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Research paper

# New application for haplotype-specific extraction: Separation of mitochondrial DNA mixtures

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ABSTRACT

A particularly challenging aspect of forensic casework is the accurate interpretation of samples that contain DNA from more than one contributor, since DNA analysis of specific identification markers often results in ambiguous mixed profiles. Physically separating the various haploid components of a diploid or polyploid DNA sample or of a specimen containing DNA from multiple individuals can resolve this problem. We adapted and combined previously developed methods of haplotype-specific extraction (HSE) to separate and analyze mitochondrial DNA (mtDNA) in samples containing DNA from two individuals. After optimizing conditions and probes, we performed HSE to separate mitochondrial DNA mixtures, which manifest as mixed nucleotide calls at single base positions. We were able to subsequently identify the DNA of the two individuals by sequencing. Based on our findings from this proof-of-concept experiment, this novel assay will be useful for distinguishing among the mtDNA of individuals in mixed DNA samples.

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## 1. Introduction

The interpretation of ambiguous mixed profiles caused by polyploid DNA samples or a specimen containing DNA from multiple individuals is still difficult in some cases. Nagy et al. [1] used haplotype-specific extraction (HSE) technology (invented and applied by Dapprich et al. [2–4]) at specific regions of the major histocompatibility complex to resolve ambiguous genotypes and overcome problems in human leukocyte antigen (HLA) sequencing and transplantation tissue typing, and they also pioneered the use of HSE for forensic applications. Rothe et al. [5–7] then applied this approach to the separation of Y-chromosomal DNA from male mixtures to extract the haploid components from a diploid sample with two contributors. The analysis of DNA mixtures is common practice in forensic casework and of fundamental importance in cases where more than one suspect is involved. There are several other well-established methods to distinguish multiple contributors in special cases, such as in the presence of sperm and/or epithelial cells in a trace after sexual assault [8]. Examples are differential lysis [9] and laser microdissection [10], chemical and physical processes that differentiate between the abundant female DNA of the victim and the DNA of the offender. Mixed DNA profiles with clear major and minor components can also be analyzed to distinguish between individual contributors. However, many mixed samples cannot be investigated by such methods.

In the present report, we outline a molecular biological approach to physically separating mixture samples into single components [1]. This approach differentiates between DNA originators by utilizing differences in the control region of mitochondrial DNA (mtDNA), with haplotype-specific probes, hybridization reagents, and an automated bead-based extraction robot [11] to capture specific magnetic beads containing immobilized DNA. Via the haplotype-specific probes, the beads can be connected to polymorphic sites of the mtDNA and used to isolate the targeted fragments from a heterozygous mixture. The aim of the HSE approach is unambiguous single typing of clear nucleotide polymorphism positions that appear heteroplasmic in a mixture. Heteroplasmy can be considered a “natural” DNA mixture (present in a single individual), which is in contrast to a “situational” mixture of body fluids all containing DNA often encountered in forensic science. The investigation of different heteroplasmic states in various tissues, as we know from Naue et al. [12], represents an important and useful area of application. Consequently, our method can be an innovative and very useful step for the accurate determination of the suspects’ origin in cases where a trace mixture is present but no further information available.

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Moreover, our mtDNA approach may be the solution for potential serious crime cases whenever an autosomal DNA mixture, which is presented to interpret the suspects' contribution to a mixed trace stain, is inconclusive or considered insufficient to resolve the case on the basis of statistics alone.

## 2. Materials and methods

The purpose of this application of HSE was to extract the mtDNA of homoplasmic contributors from a heteroplasmic mtDNA mixture. This was accomplished by using specifically designed probes that target mitochondrial polymorphisms and a bead-based DNA-extraction method (Fig. 1).

### 2.1. DNA samples and searching for suitable nucleotide positions

We first analyzed various DNA samples with Sanger sequencing to gain an overview of the variability of polymorphisms in the mitochondrial genome (data not shown). Then we chose two volunteers provided standard laboratory blood samples. Genomic DNA was extracted with the EZ1 DNA Blood 200 μl kit and the BioRobot®EZ1 workstation (both from Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The samples had different mtDNA haplotypes and similar genomic DNA concentrations which were measured by the QuantiFluor® dsDNA System (Promega GmbH, Mannheim, Germany). We pooled together both samples in same proportions to create a DNA mixture. Both donor samples and the mixture were screened for polymorphisms (Fig. 2) by amplifying two mtDNA fragments, each covering the entire control region (nucleotides 16024–576), in two independent PCRs followed by ten sequencing reactions covering the whole amplimer (adapted and optimized from Brandstätter et al. [13], shown in Fig. 2). Optimization of the initial protocol included a reduced primer concentration (in the amplification and sequencing reaction up to 0.2 μM, respectively) and an extended period for activation of the polymerase in the PCR protocol (up to 11 min). Furthermore we added just 2 μl of the purified PCR-product to the sequencing reaction. The mtDNA sequences of both donors and the mixture are shown in Supplementary Fig. S1.

### 2.2. Probe design for HSE

We designed the HSE probes (TIB Molbiol, Berlin, Germany) in accordance with the guidelines in the Qiagen supplementary protocol [14]. Probes were short oligonucleotides with a 3′ end specific to the mtDNA polymorphisms of only one of the DNA contributors and a mismatch between the probe and the non-targeted mitochondrial sequence [15] of the other contributor. Because the mitochondrial genome is short (16 kb), we designed haplotype-specific probes for mitochondrial point mutations for only one extraction point per sample, because HSE should be able to separate the whole mtDNA fragment in one piece. Optimal extraction points for HSE probe design are characterized by at least one clear differentiation between two bases at the same nucleotide position. In our approach we chose nucleotide differences at positions 72 and 73 as the extraction point. A high degree of variation of polymorphisms in the mtDNA control region is helpful in order to design highly effective separation probes that show the HSE success more effectively. Due to the high GC content that would have resulted by placing a probe in the forward direction, which is a limiting factor for analysis, we designed the probes in reverse orientation with the discriminating nucleotide at the 3′ end and at the second-to-last position. Because we knew that the length of the probe was a critical factor, we used the calculation formula of Rothe et al. [5] to determine the theoretical optimal length of the extraction probe for successful HSE (probes: N_R_nt72_17/C_R_nt72_17). The length of a probe varies depending on GC content, melting temperature and free energy. In order to test a possible influence of the probe length on HSE success, we made probes of varying lengths for each donor (for individual 1: probes of 13 up to 20 and 24 nucleotides, and for individual 2: probes of 13, 15, 18, 20 and 24 nucleotides) and tested them in HSE assays. Probes were named as follows: The first letter specifies the individual (N for individual 1 and C for individual 2), "R" signifies the reverse direction of the probe, "nt72" marks the position with the discriminating nucleotide at the 3′ end, and the final number indicates the length of the probe. Table 2 summarizes the probes that we tested.

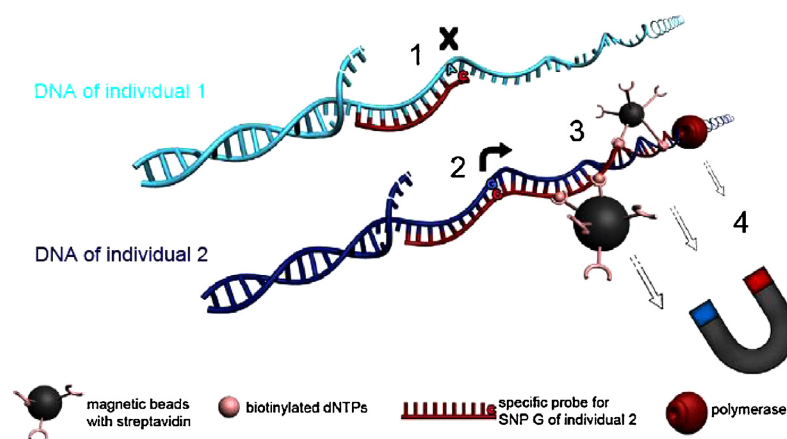


**Fig. 1.** Schematic illustration of HSE to separate the DNA of individuals in a DNA mixture (modified from Rothe et al. [5]). A DNA mixture contains two different DNA patterns from two individuals that differ in one base position (e.g. A/G). In the illustration, HSE is separating a fragment with a probe specific to base G of individual 2, which differs from the base at this position in individual 1. HSE probe has a 3′ terminal mismatch for individual 1, so that an enzymatic extension cannot be processed. The specific probe matches completely with the DNA strand of individual 2 and can be extended. Biotinylated dNTPs are incorporated by the polymerase, and streptavidin-coated magnetic beads bind to the DNA. This complex is captured with a magnetic field.

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