J Forensic Sci, January 2009, Vol. 54, No. 1
doi: 10.1111/j.1556-4029.2008.00924.x
Available online at: www.blackwell-synergy.com

TECHNICAL NOTE

Pamela G. Jarman,[1,‡] *M.Sc.; Sherri L. Fentress,*[1] *M.S.; and Daniel E. Katz,*[1,§] *M.S.F.S.*

Mitochondrial DNA Validation in a State Laboratory*,†

ABSTRACT: Because of the inception of the FBI Regional mitochondrial DNA (mtDNA) laboratories, many do not see establishing state/local mtDNA processing laboratories as a priority. Yet there is a long-term need for mtDNA processing that will exceed the capabilities of the FBI Regional mtDNA laboratories and the few other laboratories that are currently processing mtDNA, and that need can be fulfilled by state/local laboratories. Thus, the DNA Unit of the Delaware Office of the Chief Medical Examiner (OCME-DNA Unit) completed validation of in-house mtDNA testing in January 2007. The validation plan for mtDNA processing included the following sections: preliminary research, sensitivity and contamination studies, ExoSAP-IT® optimization, BigDye® optimization, sequencing and 310 optimization, sample preparation and extraction optimization, heteroplasmy, mixtures, and reproducibility. All sections of the validation were successfully completed, and mtDNA processing of skeletal remains, teeth, and hairs, as well as blood and buccal reference samples was adopted by the OCME-DNA Unit.

KEYWORDS: forensic science, mitochondrial DNA sequencing, hypervariable regions I and II, Delaware Office of the Chief Medical Examiner, validation

The majority of DNA profiling methods currently performed in forensic laboratories examine nuclear DNA (nucDNA) utilizing short tandem repeat (STR) technology. Although STR profiling has great discriminatory power and is the method of choice when possible, mitochondrial DNA (mtDNA) is a useful alternative when nucDNA processing is not possible. There are thousands of copies of mtDNA in each cell compared with two copies of nucDNA, making mtDNA analysis a more sensitive test, and thus more successful on both highly degraded specimens and those containing minimal amounts of nucDNA. Furthermore, the exclusively maternal inheritance pattern of mtDNA means that only one maternal relative, who need not be immediate family, is needed as a reference. The sensitivity of the testing and the method of inheritance make mtDNA particularly well suited for identification and re-association purposes, as well as for analysis of hair evidence (1,2).

Four FBI regional mtDNA laboratories opened for testing between 2005 and 2006 with the purpose of aiding the FBI by providing free mitochondrial testing for state and local laboratories. Many believe that the availability of these laboratories precludes the need to establish state/local mtDNA processing laboratories. However, the identification of missing persons is rapidly becoming a national priority and relies heavily upon the use of mtDNA. There is a long-term need for mtDNA processing that will exceed the capabilities of the FBI Regional mtDNA laboratories and the few other laboratories that are currently processing mtDNA, and that need can be fulfilled by state/local laboratories. Additionally, mtDNA testing at the state/local level will support the institution and success of CODIS + Mito, the software program developed for the FBI as a search tool for felons as well as missing persons. With CODIS + Mito, laboratories are given the capability to upload mitochondrial data to perform searches of unidentified human remains profiles against other unidentified human remains profiles or to perform regimented familial searches in an effort to identify the remains based on profiles of relatives that have been uploaded into the missing persons database. This capability is in addition to the standard forensic searches that could be performed with prior versions of the CODIS software (3).

The availability of grant funding allowed the DNA Unit of the Delaware Office of the Chief Medical Examiner (OCME-DNA Unit) to implement mtDNA processing to aid the medical examiner in the identification of remains and also to support criminal investigations involving hair evidence (hair shafts or hairs with insufficient roots for nucDNA testing). Because of the lack of state/local laboratories with mtDNA capabilities, the OCME-DNA Unit found itself with no "role model" laboratory. The FBI regional mtDNA laboratories and the few other laboratories that were currently processing mtDNA were equipped to process more samples than the OCME-DNA Unit expected to have as a caseload, making it impractical and cost prohibitive to mimic their laboratory set-up and mtDNA processes.

As the validation plan was initiated, it became apparent that some decisions and choices would have to be made prior to laboratory experimentation as it was often cost prohibitive to purchase multiple options to do comparison studies. Whenever possible, however, experiments were conducted to distinguish between specific techniques and methodologies. The validation plan for mtDNA processing was separated into the following sections: Preliminary Research, Sensitivity and Contamination, ExoSAP-IT®

[1]DNA Unit, Delaware Office of the Chief Medical Examiner, 200 South Adams Street, Wilmington, DE 19801.

*This work was presented in part as a poster at the 58th Annual Meeting of the American Academy of Forensic Sciences in February 2006.

†Financial support for this study was provided by Delaware Criminal Justice Council Byrne Grant.

‡Present Address: DNA Solutions Pty. Ltd., 4 Eastgate Court, Wantirna, Vic. 3152, Australia.

§Present Address: Maryland State Police—Forensic Sciences Division, Forensic Biology Unit, 221 Milford Mill Road, Pikesville, MD 21208.

Received 25 Aug. 2007; and in revised form 21 June 2008; accepted 2 Aug. 2008.

Optimization, BigDye® Optimization, Sequencing and 310 Optimization, Sample Preparation and Extraction Optimization, Heteroplasmy, Mixtures, and Reproducibility.

Preliminary Research

The first step in developing mtDNA methodology was to determine the current backlog of mtDNA cases as well as what could be expected for a typical monthly/yearly caseload once that backlog was eliminated. This caseload prediction was critical in considering finances and labor because some options for certain stages of testing had the potential to decrease labor, and therefore turnaround time, but could cost many fold more than the alternatives.

The types of samples predicted for mtDNA processing by the OCME-DNA Unit included skeletal remains, teeth, and hair (hair shafts or hairs with insufficient roots for nucDNA testing). The preparation of skeletal remains and teeth required that the sample be pulverized to extract the DNA. Two ways in which this could be accomplished were via stainless steel blender cups with a professional grade blender (Waring® Laboratory Science, Torrington, CT) or by way of a freezer mill (4) (SPEX CertiPrep®, Incorporated, Metuchen, NJ). The decision was made to purchase a freezer mill, partially for the possible benefits the freezer mill could provide for processing difficult/low template nucDNA samples, although it should be noted that both options would have been appropriate for mtDNA processing at the OCME-DNA Unit.

From the outset of this validation project, one of the few clear goals was to validate a nonorganic extraction method. The advantages would be numerous, including time efficiency and the elimination of hazardous chemicals (phenol, chloroform, and isoamyl alcohol) from the protocol that is currently used to extract nucDNA samples at the OCME-DNA Unit. The decision was made to utilize Qiagen QIAamp® DNA Blood Mini/Midi columns (Qiagen, Incorporated, Valencia, CA) based upon cost comparisons and the ability to efficiently remove contaminants and inhibitors (5).

Methods

Sensitivity and Contamination

An amplification scheme was proposed to amplify two hypervariable regions (HVI and HVII) of the mtDNA genome. The amplification plan included multiple forward and reverse primers (see Table 1 for primer sequences) that, when used in various combinations (see Table 2 for primer pairs), would yield amplicons ranging from approximately 85 base pairs (bp) in length to approximately 230 bp in length. With this amplification scheme a minimum of four amplifications would be required to amplify the entire HVI and HVII regions (primer set [PS] 1 and PS2 combine to yield HVI while PS3 and PS4 cover HVII). If the sample was more degraded such that smaller amplicons would be necessary, each PS could be amplified as two smaller amplicons termed mini primer sets (MPS) (i.e., MPS1A and MPS1B combine to yield PS1) (6,7). The efficiency and sensitivity of the Linear Array mtDNA HVI/HVII Region Sequence Typing Kit (HVI/HVII duplex; Roche Applied Science, Indianapolis, IN) was also explored, as this mix was designed to amplify the full HVI and HVII regions in a single amplification reaction (8,9).

A range of 0.01–800 pg of positive control DNA, HL60 (ATCC®, Manassas, VA), was amplified (1x AmpliTaq® Gold polymerized chain reaction [PCR] Master Mix [Applied Biosystems, Foster City, CA], 0.025 μg/μL bovine serum albumin, and 0.4 μM each primer) with each set of proposed amplification

TABLE 1—*Proposed mtDNA amplification and sequencing primer sequences.*

Region	Primer	Sequence (5′ to 3′)*
HVI	F15989	CCCAAAGCTAAGATTCTAAT
	F16112	CACCATGAATATTGTACGGT
	F16190	CCCCATGCTTACAAGCAAGT
	F16222	CCTCAACTATCACACATC
	F16268	CACTAGGATACCAACAAACC
	R16158	TACTACAGGTGGTCAAGTAT
	R16251	GGAGTTGCAGTTGATGT
	R16322	TGGCTTTATGTACTATGTAC
	R16400	GTCAAGGGACCCCTATCTGA
	R16410	GAGGATGGTGGTCAAGGGA
HVII	F15	CACCCTATTAACCACTCACG
	F34	GGGAGCTCTCCATGCATTTGGTA
	F109	GCACCCTATGTCGCAGTATCTGTC
	F140	CCTGCCTCATCCTATTATTTA
	F151	CTATTATTTATCGCACCT
	F155	TATTTATCGCACCTACGTTC
	F220	TGCTTGTAGGACATAATAAT
	R159	AAATAATAGGATGAGGCAGGAATC
	R240	TATTATTATGTCCTACAAGCA
	R285	GTTATGATGTCTGTGTGGAA
	R292	ATTTTTTGTTATGATGTCT
	R381	GCTGGTGTTAGGGTTCTTTG
	R389	CTGGTTAGGCTGGTGTTAGG

*All primers ordered HPLC purified.
Primer sequences and nomenclature obtained from the AFDIL.
AFDIL, Armed Forces DNA Identification Laboratory; mtDNA, mitochondrial DNA.

TABLE 2—*Proposed mtDNA amplification primer pairs and their associated thermal cycler parameters.*

Region	Thermal Cycler Parameters
HVI/HVII Duplex	94°C 14 min 92°C 15 sec, 59°C 30 sec, 72°C 30 sec 33–38 cycles* 72°C 10 min
PS1 (F15989/R16251), PS2 (F16190/R16410), PS3 (F15/R285), PS4 (F155/R389)	96°C 10 min 94°C 20 sec, 56°C 20 sec, 72°C 30 sec 38 cycles
Alternate PS4 (F140/R389)	96°C 10 min 94°C 20 sec, 51°C 20 sec, 72°C 30 sec 38 cycles
MPS1A (F15989/R16158)	96°C 10 min 94°C 20 sec, 50°C 20 sec, 72°C 30 sec 38 cycles
MPS1B (F16112/R16251)	96°C 10 min 94°C 20 sec, 50°C 20 sec, 72°C 30 sec 42 cycles
MPS2A (F16190/R16322)	96°C 10 min 94°C 20 sec, 46°C 20 sec, 72°C 30 sec 38 cycles
MPS2B (F16222/R16410) or (F16268/R16410)	96°C 10 min 94°C 20 sec, 48°C 20 sec, 72°C 30 sec 42 cycles
MPS3A (F34/R159)	96°C 10 min 94°C 20 sec, 54°C 20 sec, 72°C 30 sec 38 cycles
MPS3B (F109/R240) MPS4B (F220/R389)	96°C 10 min 94°C 20 sec, 46°C 20 sec, 72°C 30 sec 42 cycles
MPS4A (F151/R292)	96°C 10 min 94°C 20 sec, 45°C 20 sec, 72°C 30 sec 38 cycles

*Range set by manufacturer, optimized at 36 cycles during validation.
MPS, mini primer sets; mtDNA, mitochondrial DNA; PS, primer set.

primers to determine the efficiency and sensitivity of the primer pairs. A similar range of HL60 template amounts was amplified with the HVI/HVII duplex according to manufacturer's recommendations. Concurrently, the HVI/HVII duplex underwent optimization of the number of cycles (33, 34, 35, 36, 37, and 38) for the thermal cycler program. The results of each amplification throughout this validation were observed by running 5 μL of product on a 2% agarose gel containing ethidium bromide alongside a mass ladder (GeneChoice® DNA Ladder II; PGC Scientifics, Frederick, MD) to verify the correct size of the amplification products and to estimate the concentration of said products (verbal communication Dr. Timothy McMahon, Armed Forces DNA Identification Laboratory [AFDIL]). If an amplification yielded an amplicon (as visualized on an agarose gel) in the negative control, the entire amplification was deemed unsuccessful and repeated. These negative controls that were positive were sequenced and analyzed to track contamination (no associated samples were processed alongside these negatives). Similarly, if an amplification failed to yield correctly sized amplicons for HL60, the entire amplification was deemed unsuccessful and repeated. All successful (proper negative and positive controls) samples and associated controls underwent ExoSAP-IT® purification, followed by dideoxy termination sequencing utilizing BigDye® v1.1 (Applied Biosystems) (both processes are detailed further on). HL60 samples were sequenced utilizing 5 μL of template DNA in each sequencing reaction, while negative controls were sequenced with the maximum proposed template amount (11 μL). All samples underwent Performa dye terminator removal (DTR) (Edge Biosystems, Gaithersburg, MD) clean-up following sequencing and were then dried-down and resuspended in 10 μL HiDi Formamide. Samples were run on a 310 Genetic Analyzer (Applied Biosystems) with a 30-sec injection for HL60 samples and 60-sec injection for negative controls. Following analysis of the sequenced amplifications, samples that yielded low sequence data were reinjected for 60 sec. Conversely, all samples that yielded blown-out sequence data were reinjected for 10 sec. All negative controls throughout the validation were deliberately sequenced with the maximum proposed template amount (11 μL) and injected for the longest proposed injection time (60 sec) to explore the upper boundary of the sensitivity and contamination of the system. Analysis of all data during the validation was performed with Sequencing Analysis version 5.2 (Applied Biosystems) and Sequencher version 4.1.4 (Gene Codes Corporation, Ann Arbor, MI). Chromatograms were printed using Sequence Scanner version 1.0 (Applied Biosystems). Interpretation guidelines were developed according to the guidelines established by the Scientific Working Group on DNA Analysis Methods published in 2003. These guidelines were strictly applied to ensure uniform analysis between analysts (10).

ExoSAP-IT® Optimization

Amplicons were enzymatically purified using ExoSAP-IT® (USB Corporation, Cleveland, OH) (11). ExoSAP-IT® consists of Exonuclease I which degrades residual single-stranded DNA produced in the PCR while shrimp alkaline phosphatase hydrolyzes remaining dNTPs from the PCR mixture. It has been observed that ExoSAP-IT® can be used in a diluted form (verbal communication Dr. Timothy McMahon, AFDIL), so this possible dilution was explored.

For this study, a total of 37 amplicons representing five PS, MPS, and HVI/HVII duplex regions and consisting of HL60 and casework-like samples (hair shaft extracts) were analyzed. Amplification products were split equally and subjected to two different methods of ExoSAP-IT® purification. PS and MPS amplification products were separated into two allotments of 10 μL each (original reaction volume = 25 μL − 5 μL for product gel = 20 μL remaining) while the HVI/HVII duplex amplification products were separated into two allotments of approximately 22 μL each (original reaction volume = 50 − 5 μL for product gel = 45 μL remaining). One allotment of each amplicon was subjected to ExoSAP-IT® purification as per the manufacturer's suggestions. This consisted of adding 4 μL ExoSAP-IT® per 10 μL of amplification reaction mixture, incubating for 15 min at 37°C and then 15 min at 80°C. The other allotment of each amplicon was subjected to ExoSAP-IT® purification as per the AFDIL validation. This involved combining 0.335 μL ExoSAP-IT® with 4.1 μL dilution buffer (50 mM Tris, pH 8.0) per 10 μL of amplification reaction mixture, incubating for 30 min at 37°C and then 15 min at 85°C. All samples were sequenced and run on a 310 Genetic Analyzer as previously described.

BigDye® Optimization

For this study, a total of 51 amplicons representing seven PS, MPS, and HVI/HVII duplex regions and consisting of HL60 and casework-like samples (hair shaft extracts) were analyzed. Following ExoSAP-IT® purification (AFDIL method), each sample underwent duplicate sequencing, once with the manufacturer's recommended amount of BigDye® (full reaction) and the second time with half the recommended concentration of BigDye® (half reaction), utilizing 5 μL of template DNA per sequencing reaction. All samples were then run on a 310 Genetic Analyzer as previously described.

Sequencing and 310 Optimization

This portion of the validation focused on optimizing the amount of template DNA utilized in sequencing reactions, injection times, and the sequencing primers. Samples consisted of HL60 as well as staff buccal and blood references on FTA (patented filtration matrix) and non-FTA paper. Reference specimens were extracted using Chelex resin as per Walsh et al. (12) and amplified with the HVI/HVII duplex. A total of 116 amplicons representing six PS, MPS, and HVI/HVII duplex regions were analyzed.

Amplicon bands on the product gels were compared with the mass ladder to give an estimate of the quantity of DNA within the amplification product. A starting point was established whereby any amplicon that was equivalent to the 60 ng mass band or brighter was sequenced using 1 μL of template DNA and injected for 30 sec. Any amplicons that were lighter than the 60 ng band were sequenced (half reaction BigDye®) using 5 μL of template DNA and injected for 30 sec. All samples that yielded low sequence data were reinjected for 60 sec and/or resequenced with more template DNA (on a sample-specific basis) and injected for 30 sec. Conversely, all samples that yielded blown-out sequence data were reinjected for 15 sec and/or resequenced with less template DNA (on a sample-specific basis) and injected for 30 sec.

Sample Preparation and Extraction Optimization

Variations in preparation and extraction methods were explored for skeletal remains, teeth, and hair. Two sets of degraded skeletal remains, including associated teeth, were approved for use in validation studies by the OCME. Both sets of skeletal remains were on hand at the OCME as they had been previously submitted as unidentified human remains. These remains were skeletal remains (no adhering tissue), but were not visibly severely degraded as

there was no evident burning, nor were they found in a particularly harsh environment such that excessive degradation of the DNA was expected. An additional three sets of teeth were obtained from adults who had wisdom teeth extracted at some point in the past. Fresh hair shafts were obtained from eight individuals, attempting to ensure a range of hair colors, textures, and treatments. All experiments were performed in duplicate by two independent analysts to ensure reliability and reproducibility of results.

Femur specimens from each of two unknown individuals were used to explore the sample preparation of skeletal remains. Specimens were sanded utilizing a handheld Dremel tool (Dremel, Racine, WI) with a new sanding bit for each specimen and broken into 2 g samples by use of a sterilized mortar, chisel, and hammer. One 2 g sample of each skeletal specimen was cleaned by a series of three water washes with sonication followed by a 100% ethanol wash with sonication. All sonications were performed for 3 min at room temperature with a speed of 40 kHz (conventional method) similar to the method used by the AFDIL for hair shaft extraction (13). The second 2 g sample of each skeletal specimen was cleaned by a modified series of washes which included an additional 10% bleach wash (agitation by hand) preceding the sonicated water and 100% ethanol washes outlined above (developed based on verbal communication with Dr. Timothy McMahon, AFDIL). All samples were pulverized using a SPEX SamplePrep 6750 Freezer/Mill® according to the following parameters: 10-min precool, 30 sec at grinding speed 10. The pulverized samples were extracted according to the manufacturer's protocol (based on reagent volumes for 1–2 mL of whole blood) using the QIAamp® DNA Blood Midi columns (14).

To investigate tooth sample preparation, molar specimens from the same two unknown sets of remains were processed along with two known specimens. The tooth specimens were prepared with the modified series of washes previously described for skeletal samples (13). For the latter preparation, the tooth was subsequently pulverized in the freezer mill (10-min precool, 1 min at grinding speed 10) (15). All tooth samples were extracted via QIAamp® DNA Blood Midi columns as per the manufacturer's protocol.

Collected known hair specimens from a single individual were prepared either by microtissue grinder or enzymatic digestion. Two centimeter cuttings from both sets of hairs were first cleaned using a series of washes that consisted of two 5% Terg-a-zyme® (Alconox, Inc., New York, NY) washes with sonication (10 min, 56°C, 40 kHz), a 100% ethanol wash (agitation by hand), and a water wash (agitation by hand). One set of hairs was then ground using a microtissue grinder and subsequently extracted using the QIAamp® DNA Blood Mini columns. The other set was extracted according to the manufacturer's protocol for the QIAamp® DNA Blood Mini columns, whereby enzymatic digestion occurred during the overnight incubation in tissue lysis (ATL) buffer and 10 mg/mL Proteinase K (16,17).

The Qiagen manual states that a second AW2 spin can increase the DNA yield, and also holds that this is the case for reloading the eluate after the first elution (AE) buffer spin. Additional hair shaft extractions were performed with these variables independently removed in order to determine if the steps do increase the DNA yield.

Heteroplasmy

Non-FTA bloodstain cards, buccal swabs, and hair references from three previously sequenced, known heteroplasmic individuals (individuals A, B, and C) were obtained. Blood and buccal specimens were extracted via Chelex resin, using a 1/8″ punch from each of the bloodstain cards or half of each buccal swab. Hair shafts were extracted enzymatically. Extracts were amplified with a minimum of the HVI/HVII duplex (2 μL template DNA, cycling for 36 cycles), while some extracts underwent amplification for PS and MPS amplicons as well. Results were assessed for consistency between specimens within the same individual and compared with the results obtained by the AFDIL which originally processed blood samples from the three individuals.

Mixtures

Mixtures were prepared using Chelex extracts from the staff sequence and heteroplasmy projects. Two sets of mixtures were prepared using the following mixture ratios: 1:19, 1:14, 1:9, 1:4, 1:3, 1:2, 1:0, 1:1, 0:1, 2:1, 3:1, 4:1, 9:1, 14:1, and 19:1. It should be noted that the range was initially set at 1:19, 1:14, 1:9, 1:0, 9:1, 14:1, and 19:1, but results indicated that a mixture could not be reliably detected at any of these ratios so the range was refined to lower values. The first mixture consisted of two individuals who were non-heteroplasmic in HVI but one individual displayed length heteroplasmy in HVII. The second mixture contained two individuals who were also non-heteroplasmic in HVI, but one individual had a length heteroplasmy in HVII while the other individual displayed a point heteroplasmy in HVII.

Reproducibility

Reproducibility studies were conducted to determine the reliability of the entire mtDNA system from beginning to end. Two scientists independently processed specimens originating from three individuals (Individuals D, E, and F) that depicted a realistic range of degradation that was expected to be encountered at the OCME-DNA Unit. The samples consisted of hairs, femurs, and teeth to demonstrate reproducibility of sequence data from different types of specimens within the same set of remains as well as reproducibility between scientists. All processing was based upon previously optimized conditions from sample preparation through to attaining quality sequence data.

TABLE 3—*Lower amplification sensitivity levels for each mtDNA amplification primer pair.*

	Amplification Lower Limit	
Primer Pair	HVI (pg)	HVII (pg)
HVI/HVII Duplex (33 cycles)	1	1
HVI/HVII Duplex (34 cycles)	0.1	0.01
HVI/HVII Duplex (35 cycles)	1	0.01
HVI/HVII Duplex (36 cycles)*	0.1	0.01
HVI/HVII Duplex (37 cycles)	>0.1	>0.1
HVI/HVII Duplex (38 cycles)	>0.1	>0.1
PS1 (F15989/R16251)	0.1	
PS2 (F16190/R16410)	0.1	
PS3 (F15/R285)	0.01	
PS4 (F155/R389)	0.1	
altPS4 (F140/R381)	0.1	
MPS1A (F15989/R16158)	0.01	
MPS2A (F16190/R16322)	0.1	
MPS2B (F16222/R16410)	0.1	
MPS2B (F16268/R16400)	0.01	
MPS3A (F34/R159)	0.01	
MPS3B (F109/R240)	0.01	
MPS4A (F151/R292)	0.01	
MPS4B (F220/R381)	0.01	

*Thirty-six cycles determined to be optimal during validation.

HVI and II, hypervariable region I and II; MPS, mini primer sets; mtDNA, mitochondrial DNA; PS, primer set.

Results and Discussion

Sensitivity and Contamination

The amplification sensitivity for each primer pair that would be used for mtDNA processing was assessed (Table 3). The HVI/HVII duplex amplification was optimized for 36 cycles based upon amplification and sequencing result quality (data not shown) and provided an amplification sensitivity down to 0.1 and 0.01 pg for HVI and HVII, respectively. All PS were capable of yielding a detectable PCR product at 0.1 pg and PS3 could do so as low as 0.01 pg. Six of the eight MPS detected PCR product at 0.01 pg. At no point throughout validation did any of the clean negative controls (as visualized on an agarose gel) produce readable sequence data.

Eleven amplifications were classified as unsuccessful out of a total of 55 amplifications (20%) because of a positive negative control. Five of the 11 unsuccessful amplifications were associated with one particular primer pair, MPS1B, and it was decided that MPS1B would not be included in the amplification scheme for mtDNA processing at this time. Future studies shall include re-examining this primer pair starting with re-ordering the primers. The remaining six unsuccessful amplifications (11% overall) were associated with seemingly random primer pairs: PS1, PS3, MPS2B, MPS3A, MPS3B, and MPS4B. All amplifications that were classified as unsuccessful were repeated.

All sequences obtained from negative controls were compared with the sequences of the staff handling the samples and to that of HL60 (the sample that was used throughout these sensitivity amplifications), and no conclusive matches were found. If a reagent was contaminated, more unsuccessful amplifications would have been expected and the sequence results from positive negative controls would have been consistent, but this was not the case. The lack of regularity attaining the contaminant and the lack of uniformity between the sequences of the negative controls prevented the determination of the source of this contamination. Given the inherent sensitive nature of mtDNA amplification, some amount of sporadic amplification is to be expected, and it was felt that the amount that was obtained during this section of the validation (11% overall) was not excessive and could provide a basis of expectations for future mtDNA amplifications.

ExoSAP-IT® Optimization

Upon comparing sequence data (data not shown) following PCR product clean-up with ExoSAP-IT® both according to the manufacturer's recommendations and the AFDIL's recommendations, it was determined that the data obtained when following the AFDIL's protocol was of a better quality with lower DNA template amounts. When processed according to the manufacturer's recommendations, HL60 sequence data was obtained for 150 and 800 pg samples only, while readable sequence data was obtained down to 0.1 pg with the AFDIL's protocol. Conversely, similar sequence data was obtained with both methods for the casework-like samples amplified with the HVI/HVII duplex (hair shaft extracts). The decision was made to implement the AFDIL method of ExoSAP-IT® purification.

BigDye® Optimization

Upon comparing sequence data (data not shown) following sequencing with the manufacturer's recommended full reaction of BigDye® alongside half reaction BigDye®, it was determined that the HL60 data was consistent with both sequencing reactions. The quality of data obtained from the casework-like samples (hair shaft extracts), however, was consistently better with the half reactions. The sequence data obtained from both sequencing reactions were closely compared to ensure that the different amounts of sequencing chemistry present did not pull up varying amounts of background, that the peak height ratios were consistent, and that there were no questionable positions (possible mixture/heteroplasmy) different between the two studies. No evidence of any such differences was found. As a result, the more economical half reaction BigDye® was implemented.

Sequencing and 310 Optimization

It was determined that optimal sequence results (data not shown) were most likely to be obtained when the following sequencing template guidelines were applied: amplicons that were equivalent to or less bright than the 20 ng band of the mass ladder (as visualized on an agarose gel) were sequenced with 5 μL; amplicons that were equivalent in brightness to, or in between, the 40 and 60 ng bands of the mass ladder were sequenced with 1 μL; amplicons that were equal to or brighter than the 80 ng band of the mass ladder were sequenced with 0.5 μL. Amplicons that were extremely faint were sequenced with either 5 or 11 μL at the analyst's discretion. All samples with no visual band on the product gel were also sequenced with 11 μL.

Hypervariable region I and HVII amplicons were considered independently of each other when evaluating HVI/HVII duplex amplifications. If only one of the two amplicons was present for the HVI/HVII duplex amplification, both HVI and HVII were sequenced, utilizing 11 μL for the region that was not visible. It should be noted that observations were made of usable sequence being obtained when no HVI band was seen and also of no usable sequence being obtained when a HVII band was present. As a result, while HVI appeared less sensitive than HVII on product gels, the opposite held true at the level of sequencing.

It was determined that a default injection time of 30 sec was optimal for all samples except the HVI portion of the HVI/HVII duplex amplifications, which was optimal at 15 sec because of the HVI band intensity on an agarose gel indicating less DNA than was actually present, which subsequently resulted in sequencing with excessive template. Troubleshooting of sequence data was handled by decreased injection times of 15 sec or increased injection times of up to 60 sec, as appropriate.

The most productive sequencing strategy was determined to be the use of primers F15989, R16251, and R16400 for HVI and primers F15, R285, and R389 for HVII when sequencing the HVI/HVII duplex amplifications. While additional amplifications or sequencing with additional primers may still be necessary in some instances, this strategy was generally found to provide the best chance of obtaining a full sequence without additional measures.

Sample Preparation and Extraction Optimization

The bone preparation experiment examined the use of an additional bleach wash prior to the conventional method of washes to further reduce contamination from the surface of the bone without significantly decreasing the yield of authentic DNA. When observed on an agarose gel, the amplicons from the bleach washed and nonbleach washed samples were similar (data not shown). Relative DNA quantities were further compared in the resultant sequence data to ensure that there was no significant decrease of DNA yield noted in the bleach washed samples. The sequence data (data not shown) was closely examined to ensure that the peak height ratios were consistent and that there were no questionable positions (possible mixture/heteroplasmy base call) different

between the two studies. All results were found to be consistent between the two preparations and the decision was made to incorporate a bleach wash into the bone specimen preparation protocol.

Teeth can provide a remarkably clean, high yield of DNA as the enamel provides a natural protection for the interior portion of the tooth. One established method for DNA extraction from teeth involves cutting off the crown and removing the dentin (18). A simpler, less time-consuming alternative consists of washing the tooth vigorously through a series of sonicated washes and subsequently grinding the entire tooth. The removal of dentin method was attempted during this validation, but it was immediately apparent that the laboratory staff would need better equipment and a higher dental skill level to cut open the tooth to cleanly and efficiently obtain the interior. Therefore, by default, all tooth extractions were performed by washing (modified series of washes) and grinding the entire tooth in the freezer mill. Amplicons on an agarose gel were strong for both HVI and HVII when amplified with the HVI/HVII duplex. Resultant sequence data (data not shown) were critically analyzed to ensure that the samples were not contaminated. The samples were determined to be clean, so this tooth preparation method was adopted.

Utilization of a microtissue grinder is common practice to ensure complete digestion of hair shafts. However, use of a microtissue grinder involves more sample handling and is more time-consuming than enzymatic digestion. These preparations were performed in parallel to observe the relative quantity and quality of DNA yielded by each method. Based upon observation of the agarose gel, results were stronger for the enzymatic procedure, which yielded a visible amplicon for both HVI and HVII, while the microtissue grinder procedure only yielded a visible HVI and HVII amplicon for one of the three extractions (data not shown). For the remaining two extractions, the band for HVI was either not observed or very faint. Relative DNA quantities were also observed in the sequence data with no notable differences. Finally, the sequence data were closely compared for amounts of background, differences in peak height ratios, and differences in the presence of questionable positions (possible mixture/heteroplasmy base call) between the two studies (Fig. 1). Once again, no evidence of any significant differences was found so the decision was made to utilize enzymatic digestion for hairs.

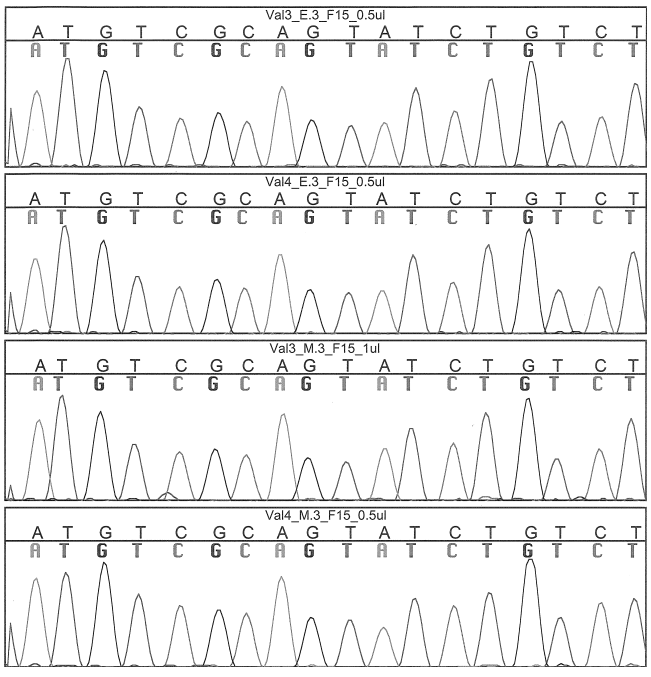

FIG. 1—*Sequence data from hair extractions. The data shown was sequenced with the F15 primer. The top two sequences are the data from two separate extractions of the hairs using enzymatic digestion; the bottom two sequences are from the hairs extracted (during the same two extraction procedures) using the microtissue grinder.*

Agarose gel results for the additional hair extractions in which the AW2 spin and the reloading of the eluate were independently removed indicated that the most product was obtained when doing a second AW2 spin and reloading the eluate (data not shown). These two additional steps were included in the extraction method as standard practice.

Future studies will ideally involve a greater range of degraded samples to further test the capabilities of this processing system. It may be necessary to explore more sensitive methods of DNA extraction for difficult hairs and skeletal remains. It should be noted that the sample set of degraded skeletal remains is arguably low for a complete validation. However, practicality of obtaining validation samples that cannot be easily donated must be accounted for. Also, the testing consumes a piece of bone, so not all skeletal remains held by an OCME or equivalent department may be eligible for such validation studies. One should also note that the hairs utilized for this study were not examined microscopically for evaluation of differentiating morphological characteristics such as pigmentation or stage of growth nor were such data as age of the donor and treatments used by the donor obtained prior to extraction and analysis. These factors have been shown to have an effect on the overall efficiency of amplification and sequencing (as discussed by Roberts and Calloway [19] and Melton et al. [20]), but for this study the effects of such characteristics could not be definitively determined to be a factor when evaluating the success of the hair processing scheme.

During these validation studies it was found that the HVI/HVII duplex successfully amplified the HVII region of most samples. Review of the data from all hairs extracted during the validation revealed that the complete HVII sequence was obtained for approximately 63% of the hair samples using HVI/HVII duplex amplification. Twelve percent of the hair samples were able to be completely sequenced (full HVI and HVII sequence) with duplex amplification. For the remaining 88%, the complete sequence data was able to be obtained for approximately 62% of the samples with further amplification using PSs and/or MPS. Complete data was unable to be obtained for the remaining 38% because of the presence of heteroplasmic sites. Upon careful consideration, it is recommended to use the HVI/HVII duplex on reference samples but to start with PS on certain nonreference samples, decided on a case by case basis, as the size of the amplicons in the HVI/HVII duplex is relatively large and useful template could be consumed with no results if the DNA is degraded.

Heteroplasmy

Heteroplasmy studies were instrumental in testing the efficiency as well as the accuracy of the proposed OCME-DNA Unit mtDNA processing scheme. Results obtained from the processed blood, buccal, and hair specimens of three known heteroplasmic individuals effectively demonstrated the ability of the system to identify heteroplasmic positions (Fig. 2). The consistency of the results was confirmed by comparing them to those obtained by the AFDIL, who had originally processed blood samples from the same three Individuals. Comparison of the blood, buccal, and hair samples from Individuals A and C and the blood and buccal samples of Individual B demonstrated that while the heteroplasmy (as well as the overall sequence) was confirmed among the three specimens, the ratio of

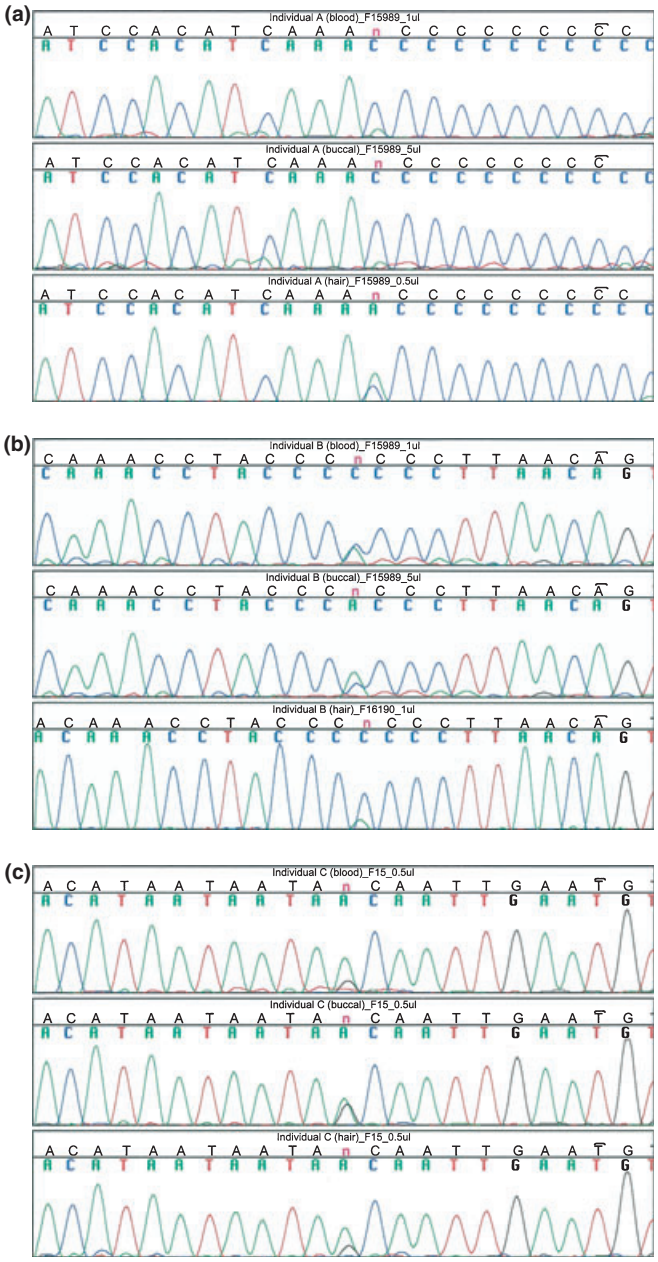

FIG. 2—*Sequence data for heteroplasmic individuals. (a) Sequence data (F15989) from blood (top), buccal (middle), and hair (bottom) samples of Individual A. Each chromatogram starts from the left at position 16171. Note the heteroplasmic base at position 16183 in the blood and hair sequences. (b) Sequence data (F15989) from blood (top) and buccal (middle) samples of Individual B and sequence data (F16190) from hair (bottom) of Individual B. Each chromatogram starts from the left at position 16282. Note the heteroplasmic base at position 16293 in each sequence. (c) Sequence data (F15) from blood (top), buccal (middle), and hair (bottom) samples of Individual C. Each chromatogram starts from the left at position 231. Note the heteroplasmic base at position 241.*

the bases at the heteroplasmic site was different between specimen types (1,21) (Table 4). It should be noted that the hair specimen for Individual B was unable to be successfully sequenced after two extractions and amping with both the HVI/HVII duplex and PSs.

Mixture

The ability of any testing system, whether nuclear or mitochondrial, to detect mixtures is crucial to determine. Results from analysis of the range of mixtures (1:19, 1:14, 1:9, 1:4, 1:3, 1:2, 1:0, 1:1, 0:1, 2:1, 3:1, 4:1, 9:1, 14:1, and 19:1) used in this study supported those of the published reports with the majority of mixture positions being detectable with both a forward and reverse sequencing primer at a 1:4 ratio (Table 5) and the detection level dropping drastically when assessing the 1:9 mixtures (Fig. 3).

TABLE 4—*Heteroplasmic detection from multiple sample types of three known heteroplasmic individuals.*

Individual	Previously Reported Heteroplasmy	Source	Observed with Forward Primer?	Ratio	Observed with Reverse Primer?	Ratio
A	16183 (A/C); 16193 C	Blood	Yes	C>A	N/A	N/A
		Buccal	Yes	C>A	N/A	N/A
		Hair	Yes	A>C	N/A	N/A
B	16293 (A/C)	Blood	Yes	A=C	Yes	C>A
		Buccal	Yes	A>C	Yes	A>C
		Hair	Yes	C>A	Yes	C>A
	309.2 C	Blood	Yes	T>C	N/A	N/A
		Buccal	Yes	C=T	N/A	N/A
		Hair	No results	N/A	No results	N/A
C	241 (A/G)	Blood	Yes	A>G	Yes	A=G
		Buccal	Yes	A=G	Yes	G>A
		Hair	Yes	A>G	Yes	A=G

Reproducibility

Reproducibility results were evaluated both between scientists and between specimen types from the same set of skeletal remains (data not shown). Results between scientists for Individuals D and E were found to be consistent. Results were also found to be consistent between the specimens within each of the tested individuals (D, E, and F). Therefore, the reproducibility studies were deemed successful.

While validating mtDNA, the OCME-DNA Unit was concurrently validating real-time quantitative PCR (qPCR). Thus it was a logical step to incorporate mtDNA quantitation by selecting a duplex nuclear and mitochondrial (nuc/mt) qPCR. To this end, Timken *et al.* (22) nuc/mt duplex was selected based upon particular aspects of the duplex design with the goal of having one quantification system that would be capable of quantifying all evidentiary samples processed by the OCME-DNA Unit. Unfortunately, when performed in our laboratory, the mtDNA quantification assay was not sensitive enough to routinely quantify samples requiring mtDNA testing. This lack of sensitivity along with the fact that mtDNA amplification has historically been performed without quantification, led to the decision to remove qPCR from mtDNA processing.

In conclusion, the OCME-DNA Unit has researched, validated, and implemented an effective and efficient mtDNA processing scheme. Protocols are in place such that degraded skeletal remains,

TABLE 5—*Mixture detection with the proposed mtDNA processing scheme.*

Mixture Ratio	Total Introduced Mixture Sites	Detected Mixture Sites (F and R primers)	Percentage Detected
1:2/2:1	23	22	95.65
1:3/3:1	23	20	86.96
1:4/4:1	23	17	73.91
1:9/9:1	23	5	21.74
1:14/14:1	23	0	0
1:19/19:1	23	0	0

mtDNA, mitochondrial DNA.

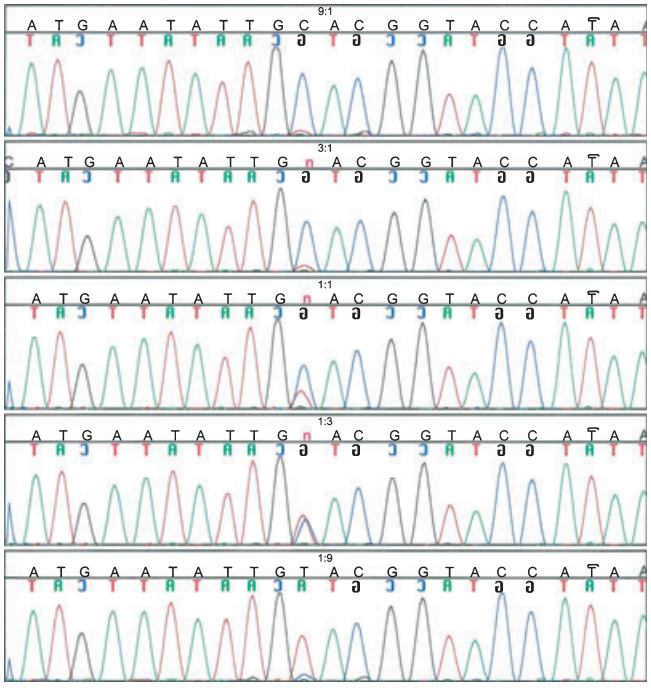

FIG. 3—*Sequence data for mixture study. Sequence data for the first mixture set (non-heteroplasmic:length heteroplasmic). Samples from top to bottom are as follows: 9:1, 3:1, 1:1, 1:3, and 1:9. Each chromatogram starts from the left at position 16116. Note the mixed base at position 16126 in the 3:1, 1:1, and 1:3 chromatograms.*

teeth, and hair evidence can be received for mtDNA testing, in addition to blood and buccal references. Following various optimizations and investigations into each aspect of the testing, the following protocols have been implemented for mtDNA testing. Skeletal remains and teeth are cleaned through a series of bleach, water, and ethanol washes followed by pulverization in a freezer mill. Hairs are cleaned through a series of Terg-a-zyme®, water, and ethanol washes followed by enzymatic degradation. All evidentiary samples are extracted using QIAamp® DNA Blood Mini/Midi columns, optimized for maximal DNA yield. All reference samples are extracted utilizing Chelex resin. Amplification of the HVI and HVII regions can be accomplished with various primers, depending upon the level of degradation. ExoSAP-IT® proved to be an effective method of postamplification clean-up, while sequencing was accomplished via BigDye®. All factors that affect the quality of the data were optimized, including injection time, sequencing primers, and sequencing template amount in order to minimize any troubleshooting. Full sensitivity and contamination, mixture, heteroplasmy, and reproducibility studies were also conducted, all proving to be successful. Hence, mtDNA processing has been successfully implemented at the OCME-DNA Unit.

Acknowledgments

The authors wish to acknowledge several individuals who provided administrative and technical support, including Department of Health and Social Services Cabinet Secretary Vincent P. Meconi, Chief Medical Examiner/Forensic Laboratories Director Richard T. Callery, MD, Deputy Director Hal. G. Brown, and DNA Laboratory Managers A. Robyn Quinn and Teri Zerbe. Vendor customer service representatives provided valuable insight into the developmental stage of the validation and with other issues encountered during this project. Similarly, Timothy McMahon, Ph.D. and the AFDIL staff provided tremendous troubleshooting assistance. Finally, we wish to acknowledge the Delaware Criminal Justice Council and the entire staff of the Delaware Office of the Chief Medical Examiner—Forensic Sciences Laboratory for their continued patience and support throughout the duration of the project.

References

1. Holland MM, Parsons TJ. Mitochondrial DNA sequence analysis-validation and use for forensic casework. Forensic Sci Rev 1999;11:21–50.
2. Wilson M, Stoneking M, Holland M, diZinno J, Budowle B. Guidelines for the use of mitochondrial DNA sequencing in forensic science. Crime Lab Dig 1993;20(4):68–77.
3. http://www.ncjrs.gov/pdffiles1/nij/grants/220297.pdf.
4. Obenauf RH, Nash D, Martin J, Bostwick R, DeStefano M, Akers J, Tucker K, editors. SPEX SamplePrep handbook of sample preparation and handling. Metuchen, NJ: SPEX SamplePrep, LLC, 2005; 134–5, 137–42.
5. Scherzinger CA, Bourke MT, Ladd C, Lee HC. DNA extraction from liquid blood using QIAamp. J Forensic Sci 1997;42(5):893–6.
6. Gabriel MN, Edwin HF, Ryan JH, Holland MM, Parsons TJ. Improved mtDNA sequence analysis of forensic remains using a ''mini-primer set'' amplification strategy. J Forensic Sci 2005;46(2):247–53.
7. Armed Forces DNA Identification Laboratory, ''9700 Validation for Casework,'' Validation Folder, 2001.
8. Chong MD, Calloway CD, Klein SB, Orego C, Buoncristiani MR. Optimization of a duplex amplification and sequencing strategy for the HVI/HVII region of human mitochondrial DNA for forensic casework. Forensic Sci Int 2005;154:137–48.
9. Divne A, Nilsson M, Calloway C, Reynolds R, Erlich H, Allen M. Forensic casework analysis using the HVI/HVII mtDNA linear array assay. J Forensic Sci 2005;50(3):548–54.
10. http://www.fbi.gov/hq/lab/fsc/backissu/april2003/swgdammitodna.htm.
11. Dugan KA, Lawrence H, Hares DR, Fisher CL, Budowle B. An improved method for post-PCR purification for mtDNA sequence analysis. J Forensic Sci 2002;47(4):511–8.
12. Walsh PS, Metzger DA, Higuchi R. Chelex® 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechnology 1991;10(4):506–13.
13. Armed Forces DNA Identification Laboratory. DNA Extraction Manual Version 3.0. Rockville, MD: Department of Defense DNA Registry, Office of the Armed Forces Medical Examiner, Armed Forces Institute of Pathology, 2004: Organic extraction of DNA from hair shafts.
14. QIAamp®. DNA blood midi/maxi handbook. Valencia, CA: Qiagen, 2005. Second Edition Purification of DNA from whole blood using the QIAamp DNA blood midi kit (spin protocol); 20–3.
15. Sweet D, Hildebrand D. Recovery of DNA from human teeth by cryogenic grinding. J Forensic Sci 1998;43(6):1199–202.
16. QIAamp®. DNA mini and blood mini handbook. Valencia, CA: Qiagen, 2007. Second Edition Appendix J: Protocol for crude cell lysates and other samples; 64–6.
17. Graffy EA, Foran DR. A simplified method for mitochondrial DNA extraction for head hair shafts. J Forensic Sci 2005;50(5):1119–22.
18. Armed Forces DNA Identification Laboratory. *DNA Extraction Manual Version 3.0*. Rockville, MD: Department of Defense DNA Registry, Office of the Armed Forces Medical Examiner, Armed Forces Institute of Pathology, 2004: Organic extraction of DNA from teeth.
19. Roberts KA, Calloway C. Mitochondrial DNA amplification success rate as a function of hair morphology. J Forensic Sci 2007;52(1):40–7.
20. Melton T, Dimick G, Higgins B, Lindstrom L, Nelson K. Forensic mitochondrial DNA analysis of 691 casework hairs. J Forensic Sci 2005;50(1):73–80.
21. Sekiguchi K, Sato H, Kasai K. Mitochondrial DNA heteroplasmy among hairs from single individuals. J Forensic Sci 2004;49(5):986–91.
22. Timken MD, Swango KL, Orrego C, Buoncristiani MR. A Duplex real-time qPCR assay for the quantification of human nuclear and mitochondrial DNA in forensic samples: implications for quantifying DNA in degraded samples. J Forensic Sci 2005;50:1044–60.

Additional information and reprint requests:
Sherri L. Fentress, M.S.
Forensic DNA Analyst
200 South Adams Street
Wilmington, DE 19801
E-mail: sherri.fentress@state.de.us