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SHORT COMMUNICATION

Developmental validation study of a 24-plex Y-STR direct amplification system for forensic application

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Abstract

In the present study, validation data for 24 Y-STR loci from the Microreader™ 24Y Direct ID System was presented. Eight Y-STR loci have PCR product sizes with less than 220 bp in this multiplex amplification system, which can better detect degraded DNA samples from a crime scene. Developmental validation studies were conducted following the SWGDAM guidelines and consisted of PCR-based studies, sensitivity testing, species specificity, stability studies, accuracy and reproducibility evaluation, mixture studies, and case-type samples. The genetic diversities and forensic parameters of the 24 Y-STR loci were also investigated in Jiangsu Han population. Results demonstrated that this kit had the characteristics of high detection accuracy, strong species specificity, favorable anti-inhibition effect, and high sensitivity, and the minimum detection amount was 125 pg. When the mixed female template amount was below 3.2 times that of the male, or the male-male mixed ratio did not exceed 1:9, the typing results produced by 24Y Direct System still exhibited a higher discriminating ability for the mixture. The system was compatible with some typical biological samples such as bloodstain, hair, buccal swab, rib cartilage, and nail. The haplotype diversity (HD) and discrimination capacity (DC) of the 24 Y-STR loci were 0.9952 and 0.8500, respectively. The results revealed that the 24 Y-STR loci were highly polymorphic in Jiangsu Han population and could be useful for forensic cases and population genetic studies.

Keywords Forensic genetics · Y-STR · Developmental validation · Jiangsu Han population

Introduction

Short tandem repeat (STR) loci are highly polymorphic genetic marker and are widely used in forensic paternity and individual identification. The STR loci are located on the Y chromosome with the characteristics of male specificity and strict paternal line inheritance, which could compensate for the deficiency of traditional autosomal STR loci to identify some special cases such as sexual assault [1]. Among the lineal and collateral consanguinity of the same paternal family, the alleles of Y-STR usually have little change through generations [2]. So, the Y-STR analysis plays an important role in tracing patrilineal migration history, identifying missing persons, population evolution study, inferring population structure, database construction, and other male-related cases [3–5]. So far, multitudinous commercial Y-STR kits have been built based on diverse research values and practical purposes. Technically, the standard operating procedures of DNA analysis methods may be affected by the practical requirement variance; therefore, forensic application value estimation must be verified by a series of validation experiments before addressing those kits to forensic application. In this study, we presented the developmental validation data of the Microreader™ 24Y Direct ID System (Suzhou Microread

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Genetics Ltd., Jiangsu, China), hereinafter referred to as 24Y Direct System, which contains 24 Y-STR loci labeled by four fluorescent dyes, and allows for forensic case investigation and database construction. All amplification products at the 24 Y-STR loci are less than 440 bp, and 8 of them are less than 220 bp, which can better detect degraded DNA samples. The general information and allelic ladder profile of the 24Y Direct System are shown in Online Resources 1 and 2. The 24Y Direct System performs PCR amplification directly without DNA extraction, reducing the possibility of DNA contamination and saving the time of DNA analysis. Previous studies proved that direct PCR amplification showed a broad prospect for forensic applications [4, 6–10]. To assess the reliability and determine the limitations of 24Y Direct System, developmental validation studies were carried out follow the update guidelines described by the Scientific Working Group for DNA Analysis Methods (SWGDAM) [11], including PCR-based studies, sensitivity studies, species specificity, stability studies, accuracy and reproducibility evaluation, mixture studies, case-type samples, and population studies.

Materials and methods

Sample preparation

Blood samples of 200 unrelated male volunteers were collected from Han population in Jiangsu Province, China, applying the principle of informed consents. Saliva samples of six male animals including dog, cow, sheep, pig, chook, and donkey were preserved in our laboratory. Common biological samples in the crime scenes such as rib cartilage, bloodstain, buccal swab, hair, gauze bloodstain, tooth, nail, cigarette butt, and saliva were selected from the Center for Forensic Science of Southern Medical University and were utilized for simulated case-type sample studies. One female DNA F312 and two male DNA M308 and M4615 were used for other developmental validation experiments. Meanwhile, the female sample of F312 was also treated as a negative control sample. The study strictly complied with the human and ethical research principles of Southern Medical University, Guangdong Province, China.

PCR amplification and capillary electrophoresis

Bloodstain sample (or template DNA) was amplified by the 24Y Direct System on GeneAmp PCR system 9700 (Thermo Fisher Scientific, Waltham, MA, USA). The 10-μL total volume, as described in the 24Y Direct System User Guide, consisted of 1 μL template DNA or about 1.2 mm^2 of bloodstain sample, 4 μL of 2.5× buffer, 2 μL of 5× primer mix, and 0.2 μL of Taq DNA polymerase II, and then nuclease-free water was added up to 10 μL. The thermal cycling parameter was set as 96 °C for 2 min, 27 cycles of 94 °C for 5 s, 60 °C for 70 s, and one cycle of 60 °C for 30 min, and then held at 4 °C. Then, 1 μL PCR product or allelic ladder mixed with 0.5-μL size standard Org500 and 8.5 μL Hi-Di™ deionized formamide (Thermo Fisher Scientific) were denatured at 95 °C for 3 min and rapidly cooled for 3 min. Finally, the mixture was genotyped on Applied Biosystems® 3500xL Genetic Analyzer (Thermo Fisher Scientific). GeneMapper ID-X software (Thermo Fisher Scientific) was used to analyze electrophoresis results with the threshold of 50 relative fluorescence unit (RFU) peak amplitude. Except for the different reaction volume studies, other validation experiments were conducted based on the 10-μL reaction volume.

Sensitivity studies

Sensitivity studies were conducted using the M308 (1 ng/μL) amplified with the following template amounts: 2 ng, 1 ng, 500 pg, 250 pg, 125 pg, 62.5 pg, 31.25 pg, and 15.63 pg, respectively.

Stability studies

Four kinds of inhibitors EDTA, hematin, tannin, and humic acid (all from Sigma-Aldrich, Shanghai, China) were utilized in stability studies. One microliter of DNA template of M308 (1 ng/μL) was amplified in accordance with the 24Y Direct System manual, but amplification reaction system was added with various concentrations of inhibitors EDTA (0 μM, 0.5 μM, 0.75 μM, 1.0 μM, 1.5 μM, and 1.75 μM), hematin (0 μM, 100 μM, 300 μM, 500 μM, 700 μM, 900 μM, and 1200 μM), tannin (0 ng/μL, 50 ng/μL, 100 ng/μL, 150 ng/μL, 200 ng/μL, 250 ng/μL, and 300 ng/μL) or humic acid (0 ng/μL, 20 ng/μL, 40 ng/μL, 60 ng/μL, 80 ng/μL, 100 ng/μL, and 150 ng/μL).

Species specificity studies

Species specificity studies of this 24Y Direct System were performed using DNA samples extracted from saliva of dog, cow, sheep, pig, chook, and donkey. Genomic DNA was extracted with QIAamp DNA Investigator Kit (QIAGEN, Hilden, Germany).

Mixture studies

Male:male DNA mixtures were created using M308 and M4615 with the mixture ratios as follows: 1:1, 1:3, 1:9, and 1:19. For male:female DNA mixtures, M308 and F312 were mixed in two different ways. First, the quantity of F312 (400 pg) remained unchanged, while amounts of M308 were decreased from 500 pg, 250 pg, 125 pg, 62.5 pg, 31.25 pg to 15.63 pg. Second, the quantity of M308 was hold constant at

500 pg, while amounts of F312 were increased from 100 pg, 200 pg, 400 pg to 800 pg.

Accuracy and reproducibility evaluation

To qualitative evaluation of accuracy of the 24Y Direct System, M308 was amplified with this system in two different laboratories by different operators.

PCR-based studies

The PCR-based studies were determined by reaction volumes (25 μL, 12.5 μL, and 6.25 μL), cycle numbers (25, 26, 27, 28, and 29 cycles), annealing temperature (56 °C, 58 °C, 60 °C, 62 °C, and 64 °C), 2.5× buffer (2 μL, 3 μL, 4 μL, 5 μL, and 6 μL), 5× primer mix (1 μL, 1.5 μL, 2 μL, 2.5 μL, and 3 μL), and Taq DNA polymerase II amounts (0.1 μL, 0.15 μL, 0.2 μL, 0.25 μL, and 0.3 μL).

Case-type sample studies

Ten different type samples commonly encountered in crime scenes, such as rib cartilage, bloodstain, buccal swab, hair, gauze bloodstain, tooth, nail, cigarette butt, saliva, and epithelial (or exfoliated cells) abrasion, were typed by 24Y Direct System, respectively. The specimens of rib cartilage and bloodstain were from the same individual, and saliva and cigarette samples were also from another individual. Epithelial abrasion (or exfoliated cells) samples were collected by rubbing with sterile cotton swab, and details of these samples can be seen in Online Resource 3. Genomic DNA of rib cartilage, tooth, nail, cigarette and epithelial abrasion was extracted with QIAamp DNA Investigator Kit.

Population study and statistical analysis

The genetic diversities and forensic parameters of these 24 Y-STR loci were determined by 200 unrelated healthy male individuals of Han population from Jiangsu Province, China. Allelic frequencies for 23 single-copy loci and the observed genotype numbers of DYS385a/b were analyzed by Genepop version 4.0 [12]. Haplotype numbers for the 24 Y-STR loci were measured by the Arlequin version 3.5 [13]. Genotype frequencies and haplotype frequencies were carried out with direct counting method. The genetic diversity (GD) at each locus and haplotype diversity (HD) are computed with the formula: GD or $HD = n\ (1 - \sum p_i^2)\quad (n-1)$ [14]. The match probability (MP) and discrimination capacity (DC) are operated with computational formula as $MP = \sum p_i^2$ and $DC = h/n$, respectively [15]. The n, p_i, and h represent the sample size, the frequency of the ith allele or haplotype, and the total number of distinct haplotypes, respectively.

Results and discussion

Sensitivity studies

Sensitivity study aims to evaluate the ability of the 24Y Direct System to obtain reliable profiles from different amounts of DNA samples and determine the minimum detectable template amount in the system. In the present research, we amplified M308 for sensitivity studies by using 8 different DNA template amounts.

As shown in Fig. 1 and Online Resources 4 and 5, with the template amounts decreased gradually from 2 ng to 15.63 pg, the mean peak height dropped from 2332.72 to 70.8 RFU. When the amount of template was greater than 125 pg, full DNA profiles (24 loci) were obtained. Nevertheless, 19 loci were obtained when the template amount decreased to 62.5 pg, with a mean height peak of 93.63 RFU. As the template DNA dropped to 31.25 pg, 15 loci were called with an average peak height of 81.34 RFU, and then 12 loci were observed at the template quantity of 15.63 pg with the mean peak height at 70.8 RFU. Therefore, the minimum detectable amount of 24Y Direct System was 125 pg when the PCR reaction was 10-μL total volume.

Stability and species specificity studies

Due to the influences of various chemical components and environmental factors, the samples collected from crime scenes were susceptible to contamination and degradation. In general, the presence of inhibitors at a relatively high concentration could interfere with the PCR reaction, resulting in inefficient PCR amplification and inability to obtain the full DNA profile. To determine tolerance of the 24Y Direct System to these factors, M308 was mixed with four different kinds of inhibitors EDTA, hematin, tannin, and humic acid, respectively, and amplified with the 24Y Direct System.

Full DNA profiles were obtained with less than 1 μM of EDTA, 500 μM of hematin, 150 ng/μL of tannin, and 80 ng/μL of humic acid, and the peak heights of the alleles reduced

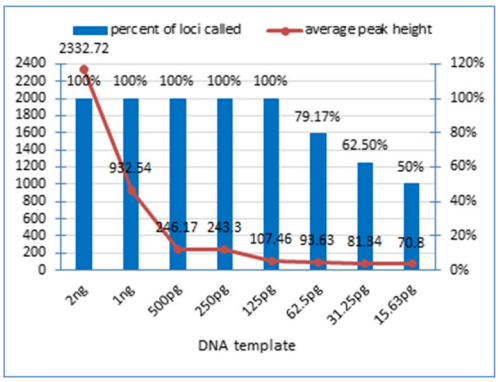

Fig. 1 Sensitivity study results of template DNA ranging from 2 ng to 15.63 pg

gradually as the inhibitor concentration increased. Almost no alleles were observed when the EDTA, hematin, tannin, and humic acid were over 1.75 μM, 900 μM, 250 ng/μL, and 150 ng/μL, respectively (Online Resource 6).

In addition, non-human samples are also encountered frequently in crime scenes. To determine the cross-reactivity of the 24Y Direct System in non-targeted species, non-human DNA samples from dog, cow, sheep, pig, chook, and donkey were typed with this system. Results showed that no specific peaks were observed in these animals with the threshold of 50 RFU peak amplitude (Online Resource 7), which suggested that the 24Y Direct System displayed good species specificity.

Mixture studies

Certainly, mixtures of multiple male samples or female and male are commonly encountered in forensic crime scenes. Usually, only one allele can be detected at single-copy Y-STR locus. When two or more alleles are observed at several single-copy Y-STR loci in the profile of a biological sample, it indicates that the sample is mixed probably by two or more than two males' DNA. Therefore, identifying the numbers of contributors in the mixtures and defining the minor and major contributor genotyping are the challenges to assisting case investigation. In order to evaluate the capability of the 24Y Direct System to detect mixed samples, M308 and M4615 were mixed in various ratios to prepare the artificial male mixtures. The respective typing results of M308 and M4615 were summarized in Online Resource 1. We also produced two sets of artificial mixture samples of female and male, which were intended to assess the influence of the presence of female DNA templates on the sensitivity of the 24Y Direct System. Then, all mixtures were amplified with the 24Y Direct System, respectively.

For the mixtures of male DNA (Table 1 and Online Resource 8), the loci called percent of minor contributor were calculated from the other 16 loci since M308 and M4615 had 8 identical genotypes, respectively. All 16 alleles were called when the M308 and M4615 ratios at 1:1 and 1:3. But the peak heights of the two contributors were similar at the mixture ratios of 1:1, making it difficult to distinguish between major contributor and minor contributor profiles. When the mixing ratio was at 1:9, the allelic peak heights of minor contributor remarkably decreased and some alleles (minor contributor template) dropped out, whereas at the ratio of 1:19 (about 50 pg minor contributor template), only 5 (31.25%) minor alleles were detected. Thus, the 24Y Direct System was able to handle the DNA mixtures in a 1:3 ratio.

For the results of the mixture samples of female and male DNA, complete profile can be obtained when the quantity of M308 (500 pg) remained unchanged, even though the amount of F312 rose from 100 to 800 pg (Table 1 and Online Resource 9). However, when the quantity of F312 (400 pg/μL) remained unchanged while amounts of M308 were decreased from 500 to 15.63 pg, whole 24 loci were available at the 250-pg template quantity and 22 loci (91.67%) of 24 loci were recognized at 125 pg based on the threshold of 50 RFU peak amplitude (Table 1 and Online Resource 9). These conclusions were inconsistent with the results of sensitivity studies, indicating that when the female template amount was 3.2 times that of male, the genotyping results of male DNA typed by the 24Y Direct System may be affected, which should be paid sufficient attention in forensic DNA analysis.

Accuracy and reproducibility evaluation

In this study, we qualitatively evaluated the accuracy of the 24Y Direct System detection result through detecting the M308 in two different laboratories and by different operators. The DNA profile results from the two different laboratories were identical except an allele of "OL" was named on DYS438 locus (Online Resource 10-b). This "OL" peak caused by the drift during capillary electrophoresis can usually be corrected manually without affecting genotyping result. Therefore, the 24Y Direct System had a good accuracy and reproducibility.

PCR-based studies

Reaction volume studies

The maximum recommended reaction volume for the 24Y Direct System was 25 μL. Herein, M308 was amplified by this system with diverse reaction volumes (25 μL, 12.5 μL, and 6.25 μL) in which each PCR component ratio remained unchanged according to the user's manual. Complete DNA profiles were obtained in three different total reaction volumes (Online Resources 11 and 12). That is to say, the system can also meet the requirements that a smaller reaction volume must be chosen to treat low copy number DNA sample.

PCR cycle number

More PCR cycle numbers are accompanied by the more non-specific products. Thus, it is quite crucial to choose the optimum cycle numbers. Herein, full DNA profiles were obtained at different cycle numbers of 25, 26, 27, 28, and 29 and the alleles' peak heights gradually raised as cycle numbers increased (Online Resources 11 and 12). However, peak heights raised drastically at 28 cycles accompanied by the increase in the non-specific products. Therefore, the suitable cycle number for the 24Y Direct System was 27 cycles. Of course, in the treatment of little amounts of DNA or old-type specimens, the yield of PCR products and the system sensitivity would be improved by increasing the PCR cycles numbers reasonably [16].

Table 1 Results of DNA mixture studies

Ratio or times	DNA inputs			Minor/male loci called (with the threshold of 50 RFU)	Percentage of loci called
	F312 (female) (pg)	M308 (male) (pg)	M4615 (male) (pg)		
M308:M4615 = 1:1		500	500	16	100
M308:M4615 = 1:3		250	750	16	100
M308:M4615 = 1:9		100	900	15	93.75
M308:M4615 = 1:19		50	950	5	31.25
F312/M308 = 0.2	100	500		24	100
F312/M308 = 0.4	200	500		24	100
F312/M308 = 0.8	400	500		24	100
F312/M308 = 1.6	800	500		24	100
F312/M308 = 0.8	400	500		24	100
F312/M308 = 1.6	400	250		24	100
F312/M308 = 3.2	400	125		22	91.67
F312/M308 = 6.4	400	62.5		19	79.17
F312/M308 = 12.8	400	31.25		8	33.33
F312/M308 = 25.6	400	15.63		3	12.50

Annealing temperature studies

For the annealing temperature studies of the 24Y Direct System, temperatures of 56 °C, 58 °C, 60 °C, 62 °C, and 64 °C were tested. At annealing temperature of 64 °C, alleles peak heights decreased, and partial alleles have dropped out, such as Y GATA H4, DYS576, and DYS643 loci. Full DNA profiles were obtained when annealing temperatures ranged from 56 °C to 62 °C, while better peak height balance was observed at 56 - 60 °C (Online Resources 11 and 12). Therefore, the 24Y Direct System had the characteristic of strong adaptability at annealing temperatures of 56 °C to 60 °C.

Concentration of PCR component studies

Concentrations of primer, buffer, and Taq DNA polymerase in PCR reaction system are the important factors influencing PCR reaction specificity and PCR product yield. In this study, M308 was amplified with different concentrations of 5× primer mix, 2.5× buffer, and Taq DNA polymerase II amount in a 10-μL PCR reaction volume (Online Resources 11 and 12). Full DNA profiles were produced when the primer mix varied from 1.5 to 2.5 μL. Partial alleles dropped out at 1 μL primer mix. While at 3 μL primer mix, the average peak height began to decrease and allelic deletion existed in one locus. Moreover, our results indicated that full DNA profiles were obtained from addition amount of 4–6 μL buffer. The allele peak heights raised with the increasing inputs of buffer and rose significantly at 6 μL buffer. Additionally, complete profiles were also obtained from different Taq DNA polymerase II amounts (0.1–0.3 μL), suggesting that these adding quantities maybe have no effect on the result analysis.

Case-type samples

Furthermore, to evaluate the reliability and adaptability of this panel for detecting different types of biological samples, 10 different types of mock evidence samples, namely, rib cartilage, bloodstain, buccal swab, hair, gauze bloodstain, tooth, nail, cigarette butt, saliva, and epithelial (or exfoliated cells) abrasion samples, were also typed by the 24Y Direct System, respectively. Full DNA profiles were obtained from 9 different type (not including abrasion samples) samples; the genotyping results of different type samples from the same individual (the rib cartilage and bloodstain samples, the saliva, and cigarette butt samples) were consistent, despite an "OL" peak appeared in the DYS458 locus of saliva and cigarette samples (Online Resource 13). Moreover, no peaks were detected in epithelial (or exfoliated cells) abrasion samples except for the buccal swab and two scalp wipe samples (Online Resource 13). These results supported the viewpoint that the 24Y Direct System can be used for genotyping of various commonly encountered samples from crime scenes, while caution should be exercised for epithelial (or exfoliated cells) abrasion samples since the system sensitivity to detect exfoliated cells may not be enough.

Population studies

In this study, we investigated the genetic diversities and forensic parameters of the 24 Y-STR loci in Jiangsu Han population

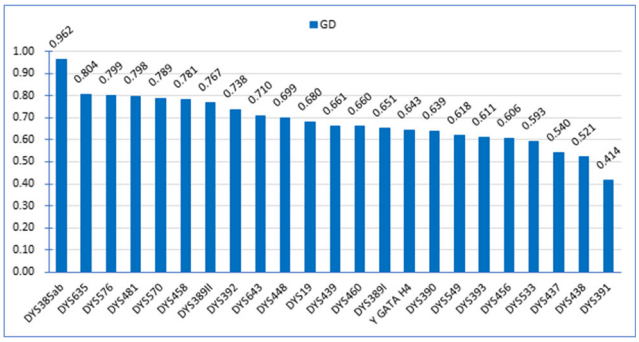

Fig. 2 24 Y-STR markers of the Microreader™ 24Y Direct ID System ranked by genetic diversity (GD) values

and the genotypes of the 200 males have provided in Online Resource 14. A total of 133 alleles in 23 single-copy loci and 48 genotypes in DYS385a/b were detected with the corresponding GD values ranging from 0.414 to 0.962 (Online Resource 15 and Fig. 2). Except for the locus DYS391 (GD = 0.414), the GD values of other loci were greater than 0.520 and with a mean GD value of 0.694. There were 170 distinct haplotypes observed, of which 154 (90.59%) carried a unique haplotype (Online Resource 16). The HD, MP, and DC values of the 24 Y-STRs in Jiangsu Han population were 0.9952, 0.0098, and 0.8500, respectively (Table 2), demonstrating that the 24 Y-STR loci were highly polymorphic in the Jiangsu Han population.

Conclusion

Validation experiments are recommended by the SWGDAM to assist laboratories in assessing the reliability of new DNA detection methods and determining their application limitations. This developmental validation experiments studied the technical performance indicators and forensic application value of the 24Y Direct System. The results indicated that the 24Y Direct System would be a reliable and robust complement tool of the existing STR panels and could contribute to Y chromosome STR database construction and forensic patrilineal investigation.

Table 2 The forensic parameters estimated for the Microreader™ 24Y Direct ID System in Jiangsu Han population

Time (s) or forensic statistical parameters of observed haplotype	Microreader™ 24Y Direct ID System
1 (unique)	154
2	14
6	1
12	1
Sample size	200
Number of different haplotypes	170
Proportion of unique haplotype	0.9059
Haplotype diversity	0.9952
Match probability	0.0098
Discrimination capacity	0.8500

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Compliance with ethical standards

Blood samples were collected with written informed consent and approved by the Ethics Committee of Southern Medical University.

The study strictly complied with the human and ethical research principles of Southern Medical University, Guangdong Province, China.

Conflict of interest The authors declared that they have no conflict of interest.

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