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A new pentaplex PCR system for forensic casework analysis

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Abstract In 1998 the Federal Criminal Police Office of Germany (BKA) established a central genetic database of offenders and suspects to facilitate comparisons with biological samples from future criminal offences. The five obligatory short tandem repeat (STR) loci in this database (TH01, SE33, vWA, FGA and D21S11) were co-amplified in a new PCR pentaplex analysing system together with the sex-specific locus amelogenin. Due to overlapping fragment sizes, amplification products were fluorescent dye-labelled with different colours, separated by electrophoresis and detected directly using the ABI PRISM 310 Genetic Analyzer. Reproducible and reliable results were obtained from as low as 125 pg template DNA, indicating high specificity and sensitivity of the assay. Environmental studies and enzymatic digest with DNase I revealed an excellent stability of the pentaplex system with typeable results even in cases of partially degraded DNA. Complete and reproducible DNA typing was possible in bloodstain mixtures with the minor component as low as 10%. Mean stutter peak intensities were analysed for all loci and ranged from 2.7 ± 0.8% (TH01) to 10.6 ± 1.6% (vWA) of the main signal intensity. Allele frequencies were determined in a North Bavarian population sample (n = 121). The combination of five systems resulted in a mean exclusion chance of 99.86% and a power of discrimination of 99.999996%. No deviation from Hardy-Weinberg equilibrium could be found.

Key words Pentaplex · Multiplex · STR · DNA typing

Introduction

Most of the approximately 400 million short tandem repeat (STR) loci in the human genome show polymorphic alleles that differ in length (Craig et al. 1988; Edwards et al. 1991; Deforce et al. 1998). These differences in length are caused by the number of repetitions of a 2–6 bp (base pair) sequence. Carefully selected STR sequences should combine all the important characteristics required for forensic purposes such as:

1. Highly polymorphic loci with a high discrimination power

2. Short STR loci allow successful typing even of degraded DNA without the risk of preferential amplification

3. Analysis of mixed samples is possible

4. Specificity and sensitivity satisfy forensic requirements (Hochmeister et al. 1991; Edwards et al. 1992; Fregeau and Fourney 1993; Kimpton et al. 1993; Urquhart et al. 1995; Rolf et al. 1997; Klintschar and Neuhuber 1998; Lee et al. 1998; Wallin et al. 1998). The co-amplification of several STR loci in one reaction saves time, material and cost with the additional benefits of conserving sample and reducing the risk of contamination (Wallin et al. 1998).

In 1998 the Federal Criminal Police Office of Germany (BKA) established a central genetic database of offenders and suspects to facilitate comparisons with biological samples of criminal offences. The five obligatory genetic loci of this database are TH01, SE33, vWA, FGA and D21S11. As no commercially available kit comprising all these systems for coamplification existed, and with regard to the large numbers of samples expected for analysis, in this study, a new pentaplex system, combined with the sex-specific locus amelogenin, was developed.

Material and methods

DNA extraction and purification

Bloodstains, buccal cell swabs, semen and hair were extracted using Chelex 100 (Bio-Rad) (Walsh et al. 1991). Purification was performed with the QIAamp Tissue kit (Qiagen) according to the manufacturer's instructions, except that 210 µl of 100% ethanol was added to each 200 µl volume of the Chelex extraction samples

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Table 1 Primer sequences employed for the multiplex PCR

Locus	Primer	Dye
Amelogenin	PE Applied Biosystems, #401992	5-FAM
TH01	PE Applied Biosystems, #401721	5-FAM
vWA	PE Applied Biosystems, #401720	JOE
FGA	PE Applied Biosystems, #401994	JOE
SE33	I: 5′ AAT CTG GGC GAC AAG AGT GA 3′ II: 5′ ACA TCT CCC CTA CCG CTA TA 3′	6-FAM
D21S11	I: 5′ ATA TGT GAG TCA ATT CCC CAA G 3′′ II: 5′ TGT ATT AGT CAA TGT TCT CCA G 3′	NED

and centrifuged for 1 min at 8000 rpm. The procedure was repeated after the addition of 500 µl AW buffer. After that, 500 µl AW buffer was added again, followed by a centrifugation step of 3 min at 14000 rpm. Elution of DNA was performed with 200 µl AE buffer (70 °C). The elution step was repeated after incubation for 5 min at room temperature and centrifugation for 1 min at 8000 rpm.

PCR amplification

The multiplex PCR was performed using the primers given in Table 1. Primer and magnesium concentration as well as the annealing temperature were systematically varied until amplification of all loci of a standardized DNA sample was well-balanced. The resulting primer concentrations were amelogenin 0.06 µM, TH01 0.1 µM, vWA 0.2 µM, FGA 0.4 µM, SE33 0.3 µM, D21S11 0.3 µM. The reaction mix contained 10 mM Tris-HCl buffer, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.16% (w/v) BSA, 0.2 mM of each nucleotide and 0.1 U/µl AmpliTaq Gold. The PCR was performed in a total volume of 25 µl with the following parameters: hot start for10 min at 95 °C, 30 cycles for 45 s at 94 °C, 30 s at 57 °C, 1 min at 72 °C, a final step of 30 min at 60 °C, and soak at 4 °C. Ladders were prepared with the same primers by pooling DNA extracts with the common alleles for each STR locus. The detection of the signals was performed with the ABI Prism 310 Genetic Analyzer. Fragment sizes were determined with the internal standard Genescan 500 (ROX) (PE Applied Biosystems).

Influence of DNA degradation, temperature, light and humidity

To evaluate the influence of degrading enzymes on DNA, the enzyme DNase I was added to a 500 µl solution of high molecular genomic DNA (40 ng/µl). At intervals of 30 s and over a time period of 15 min, 20 µl of the solution were removed and DNase I was inactivated by EDTA. After precipitation with ethanol, concentrations and fragment sizes were estimated by agarose gel (2%) analysis and 6 ng of degraded DNA from each sample was amplified using the pentaplex system.

Blood, semen and saliva specimens from four persons were stored at –20 °C, 4 °C, 21 °C, 37 °C, 55 °C and exposed to the weather under varying conditions. DNA extractions were performed after an elapsed time of 1 week, 1 month and 2 months.

Minimum sample studies

For the evaluation of the minimum quantity of DNA needed to obtain reliable results, genomic DNA from the cell line K562 was diluted with distilled water in doubling dilutions from 20 ng/µl to 16 pg/µl and 1 µl of each dilution was used for the amplification reaction.

Additionally, whole human blood samples from two persons were diluted with distilled water (dilutions 1 : 1, 1 : 2, 1 : 5, 1 : 10, 1 : 20, 1 : 60 and 1 : 100), the blood sample of one person was diluted 1 : 10, 1 : 20, 1 : 40, 1 : 60, 1 : 80, 1 : 100 and 1 : 160 and 3 µl of each dilution was used for PCR.

Mixed specimen studies

Venous blood from four persons was mixed in pairs in ratios of 1 : 1, 1 : 2, 1 : 5, 1 : 10, 1 : 20, 1 : 60 and 1 : 100, with a constant 50 µl volume of one sample component. Relative stutter peak heights were calculated by dividing the peak height of the stutter peak by the peak height of its associated allele, expressed as a percentage. This was performed for each allele from each locus in 40 saliva specimens.

Population studies

Population studies were performed with blood samples from 121 unrelated persons from north Bavaria. For the statistical evaluation, the Genepop software package, Vers. 3.1b, was used (Raymond and Rousset 1995). Analyses included possible divergence from Hardy-Weinberg expectations, observed and expected heterozygosity and possible associations between different loci. Comparison of allele frequencies was performed by using the χ^2-test for $R \times C$ contingency tables (Pearson χ^2-test and likelihood ratio test). The level of significance was 0.005 for all statistical tests (two-sided probability). The mean exclusion chance (Krüger et al. 1968) and the power of discrimination (Guo and Thompson 1992) were calculated for each locus as well as for the combined system.

Results

Minimum sample studies

Successful typing of all alleles in all systems was possible with DNA amounts of 0.125 ng up to 5 ng. In these cases, complete 3′-terminal addition of adenosine by the Taq polymerase was observed (adenylation). With DNA amounts of 10 ng and more, incomplete adenylation and broad signal peaks especially at the FGA locus were detectable. At 63 pg, only alleles of the loci TH01, SE33 and vWA were detectable, while allele 24 of FGA and allele 31 of D21S11 did not appear. DNA quantities as low as 31 pg and 16 pg did not produce typeable results. In Fig. 1 a typical electropherogram of 1 ng DNA extracted from a blood sample is shown together with the allelic ladder.

Experiments with diluted whole human blood samples showed strongly differing results. While one blood sample was completely typeable up to a dilution of 1 : 160, and another blood sample up to a dilution of 1 : 100, allelic drop-out was found in a third sample even at a dilution of 1 : 20 (locus TH01). No correlation could be observed between fragment length and amplification failure.

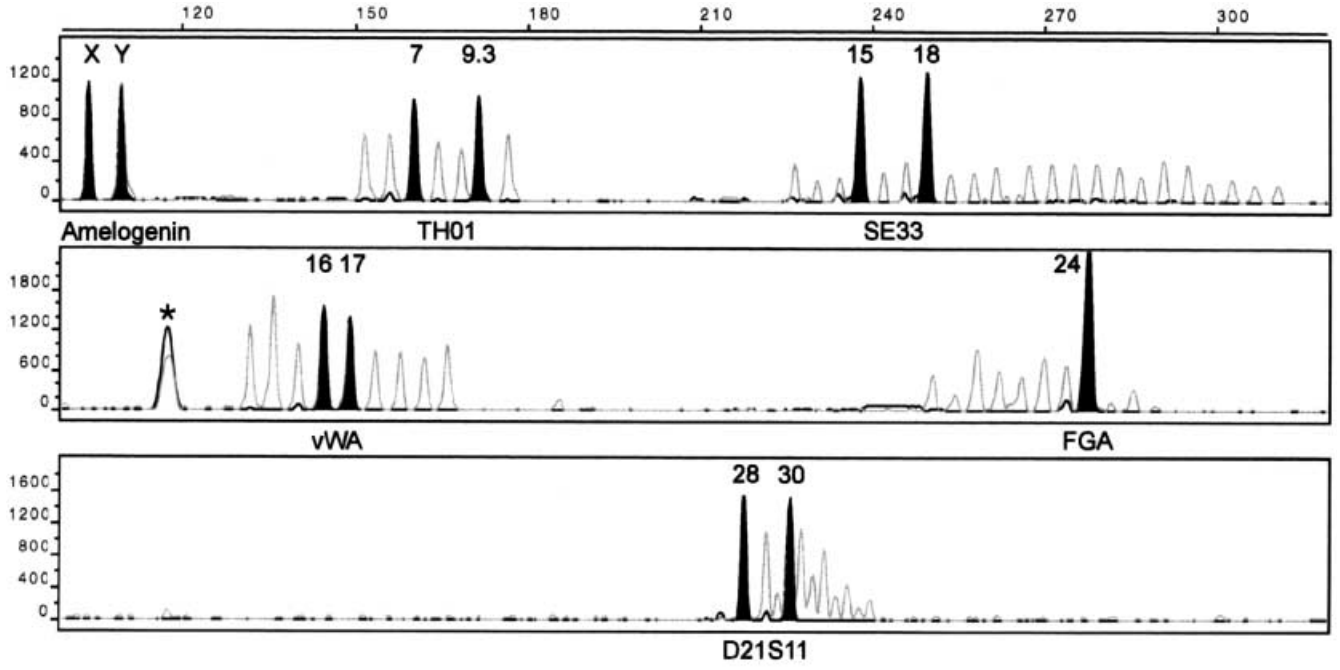

Fig. 1 Electropherogram of 1 ng amplified DNA of a human whole blood sample. Black signals represent the alleles indicated on top of the peaks. The allelic ladder is shown in grey. Different loci are marked below the respective signal range. The asterisk indicates an unspecific signal possibly resulting from green fluorescent dye molecules which were split off from JOE-labelled primers

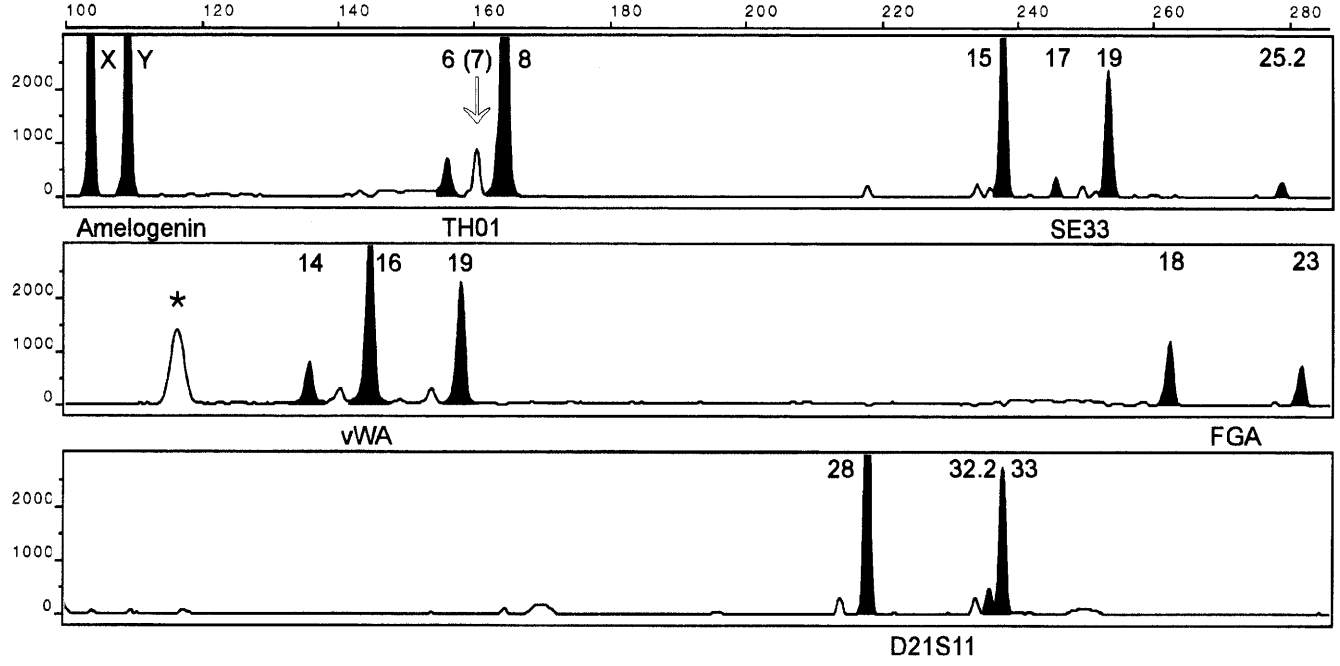

Fig. 2 Electropherogram of 1 ng amplified DNA from two human whole blood samples which were mixed prior to extraction in a ratio of 20 : 1. Black signals represent the alleles indicated on top of the peaks. The minor component was from a female blood donor with the genotype TH01: 6, 7, SE33: 17, 25.2, vWA: 14, 16, FGA: 18, 23 and D21S11: 28, 32.2. Allele 7, which is shown in brackets, could not be distinguished from the stutter peak of allele 8. The major component derived from a male blood donor with the genotype: TH01: 8, SE33: 15, 19, vWA: 16, 19, FGA: 18, 23 and D21S11: 28, 33. Black signals represent the alleles indicated on top of the peaks. The asterisk indicates an unspecific signal possibly resulting from green fluorescent dye molecules which were split off from JOE labelled primers

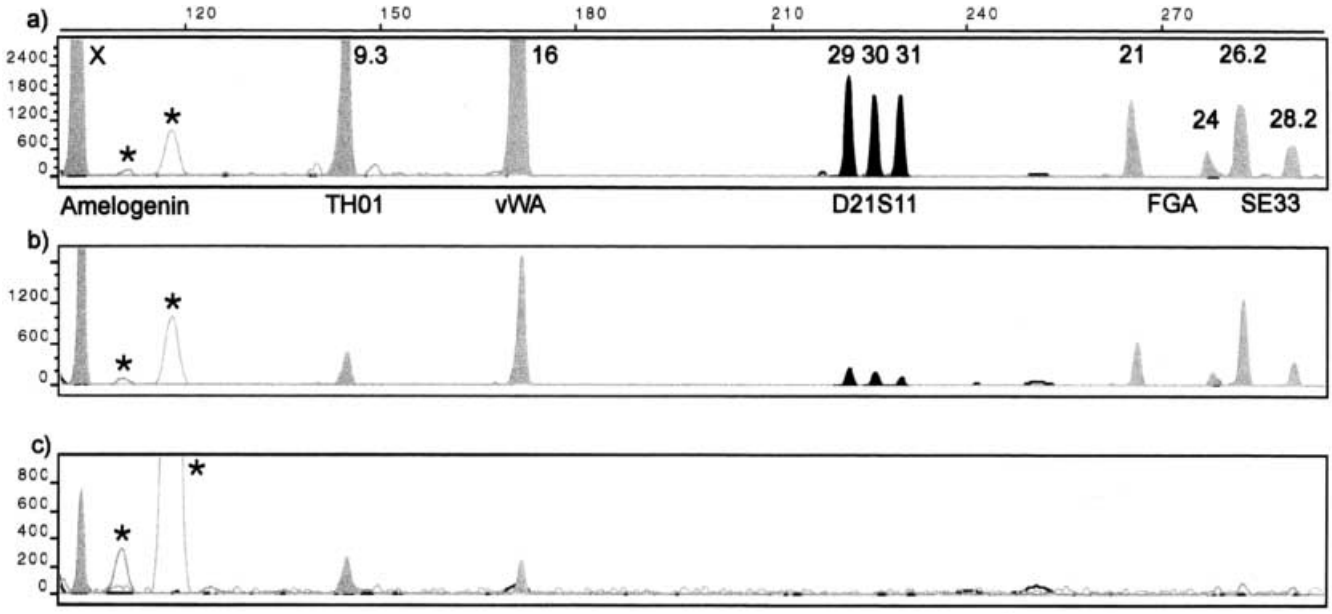

Fig. 3 Electropherograms of 1 ng amplified DNA of cell line K562 showing **a** no DNA degradation and after DNase I incubation for **b** 4 min and **c** 9 min. Grey and black signals represent the alleles indicated on top of the peaks in Fig. 3 a. The asterisks indicate unspecific signals possibly resulting from green and blue fluorescent dye molecules which were split off from JOE and FAM labelled primers

Mixed specimen studies

Correct typing of mixed blood samples was possible up to 1 : 20 mixtures for alleles which did not overlap the stutter positions (Fig. 2), in some samples up to 1 : 100 mixtures. The relative peak heights of the minor components were often larger than expected. Because the interpretation of mixed samples greatly depends on the possibility to differentiate between real allele signals and artificial stutter peaks, the mean stutter percentages were calculated for all loci and were 2.7 ± 0.8% for TH01, 10.4 ± 1.8% for SE33, 10.6 ± 1.6% for vWA, 6.6 ± 0.8% for FGA and 8.8 ± 1.4% for D21S11.

Influence of DNA degradation, temperature, light and humidity

DNA degradation with DNase I up to 1.5 min resulted in a mean DNA fragment length of about 4000 bp but after 5 min the fragment length appeared to be smaller than 100 bp after agarose gel electrophoresis. In accordance with this observation, signal intensities of the pentaplex amplification products decreased with increasing degradation time. Nevertheless, the alleles of the amelogenin locus could be detected after 15 min treatment and the alleles of the TH01 locus up to 12 min. Shorter time periods were found for vWA (9 min), FGA and SE33 (7 min) and D21S11 (5.5 min). Figure 3 shows three electropherograms for 1 ng DNA of cell line K562 each without DNA degradation (3 a) and after DNase I incubation for 4 min (3 b) and 9 min (3 c).

Environmental studies revealed correct results for all blood and saliva samples after storage under all condition for 1 week. One semen sample stored outside showed allelic drop-out for all loci, and for one semen sample stored at –20 °C only the TH01 and vWA alleles could be detected. Another semen sample stored at the same temperature showed complete allelic drop-out. While all alleles could be detected after storage at room temperature in the dark, the influence of light resulted in allelic drop-out of all loci in one sample. Repeat experiments with a slightly changed extraction protocol revealed typeable results under all conditions, indicating that the failure of amplification was due to an extraction problem.

After a storage period of 1 month, artefacts at the locus SE33 were seen in one saliva sample stored at 37 °C and in one sample stored at 4 °C. One sample stored outside showed complete allelic drop-out, except for the amelogenin system and allele 8 of the TH01 locus. Amplification of semen samples was only possible when stored at 37 °C, and only for amelogenin and TH01.

After a time lapse of 2 months, incomplete adenylation was seen in one blood sample, stored at room temperature under light. Saliva samples showed correct results, except for two samples from one person, stored at 4 °C and 55 °C.

Population studies

The allele frequencies of 121 unrelated persons from North Bavaria are given in Table 2. The most polymorphic locus was SE33 (ACTBP2) with 34 different alleles found in this study. Pearson's χ^2-test showed independence for all combinations of loci except for the combination SE33/vWA, where a p value of 0.024 revealed a slight significance for interdependence. Nevertheless, this observation may be due to the limited sample number, representing only a

Table 2 Allele frequencies of the five STR loci in a north Bavarian population sample (*n* 121, given as a percentage)

TH01		SE33		vWA		FGA		D21S11	
Allele	%	Allele	%	Allele	%	Allele	%	Allele	%
5	0.4	12	0.4	14	9.6	18	2.5	24.2	0.4
6	23.3	13	0.8	15	11.3	19	5.8	26	0.4
7	16.3	13.2	0.4	16	25.8	20	13.3	27	3.3
8	10.8	14	4.6	17	23.8	21	19.6	28	16.7
9	17.5	14.1	0.4	18	20.8	22	17.1	29	20.0
9.3	30.4	14.2	0.4	19	6.7	22.2	1.3	30	19.6
10	1.3	15	5.4	20	2.1	23	15.4	30.2	4.6
		15.2	0.4			24	12.9	31	10.4
		16	2.5			25	9.6	31.2	10.8
		17	7.9			26	2.1	32	1.3
		18	7.1			27	0.4	32.2	8.8
		18.2	0.4					33	0.4
		19	6.3					33.2	3.3
		19.3	0.4						
		20	5.0						
		21	2.9						
		21.2	1.3						
		22	1.3						
		22.2	2.9						
		23.2	4.2						
		24.2	5.0						
		25.2	3.8						
		26	0.4						
		26.2	4.2						
		27.2	8.8						
		28.2	6.3						
		29.2	4.6						
		30.2	6.3						
		31.2	3.3						
		32.2	0.8						
		33	0.4						
		33.2	0.4						
		34.2	0.4						
		35.2	0.4						

Table 3 Statistical parameters of the loci in the new pentaplex PCR system (*HWE* Hardy-Weinberg equilibrium, H_{exp} expected heterozygosity, H_{obs} observed heterozygosity, *PD* power of discrimination, *MEC* mean exclusion chance)

Locus	HWE (*p*-value)	H_{exp} (%)	H_{obs} (%)	P_D (%)	MEC (%)
TH01	0.895	78.74	80.00	91.5	56.4
SE33	0.830	95.22	96.67	99.5	89.4
vWA	0.522	81.01	87.50	96.4	61.8
FGA	0.102	86.40	80.83	93.5	71.8
D21S11	0.916	86.27	86.67	96.4	71.8
			Combined	99.999996	99.86

small portion of all possible genotypes (Chakraborty 1992). The relevant statistical parameters are summarized in Table 3. The maximum deviation between expected and observed heterozygosity rates of 6.5% was seen at the locus vWA. No significant deviations from Hardy-Weinberg expectations were found in the χ^2-test. The mean exclusion chance (MEC) of the combined pentaplex system was 99.86% and the power of discrimination (P_D) was 99.999996%.

Discussion

Typeable results were found from DNA amounts of about 125 pg. Presuming a standard cellular DNA content of about 7 pg (de Vita et al. 1994), 18 cells would be necessary for successful amplification with the pentaplex system. Recently, a minimum sample amount of 100 pg has been reported for two duplex systems (vWA/FGA and

TH01/D12S391 (Rolf et al. 1997). Wallin et al. (1998) found typeable results with the AmpF*I*STR Blue PCR amplification kit with DNA amounts of more than 125 pg, employing a peak height threshold of 150 relative fluorescence units (RFU). In our studies, peak heights of about 50 RFU were significantly above the background and were therefore considered to be clearly detectable. These observations revealed a high sensitivity of the new pentaplex system. Nevertheless, individual differences of the analysing instruments may influence the overall sensitivity of the assay. High sensitivity could also be shown with blood dilution experiments but the results of these experiments could be greatly influenced by a different content of white blood cells. Presuming a number of about 4,000–10,000 leucocytes per µl blood, the DNA amount of approximately 15–30 cells would be sufficient to obtain typeable results.

The main problem for the interpretation of mixed specimens is stutter peaks which are a result of omissions of repetitive elements by the enzyme polymerase during the amplification reaction (Walsh et al. 1996). Therefore, stutter peaks are 4 bp shorter than the major allele peak and may concur with the next 4 bp shorter allele. Consequently, the allele peak might not be distinguishable from a stutter peak at the same position.

The average standard deviation for stutter intensities was 1.3%. To facilitate quantitative interpretation of stutter and minor mixture components, a conservative threshold can be calculated by adding the greatest stutter percentage observed (10.62%) and the 3-fold average standard deviation (3.9%). Therefore, relative peak heights of more than 15% at the stutter position may indicate an additional amplification product of a minor component mixture allele at least 99.7% of the time. Although the detection of minor components of as low as 10% is theoretically possible, the correct interpretation depends on the number of contributors and alleles in stutter peak positions.

DNA degradation studies showed that in general, the amount of PCR product for a locus was reduced with higher levels of degradation. Although in agarose gel electrophoresis, no high molecular weight fragments were detectable after a DNase I incubation for 5 min or more, typeable results were obtained due to the high sensitivity of the pentaplex system. In addition, the susceptibility to allelic drop-out increased with the length of the DNA fragment, indicating a direct relationship between amplification efficiency and DNA fragment sizes. This phenomenon has also been observed for other multiplex systems (Wallin et al. 1998).

Environmental studies revealed correct typing results after various storage conditions. Nevertheless, in a few instances allelic drop-out was observed. The non-amplification of alleles in these cases may be the result of DNA degradation, induced by high temperature, microbial deterioration and the UV component of sunlight. Therefore, specimens should ideally be stored in a cool, dry and dark place.

Summarizing the results, the new pentaplex system allows rapid, sensitive and reproducible DNA typing for forensic applications. The high discrimination potential can be attributed to the addition of the locus SE33 and therefore, makes the pentaplex also suitable for paternity testing.

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