Increasing the size of PCR products without redesigning primer binding sequences

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Short tandem repeat (STR) loci in the human genome can be used for individual identification (1). Simultaneous PCR-amplification and size determination at several STR loci is desirable in order to: (i) increase the information gained when only very small amounts of template DNA are available, (ii) maximise throughput of samples and (iii) minimise costs. One means of increasing the number of loci that can be analysed together is to use fluorescently labelled primers (2), with the same dye label used on more than one STR system, provided the alleles from those systems do not overlap.

During our development of an STR–PCR octoplex system for use in routine forensic casework, we realised even from preliminary database studies (~100 individuals) that alleles from two of the constituent loci (D6S502 and D20S85) were separated from each other by <2 bp. As these loci had originally carried the same tetra chlorofluoroscein (Tet) fluorescent label (3) there was the potential that rarer alleles than those currently observed could result in overlap of the two systems. However, simple re-assignment of the fluorescent labels carried on the eight different loci was not possible due to extensive overlapping of the PCR products from several of the systems. The alternative of redesigning the primers to increase the separation between the D6 and D20 loci may have entailed a lot of work to re-optimise the PCR conditions of the octoplex, assuming the redesigned primers worked at all. It was more attractive to retain the various primer sequences which were known to co-amplify with each other under the same PCR conditions.

An increase in the size of the D6 PCR products was achieved by attaching a 'Tag' tail onto the 5' end of one of the D6 primers. This tail had to be inert in its capacity to anneal to genomic template, and should not bind any of the other primers in the octoplex reaction mixture. The M13 reverse sequencing primer *1233 (New England Biolabs, Hertfordshire, UK) which derives from Escherichia coli, was used to achieve the desired increase from -1233. Amplification was initiated by addition of -3-5 ng of chelex extracted template DNA (4). The primer which carries a specific fluorescent dye is indicated. The chimeric D6 II Tag primer has the E.coli sequence extension on the upper line, with the site specific D6 sequence on the lower.

DNA were 'snap-cooled' by boiling for 3 min and chilling on ice. AmpliTaq DNA polymerase (1.25 U) (Perkin-Elmer, Warrington, UK) was added to each Geneamp (Perkin-Elmer) tube and the resultant mixes were overlaid with one drop of mineral oil (Sigma, Dorset, UK). Amplification was initiated by addition of ~3–5 ng of chelex extracted template DNA (4).

The reaction tubes were transferred to the pre-heated block of a Perkin-Elmer Series 9600 thermal cycler at the start of the following program: 28 cycles of (93°C, 30 s; 58°C, 1 min; 72°C, 30 s), fill-in (72°C, 10 min), soak (4°C).

Once thermal cycling was complete, 1.5 µl of the product was combined with 2.5 µl of GS 500 internal lane standard (Perkin-Elmer) in 90% formamide, 1.8% dextran blue. The samples were denatured for 2 min at 90°C, chilled on ice and loaded onto a 6% denaturing polyacrylamide gel on an ABI 373A Automated DNA Sequencer. Electrophoresis was performed at 20 W (power limiting) for ~3 h 45 min.

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Figure 1. (a) Electrophoretogram of multiplex PCR products using unaltered D6 II Tet labelled primer. In this individual (SB7), the products from the D6 locus exactly match those of the FGA locus, which were also labelled with Tet in this experiment. This proximity could be overcome by re-assigning the Hex dye to the FGA products. Although distant in this individual, the potential proximity of the D20 products cannot be overcome by simple re-assignment of dyes and demanded redesign of the D6 II primer. Genetic loci and product sizes as defined by Genescan Analysis Software are shown. The ordinate scale is arbitrary, with the abscissa describing the number of base pairs. (b) Electrophoretogram of the multiplex products generated using the modified D6 II Tag Hex labelled primer. Note that the (Hex labelled) D6 products are now 24 bp larger than the (Tet labelled) FGA products which they had previously masked. There is therefore no need to alter the colour carried by the FGA products.

Results obtained from samples of 3–5 ng template DNA demonstrated clear amplification of all loci with the anticipated increase in size of the D6S502 products by 24 bp (Fig. 1). Quantitation of the peak areas with and without the ‘Tag’ tail suggested that amplification had actually been improved in the presence of the longer primer sequence (tabulated data not shown).

Analysis of results with Genescan Analysis Software (ABI, Warrington, UK), however, showed constant Hex peaks of ~94 and 100 bp in all samples amplified with the ‘Tag’ labelled primer. Although these peaks do not appear to affect the efficiency of amplification of any system in the octoplex reaction, further experimentation to remove them is currently under way.

REFERENCES