

## TECHNICAL NOTE

### CRIMINALISTICS

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# Validation of Half-Reaction Amplification Using Promega PowerPlex<sup>®</sup> 16\*

**ABSTRACT:** DNA amplification is a fundamental yet costly process used in DNA analysis. This study evaluated half-reaction amplification (12.5, 12, and 13  $\mu$ L) using the Promega Powerplex<sup>®</sup> 16 Kit with the hope of reducing sample analysis costs by half. A sensitivity study was completed, along with the testing of various blood stain samples including those with low (<0.40 ng) and high DNA concentrations (>3.0 ng), peak height imbalances, and allelic drop-out. Also, 467 samples submitted to the MUFSC laboratory for testing were analyzed. Results indicate that half-reaction amplification produced higher quality profiles than full-reactions. Average peak heights increased by 85%, peak height imbalances improved, and drop-out was eliminated in 75.8% of samples. Only eight of 467 case samples required re-amplification, a success rate of 94% was observed, and the repeat rate decreased significantly. Finally, a DNA input of 0.25–1.0 ng is ideal for half-reaction amplification.

**KEYWORDS:** forensic science, forensic DNA, validation, DNA amplification, PowerPlex<sup>®</sup> 16, CSF, D13S317, D16S539, D18S51, D21S11, D3S51358, D5S818, D7S820, D8S1179, FGA, Penta D, Penta E, TH01, TPOX, vWA

Over the last decade, laboratories have seen an increase in backlogged cases and an increase in the number and types of samples being submitted for analysis (1). To support this increased demand, laboratories can expect an increase in operational costs stemming from the purchase of more products for DNA testing, and the hiring of more analysts to handle extra caseload volumes. While the forensic community has seen an increasing success rate in sample analysis, laboratories continue to search for faster, better, and less-expensive ways to analyze samples without compromising their quality of work (2). The DNA amplification process is a possible area for such cost reduction. Specifically, the Promega (Madison, WI) PowerPlex<sup>®</sup> 16 Amplification Kit currently costs \$5,995 and supports the amplification of 400 DNA samples (approximately \$15 per sample). This study investigated the possibility of reducing the amount of amplification reagents used to increase the total reactions of each kit to 800 samples. This would reduce the cost per sample by half. The overall objective was to optimize and evaluate the performance of a half-reaction amplification protocol using the Promega PowerPlex<sup>®</sup> 16 Amplification Kit.

The DNA amplification step was chosen as an option for cost reduction because it has proven to be the most costly step throughout the DNA analysis process. A recent in-house cost analysis study conducted at the Marshall University Forensic Science Center (MUFSC) calculated the total cost of analyzing a single sample to be \$24.30. Of that total, \$15.92 is spent on PowerPlex<sup>®</sup> 16

amplification alone, accounting for nearly 66% of the total costs. Not only is DNA amplification one of the most expensive steps but it is a highly essential one as well. Many database and case-work samples with limited DNA and/or of poor quality could not be analyzed without proper PCR amplification (3). Two factors were considered for this validation; a total reaction volume that is suitable for amplification and the volume of each reaction component that will support DNA amplification. First, Promega recommends the amplification of a DNA template in a total volume of 25  $\mu$ L (4). However, amplification can be performed using a total volume in the range of 5–100  $\mu$ L (3). A reduced total amplification volume of 12.5  $\mu$ L falls within this range and should be suitable for successful amplification. Second, manufacturers' recommended protocols suggest reaction volumes of each component based on optimization studies (5). These studies have demonstrated the optimal ratio of reagents and the amplification parameters to use for the production of quality DNA profiles. Therefore, the overall goal was to keep all variables constant except the total reaction volume to collect data that was comparable to original full-reaction data.

The effects of half-reaction PCR were assessed through the analysis of five different studies using samples with various issues seen at the MUFSC laboratory. First, samples previously producing full profiles and requiring no repeat analysis when amplified using the full-reaction protocol were evaluated to determine whether amplification in 12.5  $\mu$ L would be successful on quality samples. Second, a variety of samples commonly seen at the MUFSC laboratory and identified as "problem samples" were evaluated. These included samples with high and low DNA concentrations (>3.0 and <0.4 ng, respectively), allelic drop-out, and peak height imbalances at the D5S818 locus. Total reaction volumes of 12 and 13  $\mu$ L were also assessed to allow for an easier amplification set-up. A sensitivity study was completed to determine the range of DNA template most suitable for half-reaction amplification. Finally, the half-reaction amplification procedure was used to test 456 buccal swab samples outsourced to the MUFSC laboratory for analysis.

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**Materials and Methods**

*DNA Samples and Preparation*

This study utilized single-source blood stain samples previously analyzed in the MUFSC laboratory. Original samples were spotted on FTA® cards (Fitzco Inc., Spring Park, MN), extracted on a Beckman Coulter Biomek® 2000 using Promega’s DNA IQ™ Extraction Kit, amplified with the Promega Powerplex® 16 Amplification Kit (full-reaction protocol) on a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA), run on an ABI PRISM® 3100 Genetic Analyzer and analyzed using ABI PRISM® Data Collection Software v.1.1, Genescan® v.3.7.1, and Genotyper® v 3.7 software. All procedures were performed using manufacturer’s recommended protocols. Samples used in this study were either the original extracts (not previously quantified) or the normalized samples that were previously quantified, according to manufacturer’s protocol, using the Quantifiler® Human DNA Quantification Kit (Applied Biosystems). Normalization is the automated process of diluting extracted samples to the same concentration. In this study, 25 uL of extract product was added to a variable amount of water that would bring all samples to a concentration of 1 ng/uL. Original extracts were not quantified because MUFSC validation studies of the Beckman Coulter Biomek® 2000 indicated that consistent quantities of DNA, sufficient for amplification, were obtained from FTA® cards extracted with the Promega DNA IQ™ Extraction Kit. Only a handful of samples were originally quantified; therefore, the concentration of most extracts used in this study was unknown. Known in-house DNA samples with known concentrations were used for the sensitivity study.

*PCR STR Amplification*

Three PowerPlex® 16 “half-reaction” volumes were used to amplify samples, 12 uL, 12.5 uL, and 13 uL. All three reaction volumes consisted of the following master mix components per sample: 1.25 uL Gold ST\*R 10x Buffer, 0.4 uL AmpliTaq Gold® DNA Polymerase, and 1.25 uL PowerPlex® 16 10x Primer Pair Mix. Table 1 summarizes the volumes of master mix, DNA template, and molecular biology grade (MBG) water (Thermo Fisher Scientific, Waltham, MA) used for the three total amplification volumes. The volume of DNA template amplified remained constant at 1 uL to reflect the treatment of original samples as well as assess the efficacy of half-reaction volumes on PCR and not the variation of

DNA template. Samples were loaded into a MicroAmp™ Optical 96-well Reaction Plate (Applied Biosystems), sealed with MicroAmp™ 8-cap strips, and amplified on a GeneAmp® PCR System 9700 thermal cycler. All samples were amplified using thermal cycling parameters as follows: 95°C for 11 min, 96°C for 1 min; 10 cycles of 94°C 30 sec (ramp 100%), 60°C for 30 sec (ramp 29%), and 70°C for 45 sec (ramp 23%); 22 cycles of 90°C for 30 sec (ramp 100%), 60°C for 30 sec (ramp 29%), and 70°C for 45 sec (ramp 23%); and 60°C hold for 30 min.

*Amplification of Various Sample Types*

Five studies were conducted utilizing various samples including those of good quality, low DNA concentrations, high DNA concentrations, samples with peak height imbalances, and samples with allelic drop-out. Table 2 summarizes the samples used in each of the five studies.

*Sensitivity*

A sensitivity study was performed according to SWGDAM validation guidelines (6). Four in-house, known DNA samples previously extracted from buccal swabs on the Beckman Coulter Biomek® 2000 were serially diluted and quantified, according to manufacturer’s protocol, using the Quantifiler® Human DNA Quantification Kit. Each dilution series, consisting of eight DNA concentrations ranging from 0.03125 to 5.0 ng, was amplified twice using the PowerPlex® 16 kit in a final volume of 12.5 uL. Data was analyzed at a threshold of 25 rfu (minimum), 50 rfu, and 100 rfu (maximum) to determine the optimal range of input DNA that would produce a complete profile. Results were compared to those originally obtained from the validation of PowerPlex® 16 using the full-reaction method.

*Application to Case Samples*

Finally, we evaluated the performance of the half-reaction amplification on 467 samples submitted to the MUFSC laboratory for analysis and research purposes. These samples were buccal swabs samples collected on cotton swabs. They were processed in the same manner as the blood stain samples used for this study.

*CE and Data Analysis*

Amplified products were prepared for capillary electrophoresis using 8.5 uL Hi-Di formamide, 0.5 uL Genescan 500 ROX internal size standard (Applied Biosystems), and 1 uL of the amplified product. Samples were injected on an ABI PRISM® 3130-*xl* Genetic Analyzer using a 3 kV, 5 sec injection with a run time of approximately 45 min. ABI PRISM® Data Collection Software v.1.1, Genescan® v.3.7.1, and Genotyper® v 3.7 software were used for data collection and analyzed at a maximum threshold of 100 rfu.

TABLE 1—Amplification components.

Component	Total Amplification Volumes		
	12 uL Amp	12.5 uL Amp	13 uL Amp
Master mix	3.0	2.9	3.0
DNA template	1.0	1.0	1.0
MBG water	8.0	8.6	9.0

TABLE 2—Summary of the five studies conducted.

Study	Sample Types	Number of Samples	Amp Vol. (uL)	Number of Amps.
12.5 uL reaction volume	Good quality	12	12.5	1
Extreme DNA concentrations	<0.4 ng concentration	5	12.5	2
	>3.0 ng concentration	10	12.5	2
12 uL vs. 13 uL	Good quality	14	12, 13	1 each vol.
Peak height imbalances	D5S818 peak ratio <50%	9	12, 12.5, 13	1 each vol.
Allelic drop-out	Allelic drop-out	22	12, 13	1 (12 uL); 2 (13 uL)

### Data and Statistics

The effectiveness of amplifying in a half-reaction volume was assessed primarily by evaluating peak height data. First, the mean peak height at each locus was calculated for all samples, as well as across all loci for each reaction volume. The quality of the DNA profile (drop-out, pull-up, stutter, off-scale data, etc.) was noted for samples containing high or low DNA concentrations. To assess samples with peak height imbalances, the peak height ratio at D5S818 for each sample and average ratios for each reaction volume were calculated and compared to full-reaction amplification data. The standard deviation (SD) for each reaction volume was calculated using the mean peak height ratio. Several samples in the “peak height imbalance study” were not available for testing at various times during this validation because they had yet to be submitted for analysis or were still being processed. Therefore, they are noted and were not included in calculations. To assess the success of half-reaction amplification on samples originally containing allelic drop-out, the percentage of full profiles obtained using the half-reaction method was calculated. This was carried out by dividing the number of full profiles obtained by the total number of samples tested. Data from the sensitivity study was used to determine the ideal concentration range that would yield quality profiles containing no pull-up, drop-out, off-scale peaks, or peak height imbalances of <50%.

### Results and Discussion

Results indicate that half-reaction amplification (12.5  $\mu$ L) using PowerPlex<sup>®</sup> 16 produced similar and often times improved profiles compared to full-reaction amplification (25  $\mu$ L). Average peak heights increased across all but three of the 16 STR loci (D18S51, Penta E, and D8S1179), and by an average of 155.72 rfu (Fig. 1). Only one sample amplified with the half-reaction method produced drop-out that was not observed in the full-reaction amplification.

An increase in peak heights using half-reaction amplification may be useful when working with low level DNA samples. Samples amplified at full-reaction and exhibiting drop-out just below the threshold levels could be amplified with the half-reaction method and possibly result in higher peak heights. This may be possible because average peak heights tended to increase when amplifying in a lower volume. An increase in peak heights could be significant enough to raise the original drop-out allele above threshold and result in a full profile. If low level samples were initially amplified at half-reaction, the need for re-amplification because of drop-out could be eliminated. This, in turn, could allow for the use of less DNA, which is critical when little is available.

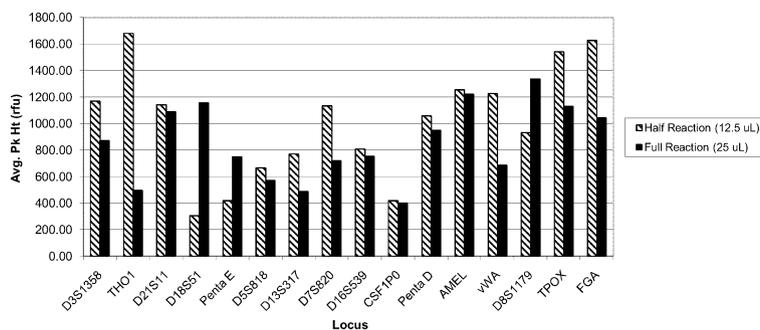


FIG. 1—Average peak heights obtained when using half-reaction vs. full-reaction amplification.

“Problem samples” containing low and high DNA concentrations were also analyzed using the half-reaction protocol. All samples with a low DNA concentration (<0.4 ng) produced full profiles when amplified in 12.5  $\mu$ L. PowerPlex<sup>®</sup> 16 DNA target studies conducted by MUFSC have indicated that “a 1–4 ng DNA amplification range would be ideal when using the PowerPlex<sup>®</sup> 16 amplification system.” Therefore, amplifying in half-reaction volumes may allow the system to work using lower concentrations of template DNA. This possibility was assessed during the sensitivity study (see further results). The ability to amplify less DNA could eliminate the need to concentrate low level samples, and in turn, the opportunity for contamination would be lessened because of reduced handling of samples.

Eight of the ten samples with a high DNA concentration produced full profiles in the first amplification while all ten had full profiles in the second amplification. The higher amount of input DNA did not result in pull-up, off-scale peaks, split peaks, enhanced stutter effects, or baseline noise often associated with the amplification of large concentrations of template DNA (7). The ability to amplify higher concentrations of DNA template would lessen the need for dilutions and, thus, the number of sample handling steps that could potentially increase the risk of contamination.

### Varying Total Reaction Volumes

After initial findings demonstrated the success of half-reaction amplification in 12.5  $\mu$ L, another study was performed to determine whether rounding the total reaction volume to 12  $\mu$ L or 13  $\mu$ L would be as effective. The half-reaction protocol using 12.5  $\mu$ L requires the addition of 2.9  $\mu$ L master mix to 8.6  $\mu$ L DNA plus water. However, Rainin repeat pipettes (Rainin Instrumentation LLC, Oakland, CA) used at the MUFSC laboratory do not have a 2.9  $\mu$ L setting, making manual amplification set-up more difficult. Therefore, this study investigated whether 3  $\mu$ L of master mix could be used in a final volume of 12  $\mu$ L or 13  $\mu$ L. Overall, results indicate that amplification volumes of either 12  $\mu$ L or 13  $\mu$ L yielded similar and often times higher quality profiles than full-reaction amplifications. Average peak heights at each locus increased as the total reaction volume decreased. Average peak heights across all loci for 25, 13, and 12  $\mu$ L reactions were 426.26, 1689.82, and 1925.50 rfu, respectively (Fig. 2). Because results for 12 and 13  $\mu$ L reaction volumes were quite similar, it should be left to the discretion of each individual laboratory as to which volume is more suitable for their amplification needs.

### D5S818 Peak Height Imbalances

A significant number of samples previously analyzed at the MUFSC laboratory have shown a peak height imbalance at the

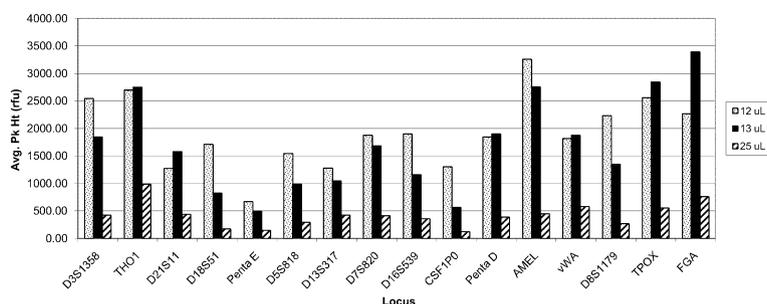


FIG. 2—Relationship between varying total amplification volumes and average peak heights.

D5S818 locus when a “10” allele was present within a heterozygote. In many cases, the “10” allele was not called in Genotyper® but was noted as a possible true allele because it was in the stutter position and above the 12.3% stutter threshold set forth by Promega (5). In other instances, the allele in question was noted because peak height ratios were very low or allelic drop-out was seen but a small peak was still visible. Re-amplification using PowerPlex® 16 produced similar, reproducible results. Amplification with the AmpF/STR Identifier® Amplification Kit (Applied Biosystems) using the manufacturer’s recommended protocol, produced two balanced peaks with proper allele calls. These observations may indicate a possible primer binding site mutation at D5S818 when amplifying with PowerPlex® 16.

Original extracts of nine samples were amplified using the three half-reaction volumes to determine whether reduced amplification volumes would have an effect on peak height imbalances at the D5S818 locus. Peak height ratios increased slightly as reaction volumes decreased (Fig. 3). The range of peak height ratios for full-reaction amplifications was 18.1–34.1%, while half-reaction amplifications ranged from 26.1% to 57%. Standard deviations decreased slightly as sample volumes increased but rose using a 25 uL volume (Fig. 3). Gaines et al. (8) conducted a similar study in which they investigated reduced reaction volumes using the AmpF/STR Profiler Plus Kit™. Their study showed that as reaction volumes decreased (with a constant DNA input of 2.0 ng), average peak height ratios did not vary significantly, and SD values remained constant. Discrepancies in results obtained from the two

studies may be because of sample size and the amount of template DNA used. Our study utilized only nine samples of unknown quantities while theirs used five replicates of each sample amplified at five different reaction volumes (5, 10, 15, 25, and 50 uL), using 2 ng of DNA. A greater sampling in this study as well as using quantified samples may yield results supporting the conclusions of Gaines et al. (8).

*Samples with Allelic Drop-Out*

Another study examined the effects of half-reaction amplification on samples containing drop-out when originally amplified at full-reaction. Forty-one percent (9 of 22) of the samples in the first amplification (13 uL) produced full profiles. However, 95.5% (21 of 22) of samples in the second amplification (also 13 uL) produced full profiles. This discrepancy may indicate that the first amplification simply was not as successful because of a variety of factors (i.e., laboratory temperature fluctuations, poor pipetting techniques of master mix and/or sample, low template volume). The third amplification (12 uL volume) produced full profiles for 91% (20 of 22) of the samples. In total, 66 amplifications were performed resulting in 50 full profiles (75.8%). Figure 4 displays profiles for one sample originally amplified at full-reaction and again after using the half-reaction protocol. Peak heights (rfu) are displayed as the second number below each allele. Average peak heights across all loci were similar for reaction volumes of 12 uL (979.01 rfu) and 13 uL (782.75 rfu) and, again, average peak

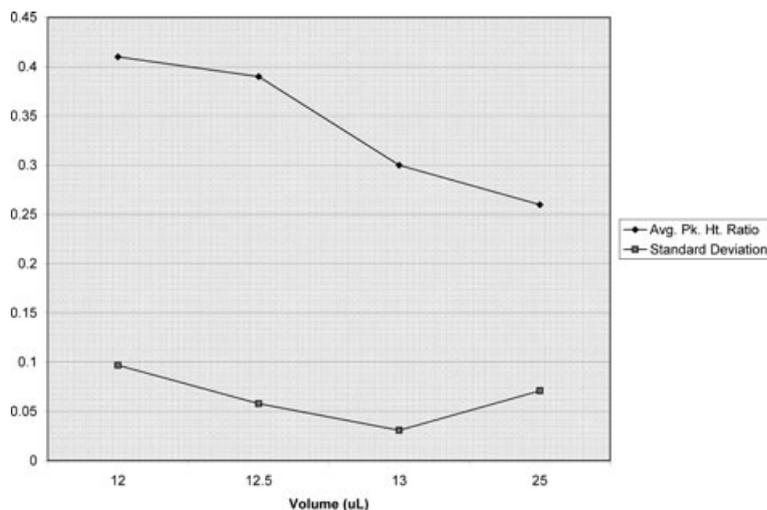


FIG. 3—Effects on peak height ratios and standard deviations when using reduced volume amplification on samples with a peak height imbalance at the D5S818 locus.

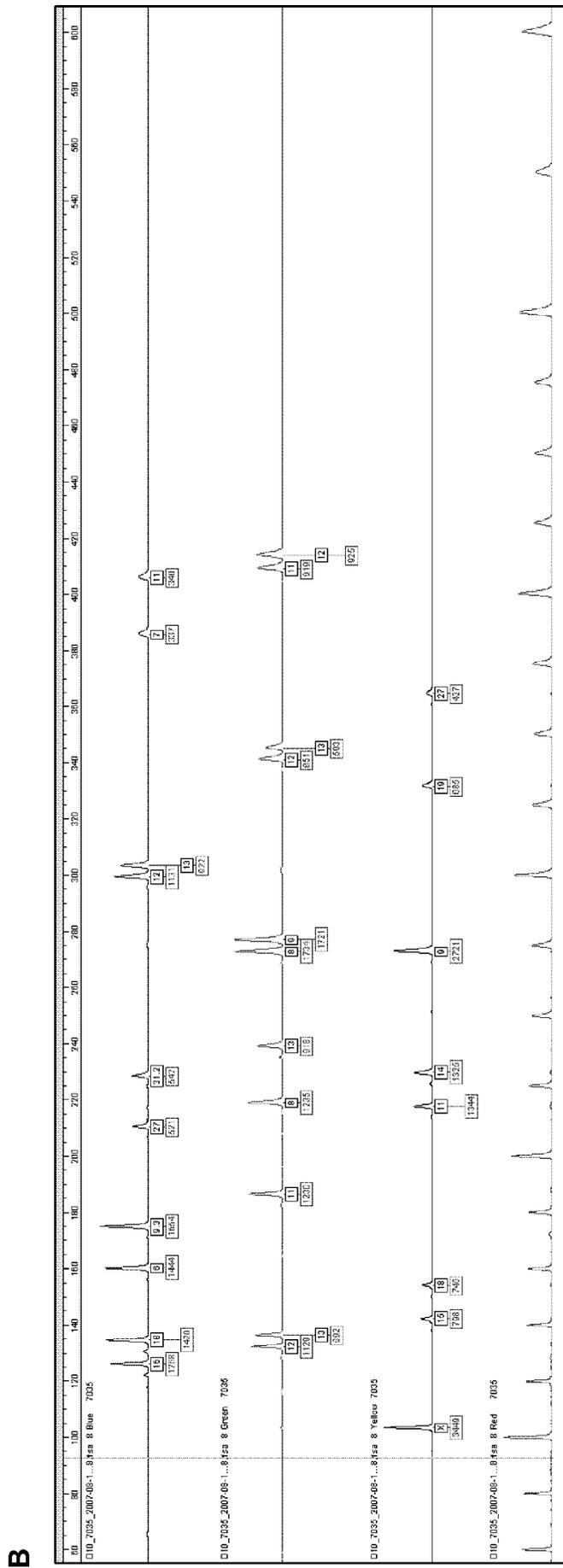
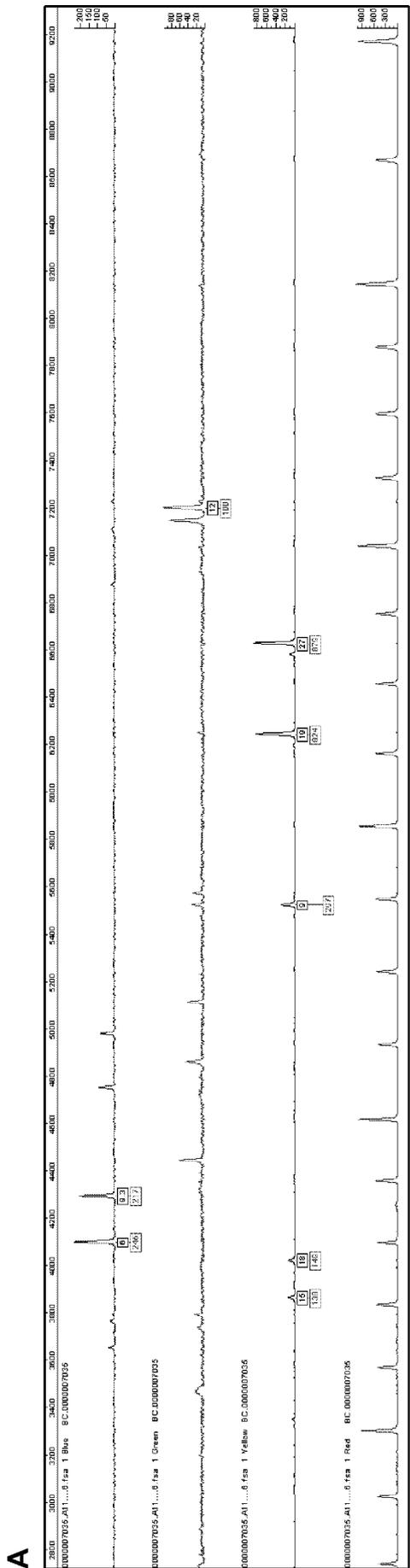


FIG. 4—PowerPlex® 16 electropherograms for sample “7035” amplified at full-reaction (A) and half-reaction (B). Panel B does not include an rfu scale; however, these values are noted as the second number below each allele.

heights increased as total amplification volumes decreased. Previous studies have found that lowering the reaction volume increases the concentration of amplified products as well as the total volume of PCR product (8). These increases allow for the amplification of less DNA template while still obtaining a full quality profile.

It is worth noting that the second amplification (13 uL) of sample "6915" contained a two-person mixed profile. The contributor of the minor profile could not be determined. Neither the extract, amplification negative control nor the first or third amplifications of the sample were contaminated. This indicates that contamination may have occurred during the second amplification set-up step, poor lab technique was involved, or samples were of low quality. This incident stresses the importance of closely following laboratory protocol to prevent contamination. It also demonstrates the high sensitivity and susceptibility to contamination associated with DNA amplification procedures.

### Sensitivity

A sensitivity study was conducted after initial findings indicated that using a reduced reaction volume may allow for the amplification of less DNA template. Data showed a DNA input range of 0.25–1.0 ng was optimal for half-reaction amplification using PowerPlex® 16. Amplifying more than 1.0 ng of DNA produced peak heights above 5000 rfu that resulted in pull-up, split peaks, and/or increased stutter. Amplifying <0.25 ng of DNA resulted in allelic drop-out and peak height imbalances with ratios less than 50%. Original sensitivity studies conducted at MUFSC showed an input range of 1–4 ng was ideal for PowerPlex® 16 full-reaction amplifications. Therefore, this study demonstrates that half-reaction amplification may be more sensitive than full-reaction amplification and may allow for the amplification of less template DNA. Results from the sensitivity study also support results seen in our initial studies in which low level DNA samples (<0.4 ng) produced full profiles. A lower sensitivity is helpful for the analysis of samples with low levels of DNA or when sample quantities are limited. Less sample volume may be consumed and more of the sample will remain for additional testing if need-be.

### Application to Case Samples

The half-reaction protocol established in this study was applied to 467 single-source samples outsourced to the MUFSC laboratory for analysis and research purposes. Unlike original blood punch samples used in the study, these samples were buccal swabs and were quantified and normalized to 1 ng. According to earlier findings in this study, 1 ng of template DNA should be sufficient for amplification using a half-reaction volume. The amplification of 467 samples produced 438 quality profiles using a 13 uL reaction volume. Of the 29 samples that failed, only six (1.3% of the total samples) required re-amplification because of drop-out, and two other samples were re-amplified because of peak height imbalances. The remaining 21 failed samples required re-setup because of low internal lane standards (ILS) in the original injection. The 1.3% repeat rate observed in this study is a significant decrease from the 24.5% repeat rate currently seen at the MUFSC laboratory for single-source samples amplified using the full-reaction amplification. Overall, the use of the half-reaction amplification method resulted in a 94% success rate for obtaining full, quality DNA profiles in one amplification.

### Conclusion

In an effort to reduce laboratory costs, this study investigated the possibility of reducing PowerPlex® 16 amplification volumes by half. Results indicate that a reaction volume of 12.5 uL produced similar if not higher quality profiles than full-reaction amplifications. In fact, the final volume can be altered to 12 uL or 13 uL, and successful amplification parameters can still be maintained. Drop-out rates decreased and peak height ratios at the D5S818 locus increased. Sensitivity results show that half-reaction amplifications may be more sensitive and allow for the amplification of less DNA template. The ideal input range of DNA was found to be 0.25–1.0 ng compared to a 1.0–4.0 ng range determined for full-reaction amplification.

The results of this study could have several positive impacts on the forensic science community and the analysis of DNA. First, half-reaction amplification is more sensitive and requires the use of less DNA template. This would allow analysts to consume less DNA for testing and retain a larger amount of sample for additional testing if needed. Second, obtaining quality DNA profiles from a single half-reaction amplification step would reduce the number of samples needing re-amplification because of drop-out or imbalanced peak heights. Because samples would not require a second or third re-amplification step, the chance for contamination is also decreased because of less sample handling. A decrease in the repeat rate and an increased success rate would result in a less time-consuming DNA analysis process and, subsequently, current DNA backlogs may be reduced. Most importantly, the use of a half-reaction protocol would allow laboratories to amplify more samples with a single amplification kit, thus lessening operational costs. Lessened costs for the laboratory could result in lower costs for the client and could possibly allow for the analysis of samples that previously proved too costly.

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