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Validation of mitochondrial DNA sequencing for forensic casework analysis

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Abstract Two sets of studies were performed to evaluate the forensic utility of sequencing human mitochondrial DNA (mtDNA) derived from various tissues and amplified by the polymerase chain reaction (PCR). Sequencing was performed on a Perkin-Elmer/Applied Biosystems Division (PE/ABD) automated DNA sequencer (model 373A). The first set of experiments included typical validation studies that had previously been conducted on forensic DNA markers, such as: chemical contaminant effects on DNA from blood and semen and the effect of typing DNA extracted from body fluid samples deposited on various substrates. A second set of experiments was performed strictly on human hair shafts. These studies included typing mtDNA from hairs that were: (1) from different body areas, (2) chemically treated, (3) from deceased individuals, and (4) deliberately contaminated with various body fluids. The data confirm that PCR-based mtDNA typing by direct automated sequencing is a valid and reliable means of forensic identification.

Key words Mitochondrial DNA · Forensics · Hair · Human polymorphisms · Sequencing

Introduction

Typing of DNA derived from forensic biological material has been facilitated by use of the PCR [1]. One particular target site on the DNA that has been reported as useful for identity testing purposes is mitochondrial DNA (mtDNA) [2–7]. Because there is a high copy number per cell, increased sensitivity is achieved when typing mtDNA [3, 5, 6, 8]. MtDNA analysis can be utilized on a range of tissues, such as hairs, which by their nature contain little nuclear DNA, and bones and teeth, which may, depending on the circumstances, also contain very little DNA. The present study shows through a variety of experiments that automated DNA sequencing of amplified mtDNA products from hair shafts and other tissues is a valid and reliable method of forensic human identity testing.

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Materials and methods

DNA extraction

Body fluid samples from six different donors were used, as described previously [9]. Hairs were obtained from both Caucasian and African-American donors. Known blood samples were obtained from each hair donor and made into blood stains for comparison with results obtained from hair shaft analysis. DNA was extracted from blood stains as described previously [10].

Extraction and amplification procedures involving hairs were performed under a laminar flow hood. All reagents, plastic tubes, Microcon-100 devices and PCR tubes placed in a microwave oven on a high setting for 2 min prior to use. Glass grinders were sterilized by the addition of 100% ethanol to the mortar, followed by a hot water and detergent wash. A cotton swab was used to clean the inside of the grinder. Care was taken to assure that the swab fitted well into the tip of the mortar. After detergent treatment, the grinders were rinsed with deionized water. Approximately 300 µl of 4 N H_2SO_4 were then added to the grinders under a vented hood and left at ambient temperature for a minimum of 20 min. Another rinse with deionized water followed. The grinders were then subjected to brief centrifugation and the excess water was removed by pipetting. The grinders were dried in a microwave oven on a high setting for 2 min. Clean grinders were stored under exposure to a UV light source until use. Reagent blanks were run for every glass grinding tube used in the hair extraction procedure. Reagent blanks are controls in which the entire extraction and amplification process is performed, from grinding to PCR amplification, without the addition of the hair to the tissue grinder.

The following protocol assumes that the hairs are first mounted in Permount on glass slides prior to DNA extraction. If unmounted hairs are used, the procedure should begin at step 9:

1. Before performing any procedures for DNA analysis, take a photograph or electronic image of the root (if present) and shaft of the hair selected for examination.

2. Carefully break the cover slip in a circular pattern around the area of the mounted hair for removal with a carborundum scorer or

similar instrument. If the hair has a root, it and about 2 cm of adjacent shaft should be targetted for extraction. If it does not, 2 cm of hair shaft should be used.

3. Place a drop of organic solvent (100% xylene or 100% Xyless) on the area.

4. After approximately 10 min carefully remove the piece of cover slip.

5. Being careful not to break the hair, pull out the hair with forceps until approximately 2 cm of the hair can be cut off.

6. Briefly place the 2-cm length of hair in a 1.5-ml plastic flip-top tube (Sarstedt) containing organic solvent (Xyless or xylene) to remove any adhering mounting medium.

7. Prepare a wash solution (approximately 50 ml) containing approximately 5% (w/v) Terg-a-zyme detergent (Alconox, New York, N.Y.) in a 250 ml Erlenmyer flask. Leave in a microwave oven on a high setting until boiling is observed. Put the cut 2-cm portion of hair in the flask. Place the flask in an ultrasonic water bath (model FS-14, Fisher Scientific) and agitate for 20 min. Remove the hair and place in a 1.5-ml plastic flip-top tube (Sarstedt) containing 1.0 ml of 100% ethanol. Tightly close the lid of the tube containing the hair.

8. If desired, re-mount the remaining portion of the hair on a new glass microscope slide. Additional hairs from the slide originally containing the particular hair may also be re-mounted on another microscope slide.

9. Place the cleaned hair fragment into a 0.2 ml Kontes glass grinder (Fisher catalogue number K885 470-0000) containing 200 µl of stain extraction buffer (10 mM Tris, 100 mM NaCl, 39 mM DTT, 10 mM EDTA, 2% SDS, pH 8.0). Note that the pestles and grinders are matched sets. Grind until all hair fragments are no longer visible. Remove the pestle from the grinder. If liquid is adhering to the pestle head, gently pass it along the inner lip of the grinder cylinder until liquid flows down into the grinder.

10. Transfer the homogenate into a 1.5-ml plastic flip-top tube and add 5 µl of proteinase K (10 mg/ml). Vortex on low speed and centrifuge briefly. Place the flip-top tube in a water bath and incubate at 56° C for 2–24 h.

11. Briefly spin the tube in a microcentrifuge to force the condensate to the bottom. In a fume hood, add 200 µl of phenol: chloroform: isoamyl alcohol (24:24:1, PCIA) to the extract. Vortex briefly. Spin the tube in a microcentrifuge for 3 minutes at 10,000 × g.

12. Add 200 µl of filtered TE buffer (10 mM Tris, 50 mM EDTA, pH 9.0) to a Microcon-100 tube (Amicon Inc., Beverly, Mass.). Using a pipette, carefully remove the aqueous (upper) phase of the PCIA/extract and place it into the Microcon-100 tube. Avoid drawing any of the proteinaceous interface into the pipette tip.

13. Centrifuge the Microcon-100 tube for 5 min at 2,000 *g*. Discard the wash and add 400 µl of filtered TE buffer to the top of the Microcon-100 tube. Centrifuge again at 2,000 *g* for 5 min. Discard the collection tube. Add 60 µl of hot (80–90° C), filtered TE buffer to the Microcon-100 and place the sample recovery tube on top of the filter apparatus in an inverted orientation. Do not flip over at this time. Vortex with the sample recovery tube pointing upward on medium speed for 30 s. Invert the filter with the sample recovery tube and centrifuge at 10,000 *g* for 3 min. The recovery volume is approximately 60 µl. Extracted DNA can be subjected to PCR immediately. However, if necessary, the DNA may be stored at –20° C.

PCR amplification

Figure 1 is a schematic depicting the human mtDNA control region showing the location of hypervariable region 1 (HV 1) and hypervariable region 2 (HV 2). With respect to the origin of replication of the heavy strand, the location of primers used to amplify overlapping segments of HVI are shown. A similar amplification scheme is used to amplify HV 2.

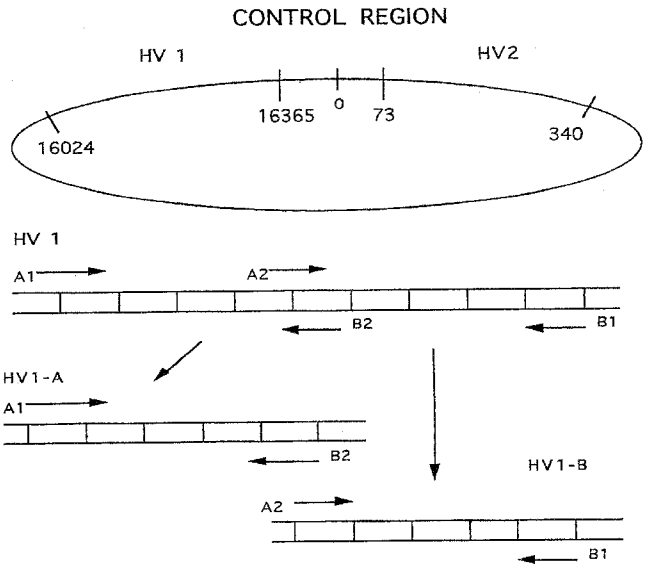

Fig. 1 Schematic of the amplification strategy of HV1, showing the control region and the approximate locations of mitochondrial DNA primers utilized to amplify overlapping segments of the region

Different amplification strategies may be employed for amplifying human mtDNA. Generally, with forensic samples amplification of small fragments is more robust (unpublished results). However, much larger amplicons may be obtained from samples where the amount of template DNA for PCR is not limited.

The mtDNA regions subjected to analysis are 16,024-16,365 (HV 1) and 73-340 (HV 2) [3]. This numbering system was described by Anderson et al. [11] and is commonly referred to as the "Anderson sequence". Two sets of PCR primers are used to amplify overlapping segments from both HV 1 or HV 2. Primer sequences used for amplifying the hypervariable regions of mtDNA for forensic purposes have been previously reported [2, 5, 12]. The specific primers used in this study and their nomenclature [3] are as follows:

HV 1
L15997
5′-CACCATTAGCACCCAAAGCT-3′ (A1)
L16159
5′-TACTTGACCACCTGTAGTAC-3′ (A2)
H16236
5′-CTTTGGAGTTGCAGTTGATG-3′ (B2)
H16395
5′-CACGGAGGATGGTGGTCAAG-3′ (B1)

HV 2
L048
5′-CTCACGGGAGCTCTCCATGC-3′ (C1)
L172
5′-ATTATTTATCGCACCTACGT-3′ (C2)
H285
5′-GGGGTTTGGTGGAAATTTTTTTG-3′ (D2)
H408
5′-CTGTTAAAAGTGCATACCGCCA-3′ (D1)

The following primer pairs were employed for the validation studies:

HV 1-A	A1/B2
HV 1-B	A2/B1
HV 2-A	C1/D2
HV 2-B	C2/D1

For known samples, larger amplified DNA fragments were obtained through the use of different primer sets, as follows:

HV 1	A1/B1
HV 2	C1/D1

Amplification was performed in 25-µl reactions using a GeneAmp System 9600 thermal cycler (Perkin-Elmer, Norwalk, Connect). Each reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 5.0 pmol of each primer (0.2 µM), 4 µg BSA (Sigma), 200 µM each dNTP, 5 U *Taq* polymerase. (Perkin-Elmer) and 10 µl of the hair extract. Thermal cycling conditions were 1 min at 95° C, followed by 36 cycles of 95° C for 10 s, 60° C for 30 s, and 72° C for 30 s. Known samples were amplified for 32 cycles instead of 36. Negative (amplification) controls and reagent blank controls were always used. In no case was amplified mtDNA sequenced from a sample if the ratio of negative control or reagent blank DNA to sample DNA, as quantitated through capillary electrophoresis (see below) exceeded 1 : 10 [12].

Post-amplification purification

Following PCR amplification the amplified fragments were purified using Microcon-100 ultrafiltration, and 500 µl of filtered TE buffer were added to Microcon-100 tubes (Amicon Inc., Beverly, Mass.). The Microcon-100 tubes were centrifuged for 5 min at 2,000 *g*. The wash was discarded and 500 µl of filtered TE buffer were added to the top of the Microcon-100 tube. The devices were centrifuged again at 2,000 *g* for 5 min. The collection tubes were discarded and 40 µl of hot (80–90° C), filtered TE buffer were added. The sample recovery tube was then placed on the filter apparatus, and the tube was vortexed on medium speed for 30 s. After inverting, the sample recovery tubes were centrifuged at 10,000 *g* for 3 min.

Quantitation by capillary electrophoresis

The purified, amplified samples were quantified using capillary electrophoresis (CE). CE electropherograms indicate not only the concentration of mtDNA but also the quality of the PCR product. If unincorporated primers are present they will interfere with the cycle sequencing reaction and produce poor sequencing results [11]. The CE instrument used was a Beckman P/ACE System 2050 (Beckman Instruments, Palo Alto, Calif.) with a Laser Module 488 argon ion laser. A 27-cm DB-17 coated capillary (J&W Scientific, Folsom, Calif.) of 50 µm i.d. with coating thickness of 0.1 µm was used. The run buffer consisted of 1% (w/v) hydroxyethyl cellulose (Aldrich 30,863-3), 100 mM Tris-borate, 1 mM EDTA and 50 ng/ml YO-PRO-1 (Molecular Probes, Eugene, Oregon) intercalating dye.

Prior to each sample injection, the column was washed with 100% methanol for 1 min and filled with run buffer for 2 min. Samples were hydrodynamically injected (0.5 psi) for 45 s. Separation took place for 4 min at 25° C with an applied voltage of -15kV. Flourescence was induced by excitation with the argon-ion laser at 488 nm and detection was at 520 nm.

A 200-bp internal standard (GenSura, DelMar, Calif.) was injected with each sample at a concentration of 10 ng/µl. The migration time and adjusted area of the mtDNA peak and 200 bp internal standard peak were collected and analysed using the software package Millenium 2010 (Waters Chromotography, Milford, Mass.). The quotient of adjusted area/migration time for each peak was calculated. The concentration of mtDNA was determined by comparing the ratio of the quotients to the known concentration of the 200 bp standard.

Generating sequencing fragments

Cycle sequencing was performed according to the instructions provided by PE/ABD in the *Taq* dye-deoxy cycle sequencing kit (# 401 150 PE/ABD) with the following exceptions: (1) ammonium chloride was substituted for ammonium sulfate in the 5X buffer (400 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NH_4Cl, pH 9.0) and (2) half the amount of dye-labelled terminators was used, and the difference in volume was made up with deionized water. The cycle sequencing pre-mix was prepared as follows (cf. pp. 6-7, PE/ABI cycle sequencing kit protocol number 401 150):

5X TACS Buffer	4.0 µl	
dNTP mix	1.0 µl	
Each ddNTP (fluorescent terminators)	0.5 µl	
Taq DNA polymerase	0.5 µl	(4.0 units)
Deionized water	2.0 µl	

Then 9.5 µl of the sequencing pre-mix were added to a Perkin Elmer 9600 PCR tube, after which 3.5 µl of appropriate sequencing primer (1.0 µM concentration) were added, followed by 7.0 µl of purified DNA template from the Microcon-100 retentate. An appropriate dilution of the amplified sample was prepared such that approximately 20–35 ng of template were added to the sequencing reaction [12]. Cycle sequencing was performed as described in the PE/ABD kit protocol (p.7), with the exception that the extension time at 60° C was shortened from 4 min to 1 min per cycle. All other operations, including gel, loading buffer, and spin column preparation, were performed as described in the PE/ABD kit protocol.

Sequencing results and interpretation

Upon completion of a sequence run, the PE/ABD software program Analysis 1.2.0 was used to analyse the sequence data. Because sufficient template for sequencing exists after PCR amplification, numerous sequencing reactions may be performed on each amplified template [12]. The SeqEd software from PE/ABD (version 1.0.3) allows multiple chromatograms to be imported and observed together. Sequencing reactions from opposing strands of the double-stranded PCR template can be observed in the same orientation due to the ability of the software to reverse complement any given chromatogram. The process of sequence editing and interpretation begins by importing chromatograms from the same template into a SeqEd file. After the sequences have been placed in the same orientation and aligned, the editor can examine multiple chromatograms and make nucleotide designations at each position. If additional sequence reactions are desired by the examiner, they are performed and the information imported into the same SeqEd file.

Results and discussion

Environmental insults

Since substantial data now exist for the reliability of PCR-based DNA typing from environmentally insulted sam-

Table 1 The HV1 control region sequences of the donors in this study, shown as deviations from a reference sequence, commonly referred to as the Anderson sequence

	16090	16093	16111	16124	16153	16189	16223	16261	16290	16296	16304	16311
Anderson	C	T	T	G	G	T	T	C	C	C	T	T
Donor 1												C
Donor 2	T		C	A			C	T				
Donor 3		C				C						C
Donor 4			C						T	T	C	
Donor 5			C		A				T	T		
Donor 6												

Table 2 The HV1 sequences and the corresponding matching donor number of the validation samples utilized in the environmental insult study (see Table 1). The sequences are again listed as deviations from the reference Anderson sequence

Sample	16069	16075	16126	16145	16153	16189	16231	16266	16294	16296	16304	16356	Equals seq. of donor
Anderson	C	T	T	G	G	T	T	C	C	C	T	T	
02-Oil												C	1
04-Oil	T		C	A			C	T					2
18-Gas		C				C						C	3
20-Gas			C						T	T	C		4
34-Dirt			C		A				T	T			5
36-Dirt													6
54-Detergent		C				C						C	3
56-Detergent			C						T	T	C		4
69-NaOH			C		A				T	T			5
71-NaOH													6
76-HAc	T		C	A			C	T					2

ples [13–24], a subset of previously prepared validation sample stains [9] was used in the current study. Replicate 5 µl bloodstains and semen stains were prepared on a variety of substrates, including carpet, denim, leather, nylon, wallboard, and wood. Briefly, the panels containing the stains were maintained at room temperature for 16 weeks and the DNA extracted thereafter. The known mtDNA HV 1 types of the sample donors, as determined by sequence analysis of known blood stains performed separately, are shown in Table 1. No two donors in this study had identical HV 1 mtDNA sequences. Of a total of 25 reactions attempted on these samples, 17 typed correctly on the first attempt, for a success rate of 68%.

As shown in Table 2, 11 environmentally insulted samples from these six individuals typed correctly after amplification and direct sequencing. Common substances were evaluated for their influence on the reliability of typing. Washed cotton sheeting panels were stained with used automobile oil, gasoline, dirt, detergent, NaOH or acetic acid. The following day, 5-µl aliquots of blood, semen, or saliva were applied to each of the stained panels. After a further 24 h, each body fluid stain was cut out and stored at –70° C until extraction. The sequences from the environmentally insulted samples matched the known sequence from each donor in every case. All 11 reactions typed on the first attempt, for a success rate of 100%.

Animals

In order to assess the cross-reactivity of the mtDNA-typing technique on non-human samples, DNA was extracted and amplified from the following animals: sheep, horse, chicken, cat, dog, rabbit, goat, pig, deer and cow. In addition, the following primates were tested: Japanese macaque, orangutan, Celebes ape, and gorilla. Japanes macaque, gorilla, and orangutan yielded amplified products that could be sequenced. The primate sequence was difficult to interpret in all cases, most probably because the primer regions have diverged, leading to less than optimal primer-template interaction. The sequence was clearly not of human origin. For example, 215 bases from HV 2 sequence from gorilla yielded 37 polymorphisms and 5 insertions with respect to the Anderson sequence. Over the same region in a search of approximately 300 published and unpublished human sequences (data not shown), the greatest number of polymorphisms observed thus far is 13. With respect to the non-primate animals, one sample from cat blood initially amplified and sequenced, but the result appeared to be of human origin. This suggests that the initial stain was contaminated with human DNA.

Hairs

The study focusing on human hairs was organized into six categories. The experiments and specific treatments were designed to address situations that would be expected to be generally encountered in forensic casework. The six areas of study were:

1. The effects of hair treatments, such as conditioners and permanent dyes.
2. The effects of microscopic mounting media (Permount).
3. The effects of dirt.
4. Typing hairs from various areas of the head and body.
5. Typing hairs from decease individuals.
6. Typing hairs contaminated with body fluids from other individuals.

Whether amplification was successful was assessed by careful comparison with negative controls and reagent blanks, as previously described [12]. Failures consisted of two types: those hair extracts that did not amplify, and those whose reagent blank was quantitated at greater than 10% of the amplified hair extract. Although not necessary for forensic casework, typing in this study involved performing cycle sequencing reactions on both strands of the amplified product until all ambiguities were resolved at each nucleotide position. In the hair study additional attempts were made to amplify the extract in some cases. These were necessary for two reasons: failure to achieve amplification in the first reaction, or the presence of a more than 1 : 10 ratio of contaminant to amplified product from the sample. If reamplification was not successful, on some occasions the sample was re-extracted, reamplified, and subsequently typed. In all cases, the sequence obtained from hair shaft matched that of the donor as determined by separate sequencing of DNA extracted from bloodstains.

Since relatively little information exists on the possible effects of hair treatments on the reliability of DNA analysis, hairs were subjected to a variety of treatments, collected and analysed. Table 3 shows that the treatments included shampoo, conditioner, permanent wave, airset, Bantu permanent, and dye. Donors were of Caucasian and

Table 3 Summary of kinds of mtDNA samples typed including both hair shaft and environmental insult samples. Totals show number of samples typed, percentage that typed at initial attempt and percentage that typed on subsequent attempts

	Total reactions	Success rate at initial attempt	Success rate for all attempts
Hair samples			
Cosmetic treatments	24	71%	92%
Shampoo/conditioner	8	63%	88%
Permanent wave	8	63%	88%
Dye	4	75%	100%
Set	4	100%	100%
Permount/aged	16	50%	56%
Dirty hair (soil and oil)	16	63%	63%
Body/head area	76	54%	76%
Body	36	61%	75%
Head	40	48%	78%
Deceased individual	8	75%	100%
Contamination	48	60%	88%
Blood	16	56%	81%
Semen	20	70%	85%
Saliva	12	50%	100%
Environmental insults	11	100%	NA
Oil	2	100%	
Gas	2	100%	
Dirt	2	100%	
Detergent	2	100%	
Base	2	100%	
Acid	1	100%	
Substrates	25	68%	NA
Carpet	1	100%	
Denim	3	33%	
Leather	6	83%	
Nylon	3	100%	
Wallboard	6	66%	
Wood	6	50%	
Totals	224	62%	79%

African-American ethnic origin. Twenty four total reactions were attempted on these hairs, and 17 typed successfully on the first attempt, for a 71% success rate. On subsequent attempts, a success rate of 92% was achieved.

The microscopic mounting medium Permount, and the solvents xylene and Xyless, were studied for any possible negative effects on amplification and sequencing. Head hairs and pubic hairs mounted for up to 2 years were dissolved from the mounting media, extracted, amplified and sequenced. Of the 16 hairs extracted and typed, 8 typed on the first attempt, for a success rate of 50%. Subsequent attempts only slightly raised this success rate, to 56%. Further research would be desirable in this area, as microscopic examination is crucial to forensic hair analysis.

Head hairs from four different donors were contaminated with soil and used automotive oil. After a period of time ranging from 2 to 5 days, the hairs were washed, and the DNA was extracted, amplified, and sequenced. Of a total of 16 hairs analysed and typed, 10 were successfully typed on the first attempt, for a success rate of 63%. Additional typing attempts were not successful on this limited set of samples.

Head, chest, pubic, limb and axial hairs were collected from one donor in order to test whether the specific body area had any effect on the amplified mitochondrial DNA sequence. Additionally, hairs were collected from two individuals from the front, crown, right, left, and back of the scalp. Seventy-six reactions were performed, and 41 typed on the first attempt, for a 54% success rate. Follow-up analyses on these samples raised the success rate to 76%. All typings from the same individual matched each other and also matched the known mtDNA sequence obtained from DNA extracted from bloodstains.

Hairs were collected from two individuals who were deceased for a period of approximately 2 years. Blood and tissue samples from these individuals had been collected post mortem. Sequences from head hairs matched the known sequence derived separately from the blood and tissue. Six of eight reactions typed on the first attempt, for a 75% success rate. All of these samples typed upon subsequent analysis, for a 100% success rate.

In a more relevant and important set of experiments from a forensic standpoint, hairs were deliberately contaminated with body fluids from individuals who were known to have a different mitochondrial DNA sequence from the hair donors. Head and pubic hairs from both Caucasian and African-American donors were collected and immersed in blood, semen and saliva. The contaminated hairs were then allowed to air-dry. Initially, a mild cleaning procedure using simple immersion in 100% ethanol was attempted prior to DNA extraction. In one initial experiment, the mitochondral DNA type from the extracted hair matched that of the blood donor rather than that of the hair donor. Thereafter, much more rigorous cleaning methods were investigated. Using the detergent Terg-a-zyme and ultrasonic cleaning as reported herein resulted in correct typing (the known type from the hair donor) from all contaminated hairs, regardless of the contaminating body fluid. A total of 48 reactions were performed on 12 contaminated hairs: 4 hairs were contaminated with blood, 3 with saliva, and 5 with semen. We found that 29 typed on the first attempt, for a success rate of 60%. Additional testing on these samples raised the success rate to 88%, all matching the known sequence from the hair donor.

The studies reported in this paper show that sequencing amplified mtDNA is a reliable method of DNA typing from human hairs, blood and semen. Careful attention must be given to the possibilities of external contamination and mixtures. Proper controls must be used to assess contamination in extraction and PCR reagents. Consistent with previous validation studies utilizing PCR-amplified DNA, reliable typing of mtDNA can be achieved from DNA extracted and amplified from environmentally challenged samples. It should be noted that in the present study, the vast majority of instances in which successful typing on the first attempt was not achieved was not due to amplification failure, but to the fact that contamination levels exceeded the 10% limit, and hence analysis was not

Table 4 MtDNA typing success rates for hairs shown by PCR reaction

	HV1A	HV1B	HV2A	HV2B	Totals
1st attempt	37/47 = 0.79	13/47 = 0.28	25/47 = 0.53	36/47 = 0.77	111/188 = 59%
All attempts	43/47 = 0.91	29/47 = 0.62	40/47 = 0.85	37/47 = 0.77	149/188 = 79%

attempted. Also, in the present study a dedicated mtDNA extraction area was not used. With heightened awareness of potential contamination and additional safeguarding measures, such as those listed below, it is possible to reduce the level of external contamination significantly and thereby increase the success rate of mtDNA analysis in forensic casework.

In the present study only one instance (cat blood) of undetected human DNA contamination was found. It is anticipated that more stringent quality control measures will reduce this rate further. Quality control measures recommended to reduce the incidence of contamination when amplifying mtDNA are as follows:

1. A specific low-level DNA extraction area, such as a dedicated hood or dead space, which is not used for other, non-mtDNA extractions.
2. Dedicated reagents and supplies, which are used strictly for mtDNA analysis and are not shared with other PCR-based markers.
3. Off-hour or intermittent UV irradiation of extraction space, reagents, and supplies.

In our experience, the most important step in mtDNA analysis is DNA extraction. If the DNA extraction is (1) efficient and (2) not contaminated, the remainder of the typing process is normally straightforward. In our experience, the second most important condition for success of mtDNA typing is the efficiency of the particular PCR reaction. For example, while four separate PCR reactions are performed in our mtDNA protocol, not every reaction has the same efficiency. When the success rate is calculated for each of the four reactions, the percentages of successful typing are as shown in Table 4. The total success rate of 79% shown in Table 4 is a better estimate of the mtDNA typing success rate at this time, since in casework additional attempts will be made when enough evidence exists to warrant them.

As seen in Table 4, the HV1B PCR reaction has a lower efficiency than the other three. Future research should be aimed at balancing these efficiencies so that each reaction can be relied upon with equal confidence. The principle to be noted from the results presented herein is that mtDNA typing reliability and efficiency are primarily dependent on the quality of the DNA extract and the quality control measures used to limit external contamination. Judicious use of controls, as described in this paper, is essential to ensure reliable typing results.

A general principle of PCR-based analysis can now be put forward. Through numerous validation studies, including those described herein, it has become obvious that PCR-based DNA-typing methods are robust and reliable. This observation follows reasonable expectation. If the DNA in the sample is altered or degraded, enzymatic amplification would not be possible without specific DNA repair steps. Without these steps, it can be inferred from successful amplification that the DNA from the sample is physically intact. The more important question in forensic mtDNA analysis is the question of possible contamination, both external and laboratory-induced. Provided proper quality assurance procedures have been designed and implemented, PCR-based DNA typing, to include mtDNA analysis, should be embraced by the forensic science and legal community without the necessity for repetitive, lengthy validation studies for each new genetic locus.

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