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The validation of short tandem repeat (STR) loci for use in forensic casework

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Abstract A quadruplex reaction has been developed which amplifies the short tandem repeat (STR) loci HUMVWA31/A, HUMTHO1, HUMF13A1 and HUMFES/FPS. Detection of the PCR products employs denaturing polyacrylamide gels coupled with fluorescent-based technology. This system has been evaluated for use in routine forensic casework and has been shown to be both robust and reproducible. The quadruplex reaction is as sensitive as the commercially available HLA DQα Amplitype typing system and can be used on both degraded and aged material. The problems of environmental contamination have been shown to be limited provided strict procedural practices are followed – i.e. physical separation of sample extraction and amplified products; the use of dedicated equipment such as pipettes; the separation of amplification preparation area. The ability of the system to detect mixtures and the successful analysis of case stains has shown that this system is well suited as a tool for forensic investigation.

Key words DNA · PCR · Short tandem repeats
Forensic Identification

Zusammenfassung Eine Quadruplex-Reaktion wurde entwickelt, die die Short tandem repeat loci (STR) HUMVWA31/1, HUMTHO1, HUMF13A1 und HUMFES/FPS amplifiziert. Zum Nachweis der PCR-Produkte werden denaturierende Polyacrylamid-Gele benutzt, in Kombination mit einer Fluoreszenz-basierten Technologie. Dieses System wurde für den Gebrauch in der routinemäßigen Fallbearbeitung evaluiert und hat sich als robust und reproduzierbar erwiesen. Die Quadruplex-Reaktion ist genauso empfindlich wie das kommerzielle HLADQα-Amplitype-Typisierungssystem und kann sowohl an degradiertem als auch an gealtertem Material verwendet werden. Die Probleme einer umgebungsbedingten Kontamination haben sich als begrenzt erwiesen, vorausgesetzt, daß strikte prozedurale Praktiken benutzt werden, d.h. physische Trennung der Extraktion und der amplifizierten Produkte; Benutzung persönlicher Einrichtungen, wie Pipetten; Abtrennung des Bereichs der Amplifikationsvorbereitung; Benutzung der Laminar Flow-Einrichtung für die Vorbereitung der Amplifikation. Die Fähigkeit des Systems, Mischungen zu erkennen und die erfolgreiche Analyse von Spurenfällen haben gezeigt, daß dieses System gut geeignet ist als ein Werkzeug für forensische Untersuchung.

Schlüsselwörter DNA · PCR · Short tandem repeats
Forensische Identifikation

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Introduction

Short tandem repeat (STR) loci consist of tandemly repeated sequences, between 2 and 6 base pairs (bp) in length, which exhibit a high degree of length polymorphism due to variation in the number of repeat units. The high abundance of STR loci in the human genome [1] has resulted in a wide choice of loci. However, the most useful for forensic analysis consist of repeat units of at least 4 bp since amplification artifacts due to enzyme slippage are reduced [2, 4].

The analysis of STR loci is based on the technique of DNA amplification or polymerase chain reaction (PCR). Therefore, the same advantages of sensitivity currently obtained using the commercially available Amplitype kit (Roche) for the analysis of the HLA DQα locus [3], are also applicable, making the system ideal for the analysis of old and degraded samples [9, 10]. The STRs studied in this paper have between 5 and 14 alleles per loci [12] and individually have discriminating powers similar to HLA DQα typing. However, when the average probabilities for each locus are multiplied together the overall probability of

a match is 1.3×10^{-4} for White Caucasian populations [12].

Detection of STR products is achieved by the incorporation of fluorescently labelled primers during amplification. These fluorescent dyes are then detected by laser scanning during electrophoresis using an automated sequencer [8, 23, 28]. The use of polyacrylamide gels enables resolution of PCR products differing in size by just one base pair. Thus precise allele designations can be determined eliminating the need for continuous allele distribution models currently required with single locus probe (SLP) analysis [7].

Previous studies have shown that multiple STR loci can be amplified in a single multiplex reaction [4, 15, 21]. In addition by utilising different fluorescent dye markers, loci which have overlapping allele size ranges can easily be detected. In this laboratory 4 STR loci; HUMTHO1 [16], HUMVWA31/A [11], HUMF13A1 [17] and HUMFES/FPS [18], have been combined into a single quadruplex reaction. The physical properties and parameters of the system are described by Kimpton et al. [13], this paper describes casework validation of this STR quadruplex for use in forensic analysis.

Materials and methods

Preparation of samples. Bloodstains, semen stains and saliva stains were prepared on boiled cotton from fresh liquid samples. All stains and samples were air-dried before storage at –20° C. Samples supplied for the aging study had previously been prepared as above and stored at room temperature. Bloodstains and semen stains were degraded by placing in 100% humidity at 56° C.

Preparation of DNA. DNA was prepared from samples using a rapid Chelex extraction procedure [25]. All samples of DNA (except the DNA samples from non-humans) were quantified by a slot-blot system detailed by Walsh et al. [26] which uses a biotinylated probe complementary to a primate-specific alpha satellite DNA sequence (D17Z1) on chromosome 17 [27]. Some casework DNA samples and all non-human DNA had previously been purified with phenol/chloroform. Non-human DNA quantification was carried out using spectrophotometry.

Amplification conditions. The repeat unit and primer sequences for the STR loci have been described previously [12]. Human DNA (100 pg-5 ng) or non-human DNA (5 ng) was amplified in 0.5 ml thin-walled tubes in a total reaction volume of 50 µl consisting of 1 × PARR buffer (10 m*M* Tris-HCl pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$ 1% Triton-X-100 – Cambio Laboratories, England), 1.25 units Taq polymerase, 200 µ*M* of each dNTP, and primers 0.18 µ*M* VWA/1&2, 0.08 µ*M* THO1/1&2, 0.16 µ*M* F13/1&2 and 0.125 µ*M* FES/1&2, sealed with one drop of mineral oil (although the 9600 thermocycler can be used without the addition of oil to tubes, in our experience this results in increased primer dimer formation in multiplexes). Prior to amplification, the master mixture comprising all reagents except the Taq polymerase and the DNA sample was boiled for 3 min before placing on ice. Amplification was carried out in a 9600 thermal cycler (Perkin Elmer) using the following conditions: 95° C for 60 s; 2 min cooling to 54° C for 60 s; 72° C for 60 s; for 28 cycles followed by 72° C for 10 min.

Detection system. Amplification reaction mixtures (1–4 µl) were combined with 6fmol of internal lane standard GS2500 (Applied Biosystems division, ABD) and heat denatured prior to loading onto a standard 6% polyacrylamide denaturing sequencing gel as described by Kimpton et al. [12]. Gels were electrophoresed for 5–6 h at constant power (42W) on an Applied Biosystems automated DNA sequencer model 372. Fragment sizes were determined automatically using Genescan 672 software (Applied Biosystems) employing the local Southern method [5].

Mixture experiments. Blood samples were mixted volumetrically in proportions of 1:2, 2:1, 1:10 and 10:1. Stain-cards were prepared as above and 3 mm^2 areas were removed and extracted using the Chelex method. DNA samples were mixed together in proportions of 1:2, 2:1, 1:5, 5:1, 1:10 and 10:1. Aliquots of each mixture (5 ng, 3 ng, 2 ng and 1 ng) were ampified and typed as above. Details of the genotypes of the samples mixed are detailed in Appendix I.

Validation of casework samples. A total of 31 cases were received from operational laboratories for analysis with the STR quadruplex. Of these cases, 16 had been previously processed using single locus probes and HLA DQα testing using the Amplitype kit, 2 cases had previously been processed using conventional blood grouping techniques and the remainder of cases had been processed using single locus probes only. Samples were provided either as extracted DNA and/or stain material/whole blood samples. A list of stain types and material is detailed in Appendix II. All samples were extracted, quantified and typed as detailed above.

Nomenclature. Nomenclature follows the recommendations of the DNA commission of the International Society of Forensic Haemogenetics (pers comm./in press). Alleles are designated according to the number of repeat units present. If a repeat is incomplete then the allele is designated by the number of complete repeats present followed by a decimal point; followed by the number of bases of the incomplete repeat; for example HUMTHO1 (9.3) means that this allele has 9 tetrameric repeats plus an additional repeat consisting of 3 bases.

Results

Optimisation of the system

All components of the amplification reaction mixture excluding the Taq polymerase were prepared as a master mixture in bulk (enough for 500 reactions) such that the volume per amplification reaction allowed the addition of a total of 30 µl of DNA solution. The preparation of a master mixture reduces pipetting errors, thus achieving greater standardisation between individual amplification batches. The reduction in the volume of the master mixture allows a large volume of extract to be added enabling low DNA concentrations to be amplified more effectively. However, the increase in initial primer concentration within the master mixture resulted in the formation of primer-dimer products. It was therefore found necessary to heat denature the master mixture and snap-cool prior to use. The bulk master mixture was shown to be stable at –20° C for at least 6 months.

To standardise the electrophoresis, commercially prepared 6% acrylamide (Scotlab) was compared to acrylamide prepared in house from powder and from a 40% liquid stock (Bio-rad). The results obtained were comparable, however the background noise on the 6% acrylamide was lower.

Table 1 Allelic designations and windows for the STR loci HUMVWA31/A, HUMTHO1HUMF13A1 and HUMFES/FPS the repeat analysis of allelic ladders and 24 samples

STR loci	Allele designation	Size range (bp)	
		Minimum	Maximum
HUMVWA31/A	13	133.4	136.1
	14	137.4	140.1
	15	141.4	144.1
	16	145.5	148.2
	17	150.5	152.2
	18	153.6	156.3
	19	157.7	160.4
	20	161.9	164.6
	21	166.0	168.7
HUMTHO1	5	152.0	155.0
	6	156.2	159.2
	7	160.3	163.3
	8	164.6	167.6
	9	168.8	171.8
	9.3/10	172.0	175.6
	11	177.6	180.0
HUMF13A1	3.2	180.8	182.2
	4	183.0	184.3
	5	186.2	189.2
	6	190.2	193.2
	7	194.3	197.3
	8	198.4	201.4
	9	202.4	205.4
	10	206.4	208.4
	11	210.4	213.4
	12	214.5	217.5
	13	218.6	221.6
	14	222.6	225.6
	15	226.6	229.6
	16	230.7	233.7
	17	234.9	237.9
HUMFES/FPS	8	212.0	214.9
	9	216.1	219.0
	10	220.2	223.1
	11	224.1	227.0
	12	228.1	231.0
	13	232.1	235.0
	14	236.6	239.5

bp, base pair

Allelic designations and reproducibility

Previous studies have shown that the STR loci fell into discrete groups [12] and from this data, together with further sequencing studies [19, 24], allele designations were assigned based on the number of repeat units present. Sizing errors are always greater between gels, rather than within gel comparisons [12]. The window (or size range) for each allele (Table 1) was determined by analysis of allelic ladders run 72 times across 6 gels and by the analysis of 24 samples which had been repeated on 16 differents gels, the window for each allele is simply the minimum and maximum values of all observations.

DNA samples extracted from various body fluids of 24 individuals were analysed and the STR fragments obtained were sized and allele designations made according to the pre-determined window. The electrophoretrograms displaying the PCR products from the DNA of a single individual for samples of bloodstain, semen stain, hair root, saliva stain and liquid blood are shown in Figs. 1 and 2. In all individuals, for all samples types, allelic designations were identical demonstrating somatic stability. PCR products were only scored as alleles if the height of the peak obtained (measured on an arbitrary scale using Genescan 672 software) was greater than 50. Below this threshold (or guideline), background noise may prevent reliable interpretation of bands particularly when allele peaks are low. This does not mean that all peaks above the threshold were automatically scored as alleles because stutter bands and background noise could both achieve levels higher than the default, especially if allele peaks were high. As with any scientific method, operator experience is the most important factor in reporting a result.

Variation in the signal intensity of different allele bands within heterozygous loci was observed. The average difference in signal intensity between the high and low molecular weight alleles, determined by peak area, for varying quantities of template DNA is given in Table 2. In general the differences in peak areas between alleles increases as the quantity of template DNA decreases and appears to be unrelated to the type of substrate. This observation is consistent with that made by Kimpton et al. [13]. Within an individual, variation in signal intensity of a locus was also observed not only between amplification reactions but also within an amplification reaction (Table 3). There was no distinct trend for the allele displaying the lower signal. The variation in signal intensity of alleles is therefore a function not only the amplification reaction but also of the electrophoresis system and may be due to co-migration of the allele with a stutter band from an adjacent allele and/or a result of smaller peak areas being calculated due to inaccurate assignment of the limits of the peaks (Fig. 3).

Sensitivity

DNA (1 ng) was successfully amplified and the correct allelic designations obtained. Lower quantities of DNA (500 pg) exhibited locus drop-out in 2 out of 11 samples (Table 4). Below 250–500 pg of template DNA a result could not be consistently called since fluorescent intensity of the signals were below the '50' peak height threshold and hence could not be reported.

Amplification of greater than 3 ng of template DNA resulted in an increase in amplification artefact peaks. The majority of these peaks were due to 'stuttering' caused by enzyme slippage (Fig. 4) and were therefore predictably 4 bp shorter than the true allele. This was particularly noticeable in the locus HUMVWA31/A. These stutter bands ranged up to 11% of the signal intensity of the adja-

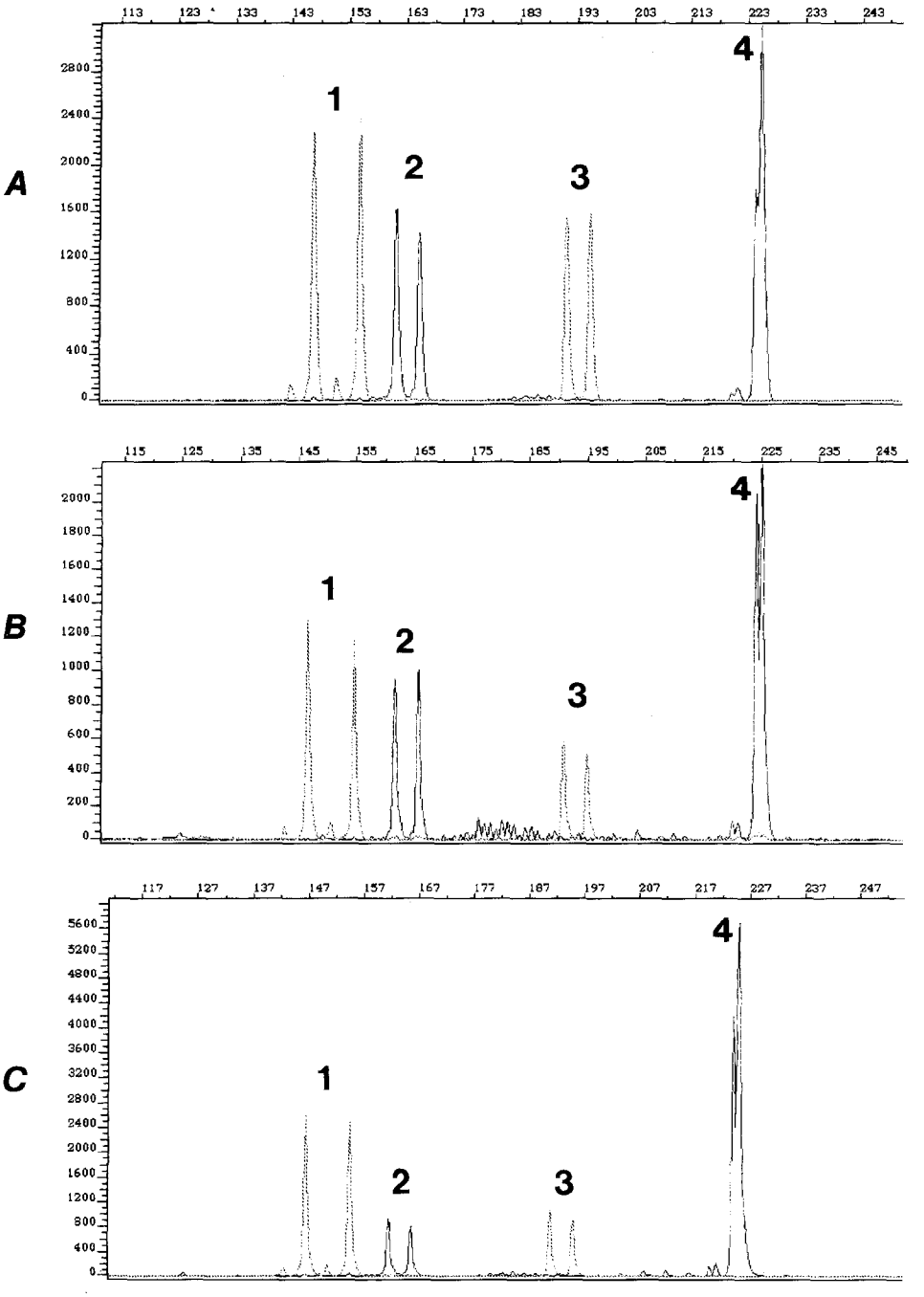

Fig. 1 Electrophoretograms displaying PCR products for samples of DNA extracted from a bloodstain (**A**), semen stain (**B**) and hair root (**C**) of a single individual. Peaks represent fluorescent intensities of dye-labelled DNA product for the loci. 1) HUMVWA31/A; type 16, 18; 2) HUMTHO1; type 7, 8; 3) HUMF13A1; type 6, 7; 4) HUMFES/FPS; type 11, 11. Peak height is measured against an arbitrary scale on the y-axis and the size of the products, in bases, is shown along the x-axis

cent allele as measured by peak area (Table 5). High background, resulting in peak identification, was also observed especially at the HUMTHO1 locus (Fig. 5). In addition, peaks greater than 4000 in height 'pulled-up' the opposite colour (Fig. 6) i.e. a blue peak would draw-up the green colour underneath.

From the above experiments, the optimal amount of DNA for amplification (which gave the best signal : noise ratio, reliably gave peaks above the threshold limit, and avoided signal 'pull-up') was within the range 1–3 ng, although with highly degraded DNA it may be advantageous to amplify a greater amount because a proportion of the DNA may be unavailable for amplification.

Aging and degradation

No spurious bands or mistyping of the allelic designations occured with age (Table 6). However for older stains a loss of sensitivity does occur and hence larger stain areas may be required (ca. 5 ng may be needed where loss of sensitivity was demonstrated).

DNA extracted from the bloodstain stored at 100% humidity at 56° C was shown to be degraded after 11 days and the semen stain after 28 days (Table 7). As the samples degraded, the high molecular weight loci such as HUMFES/FPS, followed by HUMF13A1 (Table 1) amplified less efficiently until allelic or locus drop-out oc-

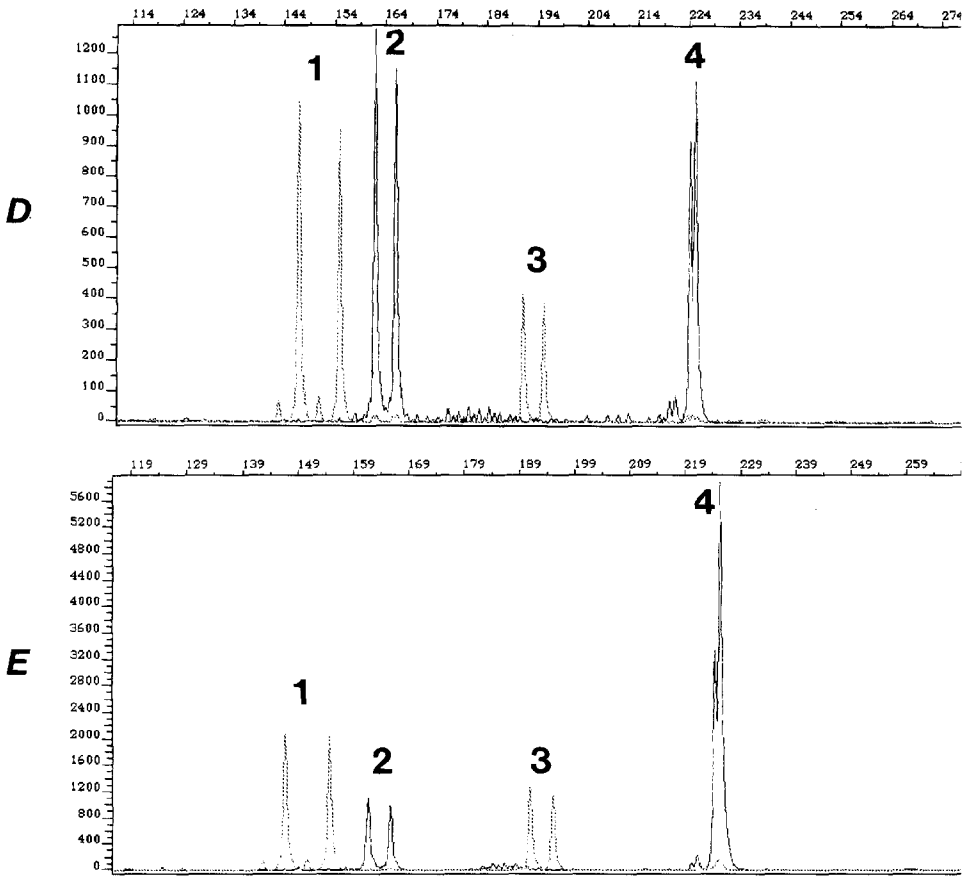

Fig. 2 Electrophoretrograms displaying PCR products for samples of DNA extracted from a saliva stain (D) and liquid blood (E) of a single individual. Peaks represent fluorescent intensities of dye-labelled DNA product for the loci HUMVWA31/A (1), HUMTHO1 (2), HUMF13A1 (3) and HUMFES/FPS (4) are the same samples as shown in Fig. 1. Peak height is measured against an arbitrary scale on the y-axis and the size of the products, in bases, is shown along the x-axis

Table 2 Pecentage average difference in peak areas between allele peaks

		HUMVWA31/A	HUMTHO1	HUMF13A1	HUMFES/FPS
Liquid blood	No. of obs	12	8	11	11
5 ng	% Average difference	6	6.26	6.73	6.73
Saliva stain	No. of obs	12	8	9	10
5 ng	% Average difference	7.86	6.18	4.89	17.68
Hair root	No. of obs	12	8	11	11
5 ng	% Average difference	8.01	6.15	3.14	7.48
Bloodstain	No. of obs	12	8	11	11
5 ng	% Average difference	4.1	10.59	6.01	6.7
Bloodstain	No. of obs	19	13	19	17
2.5 ng	% Average difference	10.33	9.18	12.31	8.12
Bloodstain	No. of obs	12	8	11	11
1 ng	% Average difference	12.54	14.77	11.06	11.63

curred (Fig. 7). In addition, peaks were observed at approximately 124 bp and 149 bp for the blue fluorescent wavelength. These peaks were outside the allele ranges for the loci HUMTHO1 and HUMFES/FPS and therefore would not be typed as alleles. Further investigations into the nature of these peaks is being conducted and will be reported elsewhere.

Amplification of non-human DNA

DNA from animals (5 ng), detailed in Table 8, failed to amplify, hence no STR products were obtained. Similarly DNA from the commonly occurring micro-organisms – *Escherichia coli* and *Candida albicans* – also failed to amplify. These results indicated that there was no need for

Table 3 A, B Difference between peak areas of heterozygous loci in the quadruplex from a single individual (i.e. only 1 DNA sample in total was used in the 2 experiments. **A** 9 replicate amplifications (1–9) using 5 ng of template DNA followed by analysis of 1.5 µl aliquots of amplified products. **B** A single amplification reaction using 5 ng of template DNA followed by the analysis of 1.5 µl aliquots of the amplified product

A	HUMVWA31/A			HUMTHO1	HUMF13A1			HUMFES/FPS		
	Peak area		% Difference	Peak area	Peak area		% Difference	Peak area		% Difference
	LMWT	HMWT		LMWT	LMWT	HMWT		LMWT	HMWT	
1	12060	11431	–5.5	18167	4500	4879	0.8	23429	21855	7.2
2	14149	14881	4.9	20199	5526	5862	5.7	30878	28987	6.5
3	15472	15109	–2.4	23881	6679	6865	2.7	29694	28230	–5.2
4	14835	15024	1.2	24108	4667	5289	11.8	24741	23273	–6.3
5	15188	15038	–1	24577	7433	7616	2.4	21497	31060	30.8
6	11926	10623	–12.3	19308	5853	6216	5.8	25941	25092	3.4
7	13222	12900	–2.5	20908	5477	3815	–43.6	19661	18771	4.7
8	11887	10861	–9.4	19002	4724	4604	–2.6	22077	19941	10.7
9	13148	13195	0.4	20402	5110	5163	1	22442	19090	17.6
Mean	13543	13229	–0.02	21172	5552	5590	0.01	24484	24033	0.02
SD	1345	1779		2270	924	1110		3551	4306	

B	HUMVWA31/A			HUMTHO1	HUMF13A1			HUMFES/FPS		
	Peak area		% Difference	Peak area	Peak area		% Difference	Peak area		% Difference
	LMWT	HMWT		LMWT	LMWT	HMWT		LMWT	HMWT	
1	9204	10993	16.3	7610	4128	4200	1.7	28454	27005	–5.4
2	13529	11951	–13.2	8228	4465	4707	5.1	40460	39494	–2.4
3	19085	17493	–9.1	12016	6618	5587	–18.4	40632	48446	16.1
4	15482	14035	10.3	9658	5248	5602	6.3	29236	29677	1.5
5	13358	13589	1.7	9218	5479	5173	5.9	36603	37595	2.6
6	15497	14262	–8.6	9844	5121	4843	5.7	38866	38033	–2.2
7	16672	7367	–126.3	5172	2298	2584	11.1	28599	29070	1.6
8	19311	18773	–2.9	12647	6243	6637	5.9	42121	42128	0
Mean	15267	13558	0.13	9299	4950	4917	0.01	35621	36431	0.02
SD	3091	3371		2238	1265	1115		5519	6881	

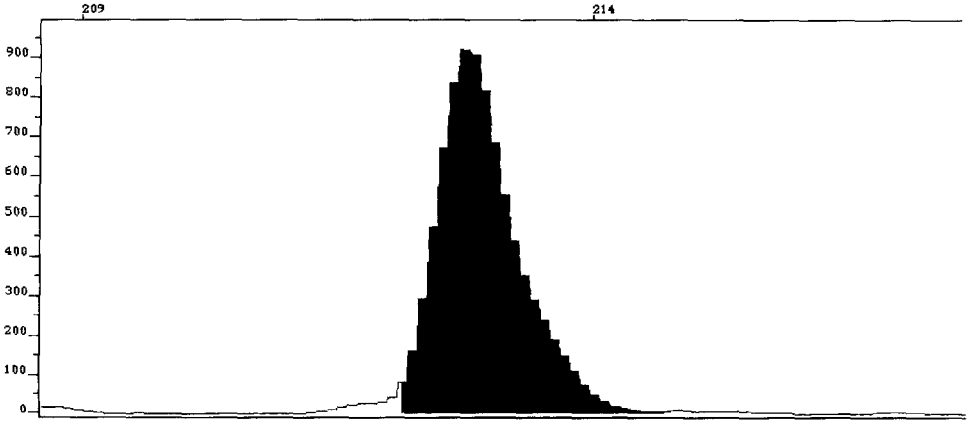

Fig. 3 Software assignment of the limits of a peak for sizing. The shaded area indicates the area of the peak which has been sized

a preliminary species test on bloodstains and suggested that the examination of bacterial or yeast contaminated vaginal swabs was possible.

Environmental contamination

With the Amplitype HLA DQα system, Comey and Budowle [3] showed that no detectable contamination was introduced onto clothing by extensive handling or coughing. To assess the effect of possible contaminants present

Table 4 The reproducibility of the quadruplex reaction with varying quantities of template DNA from 11 individuals

Individual	Quantity of DNA amplified					
	5 ng	2.5 ng	1 ng	500 pg	250 pg	100 pg
A	√	√	√	√	×	×
B	√	√	√	√	√	×
C	√	√	√	×	×	×
D	√	√	√	×	×	×
E	√	√	√	√	√	×
F	√	√	√	√	√	×
G	√	√	√	√	×	×
H	√	√	√	√	√	×
I	√	√	√	√	×	×
J	√	√	√	√	×	×
K	√	√	√	√	√	×

√, Reportable result obtained for all 4 loci; ×, reportable result not obtained at 1 or more loci

on clothing submitted in forensic cases, 20 samples cut from 'substrate control areas' were processed. These 'substrate controls' were areas of unstained material adjacent to the stained area to be analysed and provided an indication of the level of background DNA present. In addition, 13 substrate controls were analysed from simulated cases prepared in house. For each sample the substrate control was extracted by the method applicable to the sample type for which it was acting as a control.

All substrate controls processed as semen stains and saliva stains gave negative results (a total of 16 control areas). The remaining areas were processed as bloodstains. Negative results were obtained from all except one of these control areas (Table 9) which gave reportable results on all 4 loci and matched the genotype of the wearer. This substrate control area was taken from the pocket of a pair of jeans – an area prone to transfer of saliva.

Mixtures

The ABD 373 automated sequencer is able to record peak height/area, which gives a direct quantitative measure. When mixtures are present in forensic samples interpretation must proceed with care for the following two reasons:

1) If the components of a mixture are in roughly equal ratios (1 : 2 and 2 : 1; appendix 1) then it is difficult to separate into its constituent parts because allelic peak heights are similar. Usually, a mixture is identified by the presence of 3 or more prominent bands at one or more loci. If the components of the mixture have identical alleles at a locus, then the peaks will be higher as a result.

2) Alternatively, if the components of a mixture are such that the minor component is dilute (for example 1 : 10) then the presence of alleles in common to the mixture components cannot be distinguished, or the minor component may appear similar to or coincide with a stutter band at a position 1 repeat unit less than a major allele.

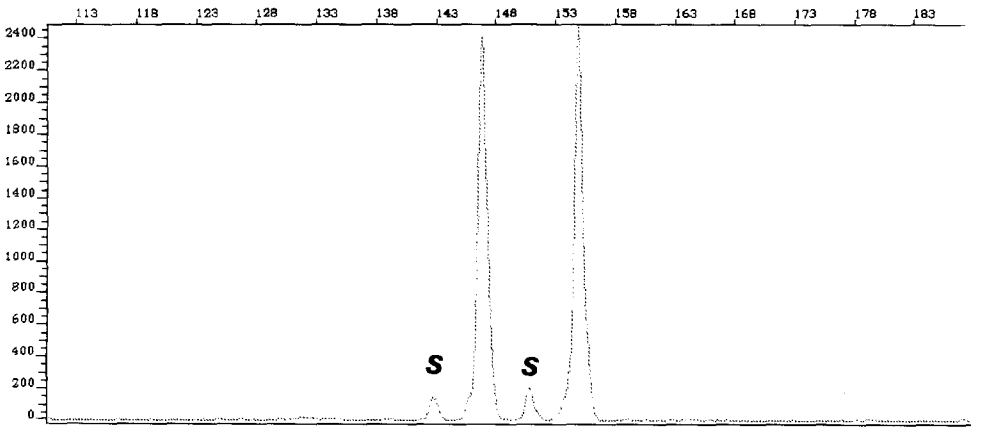

Fig. 4 Stutter peaks (s) caused by enzyme slippage at the HUMVWA31/A locus. Each stutter peak is 4 bp shorter than the true allele. The allelic designation is 16, 18

Table 5 The occurrence of stutter peaks within the STR quadruplex

Template DNA (ng)	HUMVWA31/A			HUMTHO1			HUMF13A1			HUMFES/FPS		
	% Occurence of stutter peaks	Mean % of signal intensity of stutter peak to allele peak	Max % of signal intensity of stutter peak to allele peak	% Occurence of stutter peaks	Mean % of signal intensity of stutter peak to allele peak	Max % of signal intensity of stutter peak to allele peak	% Occurence of stutter peaks	Mean % of signal intensity of stutter peak to allele peak	Max % of signal intensity of stutter peak to allele peak	% Occurence of stutter peaks	Mean % of signal intensity of stutter peak to allele peak	Max % of signal intensity of stutter peak to allele peak
5	78	8.3	10	34	2.8	4	7	1.6	2	77	2.2	4
2.5	59	8.4	11	7	4	4	0	0	0	35	2.6	3
1	4	7	7	0	0	0	0	0	0	6	6	6

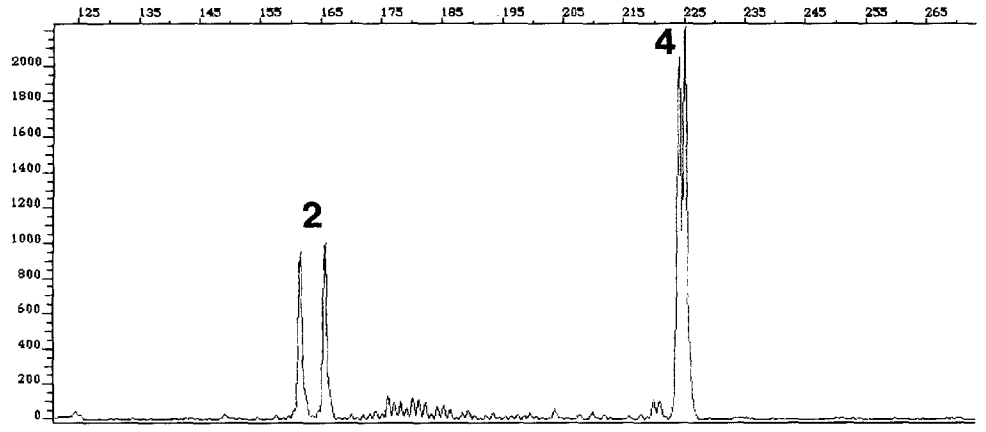

Fig. 5 Background noise due to over-amplification of the STR loci: 2) HUMTHO1; type 7, 8; 4) HUMFES/FPS; type 11, 11

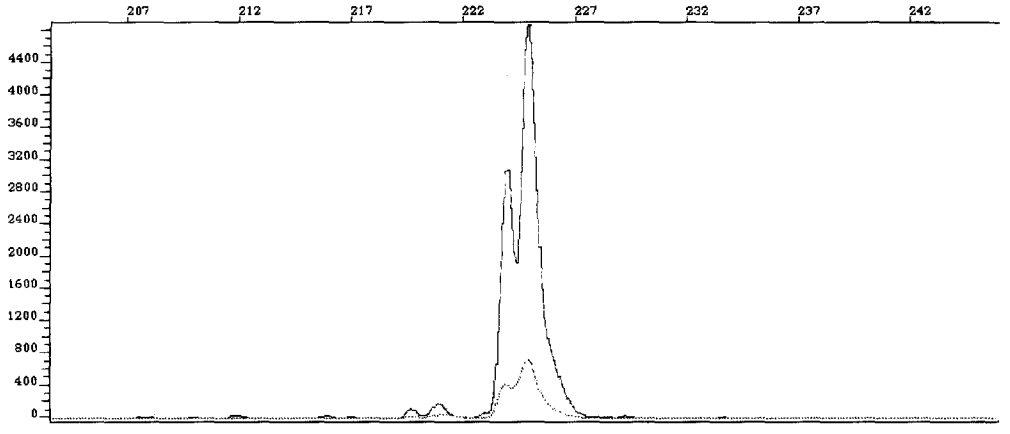

Fig. 6 "Pull-up" of the opposite colour with peaks in excess of 4000 in height. In this example the FES allele peak (blue) has 'pulled-up' the green fluorescence beneath

Table 6 The analysis and allelic typing of aged material

Sample type	Age	Allele designation WHA: THO1: F13A1: FES
Bloodstain – individual 1	15 years	19,20: 9,9: 6,6: 11,12
Semen stain – individual 1	11 years	19,20: 9,9: 6,6: 11,12
Saliva stain – individual 1	12 years	19,20: 9,9: 6,6: 11,12
Liquid blood – individual 1	Fresh	19,20: 9,9: 6,6: 11,12
Hair root – individual 2	42 days	16,19: 10,10: 5,7: 9,11
Liquid blood – individual 2	Fresh	16,19: 10,10: 5,7: 9,11

A series of mixtures were prepared in the ratios of 1:10, 10:1, 1:5, 5:1, 1:2 and 2:1 (Appendix 1) and in the majority of cases, the major constituents were readily identified, although it was easier to score the major alleles when the quantity of template was between 1–2 ng. Conversely, it was often advantageous to increase the amount of DNA template (up to 5 ng) to identify minor components of a mixture. For example, mixtures B, D, E, F and H (Appendix 1) at ratios of 1:10 or 1:5 all had minor component alleles which could have been interpreted as a stutter peak. Increasing the template concentration assists in identifying minor components by a corresponding increase in the observed signal, which could then be differentiated from a stutter peak. In practice, to resolve mixtures may require several PCRs using a range of template concentrations from 2–5 ng, where low concentrations identify the major component of a mixture, and high concentrations identify the minor component. Typical profiles obtained from a range of mixtures are shown in Fig. 8.

Validation of casework

The results for each case using STR analysis were compared to the results obtained with single locus probes (SLP), HLA DQα and conventional blood grouping (Table 10). In 29 cases the results were consistent the SLP, HLA DQα and/or conventional blood grouping, although the STR results were more discriminating than one of the conventional results and 2 of the HLA DQα results. One case yielded a result from HLA DQα and STR's which had not been obtained with SLP analysis. This case involved the identification of the body of a baby found in a dustbin. The DNA extracted from the muscle was found to be too degraded for SLP analysis.

Another case involved a fight between a number of youths of which 6 were identified. Bloodstains from items of clothing found in a suspect's house were submitted for analysis. One of these stains was shown to be of mixed origin. Two of the individuals could be resolved, the third was from an unknown source.

Table 7 The effect of degradation on allele designation

Degradation time (days)	Sample	Allele designation							
		HUMVWA31/A		HUMTHO1		HUMF13A1		HUMFES/FPS	
		LMWT	HMWT	LMWT	HMWT	LMWT	HMWT	LMWT	HMWT
1	Semen stain	√	√	√	√	√	√	√	√
	Bloodstain	√	√	√	√	√	√	√	√
3	Semen stain	√	√	√	√	√	√	√	√
	Bloodstain	√	√	√	√	√	√	√	√
5	Semen stain	√	√	√	√	√	√	√	√
	Bloodstain	√	√	√	√	√	√	√	√
8	Semen stain	√	√	√	√	√	√	√	√
	Bloodstain	√	√	√	√	√	√	√	√
11	Semen stain	√	√	√	√	√	√	√	√
	Bloodstain	√	√	√	√	√	√	√	√
28	Semen stain	√	*	*	×	×	×	×	×
	Bloodstain	×	×	×	×	×	×	×	×

√, The correct allele designation obtained; *, the correct allele designation obtained but peak height below 50; ×, no results obtained for the allele; *LMWT*, low molecular weight allele; *HMWT*, high molecular weight allele

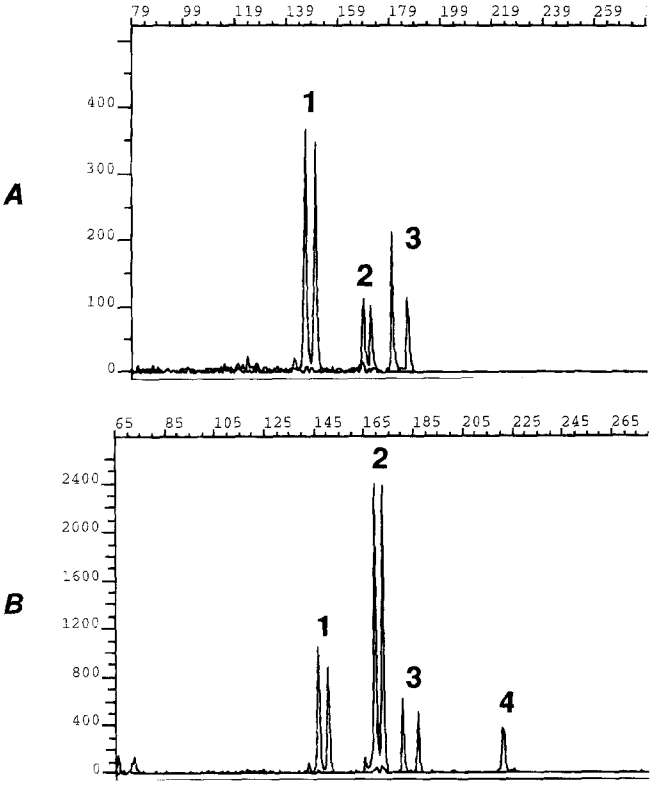

Fig. 7 Electrophoretograms displaying PCR products obtained from a degraded sample (**A**) and from a fresh blood sample from the same individual (**B**). Peak height is measured against an arbitrary scale on the y-axis and the size of the products, in bases, is shown along the x-axis. Apart from general loss of signal in the degraded sample, particularly with HUMTHO1 (2), complete locus drop-out of HUMFES/FPS (4) has occurred

Table 8 Animals tested for cross-amplification reactions

Animal type		
Mammal	Bird	Other
Cat	Chicken	Frog
Cow	Goose	Herring
Deer (red)	Partridge	
Dog	Skua	
Monkey		
Mouse		
Pig		
Rabbit		
Rat		

Two cases proved unsuccessful for STR analysis. In both of these cases the quantity of seminal staining for analysis was very limited as the majority of the seminal staining had been previously used during the SLP analysis and/or the HLA DQα analysis.

Discussion

Conventional DNA typing systems such as multilocus and single locus profiling enable biological samples to be analysed with a high degree of discrimination compared to that obtained with conventional body fluid grouping. However, the relatively poor sensitivity and requirement for good quality DNA renders these systems unsuitable for the analysis of body fluids such as saliva and highly degraded samples. To overcome these limitations, the use of PCR-based techniques have been investigated resulting in the introduction of HLA DQα testing using the commercially available Amplitype Typing Kit [3, 22]. The increased sensitivity of this technique has improved the range of samples that can be analysed but, unfortunately,

Table 9 The analysis of 'substrate controls'

Item	Case	Substrate control sample type	Result VWA: THO1: F13A1: FES
T-Shirt	Simulated, case 1	Bloodstain – area 1	Negative
		Bloodstain – area 2	Negative
		Semen stain – area 1	Negative
		Semen stain – area 2	Negative
T-Shirt	Simulated, case 2	Bloodstain – area 1	Negative
		Bloodstain – area 2	Negative
		Semen stain – area 1	Negative
		Semen stain – area 2	Negative
Pants	Simulated, case 3	Semen stain	Negative
Pants	Simulated, case 4	Semen stain	Negative
Pants	Simulated, case 5	Semen stain	Negative
Stocking mask	Simulated, case 6	Saliva stain	Negative
Woollen mask	Simulated, case 7	Saliva stain	Negative
Pyjama	Case 1	Bloodstain	Negative
Shirt		Bloodstain	Negative
Trouser fly		Bloodstain	Negative
Jeans	Case 2	Bloodstain – area 1	Negative
		Bloodstain – area 2	Negative
Jeans pocket	Case 3	Bloodstain	16,16: 9,10: 6,7: 11,12
Jacket	Case 4	Bloodstain	Negative
Trousers	Case 4	Bloodstain	Negative
Gloves	Case 5	Bloodstain	Negative
Jacket	Case 6	Bloodstain	Negative
Trousers A	Case 7	Bloodstain	Negative
Jacket		Bloodstain	Negative
Trousers B		Bloodstain	Negative
Knickers	Case 8	Semen stain	Negative
Duvet cover	Case 9	Semen stain – area 1	Negative
		Semen stain – area 2	Negative
Pants		Semen stain – area 1	Negative
		Semen stain – area 2	Negative
Cushion cover		Semen stain – area 1	Negative
		Semen stain – area 2	Negative

the discrimination power of this test is limited although the recent introduction of the Polymarker kit has served to redress this problem.

DNA from all commonly occurring forensic samples (Table 7) is suitable for analysis with STRs and the sensivity of the system is slightly greater than that achieved with HLA DQα typing using the Amplitype system [3, 23]. In addition, since only lengths of DNA are amplified, it is ideal for the analysis of old and degraded samples. However when analysing degraded samples, caution must be exercised in the interpretation of the results since the possibility of allelic or locus drop-out must be considered if limited quantities of DNA of sufficient molecular weight are present. Inevitably, there will be minor differences between batches of multiplex primer mixes; signal intensities are directly related to specific primer concentrations, but at low template concentrations HUMTHO1 was usually the first locus to drop-out [13]. Quality control procedures are needed to test new batches in order to make sure that signal intensities between loci are as close as possible in the 2–3 ng range. Allelic drop-out because of degraded DNA was more likely to occur with high molecular weight alleles of HUMFES and HUMF13A1.

The use of polyacrylamide gels and the incorporation of internal size standards enables PCR products to be sized with a high degree of precision. The limitations of lane to lane comparisons due to band shifting resulting from gel aberations which are commonly encountered in SLP analysis are also reduced, allowing accurate comparison of sizes both within and between gels. It is therefore

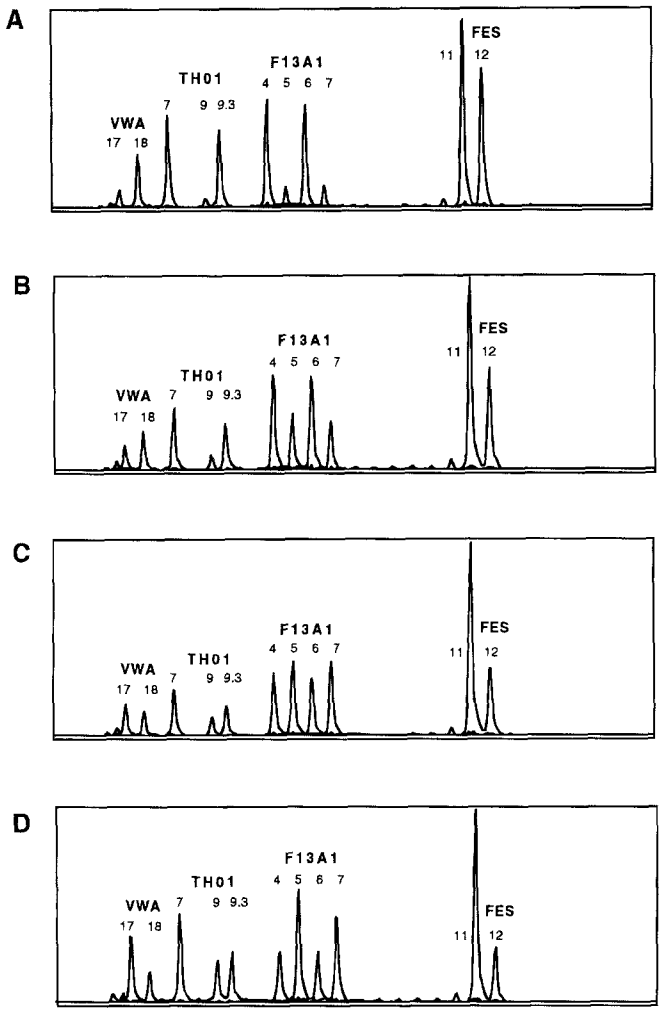

Fig. 8 An example of a mixture of 2 DNA samples which have been coamplified:

	VWA	THO1	F13A1	FES/FPS
Sample 1:	18, 18	7, 9.3	4, 6	11, 12
Sample 2:	17, 17	7, 9	5, 7	11, 11
Sample ratio	1 2			
A	5 : 1			
B	2 : 1			
C	1 : 1			
D	1 : 2			

FES/FPS 11 has a triple dose, hence the peak for this allele is relatively high. The components of the mixture are easier to distinguish in the 5 : 1 mixture

possible for alleles to be assigned discrete designations, eliminating the need for matching criteria. The typing of STR loci using these allelic designations was shown to be highly reproducible and, during this study, no alleles were mistyped.

Mixtures were readily detected using this STR quadruplex, as indicated by either the number of alleles in the sample and/or the relative peak areas of the alleles. The major component could be detected in all samples at a

Table 10 Summary of results obtained from 31 cases analysed with the STR quadruplex

Result as SLP profile and HLA DQA1	13
Result as SLP profile	10
Result as conventional grouping	2
Result as HLA DQA1, no result from SLP profile	1
Result as SLP profile and HLA DQA1 but more discriminating than conventional grouping	1
Result from SLP profiling and HLA DQA1, no results from STR's	1
Result from SLP profiling, no results from STR's	1
Total	31

ratio of 1 : 5, 5 : 1, 1 : 10 and 10 : 1 at 1–2 ng of template DNA. The major and minor components of the samples mixed in a ratio of 1 : 2 and 2 : 1 could not be resolved although the presence of a mixture could be detected. To score minor components of mixed samples was more difficult and it was found advantageous to increase the amount of template DNA to increase the signals of minor alleles, but this must also be balanced against the effect of increasing background noise from the major component alleles. The same limitations also occur in SLP analysis and in many situations, especially those deficient in circumstantial detail e.g. undetected crime, the most appropriate way of reporting the result is to present all possible options. The evaluation of the evidential strength of mixed DNA samples can be based on an analysis such as that described by Evett et al. [6]. In addition caution must be exercised when interpreting crime stain results to ensure that stutter peaks and mixed DNA samples are not confused. The amplification of smaller quantities of DNA will reduce the occurrence of stutter peaks and the comparison of relative peak areas of the alleles should also assist in the interpretation of possible mixed samples. Pattern evaluation also assists with interpretation since stutter peaks will always be minor components and their positions (1 repeat unit less than a major peak) are predictable.

Although the quantitative aspect of the system employing peak areas provides, at present, a limited basis for interpretation and may aid the interpretation of forensic stains of mixed origin, caution must be excerised in using this data since the software used (Genescan 672) can result in inaccurate calculation of the peak area resulting in a lack of reproducibility in this respect. This problem is currently being investigated by ABD and we envisage that interpretation of mixtures will improve as a result.

The increase in sensitivity renders the sysem more prone to contamination problems compared to conventional DNA profiling. There are 3 potential sources of contamination: sample contamination with genomic DNA from the environment; contamination between samples during preparation; and contamination of a sample with amplified DNA from a previous PCR reaction (PCR product 'carry-over'). The latter 2 sources of contamination can be controlled by appropriate laboratory procedures and designated working areas [14]. Suitable procedural practices include the incorporation of negative and posi-

tive controls; the analysis, whenever possible, of all samples in duplicate; and the processing of crime samples before the associated reference samples. Laboratory areas and equipment should be dedicated to different aspects of the technique. For example areas should be assigned for extracting DNA, amplification of the DNA and handling amplified products. Ideally, to prevent contamination between crime and reference samples, separate laboratory areas for these items should be used.

The studies carried out in this paper and those previously undertaken [3] have shown that environmental contamination due to adventitious body fluids is very limited and, in general, unproblematic. However, the presence of background DNA can be assessed, if neccessary, by the analysis of substrate control areas. This may be particularly relevant on outer garments in view of the reported widespread distribution of saliva staining on clothing [20]. However, it has been shown that the detection of adventitious body fluid contamination is less likely when preferential DNA extraction procedures are employed.

The STR analysis of a variety of casework samples (Appendix II) has produced identical results to those obtained by previous analyses using conventional blood grouping, SLP analysis and HLA DQα (Table 10). In addition it has been shown that mixed samples can be detected and correctly interpreted. STR analysis also yielded results where SLP analyses has failed and provides greater discrimination than HLA DQα and conventional blood grouping.

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Appendix I Genotypes of DNA and blood samples used in the mixture studies

	Genotypes VWA: THO1: F13A1: FES	Type of mixture
A	18,18: 8,10: 12,15: 10,12 + 16,19: 10,10: 5,7: 9,11	DNA and blood
B	14,16: 8.9: 7,7: 11,12 + 17,18: 6,6: 3,5: 10,11	DNA
C	14,16: 7,8: 5,7: 11,13 + 16,19: 6,6: 5,17: 10,12	DNA and blood
D	18,18: 6,6: 5,6: 9,11 + 17,17: 6,9: 5,6: 10,11	DNA and blood
E	17,18: 7,9: 6,7: 10,11 + 16,20: 9,9: 5,7: 10,11	DNA
F	14,16: 8,10: 5,7: 8,11 + 14,18: 6,8: 6,7: 10,11	DNA
G	14,16: 8,10: 5,7: 8,11 + 17,20: 6,10: 5,17: 11,11	Blood
H	14,16: 8,9: 7.7: 11,12 + 15,18: 9,10: 3,7: 10,11	Blood
I	17,18: 7,9: 6,7: 10,11 14,17: 6,6: 5,7: 10,11	Blood

Appendix II Details of the stain types and materials submitted for STR analysis

Case number	Stain material		Control samples	
	Type	Number	Type	Number
1	Bloodstain on cloth	2	Blood	1
2	DNA from semen stain	1	DNA from blood	2
3	DNA from semen stain	1	DNA from blood	2
4	Semen stain on cloth	1	Blood	2
5	Bloodstain on cloth	1	Blood	2
6	Vaginal swabs	3	Blood	2
7	DNA from muscle	1	DNA from blood	2
8	DNA from vaginal swab	1	DNA from blood	2
9	DNA from semen stain	3	DNA from blood	2
10	DNA from semen stain	1	DNA from blood	2
11	Liquid semen from condom	1	Blood	2
12	Semen on cloth	1	Blood	1
13	DNA from semen stain	1	Blood	2
14	DNA from semen stain	2	Blood	2
15	DNA from semen stain	2	Blood	2
16	Semen on tampon	1	Blood	2
17	Vaginal swabs	2	Blood	2
18	Blood scarpings	1	Blood	1
19	Semen stain on cloth	1	Blood	3
20	Bloodstain on cloth	1	Blood	2
21	Bloodstain on cloth	5	Blood	5
22	Bloodstain on cloth	1	Blood	3
23	Semen stain on cloth	1	Blood	2
24	Semen stain on cloth	1	Blood	2
25	Bloodstain on cloth	1	Blood	2
26	Semen stain on cloth	1	Blood	2
27	Semen stain on cloth	3	Blood	5
28	Semen stain on anal swab	1	Blood	2
29	Semen stain on cloth	1	Blood	2
30	Semen stain on cloth	1	Blood	1
31	Blood/body tissue	1	Blood	1
Total		45		65