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Sensitive forensic analysis using the Pyrosequencing technology

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Abstract. In forensic casework analysis, rapid and flexible systems for detection of variation found in nuclear or mitochondrial genomes are valuable. We have developed several different typing systems based on the Pyrosequencing technology to allow sensitive analysis of mtDNA as well as autosomal and Y-chromosome SNPs or STRs in short amplicons. © 2005 Elsevier B.V. All rights reserved.

Keywords: Pyrosequencing technology; Mitochondrial DNA; Nuclear DNA; SNP; STR

1. Introduction

DNA collected from casework samples are in some cases degraded and not in sufficient amounts for a routine STR analysis. Analysis of mtDNA or reduced size PCR fragments of nuclear targets is often necessary for a successful analysis of these samples.

We have developed several novel typing systems based on the Pyrosequencing technology for sequencing of mitochondrial DNA (mtDNA), as well as analysis of SNP and STR markers in the nuclear genome (nDNA). These typing systems are sensitive, rapid, flexible and easy-to-use systems that allow determination of the sequence in a region.

2. Pyrosequencing technology

Pyrosequencing is a sequencing-by-synthesis method that is non-electrophoretic, without need of dyes or specific labels [1]. It is performed in a single-tube format, in which a cascade of enzymatic reactions enables nucleotide incorporation and pyrophosphate (PPi) release to yield detectable light. The produced light is detected in real-time,

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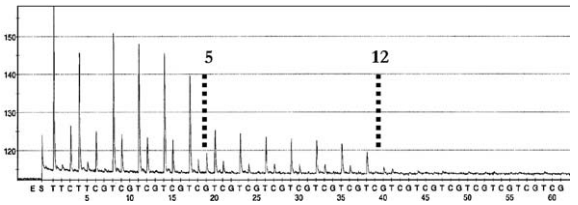

Fig. 1. Pyrogram showing a heterozygous genotype for the marker Penta E ($TCTTT_5$/$TCTTT_{12}$).

using a luminometric detection system, and is proportional to incorporated nucleotides and the released PPi.

3. Pyrosequencing-based typing systems

3.1. Autosomal STR analysis

As a complement to the routinely used STR assays, a pyrosequencing-based analysis of short PCR fragments covering only a few bases outside the actual repeat unit has been developed. Since no size separation based on overlapping fluorescence spectra is necessary using this method, all amplicons can be kept short to allow analysis of degraded DNA. Ten widely used autosomal STR markers with short repeat units and short maximum allele lengths have been analyzed in fragment between 66 and 175 using pyrosequencing [2]. Interpretation is based on the unique pattern obtained after the repeat, as well as a signal reduction by half when the shortest allele terminates for heterozygous genotypes (Fig. 1). For some markers, interpretation was possible using 25 pg of input DNA.

3.2. Analysis of STR and SNP markers on the Y-chromosome

Y-chromosome specific markers are very useful in forensic investigations involving mixtures of male/female DNA but also in investigations of paternal inheritance. We have developed two separate pyrosequencing systems for analysis of SNPs and STRs found on the Y-chromosome.

The Y-STR assay includes eight markers in PCR fragments between 72 and 233 bp, of which seven are found in the minimal haplotype [3]. Some of the markers were analyzed in duplex pyrosequencing reactions and the different repeat lengths could easily be distinguished (Fig. 2). All markers could be analyzed using 100 pg of input DNA and half of the markers were successfully interpreted using 25 pg of input DNA.

Furthermore, a system for analysis of 15 Y-SNP markers has been developed comprising 15 PCR fragments between 50 and 96 bp. These SNPs can be analyzed in

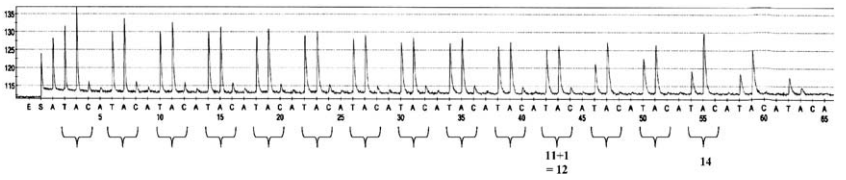

Fig. 2. Pyrogram from duplex analysis of the STR markers DYS438 and DYS392. DYS438 displays one repeat unit plus one T located in the primer, which results in 12 repeats in DYS438 and 14 repeats in DYS392.

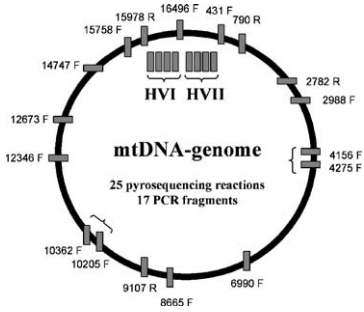

Fig. 3. Location of the 17 PCR fragments analyzed by 25 pyrosequencing reactions for mtDNA typing.

either single-, duplex- or triplex pyrosequencing reactions to save DNA, time and reagents. A few of the Y-SNP markers allowed analysis of 10 pg of input DNA.

3.3. mtDNA D-loop and coding fragment analysis

For severely degraded or limited DNA samples an mtDNA analysis system has been developed. The complete mtDNA typing system consists of 17 PCR fragments (two in the D-loop and 15 in the coding region) for rapid analysis in 25 pyrosequencing reactions [4] (Fig. 3).

The fragments in the coding region of the mitochondrial genome can be used for additional discrimination, which might be useful when none or a single difference is found between individuals in the routinely analyzed D-loop. Each fragment covers multiple polymorphic SNPs and the average read length in the pyrosequencing reactions is 74 nucleotides. Analysis of a subset of fragments can easily be performed to suit certain samples based on the information obtained in the D-loop.

In order to save valuable material multiplex PCR and pyrosequencing reactions are under development. Although it will be possible to analyze STR markers in duplex pyrosequencing reaction by further developments, the multiplex capability is the major limitation of pyrosequencing. Consequently, the pyrosequencing method is more suited for analysis of a limited set of markers in challenging samples rather than analysis of a large set of markers. Since the actual sequence is determined in addition to the repeat length in this assay, there is a possibility to achieve additional information such as the nature of a variant STR allele. Furthermore, a rapid compilation of population databases and evaluation of novel less complex STR markers can be performed. In conclusion, pyrosequencing is a robust and flexible system that can handle SNP analysis, STR analysis or sequencing of short stretches of DNA with 2 h post-PCR handling.

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