DEVELOPMENT AND OPTIMISATION OF A HIGHLY DISCRIMINATING MULTIPLEX PCR SYSTEM SUITABLE FOR FORENSIC IDENTIFICATION

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INTRODUCTION

Forensic DNA profiling in the UK has in recent years targeted the analysis of short tandem repeat (STR) loci which consist of simple tandemly repeated sequences of 1-6 bp in length. Such loci can exhibit a high degree of length polymorphism due to variation in the number of repeat units displayed and as such can be highly informative in situations of human identification.

STRs are highly abundant in the genome, occurring approximately every 6-10 kb, and as such provide a considerable resource from which to select the most promising loci for use in forensic identification. In addition, the introduction of multiple dye technology enabling co-amplification of STR loci with overlapping size ranges combined with their hypervariable nature and PCR amplification efficiency has allowed the realisation of efficient and highly discriminating multiplex PCR systems. Such systems have several advantages over existing technology in that they are more sensitive, requiring as little as 0.5 ng of DNA (SLP analysis requires in excess of 50 ng), can be used on highly degraded DNA since the maximum length of DNA amplified is usually below 400 bp compared with 1-20 kb lengths probed by SLPs, and eliminate the need for radioactivity. The intrinsically rapid nature of the protocol afforded by single step PCR reactions and computer-controlled gel-running, analysis and allele designation means that the process is ideally suited for use in high throughput situations where the number of samples precludes the use of existing and time consuming methods of DNA analysis.

The system described here was developed as a result of the evaluation of several multiplexes including the quadruplex (Kimpton *et al*, 1993), heptaplex (Urquhart *et al*, 1995) and octoplex (Oldroyd *et al*, 1995) systems. Now known as the Second Generation Multiplex (SGM), the new system contains six tetranucleotide STR loci including three which exhibit alleles differing in size by 2 bp, and retains the X-Y homologous gene amelogenin (Sullivan *et al*, 1993) described in both the heptaplex and octoplex systems. It was intended that as a minimum requirement SGM would combine an integrated sex test with PCR integrity and a discriminating provide the Forensic Science Service with a system suitable for rapid, routine analysis of a large number of samples submitted to the UK National DNA Database.

MATERIALS AND METHODS

DNA Isolation

DNA was prepared from whole blood as described previously (Gill *et al*, 1990). Blood samples were collected from unrelated Caucasians, Afro-Caribbeans and Asians residing in the UK. Quantification of DNA was carried out using a primate-specific alpha satellite probe assay (Walsh *et al*, 1992).

Optimised Amplification Conditions

Primer sequences for the loci employed have been described previously (Oldroyd *et al* 1995). All oligonucleotide primers were synthesised commercially by Oswel DNA Services (Southampton, UK.) and selected primers labelled with one of the fluorescent dye markers 6-FAM 6-carboxyfluorescein (6-FAM), hexachloro-6-carboxyfluorescein (HEX) or tetrachloro-6-carboxyfluorescein (TET) all from ABD (Warrington, UK.), coupled with an aminohexyl linker.

PCR amplification was carried out using 1-5 ng genomic DNA in a 50 µl reaction volume containing 1 x PARR-Excellence buffer (Cambio Ltd, Cambridge UK.), 1.25 U Amplitaq™ DNA polymerase (Perkin-Elmer, Norwalk, CT, USA) and 200 µM each dNTP (Boehringer Mannheim GmbH, Mannheim, Germany). Samples were amplified for 30 cycles of 30 s at 93°C, 75 s at 58°C and 15 s at 72°C followed by a 10 minute extension period at 72°C on a Perkin-Elmer 9600 thermal cycler. Primer concentrations employed were 0.05 µM Amel 1/2; 0.32 µM VWA 1/2; 0.22 µM TH01 1/2; 0.28 µM D6S502 1/2; 0.08 µM FGA 1/2; 0.20 µM D21S11 and 0.08 µM D18S11 1/2.

Electrophoresis and Analysis

1.5µl of PCR product were combined with 2 µl formamide and 6 fmol of internal size standard GS350 (ABD) comprising *Pst 1*-digested plasmid DNA ligated to a TAMRA-labelled (N,N,N',N'-tetramethyl-6-carboxyfluorescein) 22-mer oligodeoxynecleotide at the cut ends. Subsequent digestion with BstU 1 results in DNA fragments containing a single TAMRA dye yielding a single peak for each fragment under denaturing and non-denaturing conditions. Samples were denatured for 2 min at 90°C and loaded onto 6% denaturing 24 cm well-to-read polyacrylamide gels. National Diagnostics ultra-pure sequagel 6 and matched buffer batches were commercially supplied by Flowgen (Sittingbourne, UK.). Gels were electrophoresed for 8 h at 38 W constant power on an ABD 373A sequencer and fragment sizes determined automatically by GeneScan 672 software (ABD) using the Local Southern sizing algorithm. Allele designations determined by sequencing a selection of alleles from each locus were automatically assigned using the Genotyper DNA fragment analysis software (ABD).

Statistical Calculations

Discriminating power and matching probabilities were calculated by the method of Jones (1972).

RESULTS AND DISCUSSION

All of the loci contained within this system have previously been demonstrated to co-amplify effectively as part of a multiplex containing 7 STR loci previously described by this laboratory (the octoplex, Oldroyd *et al*, 1995). The system described here evolved as a result of forensic difficulties encountered with one of the loci (D20S85) in the octoplex. When multiplexing several loci together in this fashion, interaction between the primers can result not only in the production of desired allele peaks but also in the formation of artefactual bands caused by the interaction of mismatched primer pairs with the genomic DNA. In a forensic context, the presence of additional bands unrelated to the genotype of an individual could lead to confusion during interpretation of the profile. The locus D20S85 was found to be particularly problematic in this area and it was decided to remove it from the multiplex and optimise the conditions for the remaining loci in the system.

The loci in the revised system were found to co-amplify effectively and employed identical buffer, dNTP and enzyme concentrations as the heptaplex and octoplex systems previously described. Comparable band intensities for all the loci contained within the system were obtained by the adjustment of individual primer concentrations within the reaction. Optimum amplification was achieved through manipulation of PCR cycle parameters.
Genescan 672 analysis (ABD) generated electropherograms in which DNA fragments were depicted as coloured peaks. Examples shown in Fig. 1 indicate the consistency in peak areas generated both between alleles and across loci. Co-amplification of loci whose size ranges overlap was afforded by the combination of 3 dye labels.

Preliminary statistical calculations for each STR locus were carried out on a minimum of 50 unrelated individuals from each of the three British populations: Caucasians, Afro-Caribbeans and Asians (amelogenin was not included in these calculations). The individual and combined matching probabilities (pM) for each locus in each of the stated race codes are given in table 1 and correspond with previously published values. SGM produces a matching probability of between 1.2×10^{-8} and 1×10^{-9}, a value equivalent to that produced by 4 RFLP probes and

when used in conjunction with the quadruplex system (Kimpton *et al*, 1993), the value approaches 1 x 10^{-10}.

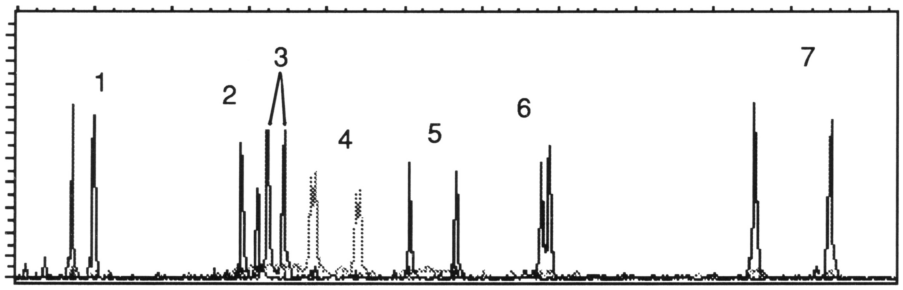

Figure 1: Example of an electropherogram generated by Genescan 672 software. Key to locus numbers: 1: Amelogenin, 2:HUMVWFA31/A, 3: HUMTH01, 4: D6S502, 5: HUMFIBRA, 6: D21S11, 7: D18S51. (Loci 1, 3, 6 & 7 labelled with 6-FAM; 2 & 5 labelled with HEX; 4 labelled with TET).

Table 1: The individual and combined matching probabilities of each locus for each of the three stated race codes. * indicates loci displaying alleles differing in size by 2bp.

Locus	Location	Probability of a Match		
		White Caucasians	Afro-Caribbeans	Indo-Pakistani
HUMVWFA31/A	12p12-pter	0.064	0.057	0.075
HUMTH01	11p15-15.5	0.086	0.100	0.084
HUMFIBRA*	4q28	0.044	0.027	0.031
D21S11*	21	0.051	0.042	0.046
D18S51*	18q21.3	0.029	0.024	0.042
D6S502	6	0.047	0.061	0.054
COMBINED (SGM)		1.7 x 10-8	9.5 x 10-9	2.0 x 10-8

SGM has been demonstrated to be robust and reproducible with profiles demonstrated to be consistent across a range of sample types (results in prep.). The system is currently used for the analysis of hair root and buccal scrape samples submitted to the UK National DNA Database.

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