**Meng Yang**[1]*
**Caiyong Yin**[2]*
**Yuexin Lv**[3]
**Yaran Yang**[1]
**Jing Chen**[1]
**Zailiang Yu**[3]
**Xu Liu**[4,5]
**Meibo Xu**[3]
**Feng Chen**[2]
**Huijuan Wu**[4,5]*,**
**Jiangwei Yan**[1]*

[1]CAS Key Laboratory of Genome Sciences and Information, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing, P. R. China
[2]Department of Forensic Medicine, Nanjing Medical University, Nanjing, Jiangsu, P. R. China
[3]Beijing Microread Genetics Co., Ltd, Beijing, P. R. China
[4]Beijing Center for Physical and Chemical Analysis, Beijing, P. R. China
[5]Beijing Engineering Technology Research Centre of Gene Sequencing and Gene Function Analysis, Beijing, P. R. China

Received November 3, 2015
Revised March 3, 2016
Accepted March 24, 2016

Research Article

# Development of a rapid 21-plex autosomal STR typing system for forensic applications

DNA-STR genotyping technology has been widely used in forensic investigations. Even with such success, there is a great need to reduce the analysis time. In this study, we established a new rapid 21-plex STR typing system, including 13 CODIS loci, Penta D, Penta E, D12S391, D2S1338, D6S1043, D19S433, D2S441 and Amelogenin loci. This system could shorten the amplification time to a minimum of 90 min and does not require DNA extraction from the samples. Validation of the typing system complied with the Scientific Working Group on DNA Analysis Methods (SWGDAM) and the Chinese National Standard (GA/T815-2009) guidelines. The results demonstrated that this 21-plex STR typing system was a valuable tool for rapid criminal investigation.

**Keywords:**
Direct amplification / DNA genotyping / Forensic validation / Rapid PCR / Short tandem repeats
DOI 10.1002/elps.201500498

Additional supporting information may be found in the online version of this article at the publisher's web-site

## 1 Introduction

With the rapid advancement of DNA analysis methods, STR genotyping technologies, including multiplex PCR with fluorescently labeled primers and capillary electrophoresis, have been commonly employed in the field of forensic science for paternity testing, individual identifications, and DNA databasing [1]. Currently, the sample testing procedure comprises DNA extraction, PCR, electrophoresis, and data analysis, which together usually takes approximately 6–8 h using traditional laboratory methods [2]. Some improved systems could shorten the process to less than 4 h [3–5]. In the past few years, significant efforts have been made to enable the rapid generation of STR profiles to meet the needs of forensic casework. Technology advancements include rapid PCR [6], direct PCR [7, 8], rapid PCR on a chip [9, 10], and integrated microfluidic devices for rapid sample-to-profile processing as alternative approaches [11–13]. In this study, we developed a new, rapid 21-plex STR typing kit to assess all Combined DNA Index System (CODIS) STR loci, as well as D2S1338, D2S441, D12S391, D19S433, D6S1043, Penta D, Penta E, and Amelogenin in a single reaction. With direct amplification, rapid PCR, and 5-dye fluorescence labeling, this STR kit was designed for rapid criminal investigation for its robust discrimination rate and rapid detection process by shortening the amplification time to 90 min and no DNA extraction was required from blood, bloodstain, or buccal samples. In addition, there were no low detection rate and poor amplification efficiency caused by the fast PCR process. We validated the kit according to Scientific Working Group on DNA Analysis Methods (SWGDAM) guidelines [2] and the Chinese National Standard (CNS) 'Basic Quality Requirements of Forensic Science Human Fluorescent STR Multiplex PCR Testing Reagent' (GA/T815-2009). Following the guidelines, we assessed the kit for PCR conditions (including number of cycles, amount of Taq polymerase, concentration of magnesium as well as dNTPs, and primers annealing temperature),

**Correspondence:** Dr. J.-w. Yan, CAS Key Laboratory of Genome Sciences and Information, Beijing Institute of Genomics, Chinese Academy of Sciences, No.7, Beitucheng West Road, Chaoyang District, Beijing, 100029, P. R. China
**E-mail:** yanjw@big.ac.cn

**Abbreviations: CNS**, Chinese National Standard; **ESS**, European Standard Set; **RFU**, relative fluorescence units; **SWGDAM**, Scientific Working Group on DNA Analysis Methods

*These authors contributed equally to this work.
**Additional corresponding author: Dr. H.-j. Wu,
**E-mail**: sunnywhj@126.com

**Colour Online:** See the article online to view Figs. 1–5 in colour.

 

sensitivity, precision and accuracy, species specificity, PCR inhibitors, stutter ratio, and effect of DNA mixtures. Our results showed that the rapid 21-plex STR typing system is a reproducible, accurate, sensitive, and robust tool for rapid forensic investigation.

## 2 Materials and methods

### 2.1 Sample preparation

We analyzed 591 blood samples and 120 buccal samples available on FTA® cards, 80 peripheral blood samples collected with EDTA-containing tubes and 100 bloodstain samples collected on cotton swabs. All samples were collected with informed consent. Control DNA samples 9947A and 9948 were purchased from Promega Corporation (WI, USA), and sample M4615 was purchased from Microread Genetics Incorporation (Suzhou, China). Non-human samples from rat, mouse, sheep, chicken, cow, pig, dog, black bear and soil microbial pool were collected for species specificity testing. Samples from the FTA cards were punched using a 1.2 mm BSD600-DUET stiletto instrument (BSD, Australia) and placed into 96-well plates for direct PCR amplification. In addition, 0.5 μL of each peripheral blood sample and approximate 3 × 3 $mm^2$ of each bloodstain sample were used for direct PCR amplification. The procedures were in performed accordance with the human and ethical research principles of the Beijing Institute of Genomics, Chinese Academy of Sciences.

### 2.2 STR locus selection and characterization

To enable DNA comparisons within or between databases, a common set of STR loci must be used among the profiles. In this study, the selection of STR core loci was as follows: (1) the 13 core CODIS loci, (2) D2S441 and D12S39 in European Standard Set (ESS), (3) Penta D, Penta E and D19S433 in the CNS 'Loci Selection of National Forensic Science DNA Database' (GA469- 2004), (4) D6S1043 and D2S1338 suggested by [14, 15] because of their high discriminating power in Chinese Han. The final set of loci included the sex-linked locus Amelogenin and 20 autosomal loci (D19S433, D5S818, D21S11, D18S51, D6S1043, D3S1358, D13S317, D7S820, D16S539, CSF1PO, Penta D, D2S441, vWA, D8S1179, TPOX, Penta E, TH01, D12S391, D2S1338, and FGA). Characterization of each locus (chromosomal location, physical position, repeat motif, and observed allele range) is given in Table 1.

### 2.3 Primers, internal size standard, allelic ladders, and matrix standard set

Reference sequences for selected loci were obtained from GenBank and Short Tandem Repeat DNA Internet DataBase (http://www.ncbi.nlm.nih.gov/genbank/ and http://www.cstl.nist.gov/div831/strbase/). Primers were designed using the web-based Primer3 (v.0.4.0) software [16]. The following factors were considered during PCR primer design: primer length of 19–28 bp; Tm value of 55–65°C; strict primer design parameters to avoid primer dimers, hairpin structures, and sequences with high homology to nearby sequences. Product size ranges were limited to 75–450 bases. Designed primers were examined for potential interactions with each other using AutoDimer v1.1 software [17]. All forward primers were labeled with a fluorescent dye for convenience.

The internal size standard was created by amplifying different fragments between nucleotide positions 927 and 1425 of the pUC18 plasmid (Takara, Dalian, China). A universal forward primer was designed and labeled with Orange500 dye. A series of reverse primers were designed to obtain 15 differently sized amplicons (50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 bp).

Allelic ladders were created for the 21 multiplexed loci using a combination of individual templates that represent the range of alleles observed in the population. Samples were amplified independently at each locus, and then the products of each locus were diluted, mixed together, reanalyzed, and balanced to produce a single allelic ladder for each locus. Finally, those single allelic ladders were mixed in appropriate proportions to create a 'cocktail' containing all allelic ladders [18].

A standard matrix set, Microreader™ 5-Dye Matrix Standards (Microread Genetics Incorporation, Suzhou, China), contains fragments labeled with the 5′ dyes used in subsequent analyses. For each dye, only one fragment should be present. We chose and labeled five amplified fragments from pUC18 DNA: 75 bp (Orange500), 90 bp (ROX), 100 bp (TAMRA), 120 bp (HEX), and 140 bp (FAM).

### 2.4 PCR amplification

Unless otherwise noted, multiplex PCR was performed in a 25 μl single-volume system, including 1X Reaction Mix (2 mM magnesium ion, 0.2 mM dNTPs, 50 mM Tris-HCl pH8.3, 90 mM KCl, 200 mM Betaine, and 0.16 mg/ml BSA), 1X primer set, 1 U/μL of Taq DNA Polymerase II (Microread Genetics Incorporation, Suzhou, China), and 2 ng of DNA. Thermal cycling was performed in the GeneAmp PCR system 9700 with a gold-plated silver block (Applied Biosystems, Foster City, CA, USA). The rapid thermal cycling conditions included pre-incubation at 96°C for 2 min, followed by 27 cycles of denaturation at 94°C for 5 s, combined the annealing and extension step at 60°C for 70 s. A final extension step was performed at 60°C for 30 min, followed by a final soak at 15°C.

### 2.5 Sample electrophoresis and data analysis

PCR products were separated and detected on the ABI 3130xl and 3500xl Genetic Analyzer™ (Life Technologies)

 

with E5 Matrix to process the data from the five dyes—FAM (blue), HEX (green), TAMRA (yellow), ROX (red), and Orange500 (orange). The PCR product or allelic ladder (1 μL) was combined with 9 μL of formamide/Org solution, 0.5 μL internal size standard and 8.5 μL of deionized Hi-Di formamide (ThermoFisher Scientific). Prior to electrophoresis, samples were denatured at 95°C for 3 min, and then chilled on ice for 3 min. Samples were injected at 3 kV for 10 s and electrophoresed at 15 kV for 1591 s in Performance Optimized Polymer-4 (Life Technologies). Following data collection, electrophoresis results were analyzed using GeneMapper ID-X v1.2 (Life Technologies). Unless stated otherwise, allele peaks were interpreted when the peak heights were ≥ 50 relative fluorescence units (RFU).

### 2.6 Assessment of PCR conditions

Optimal amplification conditions can determine the success or failure of STR genotyping reactions. It is, therefore, critical to evaluate the performance of key parameters of the reaction. We evaluated a range of PCR conditions, including annealing temperature, number of cycles, and reaction components. Amplifications of M4615 male DNA (2 ng) were evaluated in duplicate for validation of thermal cycling parameters (annealing temperature and number of cycles). Annealing temperature was tested at the standard temperature of 60°C (±2°C) and at 56, 58, 62, and 64. Cycle numbers from 25 to 29 were tested to determine the optimal signal and balance of the profile. Results were assessed for basic performance criteria such as profile completeness, average peak height, and balance.

A comparison between the rapid protocol and the normally amplified protocol with M4615 was performed to determine if the rapid protocol affected the amplification efficiency. The rapid protocol followed the thermal parameters provided above, while the normal protocol extended the denaturation at 94°C by 30 s, annealing at 60°C by 1 min, extending at 70°C by 1 min per cycle, and adjusted the number of cycles to 30; at last, the final extension step was performed at 60°C for 30 min, followed by a soak at 15°C.

In forensic analyses, DNA polymerase, magnesium, dNTPs, and primers are generally included in a reaction mix for convenience. The concentrations of the reaction mix may vary due to pipetting error. Therefore, the degree of variability should be determined for consistent and robust results. Component concentrations ±10%, ±20% and ±30% from optimized conditions were examined using control DNA M4615 (2 ng).

### 2.7 Assessment of sensitivity

Sensitivity studies were performed in triplicate using a series of dilutions (1 ng, 500 pg, 250 pg, 125 pg, 62.5 pg and 31.25 pg) of control DNA M4615. All experiments were carried out according to the parameters provided above. The numbers of successful genotyping allele peaks were counted to show the percent of the full profile and the mean peak height were determined for each template amount.

### 2.8 Assessment of precision and sizing accuracy

Size precision allows for determining reliable and accurate genotypes. Variations of runs on the same or different instruments are possible due to different types and concentrations of polymer mixture, run temperature, and electrophoretic conditions. A precision study was therefore performed, which involved running capillary electrophoresis for 32 allelic ladder samples (two successive runs) on the 3130xl and 48 ladder samples (two successive runs) on the 3500xl Genetic Analyzer. The mean values and standard deviation (SD) of sizes were calculated for each allele on each instrument.

### 2.9 Assessment of PCR inhibition

Samples obtained for forensic cases often contain various PCR inhibitors. It is hence necessary to examine the performance of the 21-plex STR typing system in the presence of a variety of PCR inhibitors. Hematin, humic acid, and tannic acid, agents which are known to affect the PCR process, were incubated with the PCR reagents at various concentrations: 200, 300, 400, 500, 600, and 700 μM hematin; 20, 40, 60, 80, and 100 ng/μL humic acid; and 5, 10, 15, 20, and 40 ng/μL tannic acid. All the experiments were carried out with the rapid thermal parameters provided above using 2 ng control DNA M4615.

### 2.10 Assessment of species specificity

Non-human DNA samples from common domestic animal species (10 ng DNA each from rat, mouse, sheep, chicken, cow, pig, dog and black bear and 2 ng DNA from soil microbial pool) were subjected to PCR amplification and electrophoresis was performed on the ABI 3130xl Genetic Analyzer. The human DNA sample M4615 (2 ng) was used as a positive control.

### 2.11 Assessment of stutter ratio

The presence of stutter peaks, reflecting the process of strand slippage, is a natural by-product of PCR amplification of STR profiles [19]. The full sets of 891 samples were used to calculate the stutter ratio. The proportion of stutter peaks relative to main allele peaks was measured by the stutter peak height divided by the main peak height.

### 2.12 Assessment of the effects of DNA mixtures on amplification

Mixed samples consisting of DNA from two or more individuals are often encountered in forensic cases, so it is necessary

 

**Table 1.** Characterization of loci and primers in the 21-plex system

| Locus | Characterization of locus | | | | Primer-related information | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | GenBank Accession (UniSTS) | Chromosomal location | Motif (category) | Allele range | Primer sequence (5′–3′) | Tm (°C) | Concentration (μM) | Amplicon size (bp) | Dye label |
| D19S433 | AC008507.11 (33588) | 19q12 | AAGG/TAGG (Compound) | 9–18.2 (14) | F-ATTCCCGAATAAAAATCTTCTCR-GTGCGAATAAGATTCTGTTGA | 55.7 55.6 | 0.180 | 94–132 | FAM |
| D5S818 | AC008580.7 (54700) | 5q23.2 | AGAT (Simple) | 7–15 (11) | F-ATTGGTGATTTTCCTCTTTGGTAR-GTGCTTTTTAGCCAAGTGATT | 58.9 56.3 | 0.105 | 145–178 | FAM |
| D21S11 | AC000433.2 (240642) | 21q21.1 | TCTA/TCTG (Complex) | 27–35 (23) | F-ATATGTGAGTCAATTCCCCAAGR-GTATTAGTCAATGTTCTCCAGA | 57.9 51.2 | 0.250 | 214–246 | FAM |
| D18S51 | AC021803.8 (44409) | 18q21.33 | AGAA (Simple) | 9–27 (18) | F-TTCTTGAGCCCAGAAGGTTAR-GTTTCTTACCAGCAACAACACAAATAAA | 57.5 62.6 | 0.140 | 285–355 | FAM |
| D6S1043 | AL132766.13 (23182) | 6q15 | AGAT/AGAC (Compound) | 10–22.3 (11) | F-ACTCCAGAGAGATAGAAGCAATAR-GTTTGTCTCTTTCTTCTGGAGCTG | 54.3 60.9 | 0.120 | 380–430 | FAM |
| Amelogenin | AC002366.1 AC013412.3 | Xp22.2 Yp11.2 | | X<br>Y | F-TCCCTGGGCTCTGTAAAGAATA<br>R-GATCAGAGCTTAAACTGGGAAG | 60.157.3 | 0.075 | 104–110 | HEX |
| D3S1358 | AC099539.2 (148226) | 3p21.31 | AGAT/AGAC (Compound) | 11–19(15) | F-ACTGCAGTCCAATCTGGGTR-GCAAGCCTCTGTTGATTTCAT | 65.459.3 | 0.110 | 125–155 | HEX |
| D13S317 | AL391354.12 (7734) | 13q31.1 | TATC (Simple) | 7–14(11) | F-ATTACAGAAGTCTGGGATGTGR-GTTGGCAGCCCAAAAAGACAGA | 54.265.7 | 0.125 | 174–202 | HEX |
| D7S820 | AC004848.1 (74895) | 7q21.11 | GATA (Simple) | 7–15(13) | F-ATGTTGGTCAGGCTGACTATGR-GATTCCACATTTATCCTCATTG | 57.756.1 | 0.200 | 218–250 | HEX |
| D16S539 | AC092327.4 (45590) | 16q24.1 | GATA (Simple) | 6–15(11) | F-TGGGGGTCTAAGAGCTTGTAAAAAR-GTTTGTGTGTGCATCTGTAAGC | 62.358.4 | 0.080 | 268–304 | HEX |
| CSF1PO | AC011382.4(156169) | 5q32 | AGAT (Simple) | 7–17(13) | F-CGGAGGTAAAGGTGTCTTAAAGR-ATTTCCTGTGTCAGACCCTGTT | 57.259.9 | 0.150 | 322–362 | HEX |
| Penta D | AP001051.1 (none reported) | 21q22.3 | AAAGA (Simple) | 5–16(13) | F-TCGGTGAAGGTCGAAGCTGAAGTGR-ATTAGAATTCTTTAATCTGGACAC | 68.953.0 | 0.350 | 380–433 | HEX |

**Table 1.** Continued

| Locus | Characterization of locus | | | | Primer-related information | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | GenBank Accession (UniSTS) | Chromosomal location | Motif (category) | Allele range | Primer sequence (5′–3′) | Tm (°C) | Concentration (μM) | Amplicon size (bp) | Dye label |
| D2S441 | AC079112.4 (71306) | 2p14 | TCTA/TCAA (Compound) | 9–19(12) | F-TGTGTGGCTCATCTATGAAAACTTCR-GAAGTGGCTGTGGTGTTATGATA | 61.759.0 | 0.160 | 82–122 | TAMRA |
| vWA | AC005904.19 (240640) | 12p13.31 | TCTA/TCTG (Compound) | 13–21(13) | F-TGCCCTAGTGGATGATAAGAATAATCR-GGACAGATGATAAATACATAGGAT | 60.853.3 | 0.145 | 136–168 | TAMRA |
| D8S1179 | AC100858.3 (83408) | 8q24.13 | TCTA/TCTG (Compound) | 8–18(12) | F-ATTGCAACTTATATGTATTTTTGTAR-ACCAAATTGTGTTCATGAGTATA | 52.453.9 | 0.180 | 209–247 | TAMRA |
| TPOX | AC105450.1 (240638) | 2p25.3 | TGAA (Simple) | 7–15(8) | F-TTCCTCTGCTTCACTTTTCACCR-GTAAGGTCTTACTCCTGTTCCCTTC | 60.860.0 | 0.160 | 273–305 | TAMRA |
| Penta E | AC027004.15 (none reported) | 15q26.2 | AAAGA (Simple) | 5–26(5) | F-ATTGAGGCCGATGCAGGTGTATTR-GGCGCATGGAAAGAATTCTCTTAT | 65.963.8 | 0.250 | 320–419 | TAMRA |
| TH01 | AC132217.15 (240639) | 11p15.5 | AATG (Simple) | 5–11(7) | F-TGTGATTCCCATTGGCCTGTTCR-GGAATATTCTTCCGAGTGCAGGTCA | 66.466.6 | 0.190 | 100–124 | ROX |
| D12S391 | AC007621.34 (2703) | 12p13.2 | AGAT/AGAC (Compound) | 15–26(19) | F-TAACAGGATCAATGGATGCATR-GGGACTGTCATGAGATTTTTCAGC | 57.963.3 | 0.380 | 150–194 | ROX |
| D2S1338 | AC010136.8 (30509) | 2q35 | TGCC/TTCC (Compound) | 15–27(21) | F-TAAAGACTTCATGGTCCTGACTACAGR-GTTTCTTCCATGAGTTATTCAGTAAGTTA | 60.158.8 | 0.140 | 216–264 | ROX |
| FGA | AC107385.4 (240635) | 4q31.3 | CTTT/CCTT (Compound) | 14-29(19) | F-ACAAATGCCCCATAGGTTTTGR-GTTTCTTAATTCTATGACTTTGCGCTT | 60.961.5 | 0.380 | 285–347 | ROX |

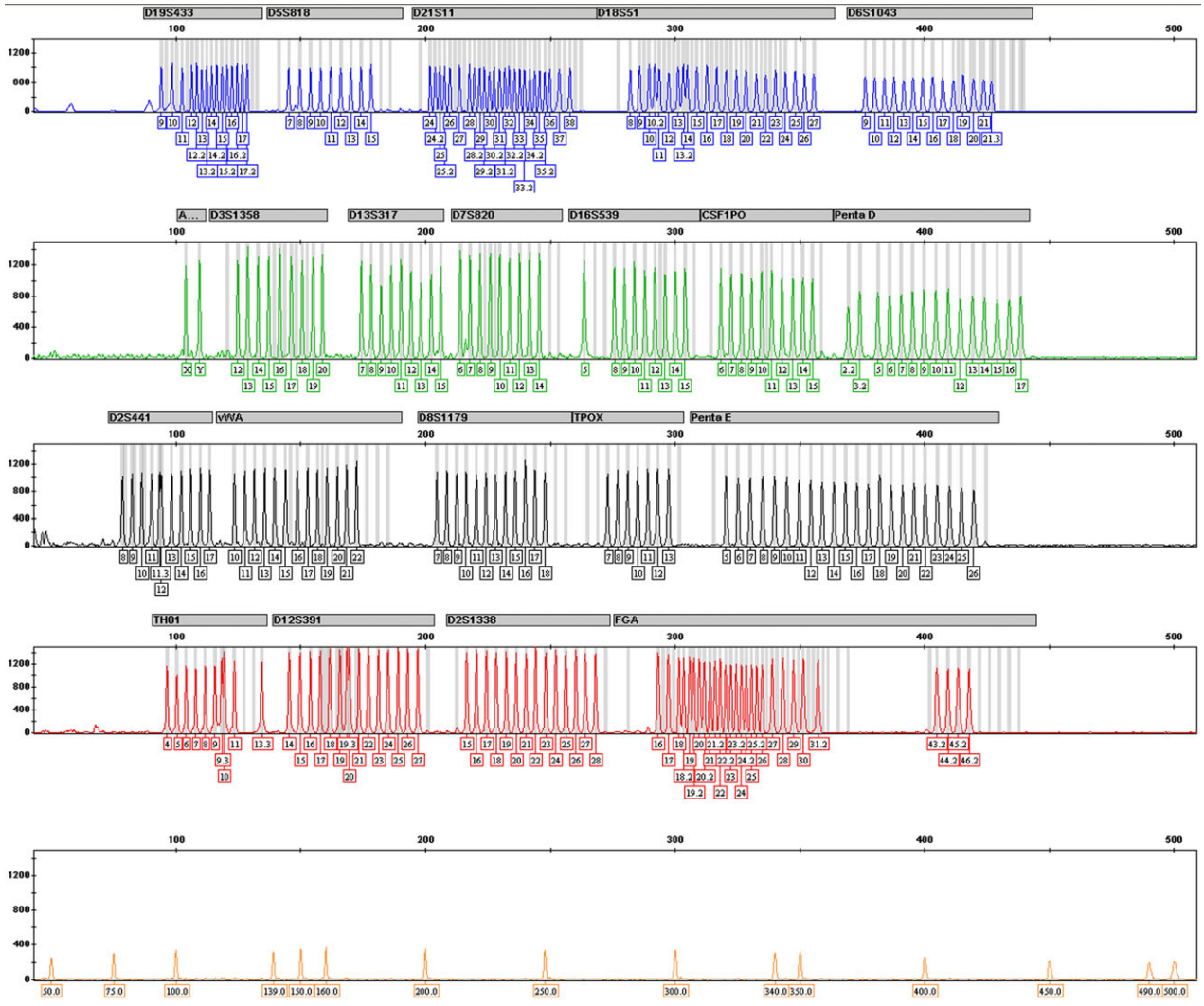


**Figure 1.** Electropherogram of allelic ladders and internal size standard in the 21-plex system. The four dye panels for the allelic ladders correspond to (from top to bottom) FAM (blue), HEX (green), TAMRA (yellow), ROX (red) dye-labeled peaks. The genotype is shown with the allele number displayed underneath each peak. The fifth panel shows the internal size standards labeled with Orange500 dye (a total of fifteen fragments: 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 bp).

to assess the ability of the STR typing system to analyze these DNA mixtures. Mixtures of two individual samples (female 9947A and male 9948) were examined in various ratios (1:0, 1:1, 2:1, 4:1, 9:1, 0:1) while keeping the total amount of DNA constant at 1 ng. The experiment was conducted with two replicates to ensure the accuracy of the results.

## 3 Results and discussion

### 3.1 Multiplex design

The 21 loci were divided into four groups and each group assigned a color dye. Internal size standards and allelic ladders are shown in Fig. 1. The set of designed primers act according to unified parameters, with a consistent temperature in order to achieve a stable and balanced amplification sensitivity and efficient amplification in multiple PCR processes. Primer-related information on each locus (sequence, melting temperature, and distance from repeat motif, concentration, PCR product size, and dye label) is listed in Table 1, and the genotype results of 9947A, 9948, and M4615 were shown in Supporting Information Table 1.

### 3.2 Assessment of PCR conditions

Full PCR profiles were obtained for reactions with an annealing temperature within the range of 56–64°C (Supporting Information Fig. S1). At 56 and 58°C, non-specific allele peaks were observed for the Penta E and FGA loci. The peak ratios (non-specific allele peak to the main peak) for Penta E and FGA loci were 30 and 25% at 56°C, and 10 and 5% at 58°C, respectively. In addition, the peak height for D19S433

 

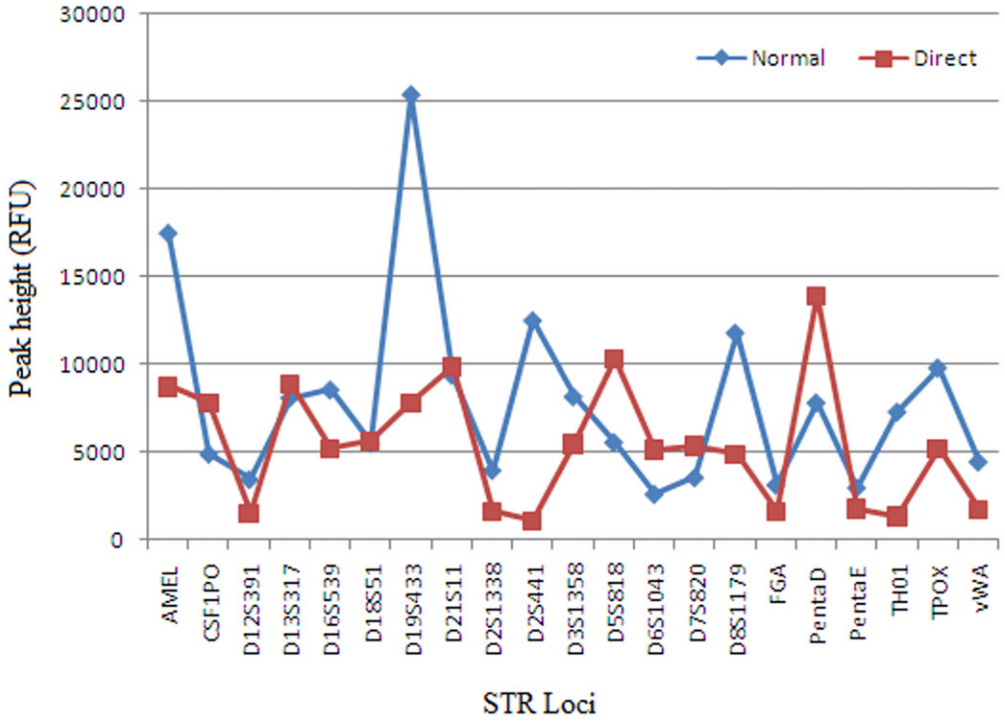


**Figure 2.** Comparison of peak heights at each locus between the rapid ("direct") protocol and normal protocol. The locus peak height is a sum of the heterozygote peaks and the right single peak for a homozygote site.

decreased by 20 and 50% at 62 and 64°C, respectively. At 60°C annealing temperature, the PCR reactions had the greatest specificity and sensitivity, as well as the best peak height balance of different alleles. Therefore, we determined that an annealing temperature of 60°C was optimal for this system.

We found that, as expected, peak heights increased as the number of cycles increased; however, locus-to-locus imbalance and non-specific allele peaks were also observed with higher numbers of cycles (Supporting Information Fig. S2). Therefore, 27 cycles are recommended for thermal cycling of the reaction.

The peak comparison between the rapid and normal PCR amplification protocols revealed that the rapid protocol still succeeded in amplifying the control DNA (9947A). For most of the loci, peak heights were reduced when using the rapid protocol compared to the normal one, although for some loci (CSF1PO, D5S818, D6S1043, D7S820, and Penta D) the peaks were higher (Fig. 2).

Full allele profiles were obtained for all tested PCR component concentrations; although there were associated peak height changes (Supplementary Supporting Information Figs. S3–S6). Overall, our results showed that the 21-plex system was robust to normal variations in PCR component concentrations.

### 3.3 Assessment of sensitivity

We assessed the sensitivity of the reaction by setting 50 RFU as the limit of detection and 60% as the stochastic threshold for peak balance using varying amounts of M4615 control DNA. We were able to obtain the full profile of sample M4615 when the DNA template provided was greater than 125 pg (Fig. 3, Supporting Information Fig. S7), although we did observe allelic imbalance at four loci (D13S317, D7S820, TPOX and TH01) at 125 pg. For samples with more than 125 pg, the mean peak height increased with increasing amounts of DNA template in the reaction. Allelic drop-out and allelic imbalance occurred when the amount of template DNA was reduced to 62.5 pg or 31.25 pg. Allelic drop-out was observed at two loci (D13S317 and D2S1338) and allelic imbalance at five loci (D19S433, D7S820, CSF1PO, TPOX, and TH01) at 62.5 pg of template DNA. Nearly half of the 21 loci (D19S433, D21S11, Amelogenin, D13S317, D7S820, D16S539, TPOX, Penta E, TH01, and D2S1338) exhibited allelic drop-out and CSF1PO exhibited allelic imbalance with 31.25 pg DNA. We thus determined that the minimum amount of DNA template for our typing system is 125 pg.

### 3.4 Assessment of precision and sizing accuracy

The average size and standard deviation of each allelic ladder were found to be less than 0.14 bases when analyzed with both the 3130xl and 3500xl Genetic Analyzers (Fig. 4). The highest SDs observed were 0.135 bp for FGA allele 24.2 on the 3130xl and 0.72 bp for D18S51 allele 18 on 3500xl. Overall, the 3130xl analyzer produced more variations than the 3500xl analyzer. However, the SDs of all alleles on the two instruments fell well below 0.14 bp, ensuring that sample alleles were rarely sized outside of the 0.5 bp window and fully complying with the QAS (2009/2011) requirements [20].

 

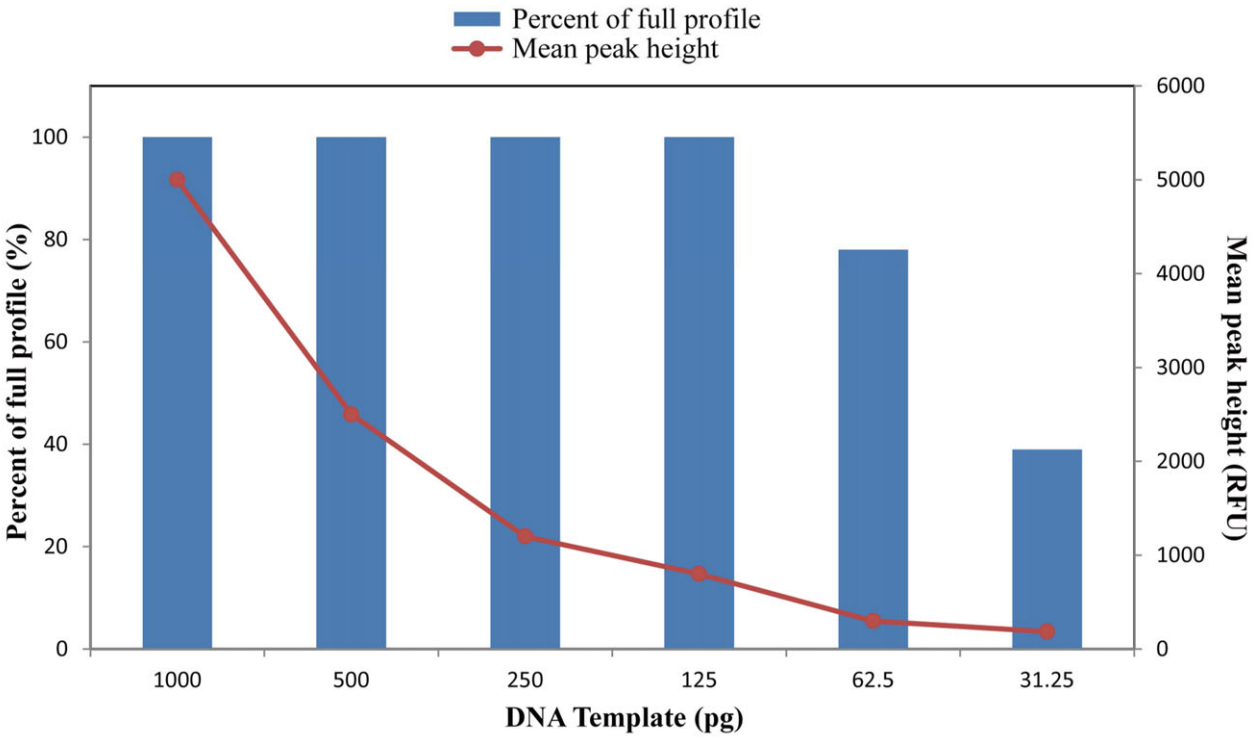


**Figure 3.** Sensitivity experiment using control sample M4615 at varying concentrations in the 25 μL reaction system. The left-hand y-axis gives the percent of full profile (the numbers of successful genotyping allele peaks vs. the full profile) and the right-hand y-axis gives the mean peak height. Full profiles were observed except at 62.5 pg and 31.25 pg of DNA.

### 3.5 Assessment of PCR inhibition

Considering the complexity of actual forensic cases and the contamination often encountered, we assessed the effects of various PCR inhibitors that can interfere with the amplification and electrophoresis of sample DNA, leading to inaccurate electropherograms. Humic acid and tannic acid from soil contamination and hematin, present in blood stains, were tested, as they are the most commonly encountered inhibitors. Full STR profiles were obtained with humic acid ≤ 60 ng/μL, tannic acid ≤ 15 ng/μL, and hematin ≤ 500 μM (Supporting Information Figs. S8–S10). Generally, with inhibitors, we observed that the larger the alleles, the easier the allele may be dropped-out.

### 3.6 Assessment of species specificity

Often, forensic crime scenes contain stains caused by animals, such as sheep, pigs, or dogs. The species specificity of the STR typing system was assessed to verify that other biological sources would not interfere with the system's ability to obtain reliable results on human samples recovered from crime scenes. Among the samples tested (rat, mouse, sheep, chicken, cow, pig, dog, black bear and microbial pool), we found no allelic peak height within the range of the tested panel (Supporting Information Fig. S11).

### 3.7 Assessment of stutter ratio

Due to replication slippage that occurs in the process of STR allele PCR amplification, there may be a relatively low stutter peak located next to the objective allele in electropherograms [19, 21, 22]. In forensic cases, the presence of stutter may lead to incorrect genotype calls, especially at homozygous loci. Thus, we calculated the stutter ratios for the 21 loci on the basis of 891 samples (Table 2). Locus Penta D exhibited the smallest mean stutter ratio (1.55%), while the largest one was observed for D12S391 (11.79%). All stutter peaks for all STR loci were below the recommended stutter filter values.

### 3.8 Assessment of the effects of DNA mixtures on amplification

Mixed samples consisting of DNA from two or more individuals are routinely encountered in many forensic cases, so we assessed the typing system's ability to analyze DNA mixtures [23]. Mixtures of two individual samples (female 9947A and male 9948) were examined in various ratios (1:0, 1:1, 2:1, 4:1, 9:1, and 0:1) with 1 ng total template DNA. Testing was performed in triplicate to ensure the accuracy of the results. We were able to call alleles for all loci for mixture ratios of 1:1 and 2:1, though the peak height of 9948 was

 

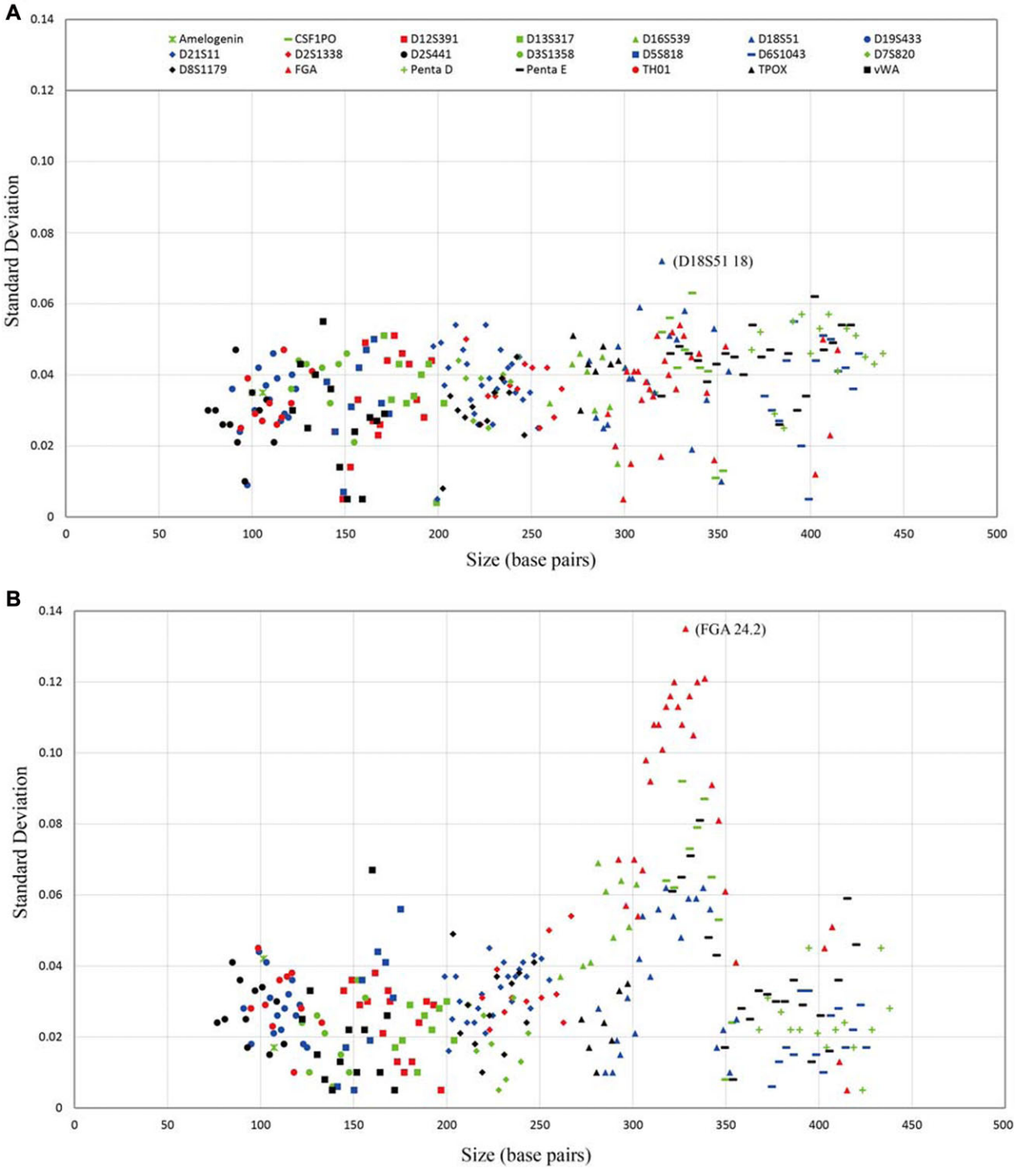


**Figure 4.** Sizing variation of all allelic ladders on 3500xl Genetic Analyzer ($n = 48$) (A.) and 3130xl Genetic Analyzer ($n = 32$) (B.). The x-axis gives the size of the allelic ladders at each locus, while the y-axis represents the variation of the standard deviation corresponding to each allelic ladder.

only 10% that of 9947A (Fig. 5). Peak height ratios in the 4:1 and 9:1 mixtures were also 10% or less. We were unable to call alleles for some of the minor profiles at the TPOX and TH01 loci in the 4:1 ratio samples and at the D2S441, TPOX, and TH01 loci in the 9:1 ratio samples. These results indicate that the 21-plex system can be used to genotype DNA samples in mixtures with relatively small mixture ratio.

 

**Table 2.** The percentage of stutter in 20 loci from 891 samples

| STR locus | Stutter range | Stutter mean | SD | Recommended filter |
|---|---|---|---|---|
| CSF1PO | 3.24–15.97% | 7.12% | 1.67% | 0.1214 |
| D12S391 | 5.99–23.68% | 11.79% | 2.09% | 0.1807 |
| D13S317 | 1.49–15.15% | 5.73% | 2.20% | 0.1232 |
| D16S539 | 2.81–16.71% | 7.05% | 1.90% | 0.1274 |
| D18S51 | 4.28–18.23% | 8.06% | 1.94% | 0.1387 |
| D19S433 | 3.85–16.30% | 9.97% | 1.79% | 0.1534 |
| D21S11 | 4.70–18.45% | 10.22% | 1.56% | 0.1491 |
| D2S1338 | 6.10–22.08% | 11.50% | 1.85% | 0.1704 |
| D2S441 | 3.10–15.13% | 7.64% | 2.29% | 0.1452 |
| D3S1358 | 5.55–19.19% | 10.23% | 1.68% | 0.1525 |
| D5S818 | 3.35–18.73% | 7.75% | 2.50% | 0.1525 |
| D6S1043 | 4.50–18.12% | 8.30% | 1.65% | 0.1324 |
| D7S820 | 2.03–13.55% | 6.06% | 1.58% | 0.1081 |
| D8S1179 | 4.15–19.71% | 8.09% | 1.77% | 0.1339 |
| FGA | 5.10–14.34% | 8.48% | 1.68% | 0.1351 |
| Penta D | 0.35–7.54% | 1.55% | 1.37% | 0.0567 |
| Penta E | 2.12–24.23% | 5.20% | 2.81% | 0.1362 |
| TH01 | 0.44–10.99% | 3.80% | 1.73% | 0.0899 |
| TPOX | 0.97–19.90% | 3.56% | 1.88% | 0.0919 |
| vWA | 1.79–15.07% | 8.28% | 2.75% | 0.1653 |

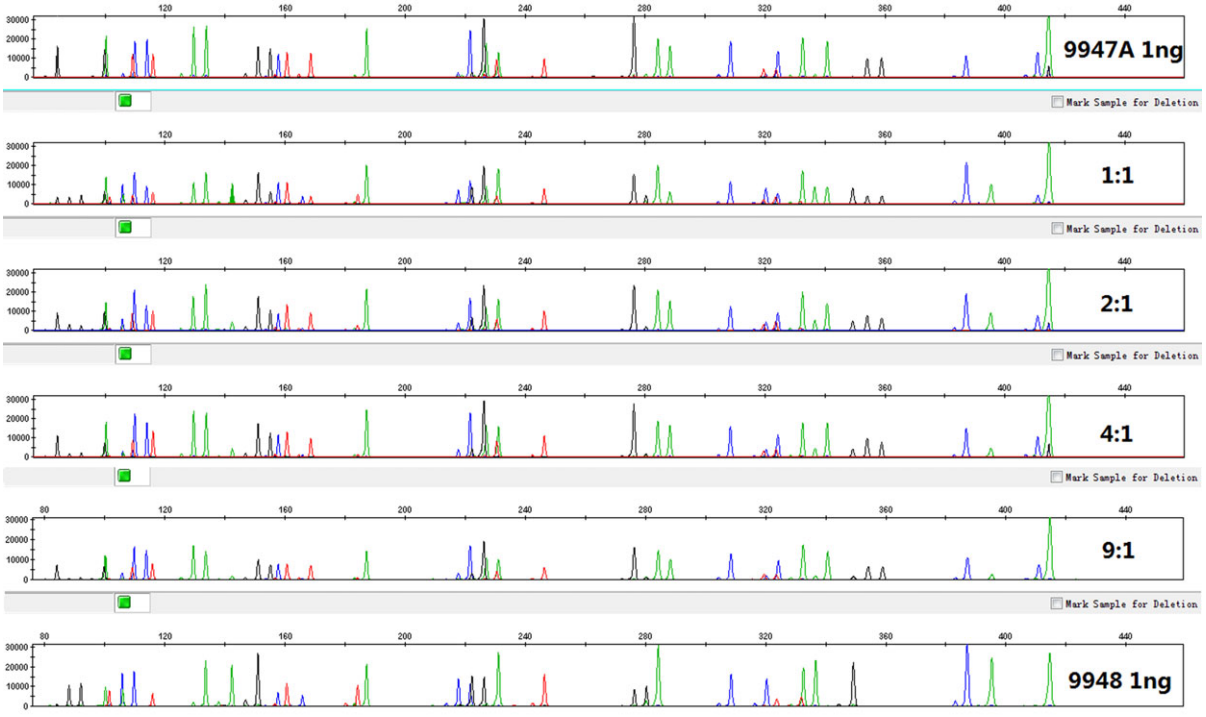


**Figure 5.** Representative Electropherogram of DNA mixture formed by 9947A and 9948 totally amount to 1 ng in the ratios 1:0, 1:1, 2:1, 4:1, 9:1, and 0:1.

## 4 Concluding remarks

This article outlines the development of a rapid 21-plex STR typing system, including all Combined DNA Index System (CODIS) STR loci, as well as D2S1338, D2S441, D12S391, D19S433, D6S1043, Penta D, Penta E, and the sex-determining locus Amelogenin in a single reaction. A modified Taq DNA polymerase has been used to substitute the commonly used AmpliTaq Gold. The modified Taq DNA polymerase demonstrated a 30-fold increase in polymerase

 

efficacy, but it also demonstrated the ability to amplify DNA with shorter extension times, thus shortening overall PCR thermal cycling times. Additionally, using this special polymerase, and furthermore improving the PCR buffer could also increase resistance to inhibition found in whole blood and tissue sample. Thus, the amplification time could be reduced to a minimum of 90 min and no DNA extraction was required from blood, bloodstain, or buccal samples. Using this 21-plex system, plus the electrophoresis, the time from sample to STR profile is within 2 h.

As suggested by SWGDAM and CNS guidelines, we conducted developmental validation studies to examine many aspects of multiplex performance, such as PCR conditions, sensitivity, precision and accuracy, species specificity, effects of PCR inhibitors, stutter ratio, and effects of mixed DNA samples. Our validations revealed that the rapid 21-plex system represents a reproducible, accurate, sensitive, and robust tool for rapid forensic investigation. This system could also be used for non-forensic scientific research, such as human population studies, as the STR loci selected for the 21-plex system were found to be highly polymorphic and informative in the Han Chinese population [24,25]. In the future, with the next generation CE instrument, we plan to add additional fluorescent dyes and/or types of markers (i.e., Y-STRs, INDELs) to improve the multiplex system further.

*This project was supported by the National Natural Science Foundation of China (NSFC, No. 81330073).*

*The authors have declared no conflict of interest.*

## 5 References

[1] Butler, J. M., *J. Forensic Sci.* 2006, *51*, 253–265.

[2] SWGDAM, *Validation Guidelines for DNA Analysis Methods*, 2010 Available at: http://swgdam.org/SWGDAM_Validation_Guidelines_APPROVED_Dec_2012.pdf, (accessed 03.19.13).

[3] Zhou, H., Wu, D., Chen, R., Xu, Y., Xia, Z., Guo, Y., Zhang, F., Zheng, W., *Forensic Sci. Int. Genet.* 2014, *11*, 31–38.

[4] Choung, C. M., Lee, D. S., Park, K. W., Han, M. S., *Forensic Sci. Int.* 2011, *3*, e475–e476.

[5] Bahlmann, S., Hughes, S. S., Gangitano, D., *Leg. Med. (Tokyo)* 2014, *16*, 227–233.

[6] Tsukada, K., Harayama, Y., Shimizu, M., Kurasawa, Y., Kasahara, K., *Forensic Sci. Int.* 2011, *3*, e190–e191.

[7] Biosystems, A., *GlobalFiler Express Kit User's manuel* 2013.

[8] Corporation, P., *PowerPlex Fusion System Technical Manual* 2012.

[9] Cheng, J., Shoffner, M. A., Hvichia, G. E., Kricka, L. J., Wilding, P., *Nucleic Acids Res.* 1996, *24*, 380–385.

[10] Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., Pease, L. R., *Gene* 1989, *77*, 51–59.

[11] Hopwood, A. J., Hurth, C., Yang, J., Cai, Z., Moran, N., Lee-Edghill, J. G., Nordquist, A., Lenigk, R., Estes, M. D., Haley, J. P., McAlister, C. R., Chen, X.-j., Brooks, C., Smith, S., Elliott, K., Koumi, P., Zenhausern, F., Tully, G., *Anal. Chem.* 2010, *82*, 6991–6999.

[12] Cedric, H., Smith, S. D., Nordquist, A. R., Lenigk, R., Duane, B., Nguyen, D., Surve, A., Hopwood, A. J., Estes, M. D., Yang, J., Cai, Z., Chen, X.-j., Lee-Edghill, J. G., Moran, N., Elliott, K., Tully, G., Zenhausern, F., *Electrophoresis* 2010, *31*, 3510–3517.

[13] Njoroge, S. K., Chen, H.-w., Witek M. A., Soper, S. A., *Top. Curr. Chem.* 2011, *304*, 203–260.

[14] Yuan, L., Jiang, C. T., Ye, J., Lu, D., Bai, X., Yang, X., *Chinese J. Forensic Med.* 2012, *27*, 181–184.

[15] Xie, B., Chen, L., Yang, Y., Lv, Y., Chen, J., Shi, Y., Chen, C., Zhao, H., Yu, Z., Liu, Y., *Forensic Sci. Int. Genet.* 2015, *19*, 205–206.

[16] Rozen, S., Skaletsky, H., *Methods Mol. Biol.* 2000, *132*, 365–386.

[17] Vallone, P. M., Butler, J. M., *BioTechniques* 2004, *37*, 226-231.

[18] Griffiths, R. A. L., Barber, M. D., Johnson, P. E., Gillbard, S. M., Haywood, M. D., Smith, C. D., Arnold, J., Burke, T., Urquhart, A. J., Gill, P., *Int. J. Legal Med.* 1998, *111*, 267–272.

[19] Schlötterer, C., Tautz, D., *Nucleic Acids Res.* 1992, *20*, 211–215.

[20] Investigation, F. B., *Forensic Sci.* Commun. 2000, *2*, 3.

[21] Levinson, G., Gutman, G. A., *Mole. Biol. Evol.* 1987, *4*, 203–221.

[22] Walsh, P. S., Fildes, N. J., Reynolds, R., *Nucleic Acids Res.* 1996, *24*, 2807–2812.

[23] Swaminathan, H., Grgicak, C. M., Medard, M., Lun, D. S., *Forensic Sci. Int. Genet.* 2015, *16*, 172–180.

[24] Xie, B.-b., Chen, L., Yang, Y.-r., Lv, Y.-x., Chen, J., Shi, Y., Chen, C., Zhao, H.-y., Yu, Z.-l., Liu, Y.-c., Fang, X.-d., Yan, J.-w., *Forensic Sci. Int. Genet.* 2015, *19*, 205–206.

[25] Yin, C.-y., Ji, Q., Li, K., Mu, H.-f., Zhu, B.-f., Yan, J.-w., Yu, Y.-f., Wang, J.-w., Chen, F., *Forensic Sci. Int. Genet.* 2015, *14*, 108–109.

 