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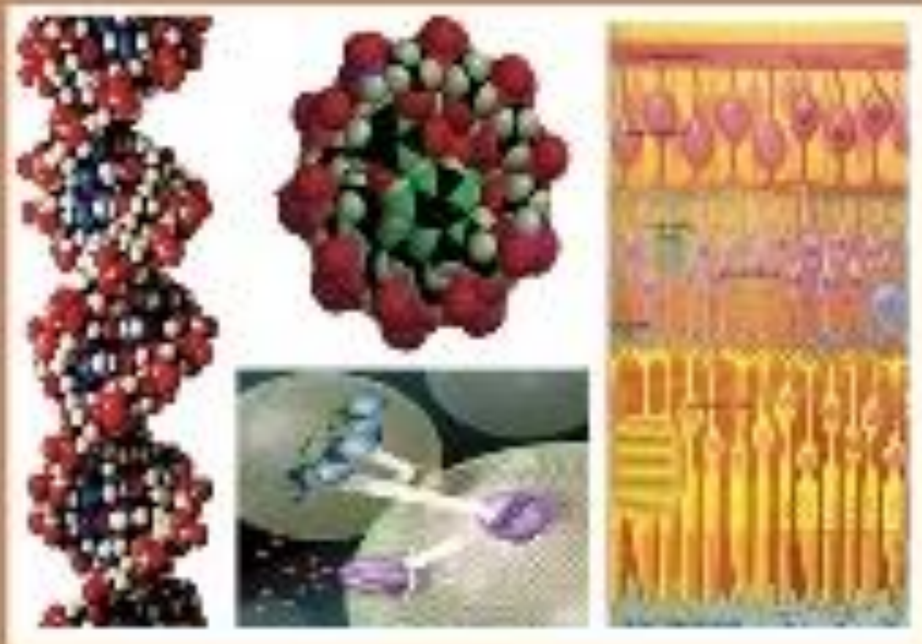

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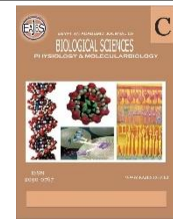

Evaluation of the Reduced Reaction Volume of PowerPlex Fusion system in DNA Profiling

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ABSTRACT

This study was conducted to examine the possibility of reducing PCR reaction volume of the PowerPlex Fusion system to produce full DNA genotyping of some forensic casework samples. Human DNA profiling was analyzed by half and quarter PCR reaction volume of the PowerPlex® Fusion system amplification kit to examine the reliability of the obtained STR profiles. Serial DNA dilutions (1 ng, 0.5 ng, 0.125 ng, 0.0625 ng) of a buccal swab were used with half PCR reaction volume to analyze STR loci via estimating their peak height and stutter ratio. The peak height ratio was above 60% in all heterozygous loci obtained and the best ratio of stutter was in the sample with a concentration of 0.0125, as it was less than 16% in all loci. Half and quarter PCR reaction volumes were evaluated by different casework samples (blood, hairs, and nails). The results showed that half reaction volumes gave 100% STR profiles of blood and nail samples and 60% of hair samples gave complete profiles. In 40% of hair samples, alleles were dropped out in the loci Penta E and Penta D. Quarter PCR reaction volumes gave 30% of blood and hair samples and 20% of nail samples produced a complete STR profile. It was possible to reduce the PCR reaction volume by more than half while still obtaining a complete STR profile suitable for human identification, despite some negative results that may have been due to DNA purity issues. Further studies are necessary to generalize this conclusion.

INTRODUCTION

DNA typing by Short Tandem Repeats (STRs) is an effective technique used in forensic laboratories for various forensic casework of human identification, paternity, kinship, and genealogy testing (Bukyya *et al*., 2021). Promega PowerPlex® Fusion System is among kits frequently used to amplify simultaneously 24 STR loci (Oostdik *et al*., 2014). The system includes the expanded CODIS - required loci besides the optional markers, Penta E, Penta D, D22S1045, and TPOX, and achieved the updated ESS requirements. Profiles obtained by the PowerPlex® Fusion System are comparable with databases of either CODIS or European Standard Set ESS (Oostdik *et al*., 2014; McCaughan, 2021). This system is compatible with the Applied Biosystems 3130 and 3500 Series Genetic Analyzer capillary electrophoresis (CE) instruments and does not require upgrades to the available collection and analysis software versions (Boavida *et al*., 2018).

Compared to AmpFℓSTR® Identifiler® and Globalfiler™ amplification kits, the PowerPlex Fusion system is comparable to Verifiler Plus providing more accurate human identification as it combines common and informative loci to overcome typical challenges faced by laboratories, including low DNA input, sample inhibitors, degradation, and mixtures.

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It provides a high level of precision, reliability, and significant gains in discriminatory power, making it suitable for demanding forensic, paternity, and relationship-testing cases (Green *et al*., 2021). The System has been shown to reliably produce complete profiles from as little as 100 pg of human DNA, demonstrating its effectiveness in amplifying minimal amounts of DNA from casework samples (Turrina *et al*., 2013; Oostdik *et al*., 2014).

Due to the great need for forensic tests, it is necessary to conduct as many tests as possible, besides minimizing the cost without compromising the quality of the results. The DNA amplification step is best suited to reduce testing costs because it is the most expensive step in DNA analysis (Mahmood *et al*., 2020). Almost half of the manufacturer's recommended PCR reaction volume (25 µl) of Globalfiler™ amplification kit (Smith, 2021), one fifth reaction volume of VeriFiler™ Express PCR Amplification Kit (Perry *et al*., 2022), half and possibly one fifth reaction volume of AmpFℓSTR® Identifiler® kit (Gaines *et al*., 2002; Bessekri *et al*., 2013; Iyavoo *et al*., 2015) and half reaction volume of Promega PowerPlex® Forensic Amplification Kits (Barbaro *et al*., 2015; Mahmood *et al*., 2020) were validated to amplify and produce reliable DNA profiles without undermining the quality of the results. The present study aimed to examine the possibility of reducing PCR reaction volume (half and one fourth) of the PowerPlex Fusion system to produce full DNA genotyping of some casework samples.

MATERIALS AND METHODS

Healthy Saudi unrelated adult volunteers inhabiting Riyadh city whose ages ranged from 15 to 50 years were requested to donate the samples of this study. They donated 40 samples in total (10 buccal swabs as reference samples, 10 blood, 10 hairs, and 10 nails). Informed consent and ethical approval were issued by the local ethical committee at Naif Arab University for Security Sciences. A single hair from each person was cut and standardized into a 1-centimeter piece. Five milligrams from each nail sample were weighed. Samples were cleaned several times with distilled water and 100% ethanol and dried before DNA extraction.

DNA was extracted from buccal swabs, blood, hair, and nail samples using the QIAamp DNA Investigator Kit according to the manufacturer protocol (Qiagen, GmbH, Hilden, Germany). Extracted DNA was quantified using the Quantifiler™ Trio DNA Quantification Kit following manufacturer's recommended procedures on a 7500 Real-time PCR System.

The sensitivity experiment was done on serial dilutions of a swab sample. The sample was diluted to 1, 0.5, 0.125, 0.0625 ng DNA and examined for a half PCR reaction volume. Internal control DNA provided with the kit was used as a positive control. The 24 STR loci of the Promega kit were amplified simultaneously by multiplex PCR using PowerPlex Fusion system in a 12.5 µL (half reaction) and 6.25 µL (one fourth reaction) containing 2.5 µL for half reaction and 1.25 µL for one fourth reaction master mix (Invitron, UK) and 2.5 µL for half reaction and 1.2 µL for one fourth reaction primer mix. Multiplex PCR was performed on an Applied Biosystems Veriti Thermal Cycler and a negative control was also run parallelly. To test the sensitivity, a positive control sample, included in the kit, was diluted to serial dilutions of 1, 0.5, 0.125 and 0.0625 ng. PCR was conducted for standard 30 cycles and the amplified loci were separated by capillary electrophoresis on an Applied Biosystems 3500 Genetic Analyzer, following the manufacturer's instructions. Voltage was adjusted to 1.2kV, injection time was 24 seconds and runtime was 1.210 seconds.

The Gene Mapper idx v.1.5. software displayed the results from different samples showing peak ratio of heterogeneous loci and stutter ratio for all loci. The executed data of STR alleles were analyzed using Excel software and the obtained results were compared. The peak height ratio (PHR) of the heterozygous loci was calculated by dividing

the height of the small peak in a heterozygote pair by the height of the large peak (Grisedale and van Daal, 2012). The stutter ratio was also calculated by the following equation: stutter1/allele peak height, stutter2/allele peak height (Dang *et al*., 2020). Box and Whisker Plots were conducted by Python3 SPSS statistics (IBM SPSS v29) for the PHR and stutter ratios.

RESULTS AND DISCUSSION

Table 1, indicates the DNA quantities of the studied samples and their corresponding Ct values together with the productivity of STR genotyping at half and one forth PCR reaction volumes. In general, a full STR profile with relative fluorescence unit (RFU) above 175 was possible at a minimum DNA input of 0.006 ng (a hair sample) and 0.014 ng (a nail sample) indicating possible reliability of obtained STR (Oostdik *et al*., 2014). Buccal swab, blood and nail samples gave full STR profiles when half PCR reaction volume was used. Hair samples showed full profiles for 60% of the examined samples and gave partial profiles for the rest. By using one fourth PCR reaction volume, a full profile for 30% of buccal swab and blood samples and 20% of nail samples were given. However, for nail samples, a full profile was obtained for 40% samples when one fourth PCR reaction volume was used. The obtained profiles are available from the authors upon reasonable request. For the samples in which partial profile was obtained by half PCR reaction, the alleles were dropout at the loci Penta D and Penta E. This is most probably because these are large base pair sized loci spanning more than 350 bp and the chance of alleles to dropout is more often. The discrepancies in the STR profiles, as delineated in Table 1, could stem from impurities in the extracted DNA or from the underestimated DNA quantities attributed to the existence of PCR inhibitors. These inhibitors have the potential to elevate the Ct value and yield undetectable PCR products (Timken *et al*., 2005; McCord *et al*., 2015), thereby precluding successful STR profiling. It was necessary to use an internal positive PCR control to detect this possibility.

Trace DNA samples frequently collected at a crime scene need to minimize their consumption by reducing PCR reaction volumes used in DNA typing. Improvement of STR loci detection sensitivity could promise the achievement of this purpose and this was initially studied by Leclair *et al.* (2003). The authors demonstrated stochiometric amplification and accurate detection. Reduction of PCR reaction volume to 10 µL and DNA down 0.5 ng, gave full profiles with the same signal intensity and heterozygous allele peak height ratio (PHR). When the PCR reaction volume was reduced to 5 µL and DNA was 0.063 ng, PHR values that were slightly affected in one to three STR loci were obtained. The authors concluded that reduction of PCR reaction volume can enhance sensitivity and detection while reducing the consumption of irreplaceable crime scene samples. One of the main targets of this study was to obtain a complete STR profile of a diluted biological sample that mimics the trace DNA found at a crime scene. Serial DNA dilutions (1 ng - 0.0625 ng) of a buccal swab reference sample were examined for possible amplification by measuring the peak heights of the obtained STR loci at a detectable analysis threshold of 175 RFU based on the lab internal validation and the catalogue adjusted for half PCR reaction volume of Promega DNA Amplification Kit (PowerPlex® Fusion system). Table 2 shows peak height and stutter ratios at the heterozygous loci. AMEL and DYS391 were excluded as they did not show data. PHR showed a general decrease with a gradual decrease of the template DNA concentration. All DNA dilutions produced full STR profiles with PHR higher than 0.6 as the heterozygote balances over 0.6 are routinely accepted to consider the locus alleles as documented by Leclair *et al.* (2004).

Table 1: DNA quantities (ng/µL) of the examined samples and their corresponding Ct values together with productivity of STR genotyping at half and one forth PCR reaction volumes.

	DNA Quantity	Ct	STR profile (half reaction)	STR profile (one fourth reaction)
Buccal swab	3.28	24.43	Complete profile	Partial profile
	1.43	25.60	Complete profile	Partial profile
	1.15	25.92	Complete profile	Negative
	10.59	22.77	Complete profile	Complete profile
	4.11	24.11	Complete profile	Partial profile
	9.71	22.89	Complete profile	Negative
	5.54	23.68	Complete profile	Complete profile
	7.001	23.35	Complete profile	Complete profile
	6.451	23.47	Complete profile	Negative
	13.22	22.45	Complete profile	Partial profile
Blood	15.78	22.20	Complete profile	Negative
	15.50	22.22	Complete profile	Negative
	29.04	21.33	Complete profile	Complete profile
	14.99	22.27	Complete profile	Negative
	16.45	22.14	Complete profile	Complete profile
	23.14	21.66	Complete profile	Negative
	23.77	21.62	Complete profile	Negative
	13.77	22.39	Complete profile	Negative
	12.95	22.48	Complete profile	Complete profile
	16.16	22.17	Complete profile	Negative
Hair	0.0024	34.64	Partial profile	Partial profile
	0.083	29.64	Complete profile	Complete profile
	0.002	35.10	Partial profile	Partial profile
	0.23	28.18	Complete profile	Complete profile
	0.01	33.53	Complete profile	Negative
	0.25	28.06	Complete profile	Complete profile
	4.17	24.09	Complete profile	Complete profile
	0.01	33.38	Partial profile	Negative
	0.0013	35.45	Partial profile	Partial profile
	0.0014	35.42	Complete profile	Negative
Nail	0.19	28.48	Complete profile	Negative
	0.03	31.26	Complete profile	Partial profile
	1.59	25.45	Complete profile	Negative
	1.27	25.77	Complete profile	Complete profile
	3.44	24.36	Complete profile	Negative
	0.013	32.19	Complete profile	Negative
	0.28	27.91	Complete profile	Negative
	0.42	27.33	Complete profile	Negative
	3.93	24.17	Complete profile	Negative
	0.60	26.84	Complete profile	Complete profile

Stutter is an artificial allele that appears as a by-product in PCR and its RFU value differs from locus to another (Albinsson *et al*., 2011). It is caused by strand slippage that takes place in pauses of the DNA polymerase complex during primer extension because of the enzyme's limited processivity and the presence of hairpin structures inside the repeated sequences (Hansen, 2018). It was calculated at each locus obtained by different DNA quantities (1, 0.5, 0.125 and 0.0625 ng) in half reaction (Table 2). The best DNA concentration used to calculate the stutter percentage was 0.125 ng because all the obtained loci had SR of approximately 16. In the 1 ng DNA concentration, the percentage of stutter peaks was approximately 16 %. The loci vWA, D12S391 and D19S433 showed SR of 19.2%, 20.8%, and 18.53%, respectively. In the 0.5 ng DNA concentration, SR was approximately 16 %. The loci D16S539, vWA, D8S1179, D12S391 and D19S433, recorded SR of 22.3 %, 44.5 %, 18.3 % 19.5% and 47.3 %, respectively. In the 0.125 ng DNA concentration, SR was ≤16%. In the 0.0625 ng DNA concentration, SR was approximately 16 % except for TH01, it was 20.19%.

Distribution of peak height intensity (Fig. 1) and stutter ratio (Fig. 2) at each heterozygous locus of the kit was also analyzed by Box and Whisker plots. The boxes indicate the 25th and 75th percentiles of the peak height intensity and stutter ration data. The median represents the central value in the dataset, while the minimum and maximum values denote the lowest and highest recorded values at each DNA concentration. Q1 and Q3 signify specific values, with IQR (Interquartile Range) calculated as the difference between Q1 and Q3, measuring the spread within the middle 50% of the data.

Table 2: The peak height (PHR) and stutter ratios (SR) for each locus, obtained at different DNA dilutions.

Locus	1ng DNA		0.5ng DNA		0.125ng DNA		0.0625ng DNA	
	PHR	SR	PHR	SR	PHR	SR	PHR	SR
D3S1358	81.12	11.30	64.99	14.71	81.04	9.14	78.95	8.88
D1S1656	--	6.33	--	8.63	--	8.85	--	8.96
D2S441	82.40	6.84	83.39	6.32	62.35	4.78	95.26	6.44
D10S1248	89.86	6.90	93.16	8.93	97.35	7.86	90.36	8.89
D13S317	84.03	5.58	83.14	9.48	90.69	5.73	99.65	6.58
Penta E	80.32	4.93	69.10	8.51	64.86	4.74	74.33	5.34
D16S539	98.86	5.44	99.48	22.34	99.09	4.54	78.26	7.18
D18S51	98.48	--	93.83	14.63	98.85	14.63	83.18	8.44
D2S1338	86.95	3.18	99.19	7.45	78.27	7.45	72.52	5.09
CSF1PO	--	7.98	--	5.84	--	5.84	--	6.49
Penta D	89.64	9.16	91.63	4.15	78.17	4.15	96.90	--
TH01	98.70	13.79	99.19	5.39	96.95	5.39	81.90	20.19
vWA	--	19.21	--	44.58	--	44.58	--	6.77
D21S11	83.36	10.08	77.12	10.75	93.17	10.75	88.66	7.718
D7S820	76.25	9.14	76.96	13.22	75.88	13.22	74.59	5.69
D5S818	99.14	5.80	91.76	6.86	93.87	6.86	83.24	4.08
TPOX	96.39	1.34	89.76	9.32	92.28	9.32	97.34	2.42
D8S1179	96.59	10.88	90.49	18.30	85.78	18.30	68.24	11.39
D12S391	91.28	20.88	93.15	19.54	96.86	19.54	96.61	16.35
D19S433	99.93	18.53	92.44	47.36	90.12	47.36	85.77	5.21
FGA	94.62	9.58	99.12	15.38	98.65	15.38	99.15	4.62
D22S1045	--	7.01	--	6.26	--	6.26	--	5.07

Several investigations confirmed similar trends for analyzing trace DNA in forensic caseworks (Oostdik *et al*., 2014; Barbaro *et al*., 2015; Green *et al*., 2021; McCaughan, 2021) and recently for single-cell profiling (Schulte *et al*., 2023). The present study obtained some readable STR profiles up to 7.5 µL PCR reaction volume and reduced DNA inputs of 0.0625. The profiles showed, in general, intensities comparable to that obtained by other investigations used the full PCR reaction volume and up to 0.5 ng DNA inputs (Oostdik *et al*., 2014) and half reaction volume of PowerPlex Fusion kit with DNA inputs of 0.5 ng (Mahmood *et al*., 2020; Barbaro *et al*., 2015). Validation studies on half PCR reaction volumes of other kits were conducted and produced reliable STR profiles at DNA inputs of 0.5 ng (Almohammed and Hadi, 2019), up to 360 ng (Mahmood *et al*., 2020) and 1 ng (Ludeman *et al*., 2018). We were unable to validate the use of ¼ of the reaction volume on the same samples that were tested with ½ volume. However, we successfully obtained STR profiles using ¼ reaction volumes for some casework samples. DNA purity may be a contributing factor to the partial and negative profiles obtained when using this reduced volume. This study is, thus, a preliminary to obtain full STR profiles by reduced PCR reaction volume. However, further investigation is necessary to be conducted on a wider variety of biological samples, particularly those of the degraded DNA (bone remains), and with a strategy of reducing DNA template. In case the obtained results are statistically meaningful, it is possible to generalize the results and recommend to consider this reduction.

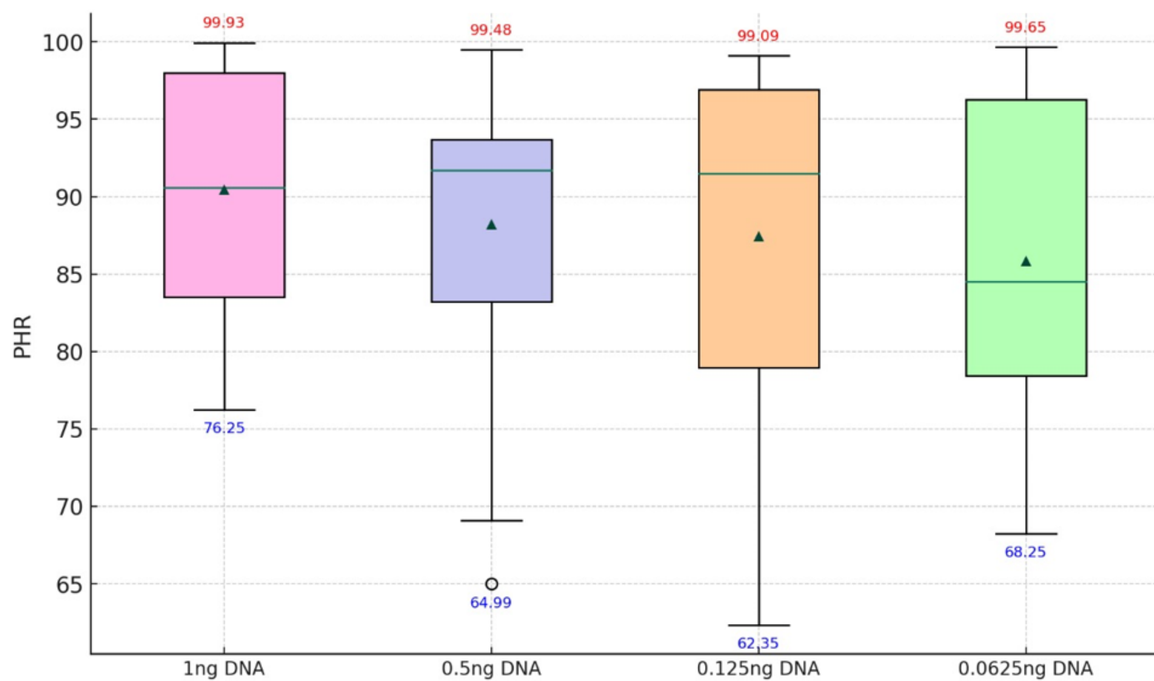

Fig. 1: Box and Whisker plots of the distribution of peak height intensity at each heterozygous locus. The boxes indicate the 25th and 75th percentiles of the peak height intensity and stutter ration data. The median represents the central value in the dataset, while the minimum and maximum values denote the lowest and highest recorded values at each DNA concentration. Q1 and Q3 signify specific values, with IQR (Interquartile Range) calculated as the difference between Q1 and Q3, measuring the spread within the middle 50% of the data.

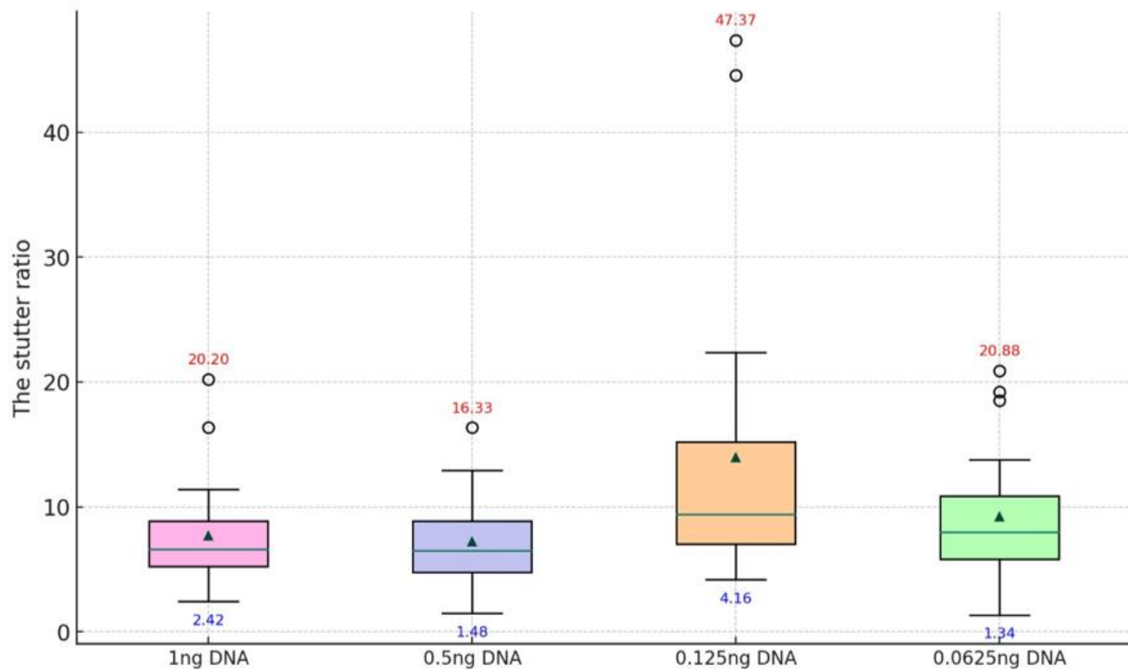

Fig. 2: Box and Whisker plots of stutter ratio at each heterozygous locus. The boxes indicate the 25th and 75th percentiles of the peak height intensity and stutter ration data. The median represents the central value in the dataset, while the minimum and maximum values denote the lowest and highest recorded values at each DNA concentration. Q1 and Q3 signify specific values, with IQR (Interquartile Range) calculated as the difference between Q1 and Q3, measuring the spread within the middle 50% of the data.

Conclusion:

Reducing the overall reaction volume of a DNA amplification kit below half could be beneficial in generating successful STR profiles with small amounts of DNA. The results obtained are preliminary and need to be compared in a subsequent study to those obtained using the full PCR reaction volume. This reduction in volume could lead to decreased operational costs, enabling the amplification of twice or four times as many samples using the same PCR kit. However, the limitations outlined in this study restrict generalizability.

Declarations:

Ethical Approval: This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Naif Arab University for Security Sciences (protocol code Nauss-Rec-23-03, June 2023).

Conflict of interests: The authors declare no conflicts of interest.

Authors Contributions: Nouf Albalawi conceived the idea, performed experimental work, and wrote the first draft of the manuscript; Sayed Amer shared in conceiving the idea, data analysis, revised and edited the final draft of the manuscript.

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Availability of Data and Materials: The data presented in this study are available on request from the corresponding author.

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