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Reduced Volume PCR Amplification Reactions Using the AmpFℓSTR® Profiler Plus™ Kit*

ABSTRACT: The forensic community continues to seek improvements in DNA typing methods on aspects such as sensitivity and efficacy. Reducing the volume of the AmpFℓSTR® Profiler Plus™ reagents offered greater sensitivity and improved the chance of obtaining useful results for samples with very low quantities of DNA and multiple source samples. On the downside, amplifications initiated with less than 0.4 ng of DNA exhibited a twofold increase in the standard deviation of peak ratios.

This research suggested a twofold approach to analyzing samples. For samples with greater than 0.25 ng of DNA, a 25 μL reaction is appropriate. Samples that did not demonstrate quantifiable results, or that have less than 0.25 ng, can be amplified by drying the sample directly in the PCR tube and amplifying in a 5 μL reaction. The analyst can expect at least limited results with as little as 0.03 ng of DNA in the 5 μL reaction.

KEYWORDS: forensic science, DNA typing, polymerase chain reaction, sensitivity, reduced volume reaction

Polymerase chain reaction (PCR)-based DNA typing has progressed significantly since its introduction to forensic analysis in the late 1980s (1,2). The technique is now universally recognized as the primary method of DNA analysis in forensic laboratories. PCR-based DNA typing systems offer three major advantages over the earlier typing methods. First, PCR-based methods are amenable to multiplexing, which permits simultaneous analysis of multiple loci with a single amplification reaction (3,4). Multiplexed reactions minimize sample consumption and maximize the probability of discrimination. Second, PCR-based typing systems are extremely sensitive and robust. Therefore, reliable typing data can be acquired from picogram quantities of DNA and badly degraded samples (5). Third, this typing method allows for significant interpretation of complex mixtures of two or more individuals in a single sample (6,7). The enhanced interpretations are due to the discrete alleles and polymorphic nature of the forensically significant genetic loci known as STRs (short tandem repeats).

The STR loci have been well characterized, extensively validated, and are universally used in the forensic community (8–10). Several PCR-based STR multiplex typing kits are now produced commercially to simplify DNA testing. However, the forensic community continues to seek techniques to further simplify the procedures and improve on aspects such as sensitivity and efficacy. Two methods that have been previously investigated for improving the sensitivity of the PCR kits involve increasing the number of cycles in the PCR reaction (11,12) and reducing the volume of the PCR reaction (13–15).

The North Louisiana Criminalistics Laboratory (NLCL) was interested in further investigating the benefits of reducing the PCR reaction volume. Potential benefits as seen by the NLCL would be a decrease in sample consumption, an increase in analytical sensitivity, and a reduction in the cost of analyses. There were two concerns about reducing the reaction volume. First, a decrease in PCR reaction volume could significantly alter the kinetics of the amplification reaction. Second, an increase in sensitivity might affect interpretation due to increased stochastic effects and low-level contamination. Often, internal laboratory validation does not adequately investigate how a reduction in reaction volume could affect the typing results. To identify and characterize benefits and pitfalls of reducing the reaction volume, extensive testing would be required. The purpose of this project was to assess the effects of reducing the reaction volume on sensitivity, kinetics, and interpretation of the STR typing results.

The effects of reduced volume PCR (RV-PCR) were assessed through a series of five experiments. First, the quantity of template DNA was reduced proportionally to the amplification reaction volume. In the second experiment, the reaction volume was reduced while holding the amount of template DNA constant. Third, the efficiency of the PCR amplifications was determined at reduced reaction volumes. Next, the minimum detection level (sensitivity) was determined for the experimental volumes. Finally, the ability to identify and interpret mixed samples was evaluated at reduced reaction volumes.

Materials and Methods

DNA Preparation

DNA for the following experiments was obtained from dried bloodstain cards and buccal swabs. The dried bloodstain cards (Life Technologies, Rockville, MD) were previously prepared from whole blood collected in purple-top tubes. The blood samples were items from past proficiency tests received at the NLCL. Buccal swabs were collected from volunteers using Sterile Omni Swabs (Whatman, Newton, MA). The DNA was extracted using an

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organic extraction method and Centricon®-100 purification (16,17). The purified DNA was stored at −20°C in TE^{-4} buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). Sufficient DNA was purified from a single source so that the same sample could be used for all experiments, except the mixture study. For the mixture study, three different DNA samples were used.

The concentration of DNA in each sample was determined by fluorometry (18). PicoGreen® (a dye from Molecular Probes, Eugene, OR) was added to an aliquot of each sample, and the fluorescence was measured in a Fluoroskan *Ascent*® FL fluorometer (Labsystems, Franklin, MA). The final DNA concentration was calculated as the average of five replicate measurements of each sample. The DNA concentration in each sample was further checked by amplifying 2.0 ng of DNA and comparing the peak height results to the 9947A Control DNA in the AmpFℓSTR® Profiler Plus™ kit. The extensive quantification performed for the stock DNA solutions was done to insure that the starting quantity of DNA in all of the reactions was accurately determined.

PCR Conditions

Master mixes containing PCR reaction mix, primer solution, and AmpliTaq Gold® DNA polymerase were used to minimize sample preparation variance. The reagents in the Applied Biosystems AmpFℓSTR® Profiler Plus™ kit were used for all experiments in the same proportions specified in the AmpFℓSTR® User's Manual (21 μL PCR reaction mix; 11 μL primer set; 1 μL AmpliTaq Gold® DNA polymerase; 20 μL sample) (19). Amplifications were carried out in 0.2 mL, thin-wall PCR tubes (Robbins Scientific, Sunnyvale, CA). The samples were diluted in sterile water (Amresco®, Solon, OH) to achieve the correct mass of DNA template for each reaction. The DNA solution was first transferred to PCR tubes and then dried using a Vacufuge™ concentrator (Eppendorf®, Westbury, NY) before adding the PCR master mix. When the samples were dried, water was added to the PCR master mix to account for the volume reserved for the sample. Mineral oil was used to overlay all 5 μL and 10 μL reactions to prevent evaporation.

Thermal cycling conditions were the same as described in the AmpFℓSTR® User's Manual (19) and are subsequently referred to as standard conditions. These conditions were as follows: initial denaturing and polymerase activation at 95°C for 11 minutes, followed by 28 cycles of one minute denaturing at 94°C, one minute annealing at 59°C, and a one minute extension at 72°C. The final, nontemplate directed extension was 45 minutes at 60°C. Twenty-eight cycles were always used, except where specifically noted. All amplifications were performed in GeneAmp® 2400 and GeneAmp® 9600 thermal cyclers (Perkin Elmer, Foster City, CA). The experimental reaction volume was set appropriately in the thermal cyclers.

Electrophoresis Conditions

Electrophoresis and data collection were performed on two ABI Prism™ 377 DNA Sequencers (Perkin Elmer, Foster City, CA). Both instruments were used interchangeably for the experiments. Denaturing gels were composed of 5% Long Ranger (FMC, Rockland, ME), 6M urea, and 1× TBE. The gels were poured in 36 cm WTR (well-to-read) glass plates (Applied Biosystems, Foster City, CA) with 0.2 mm spacers. The electrophoresis tank buffer was 1× TBE. Electrophoresis temperature, voltage, current, and power were set as described in the AmpFℓSTR® User's Manual (19). The electrophoresis parameters were set as limiting conditions at 3000 V, 60.0 mA, 200 W and 51°C. Samples were prepared for electrophoresis by mixing 1.0 μL of PCR product and 2.5 μL of loading dye solution (formamide, GeneScan™-500 ROX™, and blue dextran). After a brief denaturation at 95°C, 2.0 μL of sample was immediately loaded (still hot) onto the gel in 32-well sharkstooth combs.

GeneScan™ v3.1.1 (Applied Biosystems, Foster City, CA) was used to analyze the electrophoresis data. Baselining, matrix correction, and light smoothing were applied to all samples. The peak height, in relative fluorescent units (RFU), of all major peaks (alleles) was used for statistical analysis. Peak height was chosen as the threshold parameter for measuring the quantity of amplified product for two reasons. First, GeneScan™ software uses peak height as the parameter to identify peaks, and Genotyper® uses peak height to filter stutter peaks from the allele calling. Second, peak height and peak area are both related in a linear manner to the amount of fluorescent-labeled DNA, and either provides a reliable measurement (20).

The minimum peak height threshold was set at 50 RFU, except for the red filter, which was set at 150 RFU. The purpose of setting the threshold at 50 RFU was to allow for detection of all peaks clearly above background. The AmpFℓSTR® Profiler Plus™ v4 Genotyper® template (Applied Biosystems, Foster City, CA) was used to label all peaks, including artifact peaks. An artifact peak was defined as any peak related to PCR amplification or data collection instrumentation, except for the known allele and amelogenin peaks. Artifact peaks include split (−A) peaks, excessively high stutter peaks, and peaks caused by pull-up from another color. These peaks were not included in the data collection since forensic DNA laboratories would typically re-analyze the samples after diluting or reamplifying to eliminate the artifacts.

Statistical Analysis

To reduce variation in sample preparation and gel loading, each of the amplified samples was prepared and run five times on a gel. The mean peak height (RFU) of the five replicates was used for subsequent statistical analyses. Stutter peaks and split peaks were not used for any statistical tests, nor were they included in peak height totals. Data compilation and some calculations (replicate averaging, χ^2) were performed in Excel 2000 (Microsoft, Redmond, WA). Other statistical calculations (mean, standard deviation (SD), ANOVA, regression analysis) were performed using SYSTAT® v10 (SPSS, Chicago, IL). Results were tabulated for the sensitivity and mixture experiments, but no statistical analyses were performed.

Results

The PCR amplification conditions described in the AmpFℓSTR® Profiler Plus™ User's Manual (19) were validated for a 50 μL reaction initiated with 2.0 ng of template DNA. Two related experiments were performed to evaluate the effects of amplifying samples using the AmpFℓSTR® Profiler Plus™ kit at reduced reaction volumes. In the first experiment, subsequently referred to as the PRD experiment, the starting quantity of DNA was reduced proportionally with the reaction volume. The second experiment, hereafter referred to as the CD experiment, used a constant starting DNA quantity of 2.0 ng for each of the experimental volumes. The effects on PCR product concentration (RFU/μL), total PCR product, peak height ratios (in heterozygous loci), and variance of these ratios were evaluated for five reaction volumes (5, 10, 15, 25, and 50 μL).

Effect of RV-PCR on PCR Product Concentration

Six series of PRD reactions were amplified using standard conditions. For the PRD experiment only, the template DNA was included in the PCR master mix. The master mix was prepared so that a 50 μL reaction contained 2.0 ng of DNA, with the smaller volumes having proportionally less (25 μL=1.0 ng, 15 μL=0.6 ng, 10 μL=0.4 ng, and 5 μL=0.2 ng).

For the CD experiment, each reaction was initiated with the same amount of template DNA (2.0 ng). The PCR tubes were prepared by first transferring 10 μL of a DNA solution (diluted to 0.2 ng/μL with water) into the tube and drying down the solution in a concentrator. The appropriate volume of PCR master mix was then added to each of the PCR tubes. Six series of CD reactions were amplified using standard conditions. All of the samples for the PRD and CD experiments were prepared from the same stock DNA sample.

The amplified products from both experiments were analyzed on the ABI Prism™ 377 instruments. The DNA sample used for these experiments contained seven heterozygous loci (D3S1358, vWA, FGA, D8S1179, D18S51, D5S818, and D7S820) and three homozygous loci (D21S11, D13S317, and Amelogenin). The concentration (RFU/μL) of amplified product was calculated by summing the peak heights (RFU) of all alleles in a sample, and dividing by the volume used to prepare the electrophoresis sample (1.0 μL). The results are shown in Fig. 1.

In the PRD experiment, concentrations of amplified product did not significantly change ($p>0.05$), despite the 10-fold difference in the initial quantity of DNA (Fig. 1*a*). The electropherograms of these samples generally demonstrated balance between loci within each color and a lack of PCR artifacts, such as split peaks, excessive stutter, or off-scale data (Fig. 2). Examination of the electropherograms for these samples revealed a nearly equal value when all of the peak heights in a sample were summed. However, the smaller volumes, with the smaller quantities of template DNA, demonstrated more peak imbalance. Nevertheless, the overall amplified product concentrations in the smaller volumes were equivalent to that of the larger volume reactions. These results also illustrated that a small quantity of template DNA in a smaller reaction volume would amplify proportionally to the same concentrations as a larger amount of DNA in a larger reaction volume.

The concentration of amplified products for the CD experiment increased as the reaction volume decreased (Fig. 1*b*). The increase was inversely proportional to the volume in the 15 μL and 25 μL reactions, as compared to the 50 μL reaction. At 5 μL and 10 μL, the concentration also increased, but the increase was not proportional to the decrease in reaction volume. The electropherograms of the 5 μL samples manifested considerably more off-scale peaks and PCR artifacts than in the larger volume reactions (Fig. 3). In the 10 μL reaction, numerous artifacts and off-scale peaks were also observed, but to a lesser extent than was found in the 5 μL amplifications. The 15 μL volume reaction occasionally produced some off-scale peaks but no other artifacts. All of the amplified CD samples in the 15, 25, and 50 μL reactions were diluted 1:4 and reanalyzed by electrophoresis to lower any off-scale peaks into the analytical range of the ABI Prism™ 377 (21). The resultant peak heights were adjusted by the dilution factor and these data are shown in Fig. 1*b*. After diluting the samples, the 15 μL samples produced no off-scale data. The adjusted dilution results were consistent with the undiluted data and demonstrated the same proportional increase in PCR product concentration at 15 μL and 25 μL. The 5 μL and 10 μL reactions were not able to be reanalyzed due to insufficient remaining PCR product.

The concentrations of the amplified products in the 50 μL reactions of the PRD and CD experiments were compared to determine if there was a significant difference, possibly caused by the differences in sample preparation. The mean concentration of PCR product in the PRD 50 μL reaction (15 021 RFU/μL, SD = 2193) was not significantly different from the mean concentration of PCR product in the CD 50 μL diluted reaction (14 798 RFU/μL, SD = 2356).

Effect of RV-PCR on Total PCR Product

Total PCR product was calculated by multiplying the PCR product concentration by the reaction volume. The results of these calculations for the PRD and CD experiments are shown in Fig. 4. The total PCR product in the PRD experiment decreased proportionally

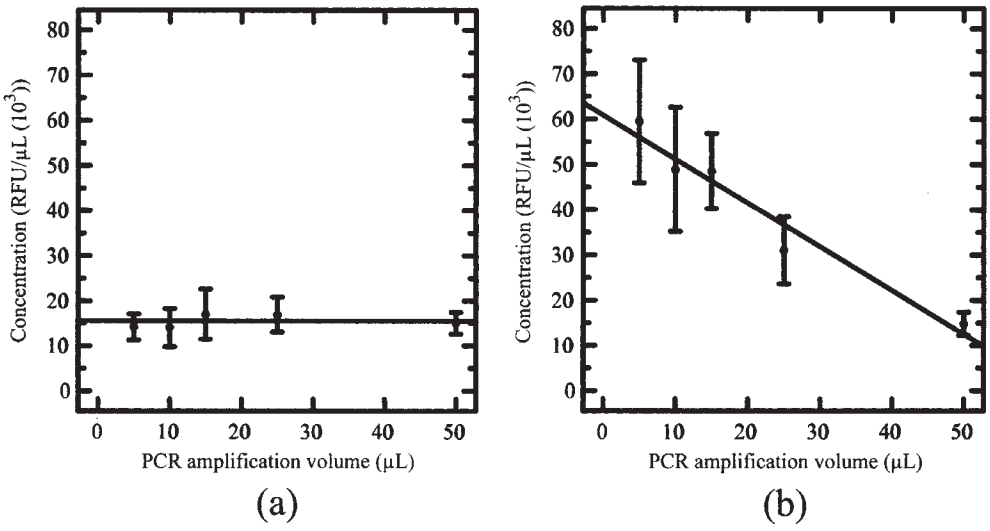

FIG. 1—*Concentration (RFU/μL) of amplified product for each sample. a) PRD experiment: 2.0 ng amplified in 50 μL, 1.0 ng amplified in 25 μL, 0.6 ng amplified in 15 μL, 0.4 ng amplified in 10 μL, and 0.2 ng amplified in 5 μL; b) CD experiment: 2.0 ng amplified in reaction volumes of 50, 25, 15, 10, and 5 μL. Error bars are ± 1 SD (N=6).*

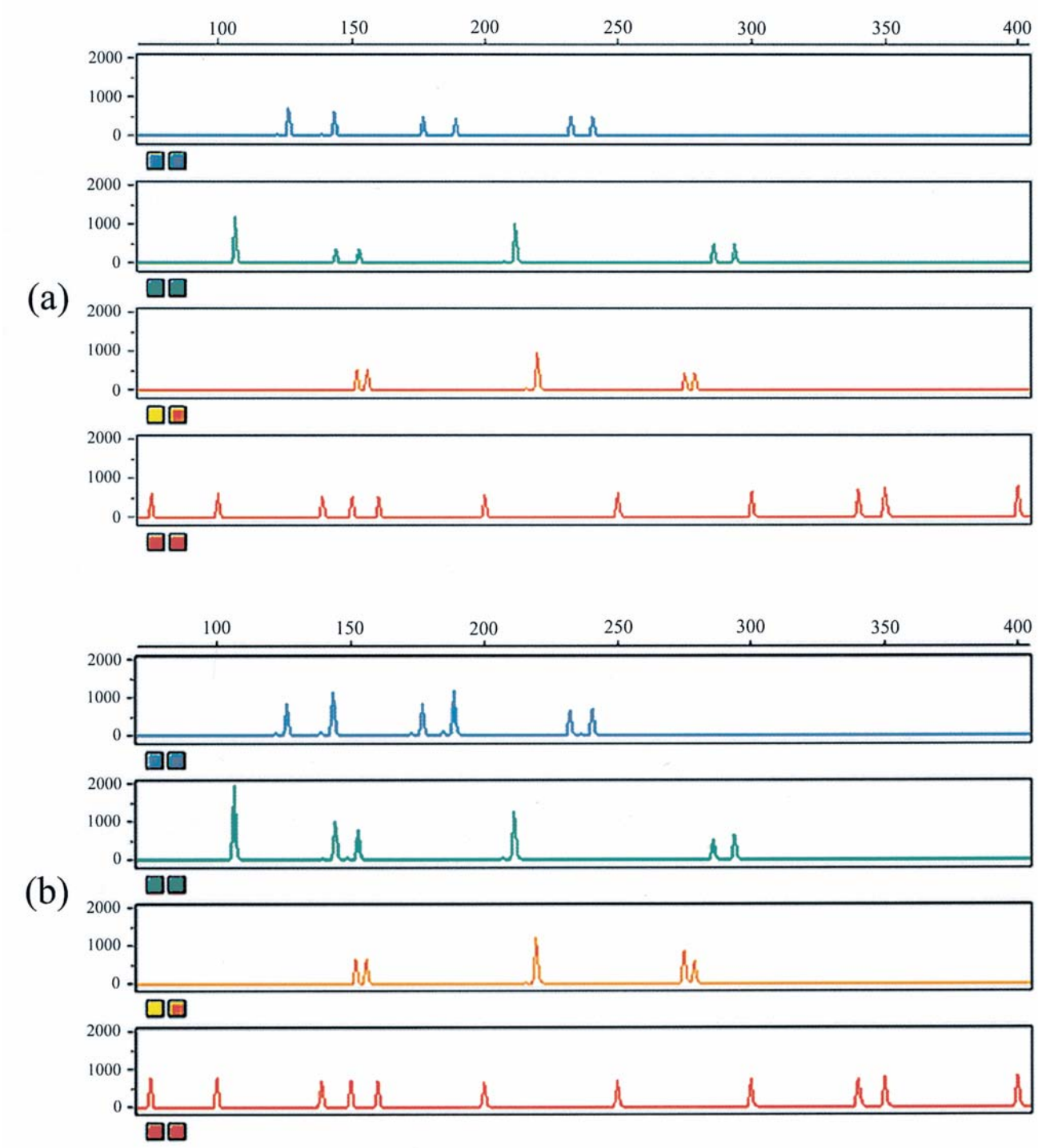

FIG. 2—*Typical electropherograms for the PRD experiment. a) 50 μL reaction amplified with 2.0 ng of template DNA (scale = 2000 RFU); b) 5 μL reaction amplified with 0.2 ng of template DNA (scale = 2000 RFU). Note the approximately equal peak heights between the two reactions. However, while the electropherogram from the 50 μL reaction showed well balanced peaks within and between loci, the electropherogram from the 5 μL reaction exhibited peak imbalance in several heterozygous loci (D3S1358, vWA, D8S1179, D18S51, and D7S820), and different peak heights among the homozygous*

to the volume, indicating that the total amplified product was proportional to the amount of template DNA used to start the reaction (Fig. 4*a*).

The total PCR product in the CD experiment showed slightly different results (Fig. 4*b*). At 50 μL, the total PCR product was equivalent to that of the 50 μL reaction in the PRD experiment. After the 1:4 dilution (described above), the total PCR product in the 15 μL and 25 μL was not significantly different from that produced in the 50 μL CD reaction. However, total PCR product dropped sharply in the 5 μL and 10 μL reactions. The decrease in product was likely due to off-scale peaks, whose true value could not be determined, and split peaks, whose RFU value was not added to the RFU value of the true allele. The sharp decrease could likely have been lessened had these amplified samples been diluted and reanalyzed to include more information lost in the off-scale peaks.

Additional data evaluated in the next experiment, determination of amplification efficiency, suggested that the total PCR product was the same in the 5, 10, and 15 μL reactions when all of the resulting peaks were on-scale. Thus, the combined information from the CD and amplification efficiency experiments pointed to a conclusion that when the same amount of DNA template was amplified, the total PCR product was the same despite the reaction volume. The off-scale data in the 5 μL and 10 μL CD reactions also indicated that both the analytical range of the ABI Prism™ 377 and the amplification range of the reagents

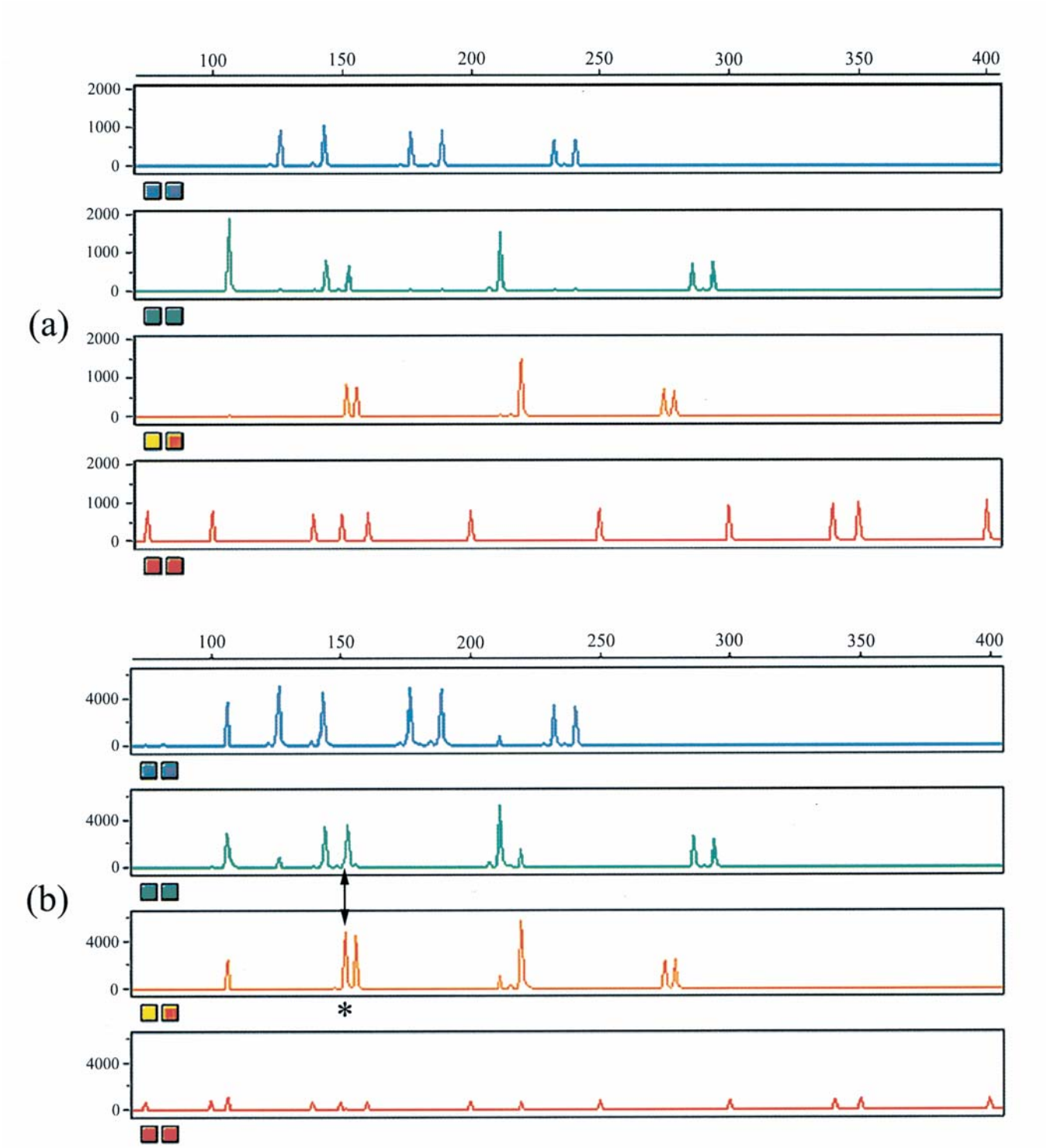

FIG. 3—*Typical electropherograms for the CD experiment. a) 50 μL reaction amplified with 2.0 ng of template DNA (scale = 2000 RFU); b) 5 μL reaction amplified with 2.0 ng of template DNA (scale = 6000). The electropherogram from the 5 μL reaction did not exhibit the peak imbalance that was observed in the PRD experiment (Fig. 2*b) *however, artifact peaks present significant interpretation concerns. One important observation was that the blue pull-up peak from the amelogenin X was larger in height than the true allele peak. Second, the green pull-up peak from the first allele in D5S818 added to the height of the split peak from the second allele in D8S1179 (indicated by *). Note also that the larger loci within each color appeared to have lower peak heights than the other loci within the same color.*

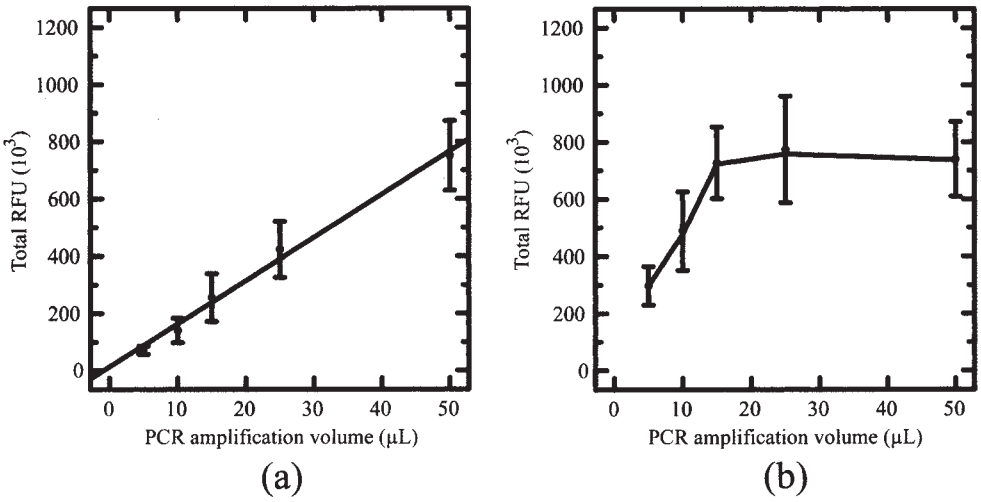

FIG. 4—*Total PCR product. a) PRD experiment: 2.0 ng amplified in 50 μL, 1.0 ng amplified in 25 μL, 0.6 ng amplified in 15 μL, 0.4 ng amplified in 10 μL, and 0.2 ng amplified in 5 μL; b) CD experiment: 2.0 ng amplified in reaction volumes of 50, 25, 15, 10, and 5 μL. Error bars are ± 1 SD (N=6). The total RFU values were calculated by multiplying the volume by the concentration (RFU/μL).*

were exceeded, when these amplifications were initiated with 2.0 ng of DNA.

Effect of RV-PCR on Peak Height Ratio

Ideally, each of the peaks in a heterozygous locus should be of equal height (balanced) without large deviations from the expected ratio of 1. Because deviations of allele balance can occur independently at any locus within a sample, the peak height ratios for all loci within a test volume were measured. The peak height results for the heterozygous loci were used to calculate within locus ratios for both the PRD and CD experiments.

Initially, the min/max ratio was used to evaluate the within locus allele balance. The min/max ratio was calculated by dividing the lower peak height by the higher peak height. The range of results will always lie between 0 and 1, inclusive, with an expected value of 1, representing equal balance of the two alleles. Restricting the range to values less than or equal to 1 meant that deviations from the expected value must always be less than 1 (one-tailed). The min/max calculation does not identify which of the two alleles in a locus was smaller or larger in height, thus losing important information about peak height variance.

A second method (PK1/PK2) was ultimately selected to analyze the within locus ratios and was used for all subsequent calculations. The PK1/PK2 ratio was calculated by dividing the height of the smaller allele by the height of the larger allele. The expected value is also 1, but the range is not limited to values less than 1 (two-tailed), and, the peak height ratios should be distributed normally about the expected mean. With the PK1/PK2 method, values greater than 1 occur when the height of the smaller allele was greater, and values less than 1 occur when the height of the larger allele was greater. Therefore, the PK1/PK2 ratios included information about which allele peak height was greater.

The PK1/PK2 ratios were averaged for all heterozygous loci, in each sample, at all volumes. The mean ratio for both the PRD and CD experiments did not change between the different volumes. The resulting mean for the PRD volumes was 1.055, and the mean for the CD volumes was 1.056 (Fig. 5). These data were consistent with observations that the smaller DNA fragments were amplified with slightly greater efficiency (7,22,23). Interestingly, Gill et al. (23) using the same method of calculating peak ratio, obtained nearly the same results using the median of the peak area. Gill et al. (23) examined the peak ratios in each locus, whereas this experiment examined the variances at all loci for a given volume and starting DNA quantity.

Although the average peak height ratios of the PRD and CD experiments were nearly identical, the variability about the mean differed noticeably (Fig. 5). The increased variability in the peak height ratios in the smaller volumes was consistent with the peak imbalance observed in the low quantity, small volume reaction in Fig. 2*b*. The apparent difference in the variability between the PRD and CD experiments led to examination of the SD at each volume in these experiments.

Effect of RV-PCR on the Variance of the Peak Height Ratios

An SD value was determined for each of the six samples at each experimental volume in the PRD and CD experiments. The SD value was the standard deviation of the peak height ratios of the seven heterozygous loci within each sample. The mean of these SD values was calculated for each volume in the two experiments and the results are shown in Fig. 6. In the PRD experiment (Fig. 6*a*), the average SD increased linearly as the reaction volume, and DNA template quantity, decreased. The average SD for the 5 μL (0.217) and 10 μL (0.173) reactions were approximately double that of the 50 μL (0.103) reaction.

The SD values in the CD experiment did not significantly change (μ=0.085) for each volume (Fig. 6*b*). These results indicated that when the same quantity of template DNA was used, the peak height ratio variability remained constant, regardless of the reaction volume.

The average SD values for the 50 μL reactions in the PRD and CD experiments were not significantly different ($p>0.05$). These results also confirmed earlier findings that preparing the samples by drying down the purified DNA in the PCR tube did not adversely affect the results as compared to when the DNA sample was not dried.

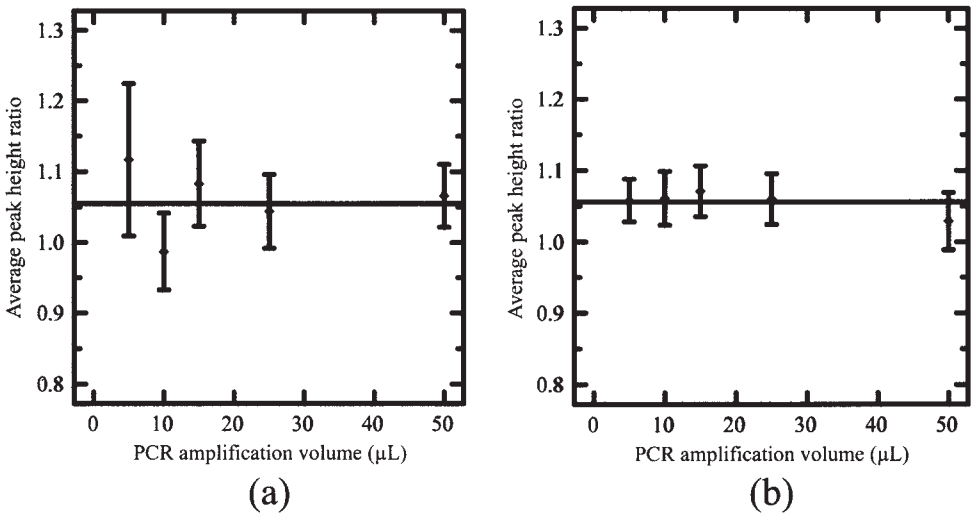

FIG. 5—*Average peak ratios (PK1/PK2) for all heterozygous loci. a) PRD experiment: 2.0 ng amplified in 50 μL, 1.0 ng amplified in 25 μL, 0.6 ng amplified in 15 μL, 0.4 ng amplified in 10 μL, and 0.2 ng amplified in 5 μL; b) CD experiment: 2.0 ng amplified in reaction volumes of 50, 25, 15, 10, and 5 μL. Error bars are ± 1 SD (N=6).*

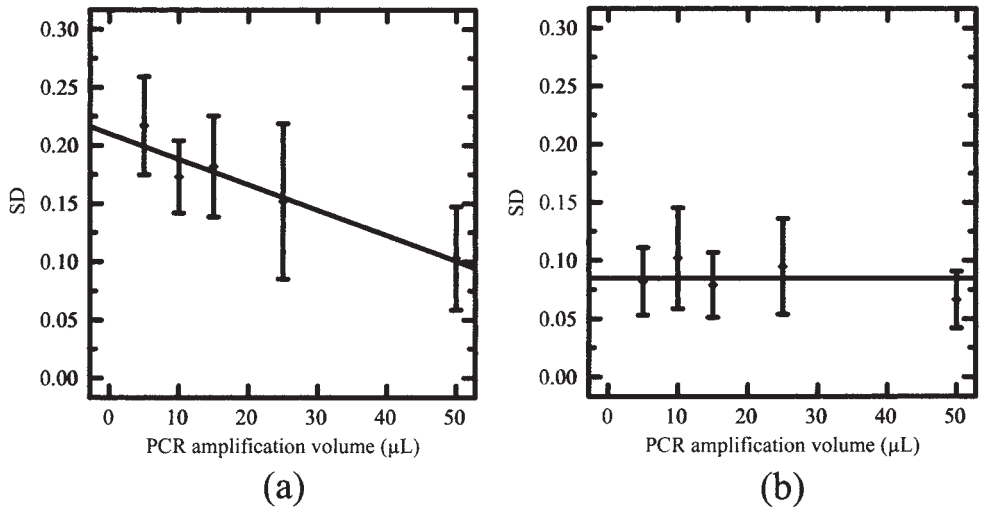

FIG. 6—*Mean SD values calculated for the heterozygous peak ratios (PK1/PK2). a) PRD experiment: 2.0 ng amplified in 50 μL, 1.0 ng amplified in 25 μL, 0.6 ng amplified in 15 μL, 0.4 ng amplified in 10 μL, and 0.2 ng amplified in 5 μL; b) CD experiment: 2.0 ng amplified in reaction volumes of 50, 25, 15, 10, and 5 μL. Error bars are ± 1 SD (N=6).*

Effect of RV-PCR on Amplification Efficiency

The amplification efficiency was measured for each experimental volume to determine if the kinetics of the amplification were affected by the reduction in reaction volume. Amplification efficiency is described by the equation below, where N is the final number of copies, N_0 is the starting number of copies, E is the efficiency, and C is the number of amplification cycles. When E equals 1 the efficiency is 100% and the number of target copies of DNA doubles at each amplification cycle.

$$N = N_0 (1 + E)^C$$

All reactions were initiated with 1.0 ng of template DNA and amplified using standard cycling conditions. The only change to the thermal cycling conditions was that the number of amplification cycles was varied from 20 to 32. The amplified products were analyzed using the ABI 377 instruments. Amplified samples that initially exhibited off-scale peaks were diluted 1:4 and reanalyzed. The diluted peak heights were used in data analysis after correcting for the dilution. The PCR product concentrations were calculated by summing the peak heights for all of the loci at one volume, amplified for a given number of cycles. RFU concentration can be used to represent copy number (N) since the quantity of fluorescent label within a peak is related to the number of target copies (20). Using RFU concentration calculated from all the peaks in the sample was intended to detect kinetic changes, such as PCR inhibition, which affects the overall rate of amplification. Changes in within-locus and between-locus amplification efficiency, which can be the result of small fragment preferential amplification or too much template DNA were not evaluated by this experiment.

Results were plotted on a semi-logarithmic graph with the ordinate being $\log_2$. Log_2 was chosen because the slope in the linear range (optimum amplification efficiency range) of the data closely approximates the amplification efficiency (E). The resulting graph

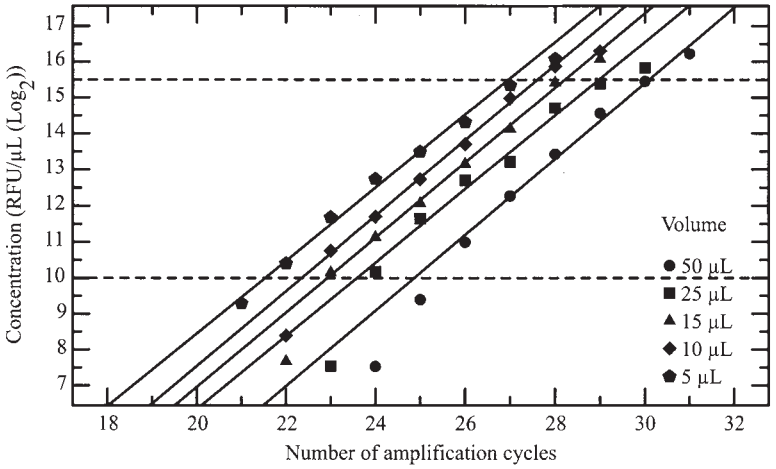

FIG. 7—*PCR amplification efficiency for the five experimental volumes. The following amplification reactions were performed: 50 μL/ 23–31 cycles, 25 μL/22–30 cycles, 15 μL/21–29 cycles, 10 μL/21–29 cycles, and 5 μL/21–28 cycles. Total RFU values were calculated by summing the peak heights for all of the loci at a given volume, amplified for a given number of cycles. The range between the dashed lines was used to calculate the efficiency. This area was selected because this was the range when the amplification rate was at a maximum.*

(Fig. 7) demonstrated a linear range between the dashed lines, a shoulder at low RFU, and a plateau at high RFU. The range between the dashed lines was used to calculate the slope, since in this range, amplification was occurring at its maximum rate and the analytical range of the instrument and the PCR kits was not being exceeded. The efficiency of amplification at all volumes was essentially the same ($\mu = 1.04$, min = 1.02, max = 1.05). Achieving comparable efficiencies for all of the experimental volumes demonstrated that the PCR reaction kinetics were not affected by the reduction in reaction volume. The plateau of the samples above $\log_2$ 15.5 was the point at which the dynamic ranges of the instrument and PCR amplification were being exceeded; that is when PCR reagents were depleted and when the instrument could no longer accurately measure the true peak heights. The shoulder at RFU values less than $\log_2$ 10 represented under-amplified samples, where some of the allele peaks may not have been identified using the 50 RFU threshold, and therefore, are not included in the RFU sum, causing the appearance of a rapid decline.

Using the slopes of the experimental volumes, the x-intercept of each experimental volume was calculated (5 μL = 11.66, 10 μL = 12.79, 15 μL = 13.31, 25 μL = 13.8, and 50 μL = 15.37). The x-intercepts represent a relative number of amplification cycles for the respective volumes for which all of the PCR product concentrations were equal. Therefore, the same peak heights (i.e., amplified product concentration) were achieved in fewer cycles for reduced volume reactions and the relative reduction in amplification cycles was proportional to the reduction in volume ($\chi^2 = 2.33$, $df = 5$, $p > 0.05$). An example of the relative equivalence of peak heights at different cycles is shown in Fig. 8. The ability to achieve the same amplified product concentration in reduced volumes was also observed in the PRD experiment where DNA template was reduced instead of relative PCR cycles.

Effect of RV-PCR on Sensitivity

The minimum detectable amount of template DNA was determined by amplifying decreasing amounts of DNA template at each experimental volume. The minimum detection limit (MDL) was defined as the lowest amount of starting DNA template in which greater than 99% of the expected alleles were identified (50 RFU). DNA solutions were prepared with the target amounts of DNA and dried directly in the PCR tubes, as previously described. The appropriate volume of PCR master mix was then added, and the samples were amplified using standard conditions. Two series of samples were prepared for each volume and quantity of template DNA. Five replicates of the amplified samples in each series were analyzed by gel electrophoresis. The amplified DNA sample had 17 allele peaks (7 heterozygous loci and 3 homozygous loci). Sensitivity was assessed by counting the number of allele peaks identified by the software for all of the loci in a sample. The counts were summed for all replicates of the sample and a percentage determined by dividing the number of labeled allele peaks by 170 (the number of allele peaks possible in two series of five replicates). The results were classified into four categories and are given in Table 1.

At 0.50 ng and greater of initial DNA template, all of the allele peaks were identified in all five experimental volumes. The results were consistent with the findings of the NLCL's validation (21) of the AmpFℓSTR® kits for routine casework, where the sensitivity of a 50 μL reaction was determined to be 0.50 ng of template DNA at 150 RFU (Fig. 9*a*). The MDL for routine casework (150 RFU) for the smaller volumes decreased consistently with the volume reduction down to 0.13 ng of template DNA for the 5 μL reaction (Fig. 9*b*). This decrease represented approximately a four-fold increase in sensitivity. At 0.13 ng of starting template, all of the experimental volumes except 50 μL provided some useful typing results at 50 RFU.

In the volumes less than 15 μL, there was usually off-scale data at the starting template values greater than 0.25 ng of DNA, and therefore, these volumes would not be recommended for reactions initiated with greater than 0.25 ng of DNA. The 5 μL and 10 μL reactions were classified similarly for both 0.03 ng and 0.06 ng of DNA. At 0.06 ng, the 5 μL and 10 μL reactions still gave useful typing results; however, at 0.03 ng, both yielded only limited typing results. This result suggested that 0.03 ng, or approximately the mass of DNA found in five diploid cells, was the lowest level for reliably detecting DNA profiles using the amplification conditions described in this project. Even though previous results in this proj-

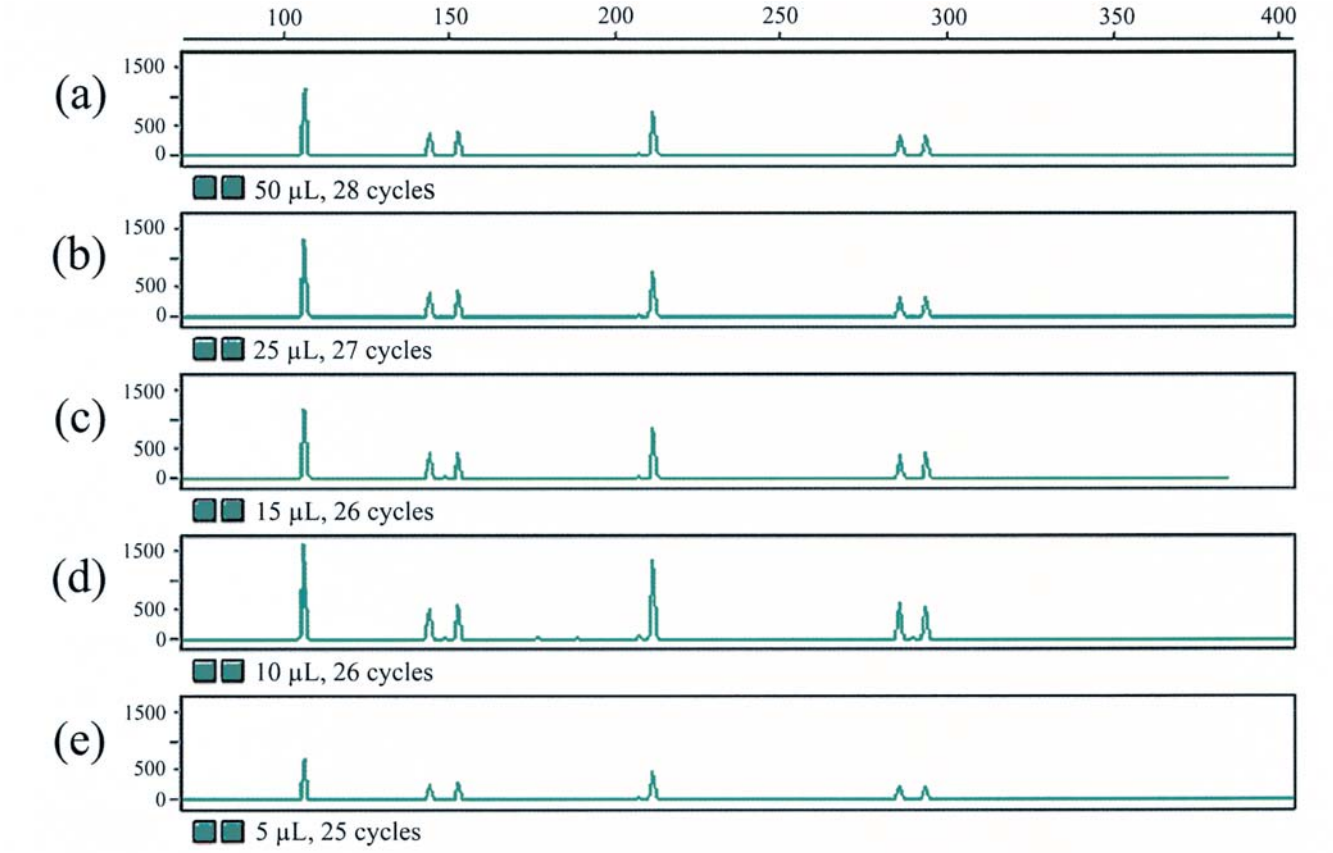

FIG. 8—*Electropherograms from the PCR amplification efficiency experiment. a) a 50 µL reaction amplified for 28 cycles; b) a 25 µL reaction amplified for 27 cycles; c) a 15 µL reaction amplified for 26 cycles; d) a 10 µL reaction amplified for 26 cycles; and a 5 µL reaction amplified for 25 cycles. All tracings are shown on a scale of 1750 RFU. The approximately equal peak heights in the 50, 25, and 15 µL reactions illustrated that as the reaction volume was reduced, the same amplified product concentration could be achieved in fewer cycles. In addition, between the 15 µL and 10 µL reactions, where the cycle number was held constant, PCR product concentration was higher in the 10 µL reaction.*

TABLE 1—*Percentage of peaks correctly labeled at varying reaction volumes and template DNA concentrations; single source sample.*

Reaction Volume (µL)	Category I		Category II		Category III		Category IV	
50	100%	100%	100%	100%	48.8%	12.9%	2.35%	ND
25	100%	100%	100%	100%	97.6%	60.0%	4.12%	ND
15	100%	100%	100%	100%	100%	91.8%	37.1%	ND
10	100%	100%	100%	100%	100%	91.8%	78.2%	32.4%
5	100%	100%	100%	100%	99.4%	99.4%	67.1%	21.8%
DNA Template (ng)	2.00	1.00	0.75	0.50	0.25	0.13	0.06	0.03

Category I—Complete typing results (MDL) >99% of the correct allele peaks identified.

Category II—Useful typing results—50% to 99% of the correct allele peaks identified.

Category III—Limited typing results—10% to 50% of the correct allele peaks identified.

Category IV—Unreliable and generally uninformative results <10% of the correct peaks identified.

ND—No alleles detected.

ect demonstrated a two-fold increase in variance of peak height ratios with the small quantities of template, the finding of limited typing results at these template quantities could potentially offer important evidence in some difficult investigations.

Effect of RV-PCR on Multiple-source Sample Interpretation

An increase in sensitivity can potentially enhance the interpretation of multiple source DNA samples by increasing the detection of peaks from minor donors. In the NLCL validation of the AmpFℓSTR® kits (21) using 50 µL reactions, the limit of reliably detecting the presence of the minor donor was approximately a 10:1 ratio of major:minor donors. At the 10:1 ratio, the minor donor would contribute about 0.2 ng of the 2.0 ng of template DNA. In the sensitivity experiment, useful typing results for the 5 µL and 10 µL reactions were obtained at 0.06 ng, suggesting that minor donors may be detectable at higher ratios with the smaller volume reactions.

The DNA samples used in this experiment were selected to produce mixtures that contained as many of the possible combinations of allele patterns as possible. DNA was extracted from buccal swabs of three individuals, two females and one male. Two experimental, two-source mixtures were set up for each volume and quantity of DNA. In one mixture, a male was the major contributor

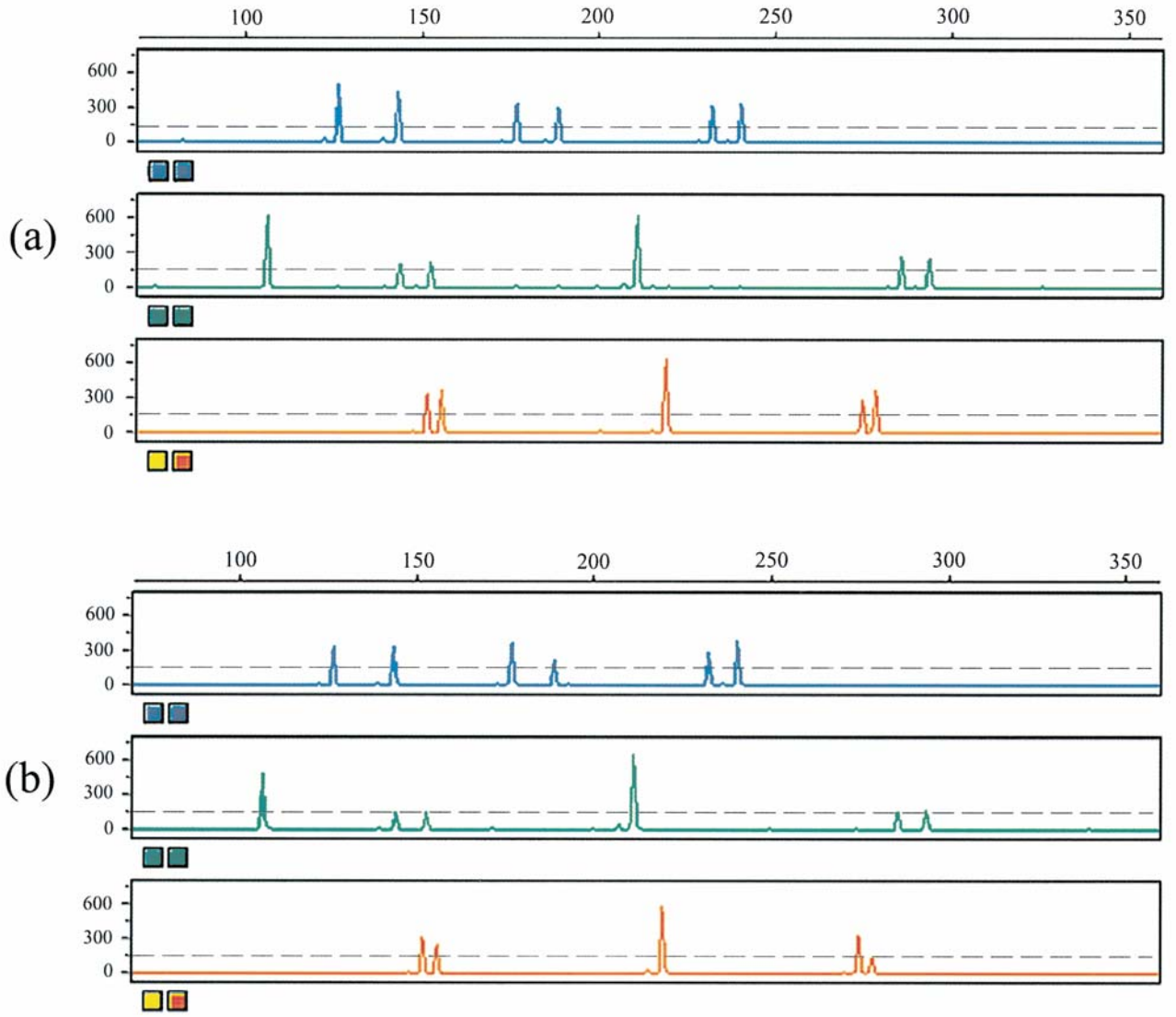

FIG. 9—*Electropherograms of a 50 μL reaction and 5 μL reaction from the sensitivity experiment. a) 0.5 ng of template DNA amplified in a 50 μL reaction (MDL for a 50 μL reaction at 150 RFU); b) 0.13 ng of template DNA amplified in a 5 μL reaction (MDL for a 5 μL reaction at 150 RFU). Both tracings are shown on a scale of 750 RFU. The dashed lines indicate the 150 RFU threshold, a value typically used for forensic casework.*

and a female the minor contributor. In the second mixture, the male to female ratio was reversed, and a different female was used. A total of 2.0 ng of DNA was amplified for each of the experimental volumes. Additional reactions were performed with 1.0 ng of template DNA in a 25 μL reaction and 0.25 ng of template DNA in a 5 μL reaction. The smaller starting DNA quantities at these two volumes represented the maximum amount of DNA that could be used in single source samples without producing off-scale or artifact peaks.

Information about the minor contributor in the mixture was assessed two ways. First, the results were examined to determine if the sample could be reliably interpreted as a multiple source sample. Second, the percent of allele peaks detected out of the total number of possible alleles in the mixture was determined. An allele peak was counted if it was from either the major or minor contributor, identified with GeneScan™ (threshold = 50 RFU), and labeled with Genotyper® using the standard filter values provided in version 4 of the Kazam macro. For example, if an allele from the minor contributor was in the stutter peak position of an allele from the major contributor, and if it were high enough to be greater than the cutoff filter for stutter peaks in the Genotyper® software, then it was counted. The number of peaks detected was combined for the two mixtures. Therefore, for each ratio at a given volume and quantity of template DNA there was a total of 56 allele peaks. These results were classified into four categories and are shown in Table 2.

As with single source samples, more complete typing results were observed at all ratios, with decreasing reaction volume. When 2.0 ng of total DNA was amplified, all of the alleles from both donors were detected at a ratio of 7:1 in all reaction volumes, except 50 μL. Thus, useful typing results could be obtained at a ratio of 7:1 for all volumes less than 50 μL (Fig. 10). The typing results for the smaller volumes had numerous artifact peaks from the major donor when 2.0 ng of template DNA was used. These off-scale data were exacerbated at reaction volumes less than 25 μL and confounded the peak identification, as shown in Fig. 10*b*. However, despite this problem, limited results could be obtained at ratios as high as 31:1 in which the minor donor would contribute only 0.06 ng of the 2.0 ng initial DNA. Often in the higher ratios, the only possible interpretation was that the sample was from more than one source, with few alleles from the minor donor being identified.

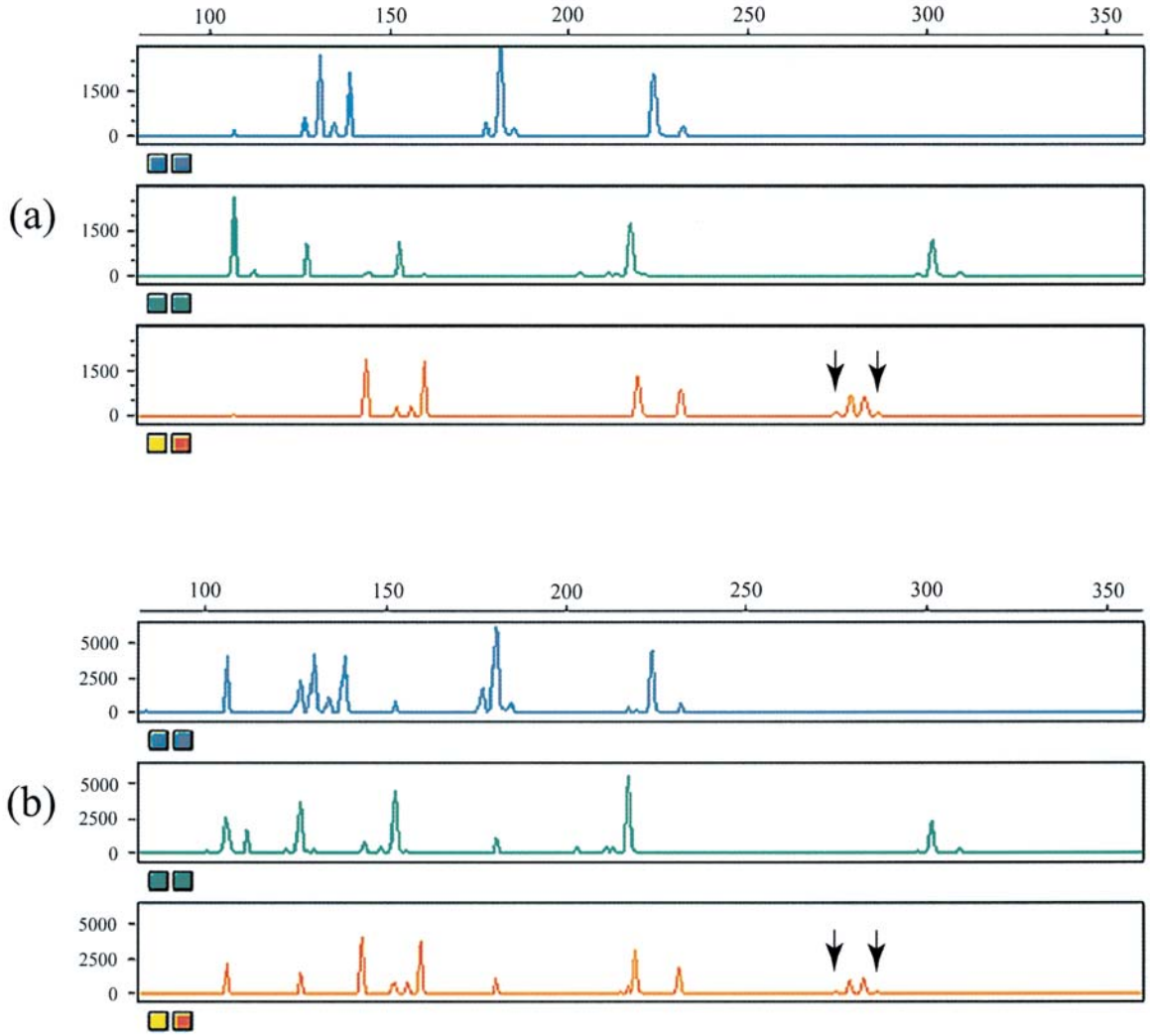

FIG. 10—*Electropherograms of a 2-source mixture with a total of 2.0 ng of input DNA. a) 7:1 ratio of female contributor to male contributor in a 25 μL reaction (scale = 3000 RFU); b) 7:1 ratio of female contributor to male contributor in a 5 μL reaction (scale = 6250). All of the alleles of the minor contributor were detected in both samples. The arrows point out the alleles of the minor contributor in the D7S820 locus.*

TABLE 2—*Percentage of peaks correctly labeled at varying reaction volumes and template DNA concentrations; two-source sample.*

Reaction Volume & DNA Template	Category I	Category II		Category III		Category IV
50 μL, 2.0ng	100%	97%	50%	25%	NM	NM
25 μL, 1.0ng	100%	100%	94%	66%	44%	NM
25 μL, 2.0ng	100%	100%	100%	90%	50%	NM
15 μL, 2.0ng	100%	100%	100%	90%	60%	NM
10 μL, 2.0ng	100%	100%	100%	90%	70%	NM
5 μL, 0.25ng	100%	100%	100%	80%	50%	NM
5 μL, 2.0ng	100%	100%	100%	93%	80%	NM
	1:1	3:1	7:1	15:1	31:1	63:1

Ratio of major:minor contributors

Category I—Complete typing results >99% of the correct allele peaks identified.

Category II—Useful typing results—50% to 99% of the correct allele peaks identified.

Category III—Limited typing results—10% to 50% of the correct allele peaks identified,

Category IV—No mixture detected (NM).

This type of result, shown in Fig. 11, may be useful in cases of sexual assault where the mere presence of the Y peak in amelogenin could be important in supporting a victim's statement. This result would also indicate the possibility of successful analysis of the sample with Y-chromosome STR typing.

The two additional experiments with 5 μL and 25 μL reactions were performed to take advantage of the increase in sensitivity and to determine if more information could be gathered at higher mixture ratios with lower starting amounts of DNA. The 25 μL reaction was initiated with 1.0 ng of template DNA and, 0.25 ng of template DNA was amplified in the 5 μL reaction. By reducing the amount of starting DNA in the 5 μL and 25 μL reactions, the presence of off-scale peaks was eliminated, and at least limited usefulness was obtained at a ratio of 31:1 (Fig. 12). Even though these reduced volume experiments resulted in only about 50% of the alleles being counted at the 31:1 ratio, some information was gained about the identity of the minor contributor. The detection of alleles from the minor contributor was more likely if the alleles were in the shorter length loci, the contributor was homozygous, or the peak overlapped a stutter peak from the major contributor. The results suggested that the amount of useful results obtained from a multiple source sample will be dependent on the genotypes of the

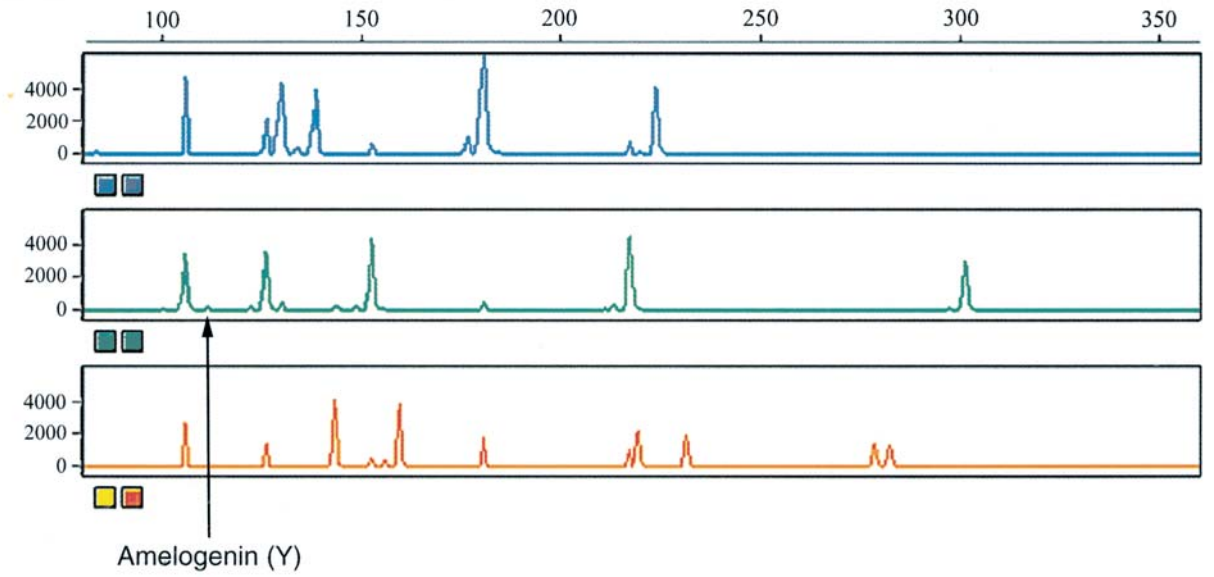

FIG. 11—*Electropherogram of a 2-source mixture with a 31:1 ratio of the female contributor to the male contributor (scale = 6000 RFU). Total input DNA was 2.0 ng amplified in a 5 μL reaction. Note the weak, but detectable Y peak in amelogenin (indicated by the arrow). Even though the major contributor was severely "overamplified," the presence of a minor male contributor is clear.*

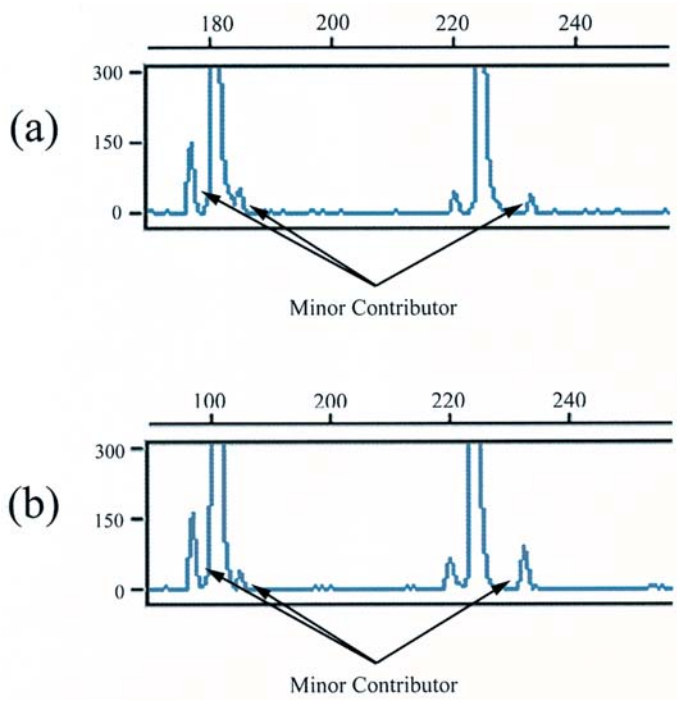

FIG. 12—*Tracings of vWA and FGA loci for a two-source mixture with a 31:1 ratio of the female contributor to the male contributor (scale = 300). a) A total of 1.0 ng of input DNA was amplified in a 25 μL reaction; b) a total of 0.25 ng of input DNA was amplified in a 5 μL reaction. The arrows indicate alleles from the minor contributor that are above 50 RFU.*

donors, but that more genetic data could be obtained by using smaller reaction volumes.

Discussion

Reducing the PCR reaction volume below the recommended 50 μL volume increased the sensitivity of the AmpFℓSTR® Profiler Plus™ reaction. Equivalent peak heights were obtained for all experimental volumes when the starting template was proportionally reduced, indicating that smaller amounts of DNA could be amplified to the same concentration in reduced volume reactions. An increase in peak heights was observed at reduced volumes when the starting DNA amount was held constant, suggesting that the same amount of DNA was amplified to higher concentrations in reduced volumes. Increased sensitivity was also observed when the amplification efficiency was examined. The efficiency results showed that the same peak heights could be achieved in fewer cycles as reaction volume was reduced. These three experiments clearly demonstrated that reducing the reaction volume increased the sensitivity, and this increase was proportional to the volume reduction. With reaction volumes less than 50 μL, useful typing results were obtained with 0.13 ng of template DNA, and limited typing results could be obtained with as little as 0.03 ng. The increased sensitivity obtained by reducing the reaction volume greatly enhances the ability to identify and interpret multiple source samples.

Gill et al. (11) reported an increase in sensitivity by increasing the number of amplification cycles and offered guidelines for interpretation of the results. Their results occasionally demonstrated inexplicable allele peaks that they attributed to extremely low-level contamination by partial DNA profiles. Whitaker et al. (12) suggested using multiple amplifications of the same sample to develop a consensus profile to help identify random contamination events. In our project, there were no unexplained peaks. Samples with extremely small amounts of starting DNA either had no identifiable peaks or only the expected peaks were present. PCR conditions were modified very little, only changing the reaction volume and the method of adding the sample to the PCR tube.

Decreasing the reaction volume to increase sensitivity does have some drawbacks. For example, reducing the reaction volume also limited the volume of sample that can be added to the reaction. This becomes a problem in 5 μL and 10 μL reactions, which have only enough volume for 2 μL and 4 μL of purified DNA, respectively. This problem can be overcome by drying larger volumes of the samples in PCR tubes prior to amplification. Up to 10 μL of sample in TE^{-4} can be dried and amplified successfully in a 5 μL reac-

tion. When the sample volume was greater than 10 μL, the EDTA inhibited the PCR reaction (data not shown). It may be possible to overcome this limitation by titration of larger sample volumes with $MgCl_2$. PCR inhibitors may also be concentrated in the drying process. These issues are being investigated and will be reported at a later date.

Another issue arose in the examination of the peak height ratios of the reduced volume reactions. This issue might not have been apparent if only peak ratio averages were calculated, because the peak ratio averages for the PRD and CD experiments were the same. However, examination of the peak height ratios in the PRD experiment demonstrated a consistent increase in variance as the amount of DNA template was decreased. The twofold increase in SD as the quantity of DNA decreased to 0.13 ng vividly demonstrated the stochastic effect attributed to low copy numbers in DNA amplification. The stochastic effect was a result of very small amounts of template DNA and not the reduced reaction volume, because reducing the volume and holding the template DNA constant at larger quantities did not result in an increase of the peak height ratio variance. The increased variance in the within locus peak ratios was also reported by Gill et al. (11) using a low copy number amplification strategy and by Gill et al. (23) when the peak areas fell below 10 000 area units.

This examination of the benefits and pitfalls of reduced volume PCR reactions has shown that these reactions can be useful in forensic analyses where extremely sensitive methods are required. Reduced volumes offered a better chance of obtaining successful results, especially for samples with low concentrations of DNA. The understanding of the effects of peak height variation becomes critical when interpreting small quantities of DNA in both single and multiple source samples. Individual laboratories must perform a thorough in-house validation to fully understand the effects of reduced volume PCR. In addition, before choosing to use reduced volume reactions, several points must be considered:

1. Decreasing the volume can substantially increase the sensitivity of the PCR reaction and can cause excessive off-scale and artifact peaks if the starting template was not also reduced.
2. Decreasing the amount of starting DNA will cause an increase in the within locus peak height variance, thus affecting interpretation of typing results when very small amounts of DNA are amplified.
3. When limited results are obtained, the possibility of allele dropout and large peak imbalance must be considered in interpreting the results. This precaution also applies to interpretation concerning the minor contributor in mixtures, who may have contributed less than 15% of the total DNA.
4. Smaller volumes of reaction mix will limit the volume of sample that can be added to the reaction unless the sample is dried, and drying samples that were stored in TE^{-4} can adversely affect amplification.

This study suggested a twofold approach to amplifying samples. For most samples, in which greater than 0.25 ng of DNA can be amplified, a 25 μL reaction is appropriate. In single source samples the analyst can expect robust amplification and useful typing results. A 25 μL reaction can increase the useful typing resuls fourfold over the 50 μL reaction and increase information available from multiple source samples. The starting amount of DNA can be safely reduced to 1.0 ng in the 25 μL reaction, thus reducing the amount of DNA consumed by 50%.

Samples that do not demonstrate quantifiable results or that have less than 0.025 ng/μL, can be successfully amplified by drying the template DNA solution directly into the PCR tubes and amplifying in 5 μL of reaction master mix. Therefore, for samples with less than 0.25 ng, the 5 μL reaction would be recommended. The analyst can expect at least limited results with as little as 0.03 ng of DNA template. Since the effects of PCR inhibitors have not been thoroughly studied on reduced volume reactions, the analyst should perform a second, larger volume reaction if no peaks at all were obtained in a 5 μL reaction.

The same amplification strategies just described for single source samples are also applicable for suspected multiple source samples. Understanding the effects of peak height variation becomes critical when interpreting small quantities of DNA in both single and multiple source samples. Integrating the twofold strategy to analyze DNA samples allows the analyst to reduce consumption of typical samples and increase the potential for obtaining useful results from forensic samples with extremely small amounts of DNA.

References

1. Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods In Enzymol 1987;155:335–50.
2. Jeffreys AJ, Wilson V, Neumann R, Keyte J. Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. Nucleic Acids Res 1988;16(23):10953–71.
3. Lins AM, Sprecher CJ, Puers C, Schumm JW. Multiplex sets for the amplification of polymorphic short tandem repeat loci—silver stain and fluorescence detection. BioTechniques 1996;20(5):882–9.
4. Urquhart A, Oldroyd NJ, Kimpton CP, Gill P. Highly discriminating heptaplex short tandem repeat PCR system for forensic identification. BioTechniques 1995;18(1):116–21.
5. Reynolds R, Sensabaugh G, Blake E. Analysis of genetic markers in forensic DNA samples using the polymerase chain reaction. Anal Chem 1991;63:2–15.
6. Gill P, Sparkes R, Pinchin R, Clayton T, Whitaker J, Buckleton J. Interpreting simple STR mixtures using allele peak areas. Forensic Sci Int 1998;91:41–53.
7. Clayton TM, Whitaker JP, Sparkes R, Gill P. Analysis and interpretation of mixed forensic stains using DNA STR profiling. Forensic Sci Int 1998;91:55–70.
8. Sprecher CJ, Puers C, Lins AM, Schumm JW. General approach to analysis of polymorphic short tandem repeat loci. BioTechniques 1996;20 (2):266–76.
9. Edwards A, Civitello A, Hammond HA, Caskey CT. DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. Amer J Hum Genet 1991;49:746–56.
10. Hammond HA, Jin L, Zhong Y, Caskey CT, Chakraborty R. Evaluation of 13 short tandem repeat loci for use in personal identification applications. Amer J Hum Genet 1994;55:175–89.
11. Gill P, Whitaker J, Flaxman C, Brown N, Buckleton J. An investigation of the rigor of interpretation rules for STR's derived from less than 100 pg of DNA. Forensic Sci Int 2000;112:17–40.
12. Whitaker, JP, Cotton, EA, Gill P. A comparison of the characteristics of profiles produced with the AmpFℓSTR® SGM Plus™ Multiplex System for both standard and Low Copy Number (LCN) STR DNA analysis. Forensic Sci Int 2001;123:215–23.
13. Ferreira-Gonzalez A, Yanovich S, Langley MR, Weymouth LA, Wilkinson DS, Garrett CT. Enhanced analytical sensitivity of a quantitative PCR for CMV using a modified nucleic-acid extraction procedure. J Clin Lab Anal 2000;14:32–7.
14. Leclair B, Fourney R. STR DNA typing: increased sensitivity and efficient sample consumption using reduced PCR reaction volumes for database analysis, and casework implications. Proceedings of the 9th International Symposium on Human Identification; 7–10 Oct. 1998; Orlando. Madison (WI): Promega Corporation, 1999, (abstract).
15. Borys S, Bowen KL, Fourney RM. PCR volume reduction study using bloodstained FTA™ collection cards and capillary electrophoresis. Proceedings of the 9th International Symposium on Human Identification; 7–10 Oct. 1998; Orlando. Madison (WI): Promega Corporation, 1999, (abstract).

16. Comey CT, Budowle B. Validation studies on the analysis of the HLA DQα locus using the polymerase chain reaction. J Forensic Sci 1991;36 (6):1633–48.
17. Sambrook J, Maniatis T, Fritsch EF. Molecular cloning (a laboratory manual). New York: Cold Spring Harbor Laboratory, 1982.
18. Hopwood A, Oldroyd N, Fellows S, Ward R, Owen SA, Sullivan K. Rapid quantification of DNA samples extracted from buccal scrapes prior to DNA profiling. BioTechniques 1997;23(1):18–20.
19. PE Applied Biosystems. AmpFℓSTR® Profiler Plus™ PCR Amplification Kit User's Manual. The Perkin Elmer Corporation, 1997.
20. Wojtkiewicz, PW. Pituitary gene expression in mice with altered growth hormone levels: quantitative RT-PCR of transcription factors *Pit-1, Zn-16, TEF*, and *Prop-1* [dissertation]. New Orleans (LA): Tulane University, 1999.
21. Gaines ML, Wojtkiewicz P. Internal validation of the AmpFℓSTR Profiler Plus™ and COfiler™ PCR amplification kits. Proceedings of the American Academy of Forensic Sciences; 15–20 Feb. 1999; Orlando. Colorado Springs: American Academy of Forensic Sciences, 1999, (abstract).
22. Walsh PS, Erlich HA, Higuchi R. Preferential PCR amplification of alleles: mechanisms and solutions. PCR Meth and Appl 1992;1:241–50.
23. Gill P, Sparkes R, Fereday L, Werrett DJ. Report of the European Network of Forensic Science Institute (ENSFI): formulation and testing of principles to evaluate STR multiplexes. Forensic Sci Int 2000;108:1–29.

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