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ORIGINAL ARTICLE

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# Validation of mitochondrial DNA minisequencing for forensic casework

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**Abstract** The validation of multiplex solid-phase fluorescent minisequencing of mitochondrial DNA (mtDNA) for use in forensic casework is presented. Validation included testing of the reliability and species specificity of the technique, analysis of mixed body fluid samples, analysis of samples and substrate controls from previous cases and somatic stability of mtDNA. Animal, bacterial and fungal species extracts were examined and the test did not show cross-reactivity with other species. Hair, blood, saliva, faeces and semen or vaginal samples were tested from five male and five female individuals. For all the samples tested, heteroplasmy was observed only at position 302/309.1. Body fluid mixtures (blood:saliva, semen:saliva, faeces:semen, vaginal:semen) and DNA:DNA mixtures were examined. In total, 189 mixtures were analysed of which one resulted in a hybrid profile consisting of peaks from each of the two donors. The semen fraction of the semen:saliva and vaginal:semen mixtures appeared to be concentrated in the supernatant fraction of the extract thus highlighting the need to extract both the pellet and supernatant fractions of a stain. Control samples, crime stains and their substrate controls from previous cases were examined. Of the 12 loci typed by minisequencing, 11 could be verified by comparison to results from the sequencing method currently in use for casework and no discrepancies were observed between the two. MtDNA minisequencing was found to be a reliable and reproducible technique and its rapid and discriminating nature make it particularly suitable as a screening technique.

**Key words** MtDNA · Minisequencing · Forensic validation

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## Introduction

Analysis of mitochondrial DNA (mtDNA) has primarily been used to determine the phylogenetic history and geographical origin of human populations [1] and to characterise disease states [2–5]. In a forensic context, analysis of mtDNA can be successfully performed where typing of genomic DNA is not possible due to degradation of the sample or low genomic DNA copy number. Examples of sample types for which mtDNA analysis is particularly appropriate are hair shafts, faeces, bone and teeth [6–10]. The rate of nucleotide substitution in the mitochondrial genome is 5–10 times faster than in the nuclear genome and as a consequence, mtDNA is highly polymorphic. The majority of sequence variation between individuals is found within the 1.1 kb control region which contains the two hypervariable regions HVI and HVII [11–12]. Because of the maternal mode of inheritance of mtDNA, sequencing of the hypervariable regions is particularly useful for identifying human remains, where the mtDNA sequence can be compared to that of a maternal relative [8, 13–16]. World-wide, the number of forensic cases involving mtDNA is increasing rapidly. Cases include an ongoing mtDNA programme to aid in "full accounting" for more than 10,000 US personnel missing from conflicts since World War II [17] and identifying skeletal remains from mass graves [8, 13]. In such cases, sequencing the entire hypervariable region is cost, time and labour intensive. A rapid screening method which can quickly exclude a number of samples has high forensic potential. Multiplex solid-phase fluorescent minisequencing is one such technique [18]. This method simultaneously identifies ten substitution polymorphisms and two length polymorphisms located in the major non-coding region of the mtDNA. The substitution polymorphisms are located at positions H00073, H00146, H00152, H00247, H16069, H16129, H16189, H16224 and H16311 and the length polymorphisms are located at H00302/309.1 and L00525/523 (numbered following [19]). The probability of a random match between two unrelated individuals using this

technique is 0.054 for British Caucasians and 0.026 for British Afro-Caribbeans, calculated from databases of 152 and 103 individuals respectively [18]. In order to validate minisequencing of mtDNA for forensic casework, the following were investigated: reliability and species specificity of the technique, analysis of mixed body fluid samples, analysis of samples and substrate controls from previous cases and somatic stability of mtDNA.

## Materials and methods

### Samples

Non human samples: Freeze dried cultures of *Candida albicans*, *Clostridium difficile*, *Enterococcus faecium*, *Escherichia coli*, *Niesseria gonorrhoea*, *Thermus aquaticus* and *Bacillius aureus* were obtained from American Type Culture Collection (Rockville, MD). Blood samples taken from cat, dog, pig, cow, rabbit, rat, chicken, turkey, sea trout, pigeon, goose, herring, pheasant, sheep and lamb were obtained from veterinary practices.

Somatic stability: Blood, saliva, hair shafts, faeces and vaginal or seminal fluid were donated by five female and five male individuals.

Mixtures: DNA samples of known genomic DNA concentration were obtained from five pairs of individuals. Mixtures were made up in the following ratios of DNA by weight; 1 : 1, 1 : 2, 1 : 5, 1 : 10, 10 : 1, 5 : 1 and 2 : 1. Liquid blood/saliva and seminal fluid/saliva were mixed in proportions of 20 : 1, 10 : 1, 5 : 1, 3 : 1, 2 : 1, 1 : 1, 1 : 2, 1 : 3, 1 : 5, 1 : 10 and 1 : 20 by volume and loaded onto clean stain cards and swabs, respectively. Postcoital vaginal swabs taken at known times after intercourse were donated by three individuals. Mock postcoital swabs were prepared by adding measured volumes of semen to vaginal swabs using samples from three pairs of individuals. Mock anal and penile swabs consisting of weight/volume faeces:semen mixtures were prepared using samples from four pairs of individuals.

Case samples: Extracted DNA was obtained from 14 reference samples, 14 crime stains and their associated substrate controls from previous cases.

Comparison of sequencing and minisequencing: Extracted DNA was obtained from 22 individuals for which sequence data were available.

### Extraction and minisequencing of DNA

Blood, saliva, vaginal swabs, seminal fluid, DNA:DNA, blood:saliva, semen:saliva and vaginal: semen mixtures were extracted using a chelex method as detailed for whole blood [20]. For the semen:saliva and the vaginal:semen mixtures both the supernatant and the pellet were extracted (250 µl supernatant was incubated with 250 µl 40% Chelex100). Faeces and faeces:semen mixtures were extracted using a standard phenol chloroform method [21]. Hair shaft samples were extracted as follows: approximately 2 cm of hair shaft was subjected to three washes (vortex mixing for 30 s in 1 ml sterile distilled water). The hair shaft was removed to a sterile tube containing 215 µl 20% Chelex, 10 µl 1 M DTT and 25 µl Proteinase K (10 mg $\mu l^{-1}$) and incubated until no hair fragments remained visible to the naked eye (either 56° C for up to 4 h or 37° C overnight). Samples were boiled for 8 min, centrifuged at 13000 rpm for 3 min and the supernatant removed to a sterile tube.

Aliquots of 1–10 µl DNA were amplified in a total volume of 50 µl. Each reaction contained 1 × polymerase assisted repair replication (PARR) buffer (Cambio, Cambridge), 200 µM of each deoxynucleotide, 2.5 U AmpliTaq (Perkin-Elmer, Norwalk, CT) and 0.5 µM of each of the following primers (Oswel DNA Service, Southampton, Hants.):

L16048 Biotin-TCATGGGAAGCAGATTTGG

H16332 GGATTTGACTGTAATGTGCTATG

L00066 Biotin-TGCATTTGGTATTTTCGTCTG

H00326 CAGAGATGTGTTTAAGTGCTGT

L00386 GAACCCTAACACCAGCCTAAC

H00537 Biotin-GGAGGTAAGCTACATAAACTGTG

Using 0.2 ml thin-walled tubes and a Perkin-Elmer GeneAmp PCR System 9600, the reaction mixtures were cycled 35 times through denaturation at 94° C for 30 s, annealing at 57° C for 30 s and extension at 72° C for 90 s. Of each amplified product 10 µl was sub-

**Table 1** Minisequencing extension primer sequences

| Location of 3′ base | Sequence 5′–3′ | Concentration (µM) |
|---|---|---|
| H00303 | GTC GTG TGG CCA GAA GCG GGG GGA GGG GGG G | 6 |
| H0074 | TTT TTT TTT CCA GCG TCT CGC AAT GCT ATC GCG TGC A | 0.3 |
| H16312 | TTT TTT TTT TGT GCT ATG TAC GGT AAA TGG CTT TAT GT | 0.06 |
| H16070 | TTT TTT TTT TTT TTT TAA GCA TTA ATT AAT TAA CAC RCT TYR RTA | 0.4 |
| H00196 | TTT TTT TTT TTT TTT TTA AGC ATT AAT TAA TTA ACA CRC TTY RRT A | 6.5 |
| H00153 | TTT TTT TTT TTT TTT TCT GTA ATA TTG AAC GTA GGT GCG ATA AAT AAT | 0.3 |
| H00147 | TTT TTT TTT TTT TTT TTA ATA TTG AAC GTA GGT GCG ATA AAT AAT RRR ATG | 1.25 |
| H00248 | TTT TTT TTT TTT TTT TTT TTT TTT TTC TGT GTG GAA AGT GGC TGT GCA GAC ATT | 0.05 |
| H16224 | TTT TTT TTT TTT TTT TTT TTT TTT TTT TTG GAG TTG CAG TTG ATG TGT GAT AGT TG | 0.1 |
| L00522 | TTT TTT TTT TTT TTT TTT TCT CAT CAA TAC AAC CCC CGC CCA TCC TAC CCA GCA CAC ACA CAC | 0.6 |
| L00524 | TTT TTT TTT TTT TTT TTT TTT TTT CTC ATC AAT ACA ACC CCC GCC CAT CCT ACC CAG CAC ACA CAC | 0.48 |
| H16130 | TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TGT ACT ACA GGT GGT CAA GT | 0.04 |
| H16190 | TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT GGT TGA TTG CTG TAC TTG CTT GTA AGC ATG RGG | 0.2 |

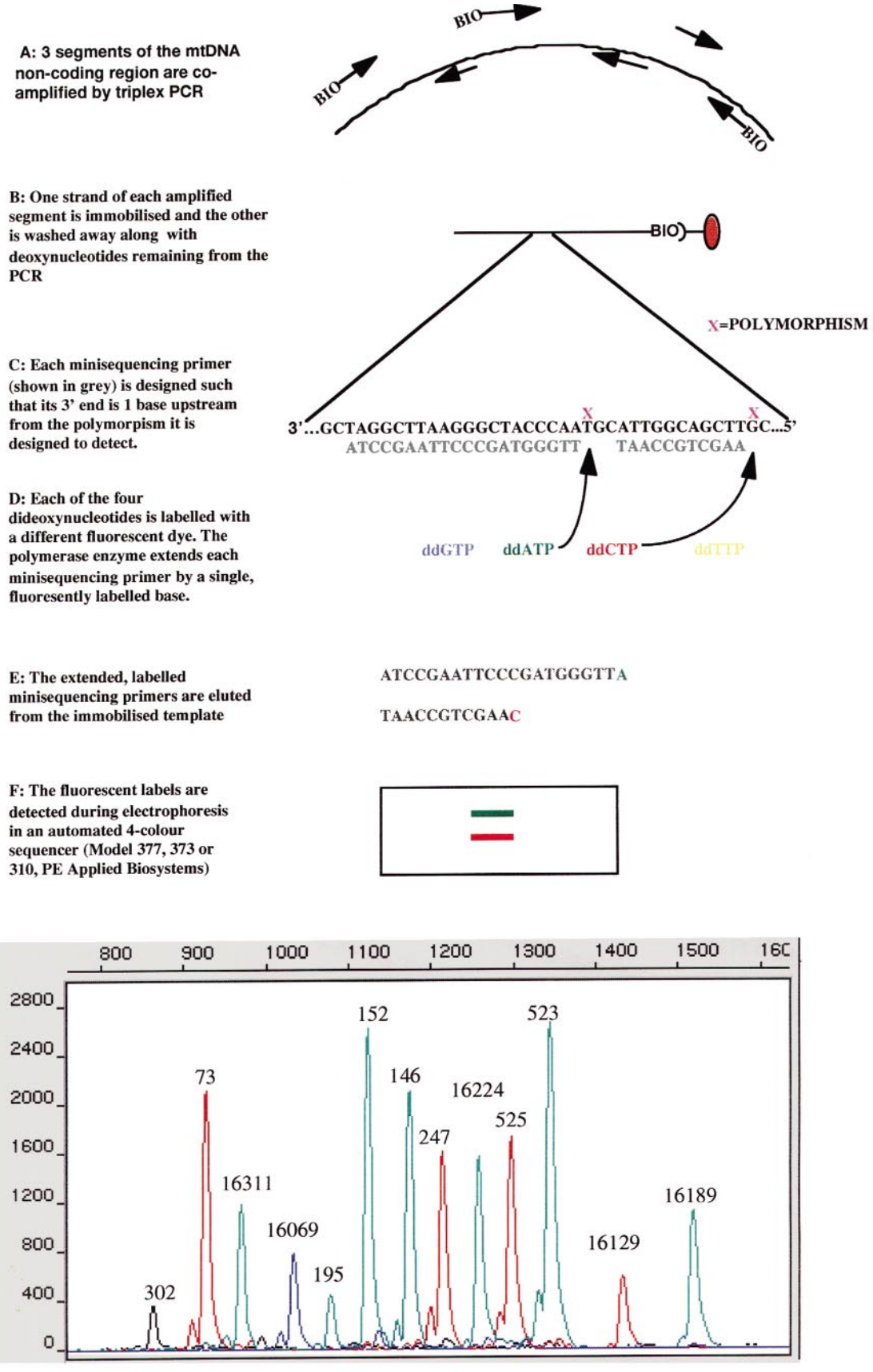


**Fig. 1** Schematic diagram of the multiplex minisequencing process and an example of a mtDNA minisequencing profile

jected to electrophoresis in a 3% NuSieve GTG agarose gel (FMC BioProducts, Rockland, ME) at 120 V for 50 min and visualised by ethidium bromide staining and short-wave UV transillumination.

Dynabeads M-280 streptavidin (Dynal, Oslo) were prewashed according to the manufacturer's instructions and resuspended in 40 µl 2 × binding and washing (BW) buffer (2 M NaCl, 1 mM EDTA and 10 mM Tris-HCL, pH 7.5). The remaining 40 µl of each PCR product was bound to 40 µl of Dynabeads by a 15 min incubation at 48° C. The Dynabead-PCR product complex was washed in 2 × BW and then in sterile distilled water (SDW). Following a 4 min incubation in 0.15 M NaOH at room temperature to denature the PCR product and a wash in 0.15 M NaOH, the samples were washed once in 250 mM Tris-HCl, pH 8, 0.1% Tween-20, once in 10 mM Tris-HCl, 1 mM EDTA and once in SDW. All washes were carried out in 100 µl volumes.

The bead-product complex was resuspended in 10 µl SDW. For each sample, 40 µl of minisequencing extension multimix consisting of 0.4 M Tris-HCl, 10 mM $MgCl_2$ pH 9, 20% dimethyl sulphoxide, 0.1 µl fluorescently labelled ddCTP and ddTTP, 0.125 µl fluorescently labelled ddATP and ddGTP, 2.5 U AmpliTaq FS (Perkin-Elmer Limited, Warrington, England) and the 13 primers listed in Table 1 was added to a fresh set of thin-walled tubes. The GeneAmp PCR System 9600 was preheated to 57° C. The programme was paused and the tubes containing the multimix and those containing the bead-product complex were placed in the wells. The programme was resumed and samples and multimix were heated at 57° C for 30 s. The programme was paused, the multimix added to the bead-product complex and the temperature reduced to 52° C for a 60 s incubation. The bead-product complex was snap-cooled on ice and the multimix decanted. Samples were resuspended in 100 µl 1 × BW buffer.

The 1 × BW buffer was decanted and the samples resuspended in 4 µl formamide and denatured at 72° C for 4 min before being snap-cooled on ice. The formamide, containing the labelled DNA was transferred to a clean tube and 1 µl loaded on a 19% denaturing acrylamide gel with a well-to-detection distance of 12 cm in a 377 automated sequencer (Applied Biosystems Division). Electrophoresis was carried out on the 2 × A setting (1680 V) for 2 h and the data collected using ABI Prism 377 Collection software version 1.1 (Applied Biosystems Division). The data were analysed using ABI Prism GeneScan Anaysis version 2.0.2 software (Applied Biosystems Division) and the peak colour and area for each extended minisequencing primer were scored for each sample (Fig. 1).

## Results and discussion

### Species specificity

For certain case sample types it was important to establish whether or not non-human species could be the source of minisequencing peaks obtained. For example, human faeces samples may contain undigested meat from animal products and vaginal or anal swabs or faeces samples may potentially contain some of the bacteria or yeast species tested. Dog or cat faeces may be recovered from a scene and submitted for analysis as putative human samples. All species extracts gave quantifiable levels of total DNA (0.16–13.85 ng $\mu l^{-1}$) and approximately 2 ng of total DNA from each sample was amplified. A human DNA positive control was included in each amplification batch. None of the bacterial or fungal species gave a peak at the minisequence loci tested. Initially partial profiles were observed in cat, dog, sheep and pork (meat from a supermarket). However, when fresh samples were extracted and analysed, no minisequence products were observed in any sample except the human DNA positive control; the possibility that a PCR inhibitor in the fresh extracts caused the failure to detect a minisequence profile was eliminated, as primer-dimer was visible in all PCR products. This indicated that human contamination had been the source of the original profiles. No detectable minisequencing loci were observed for any extract from blood of rabbit, rat, chicken, pig, turkey, sea trout, goose, pigeon, herring and pheasant. However, full or partial profiles were obtained from all extracts of bovine DNA tested. Given the fact that the profiles observed in all bovine samples, except those samples obtained from another laboratory, could be attributed to members of staff in our laboratory, it was concluded that these profiles were also due to contamination from a human source. Previous validation studies have demonstrated no cross-reactivity of a mtDNA typing technique on non-human samples including DNA extracted from cow [22]; in addition bovine mtDNA sequence is highly divergent from human mtDNA [23]. These results confirm that faeces from a domestic pet or undigested meat in a faeces sample will not give a minisequence profile. Undigested meat will not contribute to a minisequence profile as both faeces and blood samples from the same individual gave identical minisequence profiles (see somatic stability section), confirming previous results that diet does not affect results from mtDNA typing of faeces [7].

### Somatic stability

In order to demonstrate that minisequencing results from control samples can be reliably compared between tissue types likely to be encountered, the somatic stability of

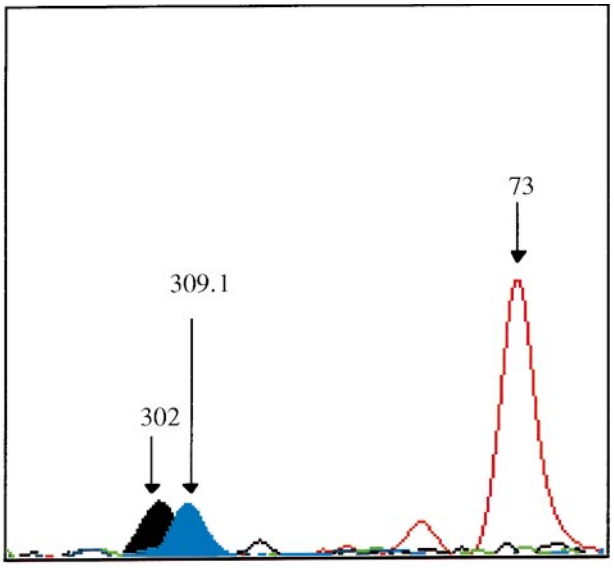


**Fig. 2** Length heteroplasmy at position 302/309.1 Heteroplasmy at this position tends to reduce the peak height of both incorporated dideoxy nucleotides relative to the peak height at the other positions. The product length is 32 bases regardless of whether the individual is 7 or > 7 Cs, however there is a mobility shift caused by the differential mobility of the dyes TAMARA (ddTTP) and dR110 (ddGTP)

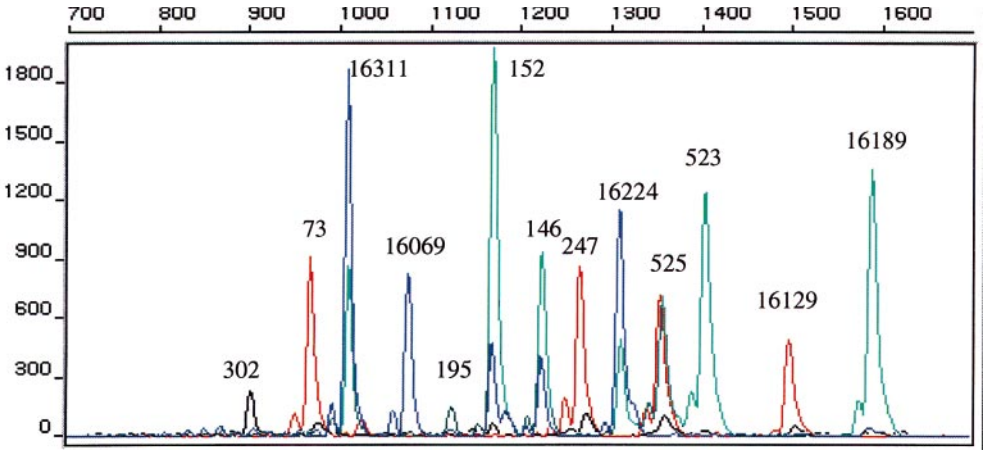


**Fig. 3** Donor 5/Donor 6 profile showing mixed peaks at positions H16311, H00152, H00146, H16224 and L00525
Donor 5. T C G G A A A C G C A C A
Donor 6. G C A G G G G C A A A C A

mtDNA at the loci determined by the minisequencing technique was investigated. In 9 of the 10 donors, no evidence of somatic variability was observed. In the tenth individual tested, heteroplasmy, in which two types of mtDNA coexist within the individual, was observed at locus 302/309.1 (Fig. 2). At this locus the primer detects a length polymorphism at a poly C (cytosine) stretch. An individual may have either 7, 8 or 9 cytosine bases, but the minisequencing technique distinguishes only between 7Cs and > 7Cs*. In order to further investigate the heteroplasmy, 15 additional hairs from this individual were processed. Length heteroplasmy was observed in the blood, saliva, semen, faeces and 6 of the 20 hairs examined. Of the hairs eight were homoplasmic for > 7Cs while one hair was homoplasmic for 7Cs. Heteroplasmy in both animal and human mtDNA is well documented [23–27] and evidence of both heteroplasmic and homoplasmic hair shafts within an individual has been documented at position 16093 [28]. On the basis of the results presented here, reporting guidelines have been formulated in order to ensure that a common maternal origin for an unknown (or crime) and a reference sample is not falsely excluded. In summary, if the only difference between samples is at position 302/309.1, an inclusion or exclusion should be confirmed by further sequencing. Furthermore if there is evidence of heteroplasmy in either the crime or control sample, a maternal origin should not be excluded. If the reference sample is from a maternal relative of the putative donor of the casework sample rather than from the putative donor, allowance should be made for the possibility of germline substitution. Therefore, if only one difference is observed between the reference sample (maternal relative of putative donor of casework sample) and the casework sample, further sequencing should be carried out for confirmation of inclusion or exclusion.

## Analysis of body fluids

If more than one individual contributes to a profile, different dideoxynucleotides may be incorporated at the same locus (Fig. 3). This would be termed a “mixture” or “mixed profile”. However, in some instances it was not possible to definitively state whether the minority peak present was due to a mixture component or “pull-up”. “Pull-up” occurs when the profiles are strong (peak heights of > approximately 2000) because the linear range of the automated sequencer will be exceeded and other colours under main peaks may be observed. If more than one individual contributes minisequence peaks to a profile but the profile does not appear to be a mixture, this would be termed a “hybrid profile”. Mixtures within a profile were identified and the peak area was noted for each component of the mixture for analysis purposes.

## DNA:DNA mixtures

For all ratios of DNA:DNA mixtures from the five pairs of individuals tested, great variability was observed in the peak area of each component in a mixture. The cause of this variability is most likely to be the fact that the DNA concentrations used in the preparation of the mixtures were genomic DNA concentrations; the mtDNA concentration in each sample was unknown. Mixed profiles were obvious in 27 out of 29 samples tested. A general trend was observed in that, as the level of template DNA was increased, the peak area of the minisequencing loci for that subject increased. In the two samples where a mixture was not obvious (no minority peaks under main peaks or the presence of “pull-up”), the profile which was apparent was that of one or other of the mixture individuals and not a hybrid of the two profiles. For both samples where a mixture was not obvious (DNA:DNA ratio of 1 : 10) the higher concentration of template DNA contributed the peak regardless of which dideoxynucleotide was incorporated. All profiles were correctly typed according to reference samples.

## Blood:saliva mixtures

In general, the blood component of all blood:saliva mixtures was dominant. Of 37 samples tested, a mixed profile

* The base determined is at position 302. The insertion occurs after 309 giving rise to the nomenclature 309.1

was clearly identified in 19. Mixed profiles could only be clearly identified when there was at least five times more saliva than blood (by volume) in the stain. This would suggest that background levels of saliva on a garment would not contribute to a profile from a blood stain extract. In the remaining 18 samples, only the blood profile was observed.

### Vaginal:semen mixtures

The pellet fraction of post coital swabs taken at measured times after intercourse and of mock post coital swabs were extracted. In all samples, only the female component contributed to the profile. If, however, the supernatant rather than the cell pellet of the recovered material was extracted, mixed profiles were obtained up to and including 14.5 h after intercourse and for 30, 75, 150, and 300 µl of added semen to vaginal swabs. It appears that extraction from the supernatant biases the extraction towards the semen component of the mixture. MtDNA resides in the mid section of the spermatozoon and breakdown of the spermatozoon head is not necessary for extraction purposes. It is not possible to conduct a preferential extraction on vaginal swabs as both fractions (vaginal epithelial and spermatozoa) are eluted into water. In our experience, extraction methods in which the spermatozoa head is ruptured does not yield any amplifiable mtDNA.

### Semen:saliva mixtures

The pellet fraction of semen:saliva mixtures was extracted. In general, the semen component of the mixture was dominant, except at ratios of 1:10 and 1:20 (semen:saliva) and eight samples showed no evidence of being mixtures. One of these (donor 1/donor 2 1:10 semen:saliva) was a hybrid profile which consisted of peaks from both donors (Table 2). Loci 302, 16311, 152 and 16224 were contributed from donor 1 and loci 195 and 525 contributed from donor 2. The remaining loci might have been contributed from either donor 1 or 2 or both.

For one of the mixture pairs, (donor 3/donor 4) a second set of swabs was prepared and both the pellet and the supernatant were extracted. At the higher concentrations of semen, 20:1, 10:1 and 5:1 (semen:saliva), there was no difference between the profiles from the pellet and supernatant fractions, with the semen component being dominant. At ratios of 1:1, 1:5, 1:10 and 1:20 (semen:saliva), the semen component was stronger in the supernatant fraction. At positions 16311 and 152, mixtures would not have been detected if only the supernatant fraction had been extracted, highlighting the need to extract both the pellet and supernatant fractions of an unknown stain.

### Faeces:semen mixtures

Of 45 samples examined, 39 showed mixed profiles, 3 showed only the semen donor's profile and 3 only the faeces donor's profile. The combination of individuals involved was the most important factor influencing which component of the mixture was dominant. For two mixture sets, the faeces component of the mixture was dominant, for one mixture set, the semen component was dominant and for the fourth mixture set there was a high incidence of sample failure. In no case was a hybrid profile obtained which would not have been recognisable as a mixture.

### General considerations for mixed stains

In total 189 mixtures were analysed, of which one resulted in a hybrid profile consisting of peaks from each of the two donors. In view of this result, caution should be exercised in interpreting results from unidentified stains.

Presumptive tests should be used where possible to identify the components of a stain. In addition both the cell pellet and the supernatant should be extracted separately to ensure that all fractions of a possible mixture can be analysed. This would minimise the chance of seeing only a hybrid profile in mixtures involving semen, as mtDNA from semen may be more concentrated in the supernatant and mtDNA from blood, saliva, vaginal material or faeces is concentrated in the cell pellet. It is unclear why only the female component gives a profile in vaginal semen mixtures when the pellet is extracted, because the pellet yields mtDNA from seminal fluid, semen stains and the semen fraction of semen:saliva mixtures. One possible explanation is that the pellet fraction in semen:vaginal mixtures shows only the female type because the epithelial cells overwhelm the male type obtained from the few available tail section mtDNA particles. In semen:saliva mixtures, there is far less saliva epithelial material, and hence, the semen type can be detected.

The absence of a second peak at a particular locus in a mixed profile cannot be taken to denote that both profiles share the same nucleotide at this locus. This is explained by the differential efficiency with which the four dideoxynucleotides are incorporated by the enzyme and the differen-

**Table 2** Minisequencing profile of donors 1 and 2 and "hybrid" profile from Donor 1/Donor 2 1:10 semen:saliva

| Locus | 302 | 73 | 16311 | 16069 | 195 | 152 | 146 | 247 | 16224 | 525 | 523 | 16129 | 16189 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Donor 1 | T | C | A | G | G | A | A | C | A | A | A | C | A |
| Donor 2 | G | C | G | G | A | G | A | C | G | C | A | C | A |
| Donor 1/Donor 2 1:10 | T | C | A | G | A | A | A | C | A | C | A | C | A |

tial sensitivity of detection of the fluorescent dyes used to label the dideoxynucleotides. For example in this study we have observed profiles where the stain donors differed at four loci, but mixtures were only evident at three of these, the fourth locus showing only one peak. However, if only loci at which a mixture is apparent are considered, and both the number of contributors to the mixture and the profile of the "victim" are known (e.g. vaginal, anal, mouth and penile swabs), suspects could be excluded or included on the basis of the profile which would be obtained after "subtraction" of the victim's profile. In this study between 0 and 7 peaks were distinguished which did not originate from the "victim". These partial profiles would primarily be of use in exclusion of a suspect, as the strength of inclusion evidence would be low.

### Samples from previous cases

Control samples from previous mtDNA cases were analysed. Of the 12 loci typed by minisequencing, 11 could be verified by comparison to results from the sequencing method currently in use for casework [29]. The final minisequence locus (L00525/523) is outside the region routinely analysed by sequencing in our laboratory. Of the seven control samples analysed by duplicate extractions four were blood and three were hair. The minisequencing results from all samples were identical to those which were determined by sequencing.

From previous mtDNA cases, 14 crime stains (on jeans, clingfilm and a rug) and their associated substrate controls were analysed and 12 of the crime stains yielded minisequencing profiles. Complete or partial mtDNA sequences had previously been obtained from three and six of these samples respectively. At the loci for which sequence and minisequence information was available (77 comparisons in all), both techniques gave identical results. Of the substrate controls two gave partial minisequencing profiles, of which one was an apparent mixture of the profile obtained from the corresponding stain and a second unidentified profile. The partial profile obtained from the second substrate control matched the profile from the corresponding stain. Neither of these samples had yielded a sequence profile, although an additional seven substrate controls yielded sequence profiles but did not yield a result using the minisequencing technique. The variability in success rate between the two techniques may be attributed to a combination of the higher sensitivity of the minisequencing technique and the age and state of degradation of the DNA extracts.

### Comparison of minisequencing and sequencing

Samples from 22 staff members were minisequenced and sequenced. A total of 170 minisequence peaks were checked against sequence data and no discrepancies were observed.

## Conclusion

This paper demonstrates that mtDNA minisequencing is a reliable and reproducible technique for analysis of forensic samples. The test may successfully yield results where genomic DNA typing has failed, with all sample types tested in this study yielding minisequencing profiles.

Although interpretation of results from "known mixtures", such as swabs in sexual assault cases, is relatively straightforward, caution should be exercised when interpreting results from unidentified stains.

From all the samples tested in this study, heteroplasmy was observed only at position 302/309.1. Heteroplasmy has been reported once at position 16129 [24]. We have not observed heteroplasmy at this position throughout the course of this study or subsequent research or casework. Therefore it is unlikely that any of the loci determined in the minisequencing test (other than 302/309.1) are true mutation "hot spots". The guidelines for inclusion or exclusion of a common maternal lineage are based on:

1) The observed heteroplasmy at position 302/309.1
2) The observed stability of the other loci in the minisequencing test
3) The high reported rate of germline mutation in mtDNA [17, 30]

The rapid and discriminating nature of multiplex solid-phase fluorescent minisequencing make it particularly suitable as a screening technique, which may be used either for elimination of multiple suspects from an inquiry or to distinguish between multiple hairs recovered from a crime scene. The two-stage strategy of screening samples using minisequencing, followed by sequencing a small number of matching samples enables mtDNA analysis to be used in a much wider range of forensic cases than would be possible using sequencing alone.

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