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A sensitive issue: Pyrosequencing as a valuable forensic SNP typing platform

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Abstract. Analysing minute amounts of DNA is a routine challenge in forensics in part due to the poor sensitivity of an instrument and its inability to detect results from forensic samples. In this study, the sensitivity of the Pyrosequencing method is investigated using varying concentrations of DNA and five autosomal single nucleotide polymorphisms in singleplex on both available instrument models; the PSQ™ 96MA and PSQ™ HS 96A. A detailed comparison of the two models was completed while establishing a lower limit of detection on both instruments to give results supporting the use of Pyrosequencing as a valuable forensic SNP typing platform.

Keywords: Pyrosequencing; Sensitivity; Forensic; Single nucleotide polymorphism (SNP)

1. Introduction

Compromised and low-quantity DNA frequently challenges those working in forensic labs. The incorporation of single nucleotide polymorphism (SNP) assays into forensic studies provided alternative approaches to working with such problems when it was suggested that their unique qualities could make them ideal forensic markers. Despite their clear value, sensitivity was still an issue and choosing a technique to detect samples of low quality and concentration was imperative to maximise the benefits that SNPs could bring to forensics.

Pyrosequencing is one technology described to analyse SNPs. Given its internal controls, quantitative properties and methods of detection [1], it was clear that the method

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was a highly sensitive SNP typing platform and may be suitable to analyse low-quantity DNA samples. This theory required establishing a threshold of detection to determine an approximate concentration that the platform could still accurately detect results. As there are two instrument models available, the PSQ™ 96 MA and the PSQ™ HS 96, it was also appropriate that identical assays be run on both instruments to determine which might perform best in a simulated forensic situation. Ideally, it would be shown that pyrosequencing was capable in handling such strict conditions and that it would be comparable or better than other popular methods for detecting SNPs. By mimicking a forensic situation and running samples with extremely low concentrations in the PCRs, it was hoped that a threshold of detection may be determined to give an indication of the sensitivity of pyrosequencing and its utility for forensic research.

2. Materials and methods

Samples were extracted using the EZ1 200 μL Blood Kit (Qiagen) on the Qiagen EZ1 Robot or with Chelex™. Quantification was completed using the Quantifiler™ Human DNA Quantification Kit (ABI) on the Applied Biosystems 7500 Fast Real-time PCR System and with a spectrophotometer A_{260} on a Biophotometer (Eppendorf). Samples were normalized to a concentration of 1 ng/μL from which serial dilutions were prepared. Between 6 ng and 8 pg of DNA were ultimately added to each PCR from these dilutions.

Five autosomal SNP loci selected by the SNP*for*ID Consortium were used to test the sensitivity in singleplex reactions at the prepared DNA dilutions. Primer Sequences were designed using Pyrosequencing Assay Design Software 1.0 (Biotage) and ordered from Biomers (Germany).

PCRs were performed in 10 μL or 30 μL volumes for the PSQ™ HS 96 or PSQ™ 96 MA on a GeneAmp 9700 PCR System (Applied Biosystems) as previously described [2] with DNA added in concentrations as specified above.

Steptavidin™ Sepharose High Performance Beads (Amersham) bound with biotin-labelled PCR products for a minimum of 30 min. The beads and DNA products were isolated on the Pyrosequencing Preparation Station (Biotage) and added to Pyrosequencing plates where they were run according to instrument parameters.

3. Results

Sample were analysed by the PSQ™ HS 96 or PSQ™ 96 MA SQA software (Biotage). Results were produced in two part: 1) a well-quality reading and 2) the called genotype. Quality, rated as Pass, Check or Fail, was determined by comparing expected and actual histograms, signal to noise ratios and width and height of peaks.

Initial results at 50 PCR cycles showed a high occurrence of positives–negative results and production of other stochastic artefacts. Cycle numbers were reduced to 40 or 34 and the issues were resolved in both cases. Trials at 40 cycles showed a reduced failure rate compared to 34, therefore 40 cycles were used for the remaining experiments.

Results showed that a pass result was achieved in more than 50% of loci on the PSQ™ HS 96 instrument when a DNA concentration equal to or greater than 8 pg was added to the PCR. A pass result was achieved in more than 50% of loci on the PSQ™ 96MA instrument when a DNA concentration greater than 23 pg of DNA was added to the PCR. Lower concentrations in the same samples resulted in greater failures than passes.

The detection limits were further investigated when 15–17 samples were diluted to a concentration in range of the threshold and amplified using 3–5 autosomal SNP loci on both instruments. A pass results was achieved on the PSQ™ HS 96 in greater than 80% of loci when a DNA concentration of 25 pg or more was added to the PCR. On the PSQ™ 96MA, a pass results was achieved in greater than 80% of loci when a DNA concentration of 30 pg or more was added to the PCR. Results also showed that when between 30–500 pg and 50–1500 pg of DNA were added the PCRs of each loci on the PSQ™ HS 96 or PSQ™ 96MA, respectively, the success rate was between 93.8% and 100%.

4. Discussion and conclusions

The recommended 50 cycles was quickly changed to 40 following initial trials. Despite reduction in peak height and intensity, the overall effects of reducing the cycles did not appear to hinder detection, nor did there appear to be any reduced signal due to unincorporated biotin-labelled primers. Instead, the 40 cycles produced strong signals while reducing the number of positive negatives, which initially caused problems.

The high sensitivity of the Pyrosequencing method is demonstrated by detecting results from as little as 8pg of DNA. In all trials, loci passed absolutely or with a high frequency when greater than 30pg or 50pg of DNA were added to the PCRs; in the majority of trials, no more than 500pg or 1.5ng DNA were added to the PSQ™ HS 96 or PSQ™ 96 MA PCRs respectively. These results were more compelling when compared to other popular primer extension assays or techniques for SNP detection such as SNaPshot™ (ABI) and MALDI TOF MS, or allele-specific hybridization assays such as TaqMan™ (ABI). Many of these techniques frequently call for greater than 1ng of high quality DNA to be added to initial PCRs [3]; quantities that are not always typical of samples received. The ability to utilise a technique for investigating and detecting low-quality DNA is on invaluable technique for forensics and certainly Pyrosequencing has demonstrated these capabilities.

The major differences between the two instrument models are cost, consumable volumes and sensitivity. Although there is a great difference in the initial cost of the apparatus, the overall working costs of the instruments is significantly reduced in the PSQ™ HS 96 due to reduced PCR and pyrosequencing minisequencing reaction volumes. The recommended volumes for the PCR are reduced to 5-μL in the PSQ™ HS 96 verses the recommended 50μL in the PSQ™ 96MA. The minisequencing reagents for each instruments are also significantly reduced from 45μL on the PSQ™ 96MA to 12μL on the PSQ™ HS 96. Probably the most important factor in forensic work is the improved sensitivity observed with the PSQ™ HS 96. Although all reagents and DNA concentrations were in the same proportions to the reaction volumes, the limit of overall detection was still slightly lower on the PSQ™ HS 96 compared to the PSQ™ 96MA making future work with the PSQ™ HS 96 for forensics more likely.

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