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Development of a control region-based mtDNA SNaPshot™ selection tool, integrated into a mini amplicon sequencing method

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ABSTRACT

Mitochondrial DNA (mtDNA) analysis is regularly applied to forensic DNA samples with limited amounts of nuclear DNA (nDNA), such as hair shafts and bones. Generally, this mtDNA analysis involves examination of the hypervariable control region by Sanger sequencing of amplified products. When samples are severely degraded, small-sized amplicons can be applied and an earlier described mini-mtDNA method by Eichmann et al. [1] that accommodates ten mini amplicons in two multiplexes is found to be a very robust approach. However, in cases with large numbers of samples, like when searching for hairs with an mtDNA profile deviant from that of the victim, the method is time (and cost) consuming. Previously, Chemale et al. [2] described a SNaPshot™-based screening tool for a Brazilian population that uses standard-size amplicons for HVS-I and HVS-II. Here, we describe a similar tool adapted to the full control region and compatible with mini-mtDNA amplicons. Eighteen single nucleotide polymorphisms (SNPs) were selected based on their relative frequencies in a European population. They showed a high discriminatory power in a Dutch population (97.2%). The 18 SNPs are assessed in two SNaPshot™ multiplexes that pair to the two mini-mtDNA amplification multiplexes. Degenerate bases are included to limit allele dropout due to SNPs at primer binding site positions. Three SNPs provide haplogroup information. Reliability testing showed no differences with Sanger sequencing results. Since mini-mtSNaPshot screening uses only a small portion of the same PCR products used for Sanger sequencing, no additional DNA extract is consumed, which is forensically advantageous.

1. Introduction

Short tandem repeat (STR)-based systems remain a problem for the forensic identification of samples such as hair shafts and charred bones that lack nuclear DNA (nDNA) or have a severely degraded state [3]. One approach to obtain genetic information for such samples is to use mitochondrial DNA (mtDNA) analysis. Mitochondria have a much higher cellular copy number due to which their DNA remains longer detectable than nDNA when samples are affected by time, fire or other compromising circumstances. Also during hair shaft formation, which involves the process of cornification that is a type of programmed cell death during which keratinocytes are converted to keratin and nucleus and mitochondria are destructed [4], mtDNA remains much longer detectable than nDNA. Besides, mtDNA provides an indispensable tool for kinship testing or familial searching due to the maternal inheritance [3].

In its current practice, mtDNA typing usually involves sequence determination of the polymorphic stretches in the control region (or D-Loop). This control region is approximately 1100 base pairs (bp) and carries three hypervariable sequence regions: HVS-I, HVS-II and HVS-III that have a length of approximately 360, 300 and 150 bp respectively. HVS-I and HVS-II are most polymorphic and for good quality samples such as reference samples most often these two HVS regions are amplified in two fragments 300 to 400 bp in length (depending on primer choice) and bi-directionally sequenced. When samples are severely degraded, smaller amplicons are needed like described in the mini-mtDNA method [1]. This method amplifies ten short (144 to 237 bp) overlapping fragments in two multiplexes and covers the entire control region and thus all three HVS regions. When the ten fragments are bi-directionally sequenced, per sample 20 sequencing reactions need to be performed, which makes the method labor intensive. In 2014, mtDNA analysis was applied in 42 cases in our laboratory comprising 130 reference samples analyzed by the HSV-I, HSV-II and HVS-III approach (six sequencing reactions per sample) and 151 compromised samples analyzed by the mini-mtDNA approach (3020 Sanger sequencing reads). Cases consisted of up to 18 compromised samples (hairs). This illustrates our need for a quicker approach to pre-assess larger sample sets and select samples for further sequencing analyses like when searching for a hair not matching the victim amongst a (large) set of recovered hairs.

An effective method to analyze Single Nucleotide Polymorphisms (SNPs) is single base extension (SBE) via SNaPshot™, a method well compatible with forensic practice and commonly applied for various purposes. Recently, Chemale and co-workers described a

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SNaPshot™-based mtDNA screening tool, targeting 14 polymorphic sites in HVS-I and HVS-II that were amplified in duplex using standard-size mtDNA amplicons [2]. Straightforward application of this assay to mini-mtDNA amplified fragments would require mixing of the fragments amplified via both multiplexes and this may invoke an imbalanced SNaPshot™ result as four of the 14 SNPs reside in two amplicons that are designed to overlap [1], which is especially a concern when compromised samples are used and amplification becomes less balanced. Thus, we decided to adapt the method to comply better with the mini-mtDNA procedure and design two mini-mtSNaPshot multiplexes, one for each mini-mtDNA multiplex. These two multiplexes allow for a slightly higher number of SNPs than the 14 SNPs described by Chemale [2] and we selected 18 SNPs using the relative frequency in a European population that has predominantly (40–50%) haplogroup H [5]. The use of degenerated bases in the SBE primers was considered to prevent loss of information due to primer binding problems at polymorphic positions. We assess whether such a screening tool is relevant for forensic casework samples.

2. Material and methods

2.1. Reference samples and DNA extraction

Buccal swabs (Omni Swab, Whatman, GE Healthcare Life Sciences) were taken from male and female volunteers with informed consent and known mtDNA and STR profiles, which allowed generated profiles to be compared and verified. DNA was extracted using the QIAamp DNA Mini Kit (QIAgen, Venlo, The Netherlands) according to the manufacturer's instructions. DNA was eluted using twice 50 μL preheated (70 °C) 25% AE buffer, incubation for 2 min at 70 °C and centrifuging 1 min at 8000 rpm. The DNA extracts (100 μL) were stored at −20 °C. To minimize the occurrence of contamination, the guidelines given by the European DNA profiling (EDNAP) group were followed [6].

2.2. Nuclear DNA (nDNA) amplification

Human-specific genomic DNA concentrations were determined using the highly sensitive Alu repeat system [7] with adaptations described in Lindenbergh et al. [8]. DNA profiling was performed using the AmpF⁄STR®NGM™ PCR Amplification Kit (Thermo Fisher Scientific, Foster City, Texas, USA (Applied Biosystems (AB)), according to the manufacturer's protocol. PCR products were detected by capillary electrophoresis (CE) with an ABI Prism 3130xl Genetic Analyzer™ (AB) as described in Lindenbergh et al. [9]. GeneMapper ID-X version 1.1.1 (AB) was used for profile analysis with a detection threshold of 50 relative fluorescence units (RFU) [9].

2.3. Control region amplification

The full mtDNA control region was amplified in two multiplexes using the primers as described in Eichmann et al. [1]. Mini-mtDNA primers were ordered (Biolegio BV, Nijmegen, The Netherlands) at a concentration of 100 μM and diluted to a working solution of 10 μM (10 pmol/μL) and stored at −20 °C. Mini-mtDNA PCR amplifications were performed in a final volume of 50 or 60 μL containing 25 or 30 μL 2x Multiplex PCR Master mix (QIAgen), 2 or 2.4 μL set 1 or set 2 mini-mtDNA 25x primer mix (Supplementary Table 1), 21 or 25.6 μL nuclease-free H_2O (Ambion® by Life Technologies a Thermo Fisher Scientific brand, Carlsbad, USA) and 2 μL DNA extract. A reaction volume of 50 μL was used for the experiments in Sections 3.1 and 3.2. A larger volume of 60 μL was used in the experiments in Section 3.3 and is suggested for casework as it allows certainly for a QIAxcel capillary electrophoresis analysis, a mini-mtSNaPshot assay, all sequencing reactions and a remainder for re-examination if needed.

PCR amplifications were performed in a GeneAmp® PCR System 9700 (AB) with the following conditions: pre-denaturation for 10 min at 94 °C, 36 cycles of 20 s at 94 °C, 20 s at 55 °C and 20 s at 72 °C, followed by a final extension of 10 min at 72 °C. PCR products were checked using the QIAxcel analyzer (QIAgen) and the QIAxcel DNA high Resolution kit (QIAgen) with the QX Alignment marker (15 bp – 1 kB). Sample preparation consisted of adding 5 μL PCR product to 5 μL QX DNA Dilution Buffer. In addition, QX DNA size marker in the 50 to 800 bp range was taken along in each run (QIAgen), for which 1 μL size marker was mixed with 9 μL QX DNA Dilution Buffer. The OM500 method with an injection time of 20 s was used to run mini-mtDNA PCR products. Analysis was performed with the QIAxcel ScreenGel Software (QIAgen).

2.4. SNP selection and SBE primer design

To integrate the screening tool with mini-mtDNA sequencing [1], SNPs had to reside within the control region targeted by the mini-mtDNA method and befit two SNaPshot™ reactions that match the amplified products from the set 1 and set 2 amplification multiplexes (Supplementary Table **1**). Both frequent and less frequent SNPs were selected, and some SNPs provide haplogroup information (see Section 3.1). In total 18 SNPs were selected that were equally distributed over the two SNaPshot™ reactions.

SBE primers were designed immediately up- or downstream of the SNP position (in forward or reverse primer orientation, respectively) [10,11] using the revised Cambridge Reference Sequence (rCRS) as reference [12,13]. All primer sequences were designed with a melting temperature (Tm) around 55 °C, and checked for the absence of primer–dimer formation, hairpin structures and complementarity to other primers in the multiplex with Autodimer, Oligo Calc and NCBI Primer-BLAST [14–17]. To spatially separate the SNPs during capillary electrophoresis, the extension primers were 5′-tailed using poly(dGACT) or poly(dC) sequences (poly(dC) tails were chosen when poly(dGACT) tails created the potential to form hairpin structures). All SBE primers were first tested individually in a SNaPshot™ assay, followed by analysis in multiplex. Artefact signals due to hairpin formation within a primer or primer–dimer formation between different primers were encountered. Also, overlapping signals for different SNPs occurred because the detected product sizes deviated (up to 15 nt (nucleotides)) from designed product sizes. Nucleotide composition, fluorescent dye label and primer size affect how much sizing deviation occurs: for instance primers larger than 50 nucleotides tend to show less deviation than the smaller primers [18]. To solve these issues, reverse orientation primers were designed and primer tails were altered. Besides, degenerated bases were included for recurring mutations in the 3′ part of the primers. No degenerate bases were included in the 5′ part of the primers as this allowed for the annealing of multiple variants with a different electrophoretic mobilities, resulting in a broad or split peak. In total 55 primers were tested to select the 18 final primers. The use of an increased annealing temperature in the SNaPshot™ PCR (60 °C instead of 55 °C) reduced the formation of broad peaks to some extent, but concomitantly signal strength was reduced when mismatches occurred with the 5′ part of the primer. Thus, it was decided to broaden the bins in the analysis software in case of broad peak signals. Finally, primer concentrations were optimized. Sequences of the selected primers, their final concentrations in the mini-mtSNaPshot assay and product sizes are indicated in Table 1. SBE primers with lengths below 50 nucleotides underwent high-performance liquid chromatography (HPLC) purification and primers with lengths of 50 nucleotides or above were purified by polyacrylamide gel electrophoresis (PAGE) after synthesis (Biolegio).

2.5. SNaPshot™ assay and electrophoretic SNP analysis

To 5 μL mini-mtDNA PCR product, 2 μL ExoSAP-IT® (according to manufacturer: Affymetrix) was added and after an incubation at 37 °C for 45 min, the enzyme was inactivated by an incubation at 80 °C for 15 min. Terminator ddNTPs, labeled with four different fluorescent