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ORIGINAL ARTICLE

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# Development of a 13-locus PCR multiplex system for paternity testing

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**Abstract** In this study the development of a 13-locus multiplex-PCR system fitting the updated demands for paternity testing in Germany is described. For this purpose an existing multiplex PCR system that allows the simultaneous amplification of eight different STR loci together with the sex-specific locus amelogenin (*genRES*MPX-2, Serac, Germany) was extended. Whereas some of the primers were taken from the underlying multiplex system, suitable primer sequences were chosen for the STR loci D19S433, TPOX, TH01, D16S539, D5S818, D2S1338 and FGA. Primers of loci resulting in potentially overlapping fragment sizes were labelled with the fluorescent dyes 6-FAM, JOE and NED. Reaction conditions, such as annealing temperature, concentrations of primers and polymerase or buffer conditions were optimised to obtain a robust amplification and reproducible genotype analysis for various sample sources. Full DNA profiles from single source samples were reliably typed from template DNA amounts of as low as 120 pg, suggesting a potential use of this system also in forensic casework analysis. With a mean exclusion chance (MEC) of 99.9989% and a power of discrimination ($P_D$) of about $1\times10^{14}$ (Caucasians), the new multiplex PCR system provides a significant and sensitive system for forensic DNA analysis. On the basis of these studies, a commercial kit system is now provided by Serac (Bad Homburg, Germany, *genRES*MPX-3).

**Keywords** Multiplex · STR · DNA typing · Paternity testing

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## Introduction

Most of the short tandem repeat (STR) loci in the human genome show polymorphic alleles that differ in length [1, 2, 3]. STR loci with a four base pair repeat motif have been found to be ideal for forensic DNA analysis purposes for a multitude of different of reasons [4].

In particular, the selected STR sequences have to combine the following important characteristics required for forensic purposes (as reviewed in [5]):

1. Individual loci have to be highly polymorphic, implicating that the probability that two randomly selected, unrelated individuals show concordant genotypes should amount to less than 10% ($P_D<0.1$).
2. In order to avoid genetic linkage of different markers, the systems should lie on different chromosomes or far from each other on the same chromosome.
3. The selected loci should exhibit low mutation rates [6, 7, 8, 9].

The simultaneous analysis of STRs (multiplex PCR) saves time, material and money with the additional benefits of conserving sample material and reducing the risk of contamination [9]. However, reduced sensitivity or the appearance of non-specific signals can be regarded as a critical handicap of multiplex systems.

Since the updating of the rules for paternity testing in Germany in the year 2002 [10], the analysis of not less than 12 different short tandem repeat (STR) loci spread over a minimum of 10 chromosomes is considered to be sufficient for expert assessments. Moreover, the selected STR systems have to provide a mean exclusion chance (MEC) of at least 99.99%. Therefore, the existing multiplex PCR system *genRES*MPX-2, which allows the simultaneous amplification of 8 different STR loci together with the sex-specific locus amelogenin, was extended to 12 loci and subsequently validated.

## Material and methods

### DNA extraction and purification

Blood samples and buccal cell swabs were obtained from employees of the Institute of Legal Medicine, University of Erlangen-Nürnberg, from completed paternity testing cases, and from stains of finished casework analyses. The samples were extracted using Chelex 100 [11] and in some cases, the DNA samples were purified using the spin columns in the QIAampDNA mini kit (Qiagen, Hilden, Germany). For this purpose 210 µl of 100% ethanol was added to each 200 µl volume of the Chelex-extracted samples, directly placed on the columns and centrifuged for 1 min at 6,000 *g*. The spin 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 and 20,000 *g*. A first elution of DNA was performed with 100 µl AE buffer (warmed up to 70°C) and the elution step was repeated after incubation for 5 min at room temperature and centrifugation for 1 min at 6,000 *g*.

### PCR amplification and electrophoresis

Primer and magnesium concentrations, as well as the annealing temperature, were systematically varied until amplification of all loci of a standardised DNA sample were well-balanced. Primer sequences of the multiplex PCR are available on request from Serac, Bad Homburg, Germany. Best performance was achieved with a reaction mix containing Gold Star 10× buffer (Promega, Mannheim, Germany) and 0.1 U/µl Thermo-StartDNA polymerase (ABgene, Hamburg, Germany). The PCR was performed on different thermal cyclers in a total volume of 25 µl with the following parameters: hot start for 12 min at 95°C, then 30 cycles of 60 s at 93°C, 60 s at 59°C, 90 s at 72°C, and a final step of 45 min at 60°C. Electrophoresis was carried out on an ABI Prism 310 Genetic Analyser (Applied Biosystems, Darmstadt, Germany) using a 47 cm× 50 µm ID capillary with polymer POP4. Electrophoresis was done using the module GS STR POP4A, 1–5 µl of each sample was mixed with 12 µl HiDi-formamide (Applied Biosystems) and 0.5 µl of the internal standard *genRES*LS500$^{ROX}$. After denaturation (5 min at 95°C), the samples were cooled on ice and immediately transferred to the autosampler.

The data were collected using the ABI PRISM 310 collection software and analysed with the 310 GeneScan software (version 2.1).

### Minimum sample studies

For the evaluation of the minimum quantity of DNA needed to obtain a complete DNA profile, genomic DNA of the cell lines K562 and 9947A (Promega, Mannheim, Germany) and photometrically quantified DNA derived from buccal cell swabs in ascending amounts from 30 pg to 50 ng were used for amplification.

### Mixture studies, stutter intensities and allele balance

For mixture studies, increasing amounts of one sample were added to a constant amount (200 pg) of another sample resulting in mixture ratios of 2:1, 5:1, 10:1, 15:1 and 20:1.

Genotypes of 88 individuals were determined using the new multiplex system. The stutter intensity was calculated from 32–71 genotypes at each locus. In the case of heterozygous genotypes, alleles were at least two repeat units apart (8 bp). For allele designations, only signals with a total height of less than 4,500 rfu were used. Relative stutter peak heights were calculated by dividing the peak height at the stutter position by the height of the associated allele signal, expressed as a percentage.

Heterozygous allele balance of 12–25 heterozygous genotypes was calculated by dividing the peak of the second peak by the height of the first allele signal. All values are given as a percentage. No off-scale signals were included in the calculation and alleles had to be separated by at least two repeat units.

## Results

### Kit design strategy

The newly developed multiplex system is based on the commercially available nonaplex typing kit *genRES* MPX-2 (Serac, Germany). The *genRES*MPX-2 kit comprises the

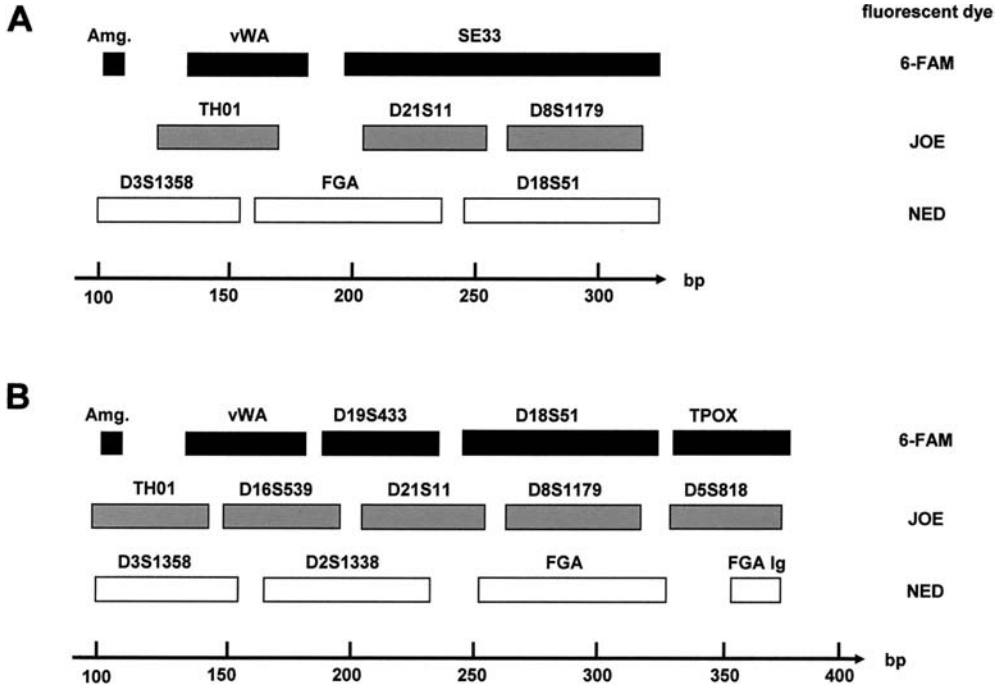


**Fig. 1** Comparison of the kit design strategy of **A** the *genRES* MPX-2 and **B** the newly developed multiplex system. The boxes represent the expected fragment sizes of each individual locus. Loci labelled with different fluorescent dyes are shown in different grey scales. The respective dyes are assigned on the right side of the figure

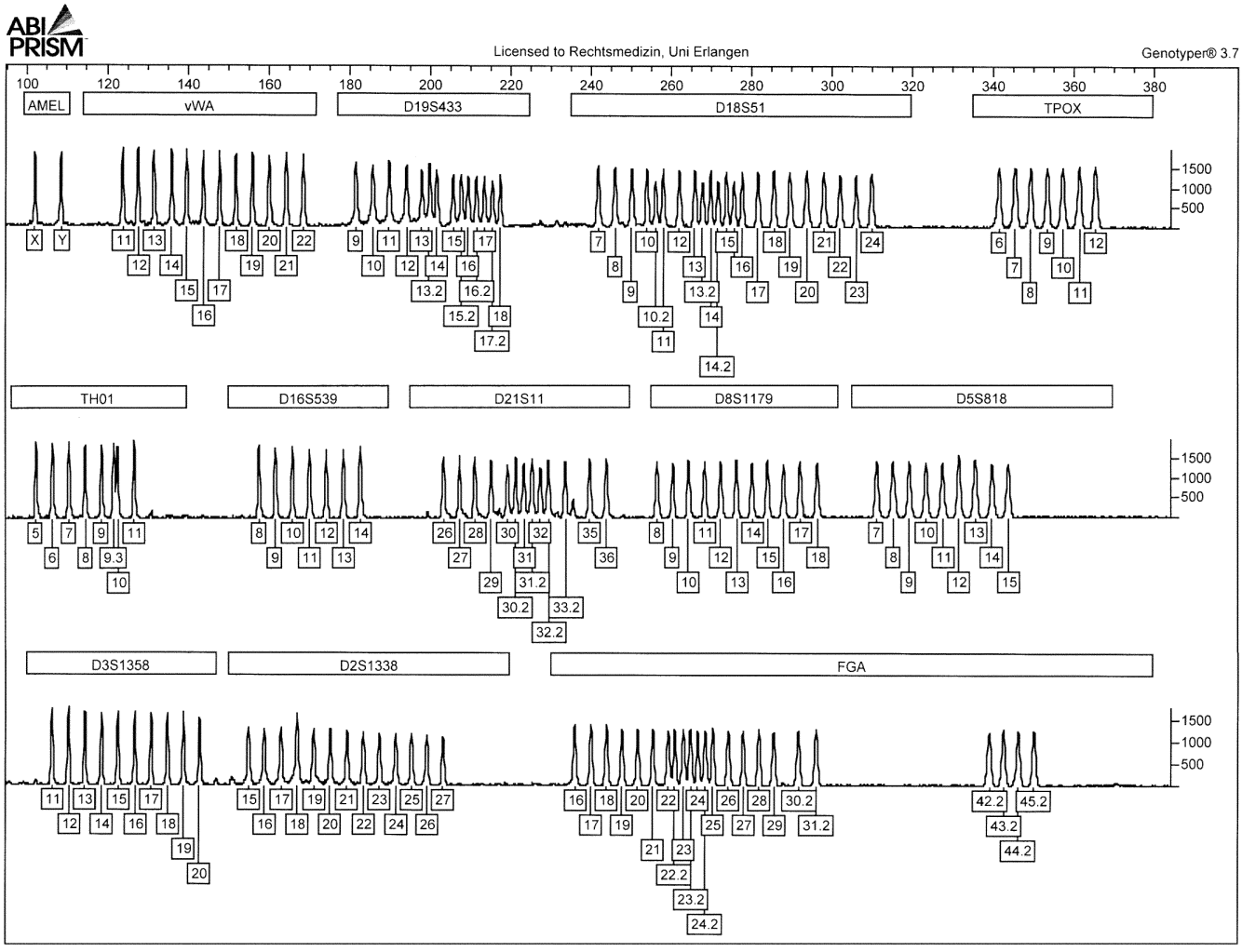


**Fig. 2** Allelic standard for the new multiplex system. The three panels display electropherograms of the allelic ladder mix. The *top panel* represents the blue channel displaying the FAM-labelled loci, the *middle panel* (green channel) shows the JOE-labelled loci, the NED-labelled loci (yellow channel) are displayed in the *bottom panel*. The allele designation of each locus is assigned under the respective signals

complete set of eight STR systems (vWA, SE33, TH01, D21S11, D8S1179, D3S1358, FGA and D18S51) which are components of the German DNA database established in 1998 by the Federal Criminal Office of Germany (BKA).

Whereas the locus SE33 (ACTBP2) was removed from the new typing system, the five loci D19S433, TPOX, D16S539, D5S818 and D2S1338 have been added. The locus SE33 spans a wide range of fragment sizes, thus blocking the space for other loci. Therefore, the elimination of this locus was necessary to be able to limit the fragment size of all other loci to a maximum of about 380 bp. In addition, the amplification products of the TH01 locus were shortened by 16 bp, in order to allow the positioning of the locus D16S539 between TH01 and D21S11. The fragments of the FGA locus were extended by 81 bp, and the fluorescent label of the locus D18S51 was changed from the NED to the FAM dye. By doing so, the locus D2S1338 could be added between D3S1358 and FGA and, furthermore, rare FGA alleles in the high molecular weight range could be included in the allelic ladder without overlapping the fragments of the D18S51 locus. The loci TPOX (FAM-labelled) and D5S818 (JOE-labelled) were added as high molecular weight markers. The fragment size and, therefore, the resulting position for all loci was chosen in such a way that no intersection can occur between different loci, even though rare alleles may occur in a genetic profile. Figure 1 shows a comparison of the kit designs of the *genRES*MPX-2 and the new system.

## Allelic ladder

Figure 2 shows the allelic ladder for the developed analysis system. The range of each allelic ladder and the corresponding allele sizes are shown in Table 1. The apparent allele sizes given in this table are one base pair larger than those determined by sequencing methods. This is a result of Taq-mediated, template-independent addition of adenosine to the 3'-hydroxyl end of amplicons, known as adenylation [12]. In order to eliminate the formation of double peaks, primer design and PCR conditions have been optimised to favour adenylated PCR products. All of the alle-

**Table 1** Ladder range and fragment sizes for each locus of the new multiplex defined by the extreme low molecular weight and high molecular weight alleles

| Locus | Fluorescent label | Low MW allele | High MW allele | Size range (bp) |
|---|---|---|---|---|
| Amelogenin | 6-FAM | X | Y | 107; 113 |
| vWA | 6-FAM | 11 | 22 | 127–171 |
| D19S433 | 6-FAM | 9 | 18 | 178–214 |
| D18S51 | 6-FAM | 7 | 24 | 243–311 |
| TPOX | 6-FAM | 6 | 12 | 345–369 |
| TH01 | JOE | 5 | 11 | 106–130 |
| D16S539 | JOE | 8 | 14 | 160–184 |
| D21S11 | JOE | 26 | 36 | 205–245 |
| D8S1179 | JOE | 8 | 18 | 258–298 |
| D5S818 | JOE | 7 | 15 | 322–354 |
| D3S1358 | NED | 11 | 20 | 109–145 |
| D2S1338 | NED | 15 | 27 | 158–206 |
| FGA | NED | 16 | 45.2 | 239–357 |

The given fragment size includes the terminal adenine added by the Taq polymerase

les in the ladder markers have been sequenced and designated in accordance with the recommendations of the ISFG DNA Commission [13, 14, 15]. As there exist two nomenclature systems for the D21S11 locus, the nowadays generally used notation published by Möller et al. [16] was employed. To allow an easy and reliable fragment assignment, common as well as rare alleles have been included. In particular, this holds true for the high molecular weight fragments of the FGA locus (alleles 42.2–45.2 [17]), which were not contained in the *genRES* MPX-2 system.

All ladder fragments were cloned in a plasmid-based form to allow large-scale preparation of the respective markers. In a first step, reamplified fragments were separately mixed for each of the 13 loci and in a second step, the individual ladders were mixed and, if necessary, adjusted to give a balanced signal intensity for all loci.

## Minimum sample studies

With DNA amounts of 30 pg of cell line K562, all alleles of the loci amelogenin, vWA, D19S433 and D18S51 were detectable. At the locus TPOX only allele 9 was detected whereas allele 8 showed a poor signal intensity and, therefore, could not be clearly distinguished from a stutter signal of allele 9. The homozygous alleles of the loci TH01, D8S1179, D3S1358 and D2S1338 as well as one allele of the locus FGA were also detected. The remaining alleles were not amplified at this low DNA amount. With an increasing DNA concentration, the profile was completed step by step, until all alleles were detectable at a DNA amount of about 120 pg. Due to background noise, incomplete terminal adenylation, unspecific amplification, and broad signals, reliable typing of all alleles was not possible at DNA template amounts above 50 ng.

With 30 pg DNA from cell line 9947A, singular signals of different loci were detected. In one case, the alleles 18 and 23 instead of 19 and 23 were detected at the locus D2S1338. As with the cell line K562, the alleles of all loci were sufficiently amplified for 120 pg DNA. At 50 ng and above, broad signals were seen due to large amounts of

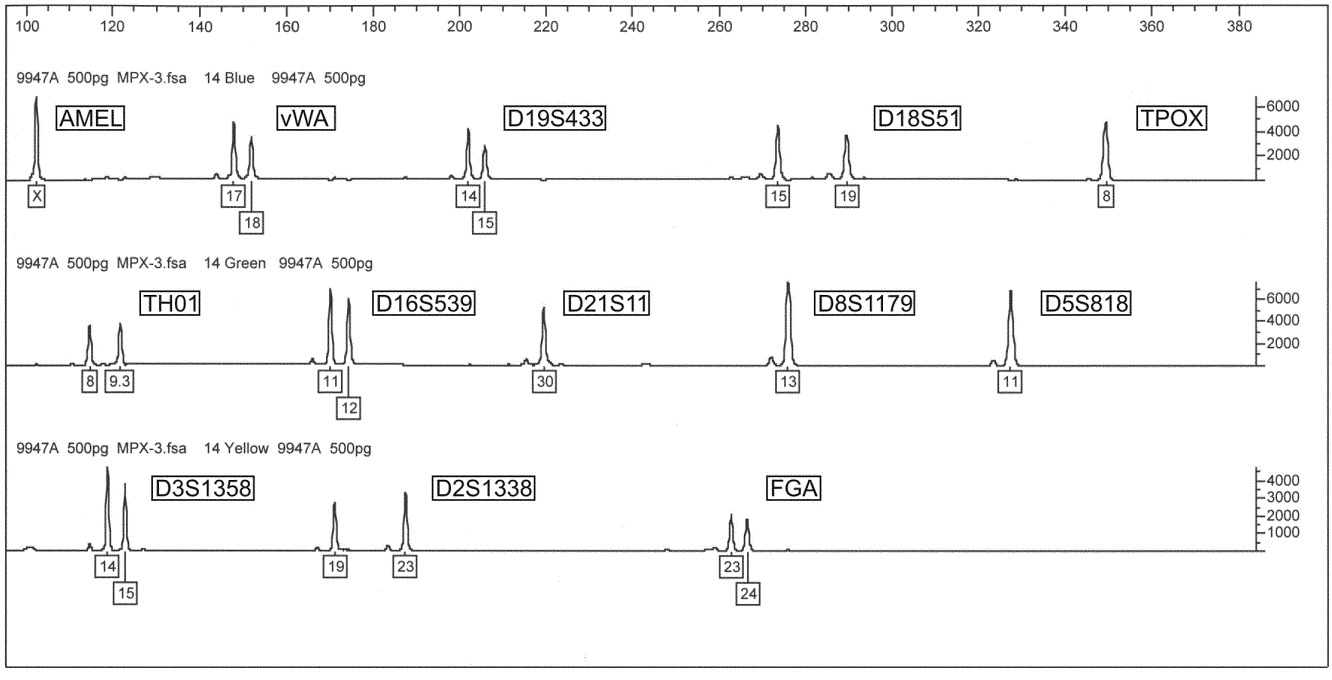


**Fig. 3** Typical electropherogram of 0.5 ng amplified DNA of cell line 9947A. Signals represent the alleles indicated below the respective peaks. *Top panel* blue channel displaying the FAM-labelled loci, *middle panel* green channel showing the JOE-labelled loci, the NED-labelled loci (yellow channel) are displayed in the *bottom panel*

amplification products. Incomplete adenylation, however, did not occur.

In Fig. 3, a typical electropherogram of 500 pg DNA from cell line 9947A is shown. It can be summarised that successful typing of all alleles in all systems was possible with DNA amounts of 120 pg up to 25 ng. However, genetic profiles have already been detected applying DNA levels below 100 pg, but preferential amplification low signal intensities of 100–150 rfu and allelic drop-out phenomena were observed in these cases.

### Preliminary studies on forensic casework samples

Typing was carried out on a selected set of different casework samples. The DNA extracts have not been quantified but were applied as known from experience with the *genRES* MPX-2 kit. With saliva samples, the new multiplex system showed a satisfactory and well balanced efficiency of amplification (up to 3,000 rfu). DNA derived from blood samples in general could be fully typed and showed an evenly high efficiency of amplification.

On average 80% of the alleles of all loci were amplified with the new multiplex system using different kinds of microstains. Depending on the type of material, however, relevant differences could be seen. In general, shorter amplicons were amplified better than the longer ones. In particular, this effect was pronounced when the existence of degraded DNA had to be assumed.

### Mixture studies, stutter intensities and allele balance

In mixture studies, all alleles of a minor component of 200 pg template DNA were clearly detectable and could be assigned to the allelic ladder up to a 5-fold excess of another DNA sample. In the case of a 10-fold excess, amplicons of the minor component, which concurred with stutter signals of the major component, could only be allocated for loci exhibiting low stutter intensities (for example TH01). All other fragments were clearly detectable even in the case of a 20-fold excess of the major component.

In Table 2 (left half) the relative intensities of stutter signals are shown for each of the amplified STR loci. The lowest average percent stutter was observed for the TH01 system (5.2±3.0%). The highest value was found at the D2S1338 locus (12.0±2.7%). For all loci, an average stutter of 9.2% was determined. Nevertheless, relative intensities of up to about 20% (TPOX, D2S1338) were detected in rare cases.

The average peak balance of the analysed loci is summarised in the right half of Table 2. The values ranged from the lowest of 52.7% (D2S1338) to the highest of 176.3% (D19S433). The average peak height ratio for all loci was 93.0%.

## Discussion

The newly developed multiplex system comprises a set of 12 STR loci and, in addition, the sex-specific locus amelogenin. The total length of amplified fragments was limited to a maximum of 380 bp, because it has been repeatedly shown that the reduction of the amplicon length can significantly enhance typing results, even in the case of degraded DNA [18, 19, 20]. To ensure the correct typing of rare alleles, which lie outside the range of individual ladders, primer pairs were used, preventing overlapping fragment sizes, or different fluorescent labels were chosen.

Consistent typing results and full genetic profiles from standard single source samples were obtained between 120 pg and 25 ng of template DNA. Presuming a standard cellular DNA content of about 7 pg [21], about 20 cells

**Table 2** Relative stutter intensities (*left half*) and peak height ratios (*right half*) of STR loci analysed with the developed amplification kit

| Locus | Stutter intensities (%) | | | | Peak height ratio (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean | S.D. | Minimal value | Maximal value | Mean | S.D. | Minimal value | Maximal value |
| Amelogenin | – | – | – | – | 104.5 | 8.5 | 85.1 | 122.6 |
| vWA | 10.1 | 2.5 | 2.1 | 15.2 | 89.9 | 15.5 | 57.6 | 130.2 |
| D19S433 | 9.5 | 1.8 | 5.3 | 13.3 | 94.8 | 19.1 | 78.6 | 176.3 |
| D18S51 | 10.2 | 2.0 | 5.4 | 13.1 | 90.1 | 14.2 | 62.1 | 116.5 |
| TPOX | 6.3 | 3.9 | 2.7 | 18.8 | 95.7 | 17.2 | 73.3 | 136.6 |
| TH01 | 5.2 | 3.0 | 2.2 | 16.5 | 103.3 | 20.2 | 79.1 | 150.3 |
| D16S539 | 8.3 | 2.4 | 4.0 | 13.7 | 88.0 | 12.4 | 70.0 | 109.9 |
| D21S11 | 10.5 | 2.1 | 5.3 | 15.4 | 93.5 | 8.2 | 81.0 | 113.2 |
| D8S1179 | 9.9 | 1.9 | 6.5 | 15.3 | 91.9 | 12.7 | 70.0 | 119.9 |
| D5S818 | 8.6 | 1.8 | 3.5 | 14.3 | 88.5 | 9.2 | 70.4 | 101.8 |
| D3S1358 | 10.9 | 2.1 | 6.7 | 14.3 | 92.7 | 11.0 | 76.5 | 115.7 |
| D2S1338 | 12.0 | 2.7 | 7.6 | 20.0 | 84.9 | 14.9 | 52.7 | 127.0 |
| FGA | 9.0 | 2.2 | 5.4 | 13.2 | 90.6 | 11.5 | 64.2 | 110.0 |

All values are given as a percentage.
*S.D.* Standard deviation.

would be necessary for successful and reliable amplification with the new multiplex system. Therefore, the overall sensitivity of the developed system turned out to be slightly higher than that of the PowerPlex16 system (>250 pg [22]) and the AmpFlSTR Profiler Plus/AmpFlSTR Cofiler multiplex systems (>160 pg [23]). A similar sensitivity (about 125 pg) was reported for some smaller multiplex systems like the AmpFlSTR Blue PCR amplification kit with three loci [9] or a pentaplex PCR system [24]. Incomplete profiles were observed using less than 100 pg template DNA, however, typing of these samples was complicated by stochastic effects, resulting in a pronounced heterozygote allelic imbalance and allelic drop-out phenomena. These effects should be kept in mind especially for the interpretation of forensic samples containing limited amounts of DNA or material from more than one individual.

Probably due to high amounts of total amplification products, some of them were incompletely adenylated at DNA amounts above 50 ng. The resulting double peaks may complicate the interpretation in special cases. Furthermore, broad and off-scale signals that can also be regarded as a system overload made an exact allocation of alleles difficult. Therefore, if possible, template DNA amounts between 0.5 ng and 5 ng DNA should be chosen to gain optimal typing results.

In preliminary studies on selected forensic stains, the new multiplex system revealed a quite satisfying performance. Due to the fact that these experiments have been done under standard conditions and that the overall sensitivity may greatly depend on a variety of analysis parameters, it can be assumed that performing the analysis under low copy number conditions [25] may further enhance the sensitivity of the assay. However, a detailed validation process has still to be done addressing these points.

As a result of omissions of repetitive elements by the enzyme polymerase during amplification [4], stutter signals are 4 bp shorter than the attendant allele peak and may concur with the main signal of any 4 bp shorter allele of a minor component. Consequently, the respective allele peak might be indistinguishable from the stutter peak at the same position, thereby influencing the correct interpretation of mixed stains. The average relative stutter intensity of all loci tested in this study was 9.2±1.9%. Nevertheless, upper and lower range values varied between the different loci. This may be a consequence of the individual sequence characteristics of the repeat structure [26]. To ensure a reliable interpretation of stutter and minor mixture components, a threshold can be calculated adding the average stutter percentage observed at a locus and the 3-fold standard deviation. For the presented multiplex system, these limits would range from 14% for the D5S818 locus to 20% for the D2S1338 system. Although the detection of minor components of as low as 5% is basically possible, the correct interpretation consequently depends on the analysed locus as well as on the number of contributors and alleles in stutter peak positions. However, in general, the differentiation between single and multi-source samples may be facilitated by extending the analysis on all loci in a multiplex profile.

The concomitance of more than one individual can also be indicated by a significant imbalance of the signal intensity of heterozygous alleles. Depending on several circumstances, the efficiency of the amplification of heterozygous alleles may substantially differ within one locus, implicating potential misinterpretation of the results. On average, the observed peak height ratio was 93%, indicating that the longer allele is amplified slightly worse than the short allele. In particular, this effect has to be considered for loci spanning a wide range of fragment lengths. The minimal observed peak height ratio was 53% at the D2S1338 locus, whereas values of >58% appeared at all other loci. Approximately similar ratios have recently been found for other multiplex PCR systems [23, 27, 28]. As variations may occur within various amplification reactions [28], repeated analysis can help to ensure a reliable interpretation. Additionally, in the case of samples which contain limited amounts of DNA, stochastic effects during amplification have to be taken into account [29], possibly resulting in signal imbalance or allelic drop-out. Therefore, a critical verification may be of particular importance for such samples.

## Conclusion

The presented multiplex system comprising a total of 12 STR loci turned out to be a robust and reliable tool for genotyping. The kit design and amplification protocol has been optimised to satisfy the requirements of different applications with DNA samples derived from a variety of sources. Due to its high level of sensitivity and a power of discrimination of about $1\times10^{14}$, the new multiplex system can be regarded as a powerful device not only for paternity testing, but also for forensic casework analysis. The mean exclusion chance (MEC) of 99.9989% (Caucasians) is one of the highest achieved in PCR multiplex systems published up to now.

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