

## Clinical Applications of Denaturing High-performance Liquid Chromatography-based Genotyping

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**Aim.** To develop and evaluate heteroduplex forming templates (HFTs) as a common set of molecular standards for genotyping by denaturing high-performance liquid chromatography (DHPLC) using hypervariable regions of human mitochondrial DNA (mtDNA) as a model system.

**Methods.** Hypervariable regions 1 and 2 from the mtDNA D-loop of 22 maternally related and unrelated human volunteers were amplified by polymerase chain reaction (PCR) and individually mixed with each of three HFTs. Following denaturation and reannealing of the mixture, the resulting hetero- and homoduplexes were separated by DHPLC using temperature-modulated heteroduplex analysis.

**Results.** Each of three HFTs, when cross-hybridized with a target mtDNA amplicon, induced the formation of an assemblage hetero- and homoduplex peaks, which were uniquely characteristic of a given mtDNA sequence variant. The mtDNA DHPLC profiles obtained in the current study were identical between maternal relatives and different between unrelated individuals – consistent with uniparental maternal inheritance of mtDNA in humans.

**Conclusions.** DHPLC in combination with a common set of HFTs targeted to a locus of interest can be used as a reliable means of genotyping. DHPLC profiles can be readily stored as a bit-coded string of hetero- and homoduplex peak retention times to form a searchable database. This approach to DHPLC genotyping will have immediate utility in extended pedigree analyses, where it will allow rapid sorting and/or confirmation of maternal lineages. Additional applications of DHPLC profiling include the discovery and scoring of clinically relevant nuclear and mitochondrial loci.

**Key words:** chromatography, high-pressure liquid; DNA mutational analysis; genetic diseases, inborn; mutation; polymerase chain reaction; polymorphism, single-stranded conformational; sequence analysis, DNA

The detection of DNA nucleotide polymorphisms has become an increasingly important aspect of basic genetic research, human identification, and molecular diagnostics (1-5). Pathology-associated DNA polymorphisms can take on various forms, including variable number tandem repeat (VNTR) sequences (6,7), insertions, deletions, and base substitutions (8-10). In recent years, the role of point mutations has attracted an increasing amount of attention in gene discovery and gene regulation studies. By examining linkage probabilities in pedigree based studies, variance at sites, known as single nucleotide polymorphisms (SNPs), can provide information regarding gene expression and/or serve as molecular flags for discovery of suspect genetic loci underlying some disease states (11,12). Dense SNP marker maps also have the potential to finely refine the genetic map in most organisms, including humans (13,14).

Screening methods for DNA polymorphisms are useful in the detection of inherited diseases in which either a single point mutation or a few known muta-

tions account for the majority of observed cases (e.g., cystic fibrosis). Hundreds of genetic disorders can now be diagnosed by using recombinant DNA-based technologies. Additionally, the field of forensic identification has been revolutionized by modern molecular biological techniques that have made it possible to characterize polymorphisms in the genomes of humans and a number of animal species (15-17). Several techniques exist for detection of polymorphic sites within a genome. These include RNase A cleavage (18), chemical cleavage (19), denaturing gradient gel electrophoresis (20,21), single-stranded conformation polymorphism (22,23), allele-specific oligonucleotide probes (24,25), ligase-mediated detection (26), and denaturing high-performance liquid chromatography (DHPLC). Most of these methods exploit characteristics of mismatched heteroduplexes formed between normal and mutant sequences.

RNase cleavage uses the ribonuclease A to cut RNA-DNA hybrids wherever a mismatch exists between a nucleotide in the fluorescent or radioactive

RNA probe strand and the corresponding nucleotide in the DNA strand. The chemical cleavage method is based upon a similar principle but uses hydroxylamine and osmium tetroxide to distinguish between mismatched C and T nucleotides, respectively. However, only about 70% of mutations are detected with the cleavage method (18).

Denaturing gradient gel electrophoresis relies on differential electrophoresis of homo- and heteroduplex double-stranded DNA under denaturing conditions of increasing concentration until the last duplex domain is denatured, and migration of the DNA through the gel is retarded. DNA sequences differing by as little as a single base pair migrate at different rates along the gel. Single-stranded conformation polymorphism screening is another gel-based approach whereby DNA is denatured, reannealed, and run on a non-denaturing gel. The secondary structures of DNA strands differing by one or more bases usually result in different electrophoretic mobilities.

Allele-specific oligonucleotide probes are designed to selectively hybridize to normal or mutant alleles. However, these probes can only detect previously known polymorphisms, and stringent hybridization conditions are necessary to accurately distinguish single base-pair mismatches. The ligation of oligonucleotide probes can also be used to detect mutations based on the fact that the ends of two single strands of DNA must be exactly aligned for DNA ligase to join them. A single base pair mismatch occurring at the junction between two oligonucleotides is sufficient to prevent the ligation reaction from occurring.

The aforementioned methods are generally time consuming, necessitate multiple handling steps, require product purification, are not readily adaptable to automation, and are typically limited to detection in small-sized nucleic acid fragments. DHPLC provides an alternative approach to mutation detection that can be easily automated and not limited to previously known mutations (27-29). This represents an especially important feature when examining genes for which a large number of sequence polymorphisms are known to exist and must be scored. The method relies on the differential separation of heteroduplex and homoduplex nucleic acid molecules (e.g., DNA or RNA). A mixture of both hetero- and homoduplex nucleic acids is formed when a mixture of DNA molecules that differ in sequence are denatured and allowed to cross-hybridize with each other. Once the sample mixture is applied to a stationary reverse-phase support, elution with a mobile phase containing an organic solvent under partially denaturing conditions results in the separation of the heteroduplex and homoduplex molecules. As originally conceived, DHPLC is employed as a simple plus/minus assay for the presence of polymorphisms relative to a given reference sequence. While this makes it possible to rapidly determine whether or not two sequences are identical, it does not allow the identification of specific sequence variants in a manner that can be easily entered into a searchable database. Here we use the hypervariable regions of human mitochondrial DNA

(mtDNA) to demonstrate a method whereby specifically engineered heteroduplex forming templates can be used as fixed standards to fully investigate a given nucleic acid target and to generate a unique bit-mapped "profile" or "signature" that can be used to rapidly characterize a target sequence for databanking or genetic polymorphism scoring for molecular diagnostic, forensic, or therapeutic purposes.

### Material and Methods

Hypervariable regions 1 and 2 (HV1 and HV2) from the mtDNA D-loop of 22 maternally related and unrelated human volunteers were amplified by polymerase chain reaction (PCR) and individually mixed with each of three heteroduplex-forming templates. Following denaturation and reannealing of the mixture, the resulting hetero- and homoduplexes were separated by DHPLC using temperature-modulated heteroduplex analysis.

All aspects of the research described herein were conducted in full compliance with U.S. Federal Policy for the Protection of Human Subjects (Basic DHHS Policy for Protection of Human Research Subjects; 56 FR 28003).

#### *MtDNA Extraction from Hair/Sheath Tissue*

Individual hair shafts with sheath tissue attached were collected from healthy subjects who had consented in writing to participation in the study. Following collection, hairs were assigned numerical codes and stored at -20°C until analysis. MtDNA was extracted from the 2-cm segment of each hair closest to the scalp. This ensured that all samples consisted of both hair shaft and sheath 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 nanopure water. After swirling briefly, each sample was placed in a boiling water bath for 10 minutes and then centrifuged for 5 minutes at 16,000 x G to pellet the Chelex® resin and any insoluble debris. The mtDNA-containing supernatant was transferred to a new 1.6-mL microfuge tube and stored at -20°C until needed. To minimize the possibility of cross-contamination between samples, each hair was individually processed in parallel with a reagent blank sample.

#### *PCR Amplification of mtDNA*

All heteroduplex forming templates and the HV1 and HV2 regions of the mitochondrial genome were amplified with primers validated previously for forensic applications (30). The primers enable the amplification of overlapping fragments of the HV1 and HV2 regions of human mtDNA. Based on the Anderson sequence (31), primer pairs A1/B2 and A2/B1 were used to amplify a 278 bp and a 271 bp fragment of the HV1 region designated HV1A and HV1B, respectively. Similarly, primer pairs C1/D2 and C2/D1 were used to amplify a 278 bp and a 277 bp fragment of the HV2 region designated HV2A and HV2B, respectively.

The amplification reactions were prepared with primers at a final concentration of 1 µmol/L each, 0.4 Units/10 µL reaction of AmpliTaq GOLD® DNA polymerase, AmpliTaq GOLD® Buffer (Applied Biosystems, Foster City, CA, USA) and 200 µmol/L of each of dNTPs (Stratagene, La Jolla, CA, USA), and 1 µL of Chelex® mtDNA extract. Reactions were carried out in an ABI 9700 thermocycler (Applied Biosystems) using a thermal profile consisting of initial denaturation at 95°C for 10 minutes, followed by 32 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s. After cycling, there was a final extension at 72°C for 15 minutes.

#### *Subcloning and DNA Sequencing*

The DNA sequence of the heteroduplex forming templates and the mtDNA HV1 and HV2 regions for each individual in the study were determined by dideoxy chain termination method (32). Subclones of the approximately 1,021 bp fragment of mtDNA amplified by primers A1 and D1, which encompass both HV1 and HV2, served as the sequencing template. The PCR reaction components were the same as described previously for amplification of the smaller HV1 and HV2 fragments. To facilitate a high yield of amplified product, a modified "Touchdown PCR" thermal profile (33) was employed on an ABI 9700 thermocycler.

Following an initial denaturation at 95 °C for 10 minutes, 4 cycles of denaturation were performed at 95 °C for 30 s, annealing at 59 °C for 45 s, and extension at 72 °C for 45 s. This was then followed by 27 touchdown cycles in which the annealing temperature was reduced by 0.1 °C/cycle, starting at 58 °C on the first cycle and ending at 55.3 °C on the last cycle. The reaction ended following a final extension at 72 °C for 20 minutes.

The resulting amplification products were purified with Wizard® PCR Preps (Promega, Madison, WI, USA), ligated into the pGEM®-T vector (Promega), and electroporated into DH5 $\alpha$  cells. Cells were plated onto Lauria-Bertani (LB) agar plates containing 50  $\mu$ g/mL ampicillin and X-gal, and colonies of transformed cells were screened by PCR to identify plasmids containing inserts of the desired size. Overnight cultures of these colonies were subjected to alkaline lysis and plasmid DNA was isolated by using glass-filter binding plates (Millipore, Bedford, MA, USA). Purified plasmid DNA was labeled with fluorescent dideoxy terminators using the DTCS DNA Sequencing kit (Beckman-Coulter Inc., Fullerton, CA, USA) and sequenced on a CEQ2000XL® automated DNA sequencer (Beckman-Coulter).

#### *Heteroduplex Formation and DHPLC Analysis*

Heteroduplex forming templates employed in the study were empirically selected on the basis of their ability to form easily discernible heteroduplexes with the target mtDNA amplicons. A total of three heteroduplex forming templates were selected for formation of heteroduplexes with each of the four target mtDNA amplicons. For DHPLC analyses, all mixtures 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 at 95 °C for 4 minutes and then allowed to reanneal for 45 minutes during which the temperature of the mixture was decreased by 1.5 °C per minute to a final temperature of 25 °C.

Temperature-modulated heteroduplex analysis by DHPLC (34) was performed on a WAVE® DNA Fragment Analysis System (Transgenomic Inc., Omaha, NE, USA) containing a DNasep analytical column packed with alkylated nonporous poly(styrene-divinylbenzene) particles (35). Aliquots (5  $\mu$ L) of the reannealed products were allowed to bind to the column in an aqueous buffer containing 0.1 mol/L triethylamine acetate (TEAA) pH 7.0, which acts as an ion-pairing reagent between the negatively charged backbone of the DNA and the alkylated column matrix.

Bound DNA was eluted from the column with an increasing gradient of acetonitrile at elevated temperature. The optimal temperature for each of the four mtDNA fragments used in the current study was estimated by nearest neighbor analysis and confirmed empirically with a temperature gradient. The optimal column temperatures for the HV1A, HV1B, HV2A, and HV2B amplicons were 58 °C, 59.2 °C, 56.5 °C, and 57 °C, respectively. Acetonitrile gradients were generated by differential mixing of solution A (0.1 mol/L TEAA) and solution B (0.1 mol/L TEAA and 25% acetonitrile). For the HV1A, HV2A, and HV2B amplicons, a linear gradient from 56% to 65% solution B was used in 3.5 minutes at a flow rate of 0.9 mL/min. For the HV1B fragment, a linear gradient from 55% to 64% solution B was used in 3.5 minutes at a flow rate of 0.9 mL/min. Eluted DNA was detected by UV absorbance at 260 nm.

DHPLC controls included zero-volume injections to screen for residual DNA on the column matrix and no-template PCR controls to check for contamination associated with the PCR process and reagent blank samples to check for contamination at all other steps in the process from DNA isolation through the DHPLC assay. All controls were run when switching between amplified fragments, column temperatures or gradient profiles.

## **Results and Discussion**

Portions of the region of the mitochondrial genome have been designated "hypervariable regions" (HV1 and HV2) because of the large number of polymorphisms that exist within human populations. Single base substitutions account for most of the base sequence variability, although insertions and dele-

tions are also seen. As such, these regions provide an excellent model system to test the reliability of heteroduplex forming template-based genotyping, especially for medically important loci where polymorphisms may be broadly distributed.

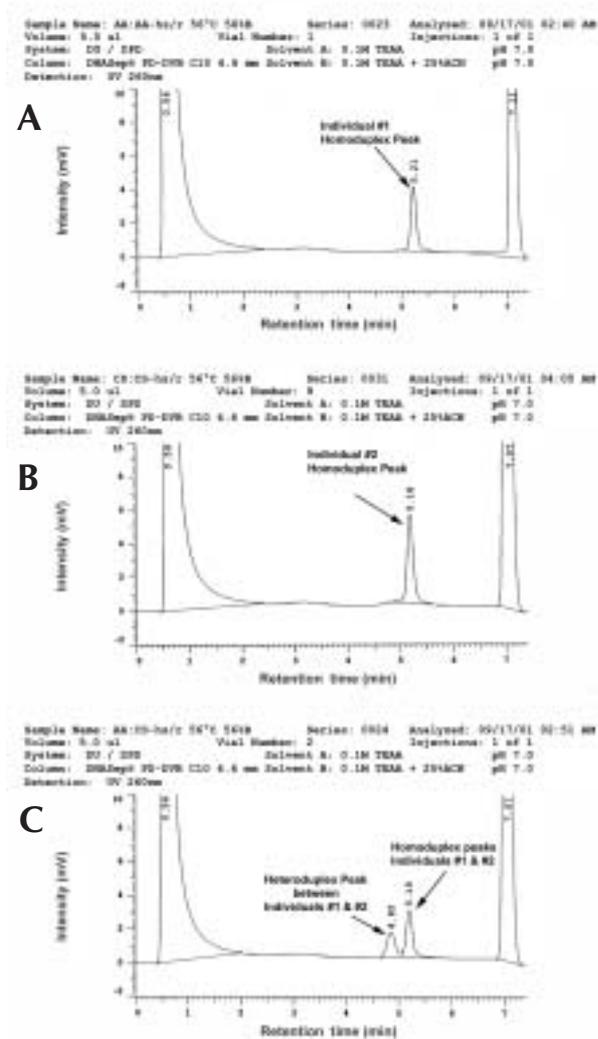
As currently used in research, DHPLC is employed as a method of rapidly determining whether or not a test sample is identical to or different from a given reference sample. When DNA amplified from a given sample is mixed with DNA of a known sequence (wild type or a specific mutant allele of choice), the appearance of a single, ie, homoduplex, peak in the resulting chromatograph indicates identity between the samples, whereas the appearance of an early eluting heteroduplex peak indicates non-identity. This simple plus-minus assay can enable clinical laboratories to sort wild-type from mutant sequences rapidly and economically and focus often expensive genotyping efforts on the characterization of mutant sequences.

In this study, mtDNA sequences amplified from the D-loop of 22 individuals served as a model system for testing the ability of DHPLC to accurately detect a broad range of sequence variants, including insertions, deletions, and substitutions in both GC and AT rich regions of DNA. In pairwise comparisons of mtDNA from eight unrelated human volunteers, sequence differences in all four of the mtDNA amplicons were identified in less than four minutes/assay. As confirmed by direct sequencing, DHPLC accurately detected every sequence variant, even in cases where only one nucleotide out of 279 bases was different, ie, it showed a single nucleotide difference in the HV2A region (36) between a reference and a test sample (Fig. 1). When the amplified fragments from the HV2A region are mixed in equimolar amounts, a heteroduplex peak resulting from a single base mismatch (T $\rightarrow$ C) at position 195 is detected (Fig. 1C).

The presence and retention time of the hetero- and homoduplex peaks is highly reproducible for any given temperature and acetonitrile gradient. Based on analyses of 10 replicate samples, the run-to-run variability in peak retention times varied with a standard deviation of 0.050 to 0.055 minutes. Normalizing peak retention times to the last eluting peak further reduced run-to-run variability to a maximum standard deviation of 0.009 minutes.

#### *DHPLC-based Genotyping*

While pairwise comparisons with wild-type make it possible to screen for non-wild type sequences, the identification of a specific mutant would require a series of pairwise comparisons between a given test sample and every known sequence polymorphism reported to date. The presence of a single homoduplex peak for any mixture would indicate identity between the test sample and the specific sequence variant with which it had been cross-hybridized. However, for highly polymorphic sequences, this could require dozens to hundreds of pairwise comparisons. Even this would not allow a novel mutation to be characterized.



**Figure 1.** Denaturing high performance liquid chromatography (DHPLC) analysis of hypervariable regions (HV) 2A defined by polymerase chain reaction (PCR) primers L048/H285. **A.** Homoduplex from individual 1 (reference sample). **B.** Homoduplex from individual 2 (test sample). **C.** Heteroduplex from equimolar mixture of the reference and test samples demonstrating detection of single base difference at position 195.

Recognizing that such exhaustive pairwise comparisons between a given test sample and all known sequence variants would be time consuming and resource intensive, the current study has used the hypervariable regions of human mtDNA as a model to examine the feasibility of using DHPLC-based strategies to generate highly reproducible chromatographic profiles that uniquely define a given genotype. Rather than relying on direct pairwise comparisons to identify matched versus unmatched samples, test samples will be cross-hybridized to a common set of control DNA molecules, ie, heteroduplex forming templates. This negates the need to run all possible pairwise comparisons and it should allow the determination of a unique DHPLC chromatographic profile for virtually any amplifiable fragment of polymorphic DNA of clinical interest. The resulting peak re-

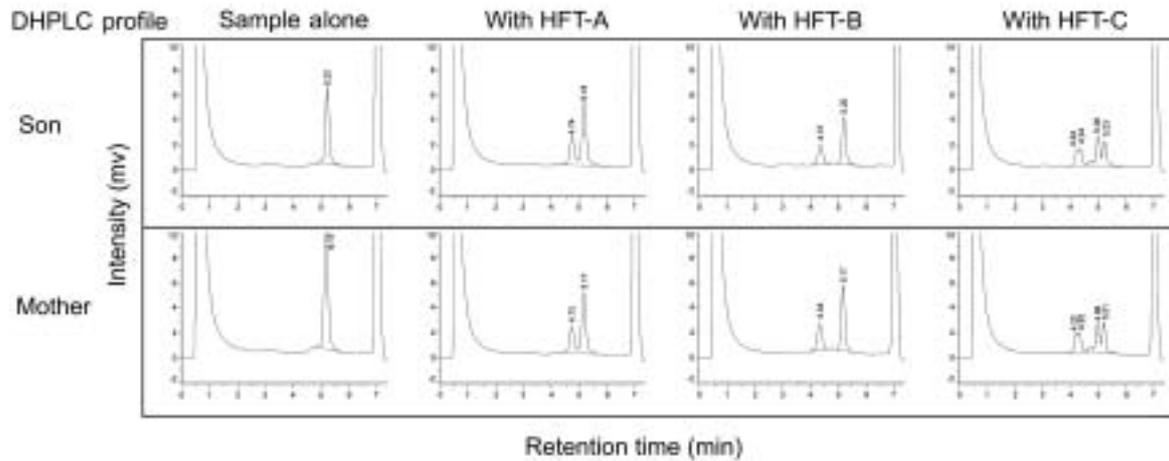
tention times of both hetero- and homoduplexes comprise a bit-coded data set capable of uniquely characterizing each mitotype, which could thus be easily compiled into searchable databanks. The use of multiple heteroduplex-forming templates per amplicon ensures optimal discrimination and internal redundancy.

An initial set of 12 heteroduplex-forming templates (three heteroduplex forming templates for each mtDNA amplicon) were empirically selected through 144 pairwise comparisons of human mtDNA based on the ability of a given fragment to induce the formation of clearly discernable (by DHPLC) homo- and heteroduplexes when paired with a broad range of sequence. These empirically selected heteroduplex-forming templates were then used in experiments with seven pairs of maternally related and unrelated individuals to demonstrate the feasibility of the DHPLC profile approach.

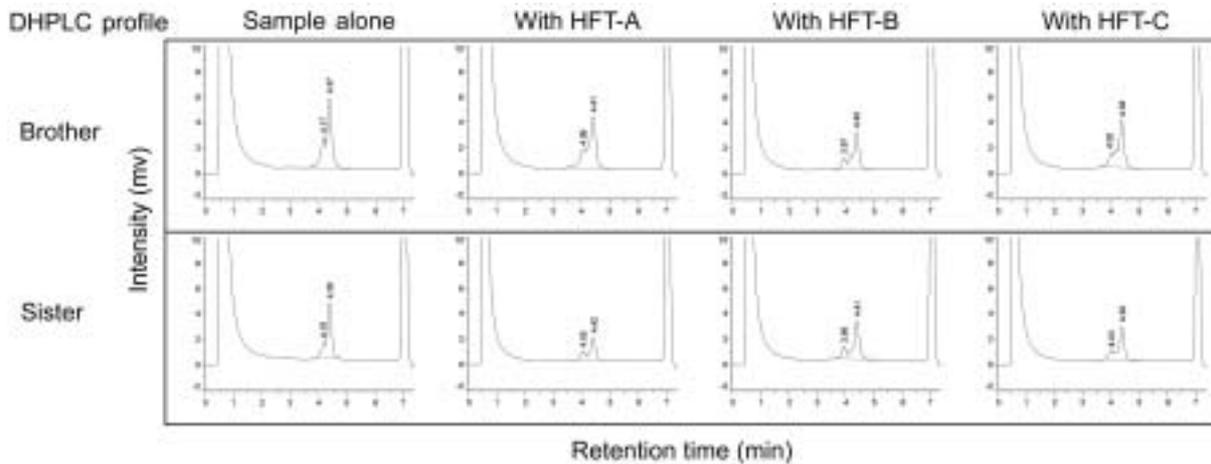
To illustrate the reproducibility of profiling using heteroduplex-forming templates in the current example, the heteroduplex-forming template profiles for mtDNA fragment HV2A were generated for a maternally-related pair, ie, mother and son. Here, both individuals would be expected to possess the same mtDNA sequences since mtDNA is uniparentally inherited through the maternal lineage. Figure 2 shows a comparison of DHPLC-profiles for the HV2A mtDNA fragment from the mother and son pair based on the use of three heteroduplex forming templates. The chromatographs for these individuals yielded identical heteroduplex forming templates profiles based on the resulting retention times for individual hetero- and homoduplexes formed through cross-hybridization between the test samples and each of three heteroduplex forming templates.

The resulting DHPLC profile for the HV2A fragment generated by heteroduplex-forming templates can be stored as a bit coded series of homo- and heteroduplex peak retention times (Table 1). Alternatively, peak-to-peak distance could also be used as a means of describing numerically the assemblage of DHPLC peaks that comprise the DHPLC profile for HV2A. However, the use of peak retention times provides more data points for comparison than the peak-to-peak distances. These numerical representations of the DHPLC profile for any sequence allow it to be compared to a library of DHPLC profiles for known polymorphisms. This makes it possible to score for specific polymorphisms without the need to actually conduct pairwise comparisons between matching sequences.

A key difference between genotyping of nuclear loci and the mtDNA is the potential for heterozygosity. Although mtDNA is generally homoplasmic, de novo mutations may arise and proliferate within the mtDNA population of a single individual. This results in the existence of two distinct mtDNA sequences within a single individual, ie, heteroplasmy. In our study, heteroplasmy can serve as a model for testing the ability of heteroduplex forming templates to characterize both nuclear heterozygosity and situations in which there may be partial loss of heterozy-



**Figure 2.** Chromatographs comprising the denaturing high performance liquid chromatography (DHPLC) profile of the hypervariable region 2A (HV2A) fragment of mitochondrial DNA (mtDNA) based on the use of three heteroduplex-forming templates (HFT-A, HFT-B, and HFT-C) for a mother and son pair with mtDNA homoplasmly.



**Figure 3.** Chromatographs comprising the denaturing high performance liquid chromatography (DHPLC) profile of the hypervariable region (HV) 2A fragment of mitochondrial DNA (mtDNA) based on the use of three heteroduplex-forming templates (HFT-A, HFT-B, and HFT-C) for a brother and sister sib pair with inherited mtDNA heteroplasmly.

gosity, as is seen in numerous clinically significant conditions. Heteroplasmly in a test sample is indicated by the presence of more than a single DHPLC peak when an amplified mtDNA fragment is denatured and

allowed to cross-hybridize with itself in the absence of any heteroduplex forming templates.

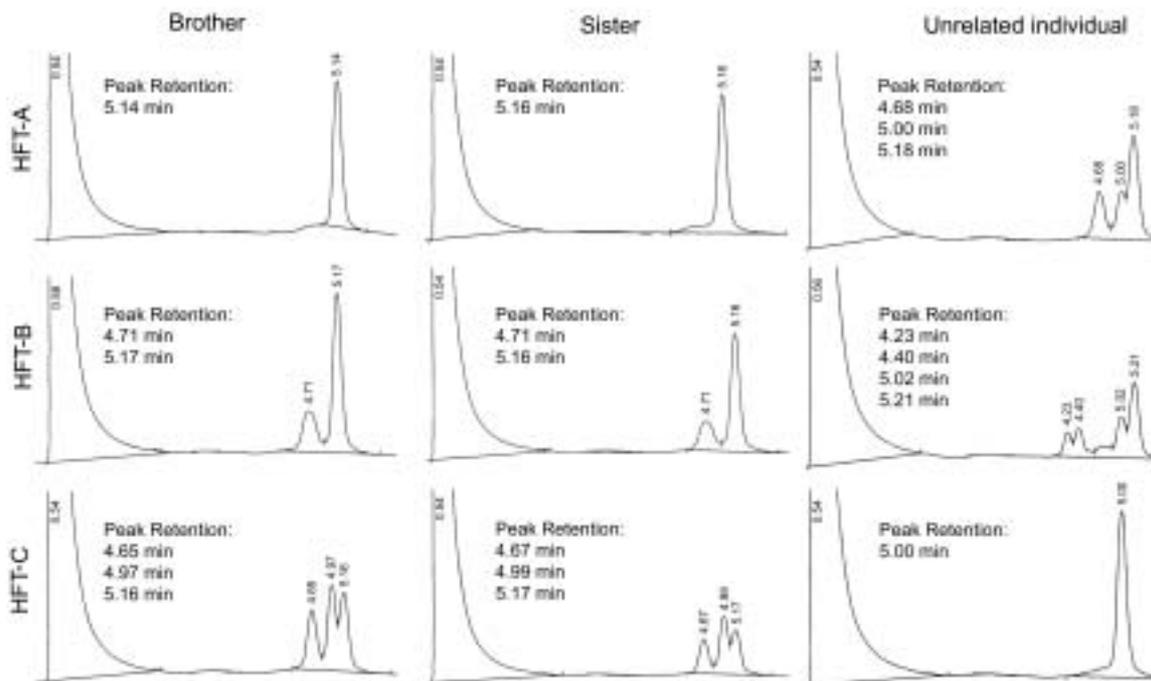
Sequence heteroplasmly is present in the test samples for both individuals in a brother and sister sib pair, as can be seen from the dual peaks in the “sample alone” DHPLC chromatographs (Fig. 3). A comparison of the DHPLC profiles for a maternally-related brother and sister for the HV2A fragment based on the use of three heteroduplex forming templates shows

**Table 1.** Bit coding of the denaturing high performance liquid chromatography (DHPLC) profile for mitochondrial DNA amplicon of the hypervariable region (HV) 2A from Figure 2

DHPLC profile for HV2A	Sample alone	With heteroduplex-forming templates (HFT)		
		A	B	C
Son's profile based on peak retention times	5.22	4.74 5.18	4.37 5.20	4.24 4.34 5.00 5.23
Son's profile based on peak-to-peak distance	n/a	0.44	0.83	0.10 0.66 0.23
Mother's profile based on peak retention times	5.17	4.73 5.17	4.34 5.17	4.22 4.32 4.97 5.20
Mother's profile based on peak-to-peak distance	n/a	0.44	0.83	0.10 0.65 0.23

**Table 2.** Bit coding of the denaturing high performance liquid chromatography (DHPLC) profile for mitochondrial DNA amplicon of the hypervariable region (HV) 2A from Figure 3

DHPLC profile for HV2A	Sample alone	With heteroduplex-forming templates (HFT)		
		A	B	C
Son's profile based on peak retention times	4.17 4.37	4.06 4.41	3.96 4.40	4.01 4.37
Son's profile based on peak-to-peak distance	0.20	0.35	0.44	0.36
Mother's profile based on peak retention times	4.19 4.39	4.05 4.42	3.96 4.41	4.02 4.39
Mother's profile based on peak-to-peak distance	0.20	0.37	0.45	0.37



**Figure 4.** Denaturing high performance liquid chromatography (DHPLC)-profiles and peak retention times generated by cross-hybridization of three heteroduplex-forming templates (HFT-A: top panel, HFT-B: middle panel, and HFT-C: bottom panel) with hypervariable region (HV) 2A amplified mitochondrial DNA from a brother/sister sib pair and a non-maternally related individual. Note the concordance within the sib pair and lack of concordance with an unrelated individual.

that, even though both individuals display heteroplasmic mtDNA, the chromatographs for these individuals yield identical DHPLC profiles with discernable hetero- and homoduplex peaks (Fig. 3). This is consistent with inheritance of the heteroplasmic mtDNA state from a maternal predecessor. Here too the DHPLC-profile can be stored as a numerical series of peak retention times (Table 2) or peak-to-peak distances that provide a bit coded representation of the assemblage of DHPLC peaks that comprise the overall DHPLC profile for these HV2A amplicons.

In the same way that identical sequences would be expected to yield identical DHPLC profiles, sequences that differ in base pair sequence would be expected to yield unique DHPLC profiles. This is the principle under which it should be possible to use DHPLC profiles generated by using heteroduplex-forming templates to score specific mutations. Figure 4 shows a comparison of the DHPLC profiles and corresponding peak retention times for the HV2A fragment generated through the use of three heteroduplex-forming templates for a second brother sister sib-pair, expected to have identical DHPLC profiles due to the maternal inheritance of mtDNA, and an unrelated individual, expected to have a non-identical DHPLC profile. Based on the total assemblage of peak retention times, the DHPLC-profiles generated by heteroduplex-forming template A (Fig. 4, top panel), heteroduplex forming template B (Fig. 4, middle panel), and heteroduplex forming template C (Fig. 4, bottom panel) are identical for the sib-pair but clearly different for the unrelated individual. Using three heteroduplex forming templates targeted to each of the three remaining amplified fragments from the

HV1 and HV2 regions of mtDNA for the two brother and sister sib pairs and the mother and son pair described above, DHPLC profiles were obtained that provided clear discrimination among all sequence variants (data not shown).

In conclusion, heteroduplex forming templates can serve as a predefined set of common "rulers", which are able to induce the formation of an assemblage of homo- and/or heteroduplexes that are a unique (ie, defining) characteristic of a given target nucleic acid sequence. Databases of DHPLC profiles stored as defined peak retention times makes it possible to associate a specific profile with a defined sequence variant, ie, to genotype samples without the need to perform exhaustive pairwise analyses. Based on the results reported in the current study, the use of DHPLC to accurately characterize sequence variants in the hypervariable regions of human mtDNA will find immediate utility in extended pedigree analyses where DHPLC profiles can be used to rapidly identify, sort, and/or confirm maternal lineages. The results of the current study also point to the potential utility of DHPLC profiling for the analysis of other clinically relevant mtDNA loci. For example, heteroduplex forming templates could be used to rapidly discriminate among the many sequence variants of the ND6 gene, a mutational hotspot linked to Leber's hereditary optic neuropathy (37). Finally, DHPLC profiles have potential utility for genotyping homozygous and heterozygous nuclear loci. The approach may be especially suited for use with highly polymorphic genes, such as connexin 26, which has been linked to nonsyndromic sensorineural hearing loss and for which at least 84 distinct polymorphisms

have already been identified (38). Future studies will focus on the development of DHPLC-based approaches to tracing paternal lineages through the Y chromosome loci and on the sensitivity of the approach for studying mtDNA heteroplasmy and nuclear loss of heterozygosity.

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