# Automation of the Amplification and Sequencing of Mitochondrial DNA

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## INTRODUCTION

Analysis of mitochondrial DNA sequence is an effective method for determining genetic individuality for the purposes of forensic investigation (Reynolds et al. 1991). It is a more sensitive test than the analysis of chromosomal loci since there are between 1000 and 10000 copies of the mitochondrial genome per human cell, and it is also useful in determining familial relationships because mitochondria are of maternal origin (Orrego and King 1990). Much of the observed sequence variation between individuals is concentrated in two hypervariable sections within the mitochondrial control region, which can be readily sequenced and is of high information content (Greenberg et al. 1983; Aquadro and Greenberg 1983).

For DNA sequencing to be used as a routine technique in forensic analysis, it is essential that the process is highly automated to maximise sequence throughput, minimise errors in data handling, and to streamline database management (Sullivan et al. 1991). This paper briefly describes the optimisation of sequence data generation from mtDNA by combining PCR amplification with solid-phase automated sequencing.

## DNA AMPLIFICATION

A 2 stage amplification process was utilised. In the first stage, a 1.3Kb fragment spanning the entire non-coding region was amplified using primers L15926 (5'-TCAAAGCTTACACCAGTCTTGTCTTGTAAACC) and H00580 (5'-TTGAGGAGGTAAGCT-ACATA). The PCR reaction comprised the following: primers at 1µM; dATP, dGTP, dCTP and dTTP each at 200µM, 1.25U Taq polymerase; 1x'PARR' buffer (CamBio), in a total volume of 25µl. DNA template amplified included in this mixture varied from 10ng of total DNA extracted from blood, to non-quantifiable amounts isolated from sections of single hair shafts. Cycling conditions were as follows: 94°C for 45 seconds, 50°C for 1 min then 72°C for 5.5 min, for 35 cycles.

0.5µl aliquots of the PCR product were added directly to a second reaction using a pair of primers internal to those used in the first PCR round: M13(-21)H16401 (5'-TGTAAAACGACGACGGCCAGTTGATTTCACGGAGGATGGTG) and L15997 (5'-CACCATTAGCACCCAAAGCT). The former primer is chimaeric comprising the M13(-21) universal sequencing primer sequence at the 5' end plus the H16401 sequence at the 3' end which is complementary to part the mtDNA D-Loop sequence. The latter primer (L15997) was biotinylated at the 5' end. The PCR reaction mix comprised 2.5U Taq polymerase, 0.5µM final concentration of each primer, 1xPARR buffer and 20µM final concentration each dNTP in a final volume of 50µl.

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## SEQUENCING REACTIONS

The biotinylated PCR product was added to 50µl LiCl and 50µl streptavidin-coated Dynal™ beads, followed by incubation at 48°C for 15 mins to immobilise the DNA. The double-stranded DNA was denatured in 0.15M NaOH for 4 mins and the non-biotinylated strand was eluted from the beads by removing the supernatant, leaving single-stranded sequencing template attached to the beads. The bead pellet was then resuspended in 60µl $dH_2O$.

By tagging the 5' end of one primer of each pair prior to amplification with the universal -21M13 primer sequence it was possible to use the immobilised PCR product in sequencing reactions using dye-labelled universal sequencing primers. Sequencing ladders were generated with an Applied Biosystems Sequenase Sequencing Kit as follows: for the dideoxyadenosine sequencing reaction, 8µl of the beads were immobilised with a magnet, and the supernatant was removed. To the beads 0.4pmol buffered -21M13 primer (labelled with dye 'JOE') was added in a final volume of 4µl. Primers were annealed to the DNA by heating to 65°C then slowly cooling to room temperature. 2U Sequenase T7 DNA polymerase (USB) plus 1µl of appropriate dideoxyadenosine extension mix were added, and the mixture was incubated at 37°C for 20 mins. Set up in parallel were the three other sequencing reactions for dideoxycytosine, dideoxyguanosine and dideoxythymidine, using FAM, TAMRA and ROX-labelled primers respectively, and under effectively identical conditions except that all quantities were doubled for the dideoxyguanosine and dideoxythymidine reactions. The four reactions were terminated by the addition of stop-salt solution; then they were pooled and the supernatant was removed from the beads. The extension products were eluted from the beads by incubation in deionized formamide for 4 mins at 37°C. The supernatant was then loaded on a 6% acrylamide gel and run in an Applied Biosystems 373A sequencer. Sequence manipulations and comparisons were performed with the SEQED software program (Applied Biosystems).

Alternatives to solid-phase sequencing of biotinylated PCR products have been evaluated for the generation of single-stranded sequence template, but have proven inferior in both reproducibility and sequence quality (data not shown). These included asymmetric PCR in the second round of amplification, lambda exonuclease digestion of one product strand which has been generated from a kinased primer, sequencing of double-stranded template, and Taq cycle sequencing.

This amplification and sequencing strategy enables high quality data to be generated from mtDNA (Figure 1). The entire sequence-generation process is highly automated; full-length amplification products (468bp) and sequence ladders can be readily generated from this hypervariable region. 10ng total DNA template is generally used for amplification and sequencing, but successful amplification has been achieved with mtDNA extracted from just 0.5cm of hair shaft.

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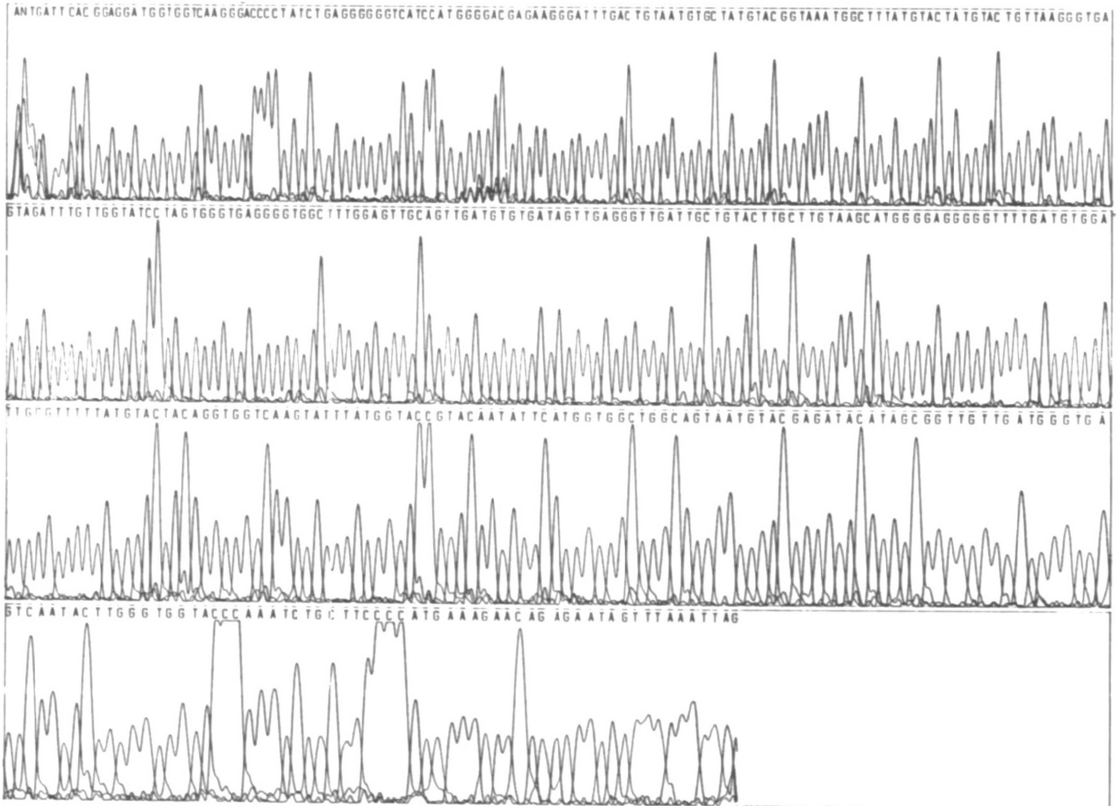

Figure 1. Mitochondrial DNA sequence: PCR product was generated with primers M13(-21)H16401 and L15997 then bound to Dynal beads and sequenced with Sequenase

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