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Forensic Utility of Mitochondrial DNA Analysis Based on Denaturing High-Performance Liquid Chromatography

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Aim. To determine the forensic utility for pairwise DNA comparisons and DNA mixture resolution with denaturing high-performance liquid chromatography (DHPLC) of human mitochondrial DNA (mtDNA).

Methods. MtDNA hypervariable regions (HV) 1 and 2 from the mtDNA D-loop were amplified by the polymerase chain reaction and mixed between known and unknown sample sources. The DNA mixtures were denatured and reannealed, and the resultant homo- and heteroduplices were evaluated by temperature-modulated heteroduplex analysis by the DHPLC method.

Results. All 144 pairwise comparisons of HV1 and HV2 mtDNA fragments were successfully resolved by the DHPLC method. Forensic proficiency test standards were successfully resolved and DHPLC match/non-match results agreed with sequencing results provided by the test providers. The DHPLC method successfully identified one questioned sample that was prepared by the test provider as a body fluid mixture. MtDNA amplicon mixtures could be separated into their constitutive components by DHPLC and fraction collection approaches.

Conclusions. DHPLC methods provide the forensic scientist with a powerful tool to rapidly screen mtDNA and may result in standardized methods to resolve mtDNA mixtures. These advances will allow mtDNA analysis in cases not previously examined by current sequencing-based approaches and could allow more forensic case samples to be entered into the proposed mtDNA Combined DNA Index System (CODIS™) databank as a result of mtDNA mixture resolution.

Key words: *base sequence; complementarity determining regions; chromatography, high pressure liquid; DNA; DNA, mitochondrial; forensic medicine*

The use of mitochondrial DNA (mtDNA) has allowed investigators to derive genetic information from forensic samples where nuclear-based analyses have failed. The size and distribution of the mitochondrial genome and the ability to amplify small target amplicons with polymerase chain reaction (PCR) make it an attractive target for forensic analysis. The high copy number of mtDNA per cell (several thousand copies/cell) facilitates a successful analysis of degraded or very limited amounts of starting material (1). Subcellular sequestration of the DNA within the mitochondrion and its exonuclease-resistant circular nature may also contribute to its molecular stability (2). MtDNA is generally inherited through the maternal lineage, allowing relationships to be inferred over several generations and between maternally related siblings (3).

Analysis of mtDNA is currently accomplished by sequencing the DNA from hypervariable regions 1 and 2 (HV1/HV2) of the displacement loop (nt 16024-16576). This approach has been used extensively to identify human remains, genotype hair shaft material in forensic cases, and explore historical questions (3-6). Developments based on these and other studies have resulted in an accepted approach to mtDNA analysis that has withstood several court challenges (See *www.denverda.org* for specific case law examples).

The analysis of mtDNA by DNA sequencing has mainly been successful, but some limitations exist with respect to sample type and composition. Individuals can have more than one species of mtDNA in varying concentrations in different body fluids or tissues. The ongoing study of this phenomenon, known as "sequence heteroplasmy," is well-known in medical research dealing with mitochondrial disease genetics (7). In forensics, both sequence and length heteroplasmy can be problematic in determining the true level of discrimination between sequences that are determined to "match" (8). Variations in the length of the HV1 cytosine-stretch can result in unreadable electropherograms because the dideoxy-terminated chains of labeled DNA fall out of register, starting at the position that displays the first base of length heteroplasmy. These length variants cannot be adequately resolved and forensic laboratories will not interpret these data. Sequence-based results can be obtained from regions outside a heteroplasmic cytosine stretch through the use of additional PCR primers, although this reduces the total amount of sequence data that can be used to characterize a given mitochondrial amplicons (1). Heteroplasmy can be considered a "natural" DNA mixture, in contrast to a "situational" mixture of body fluids containing DNA often encountered in forensic science. The mixture of bodily fluids can provide forensic scientists with valuable investigative information. For example, sexual assault cases often start as DNA mixtures between the victim's DNA and the perpetrator's DNA, with the most common situation involving the mixture of semen with vaginal fluids. However, evidence involving the mixture of the perpetrator's saliva with victim's skin cells is frequently encountered.

The current PCR and DNA sequencing-based procedures employed for mtDNA analysis are very sensitive to minute amounts of DNA and small numbers of differences in base-pair sequence. Whereas this may be advantageous in cases with limited amounts of evidentiary material, it produces difficulties when substantial heteroplasmic sequences are encountered or when the evidentiary sample presents as a DNA mixture. The ability to resolve and interpret these types of samples would substantially increase the power of mtDNA analysis by allowing its use on more types of forensic cases where the current approach is limited or fails. Several non-DNA sequencing-based technologies have been developed and tested with PCR-amplified mtDNA fragments, including hybridization to linear arrays of immobilized sequence-specific oligonucleotide probes targeted to informative single nucleotide polymorphisms commonly found within the HV1 and HV2 regions (9,10); denaturing gradient gel electrophoresis (11); single-strand conformational polymorphism analysis (12-14); time-of-flight mass spectrometry (5); and microarray-based analysis (16). These approaches have some limitations. For example, sequence-specific oligonucleotide-based strategies are limited to screening for known mitotypes, and cross-hybridization to non-target oligonucleotides (10). The accuracy with which strategies based on the use of denaturing gradient gel electrophoresis and single-strand conformational polymorphism can distinguish among different sequences is often poor compared with DNA sequencing (17). Time-of-flight mass spectrometry and single nucleotide polymorphism primers have been tested to identify mtDNA sequence variants but have been extremely limited in the number of sequence variants that can be reliably scored (15). Denaturing high-performance liquid chromatography offers some advantages to mtDNA analysis and addresses these limitations while still offering the potential to characterize mtDNA sequences in an accurate, rapid, and cost-effective manner.

Denaturing high-performance liquid chromatography allows for comparative nucleic acid sequence detection that is not compromised by the presence of salts, natural mtDNA mixtures, or situational mtDNA mixtures. In its current format, the technique allows comparative sequence analysis based on the chromatographic separation of denatured and renatured nucleic acids. A DNA sample of unknown sequence (questioned sample) is combined with a standard of known sequence (known sample). The mixture is heat denatured and then allowed to renature. Mixtures where the questioned sample is identical to the reference sample will produce only homoduplices upon DNA renaturation, whereas mixtures where the sequence of the questioned sample differs from that of the known sample will produce a combination of homoduplices representing the original components of the mixture and heteroduplices. Heteroduplices are formed as the result of cross-hybridization between the questioned and known samples. Under partially denaturing conditions and an increasing gradient of a nonpolar solvent, such as acetonitrile, it is possible to separate heteroduplices from homoduplices by high performance liquid chromatography (18). Heteroduplices, which are inherently less stable as the result of one or more base pair mismatches, appear as early eluting peaks in the chromatographic profile of a denaturing high-performance liquid chromatography assay (19,20). The sensitivity of denaturing high-performance liquid chromatography-based assays ensures that even very subtle differences in the sequence (ie, single nucleotide substitutions, insertions, and deletions) between the questioned and known samples are readily detected (21,22). When the questioned and known samples consist of identical base pair sequence, the chromatographic profile will exhibit a single (ie, homoduplex) peak, whereas differences between the questioned and known samples will result in multiple (ie, heteroduplex) peaks.

In comparative studies of denaturing high-performance liquid chromatography-based detection of sequence polymorphisms versus alternate sequence detection systems, denaturing high-performance liquid chromatography has provided better sensitivity and accuracy. Relative to denaturing high-performance liquid chromatography, the use of single-strand conformational polymorphism analysis has been shown to detect 50.0-97.5% of mutations depending upon the length and base sequence of the fragment being analyzed (23,24). Similar results were found for other methods, including denaturing gradient gel electrophoresis and protein truncation tests (19-26). The overall concordance between sequence detection by denaturing high-performance liquid chromatography and direct DNA sequencing ranges from 95% to 100%, and simple adjustments to the run conditions of denaturing high-performance liquid chromatography (temperature or acetonitrile gradient) can eliminate false negatives (27).

We studied the utility of denaturing high-performance liquid chromatography-based nucleic acid separation of mtDNA amplicons from the HV1/HV2 regions of the mtDNA D-loop in a forensic context.

Experiments conducted in our laboratory tested if this approach to mtDNA analysis could result in a rapid method for mtDNA screening between questioned and known samples and function to detect and resolve heteroplasmic and situational mtDNA mixtures.

Material and Methods

MtDNA Extraction from Hair/Sheath Tissue

All aspects of this research were conducted in compliance with U.S. Federal Policy for the Protection of Human Subjects (Basic DHHS Policy for Protection of Human Research Subjects; 56 FR 28003). Individual hair shafts (with sheath tissue) were collected from unrelated subjects who had previously provided informed consent to participate in the study. Individual hairs were assigned numerical codes and stored at -20 °C until DNA extraction. Mitochondrial DNA was extracted from 2-cm segments of each hair (proximal to the scalp), ensuring that all samples consisted of both hair shaft and follicular tissue. Individual hairs were placed in 1.6 mL microfuge tubes containing 500 μL of a 10% Chelex® 100 resin (Bio-Rad Laboratories, Hercules, CA, USA) solution (pH 10.0) prepared with Milli-Q/nanopure water. After brief vortexing, each sample was placed in a boiling water bath for 10 minutes and then centrifuged at 16,000 × G for 5 minutes to pellet the Chelex® 100 resin and any insoluble debris. The supernatant containing the mtDNA was transferred to a new 1.6 μL microfuge tube and stored at -20 °C. Each hair was processed in parallel with a reagent blank as a pair to minimize the possibility of cross-contamination.

Extraction of mtDNA from Proficiency Test Bloodstains

Extracts of mtDNA were prepared from a total of 16 whole blood samples that comprised four mtDNA proficiency tests (test codes: #9815, #99511, #99512, and #00512) prepared by Collaborative Testing Services, Inc. (Sterling, VA, USA). QIAamp® DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) was used to isolate mtDNA according to the manufacturer's protocol. Dried blood samples prepared by the test provider on sterile cotton cloth were suspended in 180 μL of Milli-Q/nanopure water, mixed with 20 μL of proteinase K solution (>600 mAU/mL) and incubated at 56 °C for one hour. Lysis of the cells was then performed in a guanidine hydrochloride proprietary solution, followed by DNA collection on a QIAamp spin column after DNA precipitation by addition of 200 μL of 100% ethanol. Immobilized DNA was washed with 500 mL of a proprietary solution and eluted into 150 μL of Milli-Q/nanopure water. Endogenous inhibitors of *Taq* DNA polymerase, normally associated with whole blood, are generally removed with this DNA isolation and purification procedure, making it possible to amplify target mtDNA fragments in the absence of added bovine serum albumin, which is incompatible with the denaturing high-performance liquid chromatography column matrix.

PCR Amplification of mtDNA

There are previously validated primer sets used in forensics to amplify the HV1 and HV2 regions of the human mtDNA genome (Table 1, ref. 28). These primers result in the amplification of overlapping fragments for the HV1 and HV2 regions with a 278 bp fragment (HV1A) amplified via primer pair A1/B2 and a 271 bp fragment amplified via primer pair A2/B1 (HV1B). Similarly, primer pairs C1/D2 and C2/D1 were used to amplify 278 bp and a 277 bp fragments of the HV2 region designated HV2A and HV2B, respectively. These primer pairs were used for all mtDNA amplifications with a single exception. In one control individual, the docking site for the D2 primer contained a single 3'nucleotide substitution that created a terminal mismatch with the D2 primer, preventing amplification. For this individual, a modified D2 primer was created by shortening the primer on the 3'-end by one nucleotide (ie, 5' GGG GTT TGG TGG AAA TTT TTT 3'), making it possible to amplify the HV2A fragment.

MtDNA amplification reactions were prepared with primers at a final concentration of 1 μmol/L, 0.4 U/10μL reaction of AmpliTaq GOLD® DNA polymerase, AmpliTaq GOLD® Buffer (Applied Biosystems, Foster City, CA, USA) and 200 μmol/L of each dNTP (Stratagene, La Jolla, CA, USA) with 1 μL of mtDNA extract (Chelex® 100 extract for hair or QIAamp® extract for blood). Reactions were carried on an ABI 9700 thermocycler (Applied Biosystems), with a thermal profile consisting of denaturation at 95 °C for 10 minutes, followed by 32 cycles of denaturation at 95 °C for 20 seconds, annealing at 60 °C for 30 seconds, extension at 72 °C for 45 seconds, and final extension at 72 °C for 15 minutes.

Subcloning and DNA Sequencing

The DNA sequence of the mtDNA HV1 and HV2 regions for each individual in the current study was determined by dideoxy chain termination method (29). Subclones, prepared from each sample, consisted of a 1,021 bp fragment containing both HV1 and HV2 regions by using primers A1 and D1. This subclone was used later as a "pure" sequencing template making it possible to obtain the maximum amount of native sequence. PCR reaction components were identical as described above for amplification of the HV1 and HV2 fragments. High-yield PCR products were obtained by using a modified "Touchdown PCR" approach on an ABI 9700 thermocycler (30). PCR conditions for this strategy included a primary PCR step consisting of denaturation at 95 °C for 10 minutes, 4 cycles of denaturation at 95 °C for 30 seconds, annealing at 59 °C for 45 seconds, and extension at 72 °C for 45 seconds. This was followed by 27 touchdown PCR cycles where the annealing temperature was reduced stepwise by 0.1 °C/cycle beginning at 58 °C for cycle one and ending at 55.3 °C on the last cycle. The PCR was completed by final extension incubation at 72 °C for 20 minutes.

Resulting amplification products of 1,021 bp were purified with Wizard® PCR Preps (Promega, Madison, WI, USA), ligated into the pGEM®-T vector (Promega) and electroporated into DH5α electrocompetent cells. Lauria-Bertani agar plates were used to plate the transformed cells. Fifty μg/mL ampicillin and X-gal were used for transformant selection, followed by PCR to identify plasmids containing the proper DNA inserts. Total DNA was isolated from these colonies with alkaline lysis, and plasmid DNA was selected by using glass-filter binding plates (Millipore, Bedford, MA, USA). Plasmid DNA sequencing was done on a CEQ8000® automated DNA sequencer (Beckman-Coulter Inc., Fullerton, CA, USA) with fluorescent dideoxy termination reactions prepared with the DTCS DNA Sequencing Kit (Beckman-Coulter Inc.).

Table 1. Polymerase chain reaction primers targeted to mitochondrial DNA (mtDNA) hypervariable (HV) regions 1 and 2

mtDNA region	Position of the terminal 3' base of the primer	Primer sequence
HV region 1A		
Primer A1	(L15997)	5' CAC CAT TAG CAC CCA 3'AAG CT 3'
Primer B2	(H16236)	5' CTT TGG AGT TGC AGT TGA TG 3'
HV region 1B (HV1B)		
Primer A2	(L16159)	5' TAT TTG ACC ACC TGT AGT AC 3'
Primer B1	(H16391)	5' GAG GAT GGT GGT CAA GGG AC 3'
HV region 2A (HV2A)		
Primer C1	(L48)	5' CTC ACG GGA GCT CTC CAT GC 3'
Primer D2	(H285)	5' GGG GTT TGG TGG AAA TTT TTT G 3'
HV region 2B (HV2B)		
Primer C2	(L172)	5' ATT ATT TAT CGC ACC TAC GT 3'
Primer D1	(H408)	5' CTG TTA AAA GTG CAT ACC GCC A 3'

Heteroduplex Formation and Denaturing High-performance Liquid Chromatography Analysis

Pair-wise mixtures of mtDNA amplicons for denaturing high-performance liquid chromatography analyses were prepared at a 1:1 ratio based on the peak area of the PCR products as determined by high-performance liquid chromatography under non-denaturing conditions. The mixtures were denatured by heating initially to 95 °C for 4 minutes and then reannealed over a period of 45 minutes to a final temperature of 25 °C, with decreasing temperature steps of 1.5 °C/min.

A WAVE® DNA Fragment Analysis System (Transgenomic Inc., Omaha, NE, USA) was used to perform temperature-modulated heteroduplex analysis by denaturing high-performance liquid chromatography (21) using a DNAsep analytical column packed with alkylated nonporous poly(styrene-divinylbenzene) particles (18). Five-μL samples of reannealed DNA products were allowed to bind to the column in an aqueous buffer containing an ion-pairing reagent (0.1 mol/L triethylamine acetate [TEAA], pH 7.0), which acts as a "coupler" between the negatively charged DNA backbone and the alkylated column matrix.

An increasing gradient of acetonitrile at an increased temperatures eluted bound DNA from the column. Optimal temperatures for each of the four-mtDNA fragments used in the study were determined by nearest neighbor analysis with empirical confirmation using a temperature gradient. The HV1A, HV1B, HV2A, and HV2B amplicons were determined to have optimal column temperatures of 58.0 °C, 59.2 °C, 56.5 °C, and 57.0 °C, respectively. Generation of acetonitrile gradients was accomplished by differential mixing of solution A (0.1 mol/L TEAA) and solution B (0.1 mol/L TEAA and 25% acetonitrile). A linear gradient was prepared for the HV1A, HV2A, and HV2B amplicons. It consisted of a 3.5-minute flow from 56% to 65% in solution B at a flow rate of 0.9 mL/min. For the HV1B fragment, a linear gradient from 55% to 64% solution B was prepared in 3.5 minutes at a flow rate of 0.9 mL/min. Eluted DNA was detected by UV absorbance at 260 nm.

The study included several controls, including no-DNA-template PCR control for contamination associated with the PCR process and reagent blank samples to control for contamination from the DNA extraction steps through the denaturing high-performance liquid chromatography assay. Zero-volume injections were used with the instrument for denaturing high-performance liquid chromatography to control for bound residual DNA on the column matrix. All of the above controls were run when analyzing different amplified fragments, or when changing between different column temperatures or gradient profiles.

Mixture Separation and Peak Capture

Mixtures of two mtDNA amplicons for separation by denaturing high-performance liquid chromatography were prepared at a 1:1 ratio, based on the peak area of the PCR products as determined by denaturing high-performance liquid chromatography under non-denaturing conditions. Primer pair C1/D2 was used to amplify the 278 bp HV2A fragment from Chelex® 100 extracts of mtDNA from two maternally unrelated individuals. PCR amplification conditions were identical to those described above *(PCR amplification of mtDNA)*. The mixture of mtDNA amplicons was denatured by heating to 95 °C for 4 minutes and then allowed to cross-hybridize by gradual cooling (1.5 °C/min) over a period of 45 minutes to reach a final temperature of 25 °C. The resulting mixture of HV2A homo- and heteroduplicies was separated with a 3.5-minute linear gradient from 56% to 65% solution B (0.1 mol/L TEAA and 25% acetonitrile) at a flow rate of 0.9 mL/min. Eluted DNA was detected by UV absorbance at 260 nm. Automated fraction collection of the leading edges of first heteroduplex and homoduplex peaks and the trailing edges of second heteroduplex and homoduplex peaks were collected with a FCW-200 Fragment Collector (Transgenomic Inc.). The trailing and leading edges of the aforementioned peaks were not collected to minimize the potential for cross-contamination between adjacent peaks. DNA recovered from individual heteroduplex or homoduplex peaks was dried under vacuum to volatilize all residual TEAA and acetonitrile in preparation for direct DNA sequencing. Sequencing of all four mtDNA fragments was done as described above by using either the C1 or D2 primers.

Results and Discussion

Initial experiments to test the accuracy of denaturing high-performance liquid chromatography for the detection of sequence differences in amplified fragments of human mtDNA with forensic utility were conducted through pair-wise comparisons of the HV1A (278bp), HV1B (271bp), HV2A (278bp), and HV2B (277bp) fragments from multiple humans. The size of these amplicons was well suited to denaturing high-performance liquid chromatography analysis (31). In initial experiments, subclones of the mtDNA control region from human volunteers were used as amplification templates to eliminate heteroplasmy as a potential variable. With the successful completion of the experiments in which subclones were used, a second series of denaturing high-performance liquid chromatography analyses was conducted with mtDNA amplified from single human hairs containing some sheath material.

Tests on 144 pairwise comparisons of human HV1 and HV2 amplicons were used to assess the accuracy of denaturing high-performance liquid chromatography as a means of comparative sequence analysis. Based on DNA sequencing, 38 of the pairwise comparisons involved fragments that were identical to each other, and 106 comparisons were between fragments with base sequence differences at 1 to 12 positions. These differences included single-base substitutions, insertions, and deletions situated internally as well as near the terminus of the amplicons. Base substitutions were also seen in both GC- and AT-rich regions of the PCR amplicon. The 38 comparisons between identical sequences produced chromatograms consisting of a single symmetrical peak representing the homoduplex species formed when amplicon were denatured and allowed to reanneal to themselves.

All of the 106 pairwise comparisons between non-identical mtDNA amplicons were identified by denaturing high-performance liquid chromatography as containing sequence differences, although this required a rescreening of a subset of the HV1B and HV2A amplicons (only those with single chromatographic peak) at a slightly higher column temperature to identify mutations in one especially GC-rich region in each of these amplicons. In this way, denaturing high-performance liquid chromatography assays accurately scored each pair-wise comparison for identity vs non-identity in less than 7 minutes/sample, even in cases where only a single nucleotide out of 279 bases was different. Figure 1 shows an example of a single nucleotide difference (T vs C at position 195 of HV2A) between two individuals, with sequence non-identity indicated in by an early-eluting heteroduplex peak (Fig. 1C).

Plus-minus Screen Using Forensic Proficiency Standards

As an additional test of the accuracy of denaturing high-performance liquid chromatography to score identical vs non-identical fragments of amplified

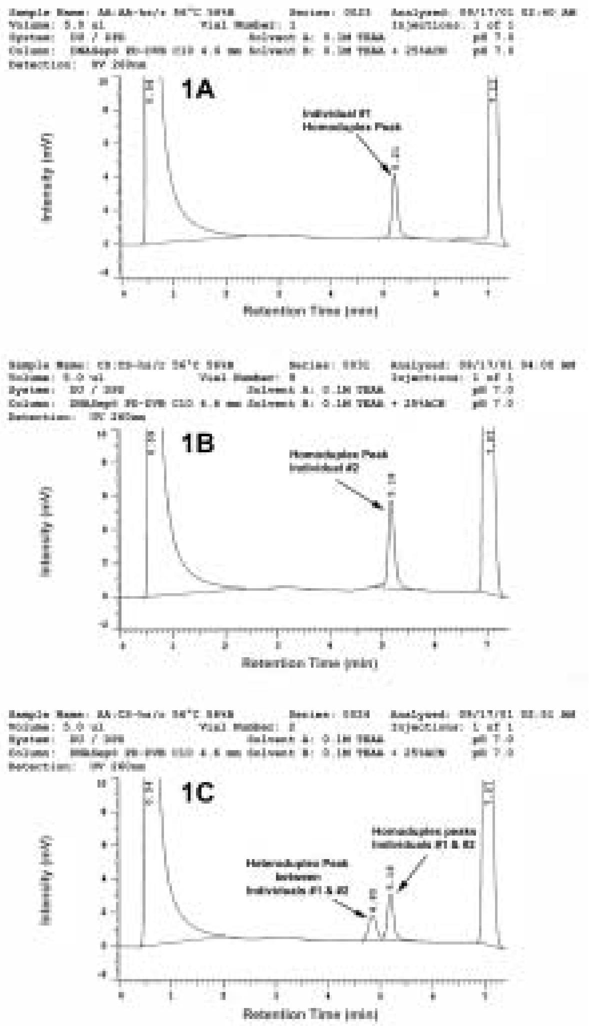

Figure 1. Single nucleotide difference between two individuals. **A.** Homoduplex from individual No. 1. **B.** Homoduplex from individual No. 2. **C.** Heteroduplex formed due to sequence nonidentity (T vs C at position 195 of HV2A) in the HV2A amplicon of individuals No. 1 and No. 2.

mtDNA, pair-wise comparisons were made by using mtDNA amplicons from proficiency test standards prepared by Collaborative Testing Services, Inc. (Sterling, VA, USA). Denaturing high-performance liquid chromatography-based analyses of 4 dried bloodstains (2 known and 2 questioned samples) were performed for each of 4 proficiency tests. This method correctly identified all matches between known and questioned DNA samples. The results were consistent with those reported by Collaborative Testing Services, Inc. and with verification by direct sequencing of the same material. In these experiments, proficiency test number 99-511 posed a special challenge. The presence of both heteroduplex and homoduplex peaks in assays where DNA amplified from the item No. 3 was denatured and allowed to reanneal to itself indicated that item No. 3 contained a mixture of DNA from at least two sources. The observation that pair-wise comparisons between items No. 1 and No. 3, and items No. 2 and No. 3 both produced the same pattern of homo- and heteroduplex peaks suggested that item No. 3 was consistent with a mixture of mtDNA from items No. 1 and No. 2. Additional support was provided by a pair-wise assay of items No. 1 and No. 2 (ie, a reconstruction of the putative mixture), which again produced a peak pattern consistent with that for item No. 3 by itself.

Analysis and Separation of mtDNA Mixtures by Denaturing High-performance Liquid Chromatography

DNA mixtures are common in the forensic analysis of body fluids, particularly samples derived from sex crimes (32). Whereas it is often possible to identify different contributors to a mixture when interpreting the results of nuclear short tandem repeat analyses, it is considerably more difficult with mtDNA (33). With short tandem repeats, different alleles are separated from each other on the electropherogram and peak height serves as an indication of the relative contribution of different alleles to a mixture. However, in mtDNA analyses, sequencing reactions involving mixtures are sensitive to subtle differences in the molecules being analyzed. Individual base substitutions between two components of a mixture yield overlapping peaks at the position of the substitution (34). This requires the forensic scientist to either make a subjective base call or to exclude the base as ambiguous with a resulting decrease in the power of discrimination. The presence of an insertion or deletion polymorphism in one component of a mixture results in sequencing electropherograms that are "out of register" and thus completely unreadable as electrophoretic peaks overlap at every position after the insertion or deletion (35,36).

Our preliminary results have demonstrated the ability of denaturing high-performance liquid chromatography to characterize heteroplasmic and forensic mixtures of DNA molecules and to identify potential contributors through pair-wise analyses and mixture reconstruction assays. Denaturing high-performance liquid chromatography is unique in that it can readily be adapted to the separation of mixtures of DNA molecules even when the molecules to be separated are identical in length. This is because the principle of separation by denaturing high-performance liquid chromatography is based on differential partial denaturation of homo- and heteroduplices, which allows for differential elution of these molecular species from the denaturing high-performance liquid chromatography column (37). Recovery of individual fractions can then be performed manually or through use of an automated platform, and the recovered DNA can be directly sequenced without further amplification.

Optimizing DNA Separation

The temperatures of denaturing high-performance liquid chromatography column and acetonitrile gradients suitable for detecting sequence differences at any position in the HV1 and HV2 mtDNA amplicons have been empirically determined. This has allowed the detection of the heteroduplices in the

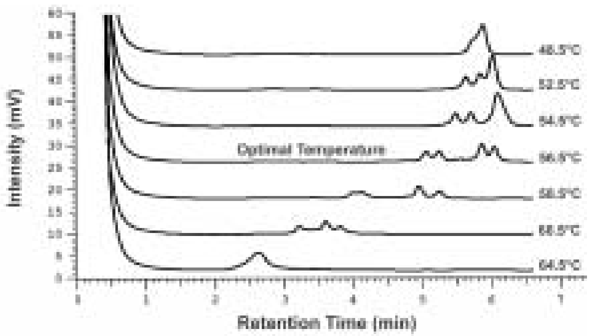

Figure 2. Temperature scan (48.5-64.5 °C) for optimal resolution of the homo- and heteroduplices formed from a mixture of two HV2A fragments that differ at 3 positions.

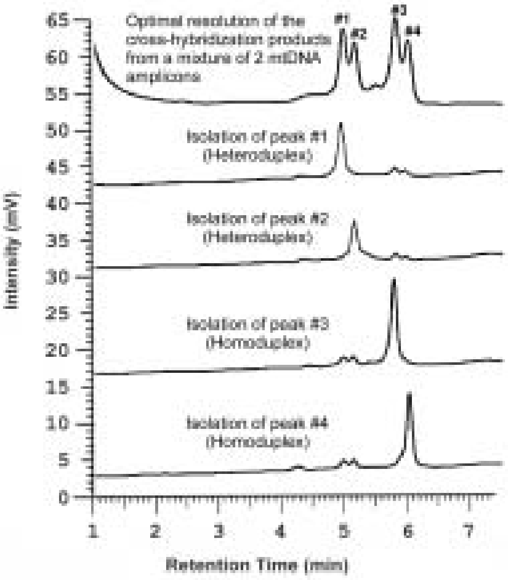

Figure 3. Separation of a 2-contributor DNA mixture into individual homo- and heteroduplex fractions in preparation for DNA sequencing. The presence of single peaks in the denaturing high-performance liquid chromatography assay of individual fractions indicates successful peak capture.

chromatograms of each of the >380 denaturing high-performance liquid chromatography assays that we have conducted to date on mixtures of two different mtDNA amplicons. For any pair-wise comparison, a screen of flanking column temperatures usually results in improved resolution between hetero- and homoduplices (38). In this way, conditions can be identified for the optimal separation of peaks with minimal carryover from adjacent peaks. Figure 2 illustrates a 48.5-64.5 °C temperature scan of the homo- and heteroduplices formed from a mixture of two HV2A fragments that differ by single base substitutions at 3 positions. The resolution of the heteroduplices from each other and from the homoduplices generally improved with increasing temperature. At 48.5 °C, the heteroduplices formed a broad shoulder on the homoduplex peak. Both heteroduplices were individually discernible at or above 52.5 °C and optimal resolution was attained at 56.5 °C. Above 58.5 °C, the resolution was lost as the hetero- and homoduplices denatured.

Fractions containing the mtDNA amplicons present in the individual peaks were collected on a 96-well plate and then desiccated to remove acetonitrile. To minimize carryover between adjacent peaks, the Navigator™ software (VerdaSee Solutions, Inc., Langhorne, PA, USA) made it possible to capture the leading edge of the first peak and the lagging edge of the adjacent peak. Capture of the target peak was confirmed by reanalyzing a sample of the collected material by denaturing high-performance liquid chromatography. Figure 3 illustrates the separation of two homoduplices and two heteroduplices formed by cross-hybridization of the mixed HV2A fragments shown in Figure 2. Following vacuum desiccation, the DNA was ethanol-precipitated and directly labeled with fluorescent dye-terminators for DNA sequencing by capillary electrophoresis. The resulting sequence data confirmed the sequence of the parent components of the mixture with zero ambiguous base calls (data not shown).

These preliminary results demonstrated the power of denaturing high-performance liquid chromatography to rapidly screen the HV1 and HV2 regions of the mtDNA D-loop for match vs non-match characteristics, and more importantly, to resolve mtDNA species amplified from an initial body fluid mixtures often encountered in forensic casework. More research is required to fully determine if this approach based on denaturing high-performance liquid chromatography will be applicable to most types of evidentiary samples encountered in forensic investigations. Of most concern is the ability of the technique to resolve mixtures where there are several differences in primary sequence, ie, greater than 5 differences per amplicon, and if complete four-peak resolution will be possible in most cases. Research is continuing into technology that will allow artificial DNA "heteroduplex forming templates" to be used as standard "rulers" against which all amplified DNA will be compared (ie, from known and questioned samples). Preliminary data suggest that denaturing high-performance liquid chromatography profiles can be constructed from standardized peak retention times, allowing direct comparisons to be made between samples in a digital manner.

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