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Rapid preparation of SNP multiplexes utilising universal reporter primers and their detection by gel electrophoresis and microfabricated arrays

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1. Introduction

Single nucleotide polymorphisms (SNPs) are the most common type of genetic variation observed with a reported frequency of approximately one in every thousand base pairs [1]. Consequently, much effort is being devoted to the study of human SNPs particularly with the aim of gaining a better understanding of their association with disease. A map of human genome sequence variation now containing over 1.0 million SNPs is publicly available and is maintained and updated regularly by The SNP Consortium (http://www.snp.cshl.org) to further these endeavours.

The existence of a large and freely available database of human SNPs has acted as a stimulus for the exploitation of SNPs for many other fields of study. Gill [2] has reported on the feasibility of SNPs for forensic purposes and has noted that there will be a major requirement to create very large, balanced SNP PCR multiplexes with the minimal amounts of template DNA that are available from forensic samples.

We describe a novel multiplex SNP assay that can be employed to generate fluorescent-labelled PCR amplicons for detection by PAGE or CE and that can be modified to produce unlabelled products for detection in microarrays or other detection systems.

2. Multiplex bi-allelic SNP detection using the universal reporter primer (URP) principle

The URP technique enables the rapid and routine creation of large, single-tube, bi-allelic SNP multiplexes. The method utilises the ARMS principle first described by Shuber

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et al. [3] coupled with the use of universal reporter sequences and universal reporter primers [4,5]. A three-phase PCR cycling regime is employed to promote specificity and minimise primer–dimer formation.

2.1. Phase 1

TAQ-Gold activation 95 °C—11 min

94 °C—30 s, 60 °C—15 s, 72 °C—15 s, 60 °C—15 s, 72 °C—15 s, 60 °C—15 s, 72 °C—30 s: two to six repeats

This is the phase during which unique, allele-specific PCR amplicons are produced. Two forward primers and one reverse primer are required for each bi-allelic SNP locus to be studied. These primers have a 3′ region of 18–25 bases (T_m 60 °C) which is complementary to template DNA but also carry a 20-base, noncomplementary, URP sequence (T_m 60 °C) at the 5′ region. The complementary regions of the two forward primers differ only at the terminal 3′ base and enable both primers to anneal to template DNA at the site of the SNP. The terminal 3′ base of one of the forward primers is complementary to one of the two bases found at the bi-allelic polymorphism and the other forward primer has a terminal 3′ base which is complementary to the alternative base found at the SNP site. The forward primers detect which polymorphism is present via the ARMS principle [3]. Only the forward primer with the correct 3′ terminal base will be able to extend to form product. The forward primers carry one of two different 5′ -end, 20-base, noncomplementary universal sequences. The point polymorphism is thus "reported" back and results in the formation of unique PCR amplicons that bear different 20-base universal sequences at the 5′ ends of the forward strands. The reverse primers for the SNP loci all carry a third noncomplementary 20-base universal sequence. All of the amplicons produced will thus carry this 'reverse' universal sequence on their reverse strands. If detection by PAGE or CE is to be employed, then the reverse primers are selected to ensure that SNP loci amplicons of different sizes are produced. If other detection formats such as microarrays are to be employed, then there is no necessity to ensure that differently sized amplicons are produced in the multiplex. Temperature oscillations between 60 and 72 °C are employed to remove incorrect forward primer annealed to the SNP site. This allows the correct primer further opportunities to anneal and extend. Increasing the number of repeats in phase 1 and reducing the number of phase 2 repeats may result in increased sensitivity and greater peak signals but may also cause increased primer–dimer formation and artefact signals with large amounts of template DNA. It may be useful, however, for amplification of samples with low template copies or with degraded DNA.

2.2. Phase 2

94 °C—30 s, 76 °C—105 s: 29–33 repeats

The allele-specific amplicons that have been produced in phase 1 are amplified using a two-step PCR regime that employs a combined annealing/extension step at 76 °C. This high temperature greatly reduces the formation of primer–dimer products which would otherwise rapidly predominate in the PCR. The high temperature also

promotes primer specificity and reduces inhibition as it favours amplification via the large universal-bearing primers in their entirety. Amplification via labelled 20-mer universal primer is inhibited and thus little production of labelled products can occur. The use of large primers at a high annealing temperature does not, however, favour efficient PCR amplification and thus a large number of cycles are required during this phase.

2.3. Phase 3

94 °C—60 s, 60 °C—30 s, 76 °C—60 s: three repeats

Two 20-mer universal-sequence primers with high concentration (2×2 μM), each bearing different fluorescent labels, are allowed to complete the last three cycles. This results in the efficient production of unique, labelled, SNP-specific amplicons for detection by PAGE or CE. If unlabelled amplicons are required for detection with a microarray or other formats, then this is achieved by replacing the two labelled, forward universal-sequence 20-mers with 2 μM of a single, unlabelled 20-mer bearing the reverse universal sequence.

2.3.1. Microarray detection

Purified, unlabelled PCR products were spotted onto coated glass slides using an Amersham Gen III microarray system. Two duplicate slides were required for each SNP locus in the multiplex. Two dye-labelled (Cy 5) allele-specific hybridisation probes and one dye-labelled (Cy 3) locus-specific probe (PCR positive control) were employed for each locus. Each allele-specific probe was 20 bases in length. Bases 1–10 (5′ -end) consisted of forward URP sequence and bases 11–20 consisted of forward primer sequence. A probe to detect allele 1 would carry 10 bases from URP sequence 1 and the probe for allele 2 would differ in that it would carry 10 bases from URP sequence 2. Probes for different SNP loci differed in the sequence of the 10 forward primer bases present. The locus-specific probes were employed to indicate a PCR failure. These consisted of 20-base probes with sequences complementary to the sequences of the reverse primers.

3. Results

3.1. Gel electrophoresis

In our hands, multiplexes of 12–15 SNP loci could be created routinely, each multiplex requiring only 1–2 man-days of effort to produce. The largest multiplex created to date contains 17 SNP loci plus a SNP-based Amelogenin sex test. All 18 SNP loci could be co-amplified and scored with as little as 0.25 ng of DNA present in a reaction volume of 25 μl. Work is continuing to establish the limits of this system with regards to the largest multiplex that can be created. The products from the 18-plex above can be easily separated and visualised with a 377 gel sequencer and allele designation has proved to be a simple matter requiring very little operator experience.

3.2. Microarray detection

It has proven possible to score correctly all of the 10 alleles co-amplified in a SNP pentaplex. The scoring obtained from a microarray technique was compared to the results obtained with a 377 gel sequencer and complete concordance was observed. Work is continuing with larger multiplexes to determine the limits of the microarray-based technique.

4. Conclusions

The URP system offers a convenient method for the rapid creation of SNP multiplexes with the limited quantities of DNA typical of forensic samples. The system can be employed as a gel-based or CE system or can be modified to produce nonlabelled products for detection by other platforms. Using this technique, we have been able to produce 12–15 plexes with ease. An 18-plex has been produced and work is continuing on the development of larger multiplexes. A microarray format assay technique that can determine the alleles co-amplified in a SNP pentaplex has been developed and work is ongoing on the development of larger microarray SNP multiplex assays.

The views expressed in this paper are not necessarily the policy of the Forensic Science Service.

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