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Developmental validation of the EX20+4 system

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ABSTRACT

The EX20+4Y System is a polymerase chain reaction (PCR)-based amplification kit that enables typing of 19 autosomal short tandem repeat (STR) loci (i.e., CSF1PO, D13S317, D16S539, D18S51, D21S11, D3S1358, D5S818, D7S820, D8S1179, FGA, TH01, TPOX, vWA, Penta D, Penta E, D2S1338, D19S433, D12S391, D6S1043), four widely used Y chromosome-specific STR (Y-STR) loci (DYS458, DYS456, DYS391, DYS635), and amelogenin. In this study, this multiplex system was validated for sensitivity of detection, DNA mixtures, inhibitor tolerance, species specificity based on the Scientific Working Group on DNA Analysis methods (SWGDAM) developmental validation guidelines, and the Chinese criteria for the human fluorescent STR multiplex PCR reagent. The results show that the EX20+4 System is a robust and reliable amplification kit which can be used for human identification testing.

1. Introduction

The rapid growth of DNA databases require more markers to be included in multiplex kits to meet the challenges of adventitious hits, missing person identification, international data sharing, etc. [1,2]. With the current short tandem repeat (STR) and capillary electrophoresis (CE) technology, which can multiplex up to about 20–25 STR loci, a combination of autosomal STR loci and several Y chromosome-specific STR (Y-STR) loci can provide a higher kinship index with sufficient discriminating power for single-source comparisons compared with a similar number of only autosomal STR loci [2]. With a nominal number of Y-STR loci, gender of the sample donor can be determined with high accuracy, even with amelogenin-Y dropout [1,3]. In samples where there is a much larger contribution of female DNA compared with male DNA, Y-STR typing can be beneficial [4].

In this study, we developed and validated the EX20+4 System. This multiplex is a 5-dye system that enables typing of 19 autosomal STR loci, four Y-STR loci, and amelogenin. The blue channel consists of fluorescein-labeled STR amplicons for the D3S1358, D13S317, D7S820, D16S539, Penta E, and DYS635 loci. The green channel includes DNA fragments labeled with hexachlorofluorescein (HEX) for the DYS456, TPOX, TH01, D2S1338, CSF1PO, Penta D, and DYS458 loci. The yellow channel consists of amplification products for the DYS391, D19S433, vWA, D21S11, D18S51, and D6S1043 loci that are labeled with tetramethylrhodamine (TAMRA). The amplified products for amelogenin, D8S1179, D5S818, D12S391, and FGA loci are in the red channel and labeled with 6-carboxy-X-rhodamine (ROX). Fragments included in the internal lane standard are detected in the orange channel and are labeled with SIZ-500.

Scientific Working Group on DNA Analysis methods (SWGDAM) developmental validation guidelines [5] and the Chinese criteria [6] for human fluorescent STR multiplex polymerase chain reaction (PCR) reagent are followed in the development and validation of the EX20+4 System.

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2. Materials and methods

2.1. Samples

For the sensitivity study, male 2800M and male 9948 human genomic DNA (Promega, Madison, WI, USA) were amplified in a serial dilution with the following template amounts: 1 ng, 500 pg, 250 pg, 125 pg, 62.5 pg, and 31.25 pg.

Species specificity studies were conducted using 1 ng each of purified DNA from human, chimpanzee, and macaque; 10 ng each of dog, pig, cat, sheep, chicken, mouse, rabbit, fish, and a microbial pool (1 ng each purified DNA from *Escherichia coli*, *Lactobacillus acidophilus*, *Salmonella*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Streptococcus salivarius*, *Corynebacterium pyogenes*, *Saccharomyces cerevisiae*, *Candida albicans*, and *Aspergillus flavus* mixed together); and a negative control. These samples were kindly donated by the Guangzhou Zoo (Guangzhou, China) and the Institute of Human Virology of Sun Yat-Sen University (Guangzhou, China). The quantity was determined by Qubit® ssDNA Assay Kit with the Qubit® 2.0 Fluorometer (Invitrogen/Life Technologies, San Francisco, CA, USA).

Several common forensic inhibitors were tested including hematin, indigo, humic acid, calcium ion, hemoglobin, and ethylenediaminetetraacetic acid (EDTA). The male DNA template quantity was held constant at 500 pg, while the concentration of inhibitors was varied: 0, 120, 160, or 200 μmol/L of hematin; 0, 500, 750, or 1000 μmol/L of indigo; 0, 5, 10, or 20 ng/μL of humic acid; 0, 1.0, 1.5, or 1.75 mmol/L of calcium ion; 0, 500, 750, or 1000 μmol/L of hemoglobin; and 0, 0.8, 1.0, or 1.2 mM of EDTA.

Male/male mixtures were prepared using male 9948 and male 2800 M human genomic DNA (Promega) with mixture ratios of 1:1, 1:4, 1:9, or 1:19. Male/female mixtures were prepared using male 9948 and female 9947a human genomic DNA with mixture ratios of 1:1, 1:4, 1:9, or 1:19. Each mixture was tested in triplicate. These samples were formulated at a concentration of 1 ng in 10 μL. An additional male/female mixture was set up using a constant template of 500 pg male DNA 2800 M with an increasing amount of female DNA: 50 ng, 100 ng, 200 ng, 400 ng, and 800 ng.

Reproducibility samples included buccal-indicating FTA® Cards, blood FTA® Cards (GE Healthcare/Whatman, Buckinghamshire, UK), and extracted DNA from one female donor and two male donors. The DNA was extracted by magnetic beads (DNA IQ™ System, Promega, Madision, WI, USA) on the Maxwell® 16 Research System (Promega). The quantity was determined by the Quantifiler Human DNA Quantification Kit with the 7500 Real-time PCR System (Life Technologies). Three laboratories (i.e., Guangzhou Forensic Science Institute and two of its sub-institutes) participated in the tests.

Case samples were tested at the Guangzhou Forensic Science Institute. These samples include three bloodstains, three bones, three epithelial fractions, and three buccal-indicating FTA® Cards. Buccal-indicating FTA® Cards were amplified directly while others were extracted by magnetic beads. The results were compared with a PowerPlex21 kit (Promega, Madision, WI, USA).

Stutter calculation studies contain 130 extracted DNA samples which were amplified and analyzed on a 3130xl Genetic Analyzer (AB, Foster City, California, USA). These samples were collected with informed consent from 130 donors of the Guangdong Han population. These samples were extracted by magnetic beads from cotton buccal swabs on the TECAN® Freedom EVO workstation (TECAN, Männedorf, Switzerland). Each amplification reaction contained 0.5 ng DNA in a 25-μL volume. The quantity was determined by the Quantifiler Human DNA Quantification Kit with the 7500 Real-time PCR System (Life Technologies).

Population studies were performed with samples from 500 unrelated individuals (250 males and 250 females) of the Guangdong Han population that were collected with informed consent. The blood samples were maintained on blood FTA® Cards (GE Healthcare/Whatman). Concordance testing was performed with 200 blood FTA® Card (GE Healthcare/Whatman) samples, including 100 males and 100 females. These samples were a subset of the 500 population study samples.

2.2. DNA amplification

Amplifications were performed on a GeneAmp® PCR System 9700 thermal cycler (Life Technologies) using the 9600 mode on a gold-plated silver block. Each amplification reaction contained 10 μL of reaction mix, 5 μL of EX20+4 primers, and 1 μL of C-Taq DNA polymerase (AGCU ScienTch Incorporation, Wuxi, China) with up to 9 μL of template. According to the manufacturer's technical manual, unless specifically mentioned otherwise, the following amplification setup and cycling parameters were used to amplify extracted DNA: initial denaturation of 95 °C for 2 min; 10 cycles of 94 °C for 30 s, 62 °C for 1 min, and 72 °C for 1 min; 20 cycles of 90 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min; a final extension at 60 °C for 60 min; and a 4 °C soak. Samples used for direct amplification were processed using the same cycling parameters with the exception of the cycle number 29 (the cycle number of the second step was reduced to 19). Direct amplification reactions were performed using one 1.2-mm punch from blood FTA® Cards and two punches from buccal-indicating FTA® Cards. Negative controls were included in all experiments.

2.3. Electrophoresis and analysis

Spectral resolution was established using the AGCU 5-dye Matrix Standards to assess each fluorescent dye contained in the kit. Reactions were prepared for CE by combining 12.0 μL of Hi-Di™ Formamide (AB, Foster City, California, USA), 0.5 μL of AGCU Marker SIZ-500, and 1.0 μL of the sample or allelic ladder. Samples were denatured for 3 min at 95 °C, then immediately quenched on ice. Electrophoresis was performed on the 3130xl (16-capillary) Genetic Analyzer using 36-cm capillary arrays with POP-4® Polymer (AB, Foster City, California, USA). Standard run conditions were: sample injection for 5 s at 3 kV and electrophoresis at 15 kV for 1500 s with the indicated run temperature of 60 °C. GeneMapper® ID v.3.2 (AB, Foster City, California, USA) was used to determine fragment size. A peak amplitude of 50 relative fluorescence units (RFUs) was implemented as the peak detection threshold when analyzing data from CE instruments.

2.4. Statistical analysis

Stutter ratios were calculated as stutter peak height divided by the peak height of the associated allele.

Population statistical analysis such as heterozygosity (H), discrimination power (DP), polymorphism information content (PIC), and power of exclusion (PE) were calculated using Power Stats v1.2 software (Promega, Madision, WI, USA). Total discrimination power (TDP) and cumulative probability of paternity exclusion (CPE) were calculated according to Ref. [6].

The fluorescence balance of heterozygous alleles (intra-locus balance) was calculated as lower peak height divided by higher peak height at one locus. The balance within the same fluorescent dye (intra-color balance) was the peak height ratio between the minimum peak height (average height at one locus) and the maximum height (average height at one locus) in the same group. The balance across all loci (inter-color balance) was calculated in a similar way as the intra-color balance.

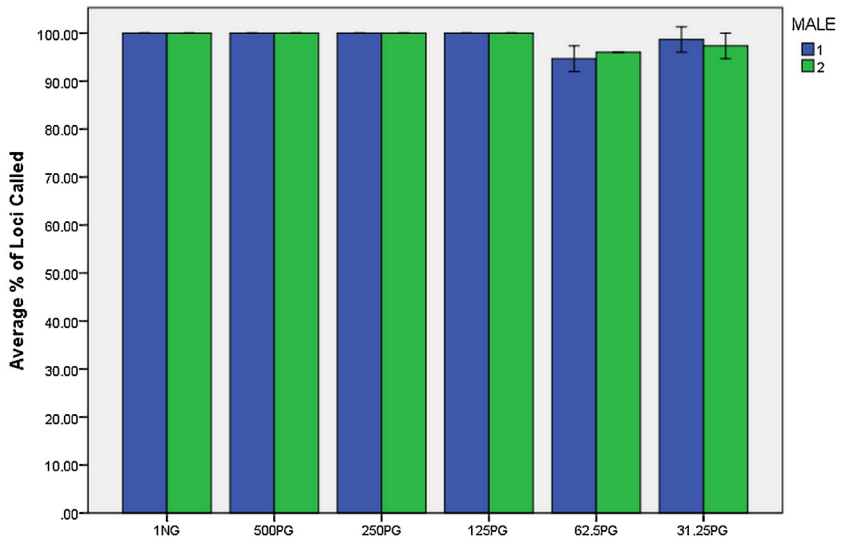

Fig. 1. Percentage of complete profiles of 9948 and 2800M human genomic DNA. Error bars show the standard deviation.

3. Results and discussion

3.1. Sensitivity studies

Sensitivity study samples were amplified with male 2800M and male 9948 human genomic DNA using three different 3130xl (16-capillary) Genetic Analyzers. The following template amounts were tested on all three 3130xl Genetic Analyzers: 1 ng, 500 pg, 250 pg, 125 pg, 62.5 pg, and 31.25 pg. Complete profiles were obtained from 1 ng to 125 pg. Allele dropouts were observed with 62.5 pg and 31.25 pg, but dropout rates were nominal (Fig. 1). Full profiles of 9948 were observed down to 31.25 pg in two 3130xl analyzers, and full profiles of 2800 M were observed down to 31.25 pg in all three 3130xl analyzers.

3.2. Species specificity

Nonhuman genomic DNA samples were tested for cross-reactivity with the EX20+4 System. Results of amplification of 1 ng each of primate samples (chimpanzee and macaque) showed expected cross-reactivity. No peak was detected at 10 ng DNA per PCR for nonprimate mammals and the microbial pool except for cat, which produced reproducible fragments at locus D8S1179 in ROX dye. The sizes of the peak in the bases are 130 and 135, and it has been proved that none of them are on the off-ladder (Supplemental Fig. 1).

3.3. Stability studies

Six common inhibitors (i.e., hematin, indigo, humic acid, calcium ion, hemoglobin, and EDTA) were tested with the EX20+4 System [7–9]. Full profiles were obtained with 0, 500, 750, and 1000 μmol/L of indigo; 0, 5, 10, and 20 ng/μL of humic acid; 0, 0.8, and1.0 mM of EDTA; 0, 80, and 120 μmol/L of hematin; 0 and 1.00 mmol/L of calcium ion; and 0 and 500 μmol/L of hemoglobin. When the concentration of EDTA was increased to 1.2 mM, the DYS635 locus dropped out. When the concentration of hematin was increased to 160 μmol/L, the DYS635 locus dropped out. When the concentration was further increased to 200 μmol/L, there was complete drop out. When the concentration of calcium ion was increased to 1.50 mmol/L, the DYS635 locus dropped out. When the concentration of hemoglobin was increased to 750 μmol/L, the DYS635 locus dropped out. When the concentration of hemoglobin was raised to 1000 μmol/L complete dropout occurred (Table 1).

3.4. Mixture studies

Male/male mixtures and male/female mixtures were prepared and tested as listed in Table 2. All alleles were called for the 1:1 and 1:4 mixtures. For the 1:9 or 1:19 mixture of the male/female samples (i.e., 9948 and 9947a), some autosomal STR alleles and/or amelogenin-Y dropped out, while no Y-STRs dropped out. No allele drop-in was observed. This system meets the requirement of the Chinese criteria for the human fluorescent STR multiplex PCR reagent, which requires that a valid kit should be able to detect all the alleles for 1:4 mixtures (Table 2).

An additional male/female mixture was set up using a constant template of 500 pg male DNA with an increasing amount of female DNA: 50 ng, 100 ng, 200 ng, 400 ng, and 800 ng. Full profiles of the four Y-STR loci were obtained for 500 pg 2800 M with 200 ng of

Table 1
Stability studies. The concentration of the inhibitors added per reaction and the corresponding percentage of the alleles called.

Hematin	Concentration	0 μmol/L	120 μmol/L	160 μmol/L	200 μmol/L
	Call rate (%)	100	100	96	0
Indigotin	Concentration	0 μmol/L	500 μmol/L	750 μmol/L	1000 μmol/L
	Call rate (%)	100	100	100	100
Humic acid	Concentration	0 ng/μL	5 ng/μL	10 ng/μL	20 ng/μL
	Call rate (%)	100	100	100	100
Calcium ion	Concentration	0 mmol/L	1 mmol/L	1.5 mmol/L	1.75 mmol/L
	Call rate (%)	100	100	96	92
Hemoglobin	Concentration	0 μmol/L	500 μmol/L	750 μmol/L	1000 μmol/L
	Call rate (%)	100	100	92	0
EDTA	Concentration	0 mM	0.8 mM	1.0 mM	1.2 mM
	Call rate (%)	100	100	100	96

Table 2
DNA mixture studies. Total amount of the templates were 1 ng per PCR. The average minor contributor allele count detected in three PCR replicates is shown in the minor allele count column. A total of 24 alleles should be observed if there was no allele drop out.

Ratio	DNA added to samples		Minor allele count
	Minor(pg)	Major(pg)	(mean)
9948:2800M = 1:1	500	500	24
9948:2800M = 1:4	200	800	24
9948:2800M = 1:9	100	900	22
9948:2800M = 1:19	50	950	20
9948:9947a = 1:1	500	500	24
9948:9947a = 1:4	200	800	24
9948:9947a = 1:9	100	900	22.5
9948:9947a = 1:20	50	950	20

female DNA. When the amount of female DNA was increased to 400 ng, only the DYS391 locus was called. For 800 ng female DNA, no Y-STR locus was detected.

3.5. Reproducibility

A reproducibility study aimed to determine if the profiles are reliable and suitable for comparison between or among laboratories when using the same DNA sample. The samples were from two male donors and one female donor. Each participating laboratory amplified three buccal-indicating FTA® Cards, three blood FTA® Cards, and three extracted DNA from buccal swabs. Buccal-indicating FTA® Cards and blood FTA® Cards were directly amplified, and DNA from the buccal swabs was extracted by magnetic beads. The loci from samples of the same donor were consistent among the participating laboratories and no peak was detected on Y-STR loci for the tested female sample (Supplemental Fig. 2).

3.6. Case samples

Guangzhou Forensic Science Institute tested 12 case samples and compared the results with those obtained using the PowerPlex21 kit (Supplemental Figs. 3.1.1–3.4.2). For the same locus of the same sample, the amplification efficiencies were similar based on their average peak heights. Peak heights were similar except for one sample (cells from a cigarette butt), which had average peak heights of 6345 and 4001 for PowerPlex21 and EX20+4, respectively (Fig. 2).

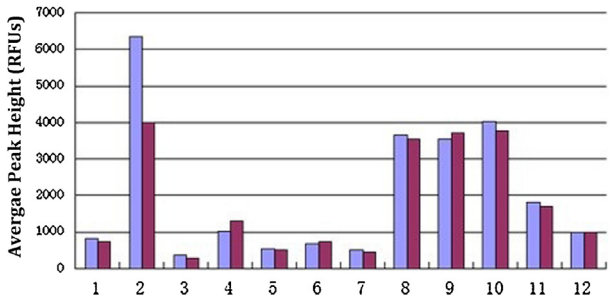

Fig. 2. Average peak heights of the PowerPlex21 kit and the EX20+4 System. Sample 1 is a bloodstain from a sheet, sample 2 is cells from a cigarette butt, samples 3, 6, and 7 are DNA from three costal cartilage bones, samples 4 and 5 are epithelial fractions from two underwear, samples 8–10 are three indicating FTA® Cards. Sample 11 is a bloodstain on the wall, and sample 12 is a bloodstain on the ground.

3.7. Electrophoresis sizing and stutter calculations

Sizing precision is critical for accurate genotyping and it was determined by running allelic ladder standards on multiple CE instruments. To determine the sizing precision of this system, an allelic ladder with internal ladder standard (ILS) was injected into 16 capillaries on a 3130xl Genetic Analyzer. The average base pair size and standard deviation were calculated for every allele. The largest standard deviation observed was 0.078, the lowest was 0.028 (Fig. 3), which was well below the target specification of 0.15 bases.

To evaluate the sizing accuracy, the size differences between sample alleles and allelic ladder alleles were investigated with 42 samples. The results showed that 98.13% alleles were sized within ±0.25 bases of their corresponding allelic ladder peaks and all sample alleles were within ±0.5 bases, which supports that there was low risk of alleles being mistyped due to insufficient sizing accuracy.

Stutter peaks are amplification by-products which are likely due to slippage [10–12]. In this study, stutter ratios were estimated from DNA samples from 130 different individuals. The average stutter plus three standard deviations were used to set the stutter filter threshold (Table 3).

3.8. Population and concordance studies

Population studies were performed with samples from 500 unrelated individuals (250 males and 250 females) of the Guangdong Han population that were collected with informed

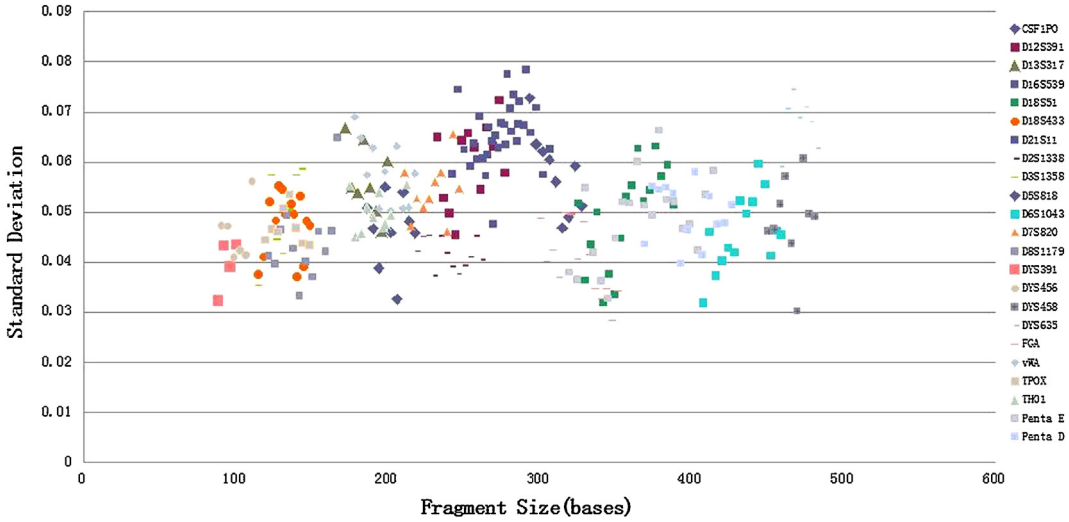

Fig. 3. Precision across 16 injections of the EX20+4 allelic ladder mix on a 3130 series instrument.

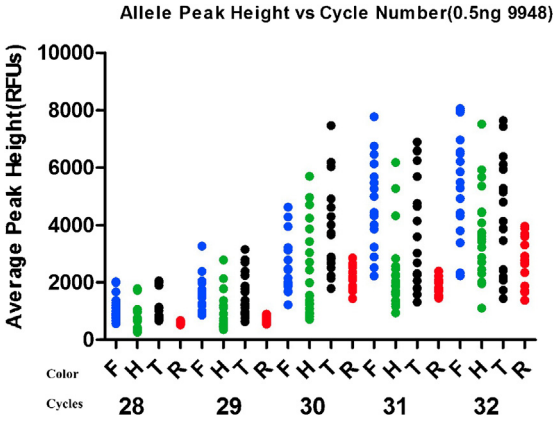

Fig. 4. Average peak height of different locus versus the number of cycles for PCRs with 0.5 ng of 9948 genomic DNA. Dye colors are FAM ("F"), HEX ("H"), TAMRA ("T"), and ROX ("R"). Each data point in that dye channel represents a different locus within the dye channel.

consent. The blood samples were maintained on blood FTA® Cards (GE Healthcare/Whatman). The related genetic parameters of 19 autosomal STR loci of EX20+4 System are shown in Supplementary Table 1. The TDP of the 19 autosomal STR loci was above 0.999999999 and the CPE was 0.999999985. There were 148 different Y-STR haplotypes among the 250 males and the haplotype diversity was 0.592.

The design of the EX20+4 System conserves the proven primer-binding sites from previously released AGCU STR systems. High confidence was placed in the EX20+4 System because of existing concordance data. However, concordance testing was performed with 200 blood FTA® Card (GE Healthcare/Whatman) samples, including 100 males and 100 females. These samples were collected with informed consent from 200 donors of the Guangdong Han population. Direct amplification was performed using a 1.2-mm punch. All 200 samples were concordant between the EX20+4 and PowerPlex®21 systems.

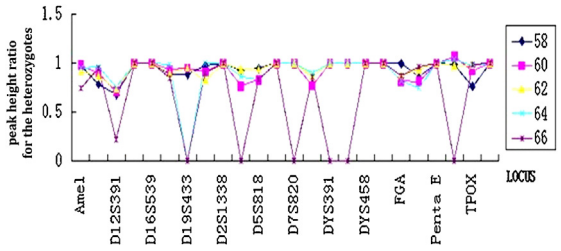

Fig. 6. The peak height ratio for the heterozygotes at 24 loci. The order in the graph is amelogenin, CSF1PO, D12S391, D13S317, D16S539, D18S51, D19S433, D21S11, D2S1338, D3S1358, D5S818, D6S1043, D7S820, D8S1179, DYS391, DYS456, DYS458, DYS635, FGA, Penta D, Penta E, TH01, TPOX, and vWA (not all loci are displayed in the graph). The ratio of 0 represented dropout of a locus which was (from left to right) D19S433, D3S1358, D7S820, DYS391, DYS456, and TH01.

3.9. PCR conditions studies

The PCR-based procedure studies included the test of reaction components, cycling number, annealing temperature, and final extension time. Concentrations of primer, DNA polymerase, and magnesium are important factors for consistent and robust results. The EX20+4 System primer mix was optimized to achieve the best balance and performance. Taq polymerase is included in the kit for its thermal stability and demonstrated application for STR typing. As an important cofactor for proper functioning of Taq polymerase, magnesium, as well as Tris–HCl, KCl, deoxynucleotide triphosphates (dNTPs), and bovine serum albumin (BSA) are included in the master mix, and the concentration cannot be changed unless the master mix is not properly mixed or chelated. As pipetting errors may cause fluctuations in the primer master mix and Taq polymerase concentrations, 0.5 ng 9948 was amplified with 0.5×, 0.75×, 1×, 1.25×, 1.5× master mix, primer, and Taq polymerase. The results showed that full profiles were obtained except at 0.5× master mix and 0.5× primer concentration. A significant reduction in signal at D16S539, D2S1338, D7S820, and Penta D at 0.75× primer occurred, as well as at D16S539, D2S1338, D7S820, vWA

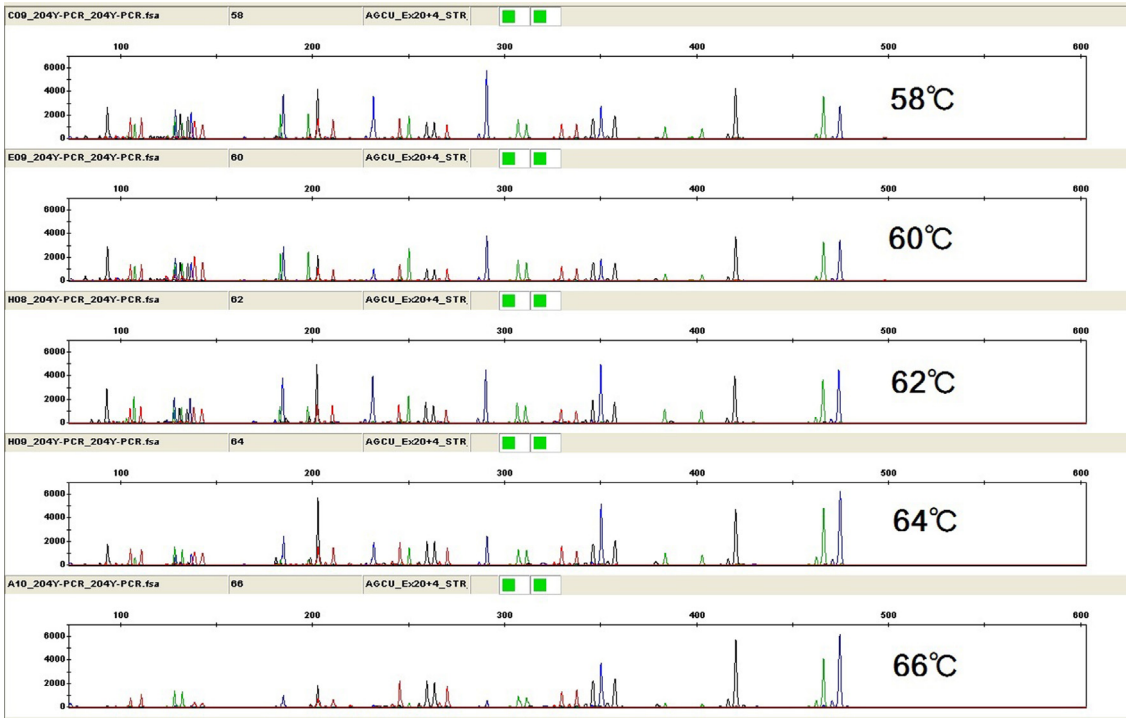

Fig. 5. Electropherograms from the PCR anneal-extend temperature study.

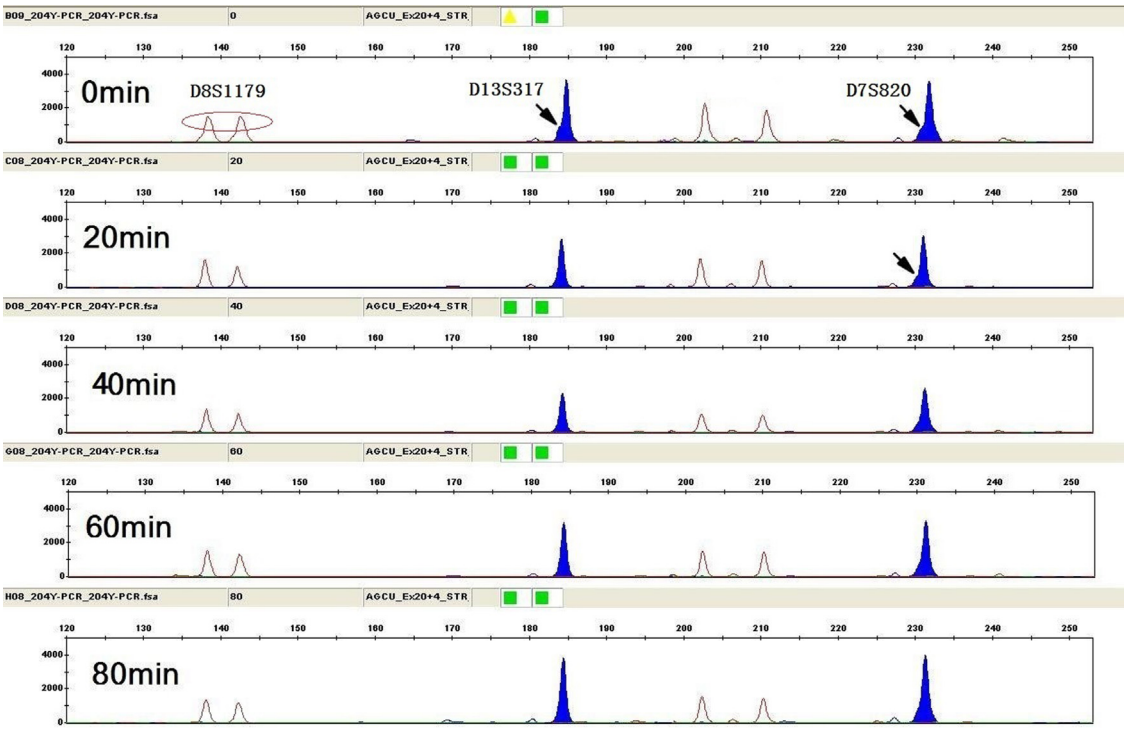

Fig. 7. Effects of shortening the final extension time after normal thermal cycling for 1 ng 9948. The black arrows show the minus A shoulder peaks for the D13S317 and D7S820 loci. Broad peaks occurred for the D8S1179 under the condition of 0 min.

and CSF1PO at 0.75× master mix. When 0.5 ng 9948 was amplified with 0.5× Taq polymerase, the average peak height was 914 RFU. When it was 0.75×, the average peak heights for the master mix was 658 RFU, 596 RFU for the primer, and 952 RFU for Taq polymerase. When it was 1.25×, the average peak heights in order were 1106 RFU, 1250 RFU, and 1454 RFU. With the altered concentration +50%, the average peak heights were 1128 RFU, 1480 RFU, and 1729 RFU in the same order. Therefore, the EX20+4 System is consistent and robust and tolerates various component concentrations (Supplemental Figs. 4–6).

The recommended optimal cycling number of the EX20+4 System is 30 cycles. As much as 500 pg 9948 and 9947a were amplified at 28 (initial 10 cycles with second 18 cycles), 29 (initial 10 cycles with second 19 cycles), 30, 31 (initial 10 cycles with second 21 cycles), and 32 (initial 10 cycles with second 22 cycles) cycles. Fig. 4 shows the mean peak heights for the 9948 DNA amplified for different cycle numbers. As expected, peak heights increased with additional cycles. Off-scale allele peaks were observed at 31 and 32 cycles (Supplemental Fig. 7).

Annealing temperature was tested in increments of 2 °C: 58 °C (58 °C for initial 10 cycles, then 56 °C for last 20 cycles), 60 °C (60 °C for initial 10 cycles, then 58 °C for last 20 cycles), 62 °C (recommended), 64 °C (64 °C for initial 10 cycles, then 62 °C for last 20 cycles), and 66 °C (66 °C for initial 10 cycles, then 64 °C for

Table 3
Observed stutter ratios per locus and recommended stutter filter threshold.

Locus	Min	Max	Stutter mean	SD	Recommended filter threshold
CSF1PO	2.61%	13.8%	8.34%	1.96%	0.1423
D6S1043	5.91%	14.81%	9.62%	1.87%	0.1524
D5S818	5.31%	15.47%	9.81%	2.47%	0.1724
D3S1358	10.01%	16.47%	12.99%	1.47%	0.1741
D7S820	3.04%	11.13%	6.89%	1.79%	0.1227
D2S1338	8.16%	17.90%	12.44%	2.10%	0.1874
D21S11	7.55%	17.60%	11.54%	1.91%	0.1728
D19S433	7.20%	16.35%	10.53%	2.22%	0.1718
D18S51	6.30%	15.59%	10.06%	2.33%	0.1705
D16S539	4.74%	12.53%	8.01%	2.12%	0.1437
D13S317	2.16%	11.65%	6.39%	2.26%	0.1318
D12S391	7.73%	18.53%	13.37%	2.47%	0.2078
vWA	3.23%	16.95%	11.48%	2.98%	0.2040
TH01	1.48%	6.21%	3.65%	1.19%	0.0722
TPOX	2.11%	9.79%	4.45%	1.50%	0.0894
Penta E	3.56%	12.99%	6.89%	2.37%	0.1400
Penta D	1.21%	3.90%	2.48%	0.66%	0.0445
FGA	4.94%	14.49%	9.55%	1.97%	0.1544
D8S1179	5.88%	17.76%	10.65%	2.78%	0.1898
DYS391	7.15%	14.67%	8.93%	1.42%	0.1319
DYS456	10.44%	19.62%	16.18%	2.66%	0.2417
DYS458	7.60%	13.52%	10.66%	1.49%	0.1513
DYS635	4.76%	11.89%	7.44%	2.05%	0.1358

last 20 cycles; Fig. 5). The peak heights dropped at 60 °C and showed a marked decrease at 66 °C (Supplemental Fig. 8). Incomplete profiles with multiple allele dropouts were observed for replicates at 64 °C and 66 °C. The D19S433 locus failed to be typed at both 64 °C and 66°C, the D3S1358, D7S820, DYS391, DYS456, and TH01 loci dropped out at 66 °C. For all tested samples, the peak height ratios for intra-locus balance was greater than 74% at 62 °C (Fig. 6), was 71% at 60 °C, and was 69% at 58 °C. At 62 °C, the peak height ratios for inter-locus balance were 78%, 80%, 49%, and 73% for loci with fluorescent fluorescein amidite (FAM), HEX, TAMRA, and ROX, respectively. The peak height ratios for balance between dye channels of inter-color balance were 44%. The results showed that the system performs well at 62 °C.

The final hold time was tested with 0 min, 20 min, 40 min, 60 min (recommended), and 80 min. As Taq polymerase has the tendency of adding an extra, non-template nucleotide at the 3′ ends of DNA strands during thermal cycling [13], sufficient final extension time is important to adenylate the fragments. One nanogram of 9948 performed well at all 40 min and 60 min extension times, but the D13S317 locus displayed small minus A peaks under the condition of 0 min and the D7S820 locus showed small minus A peaks under the conditions of 0 min and 20 min. Shoulders were detected in the direct amplification samples at 0 min, 20 min, and occasionally at 40 min. A final extension time of 60 min (or more) performed best among all tested extension times. Broad peaks were seen at the D8S1179 locus under the conditions of 0 min. Based on these results, 60 min was chosen to be the appropriate final extension time (Fig. 7).

4. Conclusion

The intent was to develop a sensitive multiplex kit for forensic laboratories with increased DP for single-source comparisons, mixtures, and kinship analyses as well as detecting male-only components in the samples. These developmental validation studies tested the EX20+4 System following the SWGDAM guidelines and Chinese criteria. The test results demonstrated that the system is robust regarding changes in annealing temperature, exposure to PCR inhibitors, sensitive, and accurately types mixed samples. In conclusion, the EX20+4 System is a robust and reliable system for analyzing both forensic casework and database samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsigen.2014.03.001.

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