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ORIGINAL ARTICLE

Developmental validation of the Ingenomics™ AutoProfiler STR system: a 6-dye STR multiplex kit for forensic DNA applications

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Abstract

The Ingenomics™ AutoProfiler system utilises a 6-dye, multiplex genotyping technology that is optimised for the simultaneous amplification of 20 Combined DNA Index System (CODIS) short tandem repeat (STR) markers, along with D6S1043, Penta D, Penta E, DYS391, SE33, and two Y-Indels (Rs2032678, Rs771783753), as well as the Amelogenin loci. This kit includes specialised internal quality control (IQC) markers, both small and large, to evaluate the quality parameters for each sample. Designed for a diverse range of applications—such as forensic DNA casework, identity establishment, database management, kinship testing, scientific research, and other analyses related to human genetic identification—this multiplex system is particularly effective for analysing low-copy number (LCN) DNA from severely compromised forensic samples, as it also evaluates two SNP markers. The multidimensional validation study aligned with the guidelines set forth by the Scientific Working Group on DNA Analysis Methods (SWGDAM) examined crucial parameters, including PCR conditions, reproducibility, analytical threshold calculation, sensitivity, species specificity, stability, mixture analysis, casework sample analysis, direct amplification, and concordance. The optimal DNA concentration range was between 60 and 500 pg/µL, with an analytical threshold established at 50 relative fluorescence units (RFUs). Overall, the findings indicate that the Ingenomics™ AutoProfiler kit is a reliable, robust, and suitable assay for human identification in casework DNA analysis and database construction.

Keywords Ingenomics™ AutoProfiler · Validation study · SWGDAM guidelines · Indian STR Kit

Introduction

Short tandem repeats (STRs) serve as gold standard genetic markers in forensic DNA analysis and are well-established for usage in such applications, since their introduction, the use of STRs has gained prominence in human identity applications. What started with the analysis of just 3 STR markers has now turned to forensic professionals working with over 25 markers, significantly increasing sensitivity discrimination capabilities between individuals, with enhanced inhibitor tolerance incorporated into commercial assay kits. It is crucial to interpret and include STR markers relevant to the specified population and those with better statistical validity to enhance discrimination capabilities in human identification (HID). This validation study is based on the recommendations of the European Network of Forensic Science Institutes (ENFSI) [7] and on the guidelines of the Scientific Working Group on DNA Analysis Methods (SWGDAM) [10] DNA Advisory Board (DAB).

The Ingenomics™ AutoProfiler assay makes use of well-established methodologies for forensic DNA analysis. It co-amplifies 20 polymorphic STR markers recommended by the CODIS (Combined DNA Index System)

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Significant statement: The present validation study of Ingenomics™ AutoProfiler demonstrates the usefulness of autosomal STR - PCR Amplification system with inclusion of CODIS markers and those relevant to Indian population too. The efficacy demonstrated by concordance studies authenticates its use in forensic case work analysis; this validation under SWGDAM guidelines establishes the same.

Extended author information available on the last page of the article

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Core Loci Working Group, the European Network of Forensic Science Institutes (ENFSI), and the European DNA Profiling Group (EDNAP). The CODIS markers included for analysis in this STR kit are CSF1PO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, FGA, TH01, TPOX, vWA, D1S1656, D2S441, D2S1338, D10S1248, D12S391, D19S433, and D22S1045, in addition to five extended STRs loci (D6S1043, Penta D, Penta E, DYS391, SE33), two Y-Indels (Rs2032678, Rs771783753), and the Amelogenin loci. In numerous studies, these genetic loci have been characterised by laboratories either in casework analysis or genetic genealogical studies [3, 8, 11]. As an additional feature, the Ingenomics™ AutoProfiler STR Kit incorporates an internal PCR control (Internal Quality Control Small and Large), which provides valuable information regarding PCR efficiency and the presence of inhibitors. The Internal Quality Sensor is present in the Primer Mix and amplified simultaneously with the polymorphic STR markers. The Internal Quality Control (IQC) system consists of two exogenous synthetic DNA sequences with primers specific for each IQC target. This helps distinguish DNA sample quality/degradation state against the amplification inhibition reactants. In addition, the IQC system serves as a positive control for PCR amplification. The kit is designed specifically for data-basing and forensics applications. We assessed and verified for directly amplifying single-source reference samples using a modified and standardised amplification protocol.

In this report, we demonstrate the robustness of the Ingenomics™ AutoProfiler STR Kit, which utilises 6-dye chemistry. The experiments demonstrate the Ingenomics™ AutoProfiler suitability for analysing forensic casework samples and reference databases.

Materials and methods

The study was approved by the Ethics Committee of Banaras Hindu University (BHU), Varanasi (Uttar Pradesh), India (vide letter no. I.Sc./ECM-*XVI*/2023- 24). Written or oral informed consent forms were obtained from all volunteers.

Collection and analysis parameters

Collection and Preparation of samples

Blood and saliva samples were collected from 500 unrelated volunteers. Saliva stains were collected on the FTA card (Whatman plc, Maidstone, UK) by transferring the epithelial cells obtained by swabbing from the buccal region. Blood samples were collected by pricking a sterilised fingertip (Accu-Chek Safe T-Pro Plus) and analysed using FTA cards [2, 5].

Human blood samples were collected from 400 anonymous donors (out of the 500 samples) and used for in-house population studies. These samples were obtained from unrelated individuals across India with self-reported ethnicities. DNA extraction of 200 samples was carried out using AllEx® Automated Nucleic Acid Extraction System (GeneAll Biotechnology Co. Ltd. Korea) [4]. The remaining 200 blood samples were analysed using a direct amplification protocol, where 10 µL of blood was applied to the FTA cards, and 1.2 mm punches were taken from the cards for analysis [6].

Evaluation studies were also performed using Genomic control DNA, including AmpFℓSTR™ 007 (male) and 9947A (female) (Thermo Fisher Scientific, Waltham, MA, USA) in addition to the use of positive control DNA included in the Ingenomics™ AutoProfiler system.

A limited set of mock casework samples were generated, with DNA extracted in-house. Drinking glass was used as a representative forensic sample obtained from a known donor with informed consent. After consumption of water orally by contact, the edges of the glass were swabbed to collect DNA traces. Other forensic casework samples such as the undergarments of a female with seminal stains, pants of female carrying vaginal fluid, aborted tissue, an umbilical cord, a blood sample from a car driver seat, a trimmer, shaver, cigarette butt, saliva after chewing tobacco, chewing gum, a glucometer, tissue block, and airbags from vehicle accidents, were examined post DNA extraction. Additionally, DNA isolated from the roots of 2 hair strands were also analysed.

For efficiency analysis, five human bone samples were used, and isolation of DNA from the bone samples was carried out using the organic method and the AllEx® Automated Nucleic Acid Extraction System using Kit AllEx® Forensic DNA Kit 96 T (GeneAll Biotechnology Co. Ltd. Korea).

For species cross-reactivity studies, control DNA samples from different animal species (Murine, Bovine, Canine) were procured from HiMedia Laboratories, India. Maccacus DNA was obtained (courtesy Dr Vivek Sahajpal). Total microbial DNA from soil samples and human gut microbial DNA were extracted using the PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific) [9].

Unless otherwise mentioned, DNA extraction of all the samples (including casework and forensic samples) was carried out using AllEx® Automated Nucleic Acid Extraction System using AllEx® Forensic DNA Kit 96 T (GeneAll Biotechnology Co. Ltd. Korea). Following extraction, DNA concentrations of manually diluted stock were confirmed using Investigator Quantiplex Pro RGQ (Qiagen GmbH,

Hilden, Germany) on the Rotor-Gene Q PCR System, following the manufacturer's protocol.

PCR amplification

The AutoProfiler STR kit contains PCR primer sets for amplification of a total of 28 loci: 24 autosomal STRs, including Penta D and Penta E, SE33 and D6S1043, one Y-STR (DYS391), Amelogenin and 2 Y-indels. Additionally, the kit includes primers for a synthetic DNA template to amplify the two IQC markers: IQC small (IQCS) of 80 bp and IQC Large (IQCL) of 500 bp. The 5 dyes used for DNA amplification were FAM™, HEX™, TAMRA™, ROX™, and PUR™. The sixth dye, LIZ™, was used to label the Size Standard, INGS600 (Ingenomics Pvt. Ltd., India). Unless stated otherwise, a QIAamplifier 96 (Qiagen, Germany) thermal cycler was used for DNA amplification in regular experiments, PCR cycles were performed on various instruments as indicated in the methodology.

The standard PCR reaction conditions for amplification were: Total reaction volume was 25 µL, including 0.5 ng template DNA, 7.5 µL, 3.3X master mix V, 2.5 µL 10 X primer mix, and nuclease free water. PCR was performed at the following conditions: 96 °C for 2 min, then 27 cycles of 95 °C for 10 s, 59 °C for 80 s, 60 °C for 10 min, and a final hold at 4 °C for further analysis.

Electrophoresis and data analysis

Most validation study experiments were performed at Ingenomics Private Limited, and for concordance studies, as detailed below, genotyping was performed at different state and central forensic laboratories across India. Amplified PCR products were detected, and electropherograms were generated on the Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific, Foster City, CA, USA), using run modules and ING6 (J6 Template) 6-dye variable binning modules, as described in the AutoProfiler STR kit User Guide. Samples were prepared by adding 1 µL of the PCR product or relevant allelic ladder to 9 µL of Hi-Di formamide (Thermo Fisher Scientific) at a ratio of 1:10 and an INGS600 size standard (Ingenomics) for electrophoresis. Samples were injected at 3 kV for 15 s and electrophoresed at 15 kV for 1350 s, run temperature was set at 60 °C. Performance Optimised polymer 4 (POP4) (Thermo Fisher Scientific, Foster City, CA, USA) was used for the fragment analysis. After data collection, electrophoresis results were analysed using GeneMapper ID Software v3.2.1 (Thermo Fisher Scientific, Foster City, CA, USA). For PCR-based, sensitivity, species specificity, inhibitor, precision and accuracy evaluation, and population-based studies, allele peaks were interpreted when the peak heights were greater than or equal to 50 relative fluorescence units (RFU).

Species specificity

The Ingenomics™ AutoProfiler kit was developed specifically for human DNA analysis. To evaluate its species specificity, DNA samples of select animals (Murine, Bovine Canine, and Primate) were used; 1 ng/µl of each non-human genomic DNA was analysed in triplicates using the AutoProfiler STR kit. Pooled genomic DNAs from several human-associated microbial species, including *Listeria monocytogenes*, *Escherichia coli*, and *Bacillus subtilis, Staphylococcus aureus* (BioZed Engineering Pvt. Ltd. Thane, Maharashtra, India), were prepared from cultures grown and purified in-house using the DNAeasy blood and Tissue kit (Qiagen, Germany). The specificity of the PCR amplification components of the Ingenomics™ AutoProfiler to human DNA was assessed by checking for cross-reactivity to DNA samples from primates, non-primate animals and pooled microorganisms. Amplified products were then electrophoresed on the Applied Biosystems 3500 genetic analyser and analysed for above-background peaks in or near the read region with GeneMapper™ ID-X 1.4 software.

Sensitivity

To analyse the detection limit of the Ingenomics™ AutoProfiler System and to further assess the sensitivity and efficiency of allele recovery, the kit was evaluated in triplicates, using Control DNA 9948 (2 ng/µL). The DNA concentrations of 1 ng/µL., 500 pg/µL, 250 pg/µL, 125 pg/µL, 62.5 pg/µL and 31 pg/µL were tested; non-template controls (NTCs) were also included. Control DNA 9948 was serially diluted into 1, 0.5, 0.25, 0.125, 0.100, 0.0625, and 0.03125 ng/µL. Amplification of DNA and further detection of alleles was performed according to the manufacturer's specifications using QiAamplifier 96. Amplified products were electrophoresed on the Applied Biosystem 3500 genetic analyser, and data was analysed using Gene Mapper™ ID-X 1.4 software. The analytical threshold was set at 50 RFU to detect alleles for sensitivity analysis.

Inhibition studies

The performance of the Ingenomics™ AutoProfiler STR Kit was tested in the presence of hematin, humic acid, tannin, melanin, indigo dye, fulvic acid and ethylenediaminetetraacetic acid (EDTA) as potential PCR inhibitors. Variable volumes of tannin (0.45 ng/µL) (Sigma-Aldrich, Shanghai,

China) were used in a mixture with Control DNA 9948 (1 ng/µL) for the amplification of STR markers in the presence of this PCR inhibitor. In a half reaction (12.5 µL), 1, 3, 5, and 7 µL of Tannin (0.45 ng/µL) was added with 1 µL of control DNA. This was repeated in triplicates using the Ingenomics™ AutoProfiler STR Kit. Hematin concentrations of 500 mM, 750 mM, and 1000 mM; for Humic acid, the concentrations were 100 ng/µL, 200 ng/µL, 300 ng/µL, Tannic acid at concentrations of 500 ng/µL, 750 ng/µL, and 1000 ng/µL and EDTA at concentrations of 750 mM, 1000 mM, and 1250 mM were used for these inhibition experiments. To assess the effect of inhibitors with respect to accuracy and consistency, triplicate samples were used following the standard protocol. The tolerance of the inhibitors was assessed via allelic calls, wherein the allelic dropout and change in notable peak height rations were tabulated.

Mixture study

Sexual assault casework samples are at times observed to be mixtures in nature, the mixture components predominantly are composed of male and female DNA, usually at different concentration levels. The variability of female and male DNA concentrations while performing genotyping can yield erroneous results. In order to study the Ingenomics™ AutoProfiler STR Kit's capability in resolving human mixture samples (Male and Female DNA) and further to ensure correct interpretation of these alleles as major and minor contributors of DNA is crucial [5]. To elucidate the useability in this regard, male and female DNA were mixed in multiple ratios, control DNA 007 (male) and 9947A (female) were utilised to simulate mixture ratios of 1:1, 1:3, 1:5, 1:7, 1:10, 10:1, 7:1, 5:1, and 3:1 (M: F). Each mixture was tested in triplicates using the Ingenomics™ AutoProfiler STR Kit and was analysed. The final concentration for the PCR reaction was maintained at 1 ng/µL.

Repeatability and reproducibility

Of the total indicated samples analysed in this study, five random saliva samples were selected and were tested for repeatability and reproducibility studies. DNA at the following concentrations were opted for the analysis: 1 ng/µl, 0.5 ng/µl, 0.15 ng/µl, 0.075 ng/µl, 0.0375 ng/µl, 0.015 ng/µl, and 0.0075 ng/µl. Repeatability was confirmed by performing PCRs three times, at least one week apart, and was executed by the same individual. Reproducibility was assessed by three different individuals perform the PCRs using the same indicated concentrations and PCR protocol. All PCR setups for both repeatability and reproducibility were carried out manually.

Stability

To evaluate the stability of the Ingenomics™ AutoProfiler STR Kit, three different sample types i.e. blood, saliva, and semen, were collected from a single male individual. PCRs were manually set up for each sample type using an optimal DNA concentration of 0.5 ng/µL. Each test was performed in triplicates, and the samples were analysed on both the Applied Biosystems 3500 and 3500xL Genetic Analyzer instruments. The stability tests incorporated the same protocol parameters as used for the sensitivity test.

Annealing extension temperature test

Annealing temperature plays an appreciable role in the primer hybridisation and a low or high annealing temperature can affect the performance of the PCR [12]. At low annealing temperature primer oligos are likely to bind non-specifically to the complementary template sequences that are found in the variable regions; this in turn might generate unwarranted allelic calls or allelic drop-outs. To evaluate the effect of annealing temperature on performance, PCR was conducted at various annealing temperatures ranging from 57 to 61 °C.

Validation of different PCR instruments

To assess the applicability of various PCR instruments routinely used in laboratories, four PCR instruments were tested for functional analysis, 9700 (ABI9700 Thermo Fisher Scientific), Veriti™ Thermal Cycler (Thermo Fisher Scientific), QiAamplifier 96 (Qiagen, Germany) and ProFlex (Thermo Fisher Scientific), the genotypes obtained by using 9948 M (commercial male control DNA), and two male DNA samples were analysed for concordance across four different PCR instruments and two operators were mandated to execute the same.

Evaluation of PCR cycles on performance

To assess the performance of the Ingenomics™ AutoProfiler STR Kit samples were subjected to varying PCR cycle numbers on a single PCR instrument. The profiles were then evaluated using DNA concentrations of 100 pg/µl. This approach was expected to determine how changes in the number of PCR cycles influenced the quality and accuracy of the generated STR profiles and would reflect on the application of the kit use, based on sample types.

Direct amplification

The buffer system of Ingenomics™ AutoProfiler STR Kit is optimised to directly amplify some sample types, such as reference samples, like saliva and blood, on FTA cards. In order to test its efficiency, direct amplification of the above-mentioned sample types, obtained from different sources, was carried out. Samples were further analysed as indicated in all instances of analysis.

Population and concordance studies

A total of 400 anonymous samples from various regions across India were analysed using the Ingenomics™ Auto Profiler STR Kit under standard conditions. A concordance study was conducted using 200 of these samples. Allele frequencies for 23 STR markers, excluding SE33 were calculated using Modified-Power State software. The random match probability, power of discrimination, power of exclusion, and cumulative probability of exclusion were also determined. To examine genotype concordance.

Results and discussion

Species specificity

The analysis of DNA samples from the indicated animal and microbial species demonstrated a high degree of human specificity for the Ingenomics™ AutoProfiler kit. DNA samples with a concentration of 1 ng/µL from Murine, Bovine, Canine, Primate, and Microbial species (*Listeria monocytogenes*, *Escherichia coli*, and *Bacillus subtilis, Staphylococcus aureus*) were used. No cross-reactivity with any of the species was observed, except for a few non-specific peaks in the primate samples (Fig. 1). The cross-reactivity analysis was repeated in triplicate, and it was confirmed that the specificity was limited to human DNA samples and did not extend to any of the unrelated species.

Sensitivity

Forensic samples are often exposed to various stress conditions and are known to yield low amounts of DNA, leading to ineffective genotyping. It becomes highly crucial to choose a reagent system that is sensitive and gives appreciable allele calls and a complete profile, even at low amounts of DNA. Ingenomics™ AutoProfiler was analysed for sensitivity, no allelic drop-outs were observed when the template DNA concentration was as low as 0.0625 ng/µL, and no more than one-third allelic disbalances were noted as the template volume was reduced to 0.0325 ng/µL (Fig. 2A, 2C). The mean peak height ratio was plotted 100% allelic recovery was observed with the concentration range was 0.625 ng/µL and over 95% of alleles were recovered when the concentration was as low as 31.25 pg/µL (Fig. 2B). The peak height ratio was set at 50 RFUs no major allelic drop out was observed (Fig. 2A), and the reagent system was observed to generate complete profiles as depicted in the heat map (Fig. 2C). The results obtained indicate that the Ingenomics™ AutoProfiler has high sensitivity, making it suitable for analysing sensitive and degraded forensic samples, along with its capabilities to analyse broad marker set on data basing samples too.

Inhibition studies

It is well documented that during multiplex PCR amplification of target DNA, various factors from the natural environmental sources or as components of DNA processing and extraction protocols cause inhibition of PCR reaction**.** Presence of PCR inhibitors often result in allelic dropout and gradual decrease in peak height of the larger amplicons during genotyping. The tolerance and sensitivity of Ingenomics™ AutoProfiler was assessed by evaluating complete profiles obtained from the Control DNA 9948 when 5 µL of tannin (0.45 ng/µL) was used to dilute the DNA. There were allele dropouts at larger markers (D6S1043, Penta D, Penta E, and SE33) and a decrease in peak height observed at a higher volume of 7 µL of 0.45 ng/µL, depicted in the (Fig. 3).With Hematin concentrations of 500 mM, 750 mM, and 1000 mM, 100%, 38% and 14% allelic calls were observed respectively, with Humic acid concentrations of 100 ng/µL, 200 ng/µL, and 300 ng/µL, 100%, 56%, and 24% allelic calls were observed respectively, with Tannic acid concentrations of 500 ng/µL, 750 ng/µL, and 1000 ng/µL, 98%, 69%, and 49% allelic calls were observed respectively and with EDTA concentrations of 750 mM, 1000 mM, and 1250 mM, 99%, 37% and 10% alleles were observed respectively (Fig. 4). Thus, the small variation in the concentration of inhibitors can lead to noticeable differences in PCR amplification, which is observed consistently with various other PCR reaction systems too [1]. These results indicate the effect of inhibitors, that needed to be evaluated and regulated while analysing samples using Ingenomics™ AutoProfiler for genotyping.

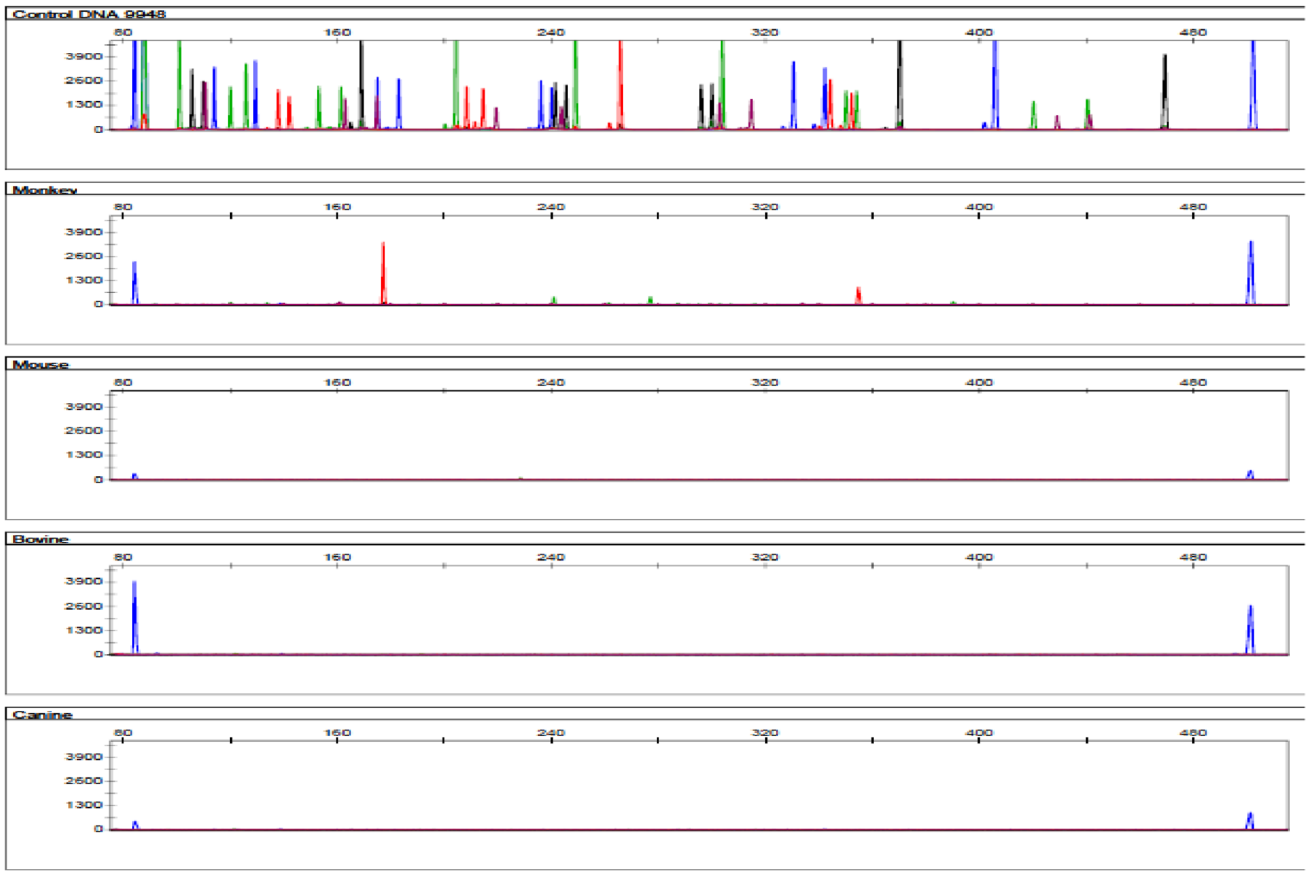

Fig. 1 Representative results of the species specificity assessment for murine, bovine, canine and Maccacus: A standard 1 ng/µl DNA from all other species was used. Results indicate the appreciable specificity of the reagent system to human DNA

Mixture study

To assess the ability of the Ingenomics™ AutoProfiler STR Kit in distinguishing between the minor and major contributor DNA, and resolve the alleles of the minor contributor efficiently, various mixture model ratios (1:1, 1:3, 1:5, 1:7, 1:10, 10:1, 7:1, 5:1, and 3:1) were analysed. Heterozygote Balance (HB) was calculated for male and female allelic profiles using the formula as per SWGDAM guidelines [4]. It was observed that 1:1 mixture ratio gave equal peak heights of both the contributor DNA (Male and Female) with an average heterozygote balance of 0.67 and 0.71 respectively (Table 1). In case of higher concentration of major contributors, i.e., 1:7, 7:1, 10:1, and 1:10 the kit successfully was able to resolve the minor contributor alleles and no allelic dropouts of either contributor was observed. Each allele of both the contributors was distinguished, without any overlapping or shutter phenomenon. Minor contributor alleles showed decrease in peak height and the major contributor allelic ratio increased, suggesting the increase in concentration of major contributor DNA (Fig. 5).

Repeatability and reproducibility

As performance consistency due to repeat freeze thaw of reagents can get affected, it becomes significant to inspect the repeatability and reproducibility and to ensure required performance due to variation in personnel handling the reagents. To test for the same replicates of the samples analysed, the Ingenomics™ AutoProfiler showed consistency in the quality of allelic call, peak heights, and sensitivity. Despite variations in personnel handling and the time gaps between processing the samples, the genotyping quality remained stable, ensuring repeatability. Uniformity in data with varied concentrations showed no difference in profile outcomes.

Stability

Different body fluids might vary qualitatively and quantitatively with regards to DNA yield. The present study evaluated three body fluids from single male individual. 1) Blood, which although comprises of sufficient nucleated

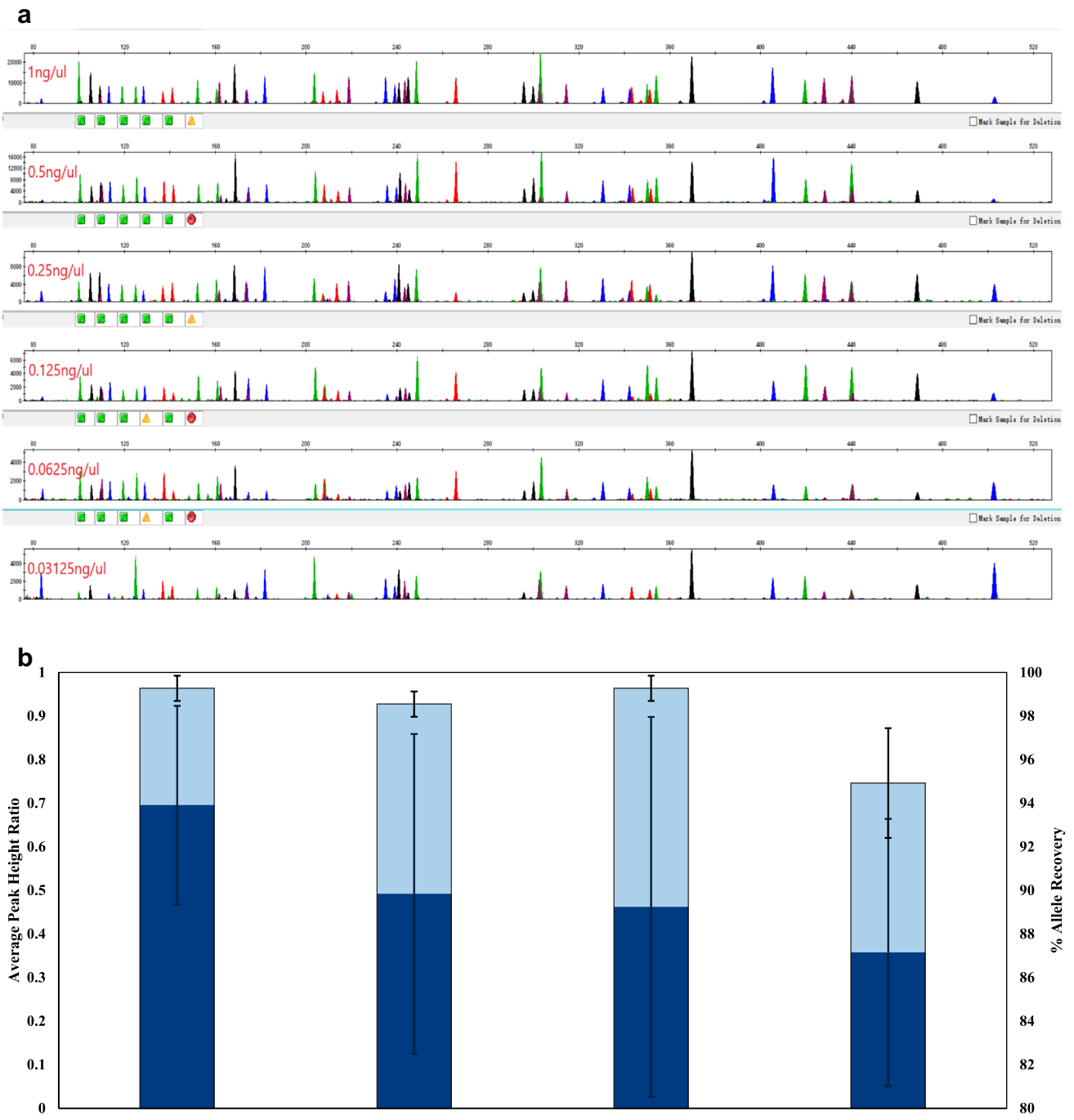

Fig. 2 **a** Sensitivity of the Ingenomics™ AutoProfiler STR Kit using the control DNA as template: The peak height ratio and allele call were analysed. Samples at various concentrations of (1 ng/µl, 500 pg/µl, 250 pg/µl, 125 pg /µl, 62.5 pg/µl, and 31 pg/µl) were analysed, the image shows allelic calls obtained at the indicated concentrations. **b** the average peak height ratios and the percentage of allele calls were demonstrated to indicate the recovery at various listed concentrations of the template DNA. **c** the analysed samples were determined based on the concentration, blue boxes indicate no allelic dropout and yellow boxes indicate that only one of the two expected heterozygote alleles dropped out

cells yet has heme which can inhibit the PCR reaction [1]. 2) Saliva has a substantial number of epithelial cells that serve as source of sizeable quantities of DNA. 3) Semen on the other hand, as a source of DNA, depends hugely on the presence of sufficient number of spermatozoa and hence at times is a tough sample to deal with. In order to check for the stability of Ingenomics™ AutoProfiler STR Kit on these three types of body fluids genotyping was

	0.03125 ng/µL	0.0625 ng/µL	0.125 ng/µL	0.25 ng/µL	0.5 ng/µL	1 ng/µL
TH01						
D5S818						
D21S11						
D18S51						
D6S1043						
Rs2032678						
AMEL						
D3S1358						
D13S317						
D7S820						
D16S539						
CSF1PO						
Penta D						
D2S441						
vWA						
D8S1179						
TPOX						
Penta E						
Rs771783						
D19S433						
D22S1045						
D2S1338						
FGA						
DYS391						
D1S1656						
D12S391						
D10S1248						
SE33						

Fig. 2 (continued)

carried out as described earlier. Results have demonstrated significant similarity in the quality and allele calls on all the body fluids tested, indicating the suitability of the test for forensic case work use.

Annealing extension temperature test

For the studied annealing temperatures analysed, no allele dropout was observed and complete profiles were generated when the annealing temperature ranged from 57 to 61 °C. Profiles can be amplified normally, and there is no noticeable discrepancies were observed, such as marker drop or additional peaks due to the temperature variance in difference of ± 2 °C (Fig. 6).

Validation of different PCR instruments

As laboratories are implementing the use of autosomal STR kits, use of PCR equipment, based on choice and availability is expected in amplifying the targets. It becomes imperative that the Ingenomics™ AutoProfiler STR Kit is also analysed for performance and profile generation, while using various PCR instruments. The results of our evaluation revealed no significant difference in intra-locus balance or peak height. The profiles were replicable, with no noticeable decrease in intensity or allelic dropout (Fig. 7).

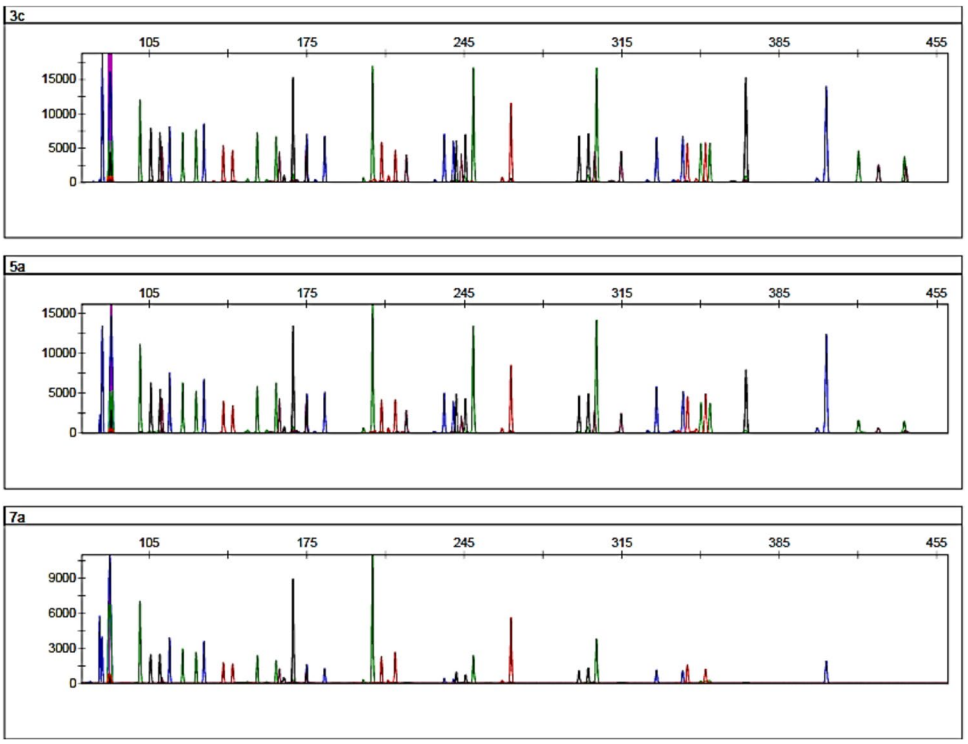

Fig. 3 Inhibition studies: Allelic drop was analysed using control DNA 9948 using inhibitors such as Tannic acid, Hematin, Humic acid and EDTA. The DNA laced with inhibitor was analysed for peaks and peak height ratios at varied concentrations of inhibitors (Panel 3C is at 3 µl of tannic acid, 5a at 5 µl of tannic acid, 7a at 7 µl of tannic acid volume used)

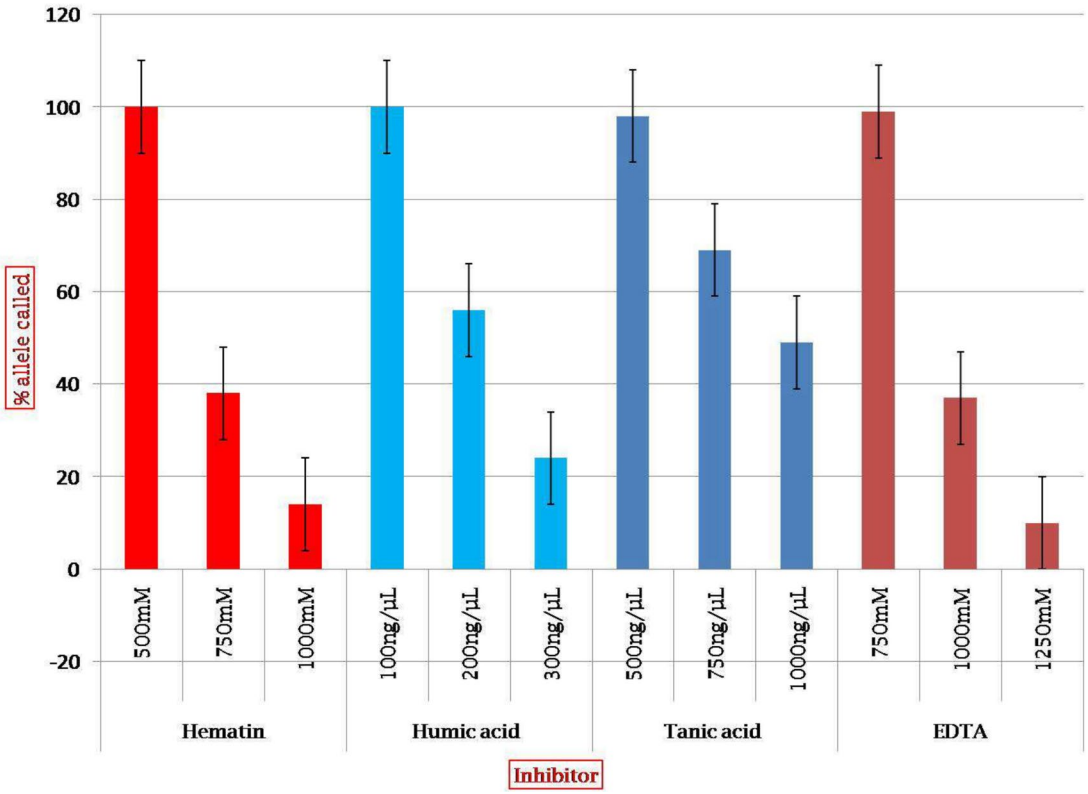

Fig. 4 Overview of Ingenomics™ AutoProfiler STR Kit inhibitor resistance: To study the robustness, the Control DNA 9948 (1 ng/µL) was used as a template with AutoProfiler STR Kit in the presence of potential PCR inhibitors at various concentrations denoted along x-axes. The average number of alleles recovered is presented in percentage along the y-axes. In all the instances, as the inhibition increased the % of allele called decreased (shown in the graph with error bars)

Table 1 Average heterozygote balance of various mixture studies

Mixture ratio (M:F)	Average heterozygote balance	
	Male contributor DNA	Female contributor DNA
1:1	0.60	0.71
1:3	0.53	0.78
3:1	0.80	0.566
1:5	0.45	0.87
5:1	0.86	0.46
1:7	0.47	0.88
7:1	0.84	0.45
1:10	0.86	0.38
10:1	0.38	0.90

Evaluation of PCR cycles on performance

Forensic case work samples are evaluated for DNA obtained from different evidential sources. Based on the type of sample, DNA concentration, state of degradation, method and materials the DNA is obtained from, it might be important to subject the samples to different number of PCR cycles, and as required to generate the correct profiles.

Profiles obtained from the analysed samples after 29 to 31 cycles were over-amplified and had some artefacts (Fig. 8). When the number of cycles was reduced to 27 and 28, the profiles were more balanced, without over-amplification; the overall signal was not reduced and showed lesser artefacts. With 27 and 28 cycles, all the alleles were correctly assigned with complete profiles and with significant peak heights (Fig. 9). These results confirmed the sensitivity of the Ingenomics™ AutoProfiler STR Kit, suggesting that an optimal number of PCR cycles should be selected based on sample characteristics to ensure accurate profiling.

Direct amplification

The buffer system of Ingenomics™ AutoProfiler STR Kit is optimised to directly amplify certain sample types, which include reference samples like saliva and blood on FTA cards. In order to test the efficiency of the same, direct amplification of the above-mentioned sample types, obtained from different sources, was carried out. Direct

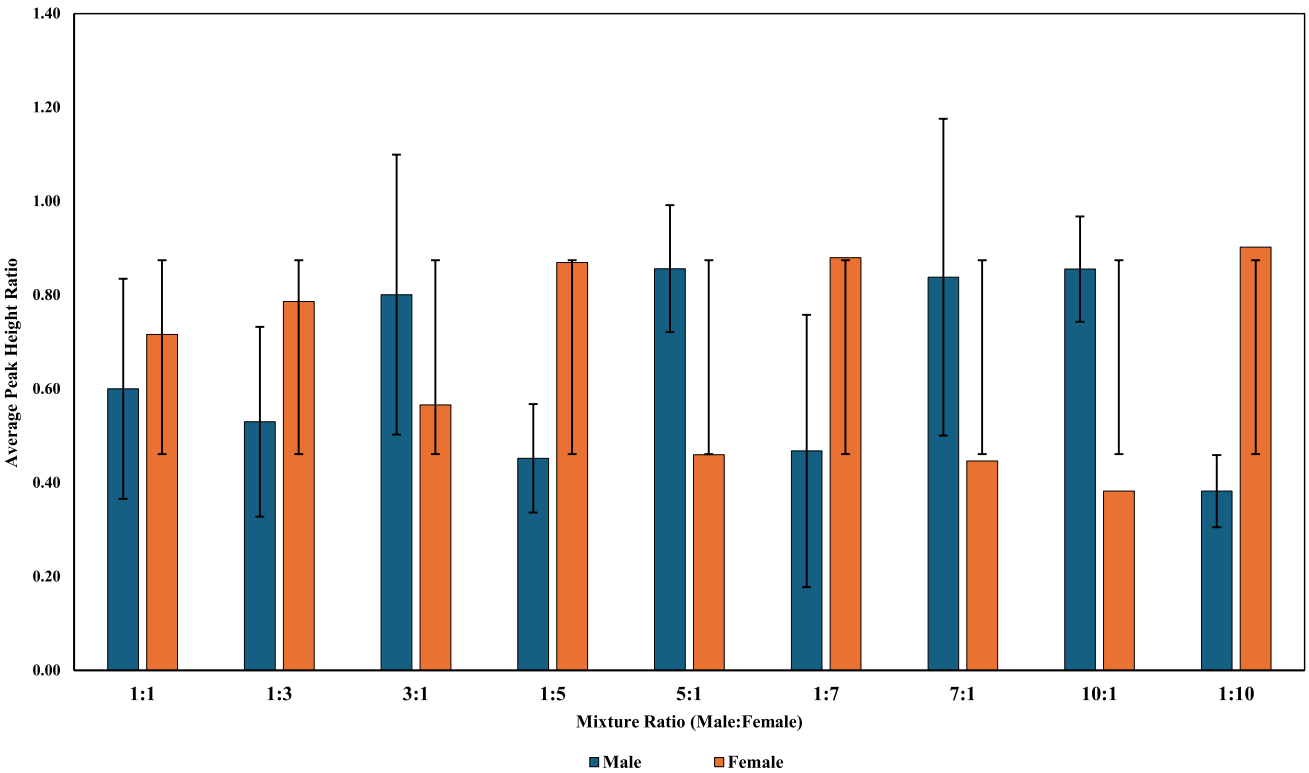

Fig. 5 The peak height ratio was determined as a reflection of the ratios of the male and female DNAs (Major and Minor contributors): The peak height of both the contributors is observed to have not gone below 0.40%. The average peak height ratio showed a very high consistency in variation as the major contributor to minor contributor shifted from being male to female or from being female to male

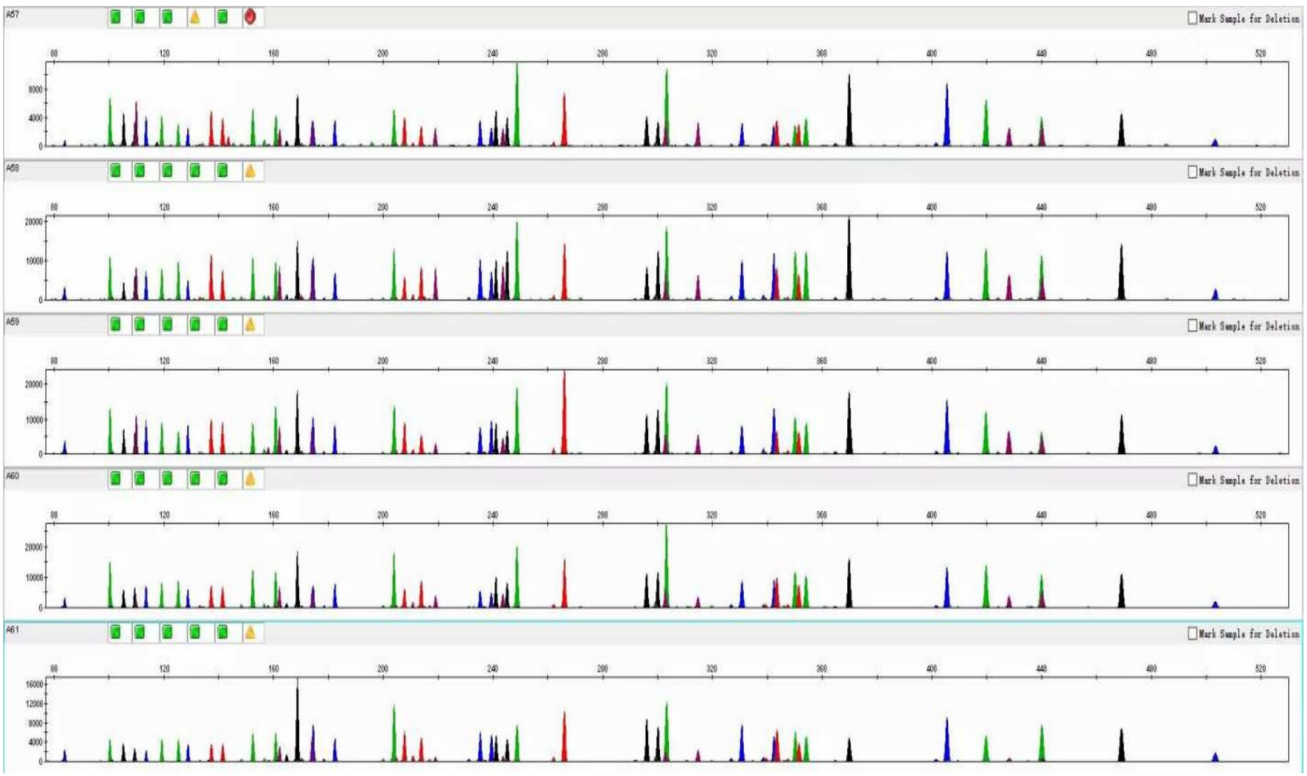

Fig. 6 Effect of annealing extension temperature: Data shows no allelic dropout as observed, complete profiles were generated when the annealing temperature ranged from 57 to 61 °C. Increase in temperature showed allelic dropout of larger amplicons

amplification protocol was tested and led to balanced and complete profiles (Fig. 10). Lowering the punch size of the FTA from 1.2 to 0.5 mm and increasing the number of cycles from 26 to 28 allowed for simultaneous amplification of extracted and direct samples with the same thermo cycler protocol. These adaptations enabled the obtainment of quality profiles, the results are in concordance with previous studies. These outcomes suggest the applicability of the kit for direct amplification and profiling.

Population and concordance studies

Allele frequencies for 23 STR markers, excluding SE33 (Supplementary Table 1), were calculated using Modified-Power State software. Additionally, the random match probability, power of discrimination, power of exclusion, and cumulative probability of exclusion were determined (data not shown). The genotyping results obtained with the Ingenomics™ Auto Profiler STR Kit were in complete agreement with those generated using the GlobalFiler™ PCR Amplification Kit, Investigator 24plex Kits, and PowerPlex® Fusion 6C System. The genotype data obtained from the Ingenomics™ Auto Profiler STR Kit was consistent with those generated from the aforementioned established systems. This large-scale study provided the basis for determining individual allele frequencies and the random match probability for the analysed samples.

Conclusion

The present study showcases the usability and validity of the Ingenomics™ AutoProfiler STR Amplification Kit for forensic casework analysis, databasing, and scientific research. This kit has been meticulously developed to incorporate the 20 primary CODIS autosomal STR markers, along with two unique internal quality controls that enable users to assess and evaluate the PCR performance of their samples, thus enhancing the quality assurance process. By including 28 markers relevant to both global and Indian populations, the kit demonstrates its broader applicability across diverse populations. It has been validated according to SWGDAM guidelines for use with both forensic and database samples. With over 500 samples analysed to evaluate its performance, the kit has proven suitable for

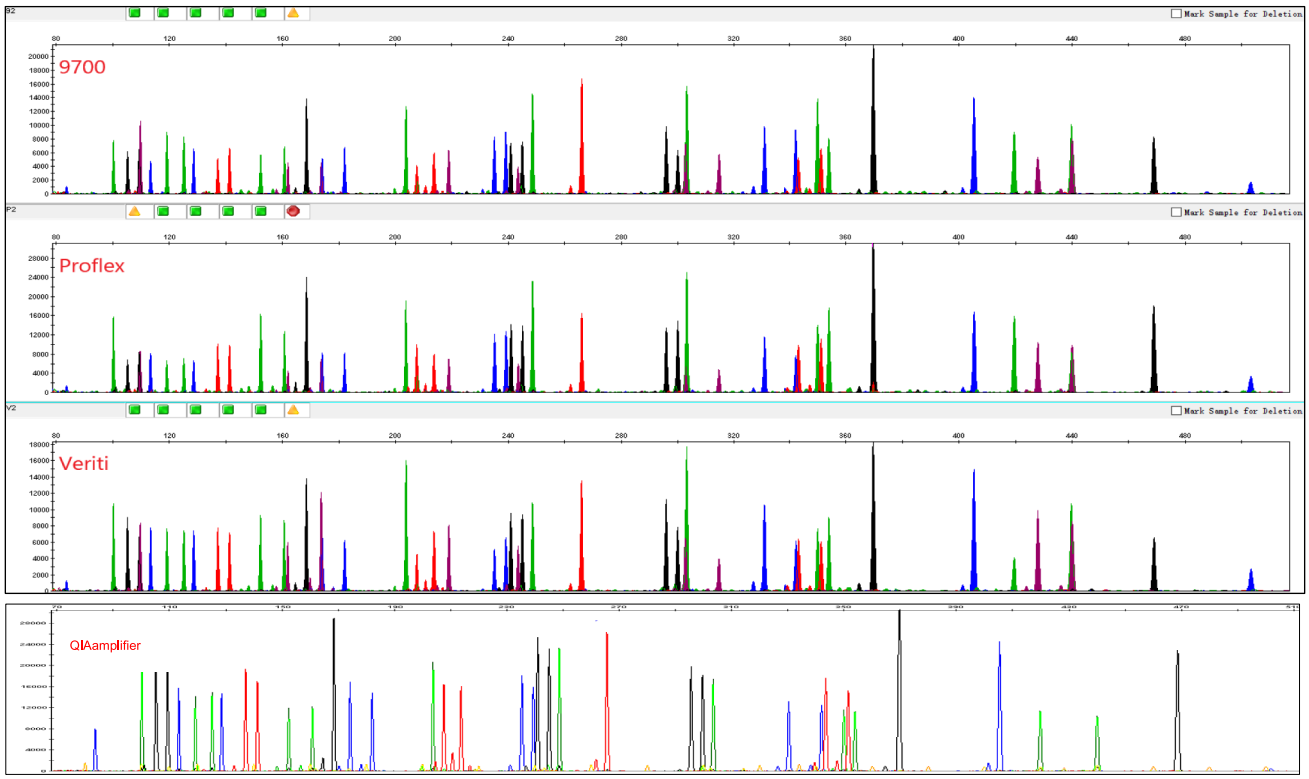

Fig. 7 Use of various PCR instruments: The expected genotypes obtained by using 4 different PCR instruments were concordant across 9948 control DNA and two male DNA, and also during different personnel operating the instruments and the kit

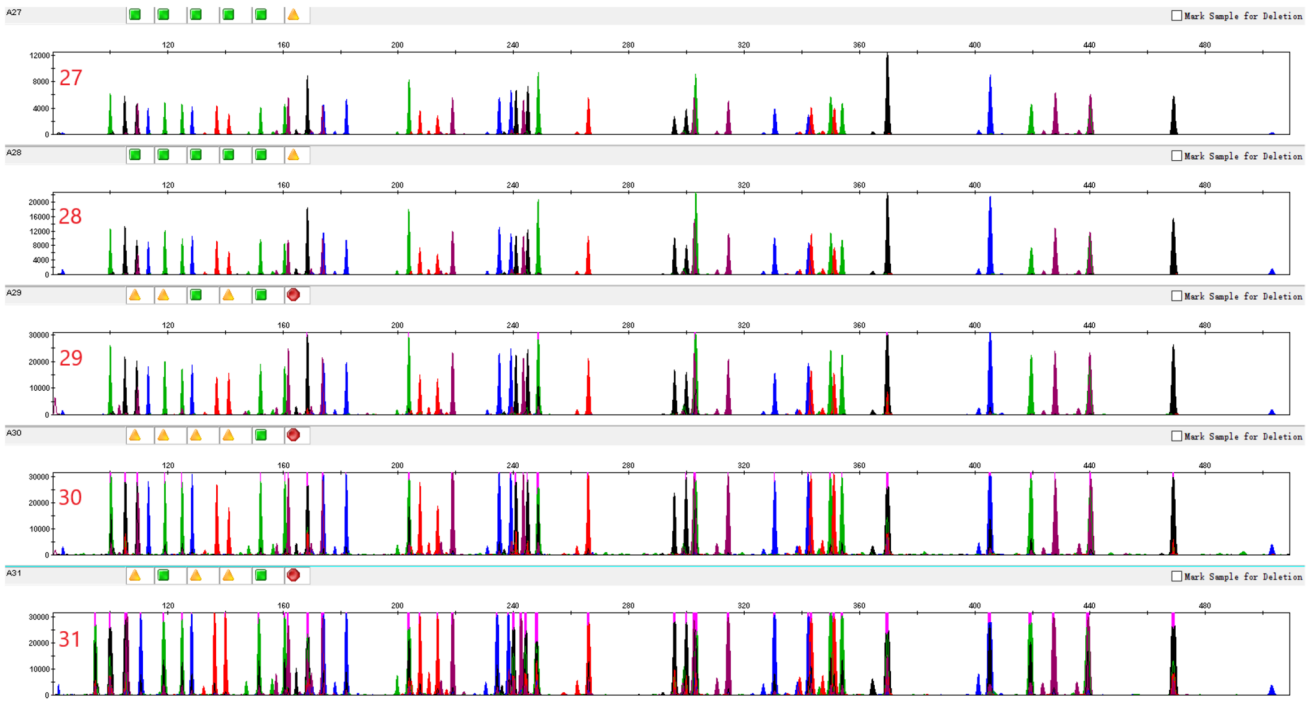

Fig. 8 Effect of different PCR Cycles: Cycle number analysis showed that complete profiles were obtained from PCR Cycle numbers 27 to 31, but there was increase in peak height ratio for the with the increase in number cycles (27–31) while at 26 cycles, there were allelic dropouts of larger amplicons

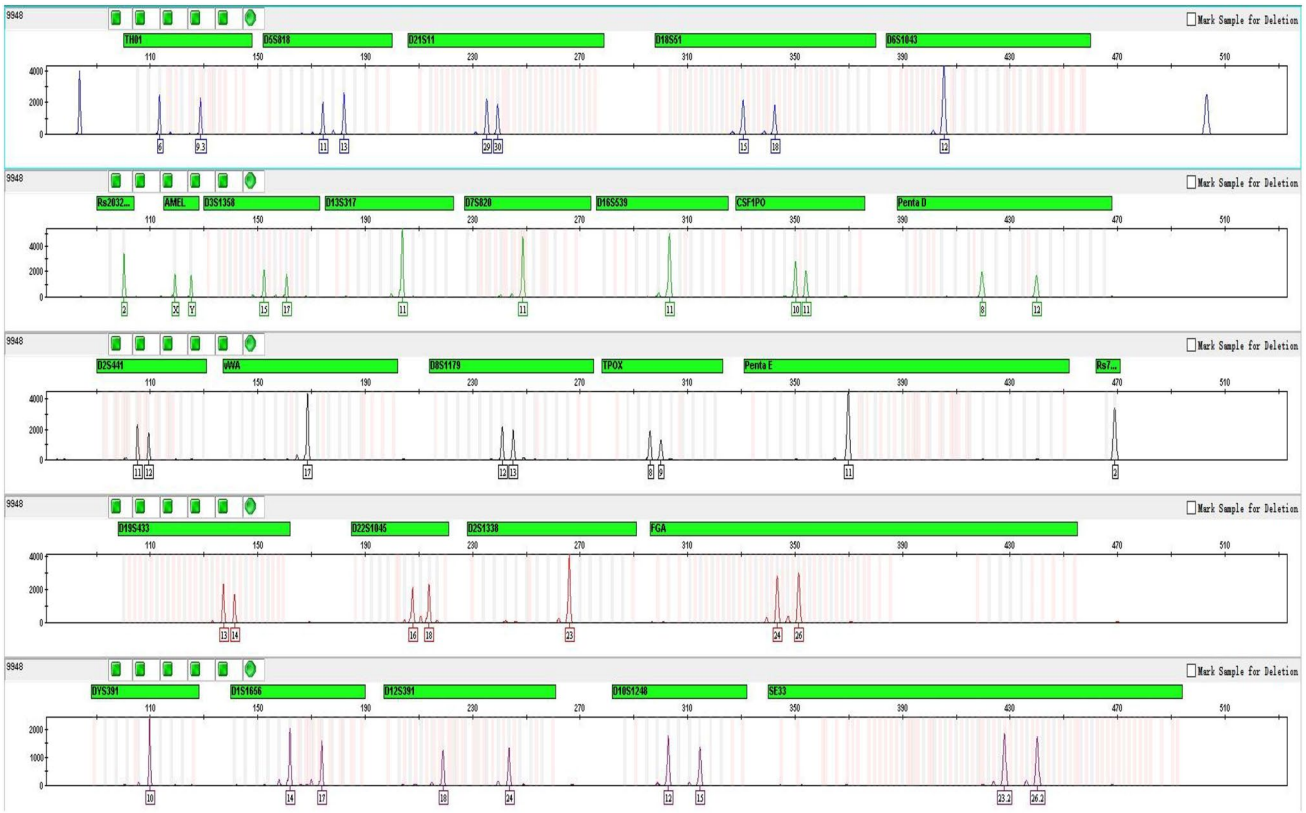

Fig. 9 Representative profile of Control DNA 9948 with 27 cycles using Ingenomics™ AutoProfiler Kit

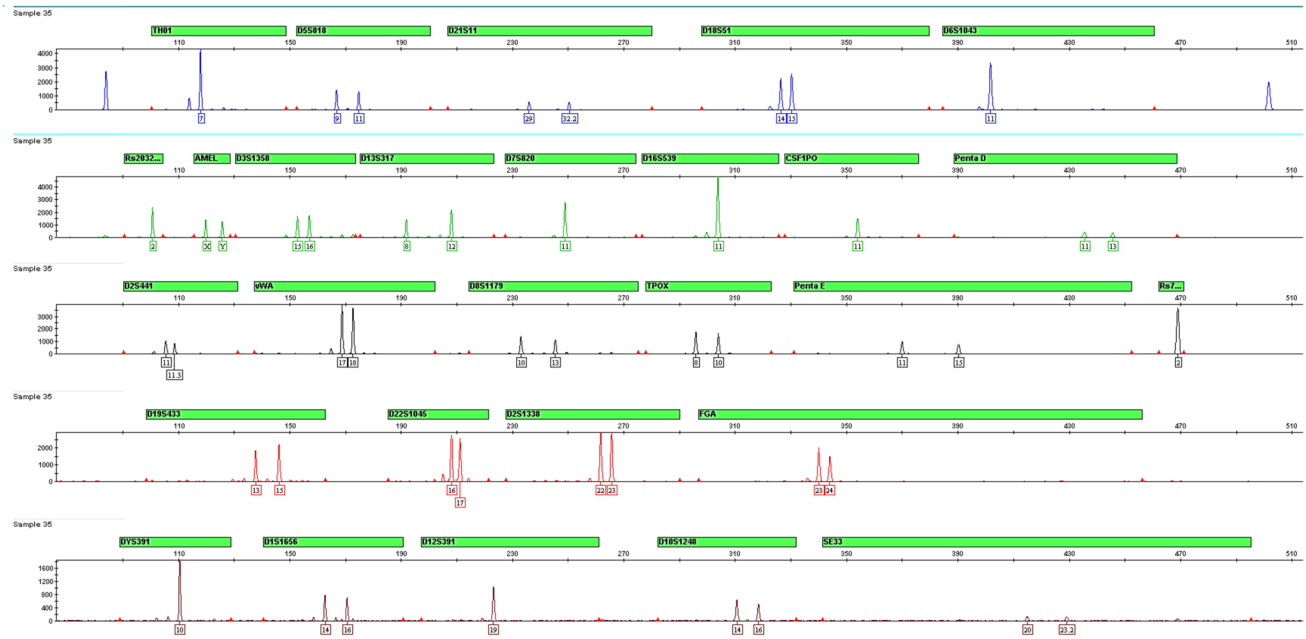

Fig. 10 Applicability in direct amplification: A balanced peak height with a complete profile was obtained upon direct amplification. The profile was generated by reducing the FTA punch size and raising the amplification cycles, which was consistent with the profile observed after the extraction

various casework analyses and is highly recommended for use in forensic laboratories.

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Authors contribution The study was conceptualized by GC, VS and SS. The study was supervised by VS, BKM, DY, KD, UA, KMY, AP and GC. Experimental works were carried out by SS, RS, CVER, data evaluation, analysis and manuscript preparation were carried out by SS, RS, CVER, RK, SD. RM, JJS edited the draft/s. All the experiments were performed at Ingenomics Private Limited, New Delhi.

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Data availability With corresponding author Dr. GC on request.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethical approval The ethical committee of Banaras Hindu University, Varanasi (Uttar Pradesh), India approved the study with Ref. No., I.Sc./ECM-XVI/2023–24).

Consent to participate Informed consent was obtained from all participants included in the study.

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