J Forensic Sci, September 2003, Vol. 48, No. 5
Paper ID JFS2002182_485
Available online at: www.astm.org

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STR DNA Typing: Increased Sensitivity and Efficient Sample Consumption Using Reduced PCR Reaction Volumes

ABSTRACT: Improvements in detection limits/sensitivity and lower sample consumption are potential benefits of reducing PCR reaction volumes used in forensic DNA typing of crime scene samples. This premise was studied first with experimental mixtures and a nine-loci megaplex, which demonstrated stochiometric amplification and accurate detection. Next, adjudicated casework samples were subjected to amplification under 15 different template DNA to PCR reaction volume ratios. Reduction of PCR reaction volume and DNA down to 10 μL and 0.500 ng, respectively, produced identical profiles with the same signal intensity and heterozygous allele peak height ratio (HR). Reduction to 5 μL and 0.063 ng yielded HR values that were slightly affected in one to three STR loci. PCR reaction volume reduction can enhance detection and sensitivity while reducing the consumption of irreplaceable crime scene samples.

KEYWORDS: forensic science, DNA typing, polymerase chain reaction, microfluidics, casework, D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820, TH01, TPOX, CSF1PO, D16S539

Clinical diagnostic laboratories usually perform analyses on biological samples that have been collected and stored in ideal conditions, making sample quality and quantity rarely an issue. Those ideal conditions are usually not met in DNA typing analysis of forensic biological evidence, as there is no control over the amount of biological material left at a crime scene or its level of degradation or contamination due to exposure to various environmental insults. Forensic biological samples may present challenges for DNA typing analysis, even with utmost care throughout crime scene evidence recovery and storage. In addition, these samples may frequently consist of mixtures of bodily substances originating from more than one individual. Therefore, beyond having to provide very high discrimination potential (DP) values, one major requirement imposed on forensic DNA typing technologies is the development of interpretable genotypes from limited, compromised samples.

In recent years, several technological improvements have contributed to making forensic DNA typing more informative and robust (1–9). The advent of PCR-based DNA typing technologies, like that used with STR loci, allows for genotypes to be derived from as little as 0.5 to 1 ng of template DNA, a 100-fold reduction in sample consumption when compared to RFLP-based assays. As amplification targets from commercially available STR DNA typing kits are below 350 bases in length, STR-based DNA typing is also more efficient at generating genotypes from degraded samples. Beyond the benefits brought about by these improvements, casework situations still arise where the available template DNA is less in quantity than the optimal amount required for a single analytical attempt. In such situations, consumption of the entire sample in a single analysis is logistically undesirable because it precludes further analysis. This limitation is further compounded when the sample involves a mixture of bodily substances originating from more than one individual, as a minor profile can prove difficult to detect if the amplicon yield is poor.

Analytical improvements that can bring about a reduction in evidential sample consumption are therefore constantly sought in forensic biology. One way to achieve this involves the concomitant reduction of both the amount of template DNA and PCR reaction volume so as to maintain the same DNA concentration and yield per unit volume (10–12). However, as STR DNA typing calls for the simultaneous amplification of bi-allelic targets, a minimum size genomic target pool is required to ensure stochiometric amplification of all alleles at any given locus. The reduction of PCR reaction volume can also impact reaction kinetics in other ways. For instance, PCR reactions without oil overlays are exposed to some solute evaporation into the ambient air above the reaction mixture, as well as potential water vapor loss if PCR vessel seals are slightly compromised. Depending upon the size of the reaction volume, PCR tube design, and ratio of solute loss/total solute, some conditions might lead to significant evaporation with resulting concentration of reactants and lower stringency for primer binding. This, in turn, can lead to the appearance of amplification artifacts such as non-target specific amplicons or non-stochiometric amplification at heterozygous loci. Whether reduced PCR reaction volumes can

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Received 20 May 2002; and in revised form 24 Nov. 2002; accepted 30 March 2003; published 4 Aug. 2003.

satisfy the competing needs of maintaining adequate template DNA concentration and minimum-size genomic target pool while consuming the least amount of template needs to be carefully determined.

The purpose of this study was to determine the extent to which PCR reaction volumes can be reduced without compromising electropherogram quality and the feasibility of an overall microfluidics approach for casework samples. The impact of volume reduction was initially assessed on a collection of pristine single source samples used in prepared mixtures. In addition, forensically relevant and challenged samples from adjudicated cases were also analyzed. All samples were assessed under various PCR reaction volume/amount of input template DNA ratios and subjected to amplification with the AmpFℓSTR©Profiler Plus™ kit (13,14). The profiles were evaluated for signal strength and quality consistency. Conditions permitting reliable use of microfluidics were identified.

Materials and Methods

Biological Samples

Liquid blood for the pristine sample study was drawn from volunteers. Casework samples ($n = 37$) were assembled from adjudicated cases and selected for having presented some difficulty with results interpretation in prior RFLP or PCR-based DNA typing casework analysis (see Table 1). Reported difficulties arose from low amplicon yield, the presence of a weak partial minor profile, suspicion of the presence of a PCR inhibitor, or locus/allele dropout. Bloodstains from a male individual displaying heterozygosity at all tested loci in this study were used as extraction controls. Extracted DNA from the female cell line GM9947A (15) was used as the amplification control. All samples were extracted according to standard operational protocols, one-step organic extraction followed by Microcon-100 concentration (16) and quantitated using the ACES 2.0 chemiluminescence kit (Whatman, Clifton NJ).

PCR Amplification, Pristine Sample Mixtures

Three DNA stocks, designated BIS A, BIS C, and BIS E, were used for the mixture study. Three different mixture ratios, 50:30:20, 65:25:15, and 70:20:10, were prepared. Each mixture ratio was prepared in triplicate through rotation of the DNA stocks as each ratio component (i.e., A-C-E, C-A-E, and C-E-A for each ratio set). A total genomic template mass of 2.4 ng was used for the amplification reactions. All samples in this study were amplified with the AmpFℓSTR©Profiler Plus™ kit (Applied Biosystems, Foster City, CA) in 50 and 25-μL reaction volumes and strip-capped thin-walled 0.2-mL Perkin Elmer MicroAmp™ reaction tubes. The cycling conditions were the following: 95°C—11 min, once; 94°C—60 s, 59°C—90 s, 72°C—90 s, for 28 cycles; 60°C—30 min, once; 22°C—overnight (10). All amplifications were carried out in Perkin-Elmer Gene Amp™ 9600 DNA Thermal Cyclers.

PCR Amplification, Casework Samples

A master mix preparation grid was built to effectively isolate the PCR reaction volume variable. The use of a two-fold reduction series for both reaction volume and input DNA quantity allowed for the preparation of one master mix stock for any given parallel reduction series (diagonals through Table 2). As an example, for Master Mix C, 75 μL of master mix stock containing 1.875 ng of DNA would be prepared, and volumes of 40, 20, 10, and 5 μL would be dispensed in individual tubes for amplification conditions Nos. 8, 9, 10, and 11, respectively, thereby maintaining rigorously identical DNA concentrations throughout a given series. In order to obtain a final volume of 5 μL under the two-fold reduction scheme, a starting volume of a 40-μL instead of the 50-μL volume recommended by the kit manufacturer (13) was used. To document that an increase in annealing segment duration from 60 to 90 s had no adverse effect on amplification beyond an increase in yield, amplification reaction grid layouts for the first four samples were prepared in duplicate to allow for both annealing segment durations to be tested. Different restricted sets of amplification conditions were used to suit sample availability. Four samples were subjected to amplification conditions Nos. 1 to 15 for both annealing segment durations, 19 samples subjected to conditions Nos. 1 to 3 as well as Nos. 16 and 18, and 14 samples subjected to only condition No. 16 (see Table 2). Amplifications were carried out under the same cycling conditions as for the pristine sample mixture study except that two annealing segment conditions were tested.

Electrophoresis

Profiles were resolved on ABD 377 DNA Sequencers. A 1.5-μL aliquot of each amplified PCR reaction was diluted in 4.5 μL of loading buffer (2X TBE, 20mM EDTA, 20 mg/mL blue dextran, 0.5 μL GS 500 ROX (ABD), 9M urea), heat-denatured at 95°C for 2 min, and snap-cooled on ice water. With allelic ladders, a volume of 0.7 μL of each of the three ladders provided with the AmpFℓSTR©Profiler Plus™ kit were mixed together with the above-mentioned volumes of loading buffer. For both amplicons and allelic ladders, a volume of 1.5 μL from these mixes was then loaded onto 0.2-mm-thick, 4% 19:1 acrylamide (Bio-Rad): bisacrylamide (BRL) 6M urea gels. All gels were cast on 36-cm WTR plates with square-tooth combs; the gels had been polymerized by making the solution 0.05% for both TEMED and APS, cured for 2 h, and pre-run for 30 min at 1000 V. Electrophoresis was carried out at 3000 V for 2.5 h at 51°C in 1X TBE. On each gel, two lanes were reserved for allelic ladder samples. Analysis of gel file data was performed with the GeneScan Analysis v. 3.1 software.

Results

Pristine Sample Mixtures

A series of three-profile mixtures assembled with pristine single source DNA samples under three component ratios, 50:30:20, 65:25:15, and 70:20:10, were prepared. These mixture ratios were selected to gauge the capabilities of the selected STR multiplex system and detection instrument to correctly reflect the contributions of two minor components of nearly identical concentrations. Three samples were prepared for every mixture ratio by rotating the DNA stocks (BIS A, BIS C, and BIS E) as each ratio component. Figures 1 and 2 show examples of 50:30:20 and 70:20:10 mixture ratios, respectively, under both 50 and 25-μL reaction volumes (shown as overlaid blue and green traces, respectively, in both figures) for a fixed template input of 2.4 ng of total DNA. Figures 1 and 2 demonstrate that, as expected, signal strength increased proportionally to the volume reduction level for a fixed amount of starting template DNA. The theoretical peak height ratio values were calculated taking into account the input ratio, homozygosity or heterozygosity at a locus, sharing of alleles between contributors, and stutter. For example, the first peak of D3S1358 in Panel A of Fig. 1 has only one contributor, BIS A, which under this mixture ratio theoretically represents 50% of the sample. BIS A being heterozygous at D3S1358, the peak height of Peak 1 should represent 25% of the total cumulative peak height. A similar calculation is carried out for the three

TABLE 1—*Casework samples examined and outcome of a reduced PCR volume on amplification. Samples 1 to 4 were subjected to Amplification Conditions 1 to 15 for both annealing segment durations. Samples 5 to 23 were subjected to Conditions 1 to 3, as well as 16 and 18. Samples 24 to 37 were subjected to Condition 16 only. The number of loci affected by locus dropout is indicated in brackets, when applicable. Gradation refers to a negative slope profile with larger-sized amplicons displaying lower intensity. Degradation refers to an overall weak profile for the amount of template amplified.*

Sample #	*Description*	*Outcome of PCR reduced volume approach*
1	bloodstain (telephone jack);low yield	very weak signal; all loci detected; HR imbalance at all volumes
2	scalp hair, 20 roots; degradation	HR imbalances (2 loci) at low template input; good profile under condition #15
3	blood on bath towel; mixture (2%)	mixture detected (see Figure 5); HR imbalance at 3 loci
4	vaginal aspirate; locus drop out (4); mixture (6%); degradation	all loci accounted for major profile; mixture detected (see Figure 6)
5	vaginal swab; degradation; mixture	no degradation; mixture detected; balanced profile under condition #18
6	bloodstain; gradation	gradation (see Figure 7)
7	semen stain; locus drop-out (3);	all loci accounted for; D3S1358 HR imbalance at 5μL; mixture detected
8	skin; gradation	light gradation, more pronounced under condition #16
9	bloodstain on cloth; gradation	light gradation
10	vaginal swab; gradation	gradation; weak profile at 5μL; profle nearly wiped-out under condition #18
11	bloodstain on mattress; gradation	light gradation
12	blood; gradation; no D7S820	no gradation; all 9 loci accounted for
13	bloodstain; gradation	no gradation
14	anal swab; gradation	HR imbalances at D3S1358, vWA & D21S11 at 5μL; no gradation
15	vaginal aspirate & pellet; degradation	HR imbalance at FGA
16	vaginal aspirate & pellet; mixture	mixture detected; ratio of contributors altered at 5μL; strong inhibition under condition #16; profile wiped-out under condition #18
17	bloodstain; gradation	gradation
18	bloodstain on blue pants; gradation;	less gradation at 5μL; mixture better detected at 5μL; strong inhibition under condition #16; profile wiped-out under condition #18
19	bloodstain; gradation	no gradation; profile nearly wiped-out under condition #18
20	pubic hair	gradation; better detection at 2ng in 5μL (condition # 18)
21	bloodstain on steering wheel;	mixture detected; strong inhibition under condition #16; profile wiped-out under condition #18
22	vaginal aspirate; mixture; degradation	gradation; mixture detected
23	semen stain; F2 fraction; mixture	mixture detected; HR imbalances at FGA and D18S51at 5μL; perfect profile under all conditions
24	bloodstain; gradation	strong profile; incomplete extra A addition, gradation
25	bloodstain on black pants;	gradation; mixture detected; no HR imbalances
26	blood; gradation	gradation; no HR imbalances
27	bloodstain on pillowcase; gradation	no signal
28	nail clippings; gradation	gradation; no HR imbalances
29	semen stain on blue jeans; gradation;	gradation; locus drop-out (3); mixture detected
30	vaginal swab (semen found);	gradation; locus drop-out (7)
31	bloodstain; gradation	no signal
32	bloodstain on blanket; gradation	no signal
33	blood on pajamas; gradation	gradation; mixture detected
34	blood on pillowcase; gradation	gradation; no imbalances
35	vaginal aspirate; mixture	no mixture detected (absence of amelogenin peak at 112 bases)
36	vaginal aspirate; gradation; mixture	no signal
37	bloodstain; low yield	no signal

TABLE 2—*PCR master mix preparation grid. Numbers (marked with an asterisk) refer to the amplification condition designation. Conditions throughout diagonals refer to concomitant reduction of both PCR reaction volume and DNA template, therefore maintaining constant DNA concentration through diagonals. Conditions have been numbered across diagonals, as the reduction of both input DNA and PCR reaction volumes is the main focus of this study. Master mix Stocks A through D were prepared for dispensing through the diagonals of this table. Master mixes for Conditions 16 and 18 (marked with an ¶) were prepared and dispensed individually. The number of genomic copies was calculated as per the following: number of base pairs in the human haploid genome: 3.2×10^9 (26); diploid genome: 6.4×10^9; average molecular weight of nucleotide base pairs: 660 g (1 mole); number of molecules per mole: 6.02×10^{23}; therefore, weight of a single diploid genome = $((6.4 \times 10^9$ bp) $\times$ 660 g)/$6.02 \times 10^{23} = 7 \times 10^{-12}$ g, or 7 pg.*

Input DNA (ng)	Approximate # of Genomic Diploid Copies	PCR Reaction Volume (μL) 40	20	10	5
2	320	4* (B)	1 (A)	16 ¶	18 ¶
1	160	8 (C)	5 (B)	2 (A)	
0.5	80	12 (D)	9 (C)	6 (B)	3 (A)
0.25	40		13 (D)	10 (C)	7 (B)
0.125	20			14 (D)	11 (C)
0.063	10				15 (D)

other peaks, yielding theoretical values of 40, 20, and 15%, respectively, of the cumulative peak height. To correct for the approximate contribution of stutter peaks, each peak's theoretical value for this locus was increased by 7% (average value for D3S1358 in our validation studies (17); locus-specific % stutter values were used for each locus) of the value of the peak in position $n + 4$ relative to the calculated peak, then normalized. The observed peak height ratio values displayed in Figs. 1 and 2 are those of the 25-μL reaction volume and were within a few percentage points of the theoretical values for nearly all tested loci. The same trend was observed for the 50-μL reaction volumes (tabulated data not shown) as well as with the remaining seven samples of this nine-sample experiment (three ratios $\times$ three samples). Both 50:30:20 and 70:20:10 ratios produced observed ratios close to expected values. The average absolute variation for all peaks was 3.7% for the 50:30:20 ratio and 3.8% for the 70:20:10 ratio, which supported the qualitative assessment. Overall, this experiment showed the capability of the tested megaplex and the ABD 377 instrument to reflect the accurate quantitation of the DNA stocks and the true contribution of each component of an electrophoretic profile, even in the presence of a challenging three-profile mixture.

Casework Samples

Many casework samples do not lend themselves to the numerous replicate amplifications required by most experimental protocols to average out potential pipetting imprecisions. To alleviate the need to consume precious sample in experimental replicates, we opted in our experimental design for two-fold reduction series of the amount of template DNA and PCR volume reductions that allowed for the preparation of few PCR reaction mixes containing DNA template (see Table 2). The preparation of relatively large-volume PCR reaction mixes benefited from the increased precision afforded by larger-volume pipetting.

As nearly all PCR reaction mixes across each diagonal from A through D of Table 2 were dispensed from single master mix stocks, the concentration of all reactants including template DNA were identical, which led to the effective isolation of the PCR reaction volume variable. It also meant that across a diagonal, the amplicon yield was expected to be the same in the absence of significant water vapor loss or stochastic effects. Panels A through D of Figs. 3 and 4 show the profiles obtained from control DNAs, GM9947A (Fig. 3), and the extraction control (Fig. 4), where the DNA template concentration is held constant while reducing the reaction volumes from 40 μL down to 5 μL. The amplicon yields are essentially the same down to 5 μL. The amplicon yields of the PCR control in Fig. 3 were nearly doubled for all conditions with the 90-s annealing segment compared to its 60-s counterpart as expected from previous studies (10), whereas that of the extraction control (a bloodstain) in Fig. 4 showed only marginal improvement. These results for the 90-s annealing experiment is considered a true increase in yield as master mixes for the two controls (extraction and amplification) were prepared in double quantity, aliquoted in PCR tubes, laid out the same way in the PCR rack, and cycled in the same cycler after the cycling of the rack containing the 60-s annealing series was completed. Similarly to the diagonals of Table 2, the two annealing segment sample sets were identical in every respect. Marginal increases in yield were observed sporadically for samples processed with the 90-s annealing segment throughout the four casework sample set tested over the first 15 conditions of Table 2.

Heterozygous peak height ratios appear balanced down to the 10-μL reaction volume level for the control DNAs, GM9947A (Fig. 3) and extraction control (Fig. 4). Some minor heterozygous peak height ratio imbalances were noticeable with 0.250 ng in 5-μL reaction volumes (equivalent to approximately 40 genomic diploid copies (see legend of Table 2 for method of calculation, see Panels D in both figures). The extent of the imbalance and number of affected STR loci increased as the DNA template concentration was reduced in two-fold increments in 5-μL reaction volumes (compare Panels G to Panels D through F in Figs. 3 and 4). The imbalance was more pronounced in Fig. 4 (in Panel F, 25 μL, seven out of nine heterozygous loci with differential greater than ±30%; see same panel in Fig. 3, no heterozygous locus with differential greater than ±30%) at the 10 genomic diploid copies level, which imbalance may be the result of a combination of stochastic effect and, as the sample is a bloodstain, PCR inhibition. However, except for some allelic imbalances, overall profile quality was still good, and all peaks were detected using Condition 15, where approximately ten genomic copies of DNA are amplified in 5-μL reaction volumes.

Several mixed samples with minor profile components described as being at the limit of analytical detection in the original casework were included in this study to evaluate the impact of volume reduction on the detection of minor profiles. For each tested sample, the qualitative outcome of amplification attempts under reduced volume conditions is listed in Table 1, next to the reason why the sample was selected for the study. The majority of samples amplified under reduced amount of template/reduced PCR reaction volume still produced nearly identical electropherograms across conditions from a given diagonal of Table 2, demonstrating the validity and value of a microfluidics approach for samples with low amounts of DNA. Some samples that initially yielded very poor signal did not do better under reduced volumes, suggestive of further DNA degradation during long-term storage or of the presence of a PCR inhibitor that increased in concentration as the reaction volume was reduced.

Three samples were selected as presenting electropherograms representative of the casework samples tested and are displayed in

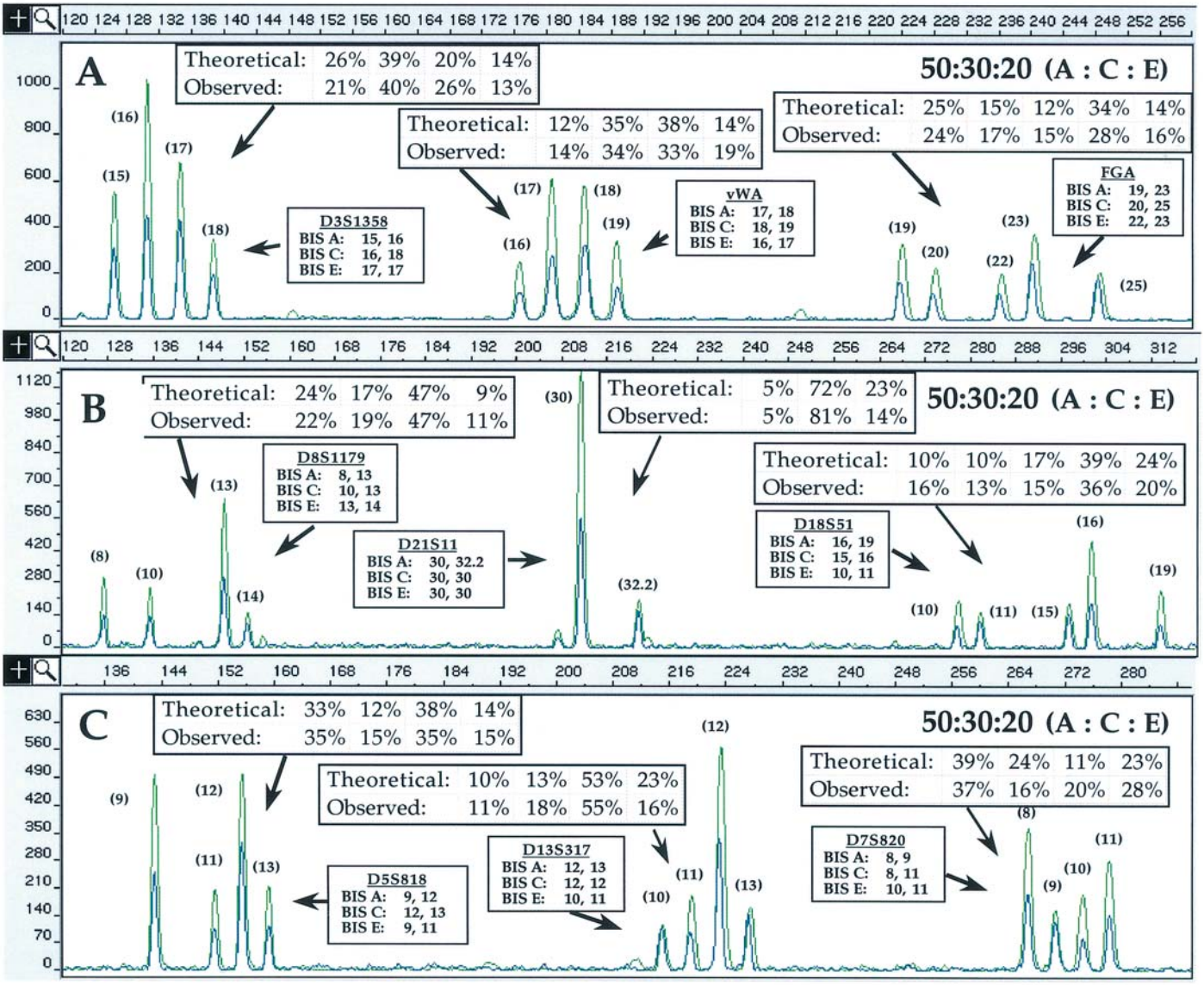

FIG. 1—*Effects of a reduction in the PCR reaction volume on peak height ratios of a three-profile mixture (50:30:20). Panels A, B, and C show the overlaid profiles obtained after the amplification of a total genomic template mass of 2.4 ng of the mixture in both 50-μL (blue-colored trace) and 25-μL (green-colored trace) PCR reaction volumes. The contributing genotypes appear boxed in the same panels. Allele designations appear as bracketed labels next to each peak. Theoretical peak height ratios were calculated as described in the text. The observed peak height ratios displayed are those from the 25-μL reaction volume profile.*

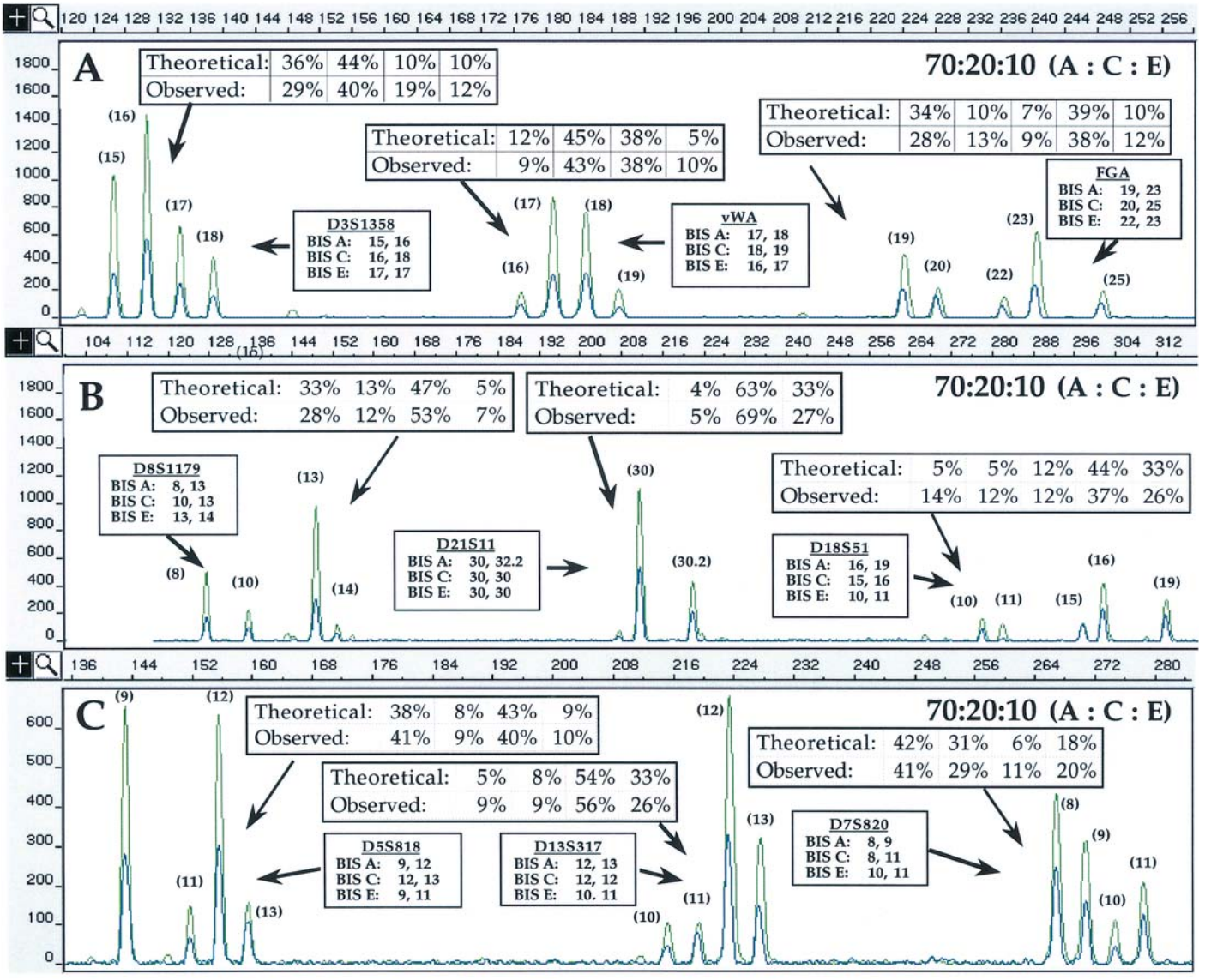

FIG. 2—*Effects of a reduction in the PCR reaction volume on peak height ratios of a three-profile mixture (70:20:10). Panels A, B, and C show the overlaid profiles obtained after the amplification of a total genomic template mass of 2.4 ng of the mixture in both 50-μL (blue-colored trace) and 25-μL (green-colored trace) PCR reaction volumes. The contributing genotypes appear boxed in the same panels. Allele designations appear as bracketed labels next to each peak. Theoretical peak height ratios were calculated as described in the text. The observed peak height ratios displayed are those from the 25-μL reaction volume profile.*

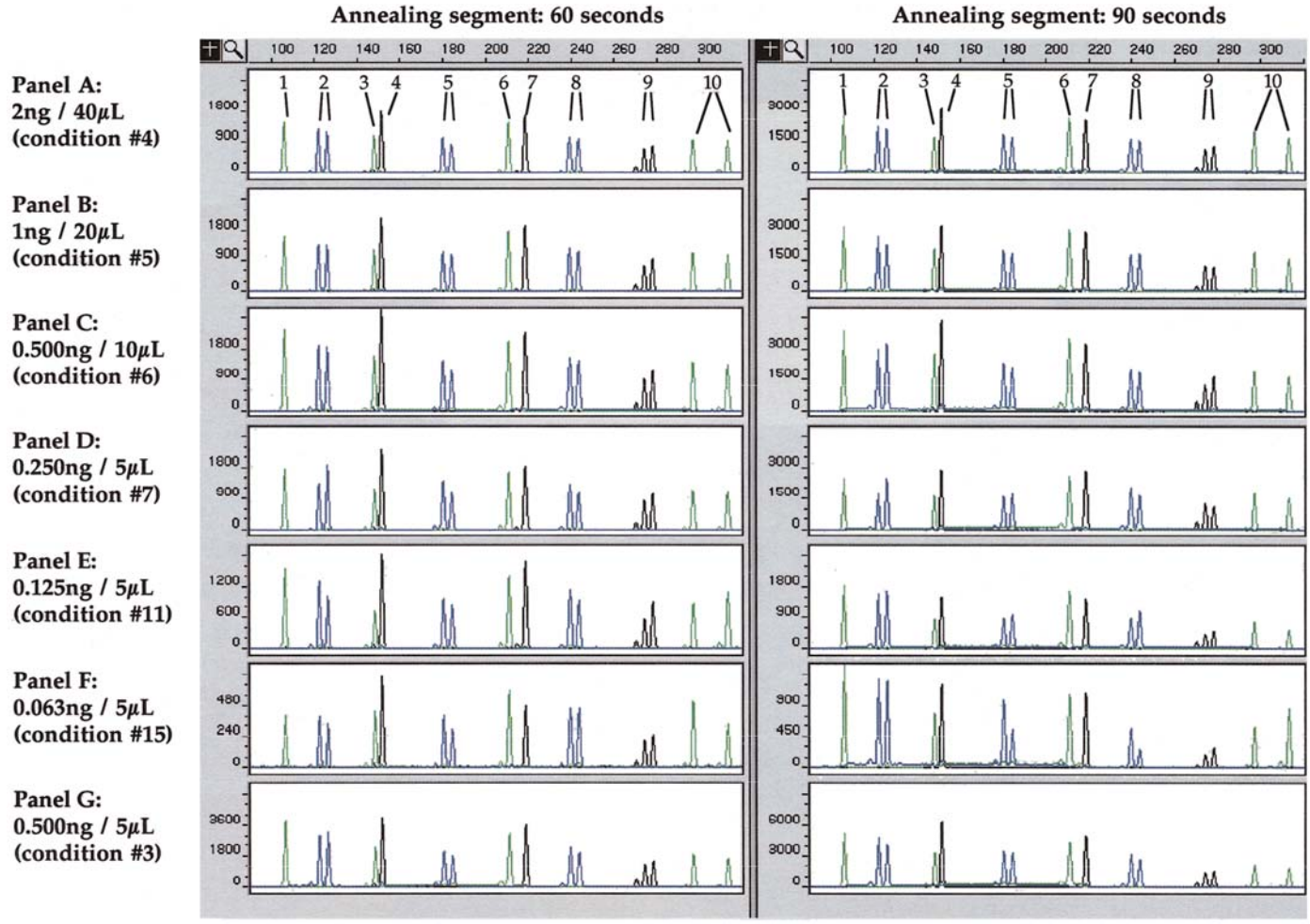

FIG. 3—*Effects of a reduction of PCR reaction volume and DNA template concentration on amplification of the control cell line GM 9947A. Panels A through F display profiles obtained with reaction Conditions 4, 5, 6, 7, 11, and 15, respectively (see Table 2). The loci are labeled as follows: 1—Amelogenin; 2—D3S1358; 3—D8S1179; 4—D5D818; 5—vWA; 6—D21S11; 7—D13S317; 8—FGA; 9—D7S820; 10—D18S51. DNA template concentration (Master Mix B) is kept constant in Panels A through D (Y-axis kept constant), whereas PCR reaction volume is kept constant in Panels D through G (Y-axis showing a two-fold incremental reduction). Panel D is common to both series and the profile acts as a control.*

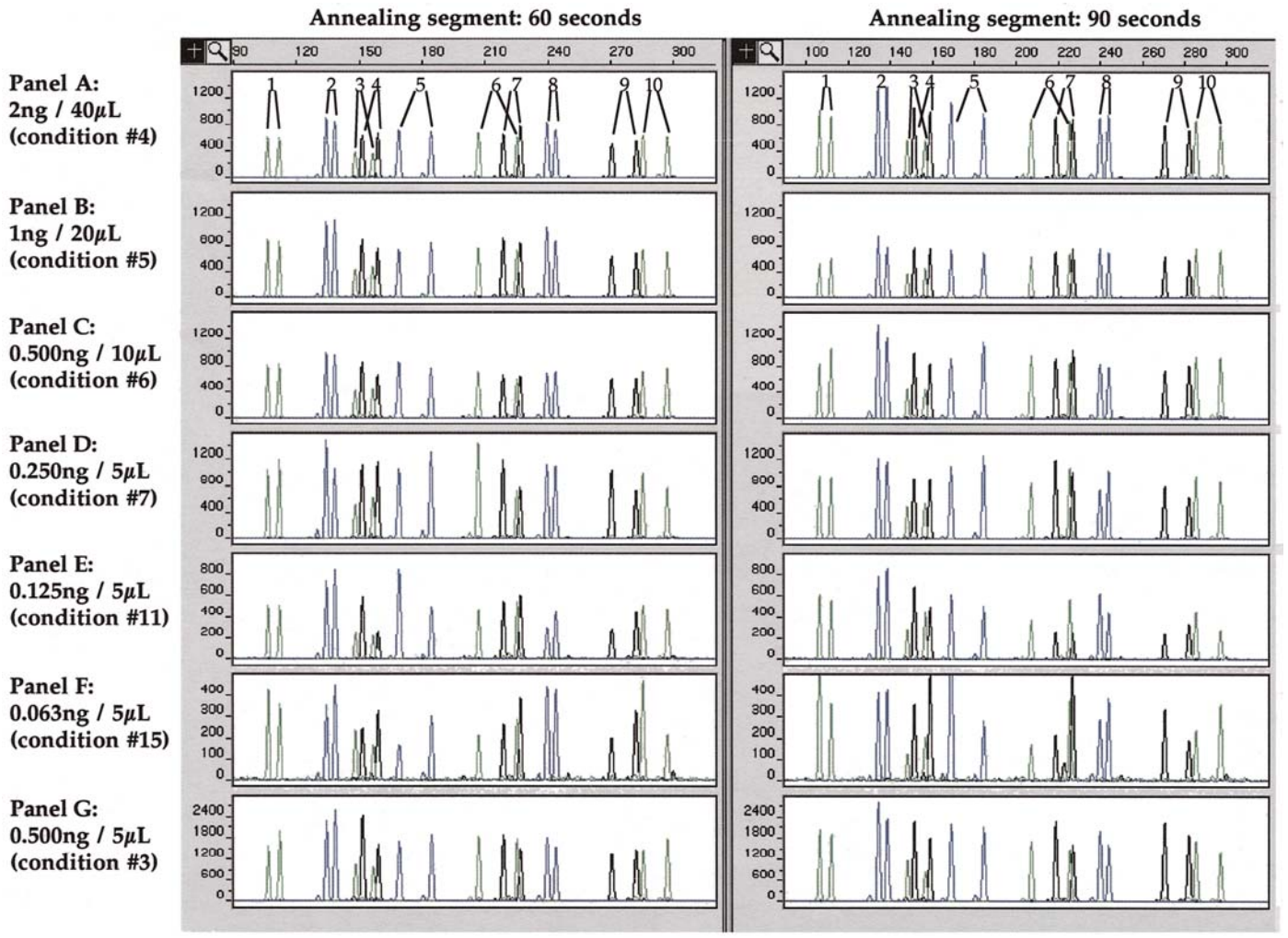

FIG. 4—*Effects of a reduction of PCR reaction volume and DNA template concentration on amplification of the extraction control (a known bloodstain). Panels A through F display profiles obtained with reaction Conditions 4, 5, 6, 7, 11, and 15, respectively (see Table 2). The loci are labeled as follows: 1—Amelogenin; 2—D3S1358; 3—D8S1179; 4—D5D818; 5—vWA; 6—D21S11; 7—D13S317; 8—FGA; 9—D7S820; 10—D18S51. DNA template concentration (Master Mix B) is kept constant in Panels A through D (Y-axis kept constant), whereas PCR reaction volume is kept constant in Panels D through G (Y-axis showing a two-fold incremental reduction). Panel D is common to both series and act as a control.*

Figs. 5 to 7. A blood sample recovered from a bath towel presenting a very minor profile (Sample 3 in Table 1) accounting for approximately 2% of the total mixture is shown in Fig. 5. In Panels A through D, amplification was carried out at an optimal and constant DNA concentration across the volume range to identify reaction volumes at which stochastic effects would be detected. Seven nonshared peaks indicated in the displays were detected down to the 5-µL level, approximately 40 genomic diploid targets being amplified under this last condition with only one or two targets originating from the contributor of the minor profile. Only very minor fluctuations in the peak heights of the minor component were observed at the 10 and 5-µL levels. In Panels E through H, amplification was carried out at a constant total amount of template DNA of 0.5 ng across the volume range to identify the reaction volumes at which minor profile dropout would be observed. None of the seven nonshared peaks were detected at the 40-µL level, but all were detected at 5 µL. Although the detected minor profile was at the detection limit of 40 relative fluorescence units (RFU) (18), for the purpose of clearing a preset detection threshold value, more amplicon could have been loaded on a subsequent electrophoresis run.

Another mixed sample, a vaginal aspirate with reported locus dropouts in green and yellow-labeled loci, is featured in Fig. 6. Although all samples were quantitated before amplification, the major profile in this case is significantly weaker than expected with very weak large-sized amplicons, suggesting significant DNA template degradation. Although all reactions in the three displayed conditions were dispensed from one single DNA-reagent mixture, significant variations in the total yield of minor components were obvious across the size range, as shown in Panels D through F, suggesting stochastic effects. A noteworthy observation is that the 10-µL volume profile proved more informative at several STR loci when compared with the 20-µL condition.

To assess whether increasing the concentration of DNA template during amplification is an efficient way of recovering dropped-out large-sized amplicons in cases where degradation is suspected, we examined the effect of increasing the DNA concentration by four- and eight-fold (Conditions 16 and 18: 2 ng of DNA in 10 and 5 µL, respectively) beyond the manufacturer recommended ratio (2 ng of DNA in 40 µL) for low-availability samples. A single-source bloodstain showing gradation in signal is featured in Fig. 7. Panels

A through C show essentially identical profiles through the displayed volume range. However, increasing the template DNA concentration from 2 ng in 20 μL to 2 ng in 5 μL did increase the yield of shorter amplicons but decreased the yield of their larger-sized counterparts. The 0.5 ng in 5-μL condition (Panel C) produced a better-balanced profile compared to the 2 ng in 5-μL condition (Panel F). This outcome suggested that PCR inhibition was at work, which was a reasonable possibility since the sample was a bloodstain. In contrast, Panels G and H show the extraction control sample used in this experiment under the 2 ng in 20-μL and 2 ng in 5-μL conditions, which provide a reference as to how a non-inhibited sample (still a bloodstain) would amplify under similar conditions.

The remaining samples in this collection of casework samples were subjected to a restricted set of conditions appropriate to sample availability. Table 1 lists the outcome of attempts at securing a profile under the range of conditions these samples were subjected to. Fewer dropped-out loci and better mixture detection are the most evident improvements, as expected under PCR-reduced volume conditions. However, it is also obvious that Conditions 16 and 18, which call for higher than optimal DNA concentrations to be used in the PCR reaction, can potentially generate a sloped profile with low signal intensity longer amplicons or generate little or no profile if a PCR inhibitor is present and in higher concentration in these conditions. This clearly demonstrates that Conditions 16 and 18 are tailored for situations where degraded DNA is encountered.

Discussion

A significant challenge in the analysis of forensic casework samples evolves around the correct interpretation of the DNA typing results from mixed forensic stains (19–23). The assignment of alleles to specific contributors relies heavily on ratios of peak height/area within each discrete locus of a profile. Imbalances in peak height ratios triggered by non-optimal PCR conditions for template concentration and quality can complicate the interpreta-

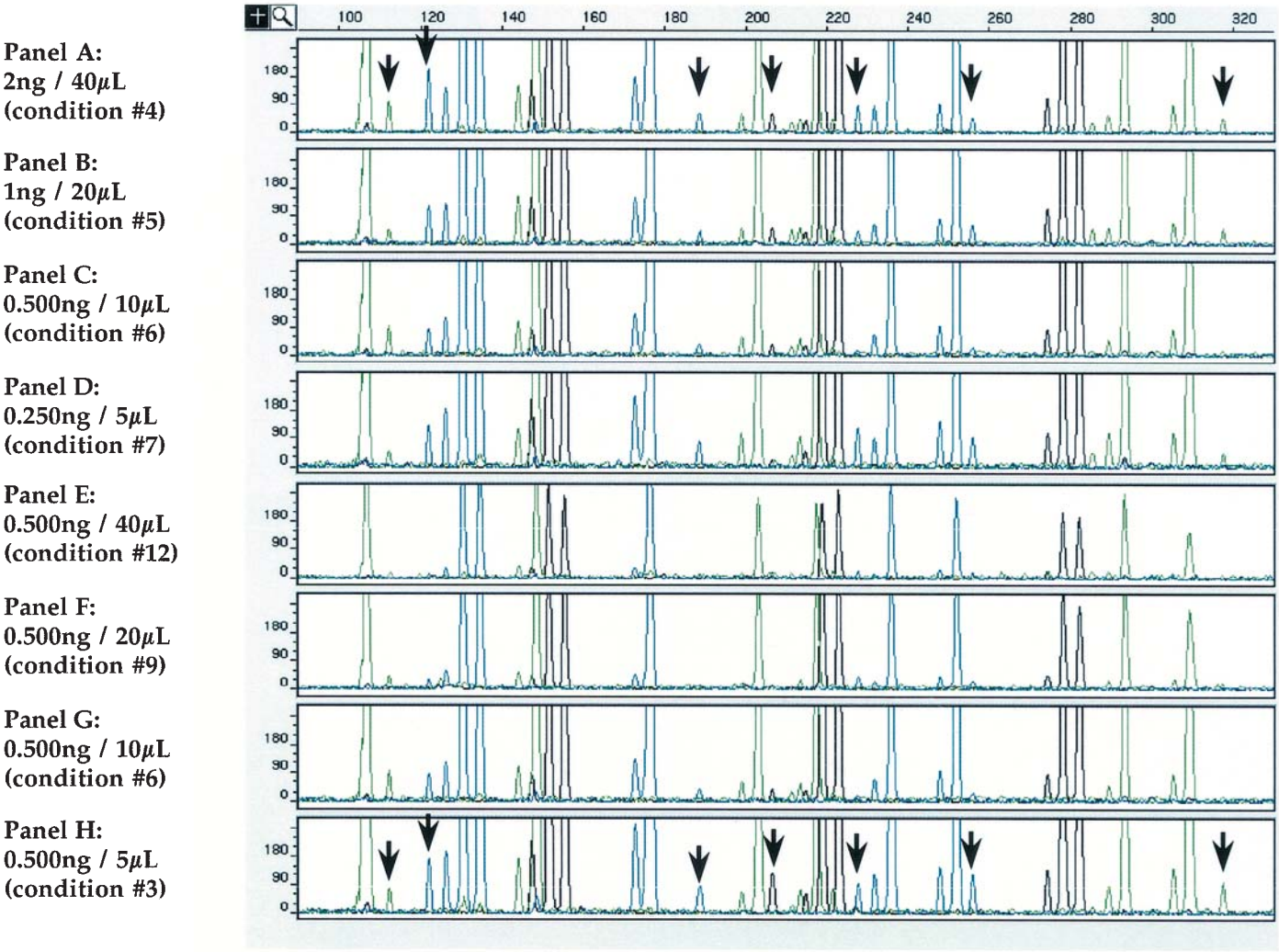

FIG. 5—*Effects of a reduction of PCR reaction volume and DNA template concentration on amplification of a casework sample with a minor profile representing 2% of the total mixture. The green-labeled loci are, from left to right, Amelogenin, D8S1179, D21S11, and D18S51. The blue-labeled loci are, from left to right, D3S1358, vWA, and FGA. The yellow-labeled (black trace) loci are from, left to right, D5S818, D13S317, and D7S820. In Panels A through D, amplification was carried out at an optimal and constant DNA concentration across the volume range to identify reaction volumes at which stochastic effects would be detected. All seven non-shared peaks that could not be attributed to stutter are indicated by an arrow in Panels A and H. In Panels E through H, amplification was carried out at a constant total amount of template DNA of 0.5 ng across the volume range to identify the reaction volumes at which minor profile dropout would be observed.*

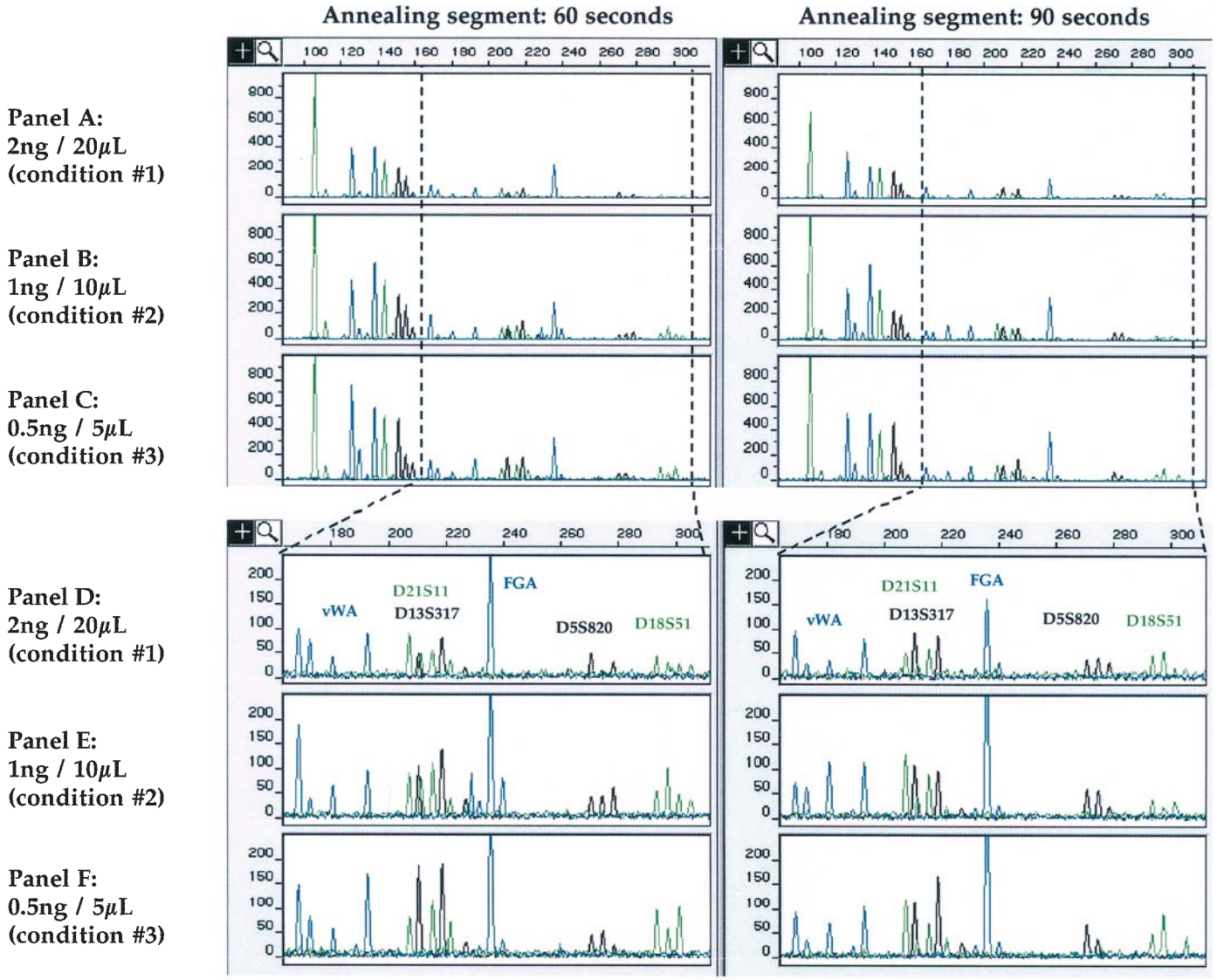

FIG. 6—*Effects of a reduction of PCR reaction volume and DNA template concentration on amplification of a casework mixture with a 10% minor profile. This sample was in limiting quantity and subjected to Conditions 1 to 3 only (see Table 2). All reactions in these three conditions were dispensed from one single DNA-reagent mixture. Panels D through F are re-scaled versions of the top panels to enhance the region of the electropherogram that displays weak amplicons.*

tion of mixed profiles if allele peaks from one contributor cannot be reliably associated because of an imbalance that is too strong. Therefore, it is important, whenever possible, to maintain stochiometry during amplification. Any attempt at enhancing signal strength must be evaluated in the context of its impact on mixture interpretation.

Fluorescent dye technology-based dynamic slab acrylamide gel electrophoresis and capillary electrophoresis are sensitive analytical platforms that permit detection of very small amounts of amplified material. Less than 1% of all amplified material from a 40-μL PCR reaction with 2 ng of DNA genomic template is actually analyzed by an ABD 377 instrument. This represents the quantity of amplicons generated from less than three genomic copies of starting material. Considering the limitations of some casework samples, the consumption during amplification of a hundred times more template than is required for instrumental detection appears inefficient and is an obvious target for improvement. In situations where scarce biological evidence is made up of a mixture including a weak minor profile component, scaling down the PCR reaction while maintaining the amount of input template DNA is expected to provide a beneficial increase in signal strength that should improve detection of a probative minor profile.

To investigate the impact of PCR reaction volume reduction on DNA typing profile interpretation of casework samples, two sample types were considered for use in this study. Pristine samples exposed to environmental insults similar in intensity and duration were considered for use as, under such pre-conditioning, replicates required for statistical compilations would have led, presumably, to similar levels of DNA degradation. Although less amenable to statistical compilations, non-selected adjudicated casework samples were also considered, as these samples show considerable heterogeneity from one sample to the next and provide a much wider variety of complexities that better reflect real life situations. For all the convenience that the simulated casework sample type provides,

it would have been difficult to select representative simulation conditions that would adequately reflect the complexities of real life samples. Therefore, adjudicated casework samples were selected as the sample type for this study because of their being the exact material caseworkers work with on a daily basis.

Before addressing ways of improving analytical detection limits, two issues needed to be addressed. First, the assumption that our PCR megaplex/detection instrumentation platform would reflect the true ratio of components in an electrophoretic profile needed to be verified. Second, in experiments designed to evaluate improvements in detection capabilities, comparison of performance of different amplification conditions generally requires statistical compilation of results from numerous replicates in order to average out differences relating to potential pipetting imprecision during sample preparation, especially at low reaction volumes. The replicate scenario can be implemented quite easily with simulated casework samples; however, most casework samples do not lend themselves to numerous replicate amplifications and, in that respect, our experimental protocol had to make best possible use of the available material.

The first point was addressed with a pristine sample mixture study designed to qualify our platform. The study made use of three-profile mixtures to test the platform's capability to resolve differences of 10% or less in contribution of minor components to the mixture. The results of this challenging test provided strong evidence that: (1) the AmpFℓSTR©Profiler Plus™ kit combined with the ABD 377 instrument perform reliably with challenging mixture ratios; (2) the reduction of the PCR reaction volume from 50 to 25

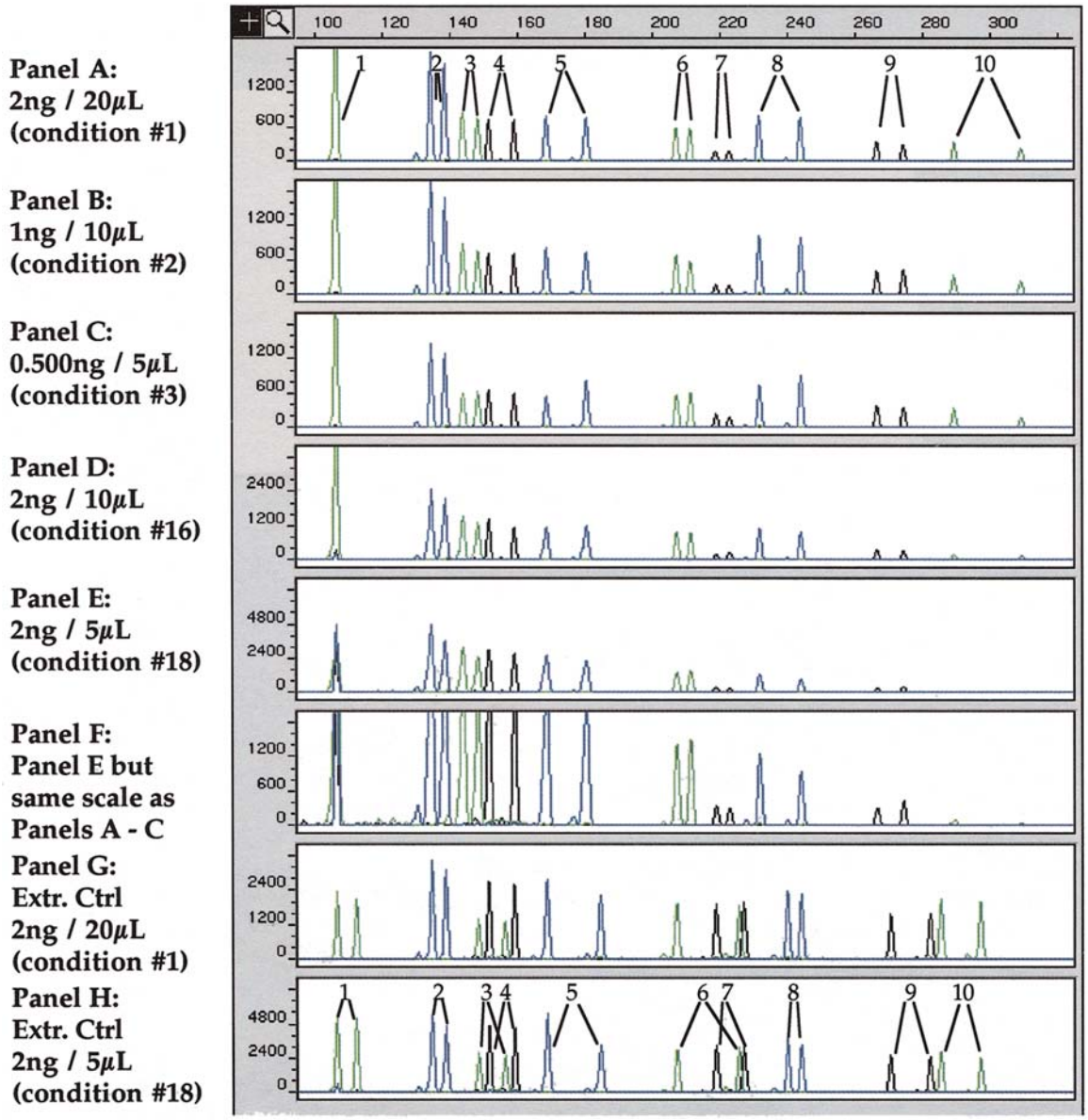

FIG. 7—*Attempt at recovering dropped-out larger-sized amplicons. A low-availability single-source bloodstain showing gradation in signal was amplified at four- and eight-fold beyond the optimal DNA concentration (Conditions 16 and 18 in Table 2) under a 60-s annealing segment. The loci are labeled as follows: 1—Amelogenin; 2—D3S1358; 3—D8S1179; 4—D5D818; 5—vWA; 6—D21S11; 7—D13S317; 8—FGA; 9—D7S820; 10—D18S51. Increasing the template DNA concentration from 2 ng in 20 μL to 2 ng in 5 μL increased the yield of shorter amplicons but decreased the yield of larger-sized amplicons.*

μL did not affect the system's performance in that context; (3) the DNA quantitation system was accurate.

The second point was addressed with the use of a two-fold reduction series for both amounts of template DNA and PCR reaction volume that allowed for minimal sample consumption and, most importantly, the effective isolation of the volume variable. Because of the varying nature of each casework sample as it pertains to its quantity, quality, level of degradation, possible presence of PCR inhibitors, number of contributors, and percentage of contribution of each individual genotype to a mixed profile, no single statistical measurement approach was deemed adequate to fully capture the degree of improvement, or lack thereof, in profile quality throughout the tested conditions and, mostly, across samples. To provide a basis for qualitative comparisons of electrophoretic data, the experimental protocol was especially designed to allow for valid column, row, and diagonal comparisons of conditions listed in Table 2. The evaluation of profile quality took into account heterozygous peak height ratios, resolution, peak height balance between loci, slope of profile, allele dropouts, split peaks, mixture ratios, and the presence of artifactual peaks.

Across the sample collection used in this study, a parallel four-fold reduction of reaction volume from 40 to 10 μL and template DNA from 1 to 0.125 ng produced profiles with increased amplitude without significant imbalance (imbalance being defined as a differential greater than ±30% at two or more loci) in heterozygote peak height ratios. An eight-fold reduction of PCR reaction volume (40 down to 5 μL) yielded heterozygote peak height ratio values that showed ratio differentials above 30% in one to three STR loci for 50% of casework samples tested. However, under these conditions, very low DNA input levels that would not have produced any detectable signal at 40-μL volumes (as little as ten genomic copies) generally produced satisfactory results, despite some imbalances in heterozygote peak height ratios. It is possible that this last result could be improved by the addition of an oil overlay on 5-μL reaction volumes, although examination of PCR vessels after amplification and before centrifugation did not reveal significant evaporation of the reaction volume. The use of oil overlays might be necessary if a particular PCR tube enclosure design is shown to be less efficient at preventing evaporation.

A previously observed and reported benefit of increasing the annealing segment duration from 60 to 90 s (10) was not observed for every sample tested under the two annealing segment protocols in this study. The increased annealing time, during which some extension presumably takes place, may be of little added value in the presence of low concentrations of PCR inhibitor, which appears to be the case of the bloodstain used as the extraction control. Similarly, when template quantity is low as with evidential samples, the PCR reaction is not subjected to limiting reactant conditions late into the cycling process and would therefore not require extended segment durations.

Although 40 to 50-μL reaction volumes and their required large amount of template material are expected to produce better-balanced profiles because of the larger DNA target population, these large-volume reactions are more prone to amplification failure when samples with low amounts of DNA are being analyzed. In that volume range, an increase in the number of cycles during amplification can presumably allow recovery of signal, although this approach can lead to heterozygote peak height imbalances as well (24,25). Even though 5-μL reactions are more prone to minor heterozygote peak height imbalances than 10-μL reaction volumes, in this study the 5 μL reaction volumes have been shown to produce complete minor profiles where 40-μL reactions with the same amount of template DNA provided only a partial, if any, minor profile. As the loss of loci data increases the number of potential hits on offender database searches and reduces the weight of the evidence in court, it might prove preferable in numerous situations to work with reaction volumes that will ensure successful production of complete profiles.

For all the benefits afforded by reduced PCR reaction volume, an optimal range of genomic template concentration (total genomic DNA mass per unit PCR reaction volume) is still required. If reduced reaction volumes are to be used, then proportionally reduced input template DNA should be used as well. The use of larger amounts of template DNA carries the potential of overcoming the PCR reaction, which can lead to preferential amplification of shorter amplicons (27–29). As most DNA quantitation procedures used for casework estimate the total amount of DNA instead of the amount of amplifiable DNA, overestimates of the amount of amplifiable DNA due to sample degradation are likely to occur that can lead to low amplicon yields. In this last situation, increasing the amount of template does not always bring about a solution to the problem as this can lead to an increase in the concentration of a PCR inhibitor, if one is present, as it was clearly seen in Table 1 for numerous casework sample situations involving bloodstains. In that respect, the 2 ng in 5-μL condition shown in Fig. 7 provided a genomic DNA concentration equivalent to 20 ng in 50 μL, beyond the optimal template concentration range. As the longer amplicons nearly vanished in conditions where shorter amplicons were not saturating the detection system, it seemed very likely that the situation with this sample reflected the presence of a PCR inhibitor instead of degraded template DNA. A dilution of the amplification reaction and electrophoretic reload to bring back the signal strength of the 2 ng in 5-μL condition within the linear dynamic range of the detector would not have corrected this problematic profile slope or brought back long amplicons into the detection range. The appropriate course of action in such cases is to work with less DNA (18), as shown in Panels F and C of Fig. 7, while being mindful that the use of too little template can also lead to stochastic effects or poor ratio of peak height over background.

In summary, 0.5 ng of DNA in a 10-μL PCR reaction volume appears to produce an identical profile to 2 ng of the same DNA in a 40-μL reaction. A further reduction down to 0.25 ng of DNA and 5-μL of PCR reaction volume produces satisfactory profiles but is more prone to HR imbalances. The combination of PCR reaction volume reduction and constant total mass of starting DNA increases signal strength. This can be beneficial when attempting to detect a minor contributor in a mixed profile. In our hands, the 5-μL reaction volume provided a reasonably balanced profile while consuming ten times less sample than a 50-μL reaction. As well, it allowed detection of a minor component estimated to contribute less than 10% to a mixture. In practice, although our data suggest that the amplification of 12 pg of DNA per μL of PCR reaction volume appears to be an appropriate threshold that will still generate a full profile with pristine samples, it seems more appropriate to envisage performing two PCR attempts on a sample available in limiting quantities: (1) a first attempt in a 5-μL reaction volume with a DNA concentration in the optimal range of 0.250 to 0.500 ng to provide an overall assessment of the sample's electrophoretic complexity and potential challenges; (2) a second attempt providing customized conditions for both reaction volume (possibly still at 5 μL) and amount of DNA template to bring out the best quantitative and qualitative data the sample can provide.

Acknowledgments

The authors wish to thank Terry Laber of the Bureau of Criminal Apprehension of Minnesota for contributing several adjudicated casework samples to this study and Dr. John Bowen of the RCMP Forensic Laboratory Services in Ottawa for his helpful comments.

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