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Sequence Detection and Comparative Analysis of the Hv1 and Hv2 Control Regions of Human Mitochondrial DNA by Denaturing High-Performance Liquid Chromatography

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SEQUENCE DETECTION AND COMPARATIVE ANALYSIS OF THE HV1 AND HV2 CONTROL REGIONS OF HUMAN MITOCHONDRIAL DNA BY DENATURED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A Thesis

Presented to

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of the Requirements for the Degree

Masters of Science

by

Sarah E. Lewis

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Advisor: Phillip B. Danielson
Abstract

The objective of this research was the evaluation and forensic validation of Denaturing High-Performance Liquid Chromatography (DHPLC) as a sequencing-independent means of detecting the presence of sequence differences in pair-wise mixtures of non-concordant amplicons of human mitochondrial DNA (mtDNA). The reproducibility and efficacy of DHPLC results, including amplification reproducibility, injection reproducibility, and column-to-column reproducibility were measured, showing negligible assay-to-assay variability. In addition, cross-contamination on the DHPLC columns demonstrated very low level DNA carryover between a high-abundance sample and subsequent zero-volume injections.

The accuracy with which DHPLC technology can be used to screen both evidence and control samples in the context of a forensic laboratory was evaluated. This was demonstrated by a number of pair-wise comparisons of each of the forensically relevant amplicons from 95 unrelated individuals in the study, and was in 100% agreement with sequencing data. Thus, DHPLC can be used to detect a diversity of sequence differences (transitions, transversions, insertions and deletions) in the mtDNA D-loop. Accordingly, DHPLC may have utility as a presumptive indicator of mtDNA sequence concordance samples.
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Chapter One: Introduction

Mitochondrial DNA: A Robust Genetic Marker

Mitochondria are often referred to as the molecular powerhouse of the cell, as they are responsible for the majority of adenosine triphosphate (ATP) synthesis in the body. In addition to supplying cellular energy, mitochondria are involved in a multitude of other processes, including cellular respiration, steroid synthesis, elongation of fatty acids, apoptosis, and heat production (McBride et al., 2006).

Mitochondria, present in almost all eukaryotic cells, were first discovered as distinct cytoplasmic organelles in 1840, and in 1963 it was determined that they in fact carry their own DNA (Robin, and Wong, 1988; Mounolou and Lacroute, 2005). However, not all eukaryotic cells contain mitochondria, including such examples as *Giardia intestinalis*, *Trachipleistophora hominis* and *Entamoeba histolytica*. Although these amitochondrial organisms lack actual mitochondria, they do appear to have remnants of mitochondria and mtDNA genes in their nuclear genome (Toval et al., 2003).

The mitochondrial DNA (mtDNA) genome is a closed, circular, double-stranded molecule consisting of typically 16,569 base pairs. A reference mtDNA genome was completely sequenced by a team of scientists at the Cambridge Research Institute in 1981 (Anderson et al., 1981). The 1981 sequence was derived primarily from a placenta of a
European individual; however, some HeLa and bovine sequence was used to fill in gaps due to early sequencing procedural problems. The original placental material was reanalyzed by another group in 1999 (Andrews et al. 1999), which found 11 nucleotides that differed from the Anderson et al. (1981) sequence. This sequence was therefore termed the revised Cambridge Reference Sequence (rCRS) and is the current standard for comparison of mtDNA sequence variations. The sequence within the genome can be broadly divided into either the coding region (about 15.5 kb, or 93% of the genome) and the non-coding control region (about 1.1 kb, or 7% of the genome).

The two strands of mtDNA differ significantly in their base composition. The heavy strand (H-strand) is purine rich, having a greater number of guanine nucleotides, whereas the light strand (L-strand) is pyrimidine rich and thus physically lighter. The sequence of the mtDNA genome codes for a total of 37 genes, all of which are essential for normal mitochondrial function. A total of 28 gene products are found on the H-strand and 9 on the L-strand. Of the 37 genes, 13 are for proteins which are necessary for cellular respiration, including the NADH dehydrogenase 6 enzyme, 22 are for mitochondrial transfer RNAs (tRNA) and the remaining two encode the 16S and 23S subunits of ribosomal RNA (rRNA) (McBride et al., 2006) (Figure 1).

In contrast to a single copy of the diploid nuclear DNA genome, the majority of human cells contain from 200 to over 1,000 copies of the mtDNA genome depending on its host tissue’s specific energy demands (Robin and Wong, 1988) (Frahm et al., 2005). This abundance of mtDNA makes it a particularly attractive target for forensic analysis because it can typically be amplified from a number of different tissues where nuclear DNA may be limiting or absent (e.g., hair shafts and aged or environmentally
compromised tissues such as skeletal remains). For example, mtDNA has been used for the identification of skeletal remains from the Vietnam War (Holland et al., 1993), identification of bone material from a grave in Yekaterinburg, Russia belonging to Tsar Nicholas II (Ivanov et al., 1996), human remains identification following the September 11, 2001 World Trade Center attack (Budimlija et al., 2003), the identification of shed hairs from a hat left behind at a robbery (Allen et al., 1998), and even in cases of sexual assault (Hatsch et al., 2007).
Figure 1: The Human Mitochondrial DNA Genome

The genes encoded by the mitochondrial DNA (mtDNA) genome are noted. Point mutations associated with mitochondrial diseases are documented in the center of the genome. Diagram provided by MitoMap (http://www.mitomap.org/).
Organization of the Mitochondrial DNA Genome

The mitochondrial genome is much more efficiently organized than the nuclear DNA genome, containing very little non-coding sequence (7%) compared to the nuclear genome where approximately 97% is not expressed. The 37 genes encoded by the mitochondrial genome are nearly contiguous with each other, lacking introns and only occasionally having one or two base non-coding sequences separating them. Because of this protein coding sequence, there are relatively few sites at which variations in sequence can be tolerated. Therefore, mtDNA is considerably less polymorphic than its nuclear counterpart, despite its higher rate of mutation. In fact, the only significant region of the genome that does not code for a gene product is the displacement loop region, also known as the D-loop. This 1.1 kb stretch of often triplex DNA, known as the “control region”, contains essential regulatory functions, including the origin of replication for the generation of multigenic transcripts and DNA replication of the heavy strand. Despite the limited amount of sites for tolerated sequence variations, the mitochondrial genome has been shown to have a mutation rate within the control region up to ten times that of comparable nuclear DNA sequences (Brown et al., 1979). Forensic analysts are particularly interested in specific portions of this control region, designated hypervariable regions 1 (HV1) and 2 (HV2), because of their non-regulatory and non-protein-coding status and the dense array of sequence variability they exhibit within human populations. Some forensic laboratories also make use of the hypervariable 3 (HV3) region on occasions where increased discriminatory power is necessary.

HV1 and HV2 regions span roughly positions 16024-16383 and 57-372, respectively, whereas the HV3 region spans positions 438-574 (numbered corresponding
to the rCRS (GenBank accession number NC_012920); (Andrews et al., 1999). Not only can there be single nucleotide mutations between individuals, but there are also two separate homopolymeric stretches of C nucleotides (poly-C repeats, or C-stretches) in both the HV1 and HV2 regions. MITOMAP, an internet mitochondrial sequence database, maintains a list of published mtDNA polymorphisms found within the D-loop and forensically informative HV1 and HV2 regions (http://mitomap.org/bin/view.pl/MITOMAP/PolymorphismsControl). As of April 2011, this list currently includes 1,013 sites at which polymorphisms have been detected between the nucleotide positions 16024-576 of the D-loop control region, most of which are associated with the HV1 and HV2 regions. The majority of these polymorphisms consist of single base substitutions, although some insertions and deletions of sequence have also been also observed. Although many of these polymorphisms are extremely rare, or are only seen in specific ethnic populations, pairwise comparisons of mtDNA sequences of both hypervariable regions between maternally unrelated individuals usually reveal multiple sequence differences. Based on a study of 1,393 unrelated individuals comprising eight population groups, most mtDNA sequence polymorphisms were observed only once within a particular population group. The highest amount of mtDNA sequence diversity was seen in African-American populations, where an average of 14.1 nucleotide differences was observed between individuals. The lowest amount of diversity was seen within the Caucasian population, where only 8.4 nucleotide differences were observed (Budowle, et al., 1999). In addition, there are a small number of common, shared HV1/HV2 haplotypes in the Caucasian database. The most common HV1/HV2 haplotype occurs at a frequency of about 7% in the population (Lutz-Bonengel et al., 2003).
Mitochondrial haplotypes can also be grouped into clusters based on evolutionary relatedness, which are defined by the presence of specific sequence polymorphisms in the mtDNA genome. These clusters, known as haplogroups, are based on descent, described by a capital letter designation, and are the result of migrations of ancient human populations out of Africa thousands of years ago (Figure 2). Continent-specific markers have been identified for Africans (Chen et al., 1995), Asians (Schurr et al., 1990), and European Caucasians (Torroni et al., 1994). African mtDNAs can be classified into one of three main haplogroups (L1, L2 or L3). Haplogroup L1 is considered to be the earliest since it is found at the root of the human mtDNA lineage tree. The Eurasian haplogroups M and N are believed to have left Africa approximately 40,000 – 60,000 years ago to give rise to the current haplogroups in Asia (A, B, C, D, F, and G) and Europe (H, I, J, K, T, U, V, W, and X).
Figure 2: Human mtDNA Migration Patterns and Haplogroups

Letters designate the names of specific haplogroups, based on published mtDNA sequence data. Arrows indicate the pattern of migration originating out of Africa. Diagram provided by MitoMap (http://www.mitomap.org/).
Mitochondrial DNA as a Forensic Tool

Mitochondrial DNA has a number of characteristics which makes it an ideal choice for forensic use. First, it has been estimated that the mtDNA genome evolves at a rate that is up to ten times that of its chromosomal counterpart (Brown et al., 1979). This is an important factor when considering that data consistently show that unrelated individuals are extremely likely to have different mtDNA haplotypes thus making mtDNA useful for purposes of human identity testing. This higher mutation rate can be accounted for by such factors as DNA repair inefficiencies, oxidative damage, and the greater number of replicate cycles that mtDNA undergoes during cell growth (Mambo et al., 2003). Evidence also suggests that in spite of such an elevated mutation rate, the majority of mtDNA molecules within a given individual will still be represented by a single sequence (homoplasmy). Occasionally, however, a de novo mutation may occur and propagate, resulting in the phenomenon known as heteroplasmy. Heteroplasmy is a state in which two distinct mtDNA haplotypes coexist within a single individual. This is thought to be due to a mtDNA genome copy “bottleneck” during the early stages of oocyte development (Marchington et al., 1998). The bottleneck theory purposes that the number of copies of mtDNA in each early oocyte is reduced to a small number of copies as compared to the mature oocyte. Thus, a small number of molecules are chosen as the founder population for all of the mtDNA molecules that are transmitted to the next generation. This set of molecules could contain a homogenous population of mtDNA, or perhaps a heterogeneous mixture due to mutations. Sometimes, such heteroplasmy may increase the discriminatory power of mtDNA identification by providing an additional inclusionary tool for the mitotype, such as situations where an evidentiary sample and a
reference sample both exhibit heteroplasmacy at the same nucleotide. Other times, it can lead to confusion when comparing two sequences that are assumed to be concordant, as it may be considered a mixture of mitotypes from more than one individual.

Second, human mtDNA is thought to be almost completely maternally inherited (Giles et al., 1980). This can be explained by the nearly 100,000 copies of the mitochondrial genome residing in the oocyte, and the fact that the few (possibly only two or three) mitochondria present in the spermatozoa are concentrated in the mid-piece and tail region, which are lost following fertilization. Additionally, if the sperm mitochondria do make it to the oocyte, they appear to be preferentially degraded. Despite this maternal preference, some research has reported a few incidences known as “paternal leakage,” where some paternal inheritance of mtDNA and recombination has occurred. A single case of paternal co-inheritance of mtDNA in humans has been reported so far, in a male individual with a mitochondrial myopathy (Schwartz and Vissing, 2002; Bandelt et al., 2005). In addition, such paternal inheritance of mtDNA has been reported in species ranging from mussels to sheep (Stewart et al., 1995; Zhao et al., 2004). Although paternal leakage may occur in rare instances, the normal detectable inheritance pattern of mtDNA is maternal. This maternal inheritance pattern, barring multiple mutations, allows for forensic identifications to be made using reference samples from within the entire maternal lineage, including those that may be separated by several generations, when those of close relatives are no longer obtainable.

Third, mtDNA is present in a high copy number within most cells. It is estimated that a single cell may contain hundreds of mtDNA genomes for every copy of nuclear DNA (Robin and Wong, 1988). Depending on the needs of the particular cell type, the
actual copy number present per cell can vary greatly among different tissue types. For instance, there are more mitochondria in muscle and brain cells than in skin cells (Veltri et al., 1990). The general abundance of mtDNA can prove vital in situations where the amount of sample may be limited or its quality may be degraded, which is often the case in forensic DNA analyses. Samples that are typical candidates for mtDNA analysis include aged bloodstains, skeletal remains, fingernails, teeth, and hair shafts lacking root tissue. The use of mtDNA typing of skeletal remains is often essential in cases of missing persons or in events such as mass disasters where small bone fragments may be the only remaining source of DNA available. In addition, mtDNA testing of hair shafts is of particular importance because shed hairs are common sources of evidentiary material at crime scenes.

**Current Approaches to Forensic mtDNA Analysis**

In a forensic setting, human mtDNA is analyzed by direct comparison of DNA sequence data of the HV1 and HV2 regions to the rCRS (Andrews et al., 1999). Standardizing alignments of sequences with the rCRS and following consistent nomenclature for sequence differences is critical to avoid unintentionally describing two sequences as different when in they are actually the same. In fact, several publications have dealt with the nomenclature of sequence data by establishing specific “rules” to follow when determining an mtDNA haplotype (Carracedo et al., 2000; Tully et al., 2001; Wilson et al., 2002). Briefly, differences are reported using the nucleotide positions and the particular base mutation. For example, a sequence that is identical to the rCRS except for having a T instead of a C at position 16150 is designated as 16150T.
In the situation of length polymorphisms in the poly-C stretches, any extra Cs are added onto the end of the poly-C stretch. The variant is named using a decimal notation to indicate the number of nucleotides that were in addition to the poly-C repeat in the rCRS. For example, if a particular mtDNA sequence has an additional C compared to the rCRS following the C-stretch of positions 303-315, it would be designated as 315.1C. A similar nomenclature is used to describe insertions or deletions of nucleotides as compared to the rCRS. For instance, if an additional T was inserted following position 294, it would be designated as 294.1T. Finally, deletions are the result of nucleotides that are missing as compared to the rCRS; an mtDNA sequence that was missing nucleotide 325 would be named 325D.

The general rules for naming profiles are as follows (Wilson et al., 2002):

- Profiles should be characterized so that the least number of differences from the reference sequence are present.

- If there is more than one way to maintain the same number of differences with respect to the reference sequence, differences should be prioritized as follows:
  1. insertions/deletions (indels)
  2. transitions (purine-to-purine or pyrimidine-to-pyrimidine changes)
  3. transversions (purine-to-pyrimidine or pyrimidine-to-purine changes)

- Because all genes have a 5’ to 3’ direction of transcription and mtDNA genes are encoded on both the heavy and light strands of the closed circular molecule, insertions and deletions should be placed 3’ with respect to the light strand of human mtDNA.

- Insertions and deletions should be combined in situations where the same number of differences from the reference sequence is maintained.
In order to determine a person’s mtDNA haplotype, total genomic DNA is extracted from the biological source material. The extracted DNA is then subjected to amplification of the HV1/HV2 regions (total of 608bp) using four primer pairs (Table 1). For the HV1 region, two primer pairs, L15997/H16236 and L16159/H16391, are used to amplify overlapping 278 and 271 base pair fragments designated HV1A and HV1B, respectively. The HV2 region is amplified by primer pairs L048/H285 and L172/H408 which typically yields overlapping products of 278 and 277 base pairs designated HV2A and HV2B, respectively (Figure 3). The “L” and “H” designation refers to the light and heavy strand of the mtDNA genome from which the primer sequence is derived and the number indicates the corresponding position of the 3’ end of the primer with respect to the rCRS (Anderson et al., 1981; Budowle et al., 2000).
Table 1: Human mtDNA Primer Pairs

The forensically-validated primers used for control region amplification of human mtDNA.
Figure 3: HV1 and HV2 Primer Overlap Scheme

The D-loop region of the mitochondrial genome is divided into two main fragments (HV1 and HV2). For universal forensic amplification and sequencing, each fragment is divided into two smaller overlapping fragments (HV1A, HV1B, HV2A and HV2B). See Table 1 for primer sequence information.
Once the overlapping products are amplified, they are sequenced using the dideoxy chain termination method, i.e., the Sanger method (Sanger et al., 1977). The Sanger method allows for differential fluorescent labeling of chain terminator ddNTPs. This allows single reaction sequencing where each label emits fluorescence at a different wavelength. In this method, DNA templates are denatured and new strands of DNA are synthesized by Taq polymerase. The incorporation of dideoxyribonucleotides creates populations of strands that are terminated with a fluorescent tag at all possible base positions along the template strand. This makes it possible to unambiguously identify the final base of each amplified mtDNA fragment. The resulting sequence product (i.e. pool of mtDNA fragments) is then fractionated by capillary electrophoresis (CE) using such commercial systems as the ABI Prism® 310, 3100, or 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). In CE, the terminated DNA chains are subjected to an electric field that separates the amplified fragments based on their size. The amplified products must be separated in order to determine the specific order of incorporated nucleotides across a target sequence. A laser excites the fluorescent dye terminators as they pass a fixed transparent window in the capillary. Light emitted by the excited fluorophores is then detected by a CCD camera. The different bases are ultimately represented as colored peaks on an electropherogram. Next, the data from each individual sequence reaction are parsed to data analysis software, such as Sequencher® (Gene Codes Corp, Ann Arbor, MI) for sequence alignment and examination by an mtDNA analyst.
Denaturing High Performance Liquid Chromatography

Analysis of mtDNA in a forensic casework context is labor intensive, expensive, and requires the expertise of skilled analysts currently employed by only a small number of public and private laboratories. Efforts are ongoing to bring about a more streamlined, efficient, and more cost effective flow of the mtDNA analysis process. A novel scheme to help accomplish these goals includes the development of a rapid and reliable screening technique for determining concordance/non-concordance between mtDNA samples prior to the laborious process of sequence analysis.

Several different techniques to increase efficiency have been explored in recent years. These include hybridization to linear arrays of sequence-specific oligonucleotides (SSO) (Reynolds et al., 2000); denaturing gradient gel electrophoresis (DGGE); (Hanekamp et al., 1996); (Steighner et al., 1999); single-strand conformational polymorphism (SSCP) analysis (Alonso et al., 1996); (Barros et al., 1997); time-of-flight mass spectrometry (Butler and Becker, 2001); and microarray-based analysis (Fukushima, 1999). While somewhat economical, these methods all have significant limitations, most importantly the fact that they often consume limited forensic evidence while not necessarily providing a more thorough, efficient evaluation of sequence differences across a pool of amplicons.

Denaturing High-Performance Liquid Chromatography (DHPLC), on the other hand, helps resolve these issues by making it possible to accurately determine whether two mtDNA sequences are concordant or non-concordant. This can be done in a rapid, accurate, cost-effective, and automated approach. Used for many years in the medical research and diagnostics fields, DHPLC has been employed to screen for a wide variety...
of genetic mutations. Based on the high-resolution chromatographic separation of DNA molecules that differ in sequence, even when they are identical in length, DHPLC has become the mainstay for rapidly determining whether the sequence of a test sample is identical to or different from a known reference sample (Rossetti et al., 2002); (Troudi et al., 2007).

The fundamental principle behind DHPLC is Temperature Modulated Heteroduplex Analysis where an unknown DNA sequence to be analyzed is mixed with a known (reference) DNA sample. The mixture is then heat denatured. Upon slow renaturation, a combination of homoduplexes and heteroduplexes are produced. The homoduplexes represent the original components of the mixture while the heteroduplexes are formed as a result of the cross-hybridization between the different mixture components (Hou and Zhang, 2000). DHPLC allows for the chromatographic separation of the homoduplexes and heteroduplexes as a function of their interaction with a proprietary DNASep® column. In this process, triethyl ammonium acetate acts as an ion pairing reagent between the negatively charged DNA backbone and the alkylated poly(styrene-divinylbenzene) particles that comprise the resin of the DNASep® column. Using partially denaturing temperature conditions and an increasing acetonitrile gradient, it is expected that DNA homoduplexes and heteroduplexes will be individually eluted from the column. Subtle differences between sequences, therefore, should be readily indicated by the appearance of distinct peaks on a chromatogram. Specifically, the earliest eluting peak (i.e., the peak with the shortest retention time) should represent the heteroduplex with the most destabilizing nucleotide mismatch. The latest eluting peak, by contrast, should have the most stabilizing basepair at the same position (Figure 4). Only
when the unknown sample and the known reference sample are an exact match should a single homoduplex peak appear. The resolution and specific retention times of the individual homo- and heteroduplex peaks is dependent upon the base composition of the amplicons which are being separated. Ideally, this should result in a unique DHPLC profile that allows the identification of concordance or non-concordance between samples.

In addition, DHPLC is easily automated, which allows for rapid detection paired with high specificity. Using a commercially available DHPLC system (WAVE® 3500 Nucleic Acid Fragment Analysis System, Transgenomic Inc., Omaha, NE), a trained user can determine the presence of one or more nucleotide polymorphisms in any one of the four forensically validated HV1/HV2 amplicons in as little as seven minutes. Furthermore, these samples can be physically collected and purified following DHPLC analysis and then used directly for downstream sequence analysis.
Figure 4: DHPLC Chromatogram of a mtDNA Mixture

Illustration of the chromatographic separation of the hetero- and homoduplicates created by cross-hybridization of two amplification products which differ in sequence by a single base.
Validation of DHPLC for use in Forensic Laboratories

In order for a novel method or technique to be implemented in a forensic laboratory, it must undergo a rigorous validation process. In July 2004, the Scientific Working Group on DNA Analysis Methods (SWGDAM) outlined the necessary steps to be taken during developmental research (Forensic Science Communications, 2004). These studies, which include accuracy, precision, and reproducibility, must show that the technique is reliable prior to use in forensic casework. In 2009, The Quality Assurance Standards for Forensic DNA Testing Laboratories document was updated, (http://www.fbi.gov/about-us/lab/codis/qas_testlabs), though the central requirements for validation studies remained unchanged.

The experiments presented in this thesis were designed to test the efficacy and reliability of DHPLC as a means of screening and/or comparative sequence analysis for the HV1 and HV2 regions of human mtDNA in forensic casework. Specific studies include accuracy, precision, and reproducibility of assay results using a broad range of samples consistent with those typically seen in the forensic laboratory. In addition, cross-contamination and assay detection sensitivity were also investigated. This technology is readily available, and if validated successfully, will help streamline the laborious process of mtDNA analysis; thereby significantly contributing to the improvement of public safety and human identification efforts.
Hypotheses and Objectives

1. It is hypothesized that developmental validation of DHPLC will support its use as a tool for rapid and accurate comparative mtDNA sequence analysis. This will make it possible to rapidly and cost effectively identify putative matches between questioned and known samples without laborious DNA sequencing.

2. It is hypothesized that the developmental validation of DHPLC will allow it to act as a tool for identifying minor source components from mixed DNA samples. This will facilitate the analysis of samples that yield low quality data when analyzed using current methods.

3. It is hypothesized that implementation of DHPLC analysis of mtDNA reference and evidentiary samples for casework will make it possible for forensic laboratories to obtain potentially useful genetic data from samples that would not otherwise be amenable to analysis.

In order to test the aforementioned hypotheses, the current study encompassed four major objectives. These were to:

1. Evaluate the reproducibility of DHPLC results, including amplification reproducibility, injection reproducibility, and column-to-column reproducibility.

2. Evaluate the degree of cross-contamination (if any) that may occur when using DHPLC technology. This will be determined by capturing the elute of DNA-free injections following the injection of PCR amplified samples.

3. Evaluate the accuracy with which DHPLC technology can be used to screen both evidence and control samples in the context of a forensic laboratory. This will be demonstrated by a number of pair-wise comparisons of each of the forensically relevant amplicons from the 95 individuals in the study.

4. Evaluate the ability of DHPLC to accurately detect a mixture of haplotypes, including heteroplasmy, within a single sample. This will be achieved by pair-wise comparisons of samples included in a Proficiency Test with a known mixture purchased from a commercial supplier.
Chapter 2: Materials and Methods

Sample Collection and Avoidance of Contamination

This research was conducted in compliance with U.S. Federal Policy for the Protection of Human Subjects (56 FR 28003), and all protocols and human subject participation was reviewed and approved by the University of Denver’s Institutional Review Board for research involving human subjects. Blood, buccal, and hair samples were collected from 103 unrelated subjects who had provided informed consent. The blood samples were collected by a finger prick using sterile disposable medical lancets (Bayer Fingerstix™), buccal swabs taken by swabbing the inner cheek with sterile cotton swabs, and head hairs individually pulled by the subjects themselves. A variety of bone samples, including femur, tibia, ribs, and skull fragments, were kindly provided by the Department of Anthropology at the University of Montana. All samples were handled carefully in order to prevent sample-to-sample contamination or extraneous DNA contamination, and were stored at -20°C until DNA extraction.

Mitochondrial DNA Extraction from Blood Stains and Buccal Swabs

Mitochondrial DNA was extracted from both blood stains and buccal swabs using the EZ1 DNA tissue kit on the Qiagen BioRobot EZ1 DNA extraction robot (Qiagen Inc.,
Valencia, CA) according to the manufacturer’s protocol as described below. First, samples were cut under sterile conditions, placed into 1.7 ml tubes, and lysed with 190 µl of Qiagen’s G2 Buffer and 10 µl Qiagen proteinase K. The samples were incubated at 56°C for 15 minutes in a shaker incubator. Following digestion, the samples were transferred to a 2 ml sample tube provided with the EZ1 tissue kit, discarding any solid material. The EZ1 DNA Forensic Card contains two protocols: “1” for reference samples, and “2” for trace samples. The trace sample protocol is used when low yields are expected (less than 2.5 µg DNA; though the protocol can yield up to 5 µg), while the reference sample protocol is used when higher yields are expected (more than 2.5 µg DNA; though the protocol will also permit isolation of lower amounts of DNA). For blood and buccal swabs, the reference sample protocol was used to isolate and purify the DNA. Samples were eluted using 200 µl of nuclease free water and the DNA was stored at -20°C until PCR amplification.

Mitochondrial DNA Extraction from Hairs

Approximately 2 cm of hair, including root tissue when available, was cut and placed in a 1.5 ml tube. In order to remove potential surface contamination, hair fragments were vigorously rinsed with 5% Tergazyme detergent, (Alconox, Inc., White Plains, NY) followed by multiple ultrapure water washes (18.2 MΩ·cm resistivity at 25 °C and < 10 ppb Total Organic Carbon) and a final 100% laboratory grade ethanol rinse. Mitochondrial DNA was extracted from hairs using the EZ1 DNA tissue kit on the Qiagen BioRobot EZ1 DNA extraction robot (Qiagen Inc., Valencia, CA) as described
above, with the addition of the manufacturer’s “pretreatment for hair” protocol prior to
e EXTRACTION. Briefly, the hairs were transferred to an EZ1 2 ml sample tube, and 180 µl of
Buffer G2 and 10 µl Qiagen proteinase K was added to each sample and vortexed.
SAMPLES were incubated for 6 hours to overnight at 56°C in a shaker in order to dissolve
the hairs and lyse the cells. An additional 10 µl Qiagen proteinase K was added to each
sample, mixed thoroughly, and incubated for 2 hours at 56°C. For hair samples, the
EZ1’s trace protocol was used to isolate and purify the DNA. Samples were eluted using
50 µl of nuclease free water and the DNA was stored at -20°C until PCR amplification.

Mitochondrial DNA Extraction from Bone

In order to remove surface contaminants from the bone samples, approximately 1-
2 g of bone was placed in a 5% Tergazyme solution and sonicated for 30 minutes. This
was repeated twice using fresh 5% Tergazyme, followed by a water and ethanol rinse,
respectively. The bone samples were then allowed to dry for a few hours in the extraction
hood. Pulverization of the bone samples was performed using the 6850 Freezer/Mill
(SPEX SamplePrep, Metuchen, NJ) according to the manufacturer’s protocol. Briefly, a
small section of each bone was added to the provided freezer mill sample vials, along
with a provided steel rod, and placed into the freezer mill coil. Liquid nitrogen was
added to the tub of the freezer mill, in order to aid in grinding the bone samples. The
freezer mill incorporates the use of a high-speed electromagnet to drive the steel rod back
and forth inside the vials, thereby pulverizing the samples. Finally, DNA was extracted
from approximately 200 mg of pulverized bone powder using the EZ1 DNA tissue kit on
the Qiagen BioRobot EZ1 DNA extraction robot (Qiagen Inc., Valencia, CA) according to the manufacturer’s “pretreatment for bones or teeth” protocol. This included placing the bone powder into a 2 ml tube and adding 600 μl 0.5 M EDTA (pH 8.3) to aid in decalcification of the bone. The samples were incubated in a shaker at 37°C for 24–48 hours. Following the incubation, 20 μl of Qiagen Proteinase K was added, and incubated again at 56°C for approximately 3 hours. The samples were then centrifuged at 10,000 x g for 4 minutes, and 200 μl of the supernatant was transferred to an EZ1 sample tube. For bone samples, the EZ1’s trace protocol was used to isolate and purify the DNA. Samples were eluted using 200 μl of nuclease free water and the DNA was stored at -20°C until PCR amplification.

**Mitochondrial DNA Amplification of HV1 and HV2 Regions**

Standard, forensically-validated PCR primers were used to amplify four forensically-relevant regions (HV1A: 278bp; HV1B: 271bp; HV2A: 278bp; HV2B: 277bp) of the human mitochondrial genome (Table 1) in a 50 μl reaction, with primers at final concentration of 1 μmol/L; 2.25 U of AmpliTaq GOLD® DNA polymerase (Applied Biosystems, Foster City, CA), supplemented with 0.25U *Pfu* DNA polymerase (Stratagene, La Jolla, CA), and 200 μmol/L of each dNTP (Stratagene) with 10 μl (approximately 0.1-1 ng) of DNA extract. Amplifications were carried out on a GeneAmp® 9700 Thermal cycler (9600 emulation mode); (Applied Biosystems) using a thermal profile consisting of denaturation at 95°C for 10 minutes, followed by 32 cycles...
of denaturation at 95°C for 20 seconds, primer annealing at 60°C for 30 seconds and extension at 72°C for 45 seconds, followed by a final extension at 72°C for 15 minutes.

**PCR Amplification and Yield Determination for mtDNA using DHPLC**

In order to confirm that the amplification reactions worked as expected and to determine the amplification yield, 7 μl of amplified sample was transferred to a sterile 0.2 ml microcentrifuge tube and placed on the sample plate of the WAVE® 3500HT DNA Fragment Analysis System (Transgenomic Inc., Omaha, NE). The PCR amplification and yield was then determined from analysis of 5 μl aliquots of each sample using a proprietary DNASep® analytical column and triethylammonium acetate (TEAA) pH 7.0 at a final concentration of 0.1M as an ion-pairing reagent. The assay employed the DS Single Fragment Application (non-denaturing conditions, 0.9ml/min flow rate, oven temperature set at 50°C) appropriate for the size fragment being assayed (HV1A: 278bp; HV1B: 271bp; HV2A: 278bp; HV2B: 277bp). Eluting DNA peaks were detected by UV absorbance (260 nm). Using the non-denaturing conditions amplified homoplasmic mtDNA typically elutes as a single peak in the chromatographic profile.

Based on previous experiments in our laboratory using a DNA sizing and concentration ladder, it was determined that the efficiency of the PCR and the amount of DNA present in a sample can be accurately determined from the area of the PCR amplicon peak. Amplified DNA was estimated from the equation $y=1230.9x + 712.99$, where $x$ is the DHPLC peak area and $y$ is the DNA concentration (Figure 5).
Figure 5: Standard curve of DNA concentration as determined from peak area of the PCR amplicon peak
Quantification of mtDNA using RT-PCR

The mitochondrial DNA Quantification protocol was adapted from Andreasson et al. (2002), and was designed to quantify the total amount of amplifiable human mtDNA in a sample to determine if sufficient human mtDNA was present to proceed with DHPLC and mtDNA sequencing analyses. The quantification assay used a primer and probe combination developed based on Andreasson et al. (Figure 6), along with the TaqMan® Universal PCR Master Mix (Applied Biosystems). The RT-PCR reaction made use of the 5´ nuclease activity of AmpliTaq Gold® DNA polymerase to cleave a TaqMan® probe during PCR. The TaqMan® probe contains a reporter dye at the 5´ end of the probe and a quencher dye at the 3´ end of the probe. During the reaction, cleavage of the probe separated the reporter dye from the quencher dye, which resulted in detectible fluorescence of the reporter. The accumulation of PCR products therefore results in an increase in fluorescence. The quantity of DNA in a sample was determined by monitoring the increase in fluorescence of the reporter dye, and compared to that of a known standard DNA curve. For each PCR reaction in this study, 8 µl total of TaqMan® Master Mix (including primers and probe) were added to a 96-well optical plate, along with 2 µl of sample (Figure 7). A total of 8 standards (10^6 – 10^1 copies/µl created by 1:10 serial dilutions) were prepared from a purified master standard stock (216,227,941 copies/ µl, quantified by UV spectroscopy) and added to the 96-well optical plate. These standards served as the basis for a DNA quantification curve. Real-Time PCR analysis was performed on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystem).
mtDNA RT- PCR Amplicon (143 bp)

```
CCACTGTAAAGCTAACTTAGCATTAACCTTTTAAGTTAAAGATT
AAGAGAACCAACACCTCTTTTACAGTGAATGCCCCAACTAAAT
ACTACCGTATGGCCACCATAATTACCCCCATACCTCCTTACACT
ATTCCTCATCAC
```

**Primers and Probe**

mt-8294F (CCACTGTAAAGCTAACTTAGCATTAACC) @ 100umol/L (55.9°C)

mt-8436R (CCATACTCCTTACACTATTCCTCATCAC) @ 100umol/L (56.2°C)

mt-8345 (VIC-CCAACACCTCTTTTACAGTGAATGCCCCA-TAMRA)

**Figure 6:** Primer and probe sequences for Real-Time PCR quantification of mtDNA.

VIC is the fluorescent reporter dye of the probe at the 5’ end of the sequence. TAMRA is the quencher dye on the 3’ end of the probe.
Figure 7: Reaction mix components for mtDNA quantification using the protocol adapted from Andreasson et al. (2002). A total of 8 µl master mix was added to 2 µl DNA for each quantification reaction.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume Per Reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan Master Mix</td>
<td>5</td>
</tr>
<tr>
<td>mtDNA Probe (mt-8345)</td>
<td>0.13</td>
</tr>
<tr>
<td>10x Forward Primer (mt-8294F)</td>
<td>0.1</td>
</tr>
<tr>
<td>10x Reverse Primer (mt-8436R)</td>
<td>5</td>
</tr>
<tr>
<td>ddH$_2$O$_2$</td>
<td>2.67</td>
</tr>
</tbody>
</table>
Cross-Hybridization Control Assay by DHPLC

In order to determine whether or not the individual samples were homoplasmic or heteroplasmic prior to the mixing of different mitotypes, 7 μl of each amplicon was transferred to a sterile 0.2 ml microcentrifuge tube and cross-hybridized to itself by heating to 95°C for 4 min and then gradually cooling over a period of 45 minutes (1.5°C decrease/min) on an MJ Research, Inc PTC-100™ Thermal cycler or on an ABI 9700 Thermal cycler (9600 emulation mode). The self-hybridized samples were then removed from the thermal cycler and placed on the sample plate of the WAVE® autosampler. The cross-hybridization control assay was carried out by DHPLC using the Mutation Detection Application (i.e., partially denaturing conditions, 0.9ml/min flow rate) and the appropriate standard fragment analysis method where oven temperature settings were dependent upon previous optimization studies performed by the laboratory (HV1A, 58°C; HV1B, 59.2°C; HV2A, 56.5°C or HV2B, 57°C).

Purification of Amplicons using DHPLC

In order to clean up PCR amplified DNA for sequencing, the remaining reaction volume was loaded onto the WAVE® autosampler plate. The DS Single Fragment Application for the appropriate size fragment (HV1A: 278bp; HV1B: 271bp; HV2A: 278bp; HV2B: 277bp) was selected and the “Fragment Collection” tab of the instrument’s Navigator™ software was used to generate a fraction collection method for PCR product purification. A total of 20 μl of sample was injected and the eluted DNA fragments were then collected into 12 wells of a 96-well plate using the WAVE®
automated fragment collector. Finally, these were combined into a single 0.6 ml microcentrifuge tube for further downstream analysis. Any unpurified DNA remaining on the WAVE® autosampler plate was stored at -20°C.

**Sequencing of Mitochondrial DNA using the ABI Prism® 310 Genetic Analyzer**

Following DHPLC purification and subsequent collection of the amplified DNA using the automated fragment collector, DNA samples were loaded into the CentriVap® Centrifugal Concentrator (Labconco, Kansas City, MO) for solvent evaporation. Concentrated samples were then reconstituted in 12 µl of ultrapure water. DNA was labeled for dideoxy terminator sequencing (Sanger et al., 1977) using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). Sequencing reactions were primed using the same PCR primers employed for amplification. Samples were prepared in 10 µl reactions (2 µl master mix, 1 µl primer, 1 µl 5X sequencing buffer) and approximately 0.8-1 ng (2 µl) of amplified DNA. Labeling reactions were carried out on a GeneAmp® 9700 Thermal cycler (Applied Biosystems) using a thermal profile consisting of denaturation at 96°C for 1 minute, followed by 25 cycles of denaturation at 96°C for 10 seconds, primer annealing at 50°C for 5 seconds and extension at 60°C for 2 minutes.

Labeled products were purified on Performa® DTR V3 96-Well Short Plates (Edge BioSystems, Gaithersburg, MD) according to the manufacturer’s protocol. Briefly, the plates were centrifuged at 850 RCF for 3 minutes in order to elute the storage buffer. Sample reactions were then pipetted directly into the center of the individual wells, and
centrifuged again at 850 RCF. The eluate was retained, which contained the purified sequence labeling reaction products. The labeled products were then resolved on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer’s protocols. Here, 2 μl of purified labeled products was added to 10 μl Hi-Di formamide (Applied Biosystems) and denatured in a GeneAmp® 9700 Thermal cycler (Applied Biosystems) at 95°C for 2 minutes. The plates were then snap-cooled for 2 minutes in a 96-well ice plate.

For electrophoresis, the plates were placed onto the ABI 310 Genetic Analyzer, using the POP-6™ polymer and 47cm x 50μm capillaries (Applied Biosystems). The raw electrophoretic traces were analyzed using the KB Basecaller software (Applied Biosystems) together with the appropriate dye set mobility file for the v1.1 kit using Sequencing Analysis Software v.5.1.1 (Applied Biosystems). The resulting sequence data were aligned using the Sequencher™ DNA analysis software (Gene Codes Corp, Ann Arbor, MI). Appropriate positive and negative controls were included at all stages of the process.

**DHPLC-Based Temperature-Modulated Heteroduplex Analysis (TMHA)**

Once DNA concentrations were determined on the basis of the peak area for each of the amplicons obtained during the PCR amplification and yield determination assay, the concentration of each of the PCR product tubes were normalized either by vacuum centrifugation or by dilution with ultrapure water to a final concentration of 4-5 ng/μl. Samples were then combined at a 1:1 molar ratio to generate a series of pair-wise
mixtures from individuals with different mtDNA haplotypes. The haplotypes for each sample had been determined previously by direct sequence analysis.

The cross-hybridized mixtures were analyzed by TMHA (Kuklin et al., 1997) on the WAVE® System using a DNAsSep® analytical column and TEAA (0.1M final concentration; pH 7.0) as an ion-pairing reagent. Cross-hybridized mixtures were analyzed under partially denaturing conditions at the empirically determined optimal temperatures for each of the four mtDNA amplicons as previously described. The optimal acetonitrile linear gradient generated from differential mixing of buffer A (0.1M TEAA) and buffer B (0.1M TEAA, 25% ACN) required a 56% to 65% buffer B increase in 3.5 minutes for the analysis of HV1A, HV2A and HV2B and a 55% to 64% buffer B increase in 3.5 minutes for HV1B. All samples were eluted at a 0.9ml/min flow rate and DNA peaks were detected by UV absorbance at 260nm. Standard DHPLC controls included: zero-volume injections to screen for DNA carryover between assays; no-template PCR controls to check for reagent contamination; and manufacturer mutation control standards to ensure rigorous buffer and DNAsSep® column quality control.

The resulting chromatograms were visually examined. The presence of a single homoduplex peak was scored as consistent with sequence identity. The presence of more than one chromatographic peak or a distinct shoulder on a homoduplex peak was scored as consistent with the presence of one or more sequence differences between the two amplicons in the sample being assayed, i.e., sequence non-identity (O'Donovan et al., 1998; Hou et al., 2000); (Figure 8).
Figure 8: Example chromatograms of two samples cross-hybridized and analyzed on the DHPLC. A single homoduplex peak indicates sequence identity (A) whereas multiple peaks indicate sequence non-identity (B). The initial primer/nucleotide peak, dissolved oxygen peak, and final solvent wash peak are also indicated.
Sample-to-Sample Non-Carryover Validation Assays

To confirm that there was no cross-contamination between subsequent injections from adjacent wells on the WAVE® autosampler plate, an amplified mtDNA sample was purified on the WAVE® system along with zero volume injections (to show there was no sample remaining on the column) and adjacent blank well injections (to show that there was no cross contamination between samples on the injection plate). To accomplish this, the HV2A region (278bp) of a single mtDNA sample was amplified using the parameters previously described and injected under non-denaturing conditions. Injections consisted of 20 µl sample, a zero volume injection, and 20 µl dH₂O, followed by another sequence of 40 and 60 µl injections of sample. The presence of a visually detectable peak between approximately 2 and 6.5 minutes was considered to be evidence of contamination. The typical dissolved oxygen peak that appears at approximately 3.5 minutes was disregarded.

DHPLC Amplification Reproducibility Validation Assays

In order to assess the reproducibility of DHPLC-detected amplification, buccal swab samples from eight maternally-unrelated individuals were chosen based on sequence differences and extracted as described above. The amount of mtDNA present in each extract was quantified by real-time PCR on the ABI 7900HT Real-Time PCR System (Applied Biosystems), and sample concentrations were normalized with ultrapure water to 40,000 copies/µl (Table 2). Pair-wise mixtures representing each of the four forensically relevant mtDNA amplicons (HV1A, HV1B, HV2A, and HV2B) were then
created at a 1:1 molar ratio. These were then amplified in ten replicate 20 μl PCR reactions. The resulting PCR products were cross hybridized and sequentially assayed under partially denaturing conditions (10 μl injections) using DHPLC. In order to determine amplification reproducibility, the relative peak heights and elution peak retention times from each of the ten injections were averaged and the standard deviation determined.

**DHPLC Injection-to-Injection Reproducibility Validation Assays**

To assess the reproducibility of independent DHPLC injections, identical amplification products were assayed. The same eight DNA extractions that were used for the amplification reproducibility experiments described in the preceding section were used to prepare pair-wise 1:1 molar ratio mixtures representing each of the four forensically-relevant mtDNA amplicons. For each mixture, ten replicate 20 μl PCR reactions were prepared. Following amplification, each reaction was cross-hybridized and identical reactions were combined into a single 0.2 ml tube for a total of 200 μl of amplified and cross-hybridized sample. Each pooled sample (one pair-wise mixture representing each of four amplified mtDNA amplicons) was sequentially injected ten times (10 μl/injections) under partially denaturing conditions by DHPLC. In order to determine injection reproducibility, the relative peak heights and elution peak retention times from each of the ten injections were averaged and the standard deviation determined.
DHPLC Column-to-Column Reproducibility Validation Assays

The reproducibility of injections between different DNASEp® columns was assessed by comparison of ten two-component mixtures sequentially injected onto ten different DNASEp® columns. To do this, a total of ten pair-wise mixtures (representing both the HV2A and HV2B regions) were created at a 1:1 molar ratio from 20 maternally-unrelated individuals and amplified using the PCR thermal profile described above. Master 400 μl PCR mixtures were set up from which 50 μl aliquots were transferred into eight 0.2 ml tubes for greater amplification efficiency. Following amplification, each 50 μl reaction was cross-hybridized and then pooled into a single 0.6 ml tube. For subsequent DHPLC analysis, 10 μl of each mixture were injected under partially denaturing conditions, with columns being changed between each set of the ten injections. To determine the reproducibility of different individual columns, the profile consistency and elution peak retention times from each of the ten injections were averaged and the standard deviation determined.
Table 2: DNA sample normalization strategy for amplification and injection reproducibility experiments. Samples were normalized and mixed at a 1:1 molar ratio prior to amplification.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Copies/µL</th>
<th>Target</th>
<th>DNA stock (µL)</th>
<th>H₂O (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5</td>
<td>118373.89</td>
<td>40000</td>
<td>6.8</td>
<td>13.2</td>
</tr>
<tr>
<td>G4</td>
<td>263076.62</td>
<td>40000</td>
<td>3.0</td>
<td>17.0</td>
</tr>
<tr>
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<td>2.2</td>
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</tr>
<tr>
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</tr>
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</tr>
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<td>40000</td>
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</tr>
<tr>
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<tr>
<td>E12</td>
<td>670962.5</td>
<td>40000</td>
<td>1.2</td>
<td>18.8</td>
</tr>
</tbody>
</table>

20µL total
Validation Using Proficiency Tests

A commercial DNA mixture proficiency test was obtained from Orchid Cellmark (Dayton, OH) in order to determine whether DHPLC can accurately perform comparative sequence analysis as a means of efficiently screening and detecting the presence of mtDNA mixtures. Such proficiency tests are largely used in forensic laboratories, and can be a useful model for testing the ability of DHPLC to detect sequence non-identity between two samples.

The test kits contained a total of five samples. These included two forensic “i.e., unknowns” (including one mixture) and three reference samples “i.e., knowns.” All samples were extracted as described above, and a total of four amplicons were amplified from each sample using forensically validated primer pairs, (i.e., HV1A, HV1B, HV2A, and HV2B). The amplified products were then sequenced using dye terminator chemistry, and analyzed using DHPLC as described above for Temperature-Modulated Heteroduplex Analysis. In the resulting chromatograms, the presence of a single peak indicated sequence identity, whereas the presence of multiple peaks, or peaks with a distinct shoulder, indicated sequence non-identity. All sequencing results were later compared with the results provided by the proficiency test manufacturer.
Chapter Three: Results and Discussion

In the criminal justice system, the question of the admissibility of scientific evidence hinges on whether the evidence is reliable and is based on accurate and accepted scientific methods. In order for the scientific analysis of an item of evidence to be admissible in a court of law, it must be tested and analyzed in a manner that is generally accepted by the scientific community. This is based on the decision by the District of Columbia Court of Appeals in 1923, known as the Frye standard. The Frye standard states that expert evidence was admissible in court if it was “sufficiently established to have gained general acceptance in the particular field in which it belongs” (*Frye v. United States*, 293 F. 1013 (D.C. Cir. (1923)). In addition, the Supreme Court ruled in 1993 in the case of *Daubert vs. Merrell Dow Pharmaceuticals, Inc.* (509 U.S. 579 (1993)) that the rule for “general acceptance” be broadened to allow for admission of scientific technologies and ensuing evidence that may be lacking in broad acceptance in the scientific literature. Known as the Daubert standard, such admissibility requires that the technology rests on a sound scientific foothold; that the underlying scientific theory is valid and has been thoroughly tested; that it has representation in the peer review literature and that it has a known or potential error rate. Thus, it is critically important in the current study for newly developed methods of forensic DNA analysis to undergo a
vigorous validation process. The results presented in this study set the foundation for a complete validation of DHPLC as a tool to rapidly and accurately screen evidentiary mtDNA samples for sequence concordance/non-concordance.

**DHPLC Injection Reproducibility**

In order to demonstrate the reproducibility of DNA analysis by DHPLC and show that identical DNA samples independently injected will produce precise results, ten replicate injections were performed for DNA samples of two-component mixtures of each of four mtDNA amplicons commonly employed for forensic analyses. Amplicons consisting of database samples A7 and B7 (HV1A), D12 and E12 (HV1B), A5 and G4 (HV2A), and F5 and D6 (HV2B) were combined at equimolar ratios, cross-hybridized and sequentially assayed by DHPLC under partially denaturing conditions. The resulting data indicate that DHPLC-based chromatographic fractionation of DNA molecules is highly reproducible for each amplicon. Assay-to-assay variability was found to be negligible across independent injections of amplicons from a single amplification reaction (Figure 9). Both the peak height (Figure 11) and retention times (Figure 12) were highly reproducible for each of the four hypervariable region amplicons as evidenced by the small standard deviations for each peak among the replicates (with an average coefficient of variation of 0.98% for peak height and 0.40% for retention time).
Figure 9: Superimposed chromatograms for replicate assays of a 50:50 mixture of each four forensically relevant amplicons. Illustrated is the assay-to-assay reproducibility across ten replicate injections from a single amplification reaction on a single DNASep® column. Amplicon HV1A is a mixture of database samples A7 and B7, HV1B a mixture of D12 and E12 database samples, HV2A a mixture of A5 and G4 database samples, and HV2B a mixture of database samples F5 and D6. See Figure 10 for the haplotypes relative to the rCRS for each database mixture, indicating which positions were mismatched in the heteroduplexes.
Figure 10: Haplotypes relative to the rCRS are shown for each of the four forensically relevant mtDNA hypervariable region amplicons used for the ten replicate injections from a single amplification reaction on a single DNASep® column as shown in Figure 9. Based on these haplotypes it is possible to identify those nucleotide positions that were mismatched in the heteroduplicates.
Figure 11: Peak heights (Mean±SD) for representative 50:50 mixtures for each of four forensically relevant mtDNA hypervariable region amplicons which resolve into either two or four chromatographic peaks. Illustrated for each amplicon is the assay-to-assay variability across ten sequential injections of DNA from a single amplification reaction that were fractionated by DHPLC on a single DNASep® column. Amplicon HV1A is a mixture of database samples A7 and B7, HV1B a mixture of D12 and E12 database samples, HV2A a mixture of A5 and G4 database samples, and HV2B a mixture of database samples F5 and D6. See Figure 10 for the haplotypes relative to the rCRS for each database mixture, indicating which positions were mismatched in the heteroduplexes.
Figure 12: Retention times (Mean±SD) for representative 50:50 mixtures for each of four forensically relevant mtDNA hypervariable region amplicons which resolve into either two or four chromatographic peaks. Illustrated is the assay-to-assay variability across ten sequential injections of DNA from a single amplification reaction that were fractionated by DHPLC on a single DNAsSep column. Amplicon HV1A is a mixture of database samples A7 and B7, HV1B a mixture of D12 and E12 database samples, HV2A a mixture of A5 and G4 database samples, and HV2B a mixture of database samples F5 and D6. See Figure 10 for the haplotypes relative to the rCRS for each database mixture, indicating which positions were mismatched in the heteroduplexes.
DHPLC Amplification Reproducibility

To demonstrate independent amplification reactions using replicate samples sequentially injected onto the DHPLC show precise and reproducible results, ten replicates of each of four mtDNA amplicons commonly employed for forensic analyses were separately amplified from samples in the study population. Amplicons consisting of database samples A7 and B7 (HV1A), D12 and E12 (HV1B), A5 and G4 (HV2A), and F5 and D6 (HV2B) were combined at equimolar ratios to form two-component mixtures, cross-hybridized and sequentially assayed by DHPLC under partially denaturing conditions. The resulting data indicate that DHPLC is highly reproducible for each amplicon. Assay-to-assay variability was found to be negligible among independent amplifications (Figure 13). Both the peak height (Figure 15) and retention times (Figure 16) were highly reproducible for each of the four hypervariable region amplicons as evidenced by the small standard deviations for each peak among the replicates (with an average coefficient of variation of 7.1% for peak height and 0.32% for retention time). Variability in peak height relative to that observed in injection-to-injection reproducibility studies may be attributed to variability in the preparation and amplification efficiency of individual PCR replicates.
Figure 13: Superimposed chromatograms for replicate assays of a 50:50 mixture of each four forensically relevant amplicons. Illustrated is the assay-to-assay reproducibility across ten replicate amplification reactions sequentially injected and fractionated by DHPLC on a single DNASEp® column. Amplicon HV1A is a mixture of database samples A7 and B7, HV1B a mixture of D12 and E12 database samples, HV2A a mixture of A5 and G4 database samples, and HV2B a mixture of database samples F5 and D6. See Figure 14 for the haplotypes relative to the rCRS for each database mixture, indicating which positions were mismatched in the heteroduplexes.
**Figure 14:** Haplotypes relative to the rCRS are shown for each of the four forensically relevant mtDNA hypervariable region amplicons used for the ten replicate amplification reactions that were sequentially injected and fractionated by DHPLC on a single DNASep® column as shown in Figure 13. Based on these haplotypes it is possible to identify those nucleotide positions that were mismatched in the heteroduplexes.
Figure 15: Peak heights (Mean±SD) for representative 50:50 mixtures for each of four forensically relevant mtDNA hypervariable region amplicons which resolve into either two or four chromatographic peaks. Illustrated for each amplicon is the assay-to-assay variability across ten replicate amplification reactions sequentially injected and fractionated by DHPLC on a single DNASep® column. Amplicon HV1A is a mixture of database samples A7 and B7, HV1B a mixture of D12 and E12 database samples, HV2A a mixture of A5 and G4 database samples, and HV2B a mixture of database samples F5 and D6. See Figure 14 for the haplotypes relative to the rCRS for each database mixture, indicating which positions were mismatched in the heteroduplices.
**Figure 16:** Retention times (Mean±SD) for representative 50:50 mixtures for each of four forensically relevant mtDNA hypervariable region amplicons which resolve into either two or four chromatographic peaks. Illustrated is the assay-to-assay variability across ten replicate amplification reactions sequentially injected and fractionated by DHPLC on a single DNASep® column. Amplicon HV1A is a mixture of database samples A7 and B7, HV1B a mixture of D12 and E12 database samples, HV2A a mixture of A5 and G4 database samples, and HV2B a mixture of database samples F5 and D6. See Figure 14 for the haplotypes relative to the rCRS for each database mixture, indicating which positions were mismatched in the heteroduplexes.
DHPLC DNASep® Column Variability

To validate the reproducibility of DHPLC across multiple DHPLC DNASep® columns and demonstrate that different purchased columns produce similar results, ten two-component mixtures were prepared by mixing previously obtained database samples. Eight HV2B amplicon mixtures were created using database samples D3/E4, A1/B2, F5/A6, E10/H10, C8/G8, F7/F8, D12/G12, and C9/F9. Two HV2A amplicon mixtures were created using database samples G2/B3 and H8/D9. These amplicon mixtures were sequentially injected onto each of ten different DNASep® columns under partially denaturing conditions. Again the results show that DHPLC assay results are highly reproducible even when comparing assays across multiple DHPLC DNASep® columns. An example of the observed column-to-column variability in chromatographic traces for a single two-component mixture is shown in Figure 17. Both the peak height and peak retention times (Figure 17) as well as the peak-to-peak interval (Figure 18) were found to be highly reproducible as evidenced by the negligible standard deviations (Figures 18 and 19). The average coefficient of variation was 15.1% for peak height and 0.5% for retention time. The peak-to-peak interval was calculated in this instance in order to determine the consistency of the profiles among different columns.
Figure 17: Representative superimposed chromatograms for replicate assays of a 50:50 mixture of amplicon HV2B for database samples C9 and F9. Illustrated is the assay-to-assay reproducibility across ten DNASep® columns for the fractionation of a single two-component mixture. Haplotypes relative to the rCRS are also shown. Based on these haplotypes it is possible to identify those nucleotide positions that were mismatched in the heteroduplicates.
Figure 18: Representative DHPLC results (Mean±SD) for replicate assays of a 50:50 mixture of amplicon HV2B for the database samples C9 and F9. Shown are the individual maximum peak height (mV), peak retention time (min) and peak-to-peak interval (min) for each injection as well as the results (Mean±SD) for ten replicate injections. See Figure 17 for haplotype information for database samples C9 and F9.

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$$\Delta \text{Ret (3-1)} = 0.5733, 0.5900, 0.5767, 0.5600, 0.5733, 0.5867, 0.5833, 0.5833, 0.5833, 0.5833$$
$$\Delta \text{Ret (3-2)} = 0.1967, 0.1967, 0.1967, 0.1900, 0.2033, 0.2000, 0.2033, 0.2067, 0.2033, 0.2033$$
$$\Delta \text{Ret (3-2)} = 0.2000, 0.0050$$
Figure 19: Peak-to-peak intervals (Mean±SD in min) calculated for each series of injections as a function of one peak to any other for two sets of HV2A amplicon mixtures and six sets of amplicon HV2B mixtures. Database sample mixtures F7/F8 and C8/G8 peak-to-peak intervals were not calculated due to only one peak produced. See Figure 20 for haplotype information from each amplicon mixture set of database samples.
**Figure 20:** Haplotypes relative to the rCRS are shown for each of the ten HV2A or HV2B amplicon mixtures used for the ten replicate amplification reactions sequentially injected onto ten different DNASEp® columns as shown in figure 19. Based on these haplotypes it is possible to identify those nucleotide positions that were mismatched in the heteroduplicates.
DHPLC Assay Cross Contamination by DNA Carryover

Sample cross contamination as a function of injection-to-injection carryover was evaluated by capture of zero-volume injections (i.e., DNA-free samples) following the injection of amplified PCR samples. In these assays, samples containing high quantities (approx. 550 ng) of an amplified 143bp DNA fragment (from a previously subcloned mtDNA coding region) were injected onto the DHPLC DNASep® column yielding a single peak with maximum absorbance at 3.82 ± 0.05 min (Figure 21). This was followed by 5 zero-volume injections which were collected at the same time point where the DNA from the initial injection had eluted. Sample detection by UV absorbance revealed no evidence of DNA carryover in any of the zero-volume injections performed. However, the use of a DNA intercalating dye and fluorescence detection revealed DNA carryover in the initial zero-volume injections, ranging from 35pg - 125pg (Figure 22). Four subsequent zero-volume injections yielded 17pg, 14pg, 9pg and 8pg, respectively, as determined by DHPLC quantitation. Eluent captured from the zero volume injections was evaporated by vacuum centrifugation, followed by dye terminator labeling. DNA sequencing reactions from the captured time period, however, yielded no detectable sequence (Figure 23). It is worth noting, that the amount of DNA injected onto the DNAsep® column in this study is more than two-fold higher than the quantity of amplified mtDNA obtained from reference samples which typically yield 50ng - 200ng.
Figure 21: Representative superimposed chromatograms for DHPLC analysis of an mtDNA amplicon (mt-Std-1) and five subsequent zero-volume injections (blanks). All blanks are coincident. Using UV detection, no indication of DNA carryover is evident between the initial high-abundance mt-Std-1 sample and the five subsequent zero-volume injections.
Figure 22: Representative superimposed chromatograms for DHPLC analysis of an mtDNA amplicon (mt-Std-1) and five subsequent zero-volume injections (blanks). Using fluorescence detection and scaled to a maximum y-axis value of 0.1 mV for enhanced DNA detection sensitivity, DNA carryover between the initial high-abundance mt-Std-1 sample and the five subsequent zero-volume injections ranged from 35-125pg.
Figure 23: Representative sequencing electropherogram generated by dideoxy-terminator labeling of a captured zero volume injection. The top pane indicates the raw electrophoretic trace that was analyzed using the KB Basecaller software (Applied Biosystems) whereas the bottom pane indicates the data analyzed using the Sequencing Analysis Software v.5.1.1 (Applied Biosystems), which shows only stochastic electrophoretic “noise”. The complete lack of any electrophoretic peaks above baseline demonstrates very low-level (if any) DNA carryover between the initial high-abundance mt-Std-1 sample and subsequent zero-volume injections (i.e., below the minimum threshold required for detection of DNA by direct DNA sequencing).
Screening of Evidentiary Samples – Validation of Comparative Sequence Analysis Using Known Samples

One of the primary objectives of this study was to evaluate the potential utility of DHPLC as a means of screening amplified human mtDNA samples for sequence identity/non-identity in the context of a forensic laboratory. To thoroughly and realistically assess the reliability of DHPLC as a tool for comparative sequence analysis, the approach was tested on native mtDNA samples isolated from 95 research volunteers.

Exclusive of length polymorphisms associated with the homopolymeric cytosine-stretch in HV2, the 95 subjects in the study represented 83 distinct mtDNA haplotypes. Seventy-seven of these were unique within the study population. Of the 6 haplotypes which occurred more than once, the most common (263G, 315.1C) was observed 8 times, or approximately 8% of the total, which is comparable to the 7% frequency at which it occurs in the general population (Lutz-Bonengel et al., 2003). The remaining 5 haplotypes each occurred twice. Relative to the rCRS, the haplotypes represented in the current study encompass 84 polymorphisms in HV1 and 46 polymorphisms in HV2, including cytosine-stretch length polymorphisms.

Based on pair-wise comparisons, individual haplotypes differed from each other at 0-22 positions (0-11 in HV1A; 0-13 in HV1B; 0-13 in HV2A; and 0-12 in HV2B). On average, there were 8.71 positional differences between haplotypes. In toto, the study population encompassed a broad diversity of haplotypes and thus was well suited for evaluating the utility of DHPLC for the accurate detection of sequence polymorphisms.
throughout the mtDNA control region. This is essential for the validation of DHPLC as a tool for comparative sequence analysis.

A total of 920 pair-wise combinations of amplicons from the 95 individuals in this study were prepared, denatured and allowed to gradually re-anneal. Of these, 72 (22 HV1A, 8 HV1B, 17 HV2A, and 25 HV2B mixtures) represented combinations of amplicons that were from different individuals but which had identical DNA sequences. DHPLC analyses of these samples all produced clear chromatograms consisting of a single symmetrical homoduplex peak. This pattern is consistent with sequence identity and is 100% concordant with direct sequencing data for these amplicons.

To assess the reliability of DHPLC to detect sequence non-identity, 849 combinations of amplicons (209 HV1A, 222 HV1B, 213 HV2A, and 205 HV2B mixtures) which differed in sequence were assayed. These differences encompassed a broad diversity of polymorphisms distributed throughout the HV1 and HV2 regions including transitions, transversions, insertions and deletions (Table 3). Positional differences were located centrally as well as near the termini of amplicons and encompassed regions of varying GC richness, ranging from approximately 15-80% GC content. In all, the mixtures assayed in this study included sequence variants at 39, 62, 38 and 30 different positions in HV1A, HV1B, HV2A and HV2B, respectively. The distribution of the variant positions and the frequency with which they were assayed is illustrated in Figure 24.
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**Table 3**: Sequence polymorphisms in the HV1 and HV2 regions assayed by DHPLC.
**Figure 24:** A histogram showing the distribution of nucleotide positions within HV1A, HV1B, HV2A and HV2B amplicons and the number of times that sequence polymorphisms at each position were assayed by DHPLC. The polymorphisms assayed are broadly distributed throughout both GC and AT rich regions as illustrated by the light gray line which indicates %GC content across each fragment, as measured by a rolling 10bp average.
Using the aforementioned initial assay temperature for each mtDNA amplicon, DHPLC analyses correctly indicated the presence of a mixture of non-identical amplicons in 836 of the combinations tested (209 HV1A, 222 HV1B, 203 HV2A, and 202 HV2B). The remaining 13 mixtures of non-identical amplicons (10 HV2A and 3 HV2B) yielded chromatographic traces with a single eluent peak, a result erroneously suggesting sequence identity. Careful examination of the non-identical amplicon mixtures which were not detected by DHPLC reveals that these aberrant results are limited to a very small number of challenging positions. Specifically, it was not possible to detect non-identical combinations of HV2A amplicons that differ only at positions 72 or 73. Taken together, these two positions account for all of the undetected non-identical mixtures involving HV2A amplicons.

In HV2B, DHPLC was not able to detect non-identical combinations of amplicons that differed only at position 295. This position lies in a narrow stretch of sequence immediately adjacent to a large GC-rich region. Given the thermodynamic stability of this region, a single base mismatch may not sufficiently destabilize the surrounding helix such that an early eluting peak can be discerned. This is a postulate supported by the observation that mixtures of amplicons that possess an additional mismatch in this same region are readily detected.

An inverse relationship was generally observed between the number of positional differences associated with a given pair of non-identical amplicons and the relative heights of the hetero- versus homoduplex peak(s). The height and retention time of a heteroduplex peak is a function of the stability and base sequence of the helix. The more
stable a heteroduplex, the more readily it should form relative to the competing homoduplces and thus the greater its peak height (indicative of quantity) on the DHPLC chromatograms. The corollary of this is that the formation of less stable helices is less favored and should be associated with smaller and earlier-eluting peaks on the DHPLC chromatograms. In theory, this could compromise the ability of DHPLC to detect, as non-identical, some combinations of amplicons that differ at a large numbers of positions. In the current study, however, no examples of such “heteroduplex dropout” were observed.

Screening of Evidentiary Samples – Forensic Validation

Another primary objective of this study was to evaluate the potential utility of DHPLC as a means of screening amplified human mtDNA samples for sequence identity/non-identity using samples commonly seen in a forensic laboratory. To thoroughly and realistically assess the reliability of DHPLC as a tool for comparative sequence analysis, the approach was tested on mtDNA samples isolated from both database samples and a variety of sample types taken from 16 individuals, representing typical sample types present in forensic examinations. These included bloodstains (designated by the letter B) buccal swabs (designated by the letter S), hairs (designated by the letter H), vaginal swabs (designated by the letter V), semen swabs (designated by the letter M), saliva swabs (designated by the letter A) and a bone sample (designated by the letter N). To forensically validate the reliability of DHPLC to detect the presence of sequence variants between different mtDNA amplicons, 4x4 matrices of reference and questioned samples were created to show the varying steps in the process of data
evaluation (Figure 25). The assay components of each 4x4 matrix include an individual “PCR check” assay to indicate whether the amplification product was of appropriate quantity, the “Denatured Control” assay to demonstrate that the samples were single source and did not contain a mixture (including heteroplasmy), and finally the two-component “Mixed Sample” assays indicating sequence identity/non-identity. Also included with the 4x4 matrix is a diagram with the haplotypes relative to the rCRS, from which it is possible to determine which positions were mismatched in each of the heteroduplicates.

Prior to each sequence identity/non-identity assay, amplified products were assayed by DHPLC under non-denaturing conditions in order to determine the quantity of amplified DNA, as visualized by the PCR Check peak. The samples were then denatured, slowly allowed to reanneal, and assayed by DHPLC under partially denaturing conditions in order to check for mixtures (e.g., heteroplasmy); as seen by the Denatured Control peak. The quantity of DNA for each sample that was to be mixed was standardized to 5 ng/μl to facilitate the generation of 50:50 equimolar mixtures. After cross-hybridization, mixtures were assayed by DHPLC under partially denaturing conditions to detect sequence identity/non-identity. The presence of early eluting peaks or shoulders was taken as evidence of sequence non-identity and the results were compared to previously determined sequences for each of the four amplicons (HV1A, HV1B, HV2A and HV2B). In all, 20 separate 4x4 matrices were created, each consisting of 6 sequence identity/non-identity assays for a total of 120 individual assays. Of these assays, there were 74 base pairs differences total over the four amplicons, and encompassed a broad diversity of
polymorphisms. Consistent with the results reported here in section 5 using known samples, in almost every instance the correct conclusion was made (\textit{i.e.}, inclusion vs. exclusion); (Figure 25). In only four assays was DHPLC not able to detect non-identical combinations of amplicons, indicating a success rate of 96.7%. Here, DHPLC was not able to detect combinations of amplicons that differed only at position 73 in HV2A. This was not unexpected since the sequence difference between these amplicons (73G vs. 73A) was immediately adjacent to a short GC-rich region. The thermodynamic stability of this region is an intrinsic feature of the HV2A amplicon which interferes with the detection of base changes at position 73. Figures 26-28 show additional representative examples of 4x4 matrices.
Figure 25: Representative 4x4 matrix of DHPLC results for amplicon HV2A, samples 3B and 3S (database sample D9) and 4B and 4S (database sample E6). Green chromatograms represent PCR controls to show that the amplification worked and to allow determination of the quantity of DNA present in the sample (5μl injections, non-denaturing conditions), red chromatograms represent denatured controls to show whether or not the sample itself is a potential mixture (including heteroplasmy); (5μl injections, partially denaturing conditions) and blue chromatograms represent sequence identity / non-identity assays which can be used to detect the presence of a mixture (8μl injections, partially denaturing conditions). Single peaks are consistent with sequence identity whereas multiple peaks or shoulders are consistent with sequence non-identity.
Figure 26: Representative 4x4 matrix of DHPLC results for amplicon HV2B samples 1B and 1S (database sample H8) and 2B and 2S (new sample CE). Green chromatograms represent PCR controls to show that the amplification worked and to allow determination of the quantity of DNA present in the sample (5μl injections, non-denaturing conditions), red chromatograms represent denatured controls to show whether or not the sample itself is a potential mixture (including heteroplasmy); (5μl injections, partially denaturing conditions) and blue chromatograms represent sequence identity / non-identity assays which can be used to detect the presence of a mixture (8μl injections, partially denaturing conditions). Single peaks are consistent with sequence identity whereas multiple peaks or shoulders are consistent with sequence non-identity.
Figure 27: Representative 4x4 matrix of DHPLC results for amplicon HV1A, samples 3B and 3S (database sample D9) and 4B and 4S (database sample E6). Green chromatograms represent PCR controls to show that the amplification worked and to allow determination of the quantity of DNA present in the sample (5μl injections, non-denaturing conditions), red chromatograms represent denatured controls to show whether or not the sample itself is a potential mixture (including heteroplasmy); (5μl injections, partially denaturing conditions) and blue chromatograms represent sequence identity / non-identity assays which can be used to detect the presence of a mixture (8μl injections, partially denaturing conditions). Single peaks are consistent with sequence identity whereas multiple peaks or shoulders are consistent with sequence non-identity.
Figure 28: Representative 4x4 matrix of DHPLC results for amplicon HV1B, samples 5S and 5H (database sample H1) and 6H and 6S (database sample H7). Green chromatograms represent PCR controls to show that the amplification worked and to allow determination of the quantity of DNA present in the sample (5μl injections, non-denaturing conditions), red chromatograms represent denatured controls to show whether or not the sample itself is a potential mixture (including heteroplasmy); (5μl injections, partially denaturing conditions) and blue chromatograms represent sequence identity / non-identity assays which can be used to detect the presence of a mixture (8μl injections, partially denaturing conditions). Single peaks are consistent with sequence identity whereas multiple peaks or shoulders are consistent with sequence non-identity.
Proficiency Test - Mixture Detection Results

All DNA analysts employed in accredited forensic laboratories must undergo an external proficiency test twice a year per the Quality Assurance Standards for Forensic DNA Testing Laboratories guidelines. Proficiency tests are a quality assurance tool used to determine analyst performance, verify standard operation procedures, and fulfill accreditation requirements. These tests are administered by an external company, and consist of both “known” and “unknown” samples. All of the samples present in the kit are processed by the analyst using standard protocols, and DNA profiles and/or mtDNA haplotypes are determined. The tests are then scored by the external party on a pass/fail basis.

To establish the accuracy of DHPLC in its ability to accurately screen for sequence identity/non-identity among authentic proficiency test samples given to forensic laboratories, a commercial kit was purchased from Orchid Cellmark (Dayton, OH). This “DNA Mixture” kit comprised five samples: three bloodstain reference FTA cards (sample names provided in kit as VB, S1B, and S2B), one bloodstained evidence FTA card (sample name CB), and one mixed evidence swab (sample name CS).

Each proficiency test sample to be assayed for sequence identity/non-identity was denatured, slowly allowed to reanneal, and assayed by DHPLC under partially denaturing conditions. A mixture was detected for the HV1B amplicon of sample CS (mixed evidence swab). This was determined by visual detection of two peaks instead of one (Figure 29). Sample CS was subsequently sequenced, and clearly showed a mixed (T/C)
position at 16218 (Figure 30). This was the only mixed base position that was detected throughout the HV1 and HV2 regions using normal sequencing protocols.
Figure 29: Chromatograms generated by DHPLC under partially denaturing conditions representing 5μl injections of individual samples (HV1B amplicon) that had been heat denatured and allowed to slowly reanneal (i.e., a Denatured Control). The single peaks on chromatograms 1, 3, 4, 5 indicate a single contributor; the multiple peaks/shoulder on chromatogram 2 indicates more than one contributor. These results were as expected as sample CS is a known mixed sample provided in the proficiency test kit.
Figure 30: Representative sequencing electropherogram of mixed proficiency sample CS indicating a T/C mixture at position 16218 in the HV1B amplicon. This position is the only detected mixed position throughout the HV1 and HV2 regions of this and all other proficiency test samples. Dye terminator sequencing employed ABI BigDye v1.1 chemistry on an ABI 310 sequencing platform.
**Proficiency Test - Screening Results**

The five samples present in the proficiency test (reference samples VB, S1B, and S2B; evidence samples CB and CS) to be assayed in sequence identity / non-identity screening tests were amplified in all four HV1A, HV1B, HV2A, and HV2B regions, standardized to 3 ng/μl, mixed with each other at a 50:50 equimolar ratio, denatured and allowed to slowly reanneal, and assayed by DHPLC under partially denaturing conditions. A 5x5 matrix for each amplicon was created (Figure 31) due to comparison of five samples instead of four as previously described, and DHPLC results were compared to the sequencing results for each of the four amplicons (HV1A, HV1B, HV2A, and HV2B) to determine screening accuracy. In each instance, the correct conclusion (*i.e.* inclusion vs. exclusion) was made. These results were consistent with the DNA sequencing results for the same sample as is illustrated in Figure 32.
Figure 31: Representative 5x5 matrix of DHPLC results for amplicon HV2A, for all proficiency samples. Green chromatograms represent PCR controls to show that the amplification worked as well and to allow determination of the quantity of DNA present in the sample (5μl injections, non-denaturing conditions), red chromatograms represent denatured controls to show whether or not the sample itself is a potential mixture (including heteroplasmy); (5μl injections, partially denaturing conditions) and blue chromatograms represent sequence identity / non-identity assays which can be used to detect the presence of a mixture (8μl injections, partially denaturing conditions). Single peaks are consistent with sequence identity whereas multiple peaks or shoulders are consistent with sequence non-identity. See Figure 32 for HV2A haplotype results for each sample in the proficiency test kit.
**Figure 32:** mtDNA haplotyping results for amplicon HV2A of the five components of the proficiency test. Four out of the five samples exhibit sequence identity in HV2A, the exception being sample S1B. This is fully concordant with the results of the pair-wise sequence identity/non-identity assays which can be used to detect the presence of a mixture presented in Figure 31.

<table>
<thead>
<tr>
<th></th>
<th>CB</th>
<th>CS</th>
<th>VB</th>
<th>S1B</th>
<th>S2B</th>
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</thead>
<tbody>
<tr>
<td>146</td>
<td>t</td>
<td>*</td>
<td>*</td>
<td>C</td>
<td>*</td>
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<tr>
<td>195</td>
<td>t</td>
<td>*</td>
<td>*</td>
<td>C</td>
<td>*</td>
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<tr>
<td>263</td>
<td>a</td>
<td>G</td>
<td>G</td>
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<td>G</td>
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</table>

|    |    |    |    |    |
|----|----|----|----|
| 146| t  | *  | *  | C   |
| 195| t  | *  | *  | C   |
| 263| a  | G  | G  | G   |

The table above shows the haplotyping results for the amplicon HV2A of the five components of the proficiency test. Four out of the five samples exhibit sequence identity in HV2A, with the exception of sample S1B. This is fully concordant with the results of the pair-wise sequence identity/non-identity assays.
Chapter Four: Conclusions

The results of this study have demonstrated that DHPLC analysis of pair-wise combinations of identical mtDNA amplicons both accurately and reliably produce a single chromatographic peak consistent with sequence identity. These results were 100% concordant with DNA sequence data. Conversely, in pair-wise combinations of non-identical amplicons, DHPLC successfully detects a diversity of sequence differences throughout the HV1 and HV2 regions. These differences, which include a wide variety of base substitutions as well as insertions/deletions, are typically indicated by the presence of more than a single peak in the resulting chromatogram. DHPLC results are reproducible, and cross contamination is not detectable. As such, DHPLC may have significant forensic utility in several areas. These include a presumptive test of mtDNA identity between known and questioned samples and a screening test for mixed samples prior to direct sequencing. These results provide direct support for hypothesis 1:

1. It was hypothesized that developmental validation of DHPLC will support its use as a tool for rapid and accurate comparative mtDNA sequence analysis. This will make it possible to rapidly and cost effectively identify putative matches between questioned and known samples without laborious DNA sequencing.
Although DHPLC is not a replacement for direct sequencing of mtDNA, it does offer some advantages as a potential screening tool. First, the assay is relatively simple and fast. It uses raw PCR products thereby avoiding the time and expense associated with amplicon cleanup. Following cross-hybridization, each assay takes only seven minutes to run and interpretation of the results is straightforward. DHPLC provides a comprehensive assessment of sequence identity across an entire amplicon without the often challenging task of trying to obtain quality base sequence information immediately adjacent to primer binding sites.

Compared to alternative mtDNA screening strategies based on oligonucleotide probes or linear arrays, DHPLC consumes less DNA and is not limited by the need to design probes for the detection of known mutations at predetermined polymorphic sites. This reduces the potential for false inclusions and eliminates the need to design custom probes for unique or rare sequence variants. Similarly, DHPLC assays are not subject to the “null” or “blank” results that arise when hybridization of the target sequence is impeded by other nearby polymorphisms. On the contrary, additional sequence variants typically make it easier to detect sequence non-identity between two amplicons.

While DHPLC circumvents many of the limitations of alternate approaches to mtDNA screening, it is important to consider very carefully the types of samples for which such an approach might be indicated. Within an mtDNA sequencing laboratory, the results of the current study indicate that screening by DHPLC makes it possible to detect samples that contain mixtures of non-identical amplicons immediately after PCR amplification and without having to sequence them. For both heteroplasmic and
situational mixtures characterized by a secondary/minor source contributor, this approach allows the analyst to identify potentially challenging samples and mark them for “special handling” – whether that be the use of alternate sequencing primers to avoid C-stretch polymorphisms or the application of emerging technologies for resolving mixed samples (Danielson et al., 2005; Danielson et al., 2007). These results provide direct support for hypothesis 2:

2. It was hypothesized that the developmental validation of DHPLC will allow it to act as a tool for identifying minor source components from mixed DNA samples. This will facilitate the analysis of samples that yield low quality data when analyzed using current methods.

It has been reasonably argued by experienced practitioners in the field that it is best to avoid using an mtDNA screening method on limited or irreplaceable evidentiary material (Melton et al., 2006; Divne et al., 2005). The results of the current study support the use of a presumptive DHPLC screen for mtDNA sequence identity. In addition, the results indicate that DHPLC can serve as a useful tool for investigators in special situations such as the investigation of property crimes. The limited budgets of many law enforcement agencies make it extremely difficult for investigators to justify the expense of mtDNA testing in the majority of criminal offences; particularly when there is no assurance a priori that the test results will necessarily advance an investigation. A presumptive screen for sequence identity between a suspect and an item of evidence, however, could provide sufficient justification to submit the sample for confirmatory analysis by direct sequencing. The results of the current study have demonstrated that such screening can help to readily eliminate from consideration such non-probative
samples as hairs consistent with a victim instead of a potential suspect. In short, this could help investigators to focus their efforts on the most probative samples and thereby maximize the efficient use of investigative resources. Taken together, these results provide direct support for hypothesis 3:

3. It is hypothesized that implementation of DHPLC analysis of mtDNA reference and evidentiary samples for casework will make it possible for law enforcement agencies to obtain potentially useful genetic data from samples that would not otherwise be amenable to analysis.

Employing a presumptive test of mtDNA sequence identity in the manner described above will also shift the process of DNA extraction from the dedicated mtDNA sequencing laboratory to the local law enforcement laboratory. This necessitates that additional consideration be given to the handling of these samples. The presence of evidentiary material with large quantities of mtDNA (e.g., blood, saliva and seminal fluids, etc.) in local laboratories can pose a significant risk of cross contamination. Accordingly, an mtDNA sequencing laboratory accepting a DNA extract for analysis would almost certainly require the submission of a co-extracted reagent blank control that could be tested to detect the presence of spurious mtDNA contamination. Similarly, the submission of amplified PCR products for direct sequencing would also need to be accompanied by the appropriate positive and negative PCR controls for quality control purposes.

Forensic laboratory implementation of a commercial DHPLC analysis system can be achieved with minimal training and a maximum equipment cost of just over $135,000. The $0.50/run operating cost for DHPLC analyses is considerably less than that for
alternative approaches. Depending on the nature of casework being analyzed and level of
throughput, the acquisition of a DHPLC system may be a fiscally viable option for some
forensic laboratories as is already the case in the molecular diagnostics arena.

In summary, the current study has demonstrated the potential utility of DHPLC-
based analysis for the economical and accurate screening of the HV1 and HV2 regions of
mtDNA for sequence identity/non-identity. In a forensic case-working context, this
approach to mtDNA sequence analysis can assist analysts by rapidly identifying
potentially challenging mixed samples prior to direct sequencing. As a presumptive test
for sequence identity, DHPLC makes it possible to screen for items of evidence that are
potentially probative to an investigation, thereby saving the investigating agency
significant funds.

**Future Directions**

Ongoing research will need to focus on the development of DHPLC-based
approaches to resolving mtDNA mixtures and the subsequent validation of the method in
accordance with guidelines of the Scientific Working Group on DNA Analysis Methods,
the European DNA Profiling Group and the International Society for Forensic Genetics.
This further validation to satisfy both Frye and Daubert standards would include
performing DHPLC analysis as described in this study on samples that are from
adjudicated cases, *i.e.* those from forensic cases that have already been closed. These
samples would demonstrate that the methods outlined can handle real casework
situations. Also, non-human studies would need to be performed to show that other
biological materials using the methods discussed do not interfere with the ability to obtain reliable results. Additional samples which contain minimal amounts of DNA should also be analyzed using the methods outlined in this paper, to show any potential stochastic effects that may occur during the PCR process or poor sensitivity during DHPLC detection. All the experiments performed that are part of the validation study should also be made part of inter-laboratory studies to demonstrate that the methods used in one’s laboratory are reproducible in another laboratory. These supplementary experiments should be shared with the scientific community through peer-reviewed publications in the professional literature.

Additional research, such as more extensive studies using heteroplasmic samples encompassing a broader range of mutations, should also be performed. In addition, the use of smaller amplicons currently used by the Armed Forces DNA Identification Laboratory (AFDIL) known as “mini-primer sets” could be investigated. These mini-primer sets range in size from 126-170 bp, and divide each hypervariable region into four segments instead of two, consisting of eight overlapping amplicons instead of the typical four. Mini-primer sets are used with highly degraded DNA samples to enable greater recovery of overlapping sequence data of the HV1 and HV2 regions, due to their smaller target amplicon size. This continuing research may further increase the forensic utility of DHPLC, increasing the types of samples that can be accurately screened. Also, following the complete validation of forensic mtDNA analysis by DHPLC, standard operating procedures will need to be finalized, training manuals and quality assurance documents will need to be written, and individual laboratories will need to undergo additional
accreditation by the American Society of Crime Laboratory Directors Lab Accreditation Board (ASCLD-LAB). Once accreditation is achieved by the laboratory, full implementation of the procedures outlined in this study can be implemented in actual forensic casework.
References


Barros F., Lareu M.V., Salas A., and Carracedo A. Rapid and enhanced detection of mitochondrial DNA variation using single-strand conformation analysis of


