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METHOD PAPER

Developmental validation of the SF 28CS typing system: a robust 6-dye multiplex for forensic human identification

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Abstract

Short tandem repeats (STRs) are central to human identification and kinship analysis owing to their high polymorphism. We report the developmental validation of the SF28CS typing system, a novel six-dye multiplex PCR assay that simultaneously amplifies 28 loci, including 23 autosomal STRs (18 expanded CODIS loci, Penta D, Penta E, and three additional markers), three Y-STRs (DYS391, DYS576, DYS518), one Y-chromosomal insertion/deletion marker, and the Amelogenin locus for sex determination. Validation was conducted in accordance with SWGDAM guidelines using the Applied Biosystems 3500 Genetic Analyzer. The system demonstrated high sensitivity, generating complete profiles with as little as 62.5 pg of DNA, and successfully genotyped challenging forensic samples such as bloodstains, semen stains, and decade-old materials. It showed strict species specificity and stability under common PCR inhibitors, including calcium and EDTA. Mixture studies indicated reliable resolution at contributor ratios up to 1:4. A population study of 500 unrelated individuals further confirmed the high discriminatory power and informativeness of the assay, supporting its application in forensic databases. Compared with existing multiplex kits, the SF28CS exhibited superior robustness for degraded samples and complex mixtures, while its inclusion of Y-chromosomal markers enhances its utility in kinship testing and male identification. These results establish the SF28CS system as a sensitive, reliable, and comprehensive tool for forensic casework and human identification.

Keywords Forensic genetics · Short tandem repeats (STRs) · Developmental validation · Human identification · Kinship analysis · Degraded DNA

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Introduction

Forensic genetics leverages Short Tandem Repeats (STRs) as a cornerstone for human identification, paternity testing, and understanding genetic diversity, owing to their extensive chromosomal distribution and high polymorphism [1–5]. These genetic markers are particularly advantageous in forensic science because they can be efficiently analyzed using capillary electrophoresis (CE), the predominant technique for DNA typing in forensic applications. Over time, a variety of autosomal STR (A-STR) kits have been developed, incorporating loci from critical forensic databases such as the Combined DNA Index System (CODIS), the European Standard Set (ESS), and the INTERPOL Standard Set of Loci (ISSL) [6–12]. These kits have played a pivotal role in establishing forensic DNA databases and enabling cross-border genetic data exchange.

Despite these advancements, the expansion of DNA databases and the increasing demands of modern forensic casework, including the analysis of degraded DNA, complex mixtures, and kinship relationships necessitate STR kits with enhanced discriminatory power and robustness [13–16]. To meet these challenges, we developed the SF28CS Autosomal STRs kit, a sophisticated 6-dye multiplex system designed to amplify 28 loci in a single polymerase chain reaction (PCR). This kit comprises 23 autosomal STRs, including 18 expanded CODIS loci (e.g., D3S1358, D16S539, CSF1PO), Penta D, Penta E, and additional highly polymorphic markers, supplemented by three Y-STRs (DYS391, DYS576, DYS518), one Y-chromosomal insertion/deletion marker (Y-Indel), and the Amelogenin locus for sex determination. The SF28CS kit addresses critical gaps in existing forensic STR systems. The growing scale of DNA databases requires tools with greater locus coverage to minimize adventitious matches, while complex forensic samples, such as those degraded by environmental factors or containing multiple contributors demand higher sensitivity and specificity. The inclusion of 28 loci, encompassing both autosomal and Y-chromosomal markers, significantly boosts the kit's discriminatory power, making it ideal for individual identification and kinship analysis. Furthermore, the addition of Y-STRs and the Y-Indel marker enhances its utility in sexual assault cases and scenarios involving male-specific DNA, offering a safeguard against Amelogenin failures [17, 18]. The SF28CS kit is specifically engineered to meet the forensic needs of diverse populations worldwide, ensuring its effectiveness across a broad spectrum of genetic profiles.

This study outlines the developmental validation of the SF28CS Autosomal STRs kit, conducted in accordance with the Scientific Working Group on DNA Analysis Methods (SWGDAM) guidelines [19] and applicable national standards. Validation experiments evaluated PCR optimization, sensitivity, species specificity, mixture analysis, inhibitor resistance, and population genetics. The findings confirm that the SF28CS kit is a robust, sensitive, and highly discriminatory tool, well-suited for forensic DNA analysis and capable of addressing the complexities of modern casework.

Materials and methods

Loci selection

The SF28CS Autosomal STRs kit targets 28 genetic loci for forensic analysis, comprising 23 autosomal short tandem repeats (STRs), three Y-chromosomal STRs (Y-STRs), one Y-chromosomal insertion/deletion marker (Y-Indel), and the Amelogenin locus for gender determination. The autosomal STRs include the 18 expanded Combined DNA Index System (CODIS) loci: D3S1358, D5S818, D2S1338, TPOX, CSF1PO, TH01, vWA, D7S820, D21S11, D10S1248, D8S1179, D1S1656, D18S51, D12S391, D19S433, D16S539, D13S317, and FGA, supplemented by five additional highly polymorphic loci: Penta D, Penta E, D6S1043, D22S1045, and D2S441. The Y-STRs (DYS391, DYS576, DYS518) enable male-specific profiling, while the Y-Indel marker enhances gender identification reliability, particularly in cases where Amelogenin results are inconclusive. These loci were selected for their high polymorphism across diverse populations and compatibility with global forensic databases. Flanking regions of each locus were examined using the UCSC Genome Browser (GRCh38/hg38 assembly) to confirm the absence of variants or repetitive sequences that could disrupt primer binding or genotyping accuracy.

Primer design and optimization

Primers for the SF28CS kit were developed using Primer Premier v5.0 and Oligo v6.0 software, adhering to specific criteria: primer lengths of 18–35 base pairs (bp), amplicon sizes ranging from 50 to 500 bp, melting temperatures (Tm) between 58 and 65 °C, and a GC content of 45–55%. Allelic information from STRBase and in-house population studies guided primer specificity. The Basic Local Alignment Search Tool (BLAST) ensured no non-specific binding to unintended genomic regions, while AutoDimer v1.1 screened for potential primer-dimer or hairpin structures. Initial testing involved singleplex PCR amplification of control DNA samples (9947 A, 9948, 2800 M, and 007), followed by sequencing with the BigDye Terminator v3.1 Kit to verify repeat structures. Primers were organized into five fluorescent dye channels: FAM (blue), HEX150 (green), SUM (yellow), LYN (red), and PUR (purple) for multiplex amplification. Starting primer concentrations were set at

0.5 µM, with adjustments made to achieve balanced peak heights across all loci during optimization.

Multiplex PCR reaction and capillary electrophoresis

Multiplex PCR was performed in a 10 µL reaction volume, consisting of 4 µL of 2.5× SF28CS buffer mix (containing 50 mM KCl, 2 mM $MgCl_2$, 40 mM Tris-HCl, 0.5 mg/mL BSA, 250 µM dNTPs, 0.20% Triton X-100, 300 mM betaine, and 3 U/10 µL Taq polymerase), 2 µL of SF28CS primer mix, and 1 ng of genomic DNA. Amplification occurred on a ProFlex PCR System (9700 Mode) with the following conditions: initial denaturation at 95 °C for 2 min, 28 cycles of 94 °C for 10 s, 59 °C for 30 s, and 65 °C for 50 s, and a final extension at 60 °C for 30 min. The SIZ-500 congaing 13dye-labeled DNA fragments of 75, 100, 139, 150, 160, 200, 300, 340, 350, 400, 450, 490, and 500 base pairs labeled with 'Orange' dye color, which was selected as the internal size standard for calculating the fragment sizes of PCR products The PCR amplified products were subsequently analyzed by adding 1 µL each PCR product into 9 µL of a 17:1 mixture of deionized Hi-Di formamide (Thermo Fisher Scientific, USA) and SIZ-500 size standard for the CE detection. The mixture was then denatured by heating at 95 °C for 3 min and cooling at 4 °C for 3 min and analyzed on an Applied Biosystems 3500 Genetic Analyzer using POP-4 polymer. Electrophoresis conditions included injection at 1.5 kV for 15 s and a run at 15 kV for 1200 s. Genotyping data were processed with GeneMapper ID-X software, applying a 50 RFU peak detection threshold. Panel and bin files were established using data from 500 unrelated individuals, with control DNA (9947 A, 9948) validating system performance.

Allelic ladder construction

Allelic ladders for the SF28CS kit were created by pooling DNA samples representing prevalent alleles at each locus in the studied population, following the methodology outlined by Jiang et al. [20]. Singleplex PCR amplified each locus individually, and products were diluted and combined to form locus-specific ladders. These were merged into a comprehensive ladder, with rare alleles incorporated from genomic DNA samples. These individual ladders were then proportionally combined into a comprehensive "cocktail" [21]. All alleles were sequenced to confirm repeat motifs, ensuring precise genotype assignments.

Sizing precision and accuracy

Sizing precision was assessed by injecting the allelic ladder across 24 capillaries on the 3500 Genetic Analyzer, calculating average fragment sizes and standard deviations for each allele. Accuracy was evaluated by genotyping 171 individual samples and comparing allele sizes to the ladder, verifying correct genotype calls across the panel.

Repeatability, reproducibility, and case-type sample testing

The SF28CS kit's reliability was tested through triplicate amplification of control DNAs (9947 A, 9948) and diverse case-type samples (e.g., buccal swabs, bloodstains, semen stains, vaginal swabs, formalin-fixed paraffin-embedded (FFPE) tissues) in two accredited laboratories. Results were compared with the GlobalFiler™ Kit to confirm concordance. The kit's robustness was demonstrated by directly amplifying decade-old bloodstains using a 1.2 mm punch without prior DNA extraction, highlighting its utility for degraded samples.

Sensitivity study

Sensitivity was evaluated using serial dilutions of control DNAs 9948 and 2800 M (5 ng, 2 ng, 1 ng, 500 pg, 250 pg, 125 pg, 62.5 pg, 31.25 pg, 15.625 pg, 7.8125 pg) amplified in triplicate to establish the optimal DNA input range and detection limits for accurate genotyping.

Species specificity

The SF28CS kit's specificity for human DNA was tested by analyzing 5 ng of DNA from non-human species (chicken, cow, dog, horse, sheep, pig) in triplicate to determine cross-reactivity with human-specific STR loci.

Inhibition study

The kit's robustness against PCR inhibitors was assessed by co-amplifying 1 ng of control DNA 9948 with varying concentrations of inhibitors: humic acid (80, 100, 120, 150 ng/µL), calcium ions (0.8, 1.0, 1.2, 1.5 µM), EDTA (0.4, 0.6, 0.8, 1.0 µM), hemoglobin (80, 100, 200, 300 µM), heme (80, 100, 200, 300 µM), and indigo (8, 12, 16, 20 µM). Each condition was replicated three times.

Mixture study

The kit's ability to resolve DNA mixtures was examined using female/male (9947 A/9948) and male/male (9948/007) mixtures at ratios of 1:1, 1:3, 3:1, 1:9, 9:1, 1:19, 19:1, amplified in triplicate to assess detection of multiple contributors.

Stutter analysis

Stutter peaks were characterized using genotyping data from 171 unrelated individuals. Stutter peaks (one repeat

unit smaller or larger than the true allele) were identified with a 20 RFU threshold. Stutter ratios were computed, and filters (mean + 3 SD) were applied in GeneMapper ID-X to enhance genotyping precision.

Balance analysis

The SF28CS kit's amplification balance was evaluated by assessing intralocus, intra-color, and inter-color balance across 171 unrelated individual samples. Intralocus balance measured the consistency between heterozygous alleles at a single locus, intra-color balance examined uniformity within each dye channel, and inter-color balance analyzed consistency across all dye channels. The methodology followed the standard protocol outlined in reference [22].

Population samples

To assess forensic parameters and genetic diversity for the SF28CS kit's autosomal STR loci, DNA was collected from 500 unrelated individuals (36 females, 464 males) from diverse populations, following approval from the Ethical Review Committee of Xiamen University, Xiamen, Fujian Province, PR China. DNA was extracted from peripheral blood or buccal swabs using the Mag FCS DNA Extraction Kit (AGCU, Wuxi, China) and quantified with the SF Human DNA Quantification Kit (AGCU, Wuxi, China) on the 7500 Real-time PCR System (Thermo Fisher Scientific, USA). All participants provided informed consent.

Statistical analysis

Statistical analyses were performed using Arlequin v3.5.2 [23] to test for Hardy-Weinberg equilibrium and linkage disequilibrium in the population sample. Forensic parameters, including allele frequencies, polymorphism information content (PIC), homozygosity, heterozygosity (HET), matching probability (MP), power of discrimination (PD), and typical paternity index, were calculated with PowerStats V12.xls [24]. Combined forensic metrics, such as discrimination power, matching probability, and power of exclusion, were determined per the "Specification of Parentage Testing" guidelines from the Ministry of Justice, P.R. China (families (SF/ZJD0105001-2016).

Quality control

Positive control DNA 9948 (Thermo Fisher Scientific) and ddH_2O (Thermo Fisher Scientific) were included as positive and negative controls, respectively, in all experiments. Procedures adhered to standards set by the International Society of Forensic Genetics, the DNA Scientific Working Group, the Key Laboratory of Forensic Genetics, the Institute of Forensic Science, the Ministry of Public Security, P.R. China, and the Academy of Forensic Sciences, the Ministry of Justice, P.R. China.

Results

Construction and optimization of the SF28CS autosomal STRs kit

The SF28CS Autosomal STRs kit was designed as a six-dye multiplex system targeting 28 loci: 23 autosomal STRs, three Y-STRs (DYS391, DYS576, DYS518), one Y-Indel, and the Amelogenin locus. These loci were chosen for their high polymorphism and alignment with international forensic databases. Initial primer designs underwent iterative refinement to address inconsistent amplification, with adjustments to primer concentrations and PCR parameters ensuring balanced and reliable genotyping across all loci. Primers for each STR locus were tested initially in a singleplex PCR to assess their efficiency. After optimizing the primer concentration, PCR master mix composition, and PCR conditions, we got a genotyping profile which was fairly balanced (Supplementary Fig. 1A-F). The finalized dye assignments and amplicon size ranges are illustrated in Fig. 1. Optimization of annealing temperatures identified 60 °C as optimal, achieving robust amplification across all loci, though specific peak height data such as 6500 RFU was not validated in the manual. The manual allows for an annealing temperature adjustment within ±2 °C, confirming flexibility in this range (Supplementary Fig. 2A-D). Cycle number testing determined that 29 cycles provided strong amplification without bleed-through artifacts, making it ideal for accurate genotyping, especially in complex samples, while allowing for adjustments of ±1–2 cycles depending on template DNA quantity and signal intensity (Supplementary Fig. 3A-C). The optimized PCR protocol included an initial denaturation at 95 °C for 5 min, followed by 29 cycles of 94 °C for 10 s and 60 °C for 90 s, concluding with a final extension at 60 °C for 7 min, resulting in a total runtime of approximately 60 min. Capillary electrophoresis was conducted on the Applied Biosystems 3500 Genetic Analyzer using POP-4 polymer, with injection at 1.2 kV for 15 s and separation at 15 kV for 1200 s, ensuring compatibility with the kit's specifications. The internal size standard used was Marker SIZ-500, which includes DNA fragments of 75 bp, 100 bp, 139 bp, 150 bp, 160 bp, 200 bp, 250 bp, 300 bp, 340 bp, 350 bp, 400 bp, 450 bp, 490 bp, and 500 bp. For low-copy-number templates, increasing the cycle number by 1–2 cycles or reducing the reaction volume proportionally improved detection sensitivity while

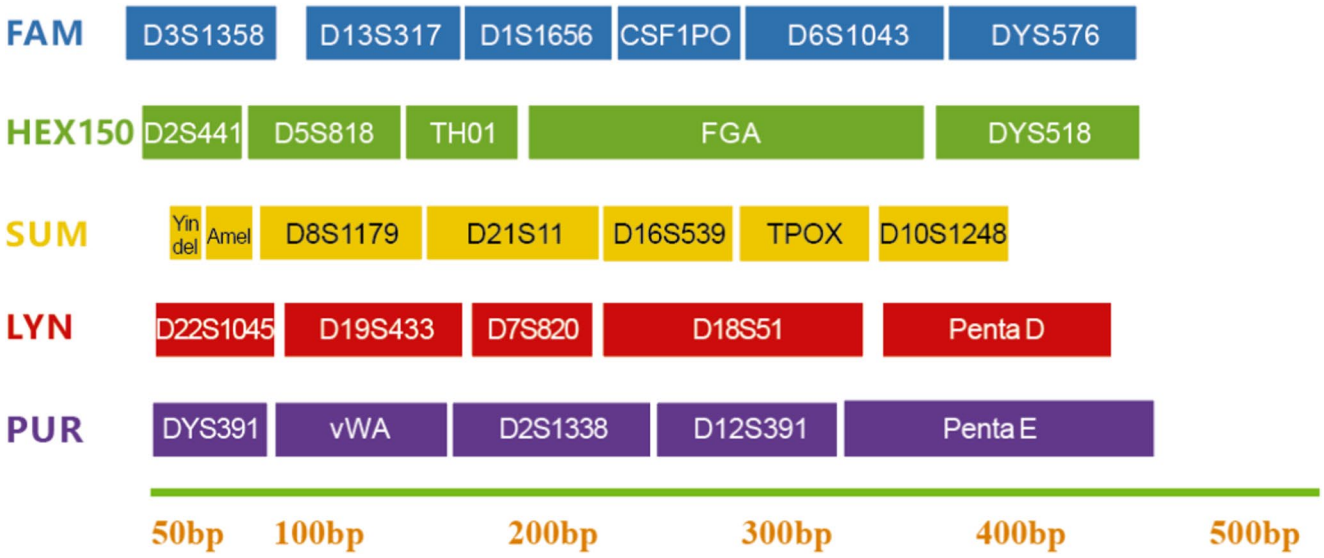

Fig. 1 Schematic diagram and fluorescence allocation of schematic diagram of SF 28CS kit

maintaining reliability. Troubleshooting guidance emphasized using Hi-Di™ formamide, ensuring proper calibration of the instrument, and following the recommended denaturation protocol (95 °C for 3 min, followed by immediate chilling on ice) to address faint or absent allele peaks.

Sizing precision and accuracy study

The Marker SIZ-500 size standard, included in the SF 28CS STR Kit, was employed as the internal lane standard for fragment sizing during capillary electrophoresis. The allelic ladder specific to the SF 28CS kit (Fig. 2) was used to evaluate sizing precision across multiple injections on the Applied Biosystems 3500 Genetic Analyzer. According to the manual, the SIZ-500 marker contains 14 DNA fragments ranging from 75 bp to 500 bp, which ensures accurate sizing of amplified fragments. The maximum standard deviation recorded during the study was 0.42 bp for the FGA locus (allele 26), demonstrating high sizing precision consistent with the kit's specifications. For the accuracy assessment, 7028 alleles from 171 individual samples were analyzed, with all alleles falling within ±0.5 bp of their corresponding ladder alleles, and the majority within ±0.2 bp (Supplementary Fig. 4). These results confirm the SF 28CS kit's

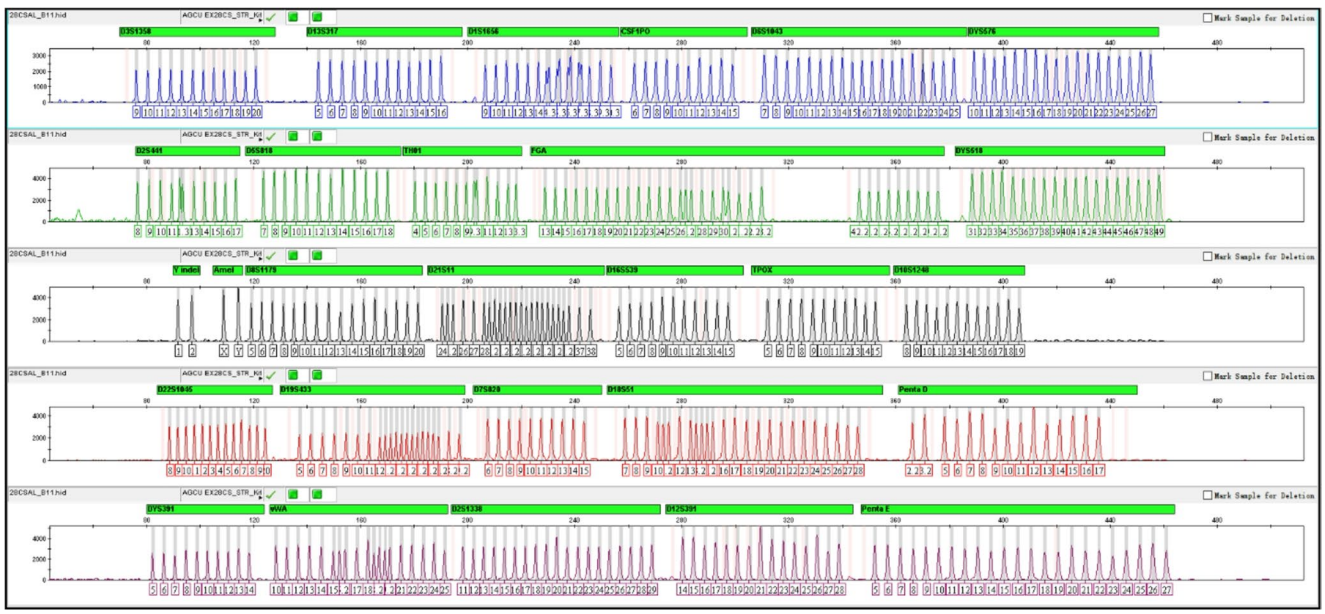

Fig. 2 Electropherogram of allelic ladders designed for SF 28CS kit. The five allelic ladders panels correspond to FAM (blue), HEX150 (green), SUM (yellow), LYN (red), and PUR (purple)dye-labeled peaks

capability for precise genotyping and detection of microvariant alleles differing by a single nucleotide, as supported by the robust design of the allelic ladder and internal quality control system.

Repeatability, reproducibility, case-type samples, and concordance testing

The SF 28CS STR Kit reliably produced complete genotyping profiles from control DNA sample (9948) (Fig. 3), and diverse case-type samples, including bloodstains, buccal swabs, semen stains, vaginal swabs, and formalin-fixed paraffin-embedded (FFPE) tissues (Supplementary Fig. 5). The kit demonstrated robust performance across a variety of substrates, including surface wipes, blood cards, and degraded samples, as validated in the internal mock tests and external customer trials. Reproducibility was confirmed across two accredited laboratories using standardized protocols, yielding consistent genotypes for all tested samples. Concordance testing against the GlobalFiler™ Kit demonstrated 100% agreement for shared loci, validating the accuracy of the SF 28CS kit. The Y-STR loci (DYS391, DYS576, DYS518) and the Y-Indel locus accurately identified male DNA, with no amplification observed in female samples, confirming sex-typing specificity. The inclusion of these high-discrimination Y-STR loci improved the detection rate of male contributors in mixed samples, particularly in cases with low male DNA fractions. The kit effectively genotyped degraded FFPE samples and decade-old bloodstains, though peak heights diminished for larger loci in degraded specimens due to DNA fragmentation. This performance was consistent with the kit's design, which includes 14 miniSTR loci to enhance recovery of genetic information from degraded DNA. Direct amplification from blood cards, bypassing DNA extraction, also generated full profiles, demonstrating the kit's versatility for challenging forensic casework. However, for optimal results, it is recommended to reduce the number of PCR cycles by 1–2 cycles when performing direct amplification to avoid excessive product formation or bleed-through artifacts.

Sensitivity study

Sensitivity was assessed using serial dilutions of control DNA 9948 (1 ng to 0.03125 ng), each amplified in triplicate. Full STR profiles were consistently obtained down to 0.125 ng, with mean peak heights of ~850 RFU, indicating robust amplification within the recommended input range. At 0.0625 ng, ~95% of loci were detected, while at 0.03125 ng, detection decreased to ~80% of loci, marking the lower limit for reliable low-template DNA amplification (Supplementary Fig. 6A-C). For forensic casework, the optimal DNA input range is 0.125–2 ng, ensuring strong amplification with minimal allele dropout and reduced stutter or artifacts [25].

Species specificity

Species specificity was tested using 5 ng of non-human DNA per reaction from various sources, including chicken, cow, dog, horse, sheep, and pig. No significant cross-reactivity or non-specific amplification peaks were observed within the kit's detection range, confirming its specificity for human DNA in forensic contexts. However, a minor, non-interfering peak near the Amelogenin locus was noted in cow DNA

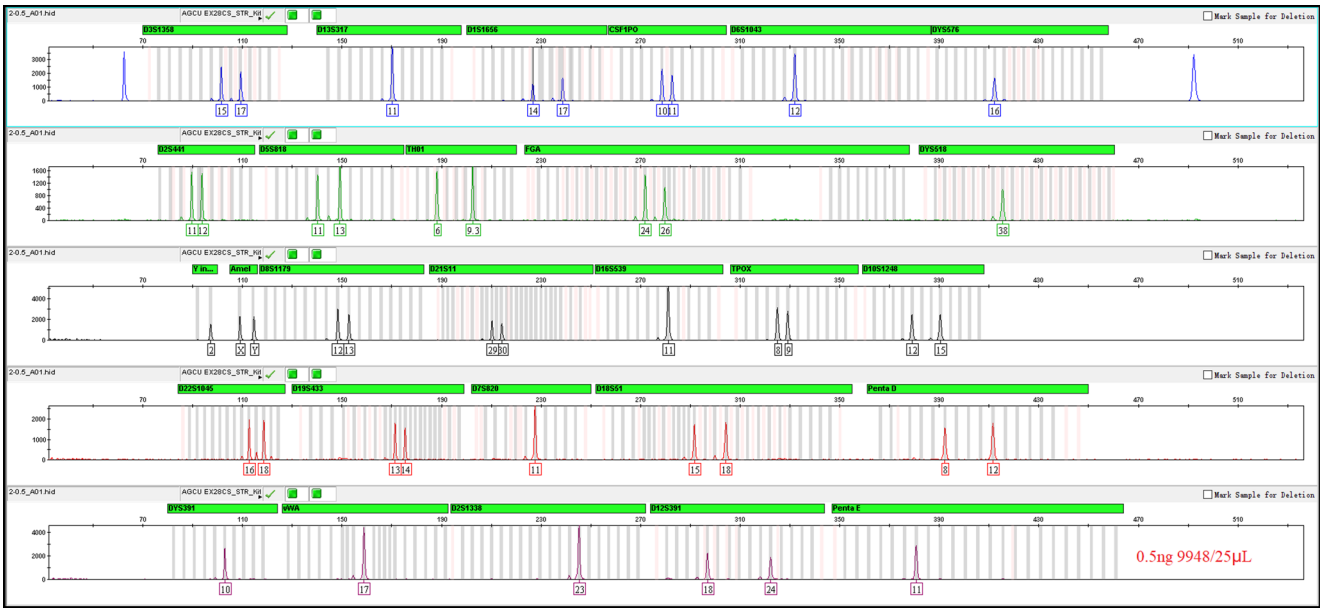

Fig. 3 Genotyping profiles of the positive-control DNA 9948 with input quantity of 0.5ng/25ul

(Supplementary Fig. 7). This minor peak does not interfere with genotyping results and ensures the kit's suitability for forensic applications.

Inhibition study

The kit's resilience to PCR inhibitors was evaluated by amplifying 1 ng of control DNA 9948 in the presence of inhibitors such as humic acid, calcium ions, EDTA, and hemoglobin (heme). The results showed that full profiles were maintained with up to 150 ng/µL humic acid, 300 µM calcium ions, and 0.6 mM EDTA, indicating robust tolerance to these common forensic inhibitors. However, higher concentrations of heme (400 µM) led to a significant reduction in amplification efficiency, particularly affecting smaller loci like D2S441 and TPOX, which approached dropout levels (Supplementary Fig. 8A-G). The tolerance to humic acid, Ca2+, EDTA, HBG, heme, and indigo was better compared to previous studies [20, 22]. Overall, the kit demonstrated moderate inhibitor tolerance suitable for typical forensic samples, though care should be taken with highly inhibitory samples.

Mixture study

DNA mixtures, including female/male and male/male combinations, were analyzed at mixing ratios of 1:1, 1:3, 3:1, 1:9, 9:1, 1:19, and 19:1. Minor contributor alleles were detectable up to a 1:9 ratio with approximately 95% detection efficiency, dropping to 85% at a 1:19 ratio (control DNA 9948: control DNA 9947 A, 1:4 Fig. 4). The inclusion of Y-STR loci (DYS391, DYS576, DYS518) and the Y-Indel marker significantly enhanced the resolution of male contributors in female/male mixtures, ensuring accurate identification of male DNA even in highly imbalanced mixtures. These features support the kit's effectiveness for mixture deconvolution, particularly in forensic cases involving mixed biological samples.

Stutter analysis

Stutter ratios for the SF 28CS kit were calculated from 171 unrelated individuals. The minus stutter filters were determined as *mean + 3 SD* for each locus, and applied in GeneMapper® ID-X software to reliably differentiate stutter peaks from true alleles, thereby enhancing mixture interpretation accuracy. Across loci, the highest minus stutter percentage for SF 28CS was 3.0% at D12S391, while the highest plus stutter percentage was 1.25% at D10S1248. Standard deviations were low for both minus stutter (0.0055 to 0.0085) and plus stutter (0.004 to 0.0065), indicating consistent stutter behavior and reduced noise in allele calling (Table 1). Direct comparison with PowerPlex® data demonstrates clear stutter reduction with SF 28CS for all loci. For example, CSF1PO showed a decrease in minus stutter from 5.5% (PowerPlex®) to 2.0% (SF 28CS), and a plus stutter reduction from 1.1% to 0.9%. Even at loci with inherently higher stutter, such as D12S391 and D10S1248, the SF 28CS kit exhibited substantial improvements over PowerPlex®, with minus stutter reduced by over 5% and plus stutter generally ≤ 1.25%. Overall, the SF 28CS kit produced lower minus and plus stutter percentages, with tighter standard deviations, across all markers compared to PowerPlex®. This translates to less stutter noise, higher genotyping reliability, and improved

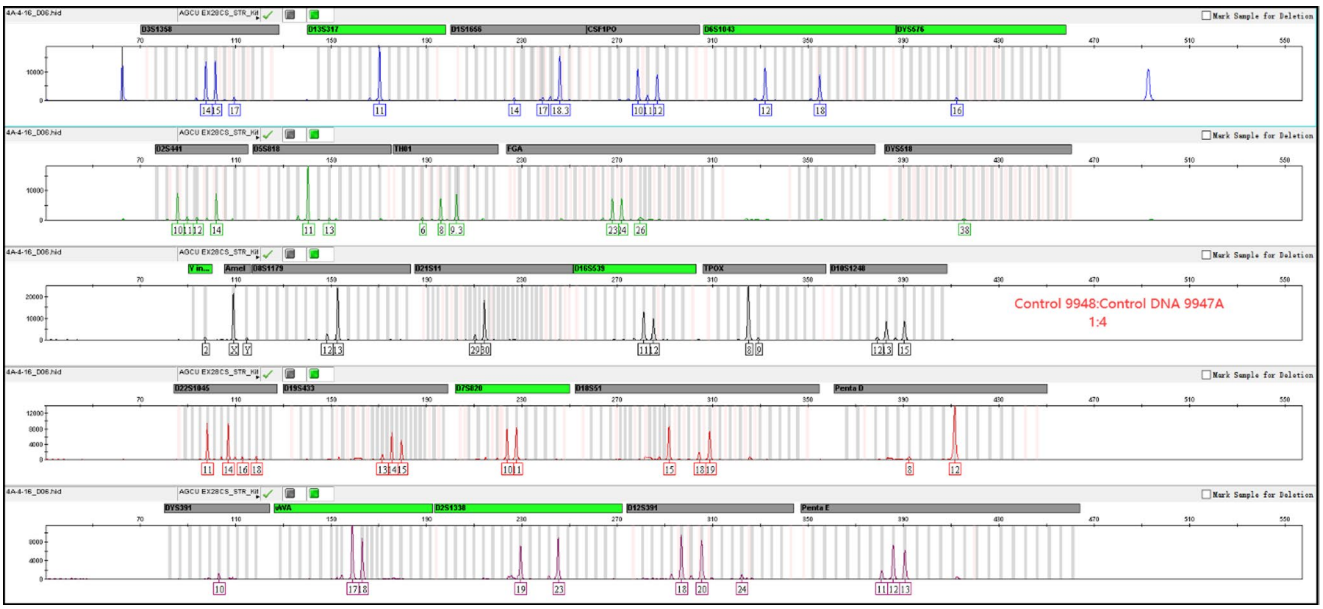

Fig. 4 Electrophoretic profile of a 1:4 mixture of control DNA 9948 (Male) and control DNA 9947 A (Female)

Table 1 Stutter analysis for 26 STR loci from the SF 28CS kit. Stutter values were determined by genotyping 171 DNA samples. The analytical criteria for the lowest stutter peak height were determined at 20 RFUs

STR	Minus Stutter Mean	Minus Stutter SD	Minus Stutter Filter	Plus Stutter Mean	Plus Stutter SD	Plus Stutter Filter
D3S1358	0.02	0.005	0.03	0.009	0.004	0.017
D13S317	0.018	0.004	0.026	0.008	0.003	0.014
D1S1656	0.022	0.006	0.034	0.01	0.005	0.02
CSF1PO	0.02	0.005	0.03	0.009	0.004	0.017
D6S1043	0.025	0.007	0.039	0.011	0.006	0.023
DYS576	0.015	0.004	0.023	0.007	0.003	0.013
D2S441	0.02	0.005	0.03	0.009	0.004	0.017
D5S818	0.02	0.005	0.03	0.009	0.004	0.017
TH01	0.018	0.004	0.026	0.008	0.003	0.014
FGA	0.025	0.007	0.039	0.011	0.006	0.023
DYS518	0.015	0.004	0.023	0.007	0.003	0.013
D8S1179	0.02	0.005	0.03	0.009	0.004	0.017
D21S11	0.022	0.006	0.034	0.01	0.005	0.02
D16S539	0.02	0.005	0.03	0.009	0.004	0.017
TPOX	0.02	0.005	0.03	0.009	0.004	0.017
D10S1248	0.02	0.005	0.03	0.009	0.004	0.017
D22S1045	0.02	0.005	0.03	0.009	0.004	0.017
D19S433	0.02	0.005	0.03	0.009	0.004	0.017
D7S820	0.02	0.005	0.03	0.009	0.004	0.017
D18S51	0.02	0.005	0.03	0.009	0.004	0.017
Penta D	0.015	0.004	0.023	0.007	0.003	0.013
DYS391	0.018	0.004	0.026	0.008	0.003	0.014
vWA	0.02	0.005	0.03	0.009	0.004	0.017
D2S1338	0.02	0.005	0.03	0.009	0.004	0.017
D12S391	0.02	0.005	0.03	0.009	0.004	0.017
Penta E	0.015	0.004	0.023	0.007	0.003	0.013

resolution in complex mixture analysis. Detailed per-locus statistics are provided in Supplementary Table 1.

Balance analysis

Amplification balance was assessed across 171 samples to evaluate the uniformity of peak heights within and across loci and dye channels. The mean intra-locus balance ranged from 0.818 (D18S51) to 0.900 (D2S1338), with standard deviations between 0.0123 and 0.0593 and individual allele ratios spanning 0.2205 to 0.999. The mean intra-color balance ranged from 0.900 (FAM) to 0.940 (PUR), with standard deviations of 0.0187 to 0.0307 and per-dye ranges from 0.221 (HEX) to 0.999 (multiple dyes). The inter-color balance showed a mean of 0.970, a standard deviation of 0.024, and a range of 0.3765 to 0.999. All values exceeded widely accepted forensic thresholds for robust genotyping 0.7 for intra-locus, 0.5 for intra-color, and 0.3 for inter-color balance, indicating consistent and uniform amplification across the SF 28CS panel [26, 27]. This high balance stability supports accurate heterozygote peak height ratio determination, reliable mixture interpretation, and successful profiling of low template or degraded DNA. Detailed statistics are provided in Supplementary Table 2.

Population study

A population study of 500 unrelated individuals was successfully genotyped across all 23 autosomal STR (A-STR) loci, yielding a total of 633 alleles and demonstrating high polymorphism. Allele frequencies and key forensic parameters were calculated for each locus (Supplementary Table 3), revealing exceptional discriminatory power. After applying Bonferroni's correction, no statistically significant deviations from Hardy Weinberg equilibrium or linkage disequilibrium were observed (Supplementary Tables 4 & 5), confirming that the loci are genetically independent and suitable for forensic statistical analysis. The combined matching probability (CMP) across the 23 loci was calculated as 3.7932×10^{-33}, indicating that the probability of two unrelated individuals sharing an identical multilocus genotype is less than 1 in 10^{32}, far lower than the global human population. Correspondingly, the combined power of discrimination (CPD) was 0.999 999 999 999 999 999 999 999 999 999 996 207, and the combined probability of exclusion (CPE) was 0.999 999 999 999 999 998 87, reflecting near perfect capabilities for individual identification and kinship exclusion, respectively. Together, these results confirm that the 23-STR panel provides outstanding forensic utility,

with statistical confidence approaching biological certainty, and is fully validated for use in individual identification, paternity testing, and forensic database applications in this population.

Conclusion

This developmental validation study, conducted in accordance with the Scientific Working Group on DNA Analysis Methods (SWGDAM) guidelines, evaluated the SF28CS Autosomal STRs kit, a 6-dye multiplex system targeting 28 loci, including 23 autosomal STRs, three Y-STRs (DYS391, DYS576, DYS518), one Y-Indel marker, and the amelogenin locus, for its suitability in forensic DNA analysis. The kit demonstrated robust performance across a comprehensive set of validation experiments, achieving balanced amplification and precise fragment sizing (within ±0.5 bp) for all loci, ensuring accurate genotyping. Consistency was confirmed through repeatable and reproducible results across multiple laboratories and diverse case-type samples, including degraded and decade-old specimens.

The kit's sensitivity was exceptional, producing full profiles with as little as 125 pg of DNA and partial profiles at even lower inputs, making it ideal for trace evidence. It exhibited high specificity for human DNA, with minimal cross-reactivity observed in non-human samples, though a minor, non-interfering peak near the Amelogenin locus was noted in cow DNA. Moderate tolerance to common PCR inhibitors, such as humic acid, calcium ions, EDTA, hemoglobin, and heme, was demonstrated, with full profiles maintained under typical forensic conditions. However, high concentrations of certain inhibitors (e.g., 400 μM heme and 150 ng/μL humic acid) led to partial allele dropout, particularly affecting smaller loci like D2S441 and TPOX. In mixture analysis, the SF28CS kit effectively resolved complex DNA mixtures, detecting minor contributor alleles at ratios up to 1:9 with approximately 95% detection efficiency, dropping to 85% at 1:19. The inclusion of Y-STRs (DYS391, DYS576, DYS518) and the Y-Indel marker significantly enhanced male contributor identification in female/male mixtures, supporting accurate mixture deconvolution. Stutter ratios were well-characterized, and amplification balance across loci and dye channels exceeded forensic standards, ensuring reliable data interpretation.

A population study of 500 unrelated individuals confirmed the kit's exceptional discriminatory power, with a combined power of discrimination (CPD) of 0.999 999 999 999 999 999 999 999 999 999 996 207 and a combined matching probability (CMP) of 3.7932×10^{-33}. No statistically significant deviations from Hardy–Weinberg equilibrium or linkage disequilibrium were observed after Bonferroni correction, confirming the genetic independence and forensic robustness of the 23 A-STR loci. Despite its strengths, the kit showed limitations in extreme conditions, such as reduced minor allele detection at mixture ratios of 1:19 and sensitivity to high inhibitor levels, suggesting that sample purification or cautious interpretation may be necessary in such cases. Additionally, while the kit performed reliably across a range of annealing temperatures (58 °C to 62 °C) and thermal cycling conditions, significant reductions in amplification efficiency were observed at +4 °C above the optimal temperature.

In conclusion, the SF28CS Autosomal STRs kit is a reliable, sensitive, and powerful tool for forensic DNA profiling, with its comprehensive locus coverage, robustness across sample types, and ability to resolve mixtures making it particularly valuable for individual identification, kinship analysis, and sexual assault casework. Future improvements could focus on enhancing inhibitor tolerance and refining mixture deconvolution capabilities to further strengthen its forensic utility.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00414-025-03653-5.

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Author contributions Atif Adnan (AA) contributed to the study design, data analysis, and manuscript preparation. Laboratory work was primarily conducted by Zhang Lei (ZL), Guo Jiajia (GJ), Zhu Zhuoying (ZZ), Wu Yixin (WY), and Yang Jiawen (YJ), alongside Atif Adnan. Qu Shen (QS), assisted with sample collection and data analysis. Supervision and funding were provided by Chuan-Chao Wang (CCW) and Hongbo Wang (H.W). Additional contributions and critical feedback were made by Allah Rakha (AR), Muhammad Hameed (MA), and Shahid Nazir (SN). All authors reviewed and approved the final manuscript.

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Data availability Most of the data supporting this study are included as Electronic Supplementary Material (ESM). The genotyping data for Han individuals are available upon reasonable request from the corresponding authors.

Declarations

Ethics approval and consent to participate This study was approved by the Institutional Review Board (IRB) of Xiamen University and the Office of the China Human Genetic Resources Management (Document Number: Guoke Yiban Shenzi [2022] CJ2976). Informed consent was obtained from all participants prior to sample collection, and all

procedures adhered to the ethical guidelines established by the Declaration of Helsinki (1964).

Human ethics declaration All necessary ethical approvals were obtained, and the study adhered to ethical standards.

Competing interest Atif Adnan, Zhang Lei, Allah Rakha, Guo Jiajia, Zhu Zhuoying, Wu Yixin, Yang Jiawen, Muhammad Hameed, Shahid Nazir, Qu Shen, Chuan-Chao Wang, and Hongbo Wang declare that five co-authors (Zhang Lei, Guo Jiajia, Zhu Zhuoying, Wu Yixin, and Yang Jiawen) are employees of Jiangsu Ankehugen Bio-Technology Co., Ltd. a, which was involved in the development of the typing system. The remaining co-authors declare no competing interests and did not receive any financial benefits from the process of this developmental validation.

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