pigment	Observed	53	5	58
	Expected	48	10	58
Total	Observed	674	136	810
	Expected	674	136	810

Demographic Factors

The amplification success rate was measured as a function of two demographic factors: the racial group and age of an individual. A separate chi-square analysis of each racial group was performed for telogen hairs, anagen hairs and as a function of both growth phases combined. The data for telogen hairs show that the results across all racial groups are significant at the 99% confidence level (table 17A). This is attributed to the higher success rates of the hairs from Caucasians compared to African-Americans, which is reflected across all success ranges but is particularly apparent at the extremes. For example, 73% of Caucasians lie within the 100% success rate compared to 26% African-Americans, whereas 7% of Caucasians lie within the 0-49% success rate compared to 37% African-Americans. The data for anagen hairs indicate that the results across all racial groups are not significant at the 95% confidence level (table 17B). Of note for this particular analysis is the higher percentage (26%) of African-Americans who lie within the 0-49% success rate compared to Caucasians (14%), Hispanics (7%) and Asians (12%). The significant differences observed for the telogen hairs within the Caucasian and African-American racial groups are reflected in the overall success rates for both growth phases combined. The results follow a similar pattern to those reflected for telogen hairs and, again, are significant at the 99% confidence level (table 17C). A cross tabulation of African American and Caucasian individuals by sex reveals that there is no significant difference in the amplification success rate for males and

females for either racial group (table 18). In this study, hairs obtained from females from both racial groups was invariably longer than 2cm, which suggests that other variables within the hair may be contributing to the lower success rates observed in hairs obtained from African Americans.

In terms of the age of an individual, the data in this study indicate that there is no statistically significant correlation (95% confidence level) between each age group and the amplification success rate (table 20). However, of note is the fact that hairs obtained from African American donors represent 61% (11/18) of the samples within the low success range (table 21).

Table 17A. Telogen Hair Amplification Success Rate by Racial Group

Racial Group	Count	# Individuals (100% Amplified)	# Individuals (50-99% Amplified)	# Individuals (0-49% Amplified)	Total
Caucasian	Observed	32	9	3	44
	Expected	24	13	7	44
African American	Observed	9	13	13	35
	Expected	19	11	5	35
Hispanic	Observed	17	9	2	28
	Expected	15	9	4	28
Asian	Observed	13	9	3	25
	Expected	13	8	4	25
Total	Observed	71	40	21	132
	Expected	71	40	21	132

Table 17B. Anagen Hair Amplification Success Rate by Racial Group

Racial Group	Count	# Individuals (100% Amplified)	# Individuals (50-99% Amplified)	# Individuals (0-49% Amplified)	Total
Caucasian	Observed	23	15	6	44
	Expected	21	16	7	44
African American	Observed	17	9	9	35
	Expected	17	13	5	35
Hispanic	Observed	12	14	2	28
	Expected	13	11	4	28
Asian	Observed	11	11	3	25
	Expected	12	9	4	25
Total	Observed	63	49	20	132
	Expected	63	49	20	132

Table 17C. Total Hair Amplification Success Rate by Racial Group

Racial Group	Count	# Individuals (100% Amplified)	# Individuals (50-99% Amplified)	# Individuals (0-49% Amplified)	Total
Caucasian	Observed	21	21	2	44
	Expected	15	23	6	44
African American	Observed	6	18	11	35
	Expected	12	18	5	35
Hispanic	Observed	11	14	3	28
	Expected	10	14	4	28
Asian	Observed	8	15	2	25
	Expected	9	13	3	25
Total	Observed	46	68	18	132
	Expected	46	68	18	132

Table 18. Total Hair Amplification Success Rate by Racial Group
(Cross Tabulation of Caucasian & African American Individuals by Sex)

Racial Group	Count	# Individuals (100% Amplified)		# Individuals (50-99% Amplified)		# Individuals (0-49% Amplified)		Total
	Sex	M	F	M	F	M	F	
Caucasian	Observed	12	9	7	14	2	0	44
	Expected	9	6	6	16	4	3	44
African American	Observed	4	2	4	14	6	5	35
	Expected	7	5	5	12	4	2	35
Total	Observed	16	11	11	28	8	5	79
	Expected	16	11	11	28	8	5	79

Table 19. Total Hair Sample in Study
(Cross Tabulation of Treatment and Racial Group of Individual)

Treatment	Count	Caucasian	African-American	Hispanic	Asian	Total[a]
No	Observed	19	17	14	15	65
	Expected	19.9	18.7	11.4	15	65
Yes	Observed	14	14	5	10	43
	Expected	13.1	12.3	7.6	10	43
Total	Observed	33	31	19	25	108
	Expected	33	31	19	25	108

Table 20. Total Hair Amplification Success Rate by Age Group

Age Range (Years)	Count	# Individuals (100% Amplified)	# Individuals (50-99% Amplified)	# Individuals (0-49% Amplified)	Total
18 – 29	Observed	16	28	10	54
	Expected	18	29	7	54
30 – 49	Observed	12	28	6	46
	Expected	15	25	6	46
50 – 69	Observed	12	9	2	23
	Expected	7	13	3	23
70 and older	Observed	3	6	0	9
	Expected	3	5	1	9
Total	Observed	43	72	17	132
	Expected	43	72	17	132

Table 21. Total Hair Amplification Success Rate by Age Group
(Cross Tabulation of Racial Group)

Age Range (Years)	Count	# Individuals (100% Amplified)				# Individuals (50-99% Amplified)				# Individuals (0-49% Amplified)				Total
Racial Group		A	AA	C	H	A	AA	C	H	A	AA	C	H	
18 – 29	Observed	7	1	5	3	8	3	8	9	0	6	2	2	54
	Expected	3	2	9	4	7	7	9	7	1	5	1	1	54
30 – 49	Observed	0	1	6	5	8	7	8	5	2	3	0	1	46
	Expected	2	2	7	3	6	6	8	6	0.6	4	0.6	0.6	46
50 – 69	Observed	0	2	9	1	0	4	3	2	0	2	0	0	23
	Expected	1	1	4	2	3	3	4	3	0	2	0	0	23
70 and older	Observed	0	2	1	0	0	2	4	0	0	0	0	0	9
	Expected	1	0	1	1	1	1	2	1	0	1	0	0	9
Total	Observed	7	6	21	9	16	16	23	16	2	11	2	3	132
	Expected	7	6	21	9	16	16	23	16	2	11	2	3	132

Mitotyping Results

Distribution of Haplotypes For Blood Samples

Using the immobilized SSOP linear array assay described in the methods and materials section, blood samples from 131 individuals were typed. Within this data set, a total of 76 different probe patterns were observed. Table 19 shows the distribution of haplotypes types within the total sample. 86% of all the types were rare, occurring once (56 types) or twice (9 types). A distinct type occurred 56 times, representing 43% of the total sample. Of the 25 haplotypes observed in the African American population, 22 of them were not observed in the other three populations. Similarly, 26 of the 31 haplotypes observed in Caucasians, 7 of the

14 haplotypes observed in Hispanics, and 13 of the 17 haplotypes observed in Asians were found exclusively within their respective populations. The most frequent haplotype observed in African Americans is A3C3D0E2A2B0C0D2189016093T (12% of the racial group). This haplotype was also unique to African Americans. The most frequent type observed in Caucasians was haplotype A1C1D1E1A1B1C1D1189A16093T (9% of the racial group). This represents the Anderson Consensus Sequence and, for this study, was found to be unique to Caucasians. The most frequent type observed in Hispanics is haplotype A1C0D2E1A2B0C1D1189A16093T (39% of the racial group). This type is also found in one individual in the African American population group and one individual in the Caucasian population group. The most frequent type observed in Asians was haplotype A1C1D1E1A2B1C1D1189A16093T (16% of the racial group). This haplotype is also observed in the Hispanic (4 individuals) and Caucasian (3 individuals) populations. This data is summarized in table 23 A & B.

Table 22. Distribution of Mitochondrial DNA SSOP Haplotype Profiles in the Study

SAMPLE #	# OBS	FREQ	BLOOD AMP'D	BLOOD SSOP HAPLOTYPE
01	11	0.083970	Y	A1C1D1E1A2B1C1D1189A16093T
02	2	0.015267	Y	A3C1D1E1A2B5C0D1189A16093T
03	1	0.007634	Y	A1C1D1E1A2B1C2D1189A16093T
04	2	0.015267	Y	A1C0D1E1A2B1C1D1189A16093T
05	1	0.007634	Y	A3C2D1E1A2B3C1D1W189A16093T
06	13	0.099237	Y	A1C0D2E1A2B0C1D1189A16093T
07	1	0.007634	Y	A2C1D1E1A2B6C1D1189A16093T
08	11	0.083970	Y	A1C1D1E1A2B1C1D1189A16093T
09	1	0.007634	Y	A1C1D1E1A2B2C1D1189A16093T
10	2	0.015267	Y	A1C1D1E1A2B3C1D1189A16093T
11	11	0.083970	Y	A1C1D1E1A2B1C1D1189A16093T
12	1	0.007634	Y	A1C2D1E1A1B1C1D1189A16093T
13	2	0.015267	Y	A1C1D2E1A2B1C0D1189016093T
14	1	0.007634	Y	A1C0D1E1A2B2C0D1189016093T
15	3	0.022901	Y	A1C1D2E1A2B1C1D1189A16093T
16	1	0.007634	Y	A3C3D1E1A2B5C1D1189016093T
17	3	0.022901	Y	A1C1D1E1A2B1C1D0189A16093T
18	13	0.099237	Y	A1C0D2E1A2B0C1D1189A16093T
19	13	0.099237	Y	A1C0D2E1A2B0C1D1189A16093T
20	1	0.007634	Y	A1C3D1E1A2B5C1D1189A16093T
21	3	0.022901	Y	A1C1D2E1A2B1C1D1189A16093T
22	3	0.022901	Y	A2C1D1E1A2B1C1D1189A16093T
23	2	0.015267	Y	A3C1D1E1A2B5C0D1189A(w)16093T
24	2	0.015267	Y	A1C0D1E1A2B1C1D1189A16093T
25	5	0.038168	Y	A1C1D1E2A2B7(w)C4D1189016093T
26	1	0.007634	Y	A0C1D1E1A2B3C0D1189G16093T
27	13	0.099237	Y	A1C0D2E1A2B0C1D1189A16093T
28	3	0.022901	Y	A1C1D2E1A2B1C1D1189A16093T
29	11	0.083970	Y	A1C1D1E1A2B1C1D1189A16093T
30	1	0.007634	Y	A1C1D1E1A1B5C1D1189A16093T
31	4	0.030534	Y	A1C3D1E1A2B1C1D1189A16093T
32	1	0.007634	Y	A1C1D1E1A1B1C1D1189A16093T/C
33	2	0.015267	Y	A2C1D1E1A2B1C1(w)D1189016093T
34	1	0.007634	Y	A1C1D1E1A2B1C0D1189G16093T
35	11	0.083970	Y	A1C1D1E1A2B1C1D1189A16093T
36	3	0.022901	Y	A2C1D1E1A2B1C1D1189A16093T
37	N/A	N/A	N/A	Not Available

SAMPLE #	# OBS	FREQ	BLOOD AMP.	BLOOD SSOP HAPLOTYPE
38	13	0.099237	Y	A1C0D2E1A2B0C1(w)D1189A16093T
39	1	0.007634	Y	A1C1D1E1A1B0C1D1189A(w)16093T
40	1	0.007634	Y	A1C1D1E1A1B1C1D1189A16093C
41	1	0.007634	Y	A1C1D1E1A2B3C1D1(w)189A16093C
42	1	0.007634	Y	A1C3D2E1A2B7C2D1189A16093T
43	2	0.015267	Y	A1C1D1E1A1B1C2D1189A16093T
44	1		Y	A1C1D1E1A1B1/w3C1D1189A16093T
45	13	0.099237	Y	A1C0D2E1A2B0C1D1189A16093T
46	2	0.015267	Y	A1C1D1E1A1B1C2D1189A16093T
47	1	0.007634	Y	A1C0D1E1A2B5C4D1189016093T
48	5	0.038168	Y	A1C1D1E1A1B1C1D1189A16093T
49	11	0.083970	Y	A1C1D1E1(w)A2B1C1D1189A16093T
50	2	0.015267	Y	A1C3D1E1A1B1C1D1189A16093T
51	2	0.015267	Y	A2C1D1E1A2B1C1(w)D1189016093T
52	3	0.022901	Y	A1C1D1E1A2B1C1D0189A16093T
53	11	0.083970	Y	A1C1D1E1A2B1C1D1189A16093T
54	4	0.030534	Y	A1C3D1E1A2B1C1D1189A16093T
55	2	0.015267	Y	A1C1D1E1A2B3C1D1189A16093T
56	11	0.083970	Y	A1C1D1E1A2B1C1D1189A16093T
57	1	0.007634	Y	A3C3D1E1A2B4C1D0189A16093T
58	1	0.007634	Y	A1C2D1E1A1B1C0D1189016093T
59	13	0.099237	Y	A1C0D2E1A2B0C1D1189A16093T
60	5	0.038168	Y	A1C1D1E1A1B1w3C1D1189A16093T
61	3	0.022901	Y	A2C1D1E1A2B1C1D1189A16093T
62	1	0.007634	Y	A1C3D1E1A2B1C1D1189A16093T/C
63	1	0.007634	Y	A1C0D2E1A2B0C1D1189A16093C
64	4	0.030534	Y	A3C3D0E2A2B0C0D2189016093T
65	1	0.007634	Y	A0C1D1E1A2B3C1D1189A16093T
66	4	0.030534	Y	A1C3D1E1A2B1C1D1189A16093T
67	11	0.083970	Y	A1C1D1E1A2B1C1D1189A16093T
68	4	0.030534	Y	A3C3D0E2A2B0C0D2189016093T
69	13	0.099237	Y	A1C0D2E1A2B0C1D1189A16093T
70	1	0.007634	Y	A3C1D1E1A2B3C0D0189A(w)16093T
71	5	0.038168	Y	A1C1D1E1A1B1C1D1189A16093T
72	1	0.007634	Y	A2C1D1E1A2B1C0D1189016093T
73	5	0.038168	Y	A1C1D1E1A1B1C1D1189A16093T
74	1	0.007634	Y	A1C3D1E3A2B5C1D1189A16093T
75	1	0.007634	Y	A1C0D2E1A2B5C0D1189G16093T
76	5	0.038168	Y	A1C1D1E2A2B7C4D1189016093T
77	1	0.007634	Y	A1C3D1E1A2B3C2D1189A16093C
78	1	0.007634	Y	A3C3D1E1A2B1C0D0189A16093T
79	13	0.099237	Y	A1C0D2E1A2B0C1D1189A16093T
80	1	0.007634	Y	A1C0D1E2A2B5C0D1189A(w)160930

SAMPLE #	# OBS	FREQ	BLOOD AMP.	BLOOD SSOP HAPLOTYPE
81	13	0.099237	Y	A1C0D2E1A2B0C1D1189A16093T
82	1	0.007634	Y	A2C2D1E1A2B0C1D1189A16093T
83	1	0.007634	Y	A1C1D1E1A2B0C0D1189A(w)16093T
84	1	0.007634	Y	A1C1D1E1A2B0C1D1189A16093T
85	11	0.083970	Y	A1C1D1E1A2B1C1D1189A16093T
86	1	0.007634	Y	A3C1(w)D1E1A1B1C1D1189A16093T
87	5	0.038168	Y	A1C1D1E2A2B7C4D1189016093T
88	3	0.022901	Y	A1C1D1(w)E1A2(w)B1(w)C1(w)D0189A(w)16093T
89	1	0.007634	Y	A0C3D2E2A2B1C1D1189A16093T
90	4	0.030534	Y	A1C3D1E1A2B1C1D1189A16093T
91	1	0.007634	Y	A2C0D1E1A2B0C1D1189A16093T
92	1	0.007634	Y	A2C3D1E1A2B1C1D0189A16093T
93	-1	0.007634	Y	A1C3D1E2A2B7C0D1189016093T
94	3	0.022901	Y	A1C1D2E2A2B1C1D1189A16093T
95	1	0.007634	Y	A1C0D1E1A2B0C1D1189A16093T
96	1	0.007634	Y	A3C3D1E2A2B0C0D2189016093T
97	1	0.007634	Y	A1C0D1E2A2B7C4D1189016093T
98	1	0.007634	Y	A0C0D1E1A2B3C1D1189A16093T
99	2	0.015267	Y	A1C1D2E1A2B1C0D1189016093T
100	3	0.022901	Y	A1C1D2E2A2B1C1D1189A16093T
101	3	0.022901	Y	A1C1D2E2A2B4(w)C2D1189A16093T
102	1	0.007634	Y	A1C1D1E1A2B5(w6)C1D1189A16093T
103	1	0.007634	Y	A3C1D1E2A2B4C2/1D1189A16093T
104	1	0.007634	Y	A3C1D1E1A2B1C1D1189A16093T
105	11	0.083970	Y	A1C1D1E1A2B1C1D1189A16093T
106	1	0.007634	Y	A3C3D0E2A2B0C0D21890160930
107	2	0.015267	Y	A1C4(w1)D1E2A2B4C2D1189A16093T
108	13	0.099237	Y	A1C0D2E1A2B0C1D1189A16093T
109	1	0.007634	Y	A3C1D1E0A2B3C1D0189A(w)16093T
110	2	0.015267	Y	A2C3D1E0A2B3C0D2189016093T
111	3	0.022901	Y	A1C1D2E2A2B4C2D1189A16093T
112	2	0.015267	Y	A1C4(w1)D1E2A2B4C2D1189A16093T
113	1	0.007634	Y	A3C3D1E2A2B6(w)C0D2189016093T
114	1	0.007634	Y	A0C1D1E1A2B3C0D1189016093T
115	1	0.007634	Y	A2C3D1E0A2B3C2D2189016093T
116	13	0.099237	Y	A1C0D2E1A2B0C1D1189A16093T
117	3	0.022901	Y	A1C1D2E2A2B4(W)C2D1189A16093T
118	5	0.038168	Y	A1C1D1E2A2B7C4D1189016093T
119	1	0.007634	Y	A3C1D0E2A2B0C0D21890160930
120	3	0.022901	Y	A1C1D2E2A2B1C1D1189A16093T
121	5	0.038168	Y	A1C1D1E2A2B7C4D1189016093T
122	1	0.007634	Y	A1C0D1E1A2B5C2D1189A16093T
123	2	0.015267	Y	A1C3D1E1A1B1C1D1189A16093T

SAMPLE #	# OBS	FREQ	BLOOD AMP.	BLOOD SSOP HAPLOTYPE
124	1	0.007634	Y	A3C3D1(W)E1A1B1C0D2189016093T
125	4	0.030534	Y	A3C3D0E2A2B0C0D2189016093T
126	2	0.015267	Y	A2C3D1E0A2B3C0D2189016093T
127	1	0.007634	Y	A1C3D1E1A2B5C0D1189016093T
128	1	0.007634	Y	A1C0D2E1A2B0C1D1189016093T
129	5	0.038168	Y	A1C1D1E1A1B1C1D1189A16093T
130	13	0.099237	Y	A1C0D2E1A2B0C1D1189A16093T
131	1	0.007634	Y	A3C3D1E1A2B1C0D0189G16093T
132	4	0.030534	Y	A3C3D0E2A2B0C0D2189016093T

Table 23A. Frequency of SSOP Haplotypes Observed in Blood Samples for Each Racial Group

AFRICAN AMERICAN N=34			CAUCASIAN N=44		
h = 0.981417			h = 0.979662		
SSOP Haplotype	**# obs**	**Freq.**	**SSOP Haplotype**	**# obs**	**Freq.**
A0C1D1E1A2B3C0D1189G16093T	1	0.02942	A2C1D1E1A2B6C1D1189A16093T	1	0.023
A1C3D2E1A2B7C2D1189A16093T	1	0.02942	A1C1D1E1A2B3C1D1189A16093T	2	0.045
A1C0D1E1A2B5C4D1189016093T	1	0.02942	A1C1D1E1A2B1C1D1189A16093T	3	0.068
A1C1D1E1A2B1C1D0189A16093T	1	0.02942	A1C2D1E1A1B1C1D1189A16093T	1	0.023
A3C3D0E2A2B0C0D2189016093T	4	0.11765	A2C1D1E1A2B1C1D1189A16093T	3	0.068
A0C1D1E1A2B3C1D1189A16093T	1	0.02942	A1C1D1E1A1B5C1D1189A16093T	1	0.023
A1C0D1E2A2B5C0D1189A(w)160930	1	0.02942	A1C3D1E1A2B1C1D1189A16093T	3	0.068
A1C0D2E1A2B0C1D1189A16093T	1	0.02942	A1C1D1E1A1B1C1D1189A16093T/C	1	0.023
A0C3D2E2A2B1C1D1189A16093T	1	0.02942	A2C1D1E1A2B1C1(w)D1189016093T	2	0.045
A1C3D1E2A2B7C0D1189016093T	1	0.02942	A1C1D1E1A2B1C0D1189G16093T	1	0.023
A3C3D1E2A2B0C0D2189016093T	1	0.02942	A1C1D1E1A1B0C1D1189A(w)16093T	1	0.023
A0C0D1E1A2B3C1D1189A16093T	1	0.02942	A1C1D1E1A1B1C1D1189A16093C	1	0.023
A3C1D1E2A2B4C2/1D1189A16093T	1	0.02942	A1C1D1E1A2B3C1D1(w)189A16093C	1	0.023
A3C3D0E2A2B0C0D21890160930	1	0.02942	A1C1D1E1A1B1/w3C1D1189A16093T	2	0.045
A1C1D2E2A2B4C2D1189A16093T	3	0.08824	A1C1D1E1A1B1C2D1189A16093T	1	0.023
A1C4(w1)D1E2A2B4C2D1189A16093T	2	0.05882	A1C1D1E1A1B1C1D1189A16093T	4	0.092
A3C3D1E2A2B6(w)C0D2189016093T	1	0.02942	A1C3D1E1A1B1C1D1189A16093T	2	0.045
A0C1D1E1A2B3C0D1189016093T	1	0.02942	A1C2D1E1A1B1C0D1189016093T	1	0.023
A2C3D1E0A2B3C2D2189016093T	1	0.02942	A1C3D1E1A2B1C1D1189A16093T/C	1	0.023
A3C1D0E2A2B0C0D21890160930	1	0.02942	A3C1D1E1A2B3C0D0189A(w)16093T	1	0.023
A1C1D1E2A2B7C4D1189016093T	3	0.08824	A2C1D1E1A2B1C0D1189016093T	1	0.023
A1C0D1E1A2B5C2D1189A16093T	1	0.02942	A1C3D1E3A2B5C1D1189A16093T	1	0.023
A3C3D1(W)E1A1B1C0D2189016093T	1	0.02942	A1C0D2E1A2B5C0D1189G16093T	1	0.023
A2C3D1E0A2B3C0D2189016093T	2	0.058823	A1C3D1E1A2B3C2D1189A16093C	1	0.023
A1C3D1E1A2B5C0D1189016093T	1	0.02942	A3C3D1E1A2B1C0D0189A16093T	1	0.023
			A1C0D2E1A2B0C1D1189A16093T	1	0.023
	Total	1	A2C2D1E1A2B0C1D1189A16093T	1	0.023
			A3C1(w)D1E1A1B1C1D1189A16093T	1	0.023
			A1C1D1E2A2B7C4D1189016093T	1	0.023
			A2C3D1E1A2B1C1D0189A16093T	1	0.023
			A3C3D1E1A2B1C0D0189G16093T	1	0.023
				Total	1

Table 23B. Frequency of SSOP Haplotypes Observed in Blood Samples For Each Racial Group

HISPANIC N=28			ASIAN N=25		
H = 0.83598			h = 0.96000		
SSOP Haplotype	**# obs**	**Freq.**	**SSOP Haplotype**	**# obs**	**Freq.**
A1C0D2E1A2B0C1D1189A16093T	11	0.39286	A1C1D1E1A2B1C1D1189A16093T	4	0.16
A1C1D1E1A2B1C1D0189A16093T	2	0.07143	A3C1D1E1A2B5C0D1189A16093T	2	0.08
A1C1D2E1A2B1C1D1189A16093T	1	0.035714	A1C1D1E1A2B1C2D1189A16093T	1	0.04
A1C0D1E1A2B1C1D1189A16093T	1	0.035714	A1C0D1E1A2B1C1D1189A16093T	1	0.04
A1C1D1E2A2B7(w)C4D1189016093T	1	0.035714	A3C2D1E1A2B3C1D1W189A16093T	1	0.04
A1C1D1E1A2B1C1D1189A16093T	4	0.14286	A1C1D1E1A2B2C1D1189A16093T	1	0.04
A1C1D1E1A1B1C2D1189A16093T	1	0.035714	A1C1D2E1A2B1C0D1189016093T	2	0.08
A3C3D1E1A2B4C1D0189A16093T	1	0.035714	A1C0D1E1A2B2C0D1189016093T	1	0.04
A1C0D2E1A2B0C1D1189A16093C	1	0.035714	A1C1D2E1A2B1C1D1189A16093T	2	0.08
A1C1D1E1A2B0C1D1189A16093T	1	0.035714	A3C3D1E1A2B5C1D1189016093T	1	0.04
A2C0D1E1A2B0C1D1189A16093T	1	0.035714	A1C3D1E1A2B5C1D1189A16093T	1	0.04
A1C0D1E1A2B0C1D1189A16093T	1	0.035714	A1C3D1E1A2B1C1D1189A16093T	1	0.04
A1C0D1E2A2B7C4D1189016093T	1	0.035714	A1C1D1E1A2B0C0D1189A(w)16093T	1	0.04
A1C0D2E1A2B0C1D1189016093T	1	0.035714	A1C1D2E2A2B1C1D1189A16093T	3	0.12
			A1C1D1E1A2B5(w6)C1D1189A16093T	1	0.04
	Total	1	A3C1D1E1A2B1C1D1189A16093T	1	0.04
			A3C1D1E0A2B3C1D0189A(w)16093T	1	0.04
				Total	1

The frequencies of the observed haplotypes were used to calculate the genetic diversity (h) values for each population using the following equation:

$$h = [1 - \sum(\text{haplotype frequency})^2] \, n/(n-1)$$

n = number of individuals in the database

Blood and Hair Characteristics and Heteroplasmy

Most of the individuals in this study exhibited a single mtDNA haplotype within their hair samples (table 22). 81 individuals (68%) were homoplasmic for both anagen and telogen hairs, whereas 38 individuals (32%) exhibited heteroplasmy for either their anagen or telogen hairs, or for both growth phases combined. Of these 38 cases, 13 individuals were exclusively anagen hairs (34.2%), 18 individuals were exclusively telogen hairs (47.4%) and 7 individuals (18.4%) overlapped in that both growth phases showed evidence of the heteroplasmic condition. The frequency of heteroplasmic hairs observed in this study is illustrated in table 25. This table shows a similar distribution for both the telogen and anagen growth phases: 86 and 82 individuals exhibited a single mtDNA haplotype for telogen (77.5%) and anagen (80.4%) hairs respectively. Within individuals, heteroplasmy most often manifests itself in one hair from a modest size sampling of between fifteen and twenty hairs, with 19 individuals falling into this category: 12 individuals exhibited the condition in one telogen hair and 7 individuals displayed the condition in one anagen hair. In addition, 2 individuals expressed a single heteroplasmic hair in both growth phases. Further, 5 individuals in this study were observed to have two hairs displaying the condition, all of which are telogen hairs. There were also 12 individuals who exhibited heteroplasmy in more than two hairs. Of these 12 individuals, 8 expressed heteroplasmy exclusively within their hairs, while the remaining 4 individuals

were heteroplasmic in both their blood and hair tissue. One individual exhibited a single heteroplasmic hair and was also heteroplasmic for the blood tissue (table 24).

Comparing the use of cosmetic treatments to the incidence of heteroplasmy, the results indicate that there is no correlation at the 95% significance level (table 27). This result also holds true when compared to the type of cosmetic treatment that was used (table 28). Similarly, as seen in table 29, the results reflect that there is no statistically significant correlation between the occurrence of heteroplasmy for living and deceased individuals (95% significance level). Finally, as seen in table 30, there is no statistical correlation between the incidence of heteroplasmy and the region of the scalp that the hair was collected.

Table 24. Observed Heteroplasmic and Homoplasmic Cases In Different Hair Growth Phases

	Count	Homoplasmy Anagen	Heteroplasmy Anagen	Total[E]
Homoplasmy Telogen	Observed	81	13	94
	Expected	78	16	94
Heteroplasmy Telogen	Observed	18	7	25
	Expected	21	4	25
Total	Observed	99	20	119
	Expected	99	20	119

Table 25. Frequency Distribution of Heteroplasmy in Hairs Observed in the Study.

# Hairs Heteroplasmic	Telogen[E] Frequency	Telogen %	Telogen Cum. Hairs	Anagen[E] Frequency	Anagen %	Anagen Cum. Hairs
0	86	77.5	0	82	80.4	0
1	14	12.5	14	9	8.8	9
2	5	4.5	24	2	1.96	13
3	1	1	27	2	1.96	19
4	2	1.8	35	4	3.9	34
5	2	1.8	45	0	0	44
6	0	0	45	1	0.98	50
7	1	0.9	52	1	0.98	57
8	0	0	52	1	0.98	57
10	0	0	52	0	0	57
Total	111	100	52	102	100	57

Table 26. Frequency of Heteroplasmic Individuals for Each Tissue Type Based on the mtDNA SSOP System

CATEGORY	Blood #	Blood %	Hair #	Hair %
Heteroplasmic	5	4	38	32
Homoplasmic	126	96	81	68
Total	131	100	119	100

Table 27. Cosmetic Treatment:
Observed Heteroplasmic and Homoplasmic Cases for Treated and Untreated Hairs

Treatment	Count	Homoplasmy	Heteroplasmy	Total[E]
No	Observed	36	21	57
	Expected	38	19	57
Yes	Observed	27	11	38
	Expected	25	13	38
Total	Observed	63	32	95
	Expected	63	32	95

Table 28. Type of Cosmetic Treatment:
Observed Heteroplasmic and Homoplasmic Cases for Treated Hairs

Type of Treatment	Count	Homoplasmy	Heteroplasmy	Total[E]
Dye	Observed	15	6	21
	Expected	15	6	21
Perm	Observed	4	2	6
	Expected	4	2	6
Highlights	Observed	6	1	7
	Expected	5	2	7
Relaxer	Observed	2	0	2
	Expected	1	1	2
Other	Observed	0	2	2
	Expected	1	1	2
Total	Observed	27	11	38
	Expected	27	11	38

Table 29. Observed Heteroplasmic and Homoplasmic Cases
for Living and Deceased Groups

Living	Count	Homoplasmy	Heteroplasmy	Total[E]
No	Observed	17	6	23
	Expected	16	7	23
Yes	Observed	64	32	96
	Expected	65	31	96
Total	Observed	81	38	119
	Expected	81	38	119

Table 30. Frequency of Heteroplasmic and Homoplasmic Hairs by Region of Scalp

Region of Scalp	Category	Frequency	Percentage (%)	Cum. (%)
Front	Homoplasmy	108	90.8	90.8
	Heteroplasmy	11	9.2	100
	Total	119	100	
Back	Homoplasmy	100	84.0	84.0
	Heteroplasmy	19	16	100
	Total	119	100	
Center	Homoplasmy	105	88.2	88.2
	Heteroplasmy	14	11.8	100
	Total	119	100	
Right-Side	Homoplasmy	99	83.2	83.2
	Heteroplasmy	20	16.8	100
	Total	119	100	
Left-Side	Homoplasmy	98	82.3	82.3
	Heteroplasmy	21	17.7	100
	Total	119	100	

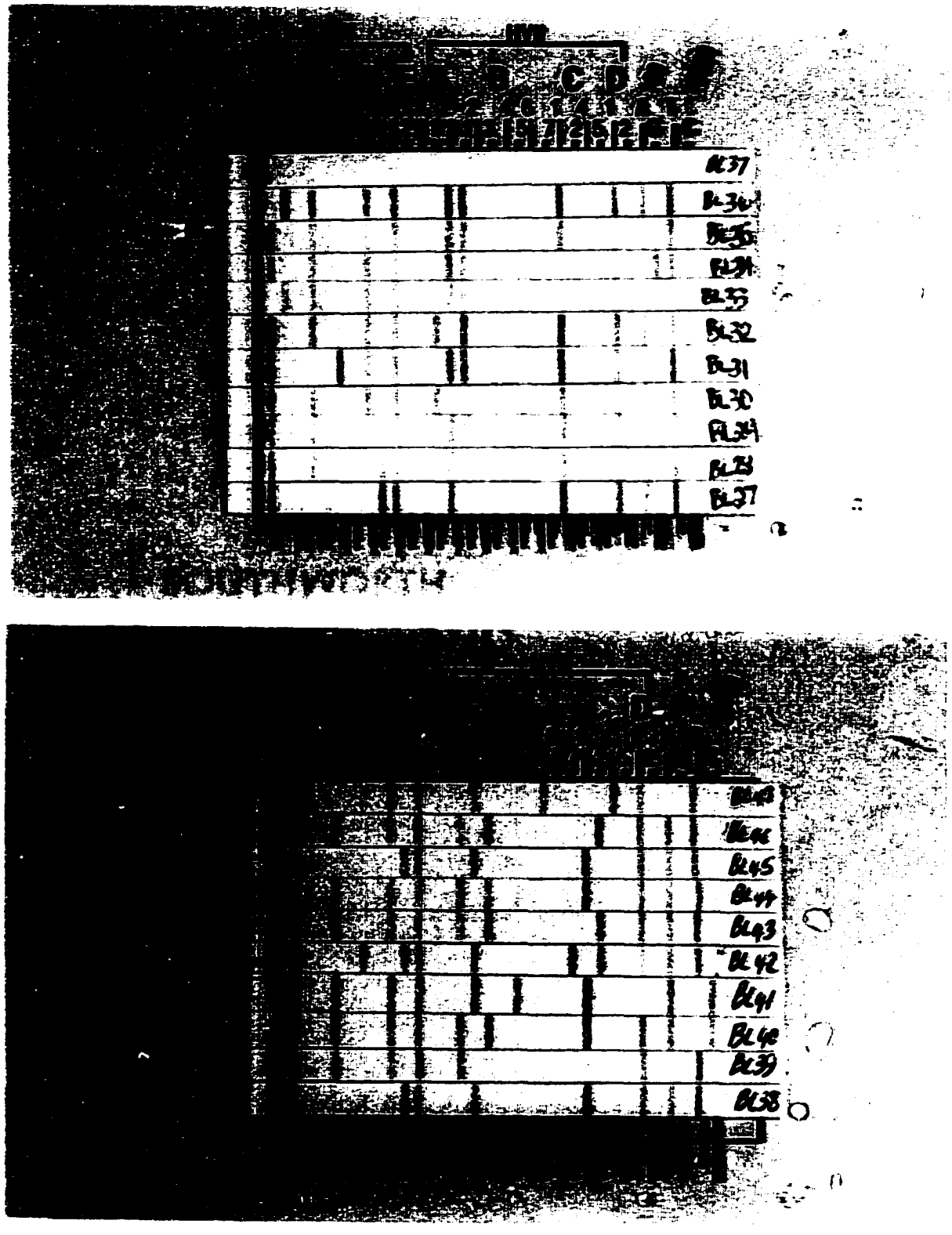


Figure 18. Immobilized SSOP Linear Assay Results
Comparison of Heteroplasmic and Homoplasmic Individuals

Demographics and Heteroplasmy

The demographic variables of the subjects were also analyzed. The data indicate that there is no significant correlation (95% significance level) between heteroplasmy and the race of an individual when comparing across all four of the population groups (table 31). However, there is a significant difference in the observed and expected cases when the comparison is restricted to the Caucasian and African American subgroups (95% significance level). 18 Caucasian individuals (43%) and 5 African-American individuals (17%) exhibit the heteroplasmic condition. Analysis of age (table 32) and sex (table 33) as a function of heteroplasmy revealed that there is no statistically significant difference between either of these independent variables and the heteroplasmic condition.

Table 31. Observed Heteroplasmic and Homoplasmic Cases in Each Racial Group

Racial Group	Count	Homoplasmy	Heteroplasmy	Total[e]
Caucasian	Observed	24	18	42
	Expected	29	13	42
African American	Observed	25	5	30
	Expected	20	10	30
Hispanic	Observed	17	7	24
	Expected	16	8	24
Asian	Observed	15	8	23
	Expected	16	7	23
Total	Observed	81	38	119
	Expected	81	38	119

Table 32. Observed Heteroplasmic and Homoplasmic Cases in Each Age Group

Age Range (Years)	Count	Homoplasmy	Heteroplasmy	Total[E]
18 – 29	Observed	26	20	46
	Expected	31	15	46
30 – 49	Observed	30	12	42
	Expected	29	13	42
50 – 69	Observed	18	4	22
	Expected	15	7	22
70 and above	Observed	7	2	9
	Expected	6	3	9
Total	Observed	81	38	119
	Expected	81	38	119

Table 33. Observed Heteroplasmic and Homoplasmic Cases by Sex

Sex	Count	Homoplasmy	Heteroplasmy	Total[E]
Female	Observed	39	23	62
	Expected	42	20	62
Male	Observed	42	15	57
	Expected	39	18	57
Total	Observed	81	38	119
	Expected	81	38	119

Detection of Mixtures Summary

Given that the mitochondrial DNA genome is haploid, the observation of two probe signals within a probe region may be due to heteroplasmy. Of the 131 samples of mtDNA extracted from blood and typed with the mtDNA linear array assay, five (~4%) appear to have two sequences present. These are summarized as follows: sample # 32 indicates a T/C mixture at 16093; sample # 44 indicates a 1/w3 mixture at HVIIB; sample # 60 indicates a 1/w3 mixture at HVIIB sample # 62 indicates a T/C mixture at 16093; and sample # 103 indicates a 1/2 mixture at HVIIC. Each of the five individuals who show a mixture of haplotypes in their blood samples also exhibits varying degrees of heteroplasmy in their hair samples. Sample # 32 indicates a T/C mixture at 16093 in six hairs; sample # 44 indicates a 1/w3 mixture at HVIIB in five hairs; sample # 60 indicates a 1/w3 mixture at HVIIB in one hair; sample # 62 indicates a T/C mixture at 16093 in thirteen hairs; and sample # 103 indicates a 1/2 mixture at HVIIC in three hairs. In addition, thirty-three individuals reveal heteroplasmy exclusively in their hair samples. These results are summarized in table 34. The heteroplasmic positions observed in this study are summarized in table 35. For the 38 individuals exhibiting heteroplasmy, 11 are observed in the HVI region and 27 are observed in HVII.

Table 34. Haplotypes of Samples in Which Two SSOP Signals Were Detected

	HVI				HVII					
	A	C	D	E	A	B	C	D	189	16093
Sample/Tissue										
Individual # 5										
Blood	3	2	1	1	2	3	1	1w	A	T
Hair (4)	3	2	1	1	2	1,3	1	1w	A	T
Individual # 8										
Blood	1	1	1	1	2	1	1	1	A	T
Hair (1)	1,3	1	1	1	1	1	1	1	A	T
Individual # 9										
Blood	1	1	1	1	2	2	1	1	A	T
Hair (1)	1	1	1	1	2	1/2	1	1	A	T
Individual # 13										
Blood	1	1	2	1	2	1	0	1	0	T
Hair (1)	1	1	2	1	2	1/w3	0	1	0	T
Individual # 15										
Blood	1	1	2	1	2	1	1	1	A	T
Hair (1)	1/2	1	2	1	2	1	1	1	A	T
Individual # 16										
Blood	3	3	1	1	2	5	1	1	0	T
Hair (1)	3	3	1	1	2	5	1/2	1	0	T
Individual # 20										
Blood	1	3	1	1	2	5	1	1	A	T
Hair (2)	1	3	1	1	2	5	1	1	A/G	T
Individual # 21										
Blood	1	1	2	1	2	1	1	1	A	T
Hair (4)	1	1/2	2	1	2	1	1	1	A	T

Table 34. Haplotypes of Samples in Which Two SSOP Signals Were Detected

	HVI				HVII					
	A	C	D	E	A	B	C	D	189	16093
Sample/Tissue										
Individual # 22										
Blood	2	1	1	1	2	1	1	1	A	T
Hair (2)	2	1,2	1	1	2	1	1	1	A	T
Individual # 23										
Blood	3	1	1	1	2	5	0	1	Aw	T
Hair (2)	1/3	1	1	1	2	5	0	1	Aw	T
Individual # 26										
Blood	0	1	1	1	2	3	0	1	G	T
Hair (1)	0	1	1	1	2	1/3	0	1	G	T
Individual # 29										
Blood	1	1	1	1	2	1	1	1	A	T
Hair (1)	1/2	1	1	1	2	1	1	1	A	T
Individual # 32										
Blood	1	1	1	1	1	1	1	1	A	T/C
Hair (6)	1	1	1	1	1	1	1	1	A	T/C
Individual # 35										
Blood	1	1	1	1	2	1	1	1	A	T
Hair (1)	1	1	1	1	2	1	1/2	1	A	T
Individual # 40										
Blood	1	1	1	1	1	1	1	1	A	C
Hair (12)	1	1	1	1	1	1	1	1	A	T/C
Individual # 41										
Blood	1	1	1	1	2	3	1	1w	A	C
Hair (8)	1	1	1	1	2	3	1	1w	A	T/C

Table 34. Haplotypes of Samples in Which Two SSOP Signals Were Detected

	HVI				HVII					
	A	C	D	E	A	B	C	D	189	16093
Sample/Tissue										
Individual # 43										
Blood	1	1	1	1	1	1	2	1	A	T
Hair (1)	1/2	1	1	1	1	1	2	1	A	T
Individual # 44										
Blood	1	1	1	1	1	1/w3	1	1	A	T
Hair (5)	1	1	1	1	1	1/w3	1	1	A	T
Individual # 46										
Blood	1	1	1	1	1	1	2	1	A	T
Hair (3)	1	1	1	1	1	1	1/2	1	A	T
Individual # 48										
Blood	1	1	1	1	1	1	1	1	A	T
Hair (1)	1	1	1	1	1	1	1/2	1	A	T
Individual # 49										
Blood	1	1	1	1w	2	1	1	1	A	T
Hair (1)	1	1	1	1w	2	1	1	1	A	T/C
Hair (1)	1	1	1	1w	2	1	1	1	A/G	T
Individual # 50										
Blood	1	3	1	1	1	1	1	1	A	T
Hair (1)	1	3	1	1	1/2	1	1	1	A	T
Individual # 60										
Blood	1	1	1	1	1	1/w3	1	1	A	T
Hair (1)	1	1	1	1	1	1/w3	1	1	A	T
Individual # 61										
Blood	2	1	1	1	2	1	1	1	A	T
Hair (1)	2	1	1	1	2	1	1	1	A/G	T

Table 34. Haplotypes of Samples in Which Two SSOP Signals Were Detected

	HVI				HVII					
	A	C	D	E	A	B	C	D	189	16093
Sample/Tissue										
Individual # 62										
Blood	1	3	1	1	2	1	1	1	A	T/C
Hair (13)	1	3	1	1	2	1	1	1	A	T/C
Individual # 63										
Blood	1	0	2	1	2	0	1	1	A	C
Hair (4)	1	0	2	1	2	0	1	1	A	T/C
Individual # 66										
Blood	1	3	1	1	2	1	1	1	A	T
Hair (1)	1	3	1	1	2	1	1	1	A	T/C
Individual # 67										
Blood	1	1	1	1	2	1	1	1	A	T
Hair (2)	1	1	1	1	2	1/3	1	1	A	T
Individual # 70										
Blood	3	1	1	1	2	3	0	0	Aw	T
Hair (2)	3	1	1	1	2	1/3	0	0	Aw	T
Individual # 74										
Blood	1	3	1	3	2	5	1	1	A	T
Hair (4)	1	3	1	1/3	2	5	1	1	A	T
Individual # 76										
Blood	1	1	1	2	2	7	4	1	0	T
Hair (1)	1	1	1	1/2	2	7	4	1	0	T
Individual # 77										
Blood	1	3	1	1	2	3	2	1	A	C
Hair (6)	1	3	1	1	2	3	2	1	A	T/C

Table 34. Haplotypes of Samples in Which Two SSOP Signals Were Detected

	HVI				HVII					
	A	C	D	E	A	B	C	D	189	16093
Sample/Tissue										
Individual # 82										
Blood	2	2	1	1	2	0	1	1	A	T
Hair (1)	2	1/2	1	1	2	0	1	1	A	T
Individual # 87										
Blood	1	1	1	2	2	7	4	1	0	T
Hair (1)	1	1	1	2	2	1/7	4	1	0	T
Individual # 100										
Blood	1	1	2	2	2	1	1	1	A	T
Hair (1)	1	1	2	2	2	1	1	1	A/G	T
Individual # 103										
Blood	3	1	1	2	2	4	2/1	1	A	T
Hair (3)	3	1	1	2	2	4	2/1	1	A	T
Individual # 108										
Blood	1	0	2	1	2	0	1	1	A	T
Hair (1)	1	0	2	1	2	0	1	1	A/G	T
Individual # 132										
Blood	3	3	0	2	2	0	0	2	0	T
Hair (2)	3	3	0	1w/2	2	0	0	2	0	T

Table 35. Summary of mtDNA Point Heteroplasmy in Study

INDIVIDUAL#	MIXTURE DETECTED	POINT MUTATION SITE
5	HVII B1,3	T→C at 152bp
8	HVI A1,3	G→A at 16129bp
9	HVII B1,2	T→C at 146bp
13	HVII B1,w3	T→C at 152bp
15	HVI A1,2	T→C at 16126bp
16	HVII C1,2	T→C at 195bp
20	HVII 189 A,G	A→G at 189bp
21	HVI C1,2	T→C at 16304bp
22	HVI C1,2	T→C at 16304bp
23	HVI A1,3	G→A at 16129bp
26	HVII B1,3	T→C at 152bp
29	HVI A1,2	T→C at 16126bp
32	HVII 16093 T,C	T→C at 16093bp
35	HVII C1,2	T→C at 195bp
40	HVII 16093 T,C	T→C at 16093bp
41	HVII 16093 T,C	T→C at 16093bp
43	HVI A1,2	T→C at 16126bp
44	HVII B1,w3	T→C at 152bp
46	HVII C1,2	T→C at 195bp
48	HVII C1,2	T→C at 195bp
49	HVII 16093 T,C HVII 189 A,G	T→C at 16093bp A→G at 189bp
50	HVII A1,2	A→G at 73bp
60	HVII B1,w3	T→C at 152bp
61	HVII 189 A,G	A→G at 189bp
62	HVII 16093 T,C	T→C at 16093bp
63	HVII 16093 T,C	T→C at 16093bp

[illegible]	[illegible]	[illegible]
67	HVII B1,3	T→C at 152bp
70	HVII B1,3	T→C at 152bp
74	HVI E1,3	C→T at 16270bp
76	HVI E1,2	C→T at 16278bp
77	HVII 16093 T,C	T→C at 16093bp
82	HVI C1,2	T→C at 16304bp
87*	HVII B1,7	T→C at 146bp C→T at 150bp T→C at 152bp
100	HVII 189 A,G	A→G at 189bp
103	HVII C1,2	T→C at 195bp
108	HVII 189 A,G	A→G at 189bp
132	HVI E 1w,2	C→T at 16278bp

Notes on Chapter 4

A. This number reflects a combined success rate for either HVI only or HVII only or HVII & HVII

B. This number reflects a combined success rate for living individuals only

C. This number reflects a combined success rate adjusted for 35 missing hairs

D. Determined based on a combination of hair length and diameter

E. This number is adjusted to included hairs that successfully typed

CHAPTER 5

Discussion

The mitochondrial DNA linear array assay typing method is a two step process that involves co-amplification of the HVI and HVII regions using a duplex primer set followed by hybridization of the PCR products to a linear array of SSO probes. PCR products that hybridize to a specific probe are visualized during a subsequent color development process. A particular probe location will turn blue if it has hybridized to a PCR product, with a pattern of lines generated for the multiple probe locations. The linear array strips are made up of thirty-one probes that reveal the haplotypes of an individual at the HVI and HVII loci within the control region of the mitochondrial DNA genome. A total of ten regions have been identified: for HVI they are A, C, D, E and 16093; for HVII they have been designated A, B, C, D and 189. Polymorphisms are recognized by three SSO probes in region AI, four probes in CI, two probes in DI, three probes in E, two probes for 16093, two SSO probes in AII, seven probes in B, four probes in CII, two probes in DII, and two probes for 189. The presence of two signals in any one of these regions indicates a heteroplasmic mixture.

PCR Amplification

The hair samples used in this study were cut at the proximal (root) end to generate a 2cm portion of hair shaft, which was extracted and amplified with the duplex primer set. For some individuals, the length of an entire hair was less than 2cm. In this situation the length was measured and the entire hair was extracted. The results indicate that one of the mitigating factors for amplification success is hair length, with shorter hairs (<2cm) showing significantly lower success. One explanation for this is that the length of the shaft may reduce the amount of template DNA available for amplification. However, the diameter of the shaft and the degree of pigmentation within the shaft may also be a factor. The PCR results indicate that there is a considerable difference in the success rate when comparing anagen with telogen hairs for the duplex primer set. One explanation for this may be that in removing the root tissue, a rich source of mitochondrial DNA may also be removed from within the hair shaft. Presumably, there are more mitochondria proximal to the root and by cutting this particular portion the amount of mtDNA in the adjacent 2cm is reduced. In addition, the distal portion may differ from the proximal portion due to the effects of UV light exposure. Presumably, the proximal end is less exposed to direct sunlight, which may manifest itself as a reduction in the number of mitochondria at the distal end compared to the proximal end of the hair shaft. It is also feasible that exposure to UV light may give rise to genetic mutations. Therefore, it is possible that different portions of the same hair shaft may give rise to different haplotypes. The increased success rate observed with the singleplex may be due to the fact that

the amplification is more efficient due to is less competition for the components of the reaction mix. The explanation for the higher success rate for HVI compared to HVII is not known at this time. The fact that HVI generates a larger amplicon (444 bp) compared to HVII (415 bp) would lead one, if anything, to expect the opposite. It may be due to the fact that the T_M values of HVII are set lower. Alternatively, it might be related to magnesium chloride concentration, since some primer pairs are more effective at different concentrations and the reaction mix may somehow bias the amplification of HVI over HVVII.

Evidence from the present research indicates that the use of cosmetic treatments, regardless of the type of treatment, has no affect on the overall amplification success rate. However, as indicated in the results section, the proportion of individuals who show a 100% success rate is much higher for untreated compared to treated hair. Therefore, chemical treatments may be a factor contributing to some degradation in mtDNA copy number. However, it is also possible that chemical treatments may be contributing inhibitory effects (see Discussion under *Future Studies*).

The data also show that there is no significant difference in the success rate when comparing living and deceased donors, indicating that postmortem changes in the tissues do not appear to affect the yield of mtDNA. In addition, the medulla structure is independent of the amplification success rate. The hypothesis here was that hairs that lack a medulla are more likely to amplify given that they contain more cellular debris compared to hairs that exhibit a continuous broad medulla. This suggests that the medulla structure is not a

useful predictor of potential amplification success. Finally, this research suggests that amplification success rate appears to be significantly correlated to the race of the donor. The findings indicate that Caucasian hairs demonstrate a significantly higher success rate when compared to African Americans. This may be attributed to the degree of pigmentation causing potential inhibition.

As indicated in the methods and materials section, a number of approaches can be taken in order to increase mitochondrial DNA yield. Initially, the mass of the PCR product should be estimated by comparing to a DNA Mass Ladder. If the amount is below the target (~3-6ng/μL) the sample should be re-amplified with an increased the number of cycles. Alternatively, the volume of template DNA in the amplification reaction may be changed (decreased if inhibitors are suspected or increased up to 20μL if the template yield <3ng/μL). In addition, two separate amplifications may be performed using the singleplex primer sets. If these approaches fail, PCR inhibition is a possibility. Potentially, inhibitors may be removed by treating the extract with bovine serum albumin (BSA), otherwise, alternative extraction methods need to be considered. In addition, if the PCR product yield exceeds 6ng/μL, it is advisable to dilute the sample prior to typing in order to minimize cross-hybridization (i.e. SSO probes may become saturated).

Mitochondrial SSOP Typing of Sequence Variants

Linear array assay typing is a simple technique, although certain precautions must be taken to minimize interpretation problems. The fact that it is a hybridization assay means that fluctuating incubation temperatures can greatly

affect stringency conditions. The water bath temperature must be maintained at 55°C in order to avoid non-specific probe hybridization (caused by lower temperatures or weak signal intensity/signal dropout observed at elevated temperatures). Two other factors may contribute to probe cross-hybridization or signal dropout: the amount of amplicon added into the typing reaction and the development time of the linear arrays. As indicated above, sample input should be monitored to avoid probe saturation (excess sample) or weak signal intensities (limited sample). Similarly, shorter development times generate weak signal intensities whereas over-development may result in the formation of weak bands at multiple locations.

When controlling for the experimental parameters described above, one of four types of probe signals is detected on the linear array assay. A single probe generates a signal; a single probe gives a weak signal; no probe signals are detected; and, two probes are visible (either with equal or uneven intensity). A weak or absent signal at a particular region is attributed to a sequence variant at the probe binding site that causes a destabilization of the probe-template interaction preventing hybridization. When the destabilization is extreme, the template fails to attach and the region is assigned a "0". If the destabilization reduces the template binding, the signal intensity is likewise reduced and the region is assigned a "w". 16 of the 131 blood samples (12%) for all four of the population groups gave a weak signal in one region. Within each population group, the frequency of weak signals is 4% (Hispanic), 14% (Caucasian) 9%

(African American) and 8% (Asian). Similarly, 71 of the 131 blood samples (54%) for all four of the population groups gave a "0" signal in at least one region. Within each population group, the frequency of "0" signals is 82% (Hispanic) 32% (Caucasian) 79% (African American) and 36% (Asian).

The results of this study indicate that the SSOP method is more discriminatory for the Caucasian and African American populations than the Asian and Hispanic population. For example, in the Hispanic database (n = 28) 11 individuals shared a common type. This type was also found once in two other population groups. Within the Asian population (n = 25) the most common type was observed 4 times. This type was also common in two other population groups: Hispanic (4 individuals) and Caucasian (3 individuals). The genetic diversity values across all four of the population groups range from 0.83598 to 0.981417 (table 23A&B). These values are consistent with those determined by Reynolds *et al.*[138] and also compare with the level of discrimination of the nuclear markers HLA DQA1 and D1S80.

When two probes within a region hybridize to the PCR product, this indicates heteroplasmy. However, it may also be an indication of a mixture of more than one contributor. In order to eliminate contamination as a source of heteroplasmy, pre-PCR and PCR controls should be performed and extraction/amplification reactions should be run in duplicate. The mitotype of the analysts should also be determined in order to rule them out as the potential source of contamination. Finally, automated sequencing of the entire coding region should be performed to confirm the SSOP results (see below *Further Studies*). Additional precautions

are necessary when analyzing hair. For example, pre-screening samples microscopically is advisable since it remains the most timely and cost-effective means of assessing hairs to determine the species, somatic origin, root morphology, possible racial origin, and whether cosmetic treatments have been used. In this study, for example, if an individual reported using a chemical dye, this was confirmed by microscopical examination. It also allows the examiner to identify outlier hairs that are not representative of the sample (i.e. hairs that may have been collected as a result of a secondary transfer). Further, it permits an examination of potential surface material attached to the hair that may contribute to the mixture (for example, traces of blood or semen). Although the hair extraction protocol calls for a sonication step in a detergent wash, it may also be advisable to perform a chemical test on the hair extract for the presence of these physiological fluids (for example, using phenolphthalein, acid phosphatase, etc). These reagents are very sensitive and can detect the biological fluids using minimal amounts of the extract.

This study further demonstrates that detectable levels of heteroplasmy can vary within an individual. For blood, the condition was observed in 5 individuals (~4%) compared to 38 individuals (~32%) who expressed the condition in at least one hair. These results were cross-referenced with the analyst's haplotypes, and in each case, the analyst was excluded as a potential contributor to the mixture observed.

When heteroplasmy was observed in a donor's blood sample at least one hair was also heteroplasmic, although typically, multiple hairs were heteroplasmic for

these individuals. For the remaining 33 individuals with homoplasmic blood, heteroplasmy manifested itself in one or two hairs (25 individuals) three to four hairs (4 individuals) and five or more (4 individuals). In most instances where five or more hairs exhibit heteroplasmy when the blood is homoplasmic, the mutated type is due to a C → T point mutation at the 16093 base pair position. All individuals whose blood exhibited a C at 16093 had multiple hairs with a T/C mixture at this position.

This information is important from an interpretational point of view because when two blood samples are compared (i.e. reference v. evidence) and are found to differ with respect to one region within the linear array, this indicates exclusionary evidence. Although a genetic switch is possible, it is unlikely. It is equally improbable that we are observing selective detection of the minor and major components for the two samples since blood mitotypes appear to be relatively stable over time. However, since inter-tissue sequence variation is possible, blood may not be the best reference for determining the type of other tissues. Therefore it is better to compare multiple hairs with each other than with the blood sample. It is still useful to type the blood because it generally reflects the major mtDNA sequence.

The most common positions for site heteroplasmy in biological samples reported in the literature are 16093 and 189. However, heteroplasmy has also been observed at positions 73, 189, 152 and 195. Therefore, the results of this study are consistent with previously reported heteroplasmic "hotspots".

The results of this study suggest that provided a sample amplifies, there is no correlation between the detection of heteroplasmy and the use of cosmetic treatments. This is true regardless of the type of treatment reported. This result suggests that if the sample amplifies chemical treatments do not reduce the level of the minor sequence below the detection threshold. For example, four individuals (3 no treatment and one chemical dye) exhibit a "C" at base pair position 16093 for their blood type and all four exhibit a T/C mixture at this position for multiple hairs. Also, one individual (chemical dye) who exhibited heteroplasmy in blood also exhibited heteroplasmy in multiple hairs.

The data also indicate that the frequency of heteroplasmy does not differ in living compared to deceased individuals. The 5 individuals (3 living, 2 deceased) who show heteroplasmy in blood exhibited similar patterns of heteroplasmy in hair. This suggests that the changes that occur in hairs postmortem do not influence the detection of heteroplasmy. In addition, the data from this study suggest that the frequency of heteroplasmy does not differ significantly with age. This contrasts with an earlier study that found heteroplasmy increases with age[73]. Mutations that increase with age indicate a somatic origin whereas heteroplasmy across multiple tissues is presumably due to a germ line mutation, with replicative segregation possibly accounting for differences in the level of heteroplasmy across tissues. They could also be due to somatic mutations very early on in the development of the zygote prior to tissue differentiation. However, in the current study, the origin of the mutation cannot be confirmed because maternal reference samples are not available.

Based on the results of the present study there is no significant difference in the incidence of heteroplasmy across different regions of the scalp. This suggests that heteroplasmic hairs are randomly distributed in the scalp rather than forming a mosaicism in one particular region. The implication is that if current hair collection procedures are followed, a representative sample of heteroplasmic hairs will also be collected. As expected, there was no difference in the frequency of heteroplasmy observed in comparing male and female subjects.

Additionally, the findings show that the incidence of heteroplasmy differs significantly when comparing the Caucasian population and the African American population groups, and is significantly higher in the Caucasian population. A logistic regression was also performed to take into account other variables, including the lower amplification success rate of the African American hair samples. Even when controlling for these variables, the statistical results revealed that the frequency of heteroplasmy was higher in the Caucasian population.

Limitations of the Study

One limitation of PCR-SSOP analysis is the reduction of discrimination power in comparison to mtDNA sequencing techniques. This is true because only discrete loci within the control region are typed. The "Roche Molecular Systems mitoTYPE" SSOP arrays are designed to detect sequence variation at these discrete nucleotide base pair positions. Therefore, they can detect sequence heteroplasmy but will not identify length heteroplasmy. The SSOP method may

also underestimate the level of heteroplasmy when a probe does not cover a sequence variation. Also, if the mutated sequence in a heteroplasmic mixture causes a destabilizing mismatch with the probe, it may not be detected and will appear as a homoplasmic sample. Finally, the technique may not be sensitive enough to pick up the minor component in a heteroplasmic mixture. This may account for the observation of intra-tissue variation in blood and hair for an individual. Heteroplasmic mixtures in peripheral blood have been detected by sequence analysis if the minor component is greater than 10-20% of the overall mixture. (i.e. sequence analysis can usually detect major and minor variants if the ratio is in the order of 10:1 to 5:1).

Despite these shortcomings, probative samples analyzed by the SSOP format can be further discriminated using direct sequencing analysis. However, since current mtDNA sequencing techniques are still based on the analysis of the HVI and HVII control region, certain haplotypes continue to be fairly frequent in the population and therefore exhibit low discrimination power. For example, in Caucasians, the most common HI/HVII type is found in approximately 7% of the population, while twelve additional types each represent approximately 0.5% of the population. In addition, as stated earlier, since the mtDNA genome is maternally inherited, it is not subject to the random segregation and assortment of genes associated with recombination. Consequently, with the exception of identical twins, unlike the nuclear genome, the mitochondrial genome is not unique to an individual. Thus, mitochondrial DNA typing will not provide the same definitive identification that STR-based typing of nuclear DNA permits. However,

the overall distribution of mtDNA types is rare in the national database. For example, of the 1175 different haplotypes identified, 982 occur only once. Therefore the greatest limitation of mtDNA testing lies with the small number of common types for which the power of discrimination is relatively low.

Future Studies

In a follow-up study, the observed frequency of heteroplasmy using the reverse SSOP method will be compared to the direct sequencing technique. This analysis will serve two main purposes. The results of the SSOP method and the direct sequencing method of double-strand sequencing the entire HVI and HVII control region using an automated sequencer (ABI Prism™ 377) will be compared to assess whether consistent typing results are obtained. The later technique distinguishes true "heteroplasmy" from DNA mixtures from more than one contributor, or from mixtures due to sequencing artifacts. The significance here is that SSOP typing determines variation at discrete locations within the HVI and HVII control region. As a result, only a limited number of haplotypes can be distinguished. The number of mutations at each specific base pair position within these two regions varies. Those that mutate at a higher rate define the "hypervariable" or "hot spot" regions that have shown a greater tendency to mutate compared to adjacent regions. The presence of two variants at any given base pair position normally indicates a heteroplasmic condition. However, since a limited number of sequence variation exist in the population, it is possible that the result may reflect a mixture of mitochondrial DNA from two individuals. In this

example, they coincidentally share the same sequence at all sites except the one indicating a mixture. Sequence analysis indicating the presence of a single base at each site along the control region and the occurrence of two bases at the heteroplasmic position provides confirmation that "true" heteroplasmy exists. This study will also permit a comparison of the sensitivity of the two techniques in detecting heteroplasmic mixtures. This is significant because although the sequencing method provides more discrimination, it may not necessarily be the most sensitive method for detecting heteroplasmy. The results of the present study indicate that different ratios of heteroplasmic mixtures are observed in tissues. In order to address the sensitivity of the technique and to semi-quantitate the line intensity (based on an estimate of the % of the minor component detected) a mixture study will be conducted. Blood samples will be mixed in varying ratios prior to amplification. The two samples selected will have minimal regions in common. This study will also be performed using hairs from the same individual.

In order to address issue of the lower discrimination potential of the technique for individuals from the Hispanic population group, "blind" validation studies will be designed to evaluate whether microscopy can enhance the discrimination power of the SSOP typing results. This will involve the co-operation of forensic experts who specialize in hair examination. Additional studies will also focus on improving the amplification success rate by evaluating alternative extraction methods. For example, a comparison will be made of the efficiency of the chelex extraction with organic-based extraction methods, while other experiments will be designed to assess the value of introducing a PCR purification step (e.g. using

Centricon® tubes) prior to the chelex extraction. Other studies will address whether the reduction in success rate associated with the use of chemical treatments is due to degradation of mtDNA or because the treatments are causing an inhibitory effect. In order to resolve this issue a positive control will be added to a series of hair extracts that failed to amplify in previous attempts.

Conclusions

This research presents a clearer understanding of some of the issues associated with the phenomenon of heteroplasmy and its detection in human hair and bloodstains. It also highlights the potential of the analysis to generate both false negative and false positive results. This insight will allow forensic scientists to frame better questions when applying mtDNA analysis to head hair and other types of evidence. The need for a greater understanding of the underlying theory of scientific knowledge was emphasized in *Daubert* because it addresses the reliability of scientific evidence and therefore, its admissibility. The study is also beneficial to the field of biology. It provides new information about the existence and prevalence of heteroplasmy in humans, and in particular, human dermis and hair. This study will directly benefit the forensic science community and the justice system at large. The ability to routinely apply this technique to mitochondrial DNA typing of evidential hairs in casework would be a valuable addition to the capabilities of a forensic science laboratory. Polymerase chain reaction (PCR), in combination with SSOP analysis of mitochondrial DNA is a more objective approach to the analysis of human hair shafts than conventional

microscopic analysis. The method is even suitable for the analysis of individual hair shafts and may also be extended to analyze other forensic samples that are known to contain low quantities of nuclear material such as, teeth and bone tissue. Due to the fact that it is a relatively simple technique, the PCR-SSOP format allows for a reasonably rapid throughput, generating preliminary results in a single day. Consequently, this allows for the timely exoneration of innocent suspects. In addition, given the subjective nature of morphological hair analysis, mtDNA testing has applications in the area of post-conviction testing. Further, in police investigations where many items of evidence are collected, there is a need to screen for the most probative evidence. This will allow investigators to focus their inquiries more efficiently. Another advantage of the SSOP mitochondrial DNA typing format is that it allows easier analysis of mixtures. Mitochondrial DNA mixtures are much less complicated to interpret due to the haploid state of the mitochondrial genome. One of the limitations of nuclear DNA analysis is the complex pattern of alleles in samples derived from two or more individuals. Interpretation can be especially confusing when the individuals in question exhibit overlapping genotypes. Further, this method provides a means of extending the application of DNA technology beyond physiological stains and into trace evidence analysis, such as the analysis of animal hairs and natural fibers. Finally, this study evaluates the applicability of the sequence specific oligonucleotide approach to mitochondrial DNA typing, which could ultimately make mitotyping more accessible to forensic science laboratories.

APPENDIX A

Human Subjects Information
(This information is reproduced as submitted in the original dissertation proposal)

Human Subjects Information

Purpose of the Study

This study will be conducted to further the understanding of the phenomenon of heteroplasmy, a condition whereby both mutant and wild type mitochondrial genomes are found to exist within a particular individual. It is anticipated that the research will lead to a greater understanding of the nature of heteroplasmy. In addition, it is anticipated that the analysis of PCR products using the reverse dot-blot format will provide an additional, cost-effective means of examining mitochondrial DNA. The proposed study will examine the following eleven research questions (see proposal methodology attached for experimental procedures):

1. How much hair sample is required for successful polymerase chain reaction (PCR) amplification? What other variables influence the success rate?
2. What is the effect of heteroplasmy on the interpretation of forensic samples obtained from the hair shafts and blood of the same individual?
3. Is the mitochondrial DNA yield from the hair shaft affected by exposure to various chemical hair treatments?
4. What is the frequency of the heteroplasmy observed using the mtDNA linear array assay method?
5. Do different hairs from the same individual exhibit different haplotypes?
6. Does detection of the heteroplasmic condition in a given individual correlate with the microscopic morphology of distinctly different hair types in the scalp of individuals?
7. Is the heteroplasmic condition correlated with age?
8. Is the heteroplasmic condition correlated to race?
9. Are there differences in the level of detection of heteroplasmy when comparing living with deceased individuals?
10. Does the heteroplasmic condition represent a mosaicism or a random distribution across the scalp of an individual?
11. Is heteroplasmy correlated to the growth stage of the hair?

Subject Recruitment

I will be distributing announcements (see attached) to local Crime Laboratory Personnel (LAPD, LA Sheriff Lab, Orange County Sheriff Lab) requesting participation in the study and the need for hair and blood samples. Participants will be asked to collect 60 head hairs in order to ensure a representative sample. The hair specimens will be obtained in a non-disfiguring manner by both combing (telogen phase), and plucking (anagen phase). As an intra-individual control sample, each participant will contribute a blood sample obtained by finger puncture. No more than 0.2 milliliters of blood (~4 drops) will be requested from each participant. Each participant will be provided with a guideline of procedures to be followed in collecting and submitting their samples (see attached). Seven thousand nine hundred and twenty head hairs (132 individuals x 60 hairs each) and 132 blood samples will be collected in total.

In addition, I have been given permission from the Los Angeles County Coroner to obtain hair and blood samples from decedents. I will be collecting hairs from each decedent as outlined for living subjects. However, the blood samples will be obtained by pipeting a small quantity from the body cavity following incision. No assumptions will be made regarding any chemical treatments applied to these hairs and they will not be used for the chemical treatment related research questions.

Subject Characteristics

Participants will be asked to indicate demographic information relating to their sex, age, and ethnicity on an index card when submitting their samples. They will also be asked a question relating to any chemical treatments they use on their hair. This information is needed because of the question of whether there is a correlation between these demographic factors and a heteroplasmic state.

The information on chemical treatments, similarly, relates to the question of whether chemical treatments alter the amount of mitochondrial DNA in the hair shaft.

All of the participants in this research project will be at least eighteen years old (not pregnant) at the time that they submit their sample. Individuals who may be immunocompromised by donating a blood sample using the finger puncture technique, individuals who are taking immunosuppressant drugs, and individuals that have blood-clotting problems will all be asked not to participate in this research. The demographic information relating to decedents will be obtained from pathology reports.

Risks to Subjects/Risk Reduction

The risk to subjects is minimal and does not exceed the risk incurred in ordinary life or tasks. Due to the possible risk of infections from obtaining blood by finger puncture, individuals who may be immunocompromised or on immunosuppressant drugs will be discouraged from participating. Samples will not be requested from individuals who have blood-clotting problems, such as hemophiliacs. Each subject will be supplied with an unused comb in order to collect telogen (inactive) hairs from their scalp. The anagen (active) hairs will be plucked from the scalp individually, causing minimum pain to the subject. A blood sample will be obtained by finger puncture using a sterile technique and stored on bloodstain cards. These cards will be refrigerated (or frozen) and the refrigerators/freezer will be marked with a "Biohazardous Materials" label. All contaminated material will be disposed of in accordance with OSHA regulations for blood borne pathogens. All contaminated material will be treated with 10% bleach prior to disposal in red biohazard receptacles located in the laboratory. Disposable gloves, a laboratory coat and eye protection will be worn at all times when working in the laboratory. Work areas and equipment will be routinely disinfected with germicidal cleaner.

Benefits of the Research

This research will benefit the criminal justice system because it will result in a greater understanding of the nature of heteroplasmy and will lead to improved interpretations of the significance of evidence analyzed by mitochondrial DNA. For example, a greater understanding might lead to a reduction in the number of false exclusions that the technique might otherwise produce. There is no apparent benefit to the individual donor.

Anonymity

Participants will be asked to sign a consent form (see attached) and to mail the form to Dr. Deborah Baskin, Dept. of Criminal Justice, California State University at Los Angeles, 5151 State University Drive, Los Angeles, CA 90032

in the stamped addressed envelope supplied. The forms will be secured in a locked filing cabinet. Only Dr. Baskin will have access to this filing cabinet. Katherine Roberts will have information on certain characteristics of the donor but their identity will remain anonymous to her. Once the consent forms and samples are sent to Dr. Baskin and Katherine Roberts respectively, there will be no way of cross-referencing the forms to the original samples. The consent forms will be destroyed following completion of the study. This will ensure anonymity.

Participant Announcement

Volunteers Needed for Research Project

Kathy Roberts, a doctoral student at the Graduate School and University Center, City University of New York, is conducting a research project to further the understanding of heteroplasmy, a condition whereby both mutant and wild type mitochondrial genomes are found to exist within a particular individual.

The objective of the research is to gain a greater understanding of the nature of heteroplasmy in human head hair.

Participation in this study is voluntary. As a participant, you will be asked to submit a blood sample (finger puncture) and head hair (60) for mtDNA analysis.*

The results generated in this study are for scientific research purposes only. The information will not be entered into any DNA databases and will not be used in any law enforcement context.

You will be asked to complete a few short questions relating to demographics and chemical treatments used on your hair.

The identity of participants will remain anonymous.

Please call Katherine Roberts at 323-343-4625 to obtain further information.

- Please note that the following individuals will be excluded from participating in this research project: individuals who may be immunocompromised by donating blood by finger puncture, individuals who are on immunosupressant drugs, and individuals who have blood-clotting problems. All human subjects must be 18 years or older (and not pregnant).

Sample Donation Packet Form

Please find the following items enclosed in this packet:

One (1) participant consent form; one (1) unused comb for hair collection; one (1) diagram showing designation of different areas of scalp; three (3) sealed sterile lancets, do not use if the seal is broken; three (3) sealed alcohol wipes, do not use if the seal is broken; one (1) bloodstain card onto which you will directly transfer your blood; one (1) small envelope labeled "Bloodstain sample" into which you will insert the dried bloodstain card containing your donated sample. Seal the envelope. **DO NOT SIGN OR MARK THE SEAL IN ANY WAY.**

Fifteen (15) small envelopes each labeled with one of the following: "Head hair sample: front/plucked", "Head hair sample: front/combed", "Head hair sample: center/plucked", "Head hair sample: center/combed", "Head hair sample: rear/plucked", "Head hair sample: rear/combed", "Head hair sample: right-side/plucked", "Head hair sample: right-side/combed", "Head hair sample: left-side/plucked", "Head hair sample: left-side/combed", "Two adjacent head hairs: front/plucked", ", "Two adjacent head hairs: center/plucked", "Two adjacent head hairs: rear/plucked", ", "Two adjacent head hairs: right-side/plucked", "Two adjacent head hairs: left-side/plucked". Place the hair that you collect into the appropriately labeled envelope. Seal the envelope. **DO NOT SIGN OR MARK THE SEAL IN ANY WAY.** One (1) index card on which you are asked to answer three demographic questions, and a question relating to your use of chemical treatments on your hair:

SEX: Male ☐ Female ☐

RACE: Asian ☐ Caucasian ☐

African American ☐ Other

DO YOU CHEMICALLY TREAT YOUR HAIR? IF YES SPECIFY_____

DATE LAST TREATMENT (APPROXIMATELY) _____

AGE ON LAST BIRTHDAY _____ YEARS*

DO NOT INCLUDE ANY OTHER INFORMATION ON THIS CARD

One (1) stamped addressed envelope. Insert your consent form in this envelope. The envelope will be pre-addressed to "Dr. Deborah Baskin, Chair, Department of Criminal Justice, California State University, 5151 State University Drive, Los Angeles, CA 90032.

One (1) large stamped addressed envelope for your donated samples. Insert the sealed "Bloodstain sample" envelope and all fifteen sealed hair sample envelopes in this large envelope. Seal the large envelope. **DO NOT SIGN OR MARK THE SEAL IN ANY WAY.**

* Participants must be at least eighteen years old (and not pregnant) in order to participate in this study.

Participant Consent Form

Heteroplasmy in the Mitochondrial DNA Analysis of Human Head Hair

To Project Participant:

You are invited to take part in a research project conducted by Katherine Anne Roberts, a doctoral student in Criminal Justice (concentration in forensic science) at the Graduate School and University Center, City University of New York. All laboratory analyses will be performed in the Criminalistics Laboratory, King Hall 1049, California State University, Los Angeles. The study will be conducted to further the understanding of the phenomenon of heteroplasmy, a condition whereby both mutant and wild type mitochondrial genomes are found to exist within a particular individual. It is hoped that the research will lead to a greater understanding of the nature of heteroplasmy. In addition, it is anticipated that the analysis of PCR products using the reverse dot-blot format will provide an additional, cost-effective means of examining mitochondrial DNA.

The benefit of this research is to the criminal justice system because a greater understanding of the nature of heteroplasmy by forensic scientists will lead to improved interpretations of the significance of evidence analyzed by mitochondrial DNA. There is no apparent benefit of the research to an individual donor. As a participant, you will be asked to submit hair samples (one time only) and a blood sample (one time only). Please follow the guidelines indicated below:

Hair Sample Collection

You will be issued a clean, unused comb in order to obtain a representative hair sample, and will collect hairs from different areas of your head (see diagram attached for designation of these areas). Collect ten hairs from each of five areas on the scalp designated: front, center, rear, left, and right side. You will obtain half of the hairs (5) from each area by plucking and the other half (5) by combing the respective areas. In addition, you will pluck two hairs that lie adjacent to each other from each of the five regions. Place the hairs in the appropriate, pre-labeled envelopes supplied. The envelopes have been pre-labeled with a notation of the area of the scalp they were removed from (i.e. front, rear, etc.) and the means used to obtain the sample (i.e. plucked v. combed). You will be supplied with a total of fifteen small envelopes, which will be pre-labeled: with one of the following: "Head hair sample: front/plucked", "Head hair sample: front/combed", "Head hair sample: center/plucked", "Head hair sample: center/combed", "Head hair sample: rear/plucked", "Head hair sample: rear/combed", "Head hair sample: right-side/plucked", "Head hair sample: right-side/combed", "Head hair sample: left-side/plucked", "Head hair sample: left-side/combed", "Two adjacent head hairs: front/plucked", "Two adjacent head hairs: center/plucked", "Two adjacent head hairs: rear/plucked", "Two adjacent head hairs: right-side/plucked", "Two adjacent head hairs: left-side/plucked". Seal each envelope once you have

completed collection but do not initial the seal. Place all fifteen envelopes in the large manila envelope supplied.

Blood Sample Collection:

You will obtain a sample of your blood by finger puncture using a sterile technique. You will be supplied with a sealed, sterile lancet and a sealed, sterile alcohol pad. Wash your hands with germicidal soap and then wipe a selected finger with the alcohol pad supplied. Allow it to air dry prior to your puncturing it. You will transfer 0.2 milliliters of your blood (~ 4 drops) directly on to the bloodstain cards supplied. Allow the bloodstain card to air dry prior to folding it and insert it into the small manila envelope supplied. Seal the envelope, **but do not sign or mark the seal in any way.**

Completion of Index Card

An index card is included in the packet supplied. Please answer the three demographic questions relating to your sex, your age on your last birthday, and your race. A fourth question inquires whether you have chemically treated your hair. **Do not include any other information on this card.** The risk to subjects is minimal to non-existent. The removal of hair samples from the root may result in slight discomfort. Also, the use of finger puncture to obtain blood samples may result in slight discomfort, soreness and possible infection. For this reason, individuals who may be immunocompromised or on immunosupressant drugs are discouraged from participating in this research project. Also, individuals who have blood-clotting problems, such as hemophiliacs, should not participate. You must be at least eighteen years old (and not pregnant) in order to participate.

The risks involved are no greater than those incurred in ordinary life or tasks. I do not expect any of these adverse medical effects to occur. However, if you do experience any medical problems as a result of your participation, the staff at the Center will provide necessary medical treatment within the scope of the services authorized by the Trustees of the California State University for Health and Personal Services. **Please feel free to contact Katherine A. Roberts at 323-343-4625 or the CSULA Health Center at 323-343-3342 if you have additional health concerns.** Your agreement to participate in this research in no way limits your legal rights in the event of a research related injury.

All information gathered in this study will remain anonymous. No participant will be identified by name in writing or orally. The samples will be subjected to DNA analysis, but the results will not be entered into a genetic database. The results will be used for research purposes only and will not be used in any law enforcement context. Please mail your consent forms to Dr. Deborah Baskin, Chair, Dept. of Criminal Justice, California State University at Los Angeles, 5151 State University Drive, Los Angeles, CA 90032 in the stamped addressed envelope supplied. The forms will be locked in a filing cabinet in her office. No other person will have access to these consent forms. Katherine Roberts will have information on certain characteristics of the donor but

donor identity will remain anonymous to her. Your decision to take part or not take part is completely voluntary. If you choose not to take part there will be no penalty or loss of benefits to which you are entitled. You are free to withdraw or discontinue participating in this study at any time.

If you have any questions pertaining to goals, procedures and/or results of this study, please call Katherine Roberts (California State University, Los Angeles) at (323) 343-4625 or Professor Peter De Forest (John Jay College of Criminal Justice, CUNY) at (212) 237-8899.

By signing this consent form you indicate that you have read the form, understand the nature of your involvement and agree voluntarily to participate in the study.

I consent to participate in "Heteroplasmy in the Mitochondrial DNA Analysis of Human Hair," as outlined above. Please make a copy of this consent form for your own records.

Signature of participant	Date	Signature of Katherine A. Roberts	Date

THIS PROJECT HAS BEEN REVIEWED BY THE CALIFORNIA STATE UNIVERSITY, LOS ANGELES INSTITUTIONAL REVIEW BOARD FOR THE PROTECTION OF HUMAN SUBJECTS IN RESEARCH (Phone number: 323.343.5366).

APPENDIX B

Abbreviations

Abbreviations

ADP	– adenosine diphosphate
AFDIL	– Armed Foreces DNA Identification Laboratory
ATP	– adenosine triphosphate
C	– cytosine
dATP	– deoxyadenosine triphosphate
dCTP	– deoxycytosine triphosphate
dGTP	– deoxyguanosine triphosphate
dTTP	– deoxythymidine triphosphate
D-loop	– displacement loop
DNA	– deoxyribonucleic acid
DTT	– dithiothreitol (Cleland's reagent)
LHON	– Leber's hereditary optic neuropathy
MELAS	– lactic acidosis with stroke-like episodes
MERRF	– myoclonic epilepsy with ragged red fibers
mtDNA	– mitochondrial DNA
NADH	– nicotinamide adenine dinucleotide (reduced state)
np	– nucleotide base pair(s)
OSHA	– Occupational Safety and Health Administration
PCR	– polymerase chain reaction
RFLP	– restriction fragment length polymorphism
rRNA	– ribosomal ribonucleic acid
SSCP	– single-strand conformation polymorphism
SSO	– sequence specific oligonucleotides
Taq	– *Thermus aquaticus* (DNA polymerase)
tRNA	– transfer ribonucleic acid
SWGDAM	– Scientific Working Group on DNA Analysis Methods

APPENDIX C

Glossary of Technical Terms

Glossary of Technical Terms

Agarose – Support medium that can be used in electrophoresis.

Adenosine triphoshate – A chemical that hydrolyzes to generate energy in the body.

Allele specific oligonucleotide probes – DNA probes that recognize different forms of a gene.

Amplification – Process that increases DNA copy number, usually by PCR.

Anagen hairs – Actively growing hairs.

Amplitype HLA DQα - Commercial typing kit that analyzes the DQα locus.

Amplitype Polymarker – Commercial typing kit that analyzes six different loci.

Anderson sequence – Reference sequence used in DNA sequencing analysis.

Anneal – Pairing of complimentary strands of DNA.

Base pairs – Pairs of complimentary DNA bases (adenine-thymine or guanine-cytosine) that are linked by hydrogen bonds.

Blastocyst – Precursor to an embryotic cell.

Cellular respiration – Breakdown and release of energy from nutrients.

Chi-square test (χ^2) – Statistical test to compare observed data with the values expected.

Coding DNA – Segment of DNA that expresses a protein product or RNA.

Cytosine – One of four nitrogenous bases that constitute DNA.

Denaturation – Conversion of DNA from a double to a single-stranded molecule usually by heat or by high pH.

Deletion – Loss of one or more bases from a DNA sequence. It can result in a mutation.

Diploid – Genes/DNA from both parents are present.

DNA polymerase – An enzyme that catalyzes the linking of nucleotides forming complementary DNA strands.

DNA sequencing – Process of reading the sequence of bases in a DNA strand.

Dot-blot analysis – Direct analysis of DNA by binding single-stranded DNA to a membrane (usually made of bonded nylon) and adding a probe.

Duplex DNA – DNA consisting of complementary strands.

Electrophoresis – Technique that separates charged molecules of different sizes due to an applied electrical field.

Enzyme – Proteins that speed up a chemical reaction.

Eukaryotic – A multicellular organism that undergoes mitosis.

Extranuclear DNA – DNA located outside the nucleus, for example, in organelles such as mitochondria.

Glossary of Technical Terms (cont.)

Gene –	Portion of a chromosome that contains the blueprint for a specific protein or for regulation of a particular process.
Genome –	Entire genetic make-up of an individual contained in the chromosomes.
Genotype –	Combination of genes present in an individual.
Germ-line cell –	The sex cell (sperm or ovum).
Guanine –	One of four nitrogenous bases that constitute DNA.
Haploid –	Only one gene or one parental source of DNA is present (either from the ovum or sperm).
Heteroplasmy –	Mixture of wild/mutant genotypes expressed in an individual.
Histones –	DNA-binding proteins.
Homoplasmy –	Single genotype (wild or mutant) expressed in an individual.
Hybridization –	Process of complimentary base pairing between two single strands of DNA.
Hypervariable region –	A segment of DNA that is characterized by considerable variation at one or more sites within the segment.
Insertion –	Addition of one or more bases into a DNA sequence. It may result in a mutation.
Meiosis (meiotic)–	Process where sex cells (sperm/ovum) divides twice to form 4 nuclei (each having half the chromosome number).
Mitochondrial DNA –	DNA that is located within a mitochondrion.
Mitochondrion –	A DNA-containing organelle present in all eukaryotes.
Mitosis (mitotic) –	Process where somatic cells divide from 2 identical cells.
Mosaicism –	Situation in which an individual is composed of two or more genetically distinct DNA types.
Mutant-type –	Form of a gene that has undergone a mutation.
Mutation –	A change in the sequence of DNA.
Non-coding DNA –	Segment of DNA that may have regulatory functions but does not appear to express a protein product or RNA.
Nuclear DNA –	DNA that is located within the nucleus of a cell.
Nucleotide –	The unit of DNA. It consists of a sugar, phosphate and base.
Oocytes –	Precursor cell to the ovum.
Organelles –	A sub-unit of a cell that has a specific function.
Origin of replication –	Site in DNA where DNA synthesis is initiated.

Glossary of Technical Terms (cont.)

Oxidative phosphorylation – Conversion of ADP to ATP coupled with electron transfer to molecular oxygen.

Pathogenic – A disease causative agent.

Phenotype – Physical characteristics of an individual expressed by genes.

Phylogenetic analysis – Study of the evolutionary trees of taxonomic groups.

Point mutations – Changes in DNA sequence involving single base pairs only.

Polymerase chain reaction – Process by which DNA is amplified to yield a larger quantity.

Polymorphism – Alternative forms of a gene that exist in the population.

Polypeptide – Single long chain of amino acids (precursor to a protein).

Primer – A short oligonucleotide that provides the initiation point for DNA replication.

Probe – Single-stranded segment of DNA. It may be labeled.

Proteinase K – An enzyme used to digest proteins.

Recombination – Combinations of genes in offspring different from those in the parents due to independent assortment and crossing-over.

Replicative segregation – Segregation of allele during meiosis.

Restriction fragment length polymorphism –Variation in the length of DNA fragments due to digestion of the DNA by restriction enzymes.

Reverse dot-blot analysis – Direct analysis of DNA by binding a probe to a membrane (usually made of bonded nylon) and adding single-stranded DNA.

Ribonucleic Acid (RNA) – Single-stranded nucleic acid. There are three types: messenger RNA, transfer RNA and ribosomal RNA.

Root sheath – Follicular tissue attached to a hair root.

Sequence specific oligonucleotide probes – DNA probes that recognize different base sequences of a gene.

Somatic cells – All eukaryotic cells except the sex cells.

Symbiotic – Association of two organisms of different species for mutually beneficial reasons.

Taq DNA polymerase – A type of DNA polymerase that is capable of withstanding high temperatures. Used in the PCR reaction.

Telogen hairs – Inactive hairs (no longer growing).

Template – Single-stranded DNA blueprint for complementary DNA strand assembly.

Thermal Cycler – Instrumentation that rapidly and automatically changes the temperatures needed to accomplish the PCR reaction.

Glossary of Technical Terms (cont.)

Transcription –	Process of producing RNA from a DNA template.
Transfer ribonucleic acid –	Involved in the synthesis of proteins from DNA.
Wild-type –	Form of a gene considered the normal type found in nature.
Zygote –	Diploid cell resulting from the fusion of an ovum and sperm.

APPENDIX D

Protocols

MtDNA PCR Amplification Protocol

1. Determine the number of samples to be amplified, including positive and negative controls.

2. Place the required number of reaction tubes into a MicroAmp Base. Label each tube appropriately (recall color coding for PCR tubes is: red-front, orange-center, yellow-back, green-right, and blue-left). Aliquot 20 µl of PCR premix into each tube. Depending on the protocol, add 10-20 µl of the duplex primer set (or, if using singleplex primer set, either HVI or HVII) into each tube. **To prevent contamination, no more than one tube should be open at a time.**

3. Prepare each tube as follows:

MtDNA Hair Samples: Add 20µl of extracted hair sample into appropriately labeled/colored PCR tube.

MtDNA Blood Samples: Add 5-10µl of extracted bloodstain sample into appropriately labeled/white PCR tube. Add 10 -15µl of distilled/deionized water.

(PCR Blank) Negative Control: Add 20 µl of distilled water into PCR tube labeled "PCR –".

(Pre-PCR Blank) Extraction Blank: Add 20 µl of reagent extraction into tube labeled "Pre-PCR –".

4. As soon as all of the samples have been added, place the tubes in the 2400 GeneAmp PCR Thermal Cycler. The instrument has been pre-programmed with one of the following parameter cycles:

Perkin-Elmer GeneAmp® 2400 PCR Profile Times and Temperatures
Amplification Parameters

Step	Temperature	Interval	Cycle
Activation	92°C	12 minutes	1
Denaturation	92°C	30 seconds	34 – 38
Annealing	60°C	30 seconds	34 – 38
Extension	72°C	30 seconds	34 – 38
Final Extension	72°C	10 minutes	1
Hold	4°C	∞	

Perkin-Elmer GeneAmp® 2400 PCR Profile Times and Temperatures
Amplification Parameters (Optimized Protocol)

Step	Temperature	Interval	Cycle
Activation	94°C	14 minutes	1
Denaturation	92°C	15 seconds	34 – 38
Annealing	59°C	30 seconds	34 – 38
Extension	72°C	30 seconds	34 – 38
Final Extension	72°C	10 minutes	1
Hold	4°C	∞	

Chelex Extraction of MtDNA from Blood Protocol

The following procedure can be used for whole blood (fresh, refrigerated or frozen) and bloodstains:

1. Pipette 1mL of sterile distilled water into a 1.5mL pre-labeled microcentrifuge tube.

2. Add one of the following, and mix gently: a) 3μLwhole blood or b) portion of bloodstain about $3mm^2$.

3. Incubate at room temperature for 15-30 min. Mix occasionally by inversion or gentle vortexing.

4. Spin in a microcentrifuge for 2-3 min. at 10,000-15,000 x g.

5. Carefully remove supernatant (all but 20-30 μL) and discard. If the sample is a bloodstain, leave the fabric/staincard substrate in the tube with the pellet.

6. Add 5% Chelex to a final volume of 200 μL.

7. Incubate at 56°C for 15-30 secs.

8. Vortex at high speed for 5-10 secs.

9. Incubate in a boiling water bath for 8 mins.

10. Vortex at high speed for 5-10secs.

11. Spin in a microcentrifuge for 2-3 mins. at 10,000 -15,000 x g.

12. The sample is now ready for the PCR amplification process.

13. It is recommended that 5-10μL of the supernatant (diluted with 10-15μL distilled/deionized water) be added to the PCR mix.

14. Store the remainder of the sample at 2-8° C or frozen.

15. To re-use, repeat Steps 9-11.

Chelex Extraction of MtDNA from Hair Protocol

1. Handling hair with clean forceps, examine the hair under a dissecting microscope for the presence of root sheath/follicular tissue material. The hair may be placed on a clean piece of white or black paper. Note any possible presence of body fluids on the hair.

2. Wash the hair to reduce surface dirt and contaminants by immersing the hair in sterile, deionized water in a clean 50mL beaker.

3. Return the hair to the dissecting microscope. For anagen hairs, use a clean scalpel to cut above the proximal (root) end of the hair and retain for separate analysis. This step is not necessary for telogen hairs.

4. Cut a 2cm portion of the shaft from the proximal end of the anagen/telogen hair and prepare as a microscope slide using a temporary mount (Cargille RI oil, 1.515). Record the morphology of the hair as a photomicrograph at x100.

5. Wash the hair in ethanol to remove excess mounting oil before placing in a microcentrifuge tube containing 500μL 2% SDS and sonicating for 10 minutes.

6. Rinse the hair in sterile, distilled and deionized water and place in a micro-tissue grinder containing 150μL TE buffer. Homogenize the hair until it is no longer visible.

7. Transfer the hair homogenate to 50μL of 20% Chelex in a 1.5mL microcentrifuge tube. Add 10μL Proteinase K to the homogenate.

8. Incubate at 56°C (at least 6-8h) or overnight

9. Vortex at high speed for 5-10 secs.

10. Spin in a microcentrifuge for 10 secs. at 10,000 - 15,000 x g.

11. Incubate in a boiling water bath for 8 mins.

12. Vortex at high speed for 5-10 secs.

13. Spin in a microcentrifuge for 2-3 mins. at 10,000 - 15,000 x g.

14. The sample is now ready for the PCR amplification process. It is recommended that 20 μL of the supernatant be added to the PCR mix.

15. Store the remainder of the sample at either 2-8°C or frozen. To re-use, repeat Steps 8 -10.

APPENDIX E

Reagents

Preparation of Reagents for MtDNA Protocols

20% Chelex (200mL)
Date_____ Prepared by_____
40g 100-200 mesh Chelex Lot # _____ Amount _____
200mL sterile nanopure water Amount _____
Mix. Aliquot 50µl with P-1000 into sterile 2mL screw-cap tubes while stirring.
Store at room temperature.

2% SDS (500mL)
Date_____ Prepared by_____
10g SDS Lot # _____ Amount _____
400mL sterile nanopure water Amount _____
Mix until clear. Add sterile nanopure water to final volume of 500mL.
Aliqout 500µl into sterile 1.5mL microcentrifuge tubes.
Store at room temperature.

20% SDS (500mL)
Date_____ Prepared by_____
100g SDS Lot # _____ Amount _____
400mL sterile nanopure water Amount _____
Mix until clear. Add sterile nanopure water to final volume of 500mL.
Aliquot 500µl into sterile 1.5mL microcentrifuge tubes.
Store at room temperature.

MtDNA Hybridization Buffer: 5X SSPE, 0.5% SDS (1000mL)
Date_____ Prepared by_____
725mL nanopure water Amount _____
250mL 20 X SSPE Lot # _____ Amount _____
25mL 20% SDS Lot # _____ Amount _____
Mix in bottle. Store at 56°C when typing.
Solution is stable for at least six months after preparation.

MtDNA Wash Buffer: 2X SSPE, 0.5% SDS (1000mL)
Date_____ Prepared by_____
875 mL nanopure water Amount _____
100mL 20X SSPE Lot # _____ Amount _____
25mL 20% SDS Lot # _____ Amount _____
Mix in bottle. Store at 56°C when typing.
Solution is stable for at least six months after preparation.

Preparation of Reagents for MtDNA Protocols

Product Gel: 2% Agarose/1 X TBE/0.5μg/mL EtBr (100mL)
Date_____ Prepared by_____
90mL nanopure water Amount _____
10mL 10X TBE Lot # _____ Amount _____
2g Agarose Lot # _____ Amount _____
5μl 10mg/mL Ethidium Bromide Lot # _____ Amount _____
Mix in bottle. Heat in microwave until dissolved. Store at 56°C.

10X Tris/Borate/EDTA (TBE) Electrophoresis Buffer (1000mL)
Date_____ Prepared by_____
1g NaOH Lot # Amount _____
108g Tris base Lot # _____ Amount _____
55g Boric acid Lot # _____ Amount _____
7.4g EDTA Lot # _____ Amount _____
Add sterile nanopure water to final volume of 1000mL.
Mix in bottle. Store at room temperature.

1X TBE Electrophoresis Buffer, (1000mL)
Date_____ Prepared by_____
100mL 10X Tris/Borate/EDTA (TBE) Electrophoresis Buffer
Add sterile nanopure water to final volume of 1000mL.
Mix in bottle. Store at room temperature.

1X TAE Electrophoresis Buffer, (1000mL)
Date_____ Prepared by_____
40mL 25X TAE Electrophoresis Buffer
Add sterile nanopure water to final volume of 1000mL.
Mix in bottle. Store at room temperature.

0.5M Ethylenediaminetetraacetic Acid (EDTA, pH 8.0)
Date_____ Prepared by_____
18.6g EDTA Lot # _____Amount _____
2.2g NaOH Lot # _____ Amount _____
Add sterile nanopure water to final volume of 100mL.
Mix in bottle. Store at room temperature.

1M Tris-HCl (pH 8.0)
Date_____ Prepared by_____
12.1g Tris base Lot # Amount _____
~5mL Conc. HCl (until pH is 8.0) Lot # _____ Amount _____
Add sterile nanopure water to final volume of 100mL.
Mix in bottle. Store at room temperature.

Preparation of Reagents for MtDNA Protocols

Tris/EDTA (TE) Buffer
Date_____ Prepared by_____
1mL 1M Tris (pH 8.0) Lot#_____ Amount _____
200µl 0.5M EDTA Lot # _____ Amount _____
Add distilled/deionized water to final volume of 100mL.
Mix in bottle. Store at room temperature.

Citrate Buffer (0.1M Sodium Citrate, pH 5)
Date_____ Prepared by_____
18.4g trisodium citrate, dihydrate Lot#_____ Amount _____
800mL of distilled/deionized water.
Adjust pH to 5.0 by adding ~6g citric acid monohydrate Lot#_____
Add distilled/deionized water to final volume of 1000mL.
Mix in bottle. Store at room temperature. Solution is stable for at least six months after preparation.

Chromogen:TMB (30mL)
Bring the bottle of Chromogen: TMB to room temperature and tap it on the lab bench to settle powder at the bottom of the bottle.
Add 30mL of room temperature reagent grade 100% ethanol to the bottle. Recap the bottle and seal the stopper with parafilm. Secure the bottle to an orbital shaker and agitate for 2 hours or until dissolved.
Store at 2-8°C. Chromogen Solution is stable for six months after preparation.

Note: Chromogen: TMB is an irritant. Avoid skin contact and inhalation.

Commercial Reagents for MtDNA Protocols

Enzyme Conjugate: Horseradish Peroxidase-Streptavidin (HRP-SA)
Enzyme Conjugate supplied in 10mM (2-[(2-Amino-2-oxoethyl)-amino] ethanesulfonic acid), pH 6.5, 2M NaCl.
Perkin Elmer (Part No. N808-0091)
Store at 2°C to 8°C.

Apex™ Gel Loading Buffer (GLB)
6X Gel loading Dye. Type II (Modified)
Doc Fugal Scientific (Cat. No. 19-123)
Store at room temperature.

Apex™ DNA Quantladder (100-1,000bp)
100µl/mL in TE buffer (250µl)
Load 0.25µl/lane.
Doc Fugal Scientific (Cat. No. 19-119)
Long term storage at 4°C or -20°C.
Stable at room temperature for > 1 year.

Chromogen: TMB
60mg 3,3',5,5'-Tetramethylbenzidine (TMB)
Add 30mL 100% ethanol.
Perkin Elmer (Part No. N808-0092)
Store at 2°C to 8°C.

Ethanol, 200 proof (100%)
Ethyl alcohol C_2H_5OH (FW 46.07)
Sigma® Chemical Company (Cat. No. E-7023)
Store at 2°C to 8°C.

Alcohol - Ethanol (95%)
Ethyl alcohol C_2H_5OH (FW 46.07)
Sigma® Chemical Company (Cat. No. E-7148)
Store at room temperature.

Boric Acid
H_3BO_3 (FW 61.8)
J.T. Baker Chemical Company (Cat. No. 1-0084)
Store at room temperature.

Commercial Reagents for MtDNA Protocols

Tris Base
$C_4H_{11}NO_3$ (FW 121.1)
Sigma® Chemical Company (Cat. No. T-6791)
Store at room temperature.

Citric Acid, Monohydrate
$C_8H_8O_7 \bullet H_2O$ (FW 210.1)
Sigma® Chemical Company (Cat. No. C-1909)
Store at room temperature.

Hydrogen Peroxide, 3%
H_2O_2 (FW 34.01)
Sigma® Chemical Company (Cat. No. H-6520)
Store at 2°C to 8°C.

Citric Acid, Trisodium Salt, Dihydrate
$C_6H_5O_7Na_3 \bullet 2H_2O$ (FW 294.1)
Sigma® Chemical Company (Cat. No. C-3434)
Store at room temperature.

Sodium Dodecyl Sulfate (SDS)/Sodium Lauryl Sulfate (SLS)
$(CH_3(CH_2)_{10}CH_2OSO_3Na)$ (FW 288.4)
Fisher Scientific (Cat. No. S529-500)
Store at room temperature.

Apex™ Agarose Powder
General purpose biotechnology grade; low EEO
Doc Frugal Scientific (Cat. No. 20-101)
Store at room temperature.

Apex™ NuSieve Agarose Powder
NuSieve GTG Agarose powder
Doc Frugal Scientific (Cat. No. 20-106)
Store at room temperature.

Apex™ SeaKem Agarose Powder
General purpose biotechnology grade; low EEO
Doc Frugal Scientific (Cat. No. 20-101)
Store at room temperature.

Commercial Reagents for MtDNA Protocols

Ethidium Bromide, 10mg/mL
$C_{21}H_{20}N_3Br$ (FW 394.32)
GIBCO BRL/Life Technologies (Cat. No. 15582-018)
Store at room temperature.

TAE Electrophoresis Buffer, (1L/25X)
Doc Fugal Scientific (Cat. No. 20-195)
Store at room temperature.

Equipment/Supplies for MtDNA Protocols

Equipment/Supplies

DNA typing tray
Aspirator Apparatus
Balance
Electrophoresis power supply
Electrophoresis apparatus with gel combs
Assorted glassware
Microcentrifuge
Microplate
Microtubes
Microscope slides
Microscope coverslips
Cargille oil immersion liquid
Microwave
Pipettors (2-20µl, 10-100µl, 1-200µl, and 1-1000µl)
Pipet tips (gel-loading tips and regular tips)
Shaker
Sonicator
Stir plate
Thermometer – total immersion
Timer
UV transilluminator
Vortexer
Water bath with cover (rotating)
Water bath (stationary)
Weight
Hot plate
DNA Thermal Cycler 2400
Thermal Cycler accessories (PCR reaction tubes with cap, PCR base/tray set)
Disposable gloves
Lab bench surface protector
Pen (for labeling probe arrays)
Permanent ink marker
Forceps
Scissors
Protective eyewear
Protective masks
Biohazard bags
Refrigerator
Freezer
Laminar flow/biological safety cabinet

Reuse of AmpliType™ DNA Typing Trays

The AmpliType DNA Typing Trays may be reused up to a maximum of five times if the following procedure is used:

1. Add approximately 5 – 10mL of 95% ethanol or 70% isopropanol to each well of the used AmpliType DNA Typing Tray.
2. Cover the tray with the lid supplied and agitate on an orbital shaker for ~ 5 minutes to dissolve residual Chromagen: TMB.
3. Repeat this step with additional alcohol until no residual blue or yellow is visible. If the color cannot be removed after repeated attempts, discard.
4. Rinse the tray and lid with sterile water. Air dry before reuse.

Preparation of 2% Product Gel

The purpose of running a product gel is twofold: to assess whether amplification of the isolated mtDNA was successful, and to evaluate the quality and estimate the quantity of the amplified product.

1. Add 2g agarose (low EEO grade) to 100mL 1X TBE electrophoresis Gel Running Buffer in a 200mL Erlenmeyer flask. Stir to suspend the agarose.

2. Heat uncovered in a microwave at high setting until all agarose is dissolved (1-1.5 minutes).

3. Seal the ends of a gel-casting tray with tape.

4. Swirl the agarose solution and check the bottom of the flask to make sure that all of the agarose has dissolved. (Just prior to complete dissolution, agarose particles appear as translucent grains). Reheat as necessary.

5. Allow solution to cool at room temperature for a few minutes prior to adding 5µL of 10mg/mL ethidium bromide to the gel solution. Swirl to mix.

6. Pour the agarose solution into the casting tray. Use a pipet tip to remove air bubbles or solid debris while the gel is still a liquid. Insert the well-forming combs and allow the gel to solidify at room temperature (~20 minutes).

7. When the agarose has set, remove the tape seals from the tray. Place the tray on the platform of the gel tank so that the comb is at the negative (black) electrode.

8. Fill the electrophoresis tank with 1X TBE buffer to a level that just covers the entire surface of the gel.

9. Gently remove the comb and ensure that the sample wells left by the comb are completely submerged. Use a pipet tip to remove any air pockets in the wells.

10. Obtain a microwell plate. Label the wells with each hair identifier #. Using a pipettor, remove 5µL from each amplified sample reaction tube and transfer into the appropriately labeled well of the reaction plate. Use a fresh tip from each reaction sample. Add 1µL glycerol loading buffer (GLB) to each well of the reaction plate. Mix the contents of each reaction well.

11. Load 6µL of each reaction sample/loading buffer into a separate well in the gel. Use a fresh tip for each reaction. Place the tip through the surface

of the buffer and center it over the well before expelling the sample into the well.

12. Use 5µL DNA Quantladder with 1µL GLB (100-1,000bp known control).

13. Use 5µL extraction reagent blank with 1µL GLB (pre-PCR negative control).

14. Use 5µL amplification reagent blank with 1µL GLB (pre-PCR negative control).

15. Close the top of the electrophoresis tank and connect the electrical leads to the power supply unit (Beckman model R-120) anode to anode, cathode to cathode. Make sure both electrodes are connected to the same channel of the power supply unit.

16. Turn on power supply and run the gel at 100 volts/80mA for 30 minutes.

17. Remove the gel and view using UV transilluminator (Sigma model T 1202). Place gel-framing mask on transilluminator. Place photographic hood assembly on mask, lining up the margins.

18. Photograph the gel using Kodak 35 mm black and white print film, ASA 400, F/5.6, shutter speed 1/8". (Pentax Honeywell SP 500 SLR/Super-Takumar 50mm lens, and Marumi 49mm +3 macro lens with attached optical bandpass EtBr filter, 590df/wratten # 22 or 23A).

Preparation of 3%/1% Product Gel

The purpose of running a product gel is twofold: to assess whether amplification of the isolated mtDNA was successful, and if so, to evaluate the quality and estimate the quantity of the amplified product.

1. Add 3g NuSieve GTG agarose and 1g SeaKem agarose to 100mL 1X TAE Gel Running Buffer in a 200mL erlenmeyer flask. Stir to suspend agarose.

2. Heat uncovered in a microwave at high setting until all agarose is dissolved (3-4 minutes).

3. Seal the ends of a gel-casting tray with tape.

4. Swirl the agarose solution and check the bottom of the flask to make sure that all of the agarose has dissolved. (Just prior to complete dissolution, agarose particles appear as translucent grains). Reheat as necessary.

5. Allow solution to cool at room temperature for a few minutes prior to adding 5µL of 10mg/mL ethidium bromide to the gel solution. Swirl to mix.

6. Pour the agarose solution into the casting tray. Use a pipet tip to remove air bubbles or solid debris while the gel is still a liquid. Insert the well-forming combs and allow the gel to solidify at room temperature (~30 minutes).

7. When the agarose has set, remove the tape seals from the tray. Place the tray on the platform of the gel tank so that the comb is at the negative (black) electrode.

8. Fill the electrophoresis tank with 1X TAE buffer to a level that just covers the entire surface of the gel.

9. Gently remove the comb and ensure that the sample wells left by the comb are completely submerged. Use a pipet tip to remove any air pockets in the wells.

10. Obtain a microwell plate. Label the wells with each hair identifier #. Using a pipettor, remove 4µL from each amplified sample reaction tube and transfer into the appropriately labeled well of the reaction plate. Use a fresh tip from each reaction sample. Add 1µL glycerol loading buffer (GLB) to each well of the reaction plate. Mix the contents of each reaction well.

11. Load 5µL of each reaction sample/loading buffer into a separate well in the gel. Use a fresh tip for each reaction. Place the tip through the surface of the buffer and center it over the well before expelling the sample into the well.

12. Use 4µL DNA Quantladder with 1µL GLB (100-1,000bp known control).

13. Use 4µL extraction reagent blank with 1µL GLB (pre-PCR negative control).

14. Use 4µL amplification reagent blank with 1µL GLB (pre-PCR negative control).

15. Close the top of the electrophoresis tank and connect the electrical leads to the power supply unit (Beckman model R-120) anode to anode, cathode to cathode. Make sure both electrodes are connected to the same channel of the power supply unit.

16. Turn on power supply and run the gel at 140 volts for 60 minutes.

17. Remove the gel and view using UV transilluminator (Sigma model T 1202). Place gel-framing mask on transilluminator. Place photographic hood assembly on mask, lining up the margins.

18. Photograph the gel using Kodak 35 mm black and white print film, ASA 400, F/5.6, shutter speed 1/8". (Pentax Honeywell SP 500 SLR/Super-Takumar 50mm lens, and Marumi 49mm +3 macro lens with attached optical bandpass EtBr filter/wratten # 22 or 23A, 590df).

APPENDIX F

Sample Sheets

PHOTOGRAPHY LOG

Date:	10-Feb-00	Case Number: Diss. Research		Analyst: K.A.Roberts
Roll #	04	Film Type/ASA: Kodak EPY 64T 35mm slide film (135-36)		
EXP	Lens	Shutter Speed	Category	Description
1	X100	1/8"	V=6 (TI)	T1(3)1C ROOT
2	X100	1/8"	V=6 (TI)	T1(3)1C MID-SHAFT
3	X100	1/8"	V=6 (TI)	T1(3)1C SHAFT END
4	X100	1/8"	V=6 (TI)	T1(3)1R ROOT
5	X100	1/8"	V=6 (TI)	T1(3)1R MID-SHAFT
6	X100	1/8"	V=6 (TI)	T1(3)1R SHAFT END
7	X100	1/8"	V=6 (TI)	T1(3)1L ROOT
8	X100	1/8"	V=6 (TI)	T1(3)1L MID-SHAFT
9	X100	1/8"	V=6 (TI)	T1(3)1L SHAFT END
10	X100	1/8"	V=6 (TI)	T1(1)1F ROOT
11	X100	1/8"	V=6 (TI)	T1(1)1F MID-SHAFT
12	X100	1/8"	V=6 (TI)	T1(1)1F SHAFT END
13	X100	1/8"	V=6 (TI)	T1(1)1L ROOT
14	X100	1/8"	V=6 (TI)	T1(1)1L MID-SHAFT
15	X100	1/8"	V=6 (TI)	T1(1)1L SHAFT END
16	X100	1/8"	V=6 (TI)	T1(1)1B ROOT
17	X100	1/8"	V=6 (TI)	T1(1)1B MID-SHAFT
18	X100	1/8"	V=6 (TI)	T1(1)1B SHAFT END
19	X100	1/8"	V=6 (TI)	T1(2)1F ROOT
20	X100	1/8"	V=6 (TI)	T1(2)1F MID-SHAFT
21	X100	1/8"	V=6 (TI)	T1(2)1F SHAFT END
22	X100	1/8"	V=6 (TI)	T1(2)1C ROOT
23	X100	1/8"	V=6 (TI)	T1(2)1C MID-SHAFT
24	X100	1/8"	V=6 (TI)	T1(2)1C SHAFT END
25	X100	1/8"	V=6 (TI)	T1(2)1R ROOT
26	X100	1/8"	V=6 (TI)	T1(2)1R MID-SHAFT
27	X100	1/8"	V=6 (TI)	T1(2)1R SHAFT END
28	X100	1/8"	V=6 (TI)	T1(2)1B ROOT
29	X100	1/8"	V=6 (TI)	T1(2)1B MID-SHAFT
30	X100	1/8"	V=6 (TI)	T1(2)1B SHAFT END
31	X100	1/8"	V=6 (TI)	T2(1)1C ROOT
32	X100	1/8"	V=6 (TI)	T2(1)1C MID-SHAFT
33	X100	1/8"	V=6 (TI)	T2(1)1C SHAFT END
34	X100	1/8"	V=6 (TI)	T2(1)1R ROOT
35	X100	1/8"	V=6 (TI)	T2(1)1R MID-SHAFT
36	X100	1/8"	V=6 (TI)	T2(1)1R SHAFT END
37	NOT USED			
38	NOT USED			

HVI/HVII mtDNA Linear Array Assay Typing Sheet I

Scientist_____ Case_____
Date_____ Samples_____

1. Label the arrays and place in wells of typing tray.
2. Denature 20µL PCR product in a microwell plate with 20µL Denaturation solution.
3. Add 3mL Hybridization Solution (5 X SSPE, 0.5% SDS) to each well.
4. Add 40µL of the denatured PCR product to corresponding well.
5. Incubate at 55°C for 15 minutes.
6. Prepare SA-HRP Conjugate Solution about 5 minutes before use:

    a. 3.3mL Hybridization Solution per array____arrays x 3.3 mL = _____
    b. 8µL of SA-HRP per array _____ arrays x 8 µL = _____

7. Aspirate and rinse the arrays with 3mL Wash Solution (2 X SSPE, 0.5% SDS)
8. Aspirate the wash and add 3mL of Conjugate Solution to each well.
9. Incubate at 55°C for 5 minutes.
10. Aspirate and rinse arrays with 3mL of Wash Solution. Aspirate.
11. Add 3mL of Wash Solution to each well.
12. Incubate at 55°C for 12 minutes.
13. Aspirate and rinse with 3mL of Wash Solution. Aspirate.
14. Add 3mL of 0.1M Citrate Buffer (pH 5) into each well.
15. Shake on an orbital shaker at 50 rpm for 5 minutes at room temperature.
16. Prepare Color Development Solution:

    a. 3mL Citrate Buffer per array-- _____arrays x 3mL = _____
    b. 4µL 3% H_2O_2 per array--_____arrays x 4µL = _____
    c. 150µL Chromogen: TMB per array ___arrays x 150µL = _____

17. Aspirate solution. Add 3mL of Color Development Solution to each well.
18. Shake for 20–30 minutes on orbital shaker at room temperature.

    Development Time _____

19. Wash arrays in 3mL DI water for 5-10 minutes on orbital shaker.
20. Pour off DI water and repeat 3 times.
21. Add fresh DI water to wells.
22. Photograph wet arrays on mtDNA interpretation sheet.

Lot Numbers:
20 X SSPE _____
20% SDS _____
SA-HRP Conjugate _____
0.1M Citrate Buffer _____
3% H_2O_2 _____
Chromagen: TMB _____

HVI/HVII mtDNA Linear Array Assay Typing Sheet II

Scientist_____ Case_____
Date_____ Samples_____

1. Label the linear arrays and place in wells of typing tray.
2. Denature 15μL PCR product in a microwell plate with 15μL Denaturation solution.
3. Add 3mL Wash Buffer (2 X SSPE, 0.5% SDS) to each well.
4. Add 30μL of the denatured PCR product to corresponding well.
5. Incubate at 55°C for 15 minutes.
6. Prepare SA-HRP Conjugate solution about 5 minutes before use:
    a. 3.3mL Wash Buffer per linear array____arrays x 3.3 mL = _____
    b. 8μL of SA-HRP per array _____ arrays x 8 μL = _____
7. Aspirate and rinse linear arrays with 3mL Wash Buffer for approximately 5-10 secs. in a rocking motion which allows the arrays to slide back and forth lengthways.
8. Aspirate Wash Buffer and add 3mL Conjugate solution to each well.
9. Incubate at 55°C for 5 minutes.
10. Aspirate and rinse linear arrays with 3mL of Wash Buffer for approximately 5-10 secs. in a rocking motion which allows the arrays to slide back and forth lengthways. Aspirate.
11. Add 3mL of Wash Buffer to each well.
12. Incubate at 55°C for 12 minutes.
13. Aspirate solution and rinse with 3mL of Wash Buffer for approximately 5-10 secs. in a rocking motion which allows the arrays to slide back and forth lengthways. Aspirate.
14. Add 3mL 0.1M Citrate Buffer (pH 5) to each well.
15. Shake on orbital shaker at 100 rpm for 5 minutes at room temperature.
16. Prepare Color Development Solution:
    a. 3.3mL Citrate Buffer per array-- _____arrays x 3.3mL = _____
    b. 4μL 3% H_2O_2 per array--_____arrays x 4μL = _____
    c. 150μL Chromogen: TMB per array_____arrays x 150μL = _____
17. Aspirate Citrate Buffer. Add 3mL Color Development Solution to each well.
18. Shake for 15 minutes on orbital shaker at room temperature.
19. Aspirate or pour off Color Development Solution. Add 3mL DI water to each linear array and rock back and forth for 20-30 seconds
20. Pour off DI water and repeat 2 times.
21. Add fresh DI water to wells.
22. Interpret wet arrays on mtDNA linear Array Template
23. Photograph wet arrays on mtDNA linear Array Template

Lot Numbers:
20 X SSPE _____
20% SDS _____
SA-HRP Conjugate _____
0.1M Citrate Buffer _____
3% H_2O_2 _____
Chromagen:TMB _____

HVI/HVII mtDNA Linear Array Interpretation Sheet

Scientist _____

Date _____

Case _____

		HVI					HVII					
	Sample	A	C	D	E	16093	A	B	C	D	189	Comments
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
16												
17												
18												
19												
20												
21												
22												
23												
24												

PCR Quantitation Worksheet

Lane #	Sample ID	Est. (ng) Product Yield	Vol. Product (µL)	Concn. of Product (ng/µL)	Concn. of Product (ng/µL)	Target Concn. (ng/µL)	Dilution Factor

Notes:

To estimate product concentration and calculate the dilution factor:

1. Estimate product yield by comparing signal intensity of the product bands to the bands of the Mass Ladders.
2. Record the gel lane number, sample name, estimated product yield (ng), and the volume of product loaded on the gel (µL) in columns A,B,C,D respectively.
3. Calculate the concentration of the product and record in column E
4. If the concentration is within the target range ~3-6ng/µL, no further manipulation of the product is required.
5. If the concentration is less than ~3ng/µL, amplify for additional cycles or repeat the amplification increasing the amount of DNA.
6. If the concentration is greater than ~6ng/µL, determine the dilution factor to be used.

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