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TECHNICAL NOTE

Criminalistics

Validation of reduced volume VeriFiler™ Express PCR Amplification Kit for buccal swab samples extracted using Prep-n-Go™ Buffer

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Abstract

The efficiency of reduced volume PCR amplification was studied using the VeriFiler™ Express PCR Amplification Kit. Full (25 μL) and reduced (5 μL) volumes were tested in parallel to identify any differences in template DNA sensitivity and other electropherogram parameters. Both volumes produced full DNA profiles down to 0.08 ng/μL DNA concentration at 26 PCR cycles; however, reduced volume produced higher peak heights due to increased signal intensities. Significant difference (p-value ≤ 0.05) in heterozygote peak height ratios was observed between both volumes, where the reduced volume threshold was lowered to 0.6 to accommodate all data points. However, no significant difference (p-value > 0.05) was identified in the stutter ratios between both volumes. The analytical threshold for reduced volume was also determined to be 150 RFU with the presence of template DNA in PCR amplification. When the optimized reduced volume parameters were tested on DNA extracted from buccal swab samples using Prep-n-Go™ Buffer, good quality DNA profiles were produced. Overall, the reduced volume not only showed better results compared to the full volume, but also enable more samples to be processed with a PCR amplification kit, thus reduced the cost.

KEYWORDS
buccal swab, DNA extraction, Prep-n-Go™ Buffer, reduced volume PCR amplification, short tandem repeats, VeriFiler™ Express kit

Highlights

- Reduction in PCR amplification volume to one-fifth without compromising the quality of DNA profiles.
- DNA extraction from buccal swabs and direct PCR amplification eliminated DNA quantification step.
- Reduction in cost and turn-around-time of DNA profile generation from buccal swab samples.

1 | INTRODUCTION

Short tandem repeats are commonly utilized in DNA profiling in both a forensic and relationship testing context. Multiplex kits have been designed to allow for multiple STR loci to be amplified and analyzed at once. It is becoming more common for users of these kits to validate using a lower PCR reaction mix volume to get more PCR reactions out of one kit. The validation of reduced volume PCR has been achieved with many common multiplex STR kits, for example, the PowerPlex® 16 System [1], AmpFLSTR™ Identifiler™ PCR Amplification Kit [2], and GlobalFiler™ PCR Amplification Kit [3]. Overall, reducing the PCR volume reduces cost [4] and has been found to increase the sensitivity and reduce the amount of template DNA required [1]. It has been found that the volume can be reduced by half without impacting the quality of the profile produced [5].

The VeriFiler™ Express PCR Amplification Kit (Applied Biosystems™) has had little to no validation for a reduced PCR volume. Therefore, this study was carried out to validate reduced volume PCR amplification with this kit, which amplifies 22 autosomal loci (D3S1358, vWA, D16S539, CSF1PO, TPOX, D8S1179, D21S11, D18S51, Penta E, D2S441, D19S433, TH01, FGA, D22S1045, D5S818, D13S317, D7S820, D6S1043, D10S1248, D1S1656, D12S391, D2S1338, and Penta D) [6]. This kit is approved by US National DNA Index System (NDIS) and was mainly developed for paternity and kinship testing, where it offers superior genotyping results from single-source samples with high discrimination power and direct amplification capability [7]. Furthermore, the effectiveness of this reduced volume method was tested on DNA extracted from buccal swabs using Prep-n-Go™ buffer; this is a simple and quick DNA extraction buffer designed for direct PCR amplification [8].

2 | MATERIALS AND METHODS

2.1 | Optimization of reduced volume PCR amplification method

2.1.1 | Sample preparation

A serial dilution from a starting DNA concentration of 10 ng/μL was prepared using the 2800M Control DNA (Promega®) giving concentrations of 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04, 0.02, and 0.01 ng/μL. DNA Control 007, included in the VeriFiler™ Express kit was used as the positive control for both full and reduced reaction volumes, while the deionized water used for the serial dilution was utilized as the negative control.

2.1.2 | PCR amplification

The VeriFiler™ Express PCR Amplification Kit (Applied Biosystems™) which includes the master mix and primer set is recommended to be used as follows: 10 μL of master mix, 10 μL of primer set, and 5 μL of DNA solution to make up the 25 μL full reaction volume. The reduced volume reaction mix maintained these ratios, using 2 μL of master mix, 2 μL of primer set, and 1 μL of DNA solution, making up the 5 μL reduced reaction volume. Each serial dilution concentration was processed in triplicate for both full and reduced volumes. PCR amplification was carried out on the GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems™) for 26 cycles with Max ramping mode.

2.1.3 | Capillary electrophoresis

Hi-Di™ Formamide (Applied Biosystems™) and GeneScan™ 600 LIZ™ dye Size Standard v2.0 (Applied Biosystems™) were used in an 8.7–0.3 μL ratio respectively for each sample and then had 1 μL of PCR product (or Verifiler™ Express Allelic Ladder) added to make up the total volume of 10 μL. Samples were run on the 3500xL Genetic Analyzer (Applied Biosystems™) with the 3500 Series Data Collection Software 4, version 4.0.1 (Applied Biosystems™) as per the manufacturer's recommended parameters for VeriFiler™ Express kit.

2.1.4 | Data analysis

GeneMapper™ ID-X v1.6 software (Applied Biosystems™) was used to analyze the raw data to determine the allele calls. Statistical analyses were carried out using the t-test on Microsoft Excel®.

2.1.5 | Analytical threshold study

Twenty-three replicates of positive and negative controls for the reduced volume PCR amplification method were prepared for the analytical threshold study. The VeriFiler™ Express DNA Control 007 was diluted with deionized water in a 15:15 μL ratio to make the concentration 1 ng/μL to be used as the DNA template for positive controls, while the deionized water employed for the dilution was used for negative controls.

For this study, the relative fluorescence units (RFU) on the GM ID-X software were set to 1 for all dye channels, with all data points in the 60–480 base pair size range captured. For the positive controls, true and stutter peaks were removed, leaving only baseline noise for calculation. The limit of quantification (LOQ) was calculated by adding 10 standard deviations to the average baseline signal [9].

2.1.6 | Sensitivity study

Serial dilution triplicates for both reaction volumes were compared to identify the lowest concentration at which full profiles were produced. Full profiles were defined as having all peaks for all alleles above the threshold determined in the analytical threshold study.

Average peak height for each locus was also calculated from the serial dilution triplicates for both reaction volumes, to identify any significant difference between them.

TABLE 1 Baseline calculations for each dye set for positive and negative controls.

Sample	Dye channel	Highest baseline noise (RFU)	Average	SD	10 SD	LOQ
Positive	Blue	108	13.46	11.71	117.15	130.61
	Green	108	20.30	9.27	92.72	113.02
	Yellow	99	10.73	9.86	98.57	109.29
	Red	111	14.56	11.95	119.54	134.09
	Purple	117	18.65	12.86	128.57	**147.21**
Negative	Blue	33	10.18	3.16	31.58	41.76
	Green	54	15.97	4.72	47.25	**63.22**
	Yellow	25	7.05	2.13	21.29	28.34
	Red	30	10.12	2.19	21.91	32.03
	Purple	37	9.17	3.84	38.41	47.58

Note: The average, standard deviation (SD), 10 SD and average + 10 SD was calculated to give the limit of quantification (LOQ). Highest LOQ for both controls in bold.

TABLE 2 The minimum DNA concentrations (ng/μL) required for the correct profile to be generated at each locus for full and reduced volumes with 150 RFU threshold. Overall minimum DNA concentration required to produce a full profile in all repeats for both volumes in bold.

	Full volume (25 μL)				Reduced volume (5 μL)			
Locus	Repeat 1	Repeat 2	Repeat 3	Minimum	Repeat 1	Repeat 2	Repeat 3	Minimum
D3S1358	0.04	0.04	0.08	**0.08**	0.04	0.04	0.04	0.04
vWA	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
D16S539	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
CSF1PO	0.02	0.01	0.02	0.02	0.01	0.01	0.01	0.01
TPOX	0.04	0.04	0.04	0.04	0.02	0.04	0.04	0.04
D8S1179	0.04	0.04	0.04	0.04	0.02	0.04	0.04	0.04
D21S11	0.04	0.04	0.04	0.04	0.02	0.04	0.02	0.04
D18S51	0.04	0.02	0.04	0.04	0.04	0.04	0.04	0.04
Penta E	0.04	0.04	0.04	0.04	0.02	0.04	0.04	0.04
D2S441	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
D19S433	0.04	0.04	0.08	**0.08**	0.04	0.04	0.08	**0.08**
TH01	0.04	0.04	0.04	0.04	0.04	0.08	0.08	**0.08**
FGA	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
D22S1045	0.02	0.02	0.01	0.02	0.01	0.01	0.02	0.02
D5S818	0.02	0.04	0.04	0.04	0.01	0.01	0.04	0.04
D13S317	0.04	0.04	0.04	0.04	0.02	0.04	0.04	0.04
D7S820	0.04	0.04	0.04	0.04	0.02	0.04	0.04	0.04
D6S1043	0.02	0.04	0.04	0.04	0.02	0.04	0.04	0.04
D10S1248	0.08	0.04	0.04	**0.08**	0.04	0.04	0.04	0.04
D1S1656	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
D12S391	0.04	0.04	0.04	0.04	0.04	0.02	0.04	0.04
D2S1338	0.02	0.04	0.02	0.04	0.02	0.04	0.04	0.04
Penta D	0.04	0.04	0.04	0.04	0.04	0.02	0.04	0.04

2.1.7 | Heterozygote peak height ratio study

From the full and reduced volume electropherograms, only heterozygotes where both peaks were in the range of 3000–12,000 RFU were used to calculate the heterozygote peak height ratios, since heterozygotes with no off-scale allele peaks or allelic dropout would be produced in this range [6]. The calculation was carried out by dividing the height of shorter allele peak by the height of taller allele peak.

2.1.8 | Stutter ratio study

For this study, all electropherogram artifacts were removed, leaving only true peaks and stutter peaks. Triplicate profiles with heterozygote peak heights within the 3000–12,000 RFU range were selected along with all homozygotes in those profiles. Stutter ratio was calculated by dividing the stutter peak height by that of the true allele peak height for each locus for all serial dilution concentrations. Then

the stutter ratio threshold was calculated by adding three standard deviations to the highest stutter ratio of the locus.

2.2 | Evaluation of reduced volume PCR amplification method on DNA from buccal swab samples extracted using Prep-n-Go™ Buffer

2.2.1 | Buccal swab samples

Buccal swab (Puritan®) samples were collected from 104 anonymous volunteers. The volunteers, who provided informed consent, were requested to not consume any food or drink for 30 min prior to sampling. Buccal samples were collected by rubbing the cotton end of the swab against the inside of the cheek for approximately 30 s; the swab was then allowed to air dry at room temperature for approximately 30 s. The swab was then placed swab-end first into a sealable envelope labeled with the details of the volunteer.

2.2.2 | DNA extraction

Prep-n-Go™ Buffer (Applied Biosystems™) was used to extract DNA from the buccal swabs. Room temperature protocol [6] was used for the DNA extraction utilizing 400 µl Prep-n-Go™ Buffer per sample. The buffer was added to the swabs in 1.5 ml Eppendorf® tubes and left to stand for 20 min at room temperature (20–25°C) to lyse the sample.

2.2.3 | DNA quantification

The QuantStudio™ 5 Real-Time PCR System (Applied Biosystems™) was used to quantify the extracted DNA using the Quantifiler™ HP DNA Quantification Kit (Applied Biosystems™) following manufacturer's recommendation to identify the amount of DNA produced by Prep-n-Go™ Buffer extraction on buccal swab samples.

2.2.4 | DNA profiling

Extracted DNA samples were directly processed with the optimized reduced volumes method for PCR amplification, capillary electrophoresis, and data analysis on GM-IDX software, as described in Section 2.1.

3 | RESULTS AND DISCUSSION

3.1 | Analytical threshold study

Limit of quantification for analytical thresholds can be calculated with only the negative amplification controls [10] where the captured data points will be without any known alleles and stutters. However, presence of template DNA in the PCR amplification can increase the baseline noise at non-allele product areas [11]. Even though the average baseline noise differences between negative amplification controls and template DNA samples are relatively small [9], inclusion

TABLE 3 Average peak height (RFU) and standard error for each locus from full (25 µL) and reduced (5 µL) PCR reaction volumes.

Locus	Average peak height (full volume)	Standard error (full volume)	Average peak height (reduced volume)	Standard error (reduced volume)
D3S1358	8483	1185.12	13,384	1694.09
vWA	9557	1445.68	14,126	1786.10
D16S539	10,203	1498.51	14,295	1812.21
CSF1PO	15,291	2478.63	17,801	2483.80
TPOX	14,048	2466.67	14,935	2446.22
D8S1179	10,040	1552.25	12,999	1647.44
D21S11	8236	1260.18	12,717	1634.71
D18S51	10,408	1380.80	13,176	1536.54
Penta E	9078	1229.37	13,567	1638.65
D2S441	8973	1362.61	11,509	1555.70
D19S433	9075	1416.30	11,433	1569.35
TH01	12,063	1651.89	12,947	1701.15
FGA	9373	1429.93	11,809	1627.47
D22S1045	14,860	2402.10	16,463	2283.40
D5S818	12,801	2454.82	15,152	2449.44
D13S317	8765	1306.21	14,470	1781.04
D7S820	8970	1291.01	13,642	1702.31
D6S1043	8953	1341.57	14,016	1750.83
D10S1248	8450	1139.68	13,970	1690.15
D1S1656	11,143	1621.74	15,157	1776.43
D12S391	9449	1281.96	14,878	1778.98
D2S1338	13,308	1704.00	14,214	1727.73
Penta D	11,511	1570.67	14,917	1776.50

FIGURE 1 Comparison of average peak heights between full (25 µL) and reduced (5 µL) PCR reaction volumes. [Color figure can be viewed at wileyonlinelibrary.com]

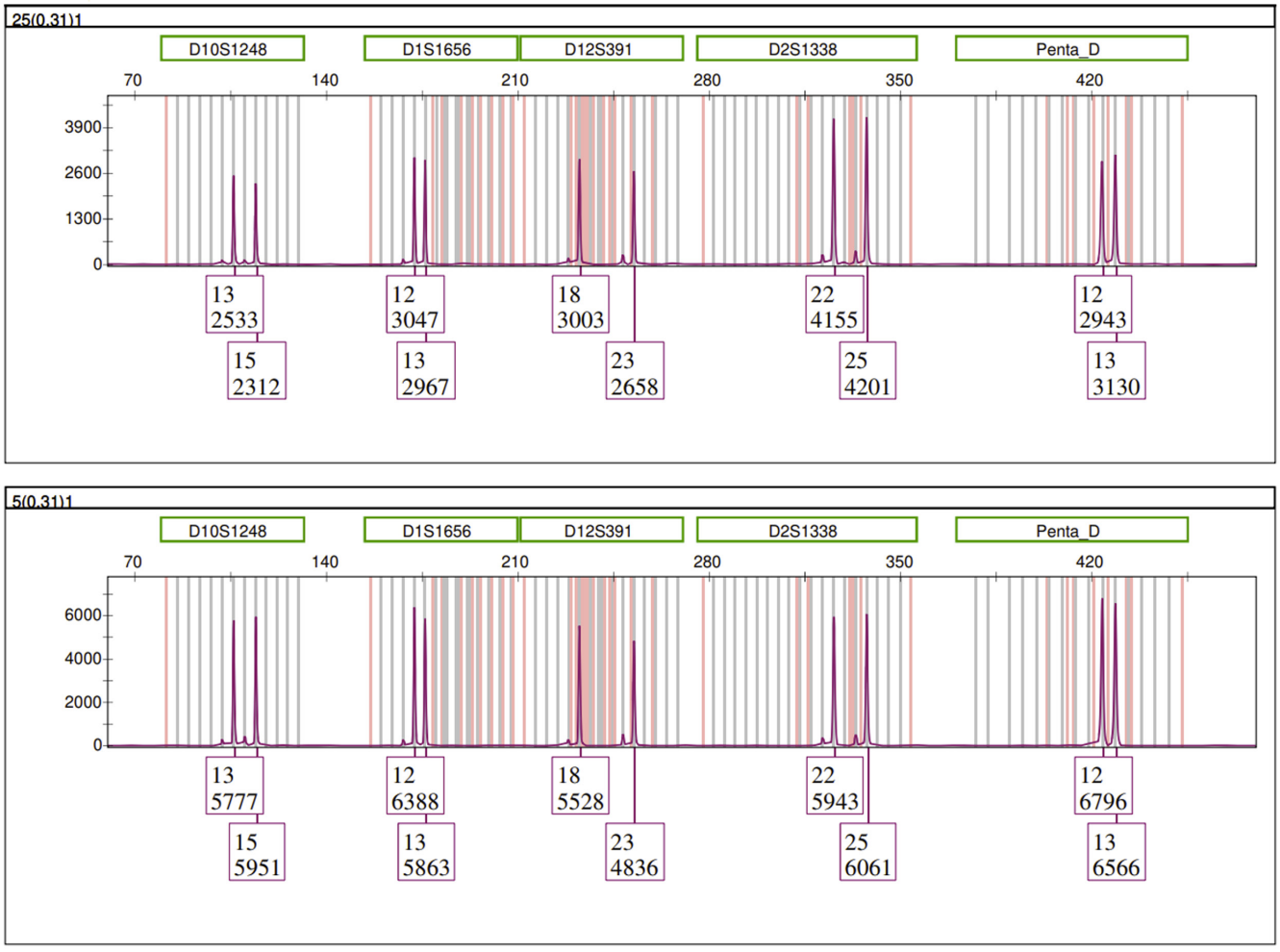

FIGURE 2 Electropherograms of full (upper pane) and reduced (lower pane) PCR reaction volumes for the purple dye channel, generated from a serial dilution sample with concentration of 0.31 ng/µL. [Color figure can be viewed at wileyonlinelibrary.com]

of template DNA samples in the analytical threshold generation is suggested when applied to samples with high levels of DNA [12], such as the buccal swab samples in this study.

The highest LOQ for the negative controls was 63.22 RFU in the green dye channel, while the highest LOQ for the positive controls was 147.21 RFU in the purple dye channel. Accordingly, the LOQ threshold for the reduced volume method was set to 150 RFU. This finding demonstrates that the presence of DNA has an impact on the baseline noise level. These results are displayed in Table 1.

3.2 | Sensitivity study

Utilizing the 150 RFU analytical threshold, both the full and reduced volume samples produced full profiles down to a DNA concentration of 0.08 ng/μL at 26 cycles. Table 2 shows the minimum DNA concentration required for the correct profile to be generated at each locus across each repeat for both full and reduced volumes. Greater sensitivity down to the lowest DNA concentration in this study (0.01 ng/μL) could be achieved by increasing the cycle number [13]. However, since this reduced volume method was being developed for buccal swab samples extracted using Prep-n-Go™ Buffer, which will provide good DNA quantity [14], 26 cycles was maintained.

Peak heights of full and reduced volumes were also compared for each locus using the 150 RFU analytical threshold. Expected allele calls were produced from the 2800M Control DNA [15], with homozygotes at loci CSF1PO, TPOX, D22S1045, and D5S818. Average peak height and standard error for each locus are shown in Table 3. The reduced volume method produced higher average peak heights for all loci from the serial dilution samples used in this study as shown in Figure 1. This observation was also supported by statistical analysis which showed significant difference (p-value ≤ 0.05) between peak heights of full and reduced volumes. Therefore, even though both reaction volumes have the same sensitivity limit, reduced volume produces electropherograms with higher signal intensities. This can be observed in Figure 2 which shows the electropherograms of full and reduced volumes for the purple dye channel, generated from a serial dilution sample with concentration of 0.31 ng/μL.

3.3 | Heterozygote peak height ratio study

Overall, the reduced volume method had a lower average heterozygote peak height ratio (0.8870) compared to the full volume method (0.9462). However, when the individual data points were analyzed, reduced volume had the highest ratio (0.9997), compared to the full volume method (0.9982). While all data points for full volume were above the manufacturer's default threshold (0.7) [16], only 2 data points (2.06%) were below this threshold in the reduced volume. However, a significant difference (p-value ≤ 0.05) was observed between both volume sample sets. The final threshold for reduced volume method was set at 0.6 to cover all heterozygote peak height ratios as shown in Figure 3 with the manufacturer's default threshold.

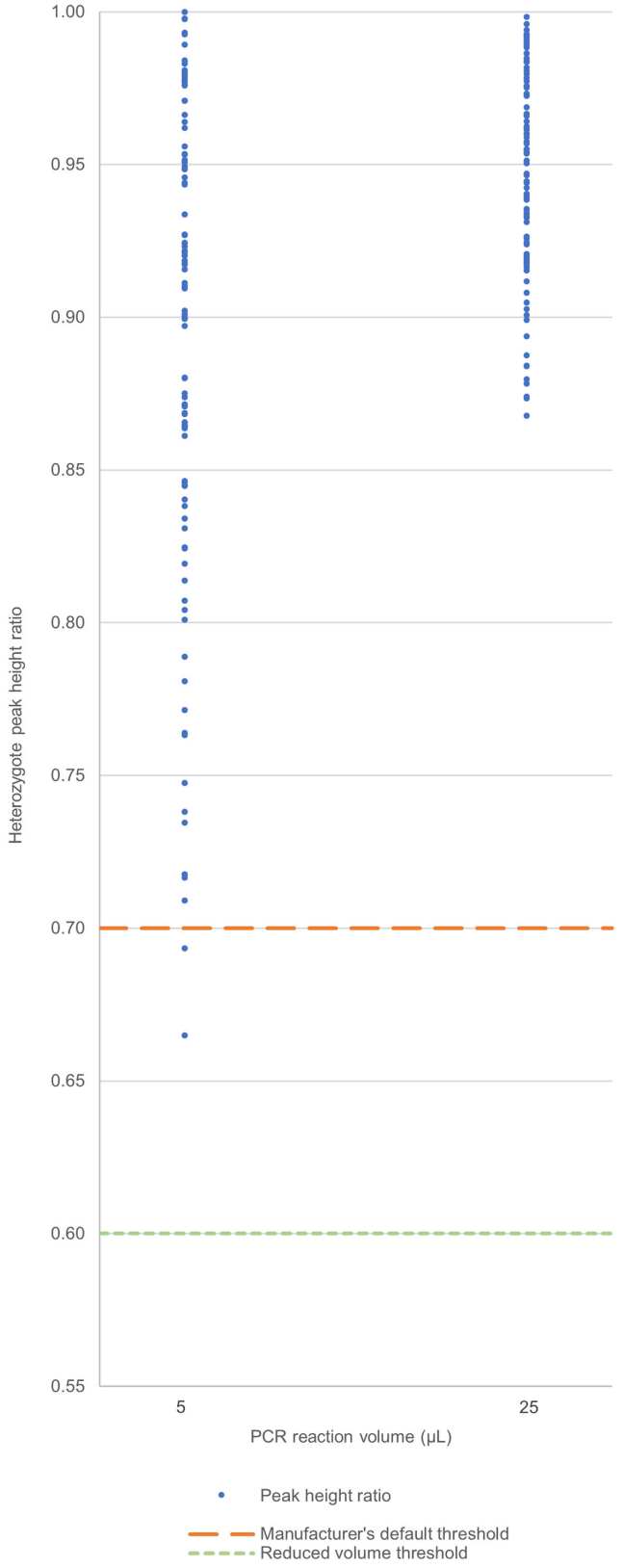

FIGURE 3 Heterozygote peak height ratio comparison between reduced (5 μL) and full (25 μL) PCR reaction volumes. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 4 Observed stutter calculated for each locus from full (25 μL) and reduced (5 μL) PCR reaction volumes.

Locus	Observed stutter for full volume	Observed stutter for reduced volume	Manufacturer's threshold
D3S1358	0.0898	0.0966	0.1216
vWA	0.1317	0.1453	0.1318
D16S539	0.1384	0.1478	0.1076
CSF1PO	0.0740	0.0884	0.1054
TPOX	0.0410	0.0564	0.0520
D8S1179	0.0878	0.1054	0.1085
D21S11	0.0985	0.1184	0.1224
D18S51	0.1412	0.1538	0.1415
Penta E	0.0548	0.0750	0.0831
D2S441	0.0580	0.0671	0.0940
D19S433	0.0633	0.0689	0.1077
TH01	0.0195	0.0265	0.0493
FGA	0.1173	0.1175	0.1227
D22S1045	0.1038 0.0575 (+3 nt)	0.1151 0.0872 (+3 nt)	0.1800 0.0805 (+3 nt)
D5S818	0.0799	0.0901	0.1053
D13S317	0.1019	0.1178	0.1010
D7S820	0.0896	0.1018	0.0939
D6S1043	0.1282	0.1436	0.1132
D10S1248	0.1284	0.1422	0.1227
D1S1656	0.0693	0.0703	0.1362
D12S391	0.1850	0.1998	0.1444
D2S1338	0.1444	0.1765	0.1329
Penta D	0.0297	0.0302	0.0409

Note: Manufacturer's threshold is also included for comparison.

3.4 | Stutter ratio study

Stutter alleles are caused by slippage when DNA polymerase pauses during primer extension [17, 18]. It could be one repeat unit smaller than the target STR allele product (minus stutter) or one repeat unit larger (plus stutter) which is less frequent. Plus stutter was observed at locus D22S1045, which is a trinucleotide repeat-containing locus and this phenomenon is more significant compared to the tetranucleotide repeats [19, 20]. When comparing the full and reduced volumes, there was no significant difference (p-value > 0.05) identified between the stutter ratio thresholds of both data sets. Results for this study are shown in Table 4, with manufacturer's threshold for comparison.

3.5 | Buccal swab samples with reduced volume PCR amplification method

The Prep-n-Go™ Buffer extraction method recommended direct PCR amplification, without quantification [6]. However, DNA quantification on all 104 buccal swab samples was carried out to determine the DNA concentrations obtained using the room temperature protocol of the Prep-n-Go™ Buffer. On average, the DNA concentration was 4.27 (1.82 SD) ng/μL with the lowest and highest DNA concentrations being 0.87 ng/μL and 10.02 ng/μL respectively.

All buccal swab samples produced full DNA profiles with the finalized reduced volume analysis parameters on the GM-IDX software. No excessive stutter or heterozygote peak height imbalance indicators were observed on the electropherograms. The lowest heterozygote peak height ratio was 0.6039 which is within the lowered threshold (0.6) for reduced volume method. Any heterozygote peak height ratio falling below this threshold will be flagged by the GM ID-X software and can be evaluated during the analysis. As size difference between heterozygote alleles increased, a reduction in heterozygote peak balance was observed. No significant change in heterozygote peak height imbalance was observed over the range of DNA concentration of buccal swab samples, but more artifacts were observed as the sample DNA concentration increased, especially pull-ups (bleed-through) and split peaks (incomplete adenylation). However, these artifacts had no effect on the true allele calls and were easily identified and corrected.

4 | CONCLUSION

This study demonstrates that reduced volume (5 μL) PCR amplification method is robust and produces reliable results similar to the full

volume (25 µL) method. The analytical threshold study results found a threshold of 150 RFU to be appropriate for the reduced volume method. Whilst this is a high threshold, it is acceptable as it takes into consideration the presence of template DNA in PCR amplification. The sensitivity study found the reduced volume method produced full profiles to the same concentration (0.08 ng/µL) as the full volume at 26 cycles. However, when comparing the peak heights of full and reduced volumes, it was shown that the reduced volume had higher signal intensities. The heterozygote peak height ratio study found the full volume method to have better heterozygote peak balance compared to the reduced volume method. However, with the new threshold (0.6), interpretation of the DNA profiles was unaffected. With all these optimized reduced volume analysis parameters, this method is suitable to be applied to samples with optimal DNA concentration.

Additionally, the Prep-n-Go™ Buffer which was used for DNA extraction from buccal swab samples showed great efficacy in extracting DNA with optimal amounts for this reduced volume method, making the whole system suitable for high-throughput sample processing workflow with reduced cost.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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