A Two Stage Strategy for the Automated Analysis of Mitochondrial DNA

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INTRODUCTION

DNA amplification and sequencing of the mitochondrial (mt) non-coding region provides a highly sensitive and discriminating test for individual identification which is ideal for the analysis of severely degraded human remains (Sullivan et al. 1994, Gill et al. 1994). A major drawback with DNA sequencing as a forensic tool is the labour-intensive nature and therefore high cost of the technique, which presently limits its application to the investigation of serious crime. Alternative strategies to DNA sequencing have been developed to characterise sequence polymorphisms in mtDNA products including hybridisation with allele-specific probes (Stoneking et al. 1991), detection of length polymorphisms (Bodenteich et al. 1992) and oligonucleotide ligation assay (Sullivan et al 1993). More recently, the novel technique of multiplex solid phase fluorescent minisequencing has been developed (Tully et al. 1995) which detects both sequence and length polymorphisms. The latter is being developed by our laboratory as a primary screen in a two stage strategy for the automated analysis of mtDNA. The minisequencing technique enables the majority of non-related individuals to be eliminated in a rapid and straightforward test so that the remaining samples can then be distinguished further by the application of full DNA sequencing, which is more discriminating than minisequencing but also much more labour-intensive to perform.

MULTIPLEX FLUORESCENT SOLID PHASE MINISEQUENCING

Sections of the non-coding region are amplified using biotinylated primers, and the PCR product is immobilised on magnetisable streptavidin-coated beads and used as the template for minisequencing. The concept of fluorescent minisequencing is to characterise a particular polymorphic site within the PCR product by annealing to it a primer whose 3' end is 1 base upstream from the site in question. A single base extension reaction is then performed utilising dideoxynucleotides labelled with 4 distinguishable dyes (Perkin Elmer). The resultant dye-labelled extension product can then be characterised by eluting the primer from the DNA followed by electrophoresis and analysis on an ABD 377 automated DNA sequencer. It is possible to characterise simultaneously several informative sites by designing a detection primer for each site with appropriate mobility-modifying homopolymer tails such that all products can be resolved by electrophoresis through a 12cm long 19% acrylamide gel.
From previously constructed British Caucasian and Afro-Caribbean mitochondrial sequence databases, minisequencing primers were designed for the characterisation of 10 highly polymorphic point mutation sites (L73, 146,

152, 195, 247, 16069, 16129, 16189, 16224, and 16311), plus one site of oligo(G) length variation (L302), and a dinucleotide repeat within the mtDNA. The latter is characterised with two oligonucleotides for sites L523 and 525, making it possible to determine whether 4,5 or >5 dinucleotide repeats are present within the polymorphic region. Preliminary results from 120 unrelated British Caucasians have been determined for which 53 different mitotypes i.e. variants at the 12 loci were detected with the commonest type present in 16.8% of the population, 34% of mitotypes are unique and the overall probability of random match (pM) is approximately 0.05.

DNA SEQUENCING OF SEVERELY DEGRADED SAMPLES

For the more detailed comparison of samples which cannot be distinguished by minisequencing, a full sequencing strategy can be used that generates sequence of excellent quality from forensic samples in a highly automated procedure. Routine analysis comprises amplification of the entire non-coding region followed by a second nested PCR reaction in which either the HV1 (403bp) or HV2 (383bp) segments of the mt non-coding region are amplified (Sullivan et al 1994). However, for highly degraded samples a single round of amplification is employed using closely spaced primers. In total 8 different pairs of primers each of which amplifies only part of 1 hypervariable section are utilised in 35 cycle reactions: (-21M13)L15997 with (Biotin)H16239; (-21M13)H16239 with (Biotin)L15997; (-21M13)H16401 with (Biotin)L16159; (-21M13)L16159 with (Biotin)H16401; (-21M13)H255 with (Biotin)L29; (-21M13)L29 with (Biotin)H255; (-21M13)L164 with (Biotin)H408; (-21M13)H408 with (Biotin)L164. This enables both strands of both hypervariable regions to be determined in solid phase sequencing reactions using universal M13 sequencing primers in conjunction with an ABD 377 sequencer. Up to 243bp of data is generated from each PCR product with sequence comparisons between complementary or overlapping strands performed using the SeqEd™ computer program to confirm the data. Comparison of different sequencing strategies indicates that use of modified T7 polymerase plus dye-labelled primers for the sequencing reaction yields data with the lowest background noise and minimal sequence context-specific variation, enabling subtle sequence characteristics such as heteroplasmy to be detected reliably.

CASEWORK APPLICATIONS

Full automated DNA sequencing is now being used on a routine basis in forensic casework within our laboratory. Applications are restricted to discrete evidential samples such as severely degraded bones, faeces and hair shafts which are refractory to chromosomal DNA analysis. Analysis of DNA from faeces has been facilitated by the magnetic capture and washing of the template in solid phase, using the Dynal DNA Direct™ kit, prior to amplification. Results are obtained from as little as 10mg wet weight of starting material (Fig. 1) Analysis of DNA from hair shafts typically requires a one round amplification of short PCR products in order to generate sufficient template for successful sequencing. In general, 2 to 4cms of head hair shaft is used per DNA extraction and where possible all casework samples are subjected to duplicate extractions, amplifications and sequence analyses to confirm the sequence for casework reporting purposes. It is envisaged that the

minisequencing technique, when introduced into casework, will provide a rapid and cheap screen making the analysis of large numbers of samples recovered from a scene of crime a more economically feasible proposition.

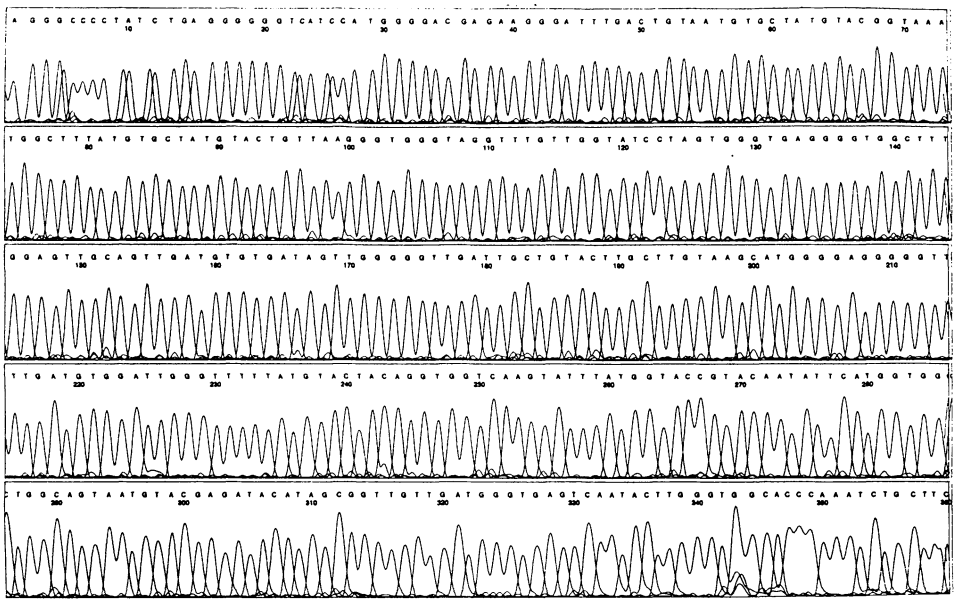

Fig 1. Mitochondrial DNA sequence from human faeces. DNA extracted from 20mg (wet weight) of material was amplified with HV1-specific primers then bound to Dynal beads and sequenced with Sequenase.

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