

Developmental validation of a 6-dye STR kit with 27 loci

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Abstract The EX27 System is a 6-dye (i.e., FAM, HEX, TAMAR, ROX, VIG, and SIZ-500) kit that combines the 24 autosomal STR loci (i.e., D3S1358, D13S317, D7S820, D16S539, D1S1656, Penta E, TPOX, TH01, D2S1338, CSF1PO, Penta D, D10S1248, D19S433, vWA, D21S11, D18S51, D6S1043, D8S1179, D5S818, D12S391, FGA, D22S1045, D2S441, SE33) with a Y-STR locus (DYS391), an Indel site (YIndel) and Amelogenin, which is designed to amplify DNA from extracts as well as direct amplification from substrates. The new system contains most of the widely used autosomal STR loci around the world and is sensitive to the male component in mixtures and is more accurate in gender determination owing to the DYS391 and YIndel.

To demonstrate the suitability for personal identification and parentage testing, the system was validated for sensitivity of detection, species specificity, inhibitor tolerance, DNA mixtures, reproducibility study, and concordance studies based on the SWGDAM developmental validation guidelines.

In view of the results, the EX27 System is a robust and reliable amplification kit which can be used for human identification testing.

Keywords Forensic Science · Short tandem repeat (STR) · 6-dye multiplexing system

Introduction

Within the past few years, a variety of commercial kits have been developed. As different regions usually choose different loci which are suitable for their own population, data sharing and comparison are becoming a problem that needs to be paid attention to. In 2012, the CODIS Core Loci Working Group recommended 25 STR loci as Expanding CODIS, focusing on facilitating greater discrimination, assisting missing person

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investigations, and promoting compatibility for international data sharing [1]. However, multiplexing such a great number of STR loci in a traditional 5-dye system seems difficult, so we conceived the idea of developing a 6-dye fluorescent multiplex STR with high tolerance which include more STR loci as comprehensive as possible [supplemental Table 1]. The kit is designed to be more sensitive to male component in mixtures and be more accurate in gender determination, even with Amelogenin-Y dropout [2].

In this study, we developed and validated the EX27 System. This is a 6-dye multiplex system that enables typing of 24 autosomal STR loci, a Y-STR loci, an Indel site, and Amelogenin. The blue channel consists of fluorescein-labeled STR amplicons for the D3S1358, D13S317, D7S820, D16S539, D1S1656, and Penta E loci. The green channel includes DNA fragments labeled with HEX for the TPOX, TH01, D2S1338, CSF1PO, Penta D, and D10S1248 loci. The yellow channel consists of amplification products for the D19S433, vWA, D21S11, D18S51, and D6S1043 loci which are labeled with TAMRA. The amplified products for Amelogenin, D8S1179, D5S818, D12S391, and FGA loci are in the red channel and labeled with ROX. The DNA fragments labeled with VIG for the D22S1045, YIndel, D2S441, DYS391, and SE33 loci are consisted in the purple channel. Fragments included in the internal lane standard are detected in the orange channel and are labeled with SIZ-500 (Supplemental Fig. 1).

SWGDM developmental validation guidelines [3] are followed in the development and validation of the EX27 System.

Materials and methods

Samples

The sensitivity studies were performed using male 9948 human genomic DNA (Promega, Madison, WI, USA) which amplified three times in a serial dilution with the following template amounts: 1 ng, 500 pg, 250 pg, 125 pg, 62.5 pg, 31.25 pg.

Species specificity studies were conducted using 500 pg purified DNA from human, 1 ng each of purified DNA from chimpanzee and macaque, which were donated from Guangzhou Zoo (Guangzhou, China), 10 ng each of dog, pig, sheep, chicken, mouse, fish, diatom [4] DNA, which were amassed in Guangzhou Forensic Science Institute over years, 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. All of them were provided by the

Institute of Human Virology of Sun Yat-Sen University (Guangzhou, China) and a negative control. The quantity was determined by NANODROP 2000 Spectrophotometer (Thermo Scientific, USA).

Six common forensic inhibitors, including hematin, indigo, calcium ion, humic acid, EDTA, and hemoglobin, were tested in stability studies. The male 9948 human genomic DNA template quantity was held constant at 500 pg while the concentration of inhibitors was varied: 0, 50, 100, 150, or 200 μ M of hematin; 0, 5.0, 7.5, 10.0, or 15.0 mM of indigo; 0, 1.0, 2.0, 3.0, or 4.0 mM of calcium ion; 0, 10, 20, 30, or 50 μ g/ml of humic acid; 0, 0.5, 0.8, 1.0, or 1.2 mM of EDTA; and 0, 300, 500, 800, or 1000 μ M of hemoglobin.

Mixture studies were tested by two sets: 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 the same ratio. These samples were formulated at a concentration of 1 ng in 10 μ l, and each of them was amplified in triplicate.

Casework samples were tested at Guangzhou Forensic Science Institute and Forensic Identification Institute of the Third Affiliated Hospital of Guangzhou Medical University. These samples include blood stains, bones, epithelial fractions, hairs, amniotic fluid, underfur, and buccal Indicating FTA® Cards. Buccal Indicating FTA® Cards were amplified directly while others were extracted by QIAamp® DNA Investigator Kit (QIAGEN GmbH, Germany).

Reproducibility samples included buccal Indicating FTA® Cards, blood FTA® Cards (GE Healthcare/Whatman), and extracted hair DNA from one male donor. The hair DNA was extracted by QIAamp® DNA Investigator Kit (QIAGEN GmbH, Germany), and the quantity was determined by NANODROP 2000 Spectrophotometer (Thermo Scientific, USA).

Stutter calculation study contains 100 extracted DNA samples which were amplified and analyzed on a 3130xl Genetic Analyzer. These samples were collected with informed consent from 100 male donors of Guangdong Han population. These samples were directly amplified by blood FTA® Cards (GE Healthcare/Whatman). These samples were a subset of the 200 population study samples.

Population studies were performed with 200 unrelated individuals (100 males and 100 females) from Guangdong Han population which were collected with informed consent. The blood samples were maintained on blood FTA® Cards (GE Healthcare/Whatman).

DNA amplification

Amplifications were performed on GeneAmp® PCR System 9700 thermal cycler (Life Technologies) using the max mode on a gold plated block. Two PCR amplification reactions were

tested: for the population studies, each amplification reaction contained 4 μl of reaction mix, 2 μl of EX27 primers, and 0.4 μl of C-Taq DNA polymerase with up to 3.6 μl of template; for other studies, each amplification reaction contained 10 μl of reaction mix, 5 μl of EX27 primers, and 1 μl of C-Taq DNA polymerase with up to 9 μl of template. According to the manufacturer's technical manual, unless specifically noted, the following amplification set-up and cycling parameters were used to amplify both extracted DNA and FTA card: initial denaturation of 95 °C for 2 min, 30 cycles of 94 °C for 15 s, 59 °C for 45 s, and 65 °C for 90s, then final extension time for 20 min, and a 4 °C soak. Direct amplification reactions were performed using one 1.2 mm punch from blood FTA® Cards and three punches from buccal Indicating FTA® Cards. Negative and positive controls were included in all experiments.

Electrophoresis and analysis

CE was performed on the 3130xl (16-capillary) Genetic Analyzer using 36 cm capillary arrays with POP-4® Polymer. Reactions were prepared for CE by combining 12.0 μl of Hi-DiTM formamide, 0.5 μl of AGCU Marker SIZ-500, and 1.0 μl of sample or allelic ladder. Samples were denatured for 3 min at 95 °C, then immediately quenched on ice. Standard run conditions involved the following parameters: sample injection for 8 s at 2 kV, electrophoresis at 15 kV for 1500s with the run temperature of 60 °C as indicated in the HID36_POP run module. Spectral resolution was established using the AGCU 6-dye Matrix Standards to assess each fluorescent dye. A peak amplitude of 50 RFU was implemented as the peak detection threshold when analyzing data from CE instruments.

Statistical analysis

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

The sizing precision of STR allele peaks were assessed by calculating the average base pair size and standard deviation of the 16 allelic ladder with ILS (Internal Ladder Standard).

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

Population statistics such as heterozygosity (H), discrimination power (DP), polymorphism information content (PIC),

and power of exclusion (PE) were calculated by Power Stats v1.2 software. Total discrimination power (TDP) and cumulative probability of paternity exclusion (CPE) were calculated according to the formula from article [5].

Results and discussion

PCR condition studies

In the PCR-based procedure studies, we tested reaction components, cycling number, annealing temperature, and final extension time of the EX27 system.

The reaction components of EX27 system involve the Primer mix, Maste Mix, C-Taq, and ddH₂O. As pipetting errors may cause fluctuations, 500 pg 9948 was, respectively, amplified with 0.5 \times , 0.75 \times , 1 \times , 1.25 \times , 1.5 \times Primer Mix, Master Mix, and C-Taq to help us see the influence. Large fragments dropout and off-ladder peaks were common at 0.5 \times (Supplemental Figs. 2–4). The full profiles were obtained at other concentrations.

For the test of cycling number, 500 pg 9948 was amplified at 28, 29, 30, 31, 32 cycles. The average peak height, respectively, was 674, 1153, 2113, 2724, and 3324 RFU (Fig. 1). As expected, peak heights increased with additional cycles. As pull-up peaks were observed at 31 and 32 cycles, the recommended optimal cycling number is 30 (Supplemental Fig. 5).

For annealing temperature test, the experiments were designed in increments of 2, 55, 57, 59, 61, and 63 °C. The average peak height of each was 1664, 1513, 2070, 1019, and 730 RFU (Supplemental Figs. 6 and 7). As the graph of 59 °C showed nice balance and suitable average peak height, we choose 59 °C as recommended temperature.

For final extension time test, 1 ng 9948 was amplified three times, respectively, with the final hold time 0, 10, 20, and 30 min. Under the conditions of 0 min, D13S317, vWA, D21S11, FGA, D22S1045, and YIndel showed small minus A peaks (Supplemental Fig. 8). As minus A peaks [6] sometimes appeared when we amplified directly with FTA cards with the final hold time of 10 and 20 min, 30 min was chosen to be the appropriate final extension time.

Sensitivity studies

Sensitivity study samples were tested with a serial dilution of following template amounts: 1 ng, 500 pg, 250 pg, 125 pg, 62.5 pg, and 31.25 pg. Each template amounts were amplified three times in three different 9700 thermal cycler. Full profiles were obtained with 9948 from 62.5 pg to 1 ng by the threshold of 50 RFU. Allele CSF1PO and TPOX dropouts were observed with 31.25 pg; obviously, the dropout rates were nominal. Though the new-building 6-sye system got full profiles with 62.5 pg, the peak height of YIndel, SE33, D8S1179,

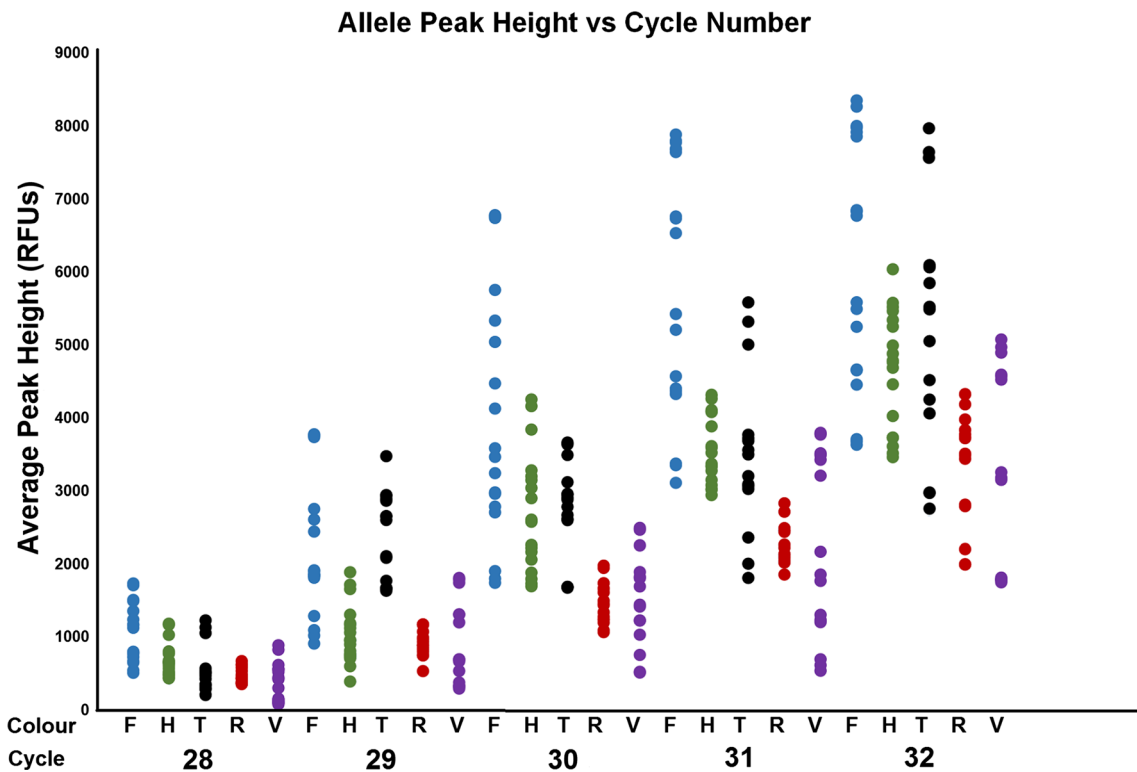


Fig. 1 Average peak height per dye channel vs the number of cycles for PCRs with 0.5 ng of 9948 genomic DNA. Dye colors are FAM (“F”), HEX (“H”), TAMRA (“T”), ROX (“R”), and VIG (“V”)

D5S818, D2S441, D12S391, TPOX, and FGA loci was detected below 100 RFU. The percentage of complete profiles is shown in Fig. 2.

Species specificity

Ten kinds of non-human genomic DNA samples were tested for cross-reactivity with the EX27 system. Excluding the chimpanzee and macaque, which showed expected

cross-reactivity, no peaks were detectable for the nonprimate mammals, fish, or the microbial samples. The result of the diatom showed one peak at 120 bases with the height of about 1500 RFU in the green channel and the other peak at 125 bases with the height of 498 RFU in the purple channel. Diatom widely exists in the air and could be checked out in the liver and lung in drowned corpse (Supplemental Fig. 9). Though the two peaks are non-specific amplifications, we still need to pay attention when testing the drown bodies.

Fig. 2 Percentage of complete profiles of 9948 human genomic DNA by threshold of 100 and 50 RFU

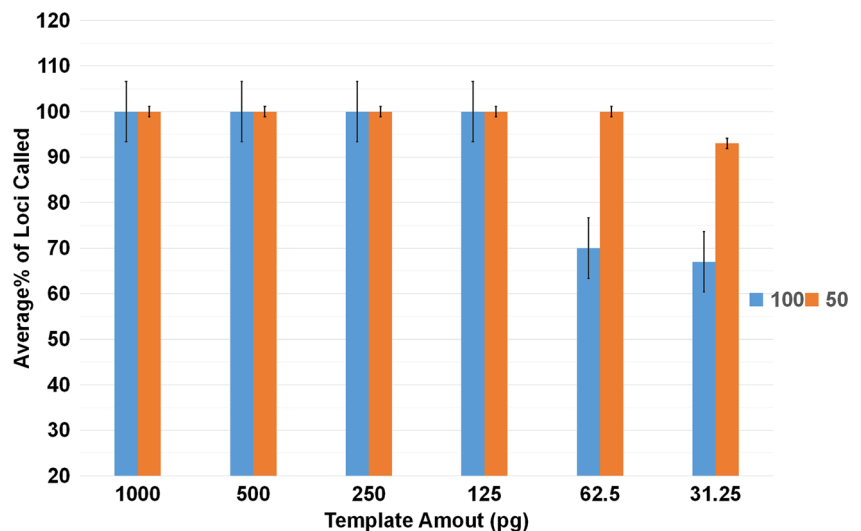


Table 1 Stability studies

Hematin	Concentration	0 μ M	50 μ M	100 μ M	150 μ M	200 μ M
	Call rate (%)	100	100	96.3	29.6	0
Indigo	Concentration	0 mM	5 mM	7.5 mM	10 mM	15 mM
	Call rate (%)	100	100	100	55.5	0
Calcium ion	Concentration	0 mM	1 mM	2 mM	3 mM	4 mM
	Call rate (%)	100	100	92.6	48.1	37
Humic acid	Concentration	0 ng/ μ l	10 ng/ μ l	20 ng/ μ l	30 ng/ μ l	50 ng/ μ l
	Call rate (%)	100	96.3	77.8	66.7	48.1
EDTA	Concentration	0 mM	0.5 mM	0.8 mM	1 mM	1.2 mM
	Call rate (%)	100	100	100	100	74.1
Hemoglobin	Concentration	0 μ M	300 μ M	500 μ M	800 μ M	1000 μ M
	Call rate (%)	100	100	77.8	55.6	0

The concentration of the inhibitors added per reaction and the corresponding percentage of the alleles called

Stability studies

Six common inhibitors (i.e., hematin, indigo, calcium ion, humic acid, EDTA, and hemoglobin) were tested with the EX27 system [7–9]. When added nothing, full profiles were obtained. With 50 μ M of hematin; 5 and 7.5 mM of indigo; 1 mM of calcium ion; 0.5 mM, 0.8 mM, and 1 mM of EDTA; and 300 μ M of hemoglobin, we also got full profiles. When the concentration of hematin was raised to 200 μ M, complete dropout occurred, the same is true for the 15 mM of indigo, 1000 μ mol/l of hemoglobin (Table 1). We compared the inhibitor tolerance for other products from Thermo, Promega, and Qiagen [10–12]. For EDTA, the EX27 kit showed a similar performance with GlobalFiler® express kit (Thermo Fisher Scientific, USA) and the Investigator 24plex QS Kit (Qiagen, Germany), for hematin, indigo, calcium ion, and humic acid, the GlobalFiler® express kit and the Investigator 24plex QS Kit showed better tolerance. As PowerPlex® Fusion System (Promega, USA) used different DNA template for validation, it is unable to compare these two kits.

Mixture studies

Male/male mixtures and male/female mixtures were prepared and tested with the ratio of 1:1, 1:4, 1:9, or 1:19. All alleles were called for the 1:1 and 1:4 mixtures. At the 9:1 ratio, 81.5–88.9% of all minor contributor alleles were detected with all six PCR replicates. For the 1:19 mixtures, some autosomal STR alleles dropped out, while no amelogenin, DYS391, or YIndel dropout were found. The call out rates were among 66.7 and 77.8% (Table 2).

Case samples

Guangzhou Forensic Science Institute tested the blood stains, bones, and epithelial fractions. Forensic Identification

Institute of the Third Affiliated Hospital of Guangzhou Medical University tested the amniotic fluid, underfur, and buccal indicating FTA® Cards (Supplemental Fig. 10). All these samples got accurate and balance profile.

Reproducibility

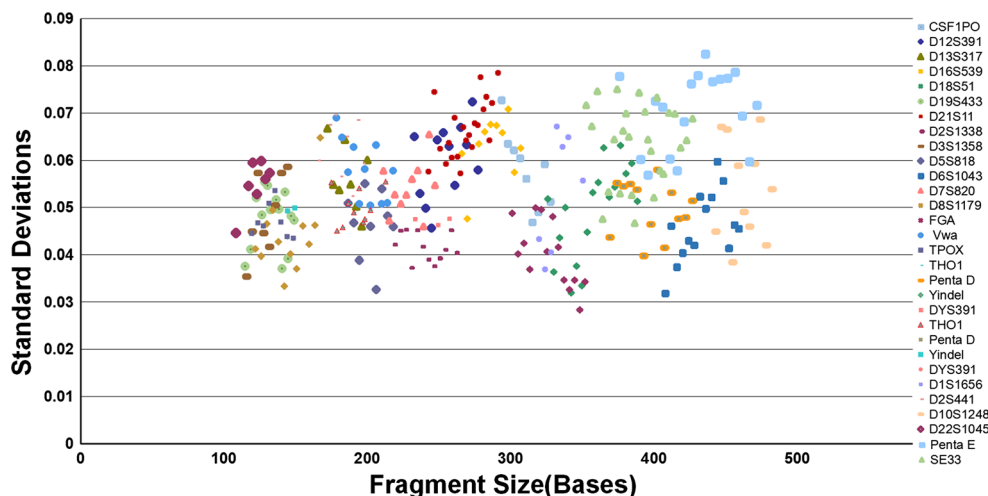
Three laboratories participated in the reproducibility study which aimed to determine if profiles are reliable and suitable for comparison among laboratories when using the same DNA sample. Each participating laboratory amplified buccal indicating FTA® Cards, blood FTA® Cards, and extracted DNA from hairs. The results show that loci from different samples of the same donor were consistent and same sample got same typing results among the three participating laboratories.

Table 2 DNA mixture studies

Ratio	DNA added to samples		Minor allele count (mean)
	Minor (pg)	Major (pg)	
9948:2800 M = 1:1	500	500	27
9948:2800 M = 1:4	200	800	27
9948:2800 M = 1:9	100	900	22
9948:2800 M = 1:19	50	950	20
9948:9947a = 1:1	500	500	27
9948:9947a = 1:4	200	800	27
9948:9947a = 1:9	100	900	22.5
9948:9947a = 1:20	50	950	20

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 dropout

Fig. 3 Precision across 16 injections of the EX27 allelic ladder mix on a 3130 series instrument



Electrophoresis sizing and stutter calculations

Sizing precision is critical for accurate genotyping, and it was determined by running allelic ladder (Supplemental Fig. 11) standards on multiple CE instruments. To determine the sizing precision of this system, allelic ladder with internal ladder

standard (ILS) was injected on 16 capillaries on a 3130 Genetic Analyzer. The average base pair size and standard deviation were calculated for every allele. The largest standard deviation observed was 0.0784, the lowest was 0.0283 (Fig. 3), which was well below the target specification of 0.15 bp.

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

locus	Min	Max	Stutter mean	SD	Recommended filter threshold
D3S1358	11.01%	16.97%	13.01%	1.54%	0.1763
D13S317	2.16%	10.65%	6.39%	2.21%	0.1302
D7S820	3.43%	11.52%	7.19%	1.97%	0.131
D16S539	4.74%	12.53%	8.21%	2.46%	0.1559
D1S1656	4.23%	10.09%	7.69%	1.65%	0.1264
Penta E	3.06%	12.99%	7.18%	2.77%	0.1549
TPOX	2.42%	10.79%	4.45%	1.58%	0.0919
THO1	1.88%	7.12%	3.49%	1.30%	0.0739
D2S1338	7.16%	16.96%	12.44%	2.36%	0.1952
CSF1PO	2.65%	13.7%	8.34%	1.94%	0.1416
Penta D	1.21%	6.92%	4.48%	0.76%	0.0676
D10S1248	3.14%	11.45%	7.22%	1.90%	0.1292
D19S433	7.26%	16.35%	10.53%	2.23%	0.1722
vWA	3.63%	16.55%	10.48%	3.29%	0.2035
D21S11	6.76%	17.60%	12.54%	1.98%	0.1848
D18S51	6.73%	15.59%	10.36%	2.58%	0.181
D6S1043	5.91%	14.67%	9.62%	1.97%	0.1553
D8S1179	5.88%	17.76%	10.68%	2.76%	0.1896
D5S818	5.13%	15.47%	9.79%	2.51%	0.1732
D12S391	7.83%	18.53%	13.47%	2.52%	0.2103
FGA	4.94%	14.52%	9.65%	2.05%	0.158
D22S1045	8.15%	16.98%	12.33%	2.08%	0.1857
D2S441	5.34%	14.69%	10.98%	1.67%	0.1599
SE33	2.97%	10.12%	7.56%	1.89%	0.1323
DYS391	7.51%	14.76%	8.93%	1.52%	0.1349
YIndel	3.05%	12.12%	8.86%	2.12%	0.1522

Table 4 The forensic population genetic parameters of 24 autosomal STR loci

Marker	H	PIC	DP	PE
D3S1358	0.745	0.674	0.858	0.501
D13S317	0.720	0.755	0.921	0.459
D7S820	0.760	0.729	0.905	0.527
D16S539	0.776	0.753	0.923	0.554
D1S1656	0.799	0.777	0.936	0.624
Penta E	0.898	0.907	0.983	0.791
TPOX	0.658	0.580	0.802	0.367
TH01	0.658	0.623	0.847	0.367
D2S1338	0.857	0.836	0.961	0.709
CSF1PO	0.765	0.688	0.879	0.536
Penta D	0.801	0.785	0.936	0.601
D10S1248	0.786	0.733	0.904	0.573
D19S433	0.806	0.782	0.927	0.610
vWA	0.796	0.752	0.910	0.591
D21S11	0.821	0.798	0.939	0.639
D18S51	0.832	0.841	0.963	0.659
D6S1043	0.811	0.865	0.972	0.620
D8S1179	0.888	0.835	0.956	0.770
D5S818	0.740	0.741	0.913	0.492
D12S391	0.878	0.839	0.959	0.750
FGA	0.883	0.849	0.963	0.760
D22S1045	0.812	0.732	0.903	0.544
D2S441	0.745	0.757	0.921	0.501
SE33	0.939	0.938	0.984	0.884

H heterozygosity, *PIC* polymorphism information content, *DP* discrimination power, *PE* power of exclusion

To evaluate the sizing accuracy, the size differences between sample alleles and allelic ladder alleles were investigated with 42 samples. The results showed that over 95% alleles were sized within ± 0.25 bp of their corresponding allelic ladder peaks and all sample alleles were within ± 0.5 bp, which supports that there was low risk of alleles being mistyped due to insufficient sizing accuracy.

DNA samples from 100 different male individuals were used to estimate the stutter ratios. Stutter peaks are amplification by-products which are likely due to slippage [13]. The average stutter plus three standard deviations were used to set the stutter filter threshold (Table 3).

Population and concordance studies

Population studies were performed with 200 unrelated individuals (100 males and 100 females) from Guangdong Han population that were collected with informed consent. The related genetic parameters such as heterozygosity (*H*), discrimination power (*DP*), polymorphism information content (*PIC*), and power of exclusion (*PE*) of 24 autosomal STR loci

are shown in Table 4. The total discrimination power of the 24 autosomal STR loci was above 0.999999999999, and the cumulative probability of paternity exclusion was 0.999999999943.

As the EX27 System multiplex 24 autosomal STR loci (i.e., D3S1358, D13S317, D7S820, D16S539, D1S1656, Penta E, TPOX, TH01, D2S1338, CSF1PO, Penta D, D10S1248, D19S433, vWA, D21S11, D18S51, D8S1179, D5S818, D12S391, FGA, D22S1045, D2S441, and SE33), DYS391, YIndel, and Amelogenin together, high confidence was placed in the new system because it conserves the proven primer-binding sites from previously released AGCU STR systems. However, five loci (D1S1656, D10S1248, D22S1045, SE33, and Y-Indel) are not contained in the existing concordance data, and further tests will be needed.

Conclusion

The intent of developing a sensitive multiplex kit for forensic testing with increased discrimination power was started from the year 2012. The aim was to facilitate single source comparisons, mixtures, and kinship analyses as well as detecting male only components in the samples. These developmental validation studies tested the EX27 System following the SWGDAM guidelines and Chinese criteria [14]. In conclusion, the new building system provides precise, accurate, and sensitive profiling data.

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