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A multiplex SNP typing approach for the DNA pyrosequencing technology

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Abstract. We have developed a multiplex Pyrosequencing assay which enables the simultaneous analyses of 23 single nucleotide polymorphisms (SNPs) from the human genome selected by the SNPforID Consortium. In our investigations we have studied the multiplex capacity of the PSQ™ 96MA instrument (Biotage AB). To test the reliability of SNP typing by Pyrosequencing the SNPs were analysed in parallel by using the SNaPshot minisequencing technique as reference method.

Keywords: Forensic; Pyrosequencing; SNPs; Multiplex

1. Introduction

Pyrosequencing technology is a sequencing-by-synthesis method relying on the luminometric detection of pyrophosphate released upon nucleotide incorporation. The reaction generates a flash of light that is detected and shown as a peak in a so-called pyrogram. The height of each peak is proportional to the number of nucleotides incorporated [1,2].

Multiplex Pyrosequencing enables simultaneous analyses of multiple DNA samples [3,4]. Single and multiplex PCR was performed to amplify target DNA templates each containing 1 of 23 single nucleotide polymorphisms (SNPs) selected by the SNPforID Consortium [5]. We have investigated the multiplex capacity of the PSQ™ 96MA

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instrument (Biotage AB), and have tested the reliability by using the SNaPshot minisequencing technique in parallel as reference method [4–7].

2. Material and methods

2.1. DNA amplification of autosomal SNPs

The SNP amplification was performed as single, triplex and sixplex reactions, respectively, using a Perkin Elmer® 2400 Thermo Cycler. The 100 μl PCR reaction mixture consisted of 1 × GeneAmp® 10 × PCR Gold buffer, 1.5 and 3 mM $MgCl_2$ for the single, triplex and sixplex reactions, respectively, 300 μM dNTP mix, 200 nM and 100 nM of forward and reverse amplification primer for the single and multiplex reactions, respectively, 2.5 unit of AmpliTaq Gold Polymerase (ABI), and 20–100 ng of DNA. PCR was carried out for 40 cycles using the following conditions: initial denaturation at 95 °C for 10 min, denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, extension at 65 °C for 30 s, followed by a final extension step at 65 °C for 6 min. PCR primers were designed to amplify short fragments ranging from 59 to 117 bp [5].

2.2. Single and Multiplex Pyrosequencing

Sequencing primers were annealed to the DNA templates according to the manufacturer's instructions. They were placed into a microtiter plate and Pyrosequencing reagents were dispensed using the PSQ™ 96MA instrument. To improve the method, a purification step with the MinElute-Kit (Qiagen®) and addition of single strand binding protein (SSB) were introduced.

Due to the lack of an adequate instrument software for our strategy the dispensation orders for the nucleotides used for pyrosequencing had to be designed manually in a time-consuming procedure. The selected sequencing primers were checked for heterodimers or mispriming and finally tested by the Multiplex SNP entry of the PSQ™ software.

3. Results and discussion

In a first step the 23 SNPs were amplified by single PCR and analysed by Pyrosequencing. 22 of them were successfully analysed with one exception (SNP 10.2). All results were controlled by minisequencing.

In the second step SNPs were amplified as seven 3plex PCRs and one duplex PCR followed by 3plex Pyrosequencing assays according to the standard protocol. About half of them could be typed successfully. But six of them failed. Purification of the PCR reaction with the MinElute-Kit (Qiagen®) resulted in a better Pyrosequencing reactions. To solve problems with secondary structures SSB was added, leading to much sharper peaks than after column purification. Finally, the combination of purification with MinElute-Kit plus the use of SSB resulted in successful typing of all SNPs in 3plex Pyrosequencing assays with the exception of another SNP than in the single assay (data not shown).

Because the amount of DNA is limited in the majority of casework samples it is necessary to amplify all relevant SNPs in one or only a few PCR reactions. Therefore we have performed a consecutive typing of two 3plex SNP Pyrosequencing reactions out of a 6plex PCR reaction. 20 SNPs could be typed successfully, but in three cases the SNP typing results were not correct. The assay could be further improved by using higher concentrations of sequencing primers.

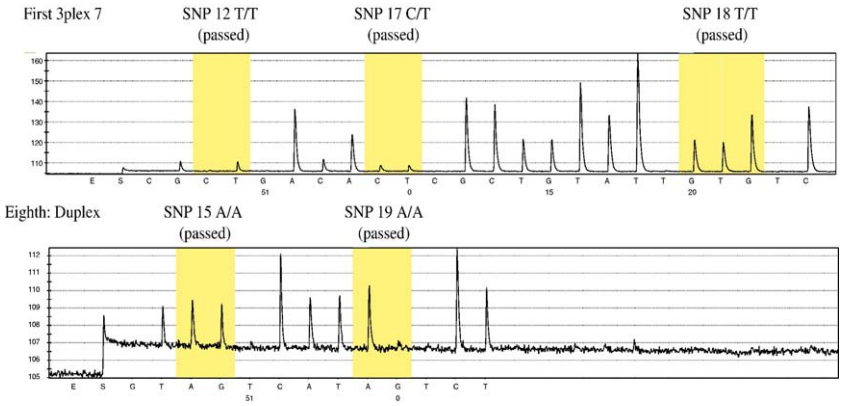

Fig. 1. SNP typing by alternating sequencing reactions for 3plex 7 and the duplex, respectively. Passed: typing is correct, failed: typing is wrong.

To elucidate whether the amount of PCR products is sufficient for a successive typing of SNPs using a single PCR reaction, the 5plex PCR was chosen to be typed in alternating sequencing reactions for 3plex 7 and the duplex, respectively. The 5plex PCR was split into two wells. Successive typing for these SNPs was performed beginning with the 3plex or the duplex, respectively. In Fig. 1 the results for the first and eighth sequencing analysis beginning with 3plex 7 are shown. The first "failed" calling (SNP 12) was observed after eight purification steps. The signal strength of the eight sequencing step was thereby much weaker than in the first one, as visible by the different scale for the *Y*-axis. From that we conclude that at least $8 \times 3 = 24$ SNPs can be typed from a single PCR reaction.

These results clearly demonstrate that the typing of 23 SNPs out of a 23plex PCR reaction seems to be possible under optimized conditions.

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