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TECHNICAL NOTE

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Short amplicon STR multiplex for stain typing

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Abstract We developed a short tandem repeat (STR) typing kit based on DNA database systems that are included in, for example, the Interpol Standard Set of Loci recommendations (i.e., TH01, VWA, D3S1358, FGA) and the gender typing system Amelogenin. Two different multiplex sets were tested using the fluorescent dyes FAM, JOE, and VIC. The PCR results were compared to the commercially available AmpFISTR Blue kit, which contains the STRs D3S1358, VWA, and FGA. The advantage of our multiplex compared with the Blue kit was the generation of shorter amplicons (<200 bp) and the higher combined power of discrimination.

Keywords DNA database · Mini STRs · PCR multiplex · Stain typing

Introduction

The number and the subset of short tandem repeat (STR) loci applied by different DNA databases can vary, but there exists a certain overlap. In Europe, for instance, there is a common core of seven STR loci, that is, VWA, TH01, FGA, D3S1358, D8S1179, D18S51, and D21S11 [1, 2].

All STR kits that have been developed have the tendency to (partially) fail if the DNA extracted is degraded, minimal, or if other disturbing factors exist [3, 4]. The amplicon length, the number of loci to be harmonized in a one-tube reaction, and the different sensitivities of the fluorescent dyes have an influence [5–10].

One exception to the complex STR kits, which include the seven STRs mentioned above as well as one to nine additional STR systems in addition to the gender typing system Amelogenin, is the so-called Blue kit [AmpFlSTR Blue PCR Amplification Kit, Applied Biosystems (ABI), Foster City, CA], which contains the three STRs D3S1358, VWA, and FGA [8]. Given the amplicon lengths and the number of loci applied, even this kit does not always give (optimal) results.

We have therefore developed a four-STR kit which seems to offer several advantages. This kit encompasses the loci VWA, TH01, FGA, D3S1358, and Amelogenin (Pentaplex).

Materials and methods

The stain samples were extracted using the GEN-IAL protocol (DNA lysis and purification without organic reagents; GEN-IAL GmbH, Troisdorf, Germany) in accordance with the manufacturer's instructions. Out of the 30-µl extraction volume, 1–5 µl was used for PCR.

DNA from telogen hair roots was isolated with the following modifications according to Hellmann et al. [5]: the extracts were additionally purified with silica-based spin columns (QIAquick, Qiagen, Hilden, Germany) if amplification had failed, and the PCR products were purified using Montage PCR Centrifugal Filter Devices (Millipore, Billerica, MA, USA) if the capillary electrophoresis gave no or weak results.

For the selection of the primer sequences, the software PrimerSelect (DNA STAR) was used. The primers were commercially synthesized and purified using polyacrylamide gel electrophoresis (PAGE; Microsynth, Basel, Switzerland) with the exception of VIC-labeled primers that can only be obtained from ABI (Weiterstadt, Germany); these primes were commercially purified using HPLC.

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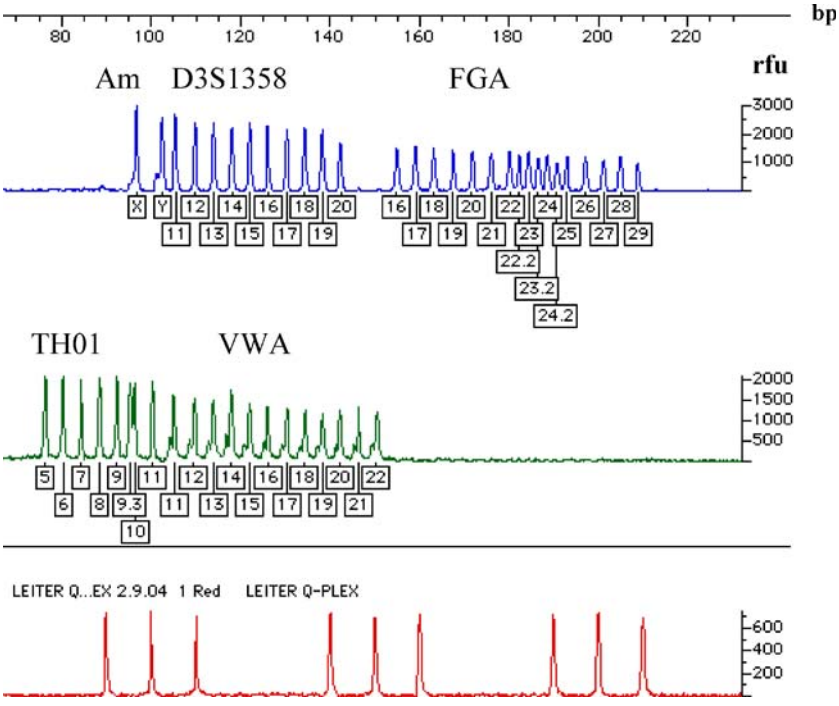

Fig. 1 Allelic ladders—short amplicon STR multiplex: Amelogenin-FAM, TH01-JOE, D3S1358-FAM, VWA-JOE, FGA-FAM. In the *bottom line*, the size marker is included. *rfu* Relative fluorescent units, *bp* base pairs

Primer sequences for the final multiplex PCR (P1, forward primer; P2, reverse primer) were the following:

Amelogenin P1-FAM: 5′-CCTGGGCTCTGTAAAGAATAGTG-3′
Amelogenin P2: 5′-AGCTTAAACTGGGAAGCTGGTGG-3′
TH01 P1-JOE/VIC: 5′-GTCACAGGGAACACAGACTC-3′
TH01 P2: 5′-ATTCCCATTGGCCTGTTCCT-3′
D3S1358 P1-FAM: 5′-ACTGCAGTCCAATCTGGGT-3′
D3S1358 P2: 5′-GAAATCAACAGAGGCTTGCA-3′
VWA P1-JOE/VIC: 5′-AGAATAATCAGTATGTGACTTGGATTG-3′
VWA P2: 5′-CAGATGATAAATACATAGGATGGATG-3′
FGA P1-FAM: 5′-GAACTCACAGATTAAACTGTAAC-3′
FGA P2: 5′-TGATTTGTCTGTAATTGCCAGC-3′

All primers were diluted to a concentration of 100 pmol/μl. After optimization of the primer concentrations, the following volumes for each primer pair/system were used to set up the multiplex: Amelogenin, 0.27 μM; TH01, 0.34 μM; D3S1358, 0.61 μM; VWA, 1.08 μM; FGA, 1.36 μM.

The final PCR protocol was applied after optimization of the annealing temperature: (initial denaturation: 95°C for 11 min) 93°C for 60 s, 60°C for 60 s, 72°C for 60 s; final

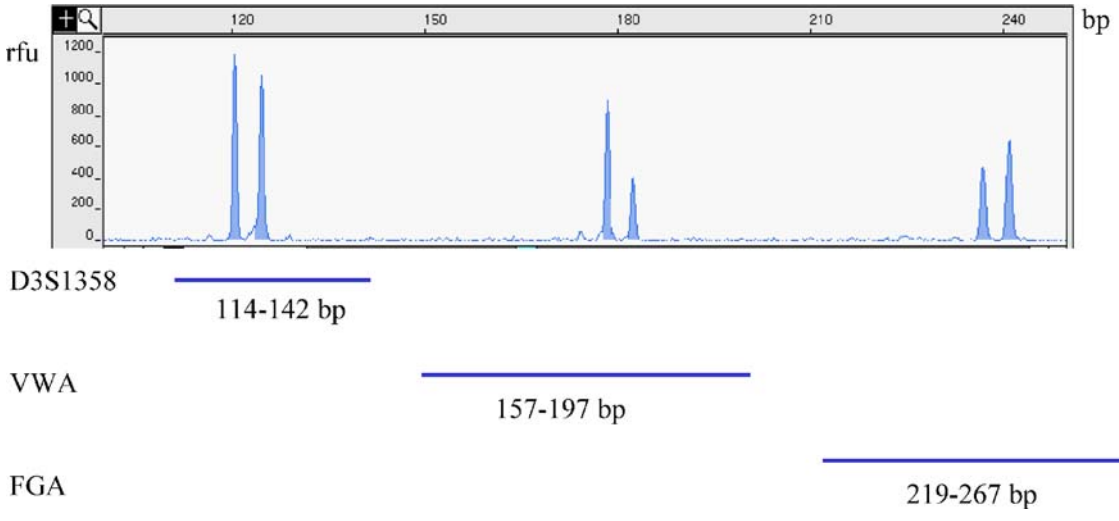

Fig. 2 STR typing using the Blue kit (ABI); 50 pg cell line DNA 9947A (Promega) was amplified using 30 PCR cycles. The profile presentation of the allelic ranges of the three STR loci are indicated *below*

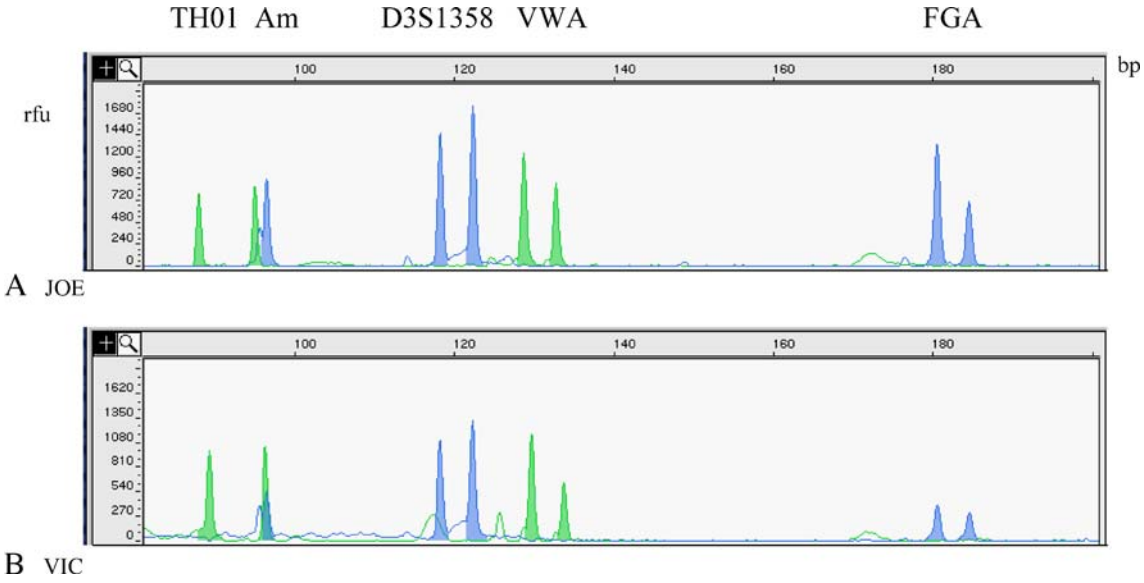

Fig. 3 STR typing using our Pentaplex system with two different dyes for the STR systems TH01 and VWA: **a** JOE, **b** VIC. Fifty picograms of cell line DNA 9947A (Promega) was amplified

extension: 72°C for 30 min; 30 cycles; 5 μl Qiagen Multiplex PCR kit, a buffer comprising of nucleotides, Hot start Taq D polymerase, and a combination of salts and additives (thus, only PCR primers, template, and bidistilled water must be added) were used in a final volume of 12.5 μl.

Alternatively, we tested further PCR reagents according to the following conditions that have been optimized based on the concentration of each parameter [8, 11]: AmpliTaq Gold (2 U; ABI), 10× buffer II (1.25 μl; ABI), dNTP (1 μl; Amersham-Pharmacia—10 mM standard concentration), $MgCl_2$ (0.7 μl; ABI—25 mM standard concentration), bovine serum albumin (0.3 μl; Sigma—10 mg/ml standard concentration); bidistilled water was added to a final volume of 12.5 μl.

Blue kit PCR was carried out in accordance with the instructions provided by the manufacturer (ABI) using 30 instead of 28 PCR cycles to be more sensitive for the typing of weak stains. Positive and negative controls were included for all PCRs. Furthermore, we used the well-established MPX2 kit, which contains the eight German DNA database systems (ACTBP2/"SE33", D21S11, VWA, TH01, FGA, D3S1358, D8S1179, D18S51), and Amelogenin (Serac, Bad Homburg, Germany, [11]) for comparing our stain typing results. In addition, this multiplex kit PCR was run with 30 cycles.

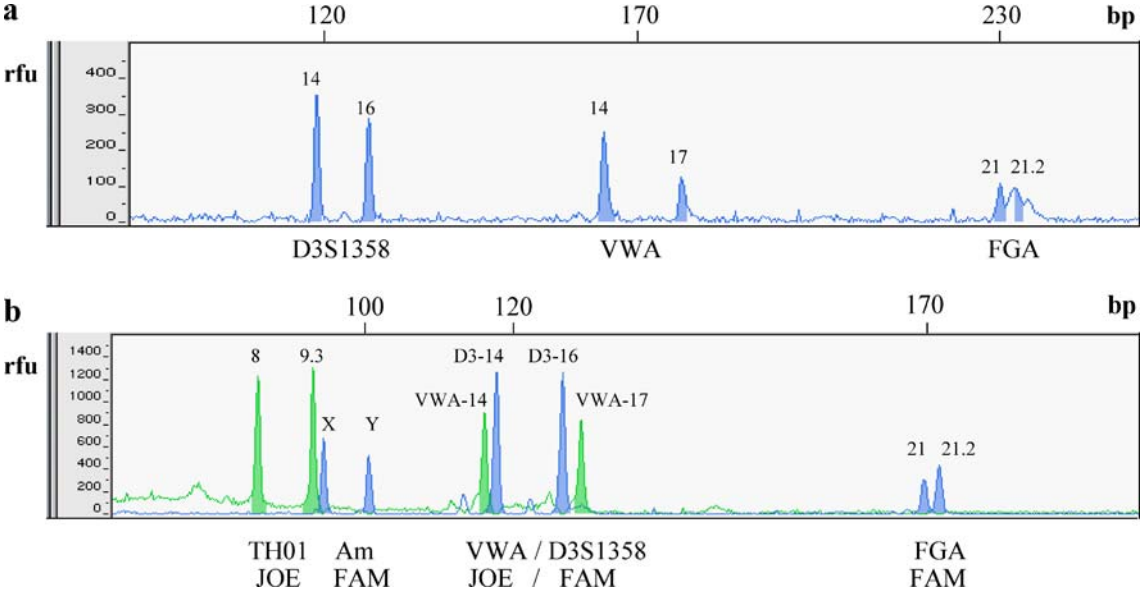

Fig. 4 Stain typing of cells collected from the steering wheel of a stolen car; the STR profile matched the pattern of the suspect. **a** Blue kit, **b** Pentaplex system

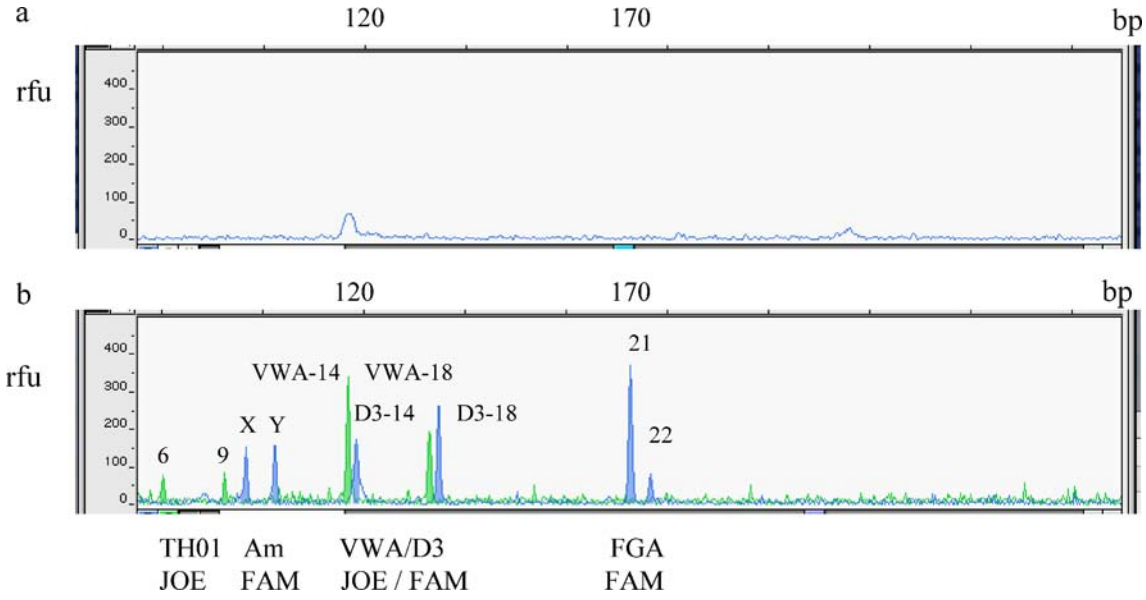

Fig. 5 PCR typing of a highly degraded saliva stain from a bottle, which was stored under humid conditions; only the Pentaplex system showed detectable PCR products. **a** Blue kit, **b** Pentaplex system

PCR products were analyzed by capillary electrophoresis (ABI PRISM 310; polymer POP4) and typed in comparison against allelic ladders that had been reamplified from MPX-2 ladders (10^6 diluted) using a GenoTyper template (Fig. 1).

Comparative PCR detection sensitivity was tested using cell line DNAs 9947A and K562 (Promega, Madison, USA) and a quantified bloodstain DNA in concentrations from 1 ng down to 0.05 ng.

Results and discussion

For the detection of the Blue kit PCR products is used the most stable and most sensitive fluorescent dye, FAM (ABI product information, [5, 7, 8]). This strategy requires a complete separation of the STR allele ranges. The FGA amplicons reach product lengths >250 bp (Fig. 2). In contrast, we used the fluorescent dyes FAM, JOE, and VIC for our multiplex to reduce the amplicon length to ≤200 bp. For the above reasons, FAM labeling was chosen for FGA typing because this dye is the most sensitive and the most stable. Additionally, we have tested the next sensitive dyes, VIC and JOE, for the shorter amplicons in our multiplex. Fifty-picogram template DNA could be typed very efficiently and reproducibly for both dye approaches (Fig. 3a,b). VIC should enable a more sensitive typing compared with JOE (ABI product information), but primer synthesis and labeling could only be carried out by ABI since VIC is a registered trademark. VIC labeling of VWA and TH01 sometimes resulted in increased peak heights, but often, no substantial difference was found (Fig. 3b). One disadvantage we observed regarding VIC labeling was the occurrence of artifact peaks, which could be attributed to unbound fluorescent dyes and may depend on insufficient primer purification or continuous hydrolytic dye liberation. To minimize this problem, we prefer an additional PAGE purification of the primers, which was commercially available for the dyes FAM and JOE. Moreover, HPLC-purified FAM and JOE primers from another company (Biomers, Ulm, Germany) led to nearly the same quality (Fig. 4).

Two different sets of PCR reagents were compared (Qiagen Multiplex PCR kit and a homemade PCR concept based on ABI standard reagents; see "Materials and methods") and found to have similar efficiencies (data not shown).

In total, more than 1,000 stains (bone, saliva, hairs with anagen and telogen hair roots, blood, epithelial cells from the skin, semen) were investigated and compared to the multiplex kit MPX2 (Serac [11]) and the Blue kit (ABI), which led to concordant results. However, if, especially, the stain DNA was highly degraded, our multiplex approach usually showed a more efficient DNA typing (Figs. 4 and 5). For instance, FGA amplicons of our

Table 1 Comparison between the success rates: our kit (Pentaplex)–MPX2 (Serac, eight STRs)–Blue kit (AmpFlSTR Blue PCR amplification kit, ABI); 100 weak and/or degraded stains (50 epithelial cell stains from different surfaces, 45 stains from cigarette butts and bottles, 5 old blood stains) from cases that took place during the last 18 months were selected

System/ Kit	Amelogenin (%)	TH01 (%)	D3S1358 (%)	VWA (%)	FGA (%)
Pentaplex	96	96	92	89	87
MPX2	73	81	65	75	48
Blue kit			78	69	54

Approximately 50% of these stains showed alleles from more than one individual (mixed stains). Allele sizing was carried out down to a detection limit of 50 rfu; success rates are given in percent/system

multiplex are approximately 60 bp shorter compared with the Blue kit. If a very low amount of DNA and especially highly degraded DNA has to be typed, Blue kit FGA amplicons have particularly shown partial or complete drop out (Table 1). Similar results were obtained for D3S1358 and FGA when we compared the MPX2 kit with our multiplex, although amplicon lengths in both approaches are similar; however, instead of the less sensitive fluorescent dye (NED) in MPX2, we used FAM. This may at least partially explain the higher success rates of our multiplex.

In an initial validation study, multiple freshly shed hairs (totaling 90) from volunteers were typed by using our novel multiplex with an overall success rate of about 50%. Some did not disclose any profile at all. In the course of stain case analysis, we have typed approximately 280 telogen hair roots, which had been stored under various conditions from 1 to 23 years, with an overall success rate of about 20% (including full and partial profiles).

In conclusion, this STR multiplex strategy enables a very sensitive DNA amplification of highly degraded DNA to include gender typing and leads to a combined degree of discrimination of approximately 1 in 100,000 based on the population data of European whites [12, 13].

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