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Evaluation of an automated DNA profiling system employing multiplex amplification of four tetrameric STR loci

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Summary. We have examined the performance and reproducibility of an automated DNA profiling system which is based on the multiplex amplification of 4 tetrameric STR loci – HUMVWFA31/A, HUMTH01, HUMF13A1 and HUMFES/FPS. The system was able to type 100 pg of purified, undegraded, genomic DNA. At lower concentrations of DNA (below 100 pg), allelec drop-out occurred due to stochastic differences in allele copy number. Minor variation of individual PCR reagent concentrations or cycling temperatures did not result in a significant effect on the efficiency of amplification of any of the 4 loci in the quadruplex system. More substantial variation of reagent concentrations or cycling temperatures outside the optimum range of the system resulted in a reduction or complete loss of signal for one or more loci. This was also observed at high ionic strength or extreme pH. However, under all reagent concentrations and conditions studied, no artefact bands that could potentially result in the mistyping of a sample were apparent within the read region (130–240 bases) of the gel. Evaluation of both native and denaturing polyacrylamide gels revealed that, although native gels displayed faster run times, the sizing precision of such gels for certain STR loci was lower than that of denaturing gels. Also, artefact bands may be present within the read region of native gels. In conclusion the quadruplex amplification system decribed, coupled with automated fluorescence-based detection on denaturing polyacrylamide gels, appeared to be a robust and reliable system for individual identification.

Key words: Forensic identification – STRs – Multiplex PCR

Zusammenfassung. Wir haben die Durchführbarkeit und die Reproduzierbarkeit eines automatisierten DNA-Profilierungs-Systems untersucht, welches auf einer Multiplex-Amplifikation von 4 tetramerischen STR-Loci beruht: HUMVWFA31/1, HUMTH01, HUMF13A1 und HUMFES/FPS. Das System war imstande, 100 pg gereinigter, undegradierter genomischer DNA zu typisieren. Bei geringeren Konzentrationen der DNA (unter 100 pg) kam Allel-Verlust zustande, aufgrund stochastischer Unterschiede in der Zahl der Repeats pro Allel. Geringere Variationen der Konzentrationen einzelner PCR-Reagenzien oder der Temperatur-Bedingungen hatten keine offensichtliche Auswirkung auf die Effizienz der Amplifikation irgendeines der 4 Loci in dem Vierfach-System. Ausgeprägtere Variationen der Reagenz-Konzentrationen oder des Temperatur-Zyklus außerhalb des Optimalbereichs des Systems führte zu einer Reduktion oder zu einem Verlust der Signal-Stärke für einen oder mehrere Loci. Dies wurde auch bei hoher Ionenstärke oder extremen pH-Werten beobachtet. Jedoch wurden unter allen untersuchten Reagenz-Konzentrationen und Bedingungen keine Artefaktbanden beobachtet, die potentiell zu einer Fehltypisierung einer Probe innerhalb der abgelesenen Region (130–240 Basen) des Gels führen konnten. Die Auswertung von nativen und denaturierenden Polyacrylamid-Gelen zeigte, daß, obwohl native Gele schnellere Laufzeiten zeigten, die Präzision der Fragment-Größen-Bestimmung solcher Gele für bestimmte STR-Loci geringer war als jene der denaturierenden Gele. Auch können Artefakt-Banden in der abgelesenen Region der nativen Gele (eher) erscheinen. Das beschriebene Vierfach-Amplifikationssystem, in Verbindung mit einer automatisierten, fluoreszenzbasierten Detektion in denaturierenden Polyacrylamid-Gelen, schien ein robustes und zuverlässiges System für die individuelle Identifikation zu sein.

Schlüsselwörter: Forensische Identifikation – STR's – Multiplex – PCR

Introduction

Short tandem repeat (STR) loci are a class of polymorphic markers which occur throughout the human genome and consist of simple tandemly repeated sequences of 1–6 bp in length. Their abundance, hypervariability and amena-

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bility to amplification by the polymerase chain reaction (PCR) make them ideal markers for use in the identification of individuals.

DNA profiling based on PCR amplification of STRs has the advantage of being more sensitive than conventional techniques. Furthermore, STR systems have been shown to be successful in typing material containing highly degraded DNA (Hagelberg et al. 1991; Gill et al. 1992; Jeffreys et al. 1992; Gill et al. 1993). Also, the ability to resolve STR amplification products on polyacrylamide gels allows precise allele sizing, thus eliminating the need for the continuous allele distribution models currently employed with VNTR systems (Balasz et al. 1989; Budowle et al. 1991; Gill et al. 1990; Evett and Gill 1991).

Tri-, tetra- and pentameric STR loci appear to be less prone to artifactual "stutter" banding caused by enzyme slippage during amplification than dimeric STRs and are therefore more suitable for routine forensic applications (Edwards et al. 1991; Fregeau and Fourney 1993; Kimpton et al. 1993; Wiegand et al. 1993). However, many of the most polymorphic loci in this group have been shown to consist of complex compound repeat regions and may display alleles which differ by only a single base (Adams et al. 1993; Urquhart et al. 1993). This makes unambiguous allele designation difficult for these complex STRs.

Recent reports have examined the use of automated fluorescence technology for the direct detection and automatic sizing of STR loci run on polyacrylamide gels (Zeigle et al. 1992; Fregeau and Fourney 1993; Kimpton et al. 1993). These systems incorporate an internal sizing marker with every sample, thus reducing differences in electrophoretic mobility both within and between individual gels. In addition, the availability of 4 different fluorescent dyes increases the potential for development of amplification reactions containing multiple loci (multiplx systems) as it is possible to use different dyes for loci with overlapping allele sizes. Such multiplex reactions can provide a highly discriminating single amplification DNA profiling system suitable for use on small samples.

We have recently documented 3 multiplex reactions containing 3–7 tri-, tetra- and pentameric STR loci (Kimpton et al. 1993). One of these systems contained the 4 STRs HUMVWFA/31 (Kimpton et al. 1992), HUMTH01 (Polymeropoulos et al. 1991a), HUMFI3A1 (Polymeropoulos et al. 1991b) and HUMFES/FPS (Polymeropoulos et al. 1991c). This system has considerable forensic potential as all 4 loci have relatively simple repeat region sequences and display regularly spaced alleles differing by 4 bases – with 2 common exceptions; one 2 base allele in F13A1 and one 3 base allele in TH01 (Puers et al. 1993; Urquhart et al. manuscript submitted). These alleles have been shown to be automatically sized with a high degree of precision using fluorescent detection, thus allowing unambiguous allele designation.

As part of the detailed evaluation of the suitability of the system for routine forensic use, we have examined the effect of variation in amplification parameters on the reproducibility and efficiency of the quadruplex system. We have also evaluated the effect of ionic strength and pH on the system and made a comparison between denaturing and native polyacrylamide gels.

Materials and methods

Standard multiplex amplification conditions. DNA was prepared from whole blood as described previously (Gill et al. 1990). Quantification of DNA was undertaken using a primate-specific alpha satellite probe assay (Walsh et al. 1992). PCR amplification was performed using 0.01–10 ng of genomic DNA in a 50 µl reaction volume. Reactions consisted of: 1 × PARR buffer (10 m*M* Tris-HCl pH 8.3; 50 m*M* KCl, 1.5 m*M* MgCl, 1% Triton-X 100 – Cambio Labortories, England); 1.25U Taq polymerase (Perkin Elmer, USA), 200 µ*M* of each deoxynucleotide triphosphate (Boehringer); 0.18 µ*M* of each HUMVWFA primer (VWA/1 5′ CCCTAGTGGATGATAAGAATAATCAGTATG3′ labelled with the fluorescent dye "JOE"-Applied Biosystems, USA – ABD, VWA/2 5′ GGACAGATGATAAATACATAGGATGGATGG3′); 0.08 µ*M* of each HUMTH01 primer (TH01/1 5′ GTGGGCTGAAAAGCTCCCGATTAT3′ labelled with the dye "FAM"-ABD, TH01/2 5′ GTGATTCCCATTGGCCTGTTCCTC3′); 0.12 µ*M* of each HUMF13A1 primer (F13A1/1 5′ ATGCCATGCAGATTAGAAA3′ labelled with the dye "JOE", F13A1/2 5′ GAGGTTGCACTCCAGCCTTT3′); 0.12 µ*M* of each HUMFES/FPS primer (FES/1 5′ GGGATTTCCCTATGGATTGG3′ labelled with the dye "FAM", FES/2 5′ GCGAAAGAATGAGACTACAT3′). All primers were synthesised and labelled commercially (Oswel DNA Services, Scotland).

Amplification reactions were carried out in 0.6 ml thin-walled Gene-Amp reaction tubes (Perkin Elmer) on a Perkin Elmer Cetus 9600 thermal cycling block and consisted of 28 cycles of 95°C for 45 s, 2 min cooling ramp to 54°C for 30 s, 72°C, for 60 s followed by a final 10 min incubation at 72°C.

Detection system. Aliquots of 1–4 µl of each amplification reaction were combined with 6 fmol of internal lane standard GS2500 (ABD), consisting of PstI restricted bacteriophage lambda DNA labelled with the dye "ROX" and diluted in formamide. PCR product and internal standard samples were heat denatured prior to loading onto standard 6% polyacrylamide denaturing sequencing gels (24 cm well to read), or loaded directly onto 6% native polyacrylamide gels (24 cm well to read), or loaded directly onto 6% native polyacrylamide gels (0.3% crosslinker). Denaturing gels were run in 1 × TBE buffer (89 m*M* Tris, 89 m*M* Borate, 2 m*M* EDTA pH 8.3) for 5.5 h at constant power (42 Watts) on an Applied Biosystems automated DNA sequencer, model 373. Native gels were run for 4.5 h at constant power (30 Watts). Fragment sizes were determined automatically using Genescan 672 software (ABD), employing the method of second order regression or Local Southern.

Results

Comparable band signal intensities for all 4 loci within the 4 locus multiplex system (Quadruplex) were initially obtained by adjustment of individual primer concentrations within the reaction (Kimpton et al. 1993). Relative concentrations of each primer set in the system were dependant upon the fluorescent dye marker employed and the efficiency of dye-labelling. The number of PCR cycles was set at the minumum required to yield detectable signals for all 4 loci with 100 pg of purified template DNA, from 3 duplicate samples, under our standard multiplex amplification conditions (see Methods). An electrophoretogram of a quadruplex DNA amplification reaction carried out under standard reaction conditions is shown in Fig. 1.

In order to calculate experimental variation, 1 ng of a single sample was amplified and detected 6 times on 4

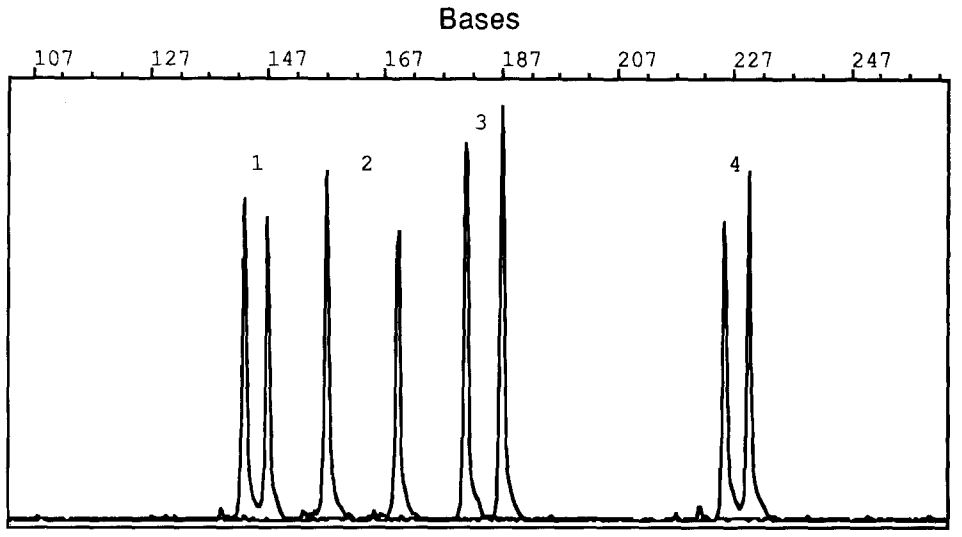


Fig. 1. Electrophoretogram of a quadruplex amplification on a random individual run on a 6% denaturing polyacrylamide gel. Peaks represent fluorescent intensities of dye-labelled DNA products. The size of the products, in bases, is shown along the *x* axis. Locus 1 HUMVWA, locus 2 HUMTH01, locus 3 HUMF13A1, locus 4 HUMFES/FPS

Table 1. Variation in fluorescent signal intensity within and across experiments

Locus	Within experiments		Across experiments	
	Range as % of mean value	S.D.	Range as % of mean value	S.D.
VWA	(±) 17%	9.20%	(±) 60%	40%
TH01	(±) 16.7%	7.80%	(±) 96%	57%
F13	(±) 23%	10.90%	(±) 88%	48%
FES/FPS	(±) 19%	11.30%	(±) 64%	40%

separate occasions under similar conditions. The range of fluorescent signal intensities for each locus within and across the 4 experiments is shown in Table 1. Within experiments each locus displayed approximately a 1.5-fold difference between the smallest and largest band signals. This variation was due to the inherent pipetting errors incurred during the small volume pipetting steps (template DNA addition, sample preparation for gel loading and gel loading), coupled with slight differences in the performance of individual PCR reactions.

The difference between the smallest and largest signals across all 4 experiments increased to 3.1 to 4.2-fold depending on the locus. This wider range reflects the additional pipetting variations incurred during the preparation of separate reaction mixtures for each individual experiment, coupled with the use of different thermal cycling blocks and automated sequencers. Although, the observed range between experiments appears fairly large it should be borne in mind that the smallest band signal observed in all 4 experiments was 18-fold greater than the minimum 'cut-off' value employed in our laboratory (50 units).

Variation in the relative peak heights of the loci within individual amplifications was also greater across the 4 experiments compared to within. This was most probably due to slight variations in the relative concentrations of individual primers between experimental set-ups, coupled with slight differences in the performance of individual PCR blocks.

We have examined the effect of variation of specific amplification parameters on the efficiency of the quadruplex amplification system. During these studies more than one batch of dye-labelled primer was employed for certain loci. For the purposes of consistency it was decided to maintain the primer concentrations throughout this study as those shown in the Materials and Methods and not make minor primer concentration adjustments with different primer batches, in order to obtain even peak signals. As a result increased variations in relative signal intensities were observed between separate experiments.

Buffer concentration

No difference was observed in absolute or relative signal intensities for all 4 loci within the multiplex when 2 commercially available buffers; PARR (Cambio Laboratories)

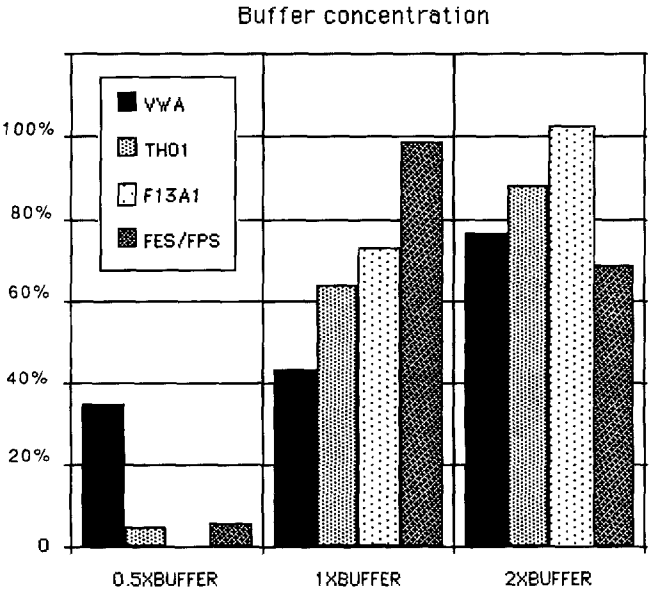
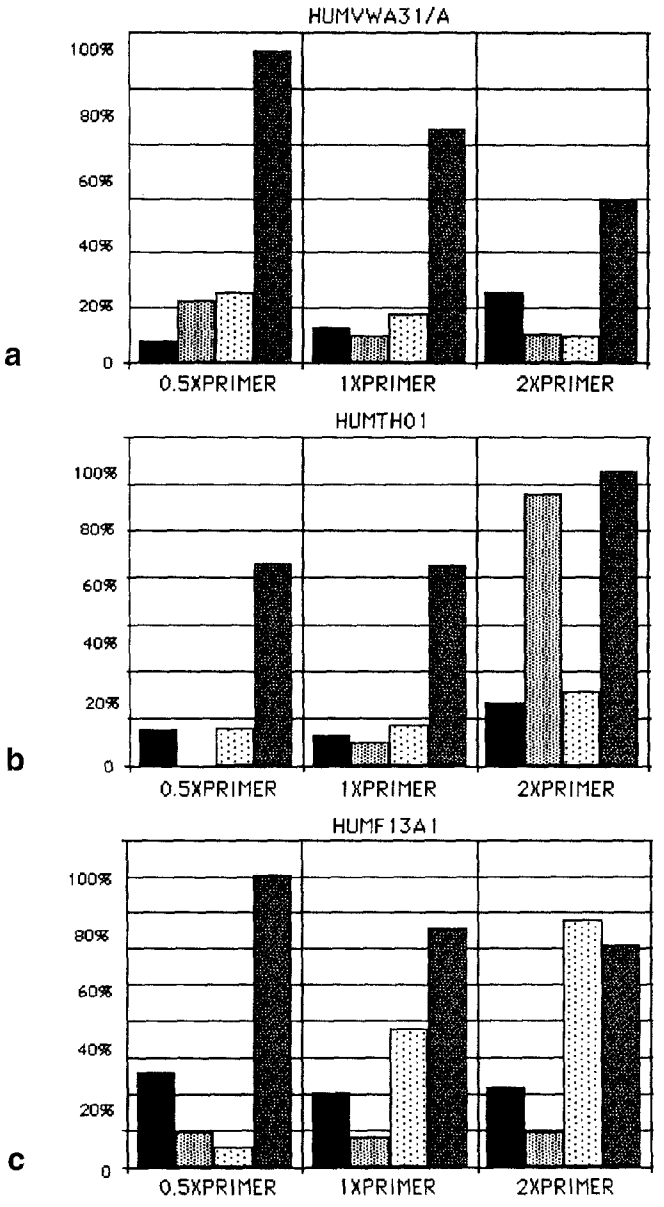


Fig. 2. Effect of buffer concentration on the efficiency of quadruplex amplification. Signal intensities (fluorescence peak areas averaged from a minimum of 6 amplifications) are measured as percentages relative to the largest signal. (Legend for columns see inset)



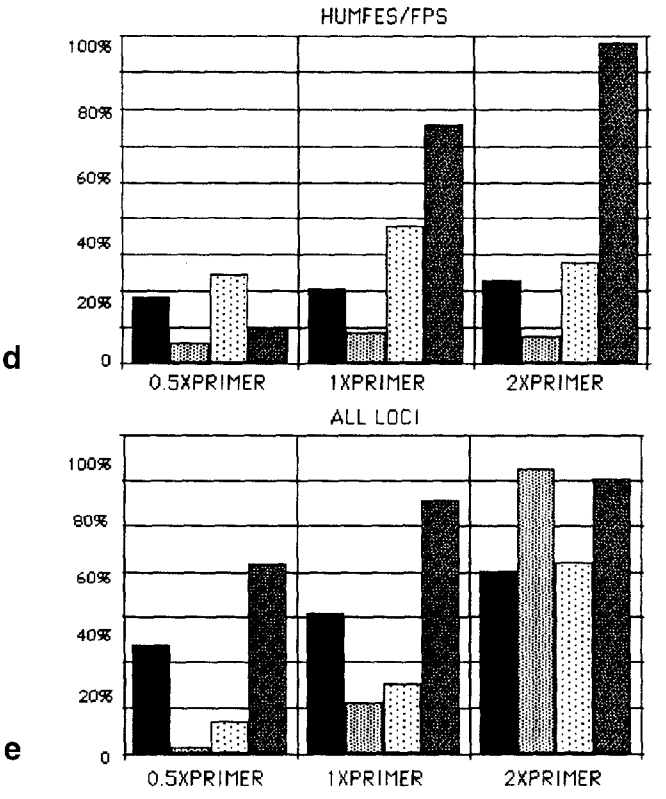


Fig. 3. Effect of primer concentration on the efficiency of quadruplex amplification. Signal intensities (fluorescence peak areas averaged from a minimum of 6 amplifications) are measured as percentages relative to the largest signal. Standard primer concentrations (1 ×) are listed in Materials and Methods. **a–d** Independant variation of individual locus primer concentration. **e** Variation of total primer concentration. (Legend for columns see inset Fig. 2)

and GeneAmp Buffer (Perkin Elmer Cetus), were directly compared. However, PARR buffer contains the detergent Triton-X-100 which may help reduce inhibition of amplification caused by protein contaminants (Kawasaki 1990) and may thus be more suitable for forensic applications. For this reason, PARR buffer was employed throughout the remainder of the study.

Amplification at 0.5 × standard buffer concentration yielded reduced amounts of amplification product of all 4 loci, although the degree of reduction was substantially less for VWA (Fig. 2). At 2 × standard buffer concentration signal intensities for VWA, TH01 and F13A1 were increased (by 37–76%) while FES/FPS showed a reduction of approximately 30%. Also, much higher background signals were apparent at 2 × buffer concentration, as was an extra (JOE labelled) artefact band which was sized at approximately 129 bases.

Primer concentration

Primer concentration for each locus within the quadruplex was independently halved and doubled from the standard concentrations (1 ×) displayed in Materials and Methods. In all cases signal intensities for each locus were found to be directly related to specific primer concentration (Fig. 3 a–d). No significant variation (greater than a 1.5-fold difference) in the relative signal intensities of the other loci present in the multiplex was apparent when individual locus primer concentrations for TH01, F13A1 and FES were varied. However, an increase in VWA signal, resulting from increased VWA primer concentration effected a slight reduction in the product yield of the other 3 loci (10–33%). This reduction was more pronounced with F13A1 and FES/FPS. In addition doubling of the standard TH01 primer concentration effected a slight increase in products for the other loci.

The effect of halving and doubling total primer concentration of all loci simultaneously was also examined (Fig. 3 e). Reduction of primer concentration by 50% resulted in a decrease of signal for all loci, with TH01 products showing the greatest reduction. Doubling of total primer concentration resulted in an increase in signal intensity for all loci, with TH01 again displaying the most substantial difference. At the higher primer concentration artefact primer-dimer bands were observed with increased frequency.

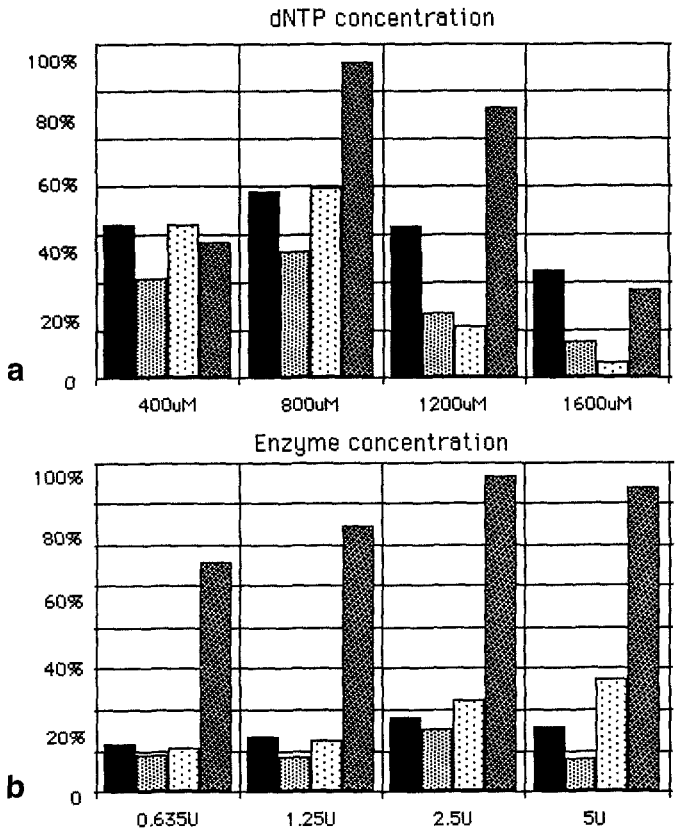


Fig. 4. Effect of **a** dNTP concentration and **b** enyzme concentration on the efficiency of quadruplex amplification. Signal intensities (fluorescence peak areas averaged from a minimum of 6 amplifications) are measured as percentages relative to the largest signal. (Legend for columns see inset Fig. 2)

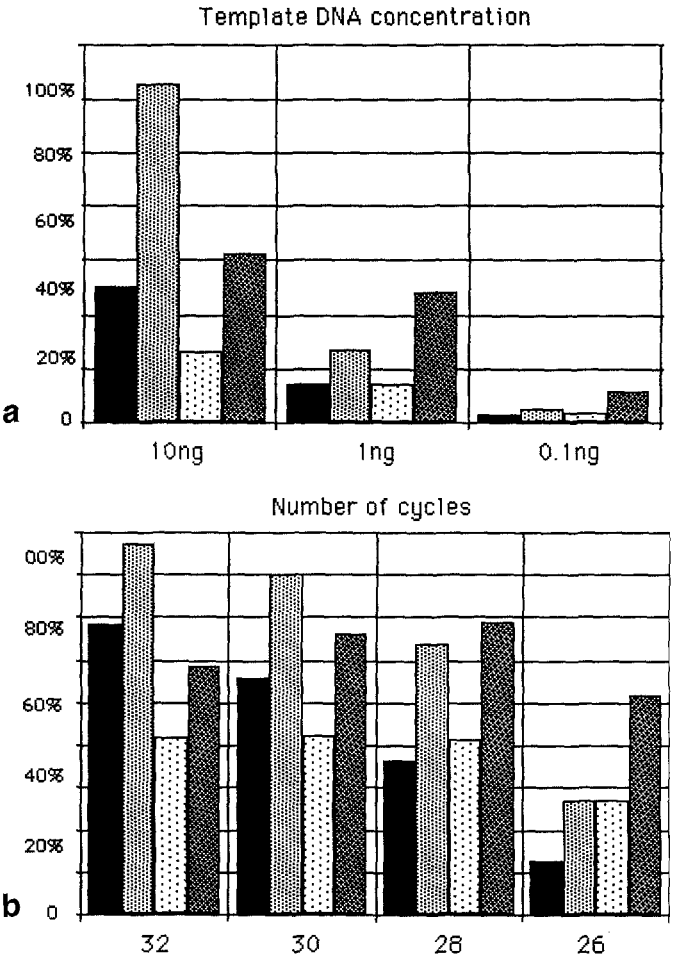


Fig. 5. Effect of **a** template DNA concentration and **b** number of amplification cycles on the efficiency of quadruplex amplification. Signal intensities (fluorescence peak areas averaged from a minimum of 6 amplifications) are measured as percentages relative to the largest signal. (Legend for columns see inset Fig. 2)

Removal of any one primer from the multiplex system resulted in loss of product for the relevant locus, but no significant variation in the relative signal intensities of the other 3 loci (data not shown).

Deoxynucleotide triphosphate concentration

The optimal deoxynucleotide triphosphate (dNTP) concentration is directly related to magnesium concentration within the PCR reaction as dNTPs quantitatively bind Mg ions. Under the standard quadruplex amplification conditions employing PARR or GeneAmp buffer the optimal dNTP concentration is 800 μM (200 μM of each of the dNTPs: Innis and Gelfand 1990).

A reduction in total dNTP concentration to 400 μM resulted in a significant (two-fold) reduction in the FES/FPS amplification product yield relative to the other loci. Amplification efficiency for all loci was also reduced to varying degrees when dNTP concentrations were increased above optimal levels (Fig. 4a) and was probably due to the resulting progressive reduction in free Mg^{2+} ions.

The relative proportion of PCR products displaying an extra base on their 3′ end, due to non-template dependant addition by Taq polymerase (Clark 1988), appeared to decrease with increased dNTP concentration. Again this was probably directly related to the amount of free Mg^{2+} ions present, with the enzyme being apparently less efficient at non-template dependant extra base addition at low free Mg^{2+} concentrations.

Taq polymerase concentration

The maximum recommended enzyme concentration for a standard 50 µl reaction is 2.5 U (Innis and Gelfand 1990). Above this concentration, non-specific background amplification may occur. The effect of varying enzyme concentration in the quadruplex between 0.625-5U/PCR is shown in Fig. 4b. At enzyme levels of 2.5 U slightly greater signals were observed for all 4 loci, although this increase was less than 1.5-fold and may thus be due to within experiment variation. However, at 5 U a significant (twofold) increase was observed in F13A1 product yield relative to the other loci. Increased non-specific background amplification was not observed at higher enzyme concentrations.

Template DNA concentration

Reduction of template DNA concentration resulted in reduced product yields of all 4 loci within the quadruplex

Table 2. Effect of template DNA concentration on variation in allele signals in heterozygous samples

Template DNA/PCR (pg)	Percentage of samples displaying > 20% difference in heterozygous allele signal intensities[a]			
	HUMVWA31/A	HUMTH01	HUMF13A1	HUMFES/FPS
100 pg	41% (46%)	46% (51%)	39% (56%)	34% (41%)
250 pg	25% (30%)	39% (44%)	17% (40%)	23% (60%)
500 pg	18% (28%)	18% (41%)	5% (21%)	5% (30%)
750 pg	27% (28%)	47% (28%)	5% (21%)	10% (28%)
1 ng	18% (28%)	6% (28%)	0% (15%)	3% (21%)

Number of observations ranged from 17–33
[a] Number in parenthesis corresponds to the maximum allele signal variation observed

(Fig. 5a). However, the relative reduction in PCR product for each locus appeared to vary. This was most pronounced with TH01. At a template concentration of 10 ng per reaction the amount of TH01 product accounted for nearly 50% of the total yield. This was reduced to approximately 30% with 1 ng of template DNA and around 20–25% with 100 pg of template DNA.

When low copy numbers of template DNA are amplified (1 diploid copy – 6 pg), stochastic variation may result in unequal amplification of individual alleles at a given locus. We therefore examined the degree of signal variation between the 2 locus-specific alleles of heterozygous individuals at DNA concentrations ranging from 100 pg to 1 ng (17–170 diploid copies) – see Table 2. For all 4 loci, reduction in the amount of template DNA present resulted in a greater proportion of samples displaying significant (greater than 20% variation in fluorescent signal intensity between the 2 alleles) differences in individual allele signals. The degree of variation in the individual allele signals of heterozygous samples increased at lower template levels to maximum difference in fluorescent signal of 56% with 100 pg of template DNA. Preliminary studies at template concentrations of less than 100 pg revealed a further increase in the amount of allele signal variation and, at template DNA levels of 50 pg or less, a small proportion of alleles completely failed to amplify, thus resulting in false homozygosity (data not shown). At all DNA concentrations the allele displaying the lower signal appeared to be completely random indicating that no preferential amplification of either allele occurred for the 4 loci.

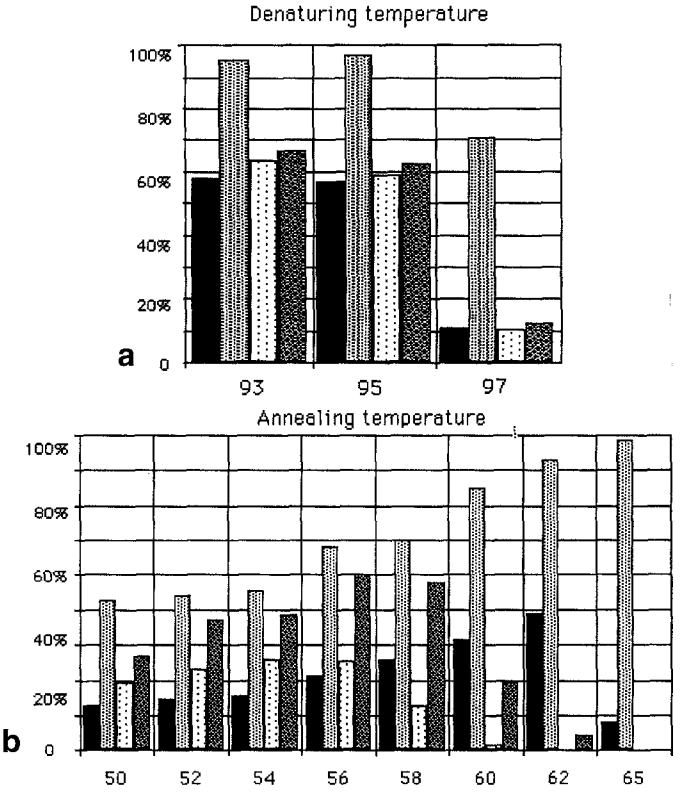


Fig. 6. Effect of **a** denaturing temperature and **b** annealing temperature on the efficiency of quadruplex amplification. Signal intensities (fluorescence peak areas averaged from a minimum of 6 amplifications) are measured as percentages relative to the largest signal. (Legend for columns see inset Fig. 2)

Number of amplification cycles

The total product yield was directly related to the number of PCR cycles employed (Fig. 5b). However, above 28 cycles the yield of F13A1 and FES/FPS remained constant while TH01 and VWA products continued to increase with cycle number. This resulted in a greater proportion of VWA and TH01 product at higher cycle numbers.

Denaturing temperature

Denaturing temperatures of 93, 95 and 97°C were examined (Fig. 6a). No significant variation in relative signal intensities between the loci was observed for all 4 loci at 93 and 95°C. However, at 97°C a fivefold reduction in VWA, F13A1 and FES/FPS signals occurred. This reduction may be related to the reduced half-life of the enzyme at elevated temperatures. For example, at 95°C the enzyme has a half-life of approximately 40 minutes. This is reduced to 5 min at 97.5°C (Gelfand and White 1990).

Effect of annealing temperature

All 4 loci multiplexed efficiently at annealing temperatures of 50–56°C (Fig. 5b). At tempertures greater than 56°C the F13A1 and FES/FPS product signals were significantly reduced. This loss of signal intensity appeared to be inversely proportional to annealing temperature, F13A1 and FES/FPS completely failed to amplify above

Table 3. Effect of ionic strength on amplification efficiency

Locus	Percentage reduction in allele signal			
	Final concentration of NaCl			
	125 m*M*	100 m*M*	75 m*M*	50 m*M*
VWA	NS	50%	0%	0%
TH01	NS	50%	25%	0%
F13A1	NS	NS	NS	0%
FES/FPS	NS	NS	NS	0%

NS, No signal

60° C and 62° C respectively. Conversely, TH01 and VWA product yields appeared to increase with increased annealing temperature up to 62° C and 65° C respectively. These results are similar to the performance of the individual loci in singleplex amplification and appear to be related to the individual optima of each locus.

Non-specific amplification products

In a small proportion of samples, non-specific low molecular weight (55–75 bases) PCR products of varying signal intensity were occasionally observed. These bands occurred in both positive and negative samples and were thought to result from non-specific primer-primer interactions – "primer-dimer".

Low levels of primer-dimer did not effect the yield of STR products. However, higher levels resulted in reduced STR product signals and in extreme cases products could not be detected.

The formation of primer-dimer in the quadruplex was directly related to the total concentration of primers which were in the presence of Taq polymerase prior to PCR. For example, primer-dimer was more likely to occur if the required volume of sterile distilled water was added directly to individual reaction tubes and omitted from the initial PCR "multimix". Primer-dimer formation also appeared to be related to the temperature of reagents and "multimix" following enzyme addition but prior to PCR cycling. When pre-PCR temperatures were increased up to approximately 70° C the amount of primer-dimer formation was greater. This is presumably related to the increased activity of the Taq polymerase at the higher temperatures.

Ionic strength and pH

Variation in ionic strength was examined by alteration of the final concentration of NaCl in the reaction tube (Table 3). Partial inhibition of amplification was observed at NaCl concentrations of 75 m*M*. At this concentration both F13A1 and FES/FPS failed to amplify and the TH01 was reduced. Both TH01 and VWA failed to amplify in the presence of 125 m*M* NaCl. The level at which NaCl inhibits amplification in the quadruplex reaction correlates with previous studies which have reported the occurrence of Taq polymerase inhibition at NaCl levels greater than 50 m*M* (Innis et al. 1988).

The efficiency of amplification in the presence of acid or alkali is dependant on the buffering capacity of the PCR buffer. Complete inhibition of amplification was observed at HCl and NaOH concentrations of 5 m*M* and 7.5 m*M* respectively. Partial reduction in signal intensity was observed at lower concentrations of these reagents (Table 4). Signal reduction was slightly more pronounced when the HCl/NaOH was added directly to the template DNA sample prior to addition of the reaction mix.

Gel types

We have evaluated the use of 6% native polyacrylamide gels run at a constant power of 30 W and compared these with our conventional 6% denaturing polyacrylamide gels run at 42 W. Under the non-denaturing conditions employed band resolution was reduced and the high molecular weight fragments of the GS2500 sizing standard appeared as very broad bands.

Sizing of STR products against GS2500 was shown to be less accurate with the non-denaturing gel system. Alleles of individual loci were sized 3.5–6 bases different from their true lengths determined by sequencing, compared to 1–2 bases for denaturing gel systems. More importantly, the sizing precision of native gels was also lower (Table 5). This was most pronounced with the largest alleles of FES/FPS which displayed sizing ranges of more than 3 bases within some gels. In addition, alleles known to differ by 4 bases were sized as differing between 2.7–5.4 bases within the same lane on native gels. This low precision may be partly due to the lower resolution of the native gel system employed in this study and may be improved by increasing the acrylamide percentage

Table 4. Effect of pH on amplification efficiency

Locus	Percentage reduction in allele signal									
	Final concentration of HCl					Final concentration of NaOH				
	7.5 m*M*	5 m*M*	2.5 m*M*	1 m*M*	0.5 m*M*	0.5 m*M*	1 m*M*	2.5 m*M*	5 m*M*	7.5m*M*
VWA	NS	NS	60%	10%	10%	0%	0%	10%	20%	NS
RH01	NS	NS	50%	0%	0%	0%	0%	0%	10%	NS
F13A1	NS	NS	80%	10%	0%	0%	0%	10%	30%	NS
FERS/FPS	NS	NS	90%	20%	10%	0%	0%	10%	50%	NS

NS, No signal

Table 5. Inter-gel allele sizing precision

Locus	Individual allele sizing range[a]			
	Native gels[b]		Denaturing gels[c]	
	min (bp)	max (bp)	min (bp)	max (bp)
VWA	0.3	1.0	0.4	0.7
TH01	0.8	2.1	0.3	1.1
F13A1	Unable to size		0.5	1.2
FES/FPS	1.4	3.3	0.6	0.9

[a] Ranges were calculated by running an allelic ladder for each locus a minimum of 12 times per gel. The figures quoted are the minimum and maximum window ranges of individual allele sizes observed for each locus
[b] Across 2 gels
[c] Across 6 gels

of the gel or modifying the electrophoresis parameters. However, this may result in longer run times.

The gel run time (to the 361 bp sizing standard) for 6% native gels run at 30 W was approximately 4.5 h as compared to 5 hours for 6% denaturing polyacrylamide gels run at 42 W. Native gel run times may be further reduced by increasing the power, although this is likely to result in increased gel temperatures and a further reduction in precision.

High molecular weight artefact bands were observed with all 4 loci on native gels but were most apparent with FES/FPS and F13A1. These artefact bands probably represent single stranded or heteroduplex PCR products and may cause significant difficulty in the interpretation of profiles for these loci.

Discussion

Prior to the introduction of a new system into routine forensic operation it must be fully validated. In the context of a PCR based system, validation must define the operating range of the system within which the following scenarios do not occur: wrongful scoring of an allele band due to band shifting; and the occurrence of a spurious band which is indistinguishable from expected allele bands, potentially leading to mis-typing. In addition, it is desirable to avoid allelic drop-out and inhibtion of amplification. Although the latter will not result in incorrect typing of samples, it may limit the usefulness of the system in certain forensic situations.

To this end, we have examined the effect of variation of amplification parameters and conditions on the efficiency and reproducibility of the VWA, TH01, F13A1, FES/FPS quadruplex STR amplification system.

In order to obtain approximately even signal intensities for all loci within the quadruplex, specific primer concentrations must be adjusted (Kimpton et al. 1993). The optimal relative primer concentrations will depend upon the method of product detection. When using the automated fluorescence detection system employed in this study, the 2 major factors involved are the fluorescent dye employed for each locus (different dyes display differing detection sensitivities) and the efficiency of primer labelling and purification. It is therefore essential that any new batch of primer, which has been re-synthesized or newly labelled and purified, is first checked to ensure that it performs to the required specification within the quadruplex reaction. In this study, primer performance was determined by checking that all 4 loci in the multiplex system yielded detectable signals with 100 pg of purified template DNA.

The potential requirement to make slight adjustments in relative primer concentrations when employing new batches of primers makes it important to know how variation in primer concentration for one locus will affect the amplification efficiency of the other 3 loci. In this study it was shown that variation in specific primer concentration resulted in only relatively small variation in signal intensity of the remaining loci. This makes re-optimisation of the system with new primer batches relatively straight forward. In addition, variation between primer batches should be substantially reduced with the employment of the recently available phosphoramidite labelling systems in which the primer is labelled directly during synthesis.

Variations in optimal individual reagent concentration and cycling temperatures of the quadruplex did not effect the production of artefact bands within the read region of the gel (130–240 bases). This indicates that sample mis-typing should not result from errors made during the setting up of quadruplex PCRs, or block failure. However, non-specific primer-dimer amplification products caused by primer-dimer interactions were occasionally observed. These products occurred more frequently when elevated levels of total primer were in the presence of enzyme (for example during multimix preparation) and in extreme cases they reduced STR product yield. It is therefore important to design routine protocols which minimise the likelihood of primer-dimer formation and also to check STR product signals when primer-dimer occurs.

The most common effect observed following employment of reagent concentrations or PCR cycling temperatures outside the optimum range was reduction in or complete loss of signal for one or more loci within the quadruplex reaction. The same observation was made at high ionic strength or extreme pH. These effects should not result in the mis-typing of a sample and, provided that operating protocols are well designed, their occurrence should be minimal.

Variation in individual reagent concentrations and cycling times affected each locus slightly differently. This is partly because the optimal parameters for the quadruplex system are different to the optimal conditions for each of the loci amplified separately. Such compromises in optimal conditions for the individual loci within the quadruplex system may result in a slight reduction in individual locus sensitivity (compared to singleplex amplification). However, increased product yields are not an issue with the quadruplex system as its sensitivity is at an acceptable level for routine casework (and in our hands slightly more sensitive than the DQalpha system). An increase in sensitivity is likely to result in increased conta-

mination problems and the possibility of allele drop-out caused by stochastic differences – demonstrated in this study.

The degree of variation in fluorescent signals across experiments was shown to be fairly large. This variation appeared as either an increase or decrease in total product yield between experiments or slight variations in relative product yields of the 4 loci. Such variations can be substantially reduced by the preparation of a bulk reaction mixture (enough for 500 or reactions – Lygo et al. 1994), thereby reducing pipetting errors. In addition, it is important that pipettes, PCR blocks and sequencers are regularly serviced and calibrated.

The use of non-denaturing (native) polyacrylamide gels has a number of advantages over denaturing gels. These include omission of the requirement for sample denaturation prior to loading and faster run times. However, although the native gel system employed in this study was able to adequately resolve alleles differing by only one repeat unit, the accuracy and precision of automatic sizing was shown to be lower than with denaturing gels. When using native gels, unambiguous allele designation was not possible for some F13A1 and FES/FPS products sized against the GS2500 internal lane standard. Therefore, at present native gels are not ideal for routine analysis of the quadruplex reaction. Alteration of gel composition or reduction of the electrophoresis power is likely to improve the precision of native gels but this may, in turn, increase gel running times.

The reduction in electrophoresis times when using native rather than denaturing gels was demonstrated to be fairly small and is unlikely to produce a dramatic increase in the sample throughout of the system. A more favourable solution would be the running of shorter denaturing gels. Indeed, preliminary studies indicate that denaturing gels with a 12 cm well-to-read distance (as opposed to the 24 cm distance employed in this study) show a significant decrease in run time without substantial reduction in sizing precision (J Robertson personal communication).

In conclusion, the quadruplex amplification system coupled with automated fluorescence-based detection on denaturing polyacrylamide gels appears to be a highly discriminating, robust and reliable system for individual identification. Furthermore, because this system is PCR based it offers the advantages of increased sensitivity, the ability to obtain results from degraded samples and the potential for further automation. Validation studies on the performance of the system with routine casework material and mixed samples has been undertaken and will be the subject of a separate publication (Lygo et al. 1994).

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