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Research Article

Internal Validation of the Investigator Argus X-12 QS Amplification System for Reduced Volume to Improve Cost Effectiveness in Forensic DNA Analysis

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Abstract
The Investigator Argus X-12 QS Kit is specifically designed for the rapid and reliable generation of DNA profiles from various sample types and particularly useful for kinship confirmation, paternity testing and analysis of mixed samples involving multiple individuals in forensic investigations. The kit enables the co-amplification of 12 X-chromosomal markers, delivering reliable and informative genetic data for complex forensic investigations. The Investigator Argus X-12 QS Kit was previously validated in the DNA laboratory of the Government Analyst's Department, following the manufacturer's guidelines for full-volume (25 µl) reactions, establishing a working range of 0.5 ng and 0.75 ng. To minimize annual chemical costs, an internal validation was conducted using a reduced reaction volume of 15 µl. This validation assessed the system's sensitivity, repeatability, reproducibility, mixture interpretation, and contamination study by amplifying a female and male DNA samples from a case study and the control DNA 9947A of the Investigator® Argus X-12 QS PCR Kit. Sensitivity studies revealed that the optimal DNA input range for achieving a complete profile was between 0.5 ng and 0.75 ng, which aligns with the manufacturer's recommended minimum DNA template input of 0.5 ng. The system's repeatability and reproducibility were confirmed through consistent results observed across repeated studies. In mixture analysis, 1:1 ratio of male: female samples successfully detected all alleles contributed by both individuals. However, when the ratio increased to 1:3, allele dropout was observed. The internal validation of the Argus X-12 QS Amplification System with a reduced reaction volume of 15 µl provides a cost-effective solution while maintaining its high effectiveness in forensic DNA analysis, offering enhanced capabilities for handling complex casework.
Keywords: Investigator Argus X-12 QS Kit, X-STR, Internal Validation, Sensitivity, Repeatability, Reproducibility, Mixture Analysis.

Introduction

X-STR analysis has been increasingly utilized in forensic settings as it provides valuable genetic insights that extend beyond what autosomal STR systems provide. Due to its unique mode of inheritance, X-STR analysis serves as valuable evidence in a court of law (1). Unlike autosomal markers, X-STRs are inherited in a unique pattern-males inherit one X chromosome from their mother, while females inherit one X chromosome from each parent. In females, the X chromosome is present in two copies, which undergo recombination along the entire chromosome during female meiosis and are transmitted to both female and male descendants (2). This distinctive inheritance makes X-STRs particularly useful in complex kinship investigations, such as confirming relationships in cases involving half-siblings, grandparents, or other relatives connected through the maternal line. Furthermore, X-STRs are highly effective in examining mixed DNA samples, especially when female DNA is present in a predominantly male sample or the other way around in sexual assault cases. Therefore, X-STR analysis serves as a powerful tool to complement autosomal and Y-chromosomal STRs in solving in criminal investigations, kinship, deficiency cases and paternity disputes (3, 4). It can also detect female DNA in mixed samples and identify genetic anomalies such as Klinefelter syndrome and

Ullrich-Turner syndrome. These advantages have led to the development of several X-STR marker multiplexes for forensic applications (5, 6).

Although commercial kits are available for analyzing a wide variety of genetic markers on both the Y chromosome and autosomes, only one commercial kit currently assays markers on the X chromosome-the Investigator Argus X-12 QS Kit (QIAGEN, Hilden, Germany). This Argus X-12 QS Kit co-amplifies 12 X chromosomal markers from four linkage groups (DXS10148, DXS10135, DXS8378, DXS7132, DXS10079, DXS10074, DXS10103, HPRTB, DXS10101, DXS10146, DXS10134, and DXS7423), along with Amelogenin and the autosomal marker D21S11. It is capable of amplifying DNA within a dynamic range of 0.2 to 2.0 ng in a 25.0 µl reaction volume (7, 8).

In forensic cases, the burden of proof requires evidence to be established beyond any reasonable doubt. The absence of comprehensive internal validation data can present challenges when defending the validity and reliability of DNA analysis results. Furthermore, laboratories must carefully consider the cost-effectiveness of the chemicals used in DNA analysis, as these often involve high-cost and advanced reagents.

This study was conducted to perform an internal validation of the Investigator Argus X-12 QS Kit (QIAGEN, Hilden, Germany) for use in the Government Analyst's Department's DNA laboratory employing a reduced reaction volume of 15µl for amplifying both reference and various forensic samples. This method aimed to lower amplification costs by allowing a greater number of samples to be processed.

Key parameters assessed during the study included sensitivity, repeatability, reproducibility, mixture analysis, and contamination evaluation. The internal validation followed the guidelines recommended by the European Network of Forensic Science Institutes (ENFSI) and the Revised Validation Guidelines established by the Scientific Working Group on DNA Analysis Methods (SWGDAM) (9).

Materials and Methods

Sample Preparation

A female DNA sample from a case study (diluted to 0.1 ng/µl), received at the Government Analyst's Department's DNA laboratory and the control DNA 9947A (concentration of 0.1 ng/µl) of the Investigator® Argus X-12 QS PCR Kit were used for the sensitivity, repeatability and reproducibility studies. In mixture studies a female and a male DNA sample (each diluted to 0.1 ng/µl) from a case study received at the Government Analyst's Department's DNA laboratory were used. The female and male DNA samples from case study were extracted using the QIAamp® DNA Investigator Kit and quantified through Real-time PCR (Applied Biosystems). A serial dilution was performed with the following template amounts: 0.95ng, 0.90 ng, 0.85 ng, 0.80 ng, 0.75 ng, 0.70 ng, 0.60 ng, 0.50 ng, 0.45ng, 0.40 ng, 0.30 ng, 0.20 ng and 0.10 ng for sensitivity, repeatability and reproducibility studies. A female: male and a male: female DNA mixture sets were prepared for mixture studies with a total DNA input of 0.5 ng and the following ratios were tested: 1:15, 1:10, 1:7, 1:3, 1:1, and 1:0.

DNA Amplification

Samples were amplified using an Applied Biosystems ProFlexTM Base thermal cycler. The following amplification set-ups and cycling parameters were used according to Technical Manual of Investigator® Argus X-12 QS PCR Kit. Each amplification reaction contains 3.75 µl of Investigator® Argus X-12 QS Reaction Mix 2.0 and 1.25 µl of Investigator® Argus X-12 QS Primer Pair Mix with respective amount of template DNA and nuclease free water. DNA amplification reactions were cycled using an initial denaturation of 95°C for 1 min, 35 cycles of 95°C for 15 s, 55°C for 15 s, and 30 s at 72°C followed up by a 7 min hold at 72°C and final 4°C soak.

Amplification Analysis

Five fluorescent dyes are used in Investigator® Argus X-12 QS PCR system. Its 5-dye Matrix Standards (Promega Corporation) were used to establish spectral resolution, allowing for the evaluation of each fluorescent dye contained in the kit. The allelic ladder mix provided with the Investigator® Argus X-12 QS PCR Kit and the DNA Size Standard 550 (BTO) were used in all analyses. Amplification products were separated using Applied Biosystems 3500 series Genetic Analyzer (Life TechnologiesTM).

A volume of 9.5 µl of Hi-Di™ Formamide (Life Technologies™) and 0.5 µl of DNA Size Standard 550 (BTO) were added to each well and 1 µl of amplified sample or allelic ladder was added to prepare the samples for separation and analysis. POP4® polymer was used in 36 cm capillary array. Samples were injected for 1.2kV,

24s injection on the Applied Biosystems 3500 series Genetic Analyzer. GeneMapper® ID-X v 1.2 (Life Technologies™) with a 150 RFU analysis threshold was used to perform allele sizing.

Table 1. Amplification set-up used for sensitivity studies with reduced total reaction volume of 15.0µl.

Concentration of the template DNA (ng)	Volume of 0.1 ng/µl template DNA (µl)	Volume of the master mix (µl)	Volume of the nuclease free water (µl)
0.10	1.0	5.0	9.0
0.20	2.0	5.0	8.0
0.30	3.0	5.0	7.0
0.40	4.0	5.0	6.0
0.45	4.5	5.0	5.5
0.50	5.0	5.0	5.0
0.60	6.0	5.0	4.0
0.70	7.0	5.0	3.0
0.75	7.5	5.0	2.5
0.80	8.0	5.0	2.0
0.85	8.5	5.0	1.5
0.90	9.0	5.0	1.0
0.95	9.5	5.0	0.5

Table 2. Amplification set-up used for mixture studies with reduced total reaction volume of 15.0 µl.

Mixture ratio	DNA amounts of binary mixtures (ng)	Volume of the 0.1 ng/µl female DNA sample (µl)	Volume of the 0.1 ng/µl male DNA sample (µl)	Volume of master mix (µl)	Volume of nuclease free water (µl)
0:1	0–0.5	0	5.0	5.0	5.0
1:1	0.25–0.25	2.5	2.5	5.0	5.0
1:3	0.125–0.375	1.25	3.75	5.0	5.0
1:7	0.0625–0.4375	0.625	4.375	5.0	5.0
1:10	0.0454–0.4546	0.454	4.546	5.0	5.0
1:15	0.03125–0.46875	0.3125	4.6875	5.0	5.0

Validation Studies

Sensitivity

A female DNA sample and control DNA 9947A of the Investigator® Argus X-12 QS PCR Kit were amplified at concentrations of 0.95 ng, 0.90 ng, 0.85 ng, 0.80 ng, 0.75 ng, 0.70 ng, 0.60 ng, 0.50 ng, 0.45ng, 0.40 ng, 0.30 ng, 0.20 ng and 0.10 ng to determine the DNA template concentration range for which full DNA profile can be obtained and also the optimal target DNA concentration range for a standard run protocol of 1 µl amplicon load. The percentage alleles called and allele drop out were observed for each sample.

Repeatability

Control DNA 9947A of the Investigator® Argus X-12 QS PCR Kit and female DNA sample (case study) in a concentration range of 0.10 ng to 0.95 ng were amplified and analyzed by the same person, same time under identical conditions on the same sample.

Reproducibility

Control DNA 9947A of the Investigator® Argus X-12 QS PCR system and female DNA sample (case study) in a concentration range of 0.10 ng to 0.95 ng were amplified and analyzed by another individual in another time under identical conditions on the same samples.

Mixture Studies

Female: male mixture samples were amplified at ratios of 1:15, 1:10, 1:7, 1:3, 1:1, 1:0 and vice versa. The number of individuals contributing to the profile was determined by assessing the number of peaks appearing at each locus that could not be identified as an artifact.

Contamination Studies

Blank samples were amplified as negative controls along with the other DNA samples and were electrophoresed on 3500 series Genetic Analyzer (Life TechnologiesTM). Clean profiles for the amplification

negatives were expected to make sure that the reagents and experiment conditions were free from any possible contaminations.

Results and Discussion

The objective of this Investigator® Argus X-12 QS PCR system validation was to assess the effectiveness of reduced amplification reagent volumes (15µl) on sensitivity, repeatability, reproducibility, mixture analysis and its potential suitability for forensic analysis conducted within the DNA Laboratory at Government Analyst's Department. The results from sensitivity, repeatability, and reproducibility studies conducted using the control DNA 9947A sample and a female DNA case study sample were analyzed across two sets of samples and two trials. The results for the control DNA 9947A sample in set 1 and set 2 across both trials demonstrate consistent allele detection across DNA input concentrations ranging from 0.1 ng to 0.95 ng. For inputs within this range, all loci exhibited a 100% allele call, confirming the reliability and reproducibility of allele detection across both sets and trials. However, at DNA inputs below 0.3 ng, peak imbalance was observed in the samples for both trials and sets. Additionally, at the highest input level of 0.95 ng, artifacts were detected at multiple loci in both trials. Despite these minor variations at the highest DNA concentration, the overall data demonstrate strong reproducibility of the expected allelic profile within the optimal DNA input range of 0.3 g to 0.9 ng.

The results for the female DNA sample (case study) in set 1 and set 2 across both trials demonstrate high consistency in allele calling across a range of DNA input concentrations from 0.1 ng to 0.95 ng. Similar to previous sample sets for control DNA 9947A, an increase in DNA template amount, the number of detected alleles also improved significantly. However, unlike the control DNA 9947A sample, at 0.1 ng, only 95% of the alleles were successfully called. From 0.2 ng onwards, 100% of the alleles were consistently detected in both trials, however, peak imbalance observed at input level below 0.45 ng. Additionally, artifacts were observed beyond 0.85 ng in both trials, whereas for the control DNA sample, artifacts appeared at 0.9 ng. Overall, the results confirm high consistency in allele calling, with 100% of alleles detected from 0.5 ng to 0.85 ng for the female DNA sample (case study) in both sets and trials.

Considering both sets of samples across both trials for the control DNA 9947A and case study female samples, the Argus X-12 QS Amplification System successfully generated complete X-STR profiles within the DNA input range of 0.50 ng to 0.75 ng. Allele dropouts were seen at lower template DNA concentrations such as 0.1 ng. However, an imbalance in peaks and low intensity peaks were observed at the input DNA of 0.45 ng and below. Stutters and off ladder peaks were emerged when the input template DNA reach 0.90 ng. Hence, in this study, the optimal input DNA range was determined to be between 0.50 and 0.75 ng. Figure 1 displays a profile obtained at input template DNA of 0.45 ng of the control DNA 9947A with imbalance in alleles and low intensity peaks. Also, Figure 2 displays a clear full profile obtained at input template DNA of 0.5 ng of the case study female DNA sample.

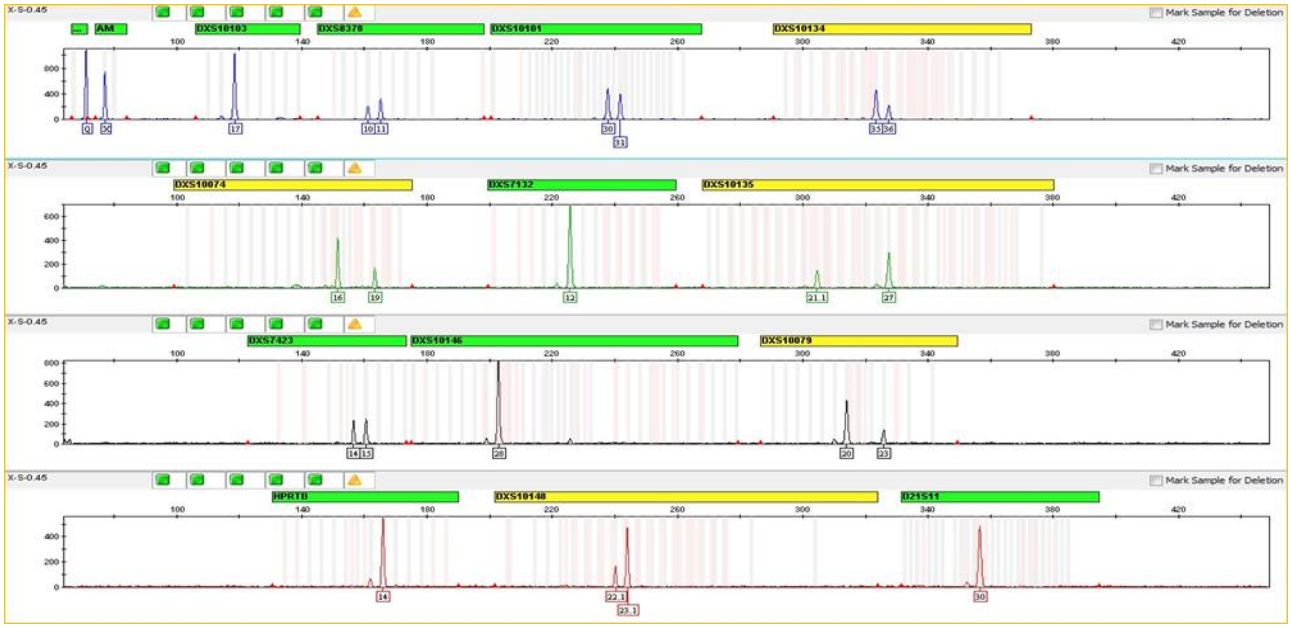

Figure 1. The Argus X-12 QS electropherogram showing imbalance in alleles for control DNA 9947A obtained at 0.45 ng template DNA.

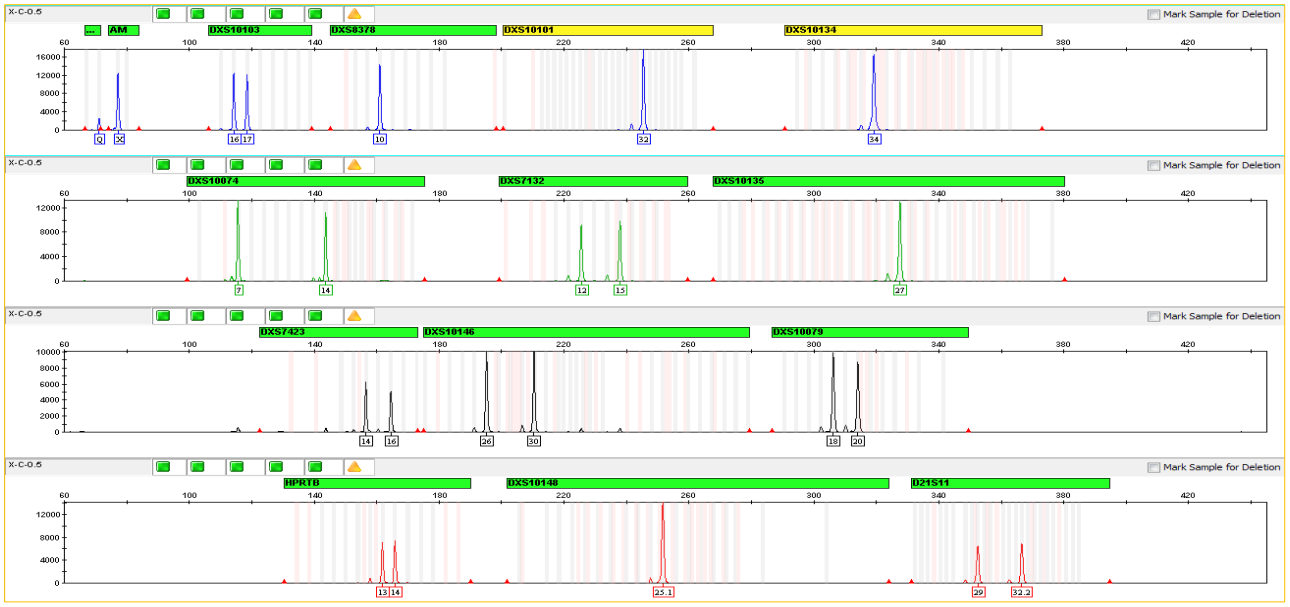

Figure 2. The Argus X-12 QS electropherogram for full profile from case study female sample obtained at 0.5 ng template DNA.

Template DNA greater than 0.75 ng started to produce various additional bands in the electropherogram like pull-up peaks, resulting from overamplification of template DNA causing saturation in fluorescent signal that cannot be resolved by the matrix files of the visualization software (10). Figure 3 displays appearing in the additional peaks in the electropherogram at a concentration of 0.95 ng for case study female DNA sample.

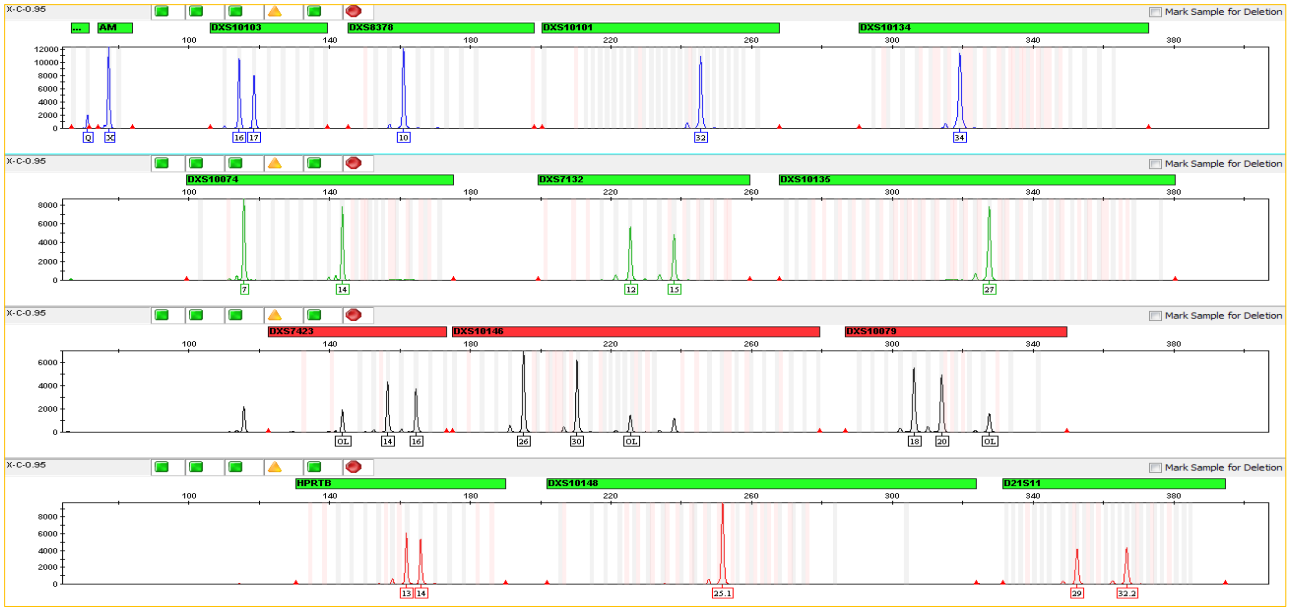

Figure 3. The Argus X-12 QS electropherogram displaying artifacts for case study female sample obtained at 0.95 ng template DNA.

Hence in this study, the optimal template DNA range was determined to be between 0.5 and 0.75 ng. According to the results, this system can be suggested to use for obtaining complete profiles for forensic samples which contain lower DNA amount. The results of set-1 and set-2 for both trial 1 and trial 2 illustrate identical complete X-STR profiles at particular input template DNA levels when it analyzed under uniform condition. A uniformity of peak heights at each input DNA level could be observed in set-1 and set-2 results conducted for both trial-1 and trial-2.

Furthermore, in majority of trials, the allele dropouts started to begin at 0.1 ng input DNA. Imbalanced in alleles and low intensity peaks were observed at the input DNA of 0.45 ng and below, stutters and off ladder peaks were started to appear when the input template DNA reach 0.90 ng and the balanced good peaks were

obtained at the input DNA ranged from 0.5–0.75 ng. Hence, the independent trials showed a high level of consistency during the validation of Argus X-12 QS Amplification System. Therefore, according to the results, the repeatability and reproducibility studies showcased the high similarity of the profiles obtained from independent trials. The generated X-STR profiles from the same sample were identical, under the similar conditions. Also, there was no difference between the test results for trial 1 and trial 2 carried out for control DNA 9947A and case study female DNA sample. Since the repeatability and reproducibility studies demonstrated a high degree of similarity in the profiles obtained from independent trials, statistical analysis was not performed for set of conducted trials. Based on the above findings, the results from both scenarios validate the suitability of the Argus X-12 QS Amplification System for forensic analysis.

The mixture study was conducted to verify the system's capacity to distinguish between female and male contributors within a single sample. The study examined the behavior of both contributors using case study female and male samples at varying ratios.

A comparison of allele detection across sample set 1, trial 1, and trial 2 for various male-to-female (M:F) DNA ratios of 1;0, 1:1, 3:1, 7:1, 10:1 and 15:1 shows consistency in allele calling. In both trials, a 1:1 M:F ratio resulted in 100% allele detection, highlighting the system's ability to accurately differentiate both contributors at equal proportions. However, as the male DNA ratio increased to 3:1, allele dropout began, with allele detection decreasing to 93% in trial 1 and 90% in trial 2. More noticeable differences were observed at higher ratios; at 7:1, the allele detection dropped to 63% in trial 1 and 60% in trial 2. Finally, at 15:1, the allele detection further decreased to 43% in trial 1 and 50% in trial 2.

When comparing sample set 2 across various male-to-female (M:F) DNA ratios of 0:1, 1:1, 1:3, 1:7, 1:10, and 1:15, similar consistency in allele calling was observed, as seen in sample set 1. In both trials, the 1:1 M:F ratio resulted in 100% allele detection, reflecting optimal performance when male and female DNA are equally represented. As the female DNA proportion increased to 1:3, allele dropout began, and the detection rate dropped to 93% in both trials. At higher ratios, the detection rate further declined, reaching 76% and 80% in trial 1 and trial 2, respectively.

The mixture studies were carried out to assess the Argus X-12 QS Amplification System's ability to differentiate multiple contributors within a single source. Overall, the results showed that at 1:1 mixture ratio, all the alleles for each contributor were produced without any allele imbalance. However, as the DNA concentration of the minor contributor decreased, number of detected alleles were reduced. Even though this was happening with the reduction of DNA amount of the minor contributor, still minor contributors' alleles were detected at lower ratios as 1:10, with reduced peak heights compared to the 1:1 ratio. This indicates that this system can be used to resolve complex mixture samples and make it as an ideal kit for forensic investigations. Figures 4 and 5 illustrate the ratios that produced clear profiles and the occurrence of allele dropouts.

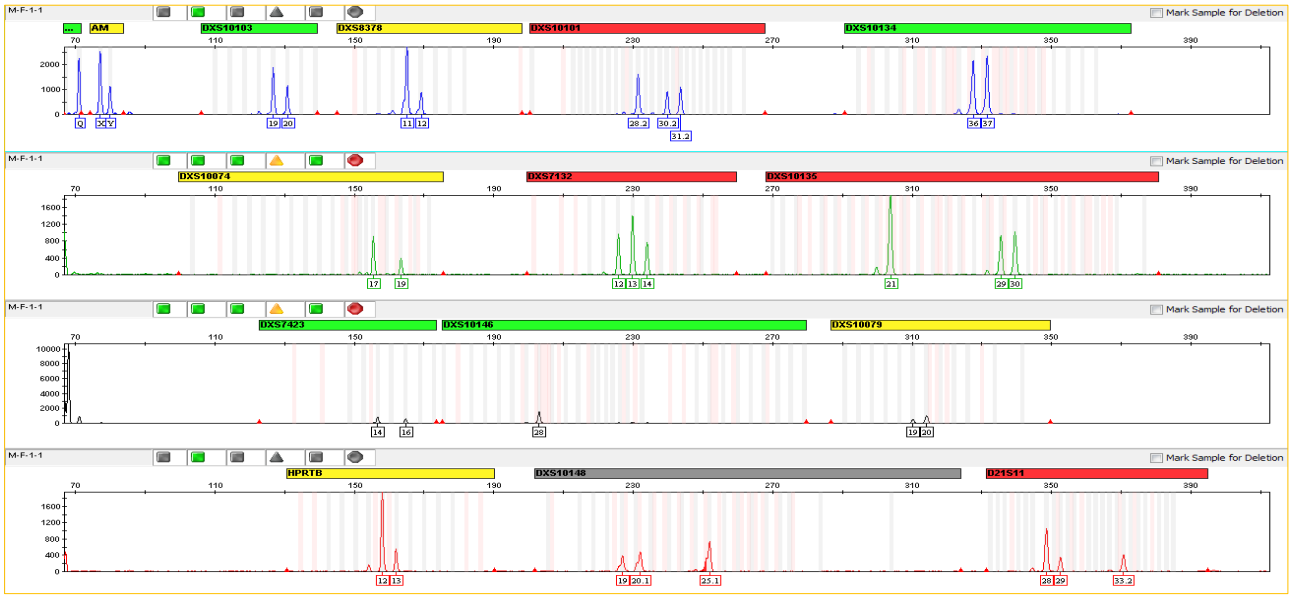

Figure 4. The electropherogram displaying all allele contribution for 1:1 input DNA ratio of male and female sample.

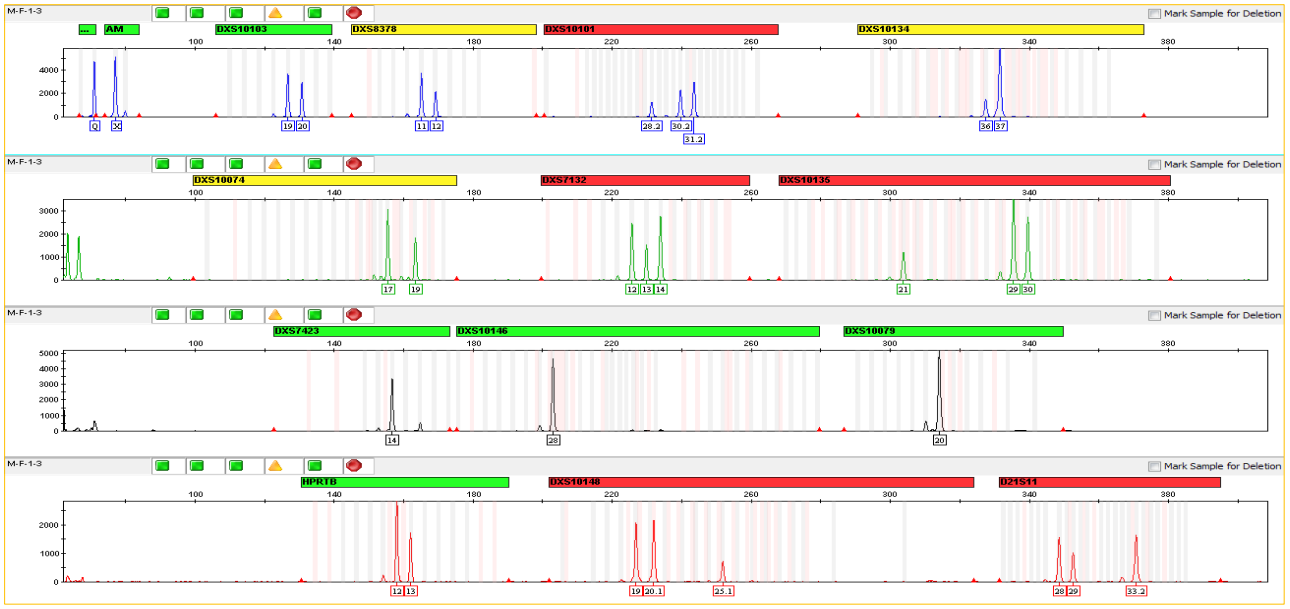

Figure 5. The electropherogram showing allele dropouts for 1:3 input DNA ratio of male and female sample.

Contamination Study

There was no amplification observed in any of the negative controls verifying the absence of foreign DNA materials throughout the validation process. We were able to achieve this as we ensure to maintain DNA free reagents and consumables and also practicing good handling to maintain the laboratory as a contamination free environment.

A negative control was run for the contamination test, and the resulting profile is presented in Figure 6.

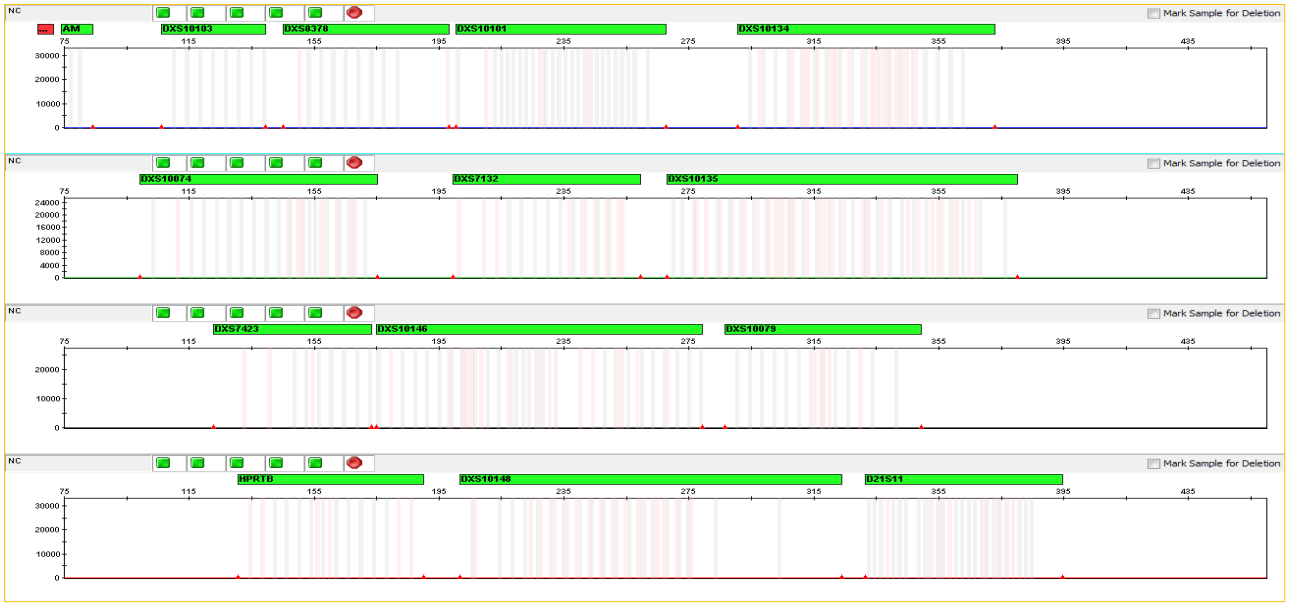

Figure 6. The electropherogram for negative control.

Conclusion

Currently X-STR analysis plays a vital role in forensic DNA investigations, particularly in identifying contributors in complex cases and important tool in both criminal investigations, kinship analysis and paternity disputes. Previously, the DNA Laboratory at Government Analyst's Department validated the Argus X-12 Kit following the manufacturer's guidelines, which recommended a full reaction volume of 25 µl. Considering that a significant portion of the Department's annual budget is allocated to purchasing chemicals for DNA analysis, an internal validation study was carried out to evaluate the kit's performance using a reduced reaction volume of 15 µl. The validation of the Investigator® Argus X-12 QS Amplification System demonstrated its reliability, sensitivity, and robustness for forensic X-STR analysis. The sensitivity studies established that the system generates complete and balanced X-STR profiles within an optimal DNA input

range of 0.50–0.75 ng, ensuring high analytical efficiency for forensic applications. Peak imbalances were observed at concentrations below 0.45 ng, with allele dropout occurring at 0.10 ng. On the other hand, at DNA concentrations ≥0.85 ng of template input, stutter artifacts and off-ladder peaks were seen, indicating overamplification effects at higher input levels.

Repeatability and reproducibility studies confirmed that the system produces consistent and concordant XSTR profiles across independent trials, with minor variations in peak heights and artifact formation. These results highlight the Argus X-12 QS System's resilience in forensic laboratory conditions. Mixture studies show the system's ability to detect multiple contributors in DNA samples, with all expected alleles successfully amplified at a 1:1 mixture ratio. Minor contributor allelic dropout was first observed at a 1:3 ratio, but alleles remained detectable at lower proportions (1:10) with reduced peak heights, reinforcing the system's suitability for forensic mixture analysis. Contamination controls confirmed the absence of detectable contamination, ensuring the reliability of laboratory protocols. Collectively, these findings support the forensic applicability of the Investigator® Argus X-12 QS Amplification System, reinforcing its role as a validated, reliable, and reproducible tool for forensic X-STR analysis in forensic casework. The internal validation results for the reduced reaction volume (15 µl) of the ARGUS X-12 System indicate that it can be effectively implemented in the Government Analyst's Department's DNA laboratory. The system demonstrated high precision and reliability for DNA analysis across various reference and forensic samples. Future studies could investigate the ability to distinguish individual alleles in complex DNA mixtures containing contributions from more than two male and female donors.

Declarations

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Author Contributions: RRWRK: Definition of intellectual content, concept, implementation of study protocol, data analysis, interpretation, review manuscript, editing, submission of article; KBA: Design of study, literature survey, implementation of study protocol, interpretation, data collection, data analysis, manuscript preparation, manuscript review; ME, GSD, PLRT, JWHMTP, TWAG: Concept, design, literature survey, interpretation, data analysis, manuscript preparation, manuscript review.

Conflict of Interest: The authors declare that they have no conflict of interest.

Consent to Publish: All authors agree to publish the paper in International Journal of Recent Innovations in Academic Research.

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Informed Consent Statement: Consent statement is not applicable, as this study does not include any personal information related to individuals in the case study.

Research Content: The research content of manuscript is original and has not been published elsewhere.

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