J Forensic Sci, May 2005, Vol. 50, No. 3
Paper ID JFS2004505
Available online at: www.astm.org

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Forensic Casework Analysis Using the HVI/HVII mtDNA Linear Array Assay

ABSTRACT: The mitochondrial hypervariable regions I and II have proven to be a useful target for analysis of forensic materials, in which the amount of DNA is limited or highly degraded. Conventional mitochondrial DNA (mtDNA) sequencing can be time-consuming and expensive, limitations that can be minimized using a faster and less expensive typing assay. We have evaluated the exclusion capacity of the linear array mtDNA HVI/HVII region-sequence typing assay (Roche Applied Science) in 16 forensic cases comprising 90 samples. Using the HVI/HVII mtDNA linear array, 56% of the samples were excluded and thus less than half of the samples require further sequencing due to a match or inconclusive results. Of all the samples that were excluded by sequence analysis, 79% could be excluded using the HVI/HVII linear array alone. Using the HVI/HVII mtDNA linear array assay, we demonstrate the potential to decrease sequencing efforts substantially and thereby reduce the cost and the turn-around time in casework analysis.

KEYWORDS: mtDNA, D-loop, immobilized SSO probe assay, linear array mtDNA HVI/HVII region-sequence typing

Analysis of mitochondrial DNA (mtDNA) is increasingly used as a complement to routine forensic identification in many forensic laboratories. The high copy number per cell and the uniparental inheritance make mtDNA analysis useful in certain types of forensic cases, especially when the amount of DNA is limited or degraded. Moreover, a high substitution rate and a high density of polymorphisms within the hypervariable regions I and II (HVI, HVII) in the non-coding D-loop allow informative sequence analysis of relatively short regions in forensic DNA analysis (1,2). Although mtDNA analysis has a lower power of discrimination than using multiple nuclear STR or SNP markers, mtDNA analysis can often be performed on limited or degraded samples when analysis of nuclear markers fails. Analysis of the mtDNA hypervariable regions is commonly performed using a direct sequencing approach (3,4). However, other probe and sequencing based technologies such as sequence-specific oligonucleotide hybridization (5), the Luminex assay (6), mini-sequencing (7) and Pyrosequencing (8) have also been used. Though well established and reliable, direct sequence analysis of mtDNA has several limitations; it is costly, labor-intensive and time-consuming. A quick pre-screening assay for forensic samples to exclude suspects and evidence materials would therefore be valuable to minimize the number of samples that require sequencing.

The reverse dot blot test is an easy to perform and rapid assay format which has been widely used to determine the extensive allelic sequence diversity among the HLA class I and class II genes in a variety of applications, including DNA identification in forensics (9–11), matching donors and recipients in organ transplants (12), disease association studies (13), population genetics and evolutionary studies (14). The method has also been used in the AmpliType PM + DQA1 Typing kit specifically developed for forensic identification (15–17). The limited parallel analysis capacity in the reverse dot blot assay has been improved substantially by the development of the linear arrays (18–22). In the linear array assay as many as 85 probes can be immobilized on the membrane, using current striping instruments. The method is based on hybridization of a biotinylated PCR product to a panel of immobilized sequence-specific oligonucleotide (SSO) probes attached to the membrane. The PCR product, hybridized to its specific probe, is then detected using a streptavidine-horse radish peroxidase conjugate (SA-HRP). The addition of a chromogenic substrate produces a blue precipitate on the filter membrane where the labeled PCR products have hybridized to the immobilized probe. The linear array results (probe reactivity pattern) can thereafter be interpreted by comparing the developed arrays to a reference guide.

Several versions of immobilized probe linear arrays targeting sequence polymorphisms located in the hypervariable regions I and II, have been developed. The reverse hybridization linear arrays based on typing of HVII region polymorphisms have previously been used in studies of heteroplasmy and population diversity (20,23,24). An extended version of the mtDNA linear array containing probes for both HVI and HVII region polymorphisms has been used for identification of human remains in mass graves in Croatia (18,19). Recently, a new version of the assay, which uses sequence-specific probes striped in 31 lines, is commercially available from Roche Applied Science. This LINEAR ARRAY mtDNA HVI/HVII Region-Sequence Typing Kit is suitable for typing or pre-screening of samples containing limited or degraded DNA not suitable for amplification of nuclear DNA. Over the past five years, we have found the linear arrays with either HVII or a combination of HVI and HVII SSO probes very useful for excluding samples prior to mtDNA sequencing. In this study the exclusion capacity has been evaluated further by a retrospective study of previously mtDNA sequenced casework samples.

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Received 30 Nov. 2004; and accepted for publication 9 Jan. 2005; published 6 April 2005.

Materials and Methods

Extraction of DNA

Forensic samples from different items or textile goods were collected on wet swabs and extracted using the Wizard® Genomic Purification System (Promega, Madison, WI). DNA from hair was extracted in a total volume of 212 μL with a final concentration of 1 × PCR Buffer II (Applied Biosystems, Foster City, Ca), 33 mM DTT and 240 μg/mL Proteinase K (SIGMA, St Louis, MO). Chelex extraction was used when a blood samples was provided as reference material (25).

HVI/HVII Linear Array Typing

Duplex amplification was performed with reagents provided in the linear array mtDNA HVI/HVII region-sequence typing kit (Roche Applied Science, Indianapolis, IN) with the addition of 160 μg/mL BSA (SIGMA). To each reaction 2–10 μL DNA extract was added depending on sample type. Amplification was performed according to the manufacturer (Roche Applied Science) using the Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA) with an initial hot start at 94°C for 14 min followed by 34 or 38 cycles at 92°C for 15 s, 59°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 10 min. The PCR product yield was estimated by electrophoresis together with the DNA Molecular Weight Marker XVI 100 bp ladder in 1:1 and 1:2 dilutions using the bands between 100–500 bp. PCR products were diluted (3–5 times) to obtain 45–105 ng of PCR product for optimal hybridization results. Alternatively, a quantification assay based on real-time PCR was used as previously described (26). The LINEAR ARRAY mtDNA HVI/HVII Region-Sequence typing was performed according to the manufacturer's protocol.

HVII Linear Array Typing

Separate amplification for HVII typing was performed as described by Reynolds et al. in 30 μL reactions with the addition of 160 μg/mL BSA (20). To each reaction 2–10 μL DNA extract was added depending on sample type. Thermal cycling (Gene Amp PCR system 9700) was performed with an initial hot start at 95°C for 10 min followed by 34 or 38 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. A final extension was carried out at 72°C for 7 min. Linear array HVII typing was performed as previously described (20).

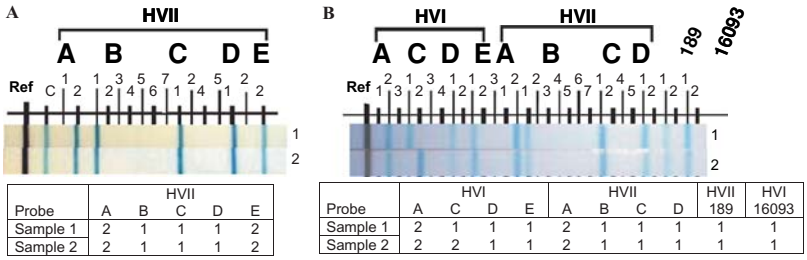
Interpretation and mtDNA Linear Array Versions

The probe reactivity patterns of the linear arrays were read using the mtDNA linear array reference guide for interpretation. Four different types of probebinding patterns can occur in each region: positive signal, weak signal, zero signal or multiple probe signals within a region due to heteroplasmy or a second contributor (mixture). The overall signal strength and the background was considered when determining these patterns. We have used three different versions of the linear array assay. The first linear array for detection of mitochondrial DNA sequence variation (Fig. 1) was comprised of 17 SSO probes targeting five polymorphic HVII regions (20). Two different versions of the combined HVI/HVII linear arrays have been used. The most recent version contains sequence-specific probes striped in 31 lines to target 10 polymorphic regions. The polymorphic positions and variants detected by the linear array mtDNA HVI/HVII Region-Sequence typing Kit are shown in Table 1. Examples of typing results using the linear array mtDNA HVI/HVII Region-Sequence typing Kit are shown in Figs. 2–5. In the first version of arrays where the HVI probe panel was added, probe number 1 and 2 were located as probe number 30 and 31 (Fig. 1).

In the HVII linear array, polymorphisms were detected in the HVII A-D regions, which correspond to the A-D regions of the linear array mtDNA HVI/HVII Region-Sequence typing Kit (Fig. 1, Table 1). In addition, the HVII arrays also had a single probe that detects the poly-C-tract between nucleotide positions 301 to 315, the E-region. However, the HVII-E region were not considered in the forensic casework interpretation due to the high frequency of length heteroplasmy reported in this region (27). This probe is specific for 8 or 9 C residues rather than the 7 residues in the CRS, Cambridge Reference Sequence (28,29). A perfect match between the HVII-E probe and the target is detected by a strong positive signal (20,23). However, a weak positive signal can also arise from a sequence containing 9 C residues or a mixture of different lengths of C residues complicating the interpretation. For this reason, the linear array mtDNA HVI/HVII region-sequence typing kit does not contain this probe.

Sequence Analysis

HVI amplification for direct sequence analysis was performed in 30 μL reactions with a final concentration of 0.4 μM of each primer HVI F15971 5′-TTAACTCCACCATTAGCACC-3′ and HVI R16410 5′-GAGGATGGTGGTCAAGGGAC-3′, 1 × TaqGold Buffer, 1.5 mM $MgCl_2$, 10% Glycerol, 160 μg/mL BSA, 0.33 U/μL AmpliTaq Gold® polymerase, 200 μM of each dNTP and 2–10 μL DNA extract. HVII amplification was performed in 30 μL reactions using primers F15 5′-CACCCTATTAACCACTCACG-3′ and R429 5′-CTGTTAAAAGTGCATACCGCCA-3′ as described for HVII membrane amplification but with a final concentration of 0.33 U/μL AmpliTaq Gold® polymerase. Cycling procedures for both the HVI and HVII target were performed as described



Probe	HVII A	B	C	D	E
Sample 1	2	1	1	1	2
Sample 2	2	1	1	1	2

Probe	HVI A	C	D	E	HVII A	B	C	D	HVII 189	HVI 16093
Sample 1	2	1	1	1	2	1	1	1	1	1
Sample 2	2	2	1	1	2	1	1	1	1	1

FIG. 1—*Probe hybridization patterns for two samples using the HVII and the HVI/HVII linear array. A) The HVII linear array demonstrates identical probe patterns for sample 1 and 2. B) The HVI/HVII linear array (earlier version) shows a single difference between sample 1 and 2 by the HVI probes C1 and C2, which differ at nucleotide position 16304.*

TABLE 1—*The HVI/HVII linear array probe panel showing detected polymorphic positions and detected sequence variants.*

	Probe Designations	Sequence Variation Detected
HVI		**16093**
	16093 1	A T **T** T C
	16093 2	· · **C** · ·
		16126 **16129**
	IA1	T G **T** A C **G** G T
	IA2	· · **C** · · · · ·
	IA3	· · · · · **A** · ·
		16304 **16309** **16311**
	IC1	A G **T** A C A T **A** G **T** A C
	IC2	· · **C** · · · · · · · ·
	IC3	· · · · · · · · · **C** · ·
	IC4	· · · · · · · · **G** · · · ·
	ICw2/ICw3*	· · **C** · · · · · · **C** · ·
		16362
	ID1	C G **T** C C
	ID2	· · **C** · ·
		16270 **16278**
	IE1	C A **C** T A G G A T A **C** C A
	IE2	· · · · · · · · · · **T** · ·
	IE3	· · **T** · · · · · · · · · ·
HVII		**73**
	IIA1	G T **A** G T
	IIA2	· · **G** · ·
		146 **150** **152**
	IIB1	C C **T** C A T **C** C **T** A T
	IIB2	· · **C** · · · · · · · ·
	IIB3	· · · · · · · · **C** · ·
	IIB4	· · **C** · · · · · **C** · ·
	IIB5	· · · · · · **T** · · · ·
	IIB6	· · · · · · **T** · **C** · ·
	IIB7	· · **C** · · · **T** · **C** · ·
		189 **195** **198** **200**
	IIC1	G A **A** C A T A C **T** T A **C** T **A** A A
	IIC2	· · · · · · · · **C** · · · · · · ·
	IIC4	· · · · · · · · **C** · · **T** · · ·
	IIC5	· · **G** · · · · · · · · · **G** · ·
		247
	IID1	T T **G** A A
	IID2	· · **A** · ·
		189
	189 1	G A **A** C A
	189 2	· · **G** · ·

* Indicates the probe pattern readout resulting from the sequence variants 16304C and 16311C detected by multiple signals from probe IC2 and IC3.

for HVII membrane typing amplification above. PCR-products for direct sequencing were purified using the QIAquick PCR-purification kit (Qiagen, GmbH, Germany). Sequencing was performed using the ABI PRISM Dye™ Terminator cycle sequencing kit (Applied Biosystems) and primers HVI F 15978 5′-CACCATTAGCACCCAAAGCT-3′/R16410 and HVII F15/R285 5′-GTTATGATGTCTGTGTGGAA-3′. The sequencing reactions were subjected to electrophoresis using an ABI 377 sequencing instrument or an ABI 3700 capillary instrument (Applied Biosystems). The sequence data were analyzed using the Sequencing Analysis 3.3 package (Applied Biosystems) and the Sequencher™ 4.1.2 software (Gene Codes Corporation, Ann Arbor, MI, USA). Each position was numbered with respect to the revised Cambridge reference sequence (29). The entire analysis procedure as well as the interpretation of inclusions, exclusions and inconclusive results was made following established guidelines for mtDNA analysis (1).

Results

In the interpretation of the linear arrays, an evidentiary sample showing a difference in only one nucleotide position in comparison to a reference sample was considered as an inconclusive result that would require further sequence analysis consistent with recommendations for mtDNA sequence analysis (1). Consequently, exclusion of a sample relies on at least two sequence differences detected by a single or several different probes (a single probe can detect several polymorphisms). Figure 1 shows an example of a reference and an evidentiary sample with identical mtDNA patterns using the older HVII linear arrays. However, using the combined HVI/HVII linear array, a single difference was detected by C1 and C2 probes. Over the past five years, we have used different versions of linear arrays with either HVII or a combination of HVI and HVII SSO probes. The linear array assay has been used on more than 300 forensic samples as a complement to our mtDNA sequence analysis. When using the first version of the linear arrays containing a probe panel for polymorphisms in the HVII region, all samples in approximately 20% of the cases were excluded. In the remaining cases the number of samples that required sequencing was reduced substantially using the linear array assay. To further evaluate the exclusion capacity of the linear arrays, a retrospective study was performed based on previously sequenced forensic samples.

HVI/HVII Linear Array Exclusion Capacity

In order to evaluate the exclusion capacity of the HVI/HVII mtDNA linear array probe panels in casework analysis, 16 cases involving 90 samples (57 evidence samples and 33 reference samples) with available HVI and HVII sequence data were investigated. Expected probe patterns were determined for each sample based on previous sequence analysis data. Further, as the linear mtDNA array has been expanded with HVI probes recently, the exclusionary capacity between the HVII and the HVI/HVII probe panels were compared. Of the 90 previously sequenced samples, 31 were typed with the HVI/HVII linear array for confirmation of the expected mtDNA probe patterns. All samples showed results in concordance with the sequencing results. Table 2 shows a summary of the expected linear array typing and the sequencing results of the evidence and reference samples in the 16 cases. A majority of the evidence materials were shed hairs while most of the reference materials were plucked hairs. Using the HVII probe panel, all materials in five cases were excluded, while all materials in one additional case were excluded using the combined HVI/HVII linear array. Sequencing would thus be required only in the remaining 10 cases due to a match or inconclusive results (a single difference). Moreover, sequencing efforts are reduced even further by linear array based exclusions of a subset of samples in six of the 10 cases. Furthermore, the sequence analysis revealed additional exclusions of samples in three of the 10 cases.

The total number of samples that were excluded based on the HVII probe panel was 41/90 (46%) while the number of excluded samples based on the HVI/HVII probe panel was 50/90 (56%). The number of samples excluded by sequence analysis was 63/90 (70%). Thus, only 13 of 63 (21%) samples excluded by sequencing could not be excluded by HVI/HVII linear array typing. A single nucleotide difference between the evidentiary sample and a suspect (inconclusive) were observed in 17 samples using the HVII probe panel and in 7 samples using the HVI/HVII probe panel. Thus, the number of samples with inconclusive results was reduced by 59% (10/17) using the additional HVI probes and the number of cases involving samples with inconclusive results was reduced from six

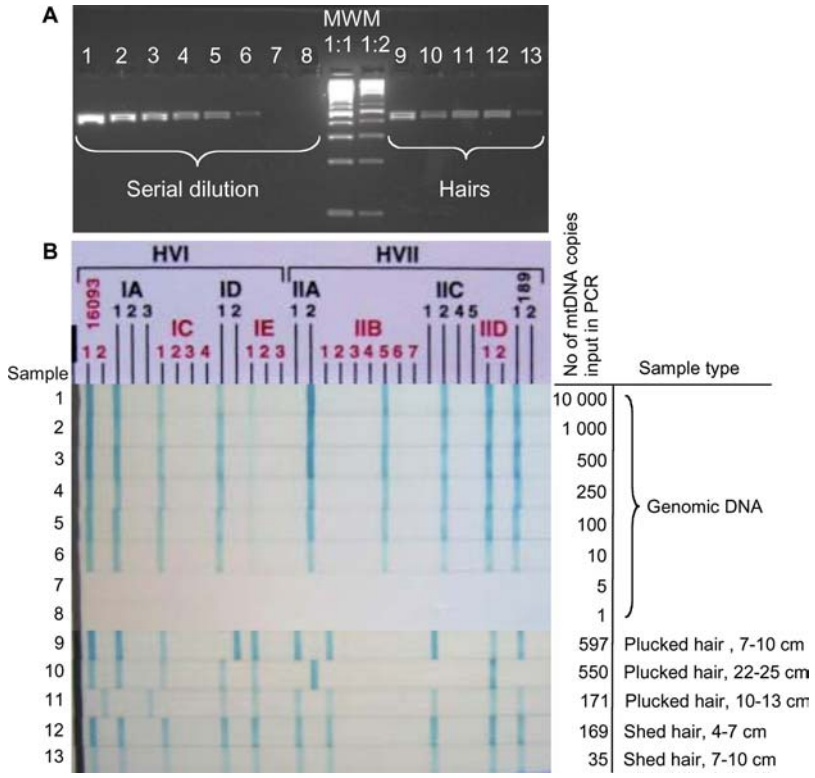
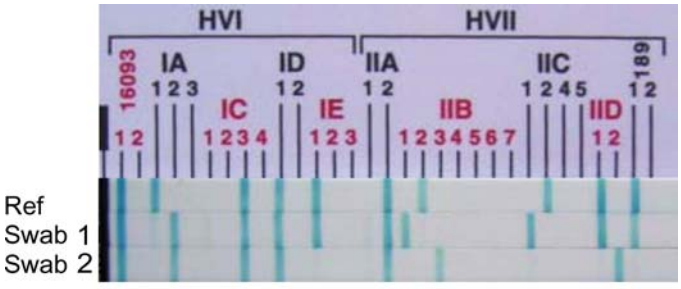


FIG. 2—*A) Agarose gel image of undiluted PCR-products from amplification of a serial dilution of genomic DNA and five distal-part hairs with different number of input mtDNA copies in the PCR (samples 1–13). The molecular weight marker for quantification is included as 1:1 (undiluted) and 1:2 dilution between sample 8 and 9. B) Linear array typing results of undiluted samples 1–13. The source of each sample and the PCR input are shown to the right of the linear arrays. The three cm distal part from which DNA was extracted is shown for the hair samples (9–13). Sample 11–13 represents the outermost part of each hair.*



	HVI					HVII				
	16093	A	C	D	E	A	B	C	D	189
Ref	1	1	3	1	1	2	2	2	1	1
Swab 1	1	2	3	1	1	2	1	1	1	1
Swab 2	1	2	3	1	0	2	3	0	2	0

FIG. 3—*Analysis of forensic samples in case 1. The two DNA samples from the knife did not match the reference. A '0' indicates no probe signal visible due to a mitochondrial variant not detected by the array.*

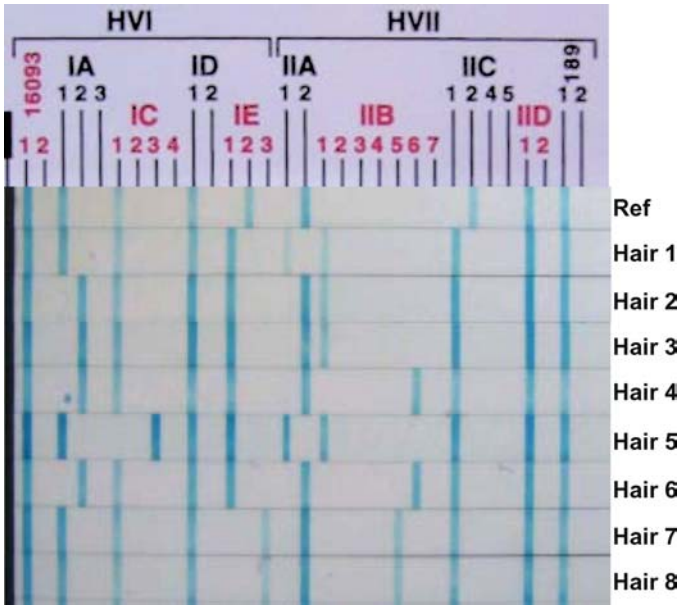


FIG. 4—*Linear array typing results for a subset of samples described in Case 2. The evidence materials (Hair 1–8) were all excluded as originating from the suspect (Ref).*

to one. In the sequence analysis, all samples showing inconclusive linear array typing results were excluded by detection of additional differences. Approximately one third of the samples (33/90) were reference materials and of these, 11 (33%) were excluded based on the HVII probe panel while 17 (52%) were excluded based on the entire HVI/HVII linear array. Overall the sequencing resulted in inclusion between 15 evidence materials and 12 suspects in nine of the 16 cases.

Quantification

The new version of the HVI/HVII linear arrays requires an estimation of the amount of PCR-product added to the immobilized probe arrays to obtain optimal results. An overload of PCR products

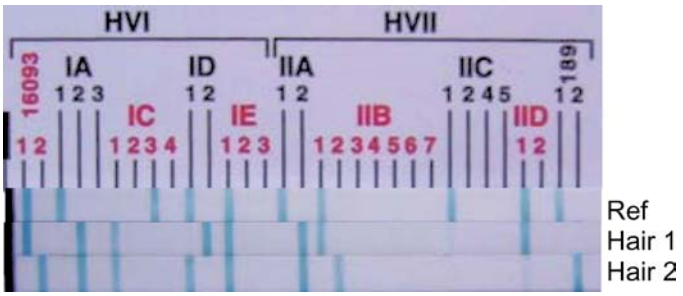


FIG. 5—*Linear array typing results for two hairs and a reference sample in case 3. The hairs in this missing person case resulted in exclusions.*

TABLE 2—*Comparison of expected HVII and HVI/HVII linear array typing and sequencing results.*

Number of Samples				HVII		HVI/HVII		Sequencing Results	
Case	Evidence	Reference	Total	0–1	≥2	0–1	≥2	0	≥2
C1	2	1	3	0	3	0	3	0	3
C2	2	1	3	3	0	3	0	3	0
C3	2	1	3	0	3	0	3	0	3
C4	2	1	3	3	0	3	0	3	0
C5	1	2	3	3	0	2	1	2	1
C6	2	1	3	0	3	0	3	0	3
C7	3	1	4	3	1	0	4	0	4
C8	14	1	15	0	15	0	15	0	15
C9	1	2	3	0	3	0	3	0	3
C10	1	2	3	2	1	2	1	0	3
C11	1	3	4	3	1	2	2	2	2
C12	1	4	5	3	2	2	3	2	3
C13	2	2	4	4	0	4	0	4*	0
C14	3	2	5	5	0	5	0	4*	1
C15	4	4	8	7	1	5	3	5*	3
C16	16	5	21	13	8	12	9	2	19
Total	57	33	90	49	41	40	50	27	63
%					46%		56%		70%

A summary of the mtDNA results from 90 forensic samples involving 16 cases. The number of evidentiary and reference samples and the total number of sequenced samples for each case are shown in the first three columns. For each case, the expected HVII and HVI/HVII probe panel results are displayed as the number of samples showing inclusions (0–1 nucleotide differences between samples) and exclusions (≥2 nucleotide differences between samples). The last two columns show the sequencing results in each case. * Denotes cases with inclusions to two suspects.

may cause cross-hybridization, a problem seen occasionally using the earlier versions of the array. However, a quantitative measurement of the PCR product yield using a molecular weight marker optimizes the outcome in the typing procedure. Alternatively, real-time PCR quantification measurements of the initial DNA content can be used to balance the input DNA in the PCR to obtain an equal product yield between samples (26). Many forensic laboratories are currently implementing real-time PCR assays for routine quantification of evidence samples. This can replace the electrophoresis-based post-PCR quantification step saving time and effort.

The sensitivity of the HVI/HVII linear arrays was investigated by typing of samples previously quantified by a real-time PCR assay (26). A quantified dilution series of genomic DNA (assuming 1 000 mtDNA copies/diploid genome) were typed using input amounts in the HVI/HVII PCR ranging from 1–10 000 mtDNA copies. Strong and easily interpretable array signals were obtained from samples containing 100–10 000 mtDNA copies, equivalent to 0.6 pg to 60 pg of genomic DNA (333 genome equivalents/ng DNA) indicating a highly sensitive typing system (Fig. 2). An identical mtDNA type was obtained from the sample containing about 10 mtDNA copies, albeit with a weaker signal. In addition, previously quantified hairs from 5 individuals were typed using the HVI/HVII linear arrays. Three of the hairs were plucked and two were shed hairs. The first centimetre including the root and a three-centimetre distal shaft part was extracted, quantified and typed for each individual. All distal shaft parts revealed typing results identical with the results obtained for the root parts. Typing of the hair shafts illustrates that it is possible to type mtDNA distal parts of shed hairs containing a few hundred mtDNA copies (Fig. 2).

Casework Examples

Case 1—A murder was committed in a restaurant where a man was stabbed with a knife. With a house search warrant of a suspect's home, the investigators found a knife as a possible murder weapon. DNA analysis was performed on material collected on swabs from the blade and the handle of the knife. A hair from the victim's mother was used as reference. The two different mtDNA types from the knife did not match the reference and the exclusion of the two evidence samples was also confirmed by sequencing. Figure 3 shows the results of the HVI/HVII linear array typing.

Case 2—In a police homicide case, two cars possibly used to escape from the crime scene were investigated. In total 14 hairs were found in the two cars. A hair from one suspect was provided as reference. Both sequence analysis and linear array typing revealed exclusionary results based on at least two nucleotide differences when comparing evidence materials with the reference material. A subset of the HVI/HVII linear array typing results (eight evidence materials and the reference sample) is shown in Fig. 4. The number of mitochondrial DNA copies in these samples was previously measured using real-time PCR quantification (26). The quantification data showed that the input DNA amounts (10 μL DNA extract) in the HVI/HVII PCR ranged between 4000–32 000 mtDNA copies with a majority of the samples containing approximately 14 000 mtDNA copies. Both the results from real-time PCR quantification data and electrophoresis using a molecular weight marker showed that the samples required a 3 to 5 fold dilution prior to typing.

Case 3—In a missing person case, a young girl was abducted and assumed to have been murdered. In a search of a lake using specially trained dogs, the police found shed hairs. HVI/HVII linear array typing and sequencing was performed on the shed hairs and a hair from the victim's mother as reference. Both linear array typing and sequencing revealed that both hairs could be excluded as originating from the victim. Figure 5 illustrates the HVI/HVII linear array results.

Discussion

Direct sequencing of the mitochondrial D-loop is a powerful and reliable method for discrimination between DNA samples containing limited amounts of DNA, but is also time consuming and labor intensive. Linear array (immobilized probe) typing of mtDNA polymorphisms proved to be a simple and quick pre-screening method with potential to substantially reduce sequencing efforts due to exclusion of samples. The linear array analysis is performed in less than 3 hours without the need for expensive equipment. Furthermore, as the PCR is performed in a duplex reaction and remaining PCR-products can be sequenced, valuable evidence material is saved. Thus, this rapid and user-friendly linear array

typing system provides a convenient and efficient pre-screening method for selection of the most relevant samples for further analyses.

Over a five-year period more than 300 forensic samples, not suitable for nuclear DNA analysis, have been successfully typed for mtDNA polymorphisms using the linear arrays. Our experience of the linear array assay in the analysis of these samples illustrates the value of this robust typing system in casework analysis. A majority of the specimens that were analyzed using the combined HVI/HVII linear array were shed hairs. However, previous use of the HVII linear array has been successful on samples obtained from a variety of items, such as epithelial cells collected from areas in close contact with the skin. Furthermore, successful linear HVI/HVII array typing results have been obtained in a cold case investigation of 15-years old hair samples, one shed head hair (3 cm long) and two reference hairs (data not shown). The assay has also proved to be useful for analysis of bone material in a previous study (19). The linear arrays are generally considered to be more sensitive than sequencing for detection of mixtures or heteroplasmy. The minor component detected by immobilized probe/linear array typing is 5–10% while the detection threshold for direct sequencing is 15–25% (18). In our experience, samples containing a mixture of DNA from several contributors are easily identified by signals from two (or more) probes in several regions. Thus, there is an added advantage to pre-screening as samples containing obvious DNA mixtures from several contributors can be identified and excluded in the further analysis.

Hybridization assays demand careful design of probes and stringent conditions to avoid formation of non-specific hybridization complexes. In the linear array assay, the critical stringency factors are temperature, time and salt concentration in the washing step. As two different versions of the HVI/HVII linear arrays have been used in our laboratory, improvement in the sensitivity and signal strength was apparent on the most recent version of the arrays. Also, the signal intensities between probes across the panel were more even. If an overload of PCR product is added to the linear array the potential risk of cross-hybridization increases. Occasionally cross-hybridization signals were observed for certain probes using the first version of the HVI/HVII linear arrays. However, no signs of cross-hybridization were detected using the new version of the HVI/HVII linear array as quantification of the initial DNA content or the PCR-product yield prior to typing was performed. In this study, a TaqMan assay was used for determination of DNA copy numbers in samples to facilitate normalization of input amounts to the PCR for equal product yields. Alternatively, an electrophoresis-based estimation of the PCR product yield was done. Real-time PCR quantification was also used to evaluate the sensitivity of the linear array assay. Typing of quantified DNA samples with limited amounts provided successful and reliable results from three centimetre pieces of distal shaft parts of shed and plucked hairs. Readable results were furthermore obtained from 10–100 mtDNA copies of a control sample.

The new combined HVI/HVII linear arrays system offers a more informative typing assay in comparison with the HVII arrays, as a consequence of the increased number of probes. The HVI region is generally considered to be slightly more polymorphic than the HVII region (24) and the selected sequence variants in the combined probe panel are highly frequent in European populations (30–33). The addition of the HVI region probe panel to the linear arrays increased the number of excluded samples by 10% and furthermore reduced the proportion of inconclusive results by 59% for the case samples presented here. While 50 samples were excluded by the HVI/HVII linear arrays, additional 13 exclusions were made in the sequence analysis. Thus, of all 63 exclusions made by sequence analysis, 79% (50/63) were excluded by using the HVI/HVII linear array probe panel alone.

Using the mtDNA linear array assay, a large number of crime scene samples can be screened simultaneously for inclusion or exclusion to suspects and thereby identify the samples of most interest for further investigation. In summary, we found the HVI/HVII linear array assay robust, rapid, accurate and sensitive with a high potential to discriminate between different mtDNA types. The use of the mtDNA linear arrays in our laboratory has served as a valuable pre-screening method and demonstrates the potential to reduce the required sequencing efforts by more than half.

Acknowledgments

We would like to thank Michael Grow, Matthew Gabriel, Natasha Stankiewicz, Jim Chou and Karen Walker for contributing to the development of the linear array mtDNA HVI/HVII region-sequence typing kit. We would like to thank Matt Fong at Roche Molecular Systems, Alameda, U.S., for reviewing the manuscript. This project was supported by grants from Kjell and Märta Beijers Foundation and the Swedish Research Council (VR grants nos. K2002-31P-12577-05C and K2003-31X-13095-05A) to M.A. and by Grant Number (1996IJCX0028) awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice to C.C and HE. Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the US Department of Justice.

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