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# Validation of Reduced-Scale Reactions for the Quantifiler™ Human DNA Kit

**ABSTRACT:** Accurate quantification of DNA samples is an important step in obtaining accurate and reproducible short tandem repeat (STR) profiles. Quantitative real-time-PCR has improved the speed and accuracy of DNA quantification over earlier methods, albeit at significantly greater cost per reaction. Here, the performance of reduced volume (10 μL) DNA quantification assays using the Quantifiler™ Human DNA Quantification Kit was evaluated using commercial standards and single source biological stains (e.g., venous blood, saliva, and semen). In addition, casework-type samples including those subjected to environmental contaminants containing PCR inhibitors and samples having undergone extensive DNA degradation were also quantified. The concentration of DNA in various forensic samples ranged from 0 to 2.9 ng/μL depending on sample source and/or environmental insult. Compared to full-scale reactions, reduced volume assays displayed equivalent to improved amplification efficiency and sample-to-sample reproducibility (±0.01–0.17 $C_{T\ FAM}$). Furthermore, the use of data from reduced-scale Quantifiler™ reactions facilitated the accurate determination of the amount of sample DNA extract needed to generate quality STR profiles. The use of 10 μL-scale Quantifiler™ reaction volumes has the practical benefit of increasing the effective number of reactions per kit by 250%; thereby reducing the cost per assay by 60% while consuming less sample. This is particularly advantageous in cases of consumptive testing.

**KEYWORDS:** forensic science, forensic DNA, validation, DNA quantification, quantitative real-time PCR, Quantifiler™

The DNA analysis of evidentiary material can make an important contribution to the outcome of a criminal investigation. The CODIS core short tandem repeat (STR) loci have become a standard for human identification (1). The quantity and/or quality of template DNA added to an STR amplification reaction, however, can significantly impact the quality of the resulting data (2,3). Numerous validation studies have demonstrated that the addition of too much or too little DNA template to an STR amplification reaction can negatively impact both the intensity of the labeled peaks and/or the level of background noise (4–6). Manufacturer recommended protocols generally rely on a 25–50 μL reaction with an input quantity of genomic DNA ranging from 0.5 to 2.0 ng per reaction. When too little DNA is used, inefficient labeling and amplification of the targeted loci lead to profiles with low signal strength, allele dropout, poor peak balance, and other stochastic effects. Conversely, if too much template is used, the resulting STR profiles often display off-scale peaks, split peaks, enhanced stutter effects, and baseline noise.

DNA quantification by forensic labs has traditionally employed slot blot hybridization or gel electrophoresis. Although these methods can be reasonably reliable, they are only able to provide an estimate of DNA concentration (7–9). They lack the ability to accurately quantify the amount of amplifiable DNA. As a result, the amount of DNA available for downstream applications is easily overestimated in cases where a sample is partially degraded. Traditional quantification methods also cannot detect the presence of PCR inhibitors in a sample and thus the adverse impact of these compounds are not seen until after downstream PCR amplification reactions. In either case, the subsequent STR profiles may fail to meet minimal quality standards for interpretation (4,10,11). Finally, both hybridization and gel-based methods also share the common drawback of relying, to some degree, on subjective human judgment; making inter-individual variability, unavoidable.

The advent of qRT-PCR has made it possible to largely circumvent the limitations of more traditional DNA quantification methods (12,13). Based on the amplification of a single-copy gene and a fluorogenic 5′ nuclease activity (14,15), the concentration of amplifiable DNA can be determined with an unparalleled degree of accuracy and sensitivity. An added advantage to this assay is that the typical 96-well plate format allows 36 unknown samples to be quantified in duplicate in just 2 h.

Applied Biosystems Inc. (ABI) has developed and validated the Quantifiler™ Human DNA kits for forensic applications (16). The Quantifiler™ kit amplifies the single copy Human Telomerase Reverse Transcriptase gene to quantify the amount of amplifiable human DNA in a sample (17–20). As few as 3–4 copies of a diploid human genome (i.e., 0.023 ng/μL) can be detected. Along with this target sequence, an Internal Positive Control (IPC) template is also included in each reaction. The amplification of this short synthetic sequence is used to detect the presence of PCR inhibitors (e.g., hematin, urine, and humic acid) that may be present in DNA extracts of forensic samples. The detection of such inhibitors prior to STR analysis provides the analyst with an opportunity to further purify samples that might otherwise be problematic (21,22). *In toto*, qRT-PCR reduces the consumption of often precious DNA extract while helping to maximize the potential for obtaining good quality STR profiles especially when working with degraded or challenging samples.

Although the benefits of qRT-PCR are widely recognized by the forensic community, the per sample cost of this approach is approximately four times that of the traditional QuantiBlot® Human DNA Quantitation assay. The objective of the current study, therefore, was to optimize and evaluate the performance of reduced scale Quantifiler™ Human DNA quantification reactions. The accuracy and precision of DNA quantification was examined using a variety of commonly encountered forensic samples. We also examined the quality of subsequent STR profiles generated from

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amplification reactions prepared using quantification data from the reduced-scale reactions. Aside from being more cost effective, the ability to successfully reduce Quantifiler™ reaction volumes will further reduce the amount of DNA that is consumed prior to STR profiling.

## Materials and Methods

All aspects of the current study were conducted in compliance with U.S. Federal Policy for the Protection of Human Subjects (Basic DHHS Policy for Protection of Human Research Subjects; 56 FR 28003).

### *DNA Sample Preparation and Extraction*

Buccal swabs, peripheral blood, saliva, semen, and hair (0.5 cm with root) were collected from individuals who had provided informed consent to participate in the study. Undiluted and 10-fold serial dilutions of peripheral blood were prepared in PBS (pH 7.4); 10 μL aliquots were applied to Pur-Wraps® sterile cotton tipped applicators (Hardwood Products, Guilford, ME) and allowed to air dry for 1 h at room temperature. To investigate the influence of PCR inhibition on reduced scale samples, simulated casework samples were prepared by applying 10 μL aliquots of undiluted and 10-fold serially diluted blood to Pur-Wraps® sterile cotton tipped applicators that had previously been dipped in a slurry of top soil in nanopure water (50:50 w/v) and then allowed to air dry for 2 h at room temperature. To test the potential impact of environmental contaminants, 10 μL aliquots of undiluted blood or semen were applied to sterile cotton tipped applicators that had previously been dipped in laundry detergent, used motor oil, gasoline, sodium hydroxide or glacial acetic acid and allowed to dry for 2 h. All samples were prepared in triplicate and then stored at −20°C until DNA quantification.

Additional simulated casework included cigarette butts from a study participant and 100 μL aliquots of blood, saliva or semen that had been applied to nylon carpeting, cotton cloth or blue denim fabric. Tests of DNA degradation used seven aged postmortem blood extraction samples from the Denver Police Department. These samples had been stored as liquid blood from 1980 to 1997 at 4°C and then spotted onto cotton cloth, dried and stored for an additional 8 years at room temperature.

All DNA samples were extracted using the EZ1 DNA Tissue kit run on the BioRobot® EZ1 with DNA Forensic Card (Qiagen Inc., Valencia, CA) in accordance with the manufacturers' recommended protocol for DNA extraction from trace samples. To maximize the recovery of genomic DNA, whole swabs, or cuttings from stained blue denim, cotton or nylon carpeting were immersed in 190 μL of the Qiagen's proprietary "G2 Buffer" and 10 μL of proteinase K solution (600 mAU/mL), vortexed for 10 sec and incubated at 56°C for 15 min. The digested supernatant was transferred to a sterile 2 mL sample tube and loaded onto the BioRobot® EZ1. Following automated DNA extraction and elution of the DNA into 200 μL of TE (10 mM Tris, 1 mM EDTA), each tube was visually inspected for the presence residual paramagnetic beads which have the potential to interfere with qRT-PCR quantification. When detected, the beads were removed by brief centrifugation and transfer to of the cleared supernatant to a new sterile 2 mL sample tube.

### *Quantitative Real-time PCR*

The human genomic DNA content of all samples was determined using Quantifiler™ Human DNA Quantification kit run on a Prism™ 7900HT Sequence Detection System (ABI, Foster City, CA). Quantification standards were prepared by serial dilution in $T_{10}E_{0.1}$/Glycogen buffer (10 mM Tris-HCl pH 8.0; 0.1 mM EDTA; 20 μg/mL glycogen) of the 200 ng/μL Human DNA Standard included in the kit. Eight serial dilutions ranging from 50 ng/μL to 23 pg/μL in three-fold increments were used.

Two Quantifiler™ reaction volumes were used in the current study, full- and reduced-scale. The manufacturer's full-scale protocol consisted of 10.5 μL of Primer Mix, 12.5 μL of PCR Reaction Mix, and 2 μL of DNA extract for a total reaction volume of 25 μL. The reduced-scale reaction consisted of 4.2 μL of Primer Mix, 5 μL of PCR Reaction Mix, and 0.8 μL of DNA extract for a final reaction volume of 10 μL. The DNA extract was aliquoted with a P2 Pipetman® (Gilson Inc., Middleton, WI). Samples were loaded into MicroAmp® Optical 96-Well Reaction plates and sealed using Optical Adhesive Covers (ABI). The same set of eight standards was used for both methods as was the manufacturer's recommended thermal amplification profile of 95°C for 10 min.; 45 cycles of 95°C for 15 sec, and 60°C for 1 min.

Data collection points were set at all stages of the PCR reaction. All standards were assayed in triplicate and all experimental samples were assayed in duplicate for statistical analyses. Standard curves were generated for each experimental set, and amplification plots were used to compare individual PCR reaction consistency, efficiency, and results. IPC results from each assay were monitored for the presence of PCR inhibitors. Negative (no template DNA) and reagent blank controls were also included on each assay plate.

### *STR Genotyping*

The AmpFℓSTR® Profiler Plus™ ID kit (ABI) was used to generate STR profiles from DNA samples that had been quantified using the reduced-scale Quantifiler™ reaction. Amplification reactions were prepared with 4.0 μL of PCR Reaction Mix, 2.0 μL of Primer Set, 0.2 μL of AmpliTaq Gold™ (5 units/μL), 0.5 ng of DNA template in DNA-free water for a final volume of 10 μL. All amplification reactions were accompanied by negative and reagent blank controls. Amplified products were electrokinetically injected for 5 sec and fractionated on an ABI Prism™ 310 Genetic Analyzer using the GS STR POP4 (1 mL) F filter set module. The electrophoresis run time of 24 min allowed detection the 500 base pair size peak of the GS500ROX sizing standard. Data Collection Software v3.1 and Genemapper ID v3.2 (Applied Biosystems, Foster City, CA) were used for data collection and analysis.

## Results and Discussion

### *Amplification of Human DNA Quantification Standards*

The Quantifiler™ Human DNA Quantification kit contains a 200 ng/μL human DNA standard (Lot No. 0602018) which is serially diluted in three-fold increments to yield eight dilutions spanning the range of 50–0.023 ng/μL. Amplification of these standards produces a standard curve from which the concentration of unknown samples is determined by linear interpolation. Accordingly, the correct amplification of these standards is absolutely critical to the accuracy of the Quantifiler™ assay. The theoretical optimum of 100% amplification efficiency would be expected to yield a standard curve having a slope of −3.333 with an $R^2$ of 1.00 and a $\Delta C_{T\ FAM}$ of 1.73 between each standard dilution.

Table 1 presents a comparison of standard curve amplification data for full-scale (25 μL) and reduced-scale (10 μL) reactions performed in triplicate. Also provided are the corresponding data from

TABLE 1—*Amplification of quantifiler™ human DNA standards.*

| Quantification Standard Dilution (ng/μL) | Quantifier™ (25 μL Reaction)* | | Quantifiler™ (25 μL Reaction)† | | Quantifiler™ (10 μL Reaction)† | |
|---|---|---|---|---|---|---|
| | $C_{TFAM}$ (Mean) | SD | $C_{TFAM}$ (Mean) | SD | $C_{TFAM}$ (Mean) | SD |
| 50 | 23.83 | 0.13 | 23.93 | 0.10 | 23.25 | 0.10 |
| 16.7 | 25.36 | 0.08 | 25.46 | 0.06 | 24.80 | 0.04 |
| 5.56 | 26.79 | 0.08 | 27.00 | 0.04 | 26.35 | 0.01 |
| 1.85 | 28.14 | 0.08 | 28.48 | 0.01 | 27.86 | 0.05 |
| 0.62 | 29.56 | 0.14 | 29.80 | 0.05 | 29.33 | 0.12 |
| 0.21 | 31.00 | 0.06 | 31.42 | 0.10 | 30.93 | 0.17 |
| 0.068 | 32.51 | 0.25 | 33.19 | 0.14 | 32.45 | 0.10 |
| 0.023 | 33.83 | 0.49 | 34.88 | 0.03 | 34.02 | 0.10 |

*Data from manufacturer's validation study.
†Data from testing in the author's laboratory.

validation studies conducted by the manufacturer (20). Full-scale Quantifiler™ reactions in the investigator's laboratory yielded a slope of −3.21 with an $R^2 = 0.998$, an average $\Delta C_{T\ FAM}$ of 1.56 between dilutions with an average standard deviation of 0.12 $C_{T\ FAM}$. Replicate assays were highly reproducible for all concentration points with standard deviations of 0.02–0.24 $C_{T\ FAM}$. Amplification of the same DNA dilution standards using reduced-scale Quantifiler™ reactions had a slope of −3.24 with an $R^2$ of 0.998, an average $\Delta C_{T\ FAM}$ of 1.60 between dilutions with an average standard deviation of 0.08 $C_{T\ FAM}$. Replicate assays were also highly reproducible with standard deviations of 0.01–0.17 $C_{T\ FAM}$. Relative to full-scale Quantifiler™ reactions both in the investigator's laboratory and as reported by the manufacturer, the performance of the reduced-scale reactions were found to be virtually indistinguishable or to trend toward slightly higher efficiency and reproducibility.

It is worth noting that the shape of the early to full plateau phase of the sigmoidal curve of the Quantifiler™ amplification plot can be improved by increasing the total number of cycles to 45 from the manufacturer's recommendation of 40 cycles. This is not essential to quantification by the cycle threshold method, but it may benefit alternate approaches to quantification based on fitting fluorescence data to four-parametric sigmoidal functions (23,24). This alternate approach has been reported to significantly improve the accuracy and reliability of qRT-PCR based assays.

### *Quantification of Peripheral Blood Samples*

Having demonstrated equivalent performance between full-scale and reduced-scale reaction volumes using the pristine 200 ng/μL Quantifiler™ human DNA quantification standard, we next compared the performance of full- and reduced-scale reactions using human blood. DNA was extracted from 42 samples with varying quantities (0.1–40 μL) of peripheral blood spotted onto cotton swabs.

Figure 1 presents the correlation between the amount of blood that had been applied to the individual cotton swabs and the resulting $C_{T\ FAM}$ values obtained for the corresponding DNA extracts. Assay reproducibility was nearly identical with $R^2$ values of 0.9711 and 0.9817 for the full-scale and reduced-scale reactions, respectively. Furthermore, subtle deviations from the line of best fit were consistent between the full- and reduced-scale assays. Such deviations primarily reflect vicissitudes in the efficiency with which the dried blood was recovered from individual cotton swabs during the DNA extraction process. Interestingly, there appeared to be trend toward higher amplification efficiency in the reduced scale reactions as evidenced by average $C_{T\ FAM}$ values that were consistently lower than those of the corresponding full-scale reactions. This

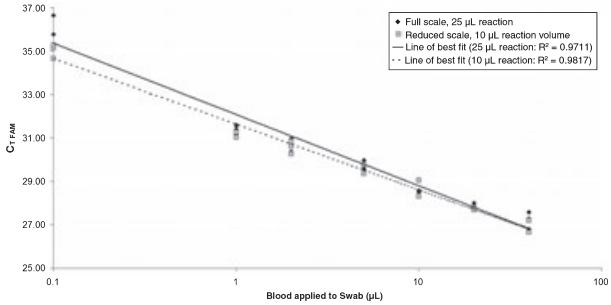


FIG. 1—*Correlation between varying amounts (0.1–40 μL) of single-source peripheral blood applied to cotton swabs and the resulting $C_{T\ FAM}$ values from full-scale (25 μL) versus reduced-scale (10 μL) Quantifiler™ reactions. The assay reproducibility was nearly identical across the full range of samples quantified. Full- and reduced-scale reactions had $R^2$ values of 0.9711 and 0.9817, respectively. All samples were prepared in triplicate and each DNA extract was assayed in duplicate.*

trend was also clearly evident in the comparisons of the $C_{T\ VIC}$ results from the IPC for the same samples. Increased amplification efficiency in reduced scale reactions is not atypical and is thought to reflect improved thermal transfer (25). Thus, the performance of reduced-scale Quantifiler™ assays across a broad range of peripheral blood quantities was indistinguishable or trended slightly better than that of full-scale reactions.

### *Quantification of Peripheral Blood/Soil Mixtures*

Forensic casework samples may contain a variety of PCR inhibitors that reduce amplification efficiency. Common inhibitors include hematin and IgG from blood (26); indigo dye from blue denim (27); and humic acid from soil (28). The Quantifiler™ assay makes it possible to detect PCR inhibition by monitoring the amplification of the IPC present in each reaction. To conduct a realistic evaluation of the performance of full- versus reduced-scale reactions in the presence of a PCR inhibitor, DNA extracts were prepared from 42 samples of varying quantities (0.1–40 μL) of peripheral blood spotted onto cotton swabs that had previously been dipped in a slurry of top soil and sterile water. The use of top soil as the source of possible PCR inhibitors was based on the prior experience of the investigators' laboratory which prepares DNA extracts using the EZ1 DNA Tissue kit (Qiagen Inc.). This system efficiently washes away most inhibitors during the extraction process. With soil-contaminated samples, however, residual soil particles occasionally carryover to the eluted DNA. In some cases, this can result in PCR inhibition.

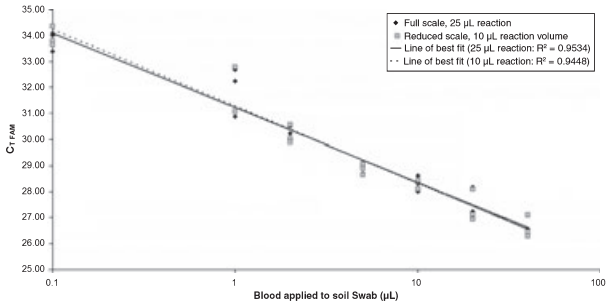


FIG. 2—*Correlation between varying amounts (0.1–40 µL) of single-source peripheral blood applied to cotton swabs stained with top soil and the resulting $C_{T\ FAM}$ values from full-scale (25 µL) versus reduced-scale (10 µL) Quantifiler™ reactions. The assay reproducibility was nearly identical across the full range of samples quantified. Relative to samples that were free of soil contamination (Fig. 1), both full- and reduced-scale reactions, deviated almost equally from the line of best fit. This is reflected by their respective $R^2$ values of 0.9534 and 0.9448. All samples were prepared in triplicate and each DNA extract was assayed in duplicate.*

Figure 2 presents the correlation between the amount of blood that had been applied to the individual soil stained cotton swabs and the resulting $C_{T\ FAM}$ values obtained for the corresponding DNA extracts. The reproducibility of assays for individual swabs was nearly identical between full- and reduced-scale reactions. In some cases, however, both the full- and reduced-scale reactions deviated significantly from the line of best fit as reflected by $R^2$ values of 0.9534 and 0.9448 for the full-scale and reduced-scale reactions, respectively. This greater variability in amplification efficiency reflects the effect of random carry over of inhibitory compounds from top soil in some of the blood/soil extracts (e.g., the soil-stained swabs stained with 1 µL of blood).

The presence of varying levels of inhibitors is evident in the $C_{T\ VIC}$ data for the IPC amplicon presented in Fig. 3. The Mean ± SD $C_{T\ VIC}$ value for the IPC associated with the pristine human DNA dilution standards was 26.45 ± 0.17. By contrast, the Mean ± SD $C_{T\ VIC}$ values for the IPC associated with the blood/soil samples ranged from 26.57 ± 0.02 to 34.51 ± 0.86 for the full-scale reactions and from 26.38 ± 0.08 to 33.93 ± 0.14 for the reduced-scale reactions. Regardless of the degree of inhibition, however, the performance of the reduced-scale reactions closely tracked that of the full-scale reactions. Thus, performance of reduced-scale reactions is consistent with that of full-scale reactions even under conditions of PCR inhibition.

### *Quantification with Simulated Casework Samples*

In a forensic context, the primary value of qRT-PCR is its ability to enable an analyst to accurately determine the amount of a DNA extract that is needed to obtain a satisfactory STR profile. To evaluate the practical applicability of using reduced-scale reactions for the range of sample types encountered in a forensic laboratory, we used the reduced scale reactions to determine the DNA concentration of a wide variety of simulated casework samples. The assemblage of simulated casework samples were designed to test a variety of tissue sources, substrates from which stains were recovered and environmental contaminants including strong acids and bases, and several common household reagents.

Table 2 presents the results from a series of reduced-scale Quantifiler™ assays on these simulated casework samples. The reduced-scale reactions quantified the amount of human DNA in all but two samples (i.e., glacial acetic acid-treated cotton swab-1 and -2).

Based on the IPC results for each sample neither the source of the DNA nor the substrate from which a stain was cut appeared to have any adverse impact on amplification efficiency. This included reduced-scale assays of blood, saliva or semen stains on blue denim containing indigo dye, an inhibitor of PCR. This was not unexpected as this inhibitor is effectively removed by the DNA extraction process used in the current study. Additionally, reduced-scale reactions worked with a broad range of environmentally challenged samples. Relative to control cotton swabs, each containing a clean 10 µL aliquot of blood, the amounts of DNA quantified from blood swabs contaminated with laundry detergent, gasoline, used motor oil, sodium hydroxide or glacial acetic acid were all significantly lower. The lowest concentrations of DNA were observed for blood treated with sodium hydroxide or glacial acetic acid and the highest, on average, were for blood mixed with laundry detergent. Comparisons of the IPC results (i.e., Mean ± SD $C_{T\ VIC}$) between control (26.592 ± 0.070) and contaminated (26.564 ± 0.075) swabs containing equivalent amounts of blood do not show significant differences. This indicates that the lower concentrations of DNA in the contaminated samples are not the result of an inhibition-associated reduction in amplification efficiency. Rather, it appears to reflect either degradation of the DNA in the sample itself or a reduction in the efficiency of the DNA extraction and isolation process.

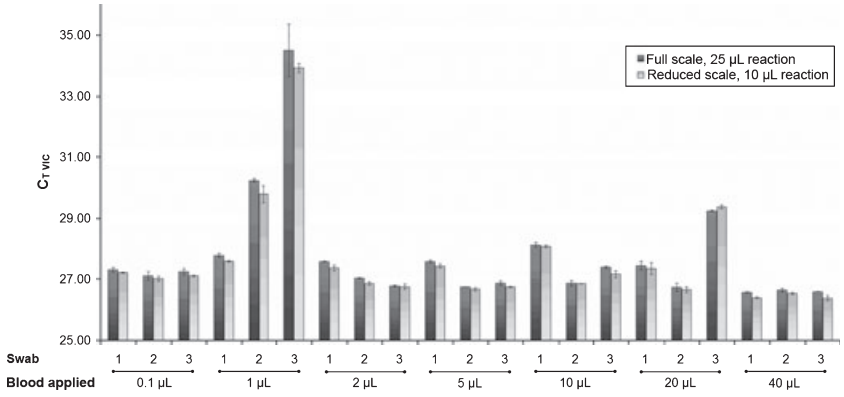


FIG. 3—*Histogram illustrating the effects of PCR inhibitors from blood/soil extractions on the IPC for full-scale (25 µL) versus reduced-scale (10 µL) Quantifiler™ reactions. The Mean ± SD $C_{T\ VIC}$ values for the IPC associated with the blood/soil samples ranged from 26.57 ± 0.02 to 34.51 ± 0.86 for the full-scale reactions and from 26.38 ± 0.08 to 33.93 ± 0.14 for the reduced-scale reactions. Regardless of the degree of inhibition, the performance of the reduced scale reactions closely tracked that of the full scale reactions.*

TABLE 2—*Quantification of casework-type samples using 10μL reactions.*

| Substrate | DNA Source | Environmental Contaminant | Quantity (ng/μL) | SD | IPC ($C_T$) | SD |
|---|---|---|---|---|---|---|
| None | 100 μL saliva | None | 14.62 | 0.570 | 25.909 | 0.017 |
| None | 100 μL saliva | None | 15.09 | 0.660 | 25.799 | 0.102 |
| None | 1.5 cm Hair with root | None | 0.607 | 0.039 | 26.478 | 0.028 |
| Blue denim | Semen stain | None | 2.418 | 0.021 | 26.383 | 0.200 |
| Blue denim | Blood stain | None | 2.217 | 0.266 | 26.537 | 0.072 |
| Blue denim | Saliva stain | None | 2.892 | 0.290 | 26.339 | 0.088 |
| Cotton cloth | Semen stain | None | 2.724 | 0.021 | 26.333 | 0.032 |
| Cotton cloth | Blood stain | None | 1.084 | 0.266 | 26.638 | 0.270 |
| Cotton cloth | Saliva/buccal cells | None | 2.008 | 0.058 | 26.580 | 0.325 |
| Nylon carpet | Blood stain | None | 2.492 | 0.250 | 26.289 | 0.021 |
| Cigarette butt | Trace saliva | None | 1.688 | 0.302 | 26.590 | 0.197 |
| Cotton swab-1 | 10 μL blood | None | 1.841 | 0.074 | 26.523 | 0.027 |
| Cotton swab-2 | 10 μL blood | None | 1.834 | 0.111 | 26.589 | 0.087 |
| Cotton swab-3 | 10 μL blood | None | 1.092 | 0.115 | 26.663 | 0.088 |
| Cotton swab-1 | 10 μL blood | Laundry detergent | 0.297 | 0.000 | 26.515 | 0.020 |
| Cotton swab-2 | 10 μL blood | Laundry detergent | 0.256 | 0.003 | 26.462 | 0.061 |
| Cotton swab-3 | 10 μL blood | Laundry detergent | 0.244 | 0.026 | 26.523 | 0.049 |
| Cotton swab-1 | 10 μL blood | Gasoline | 0.309 | 0.026 | 26.662 | 0.172 |
| Cotton swab-2 | 10 μL blood | Gasoline | 0.120* | 0.009 | 26.561 | 0.107 |
| Cotton swab-3 | 10 μL blood | Gasoline | 0.111* | 0.005 | 26.668 | 0.278 |
| Cotton swab-1 | 10 μL blood | Sodium hydroxide | 0.066* | 0.013 | 26.550 | 0.083 |
| Cotton swab-2 | 10 μL blood | Sodium hydroxide | 0.087* | 0.006 | 26.538 | 0.092 |
| Cotton swab-3 | 10 μL blood | Sodium hydroxide | 0.081* | 0.028 | 26.499 | 0.011 |
| Cotton swab-1 | 10 μL blood | Used motoroil | 0.591 | 0.024 | 26.530 | 0.067 |
| Cotton swab-2 | 10 μL blood | Used motoroil | 0.191 | 0.012 | 26.612 | 0.088 |
| Cotton swab-3 | 10 μL blood | Used motoroil | 0.293 | 0.030 | 26.613 | 0.237 |
| Cotton swab-1 | 10 μL blood | Glacial acetic acid | ND | ND | 26.528 | 0.171 |
| Cotton swab-2 | 10 μL blood | Glacial acetic acid | ND | ND | 26.476 | 0.036 |
| Cotton swab-3 | 10 μL blood | Glacial acetic acid | 0.046* | 0.014 | 26.720 | 0.209 |

*Samples were concentrated by vaccum centrifugation prior to STR amplification

### *Quantification of Degraded Samples*

Among the most challenging samples are those in which the DNA is highly degraded due to age or improper storage. The Quantifiler™ assay cannot be used as a precise measure of the extent of DNA degradation, but it can provide useful information on how much if any amplifiable DNA remains in a sample. To evaluate the applicability of using reduced-scale reactions on casework-type samples likely to have undergone significant DNA degradation, seven aged postmortem blood samples were generously provided by the Denver Police Department. These samples had been stored in liquid form at 4°C from 1980 to 1997 before being spotted on to clean cotton and stored dry at room temperature for an additional 8 years. Table 3 presents the quantification results from reduced-scale Quantifiler™ assays for approximately equal size cuttings (1 $cm^2$) of each of the seven postmortem blood samples. While recognizing that it is impossible to consider the cuttings to be quantitatively equivalent with regard to the amount of dried blood present on each one, it appears that these samples have undergone variable but extensive DNA degradation based on the low concentrations of amplifiable DNA obtained (i.e., 0.237 ng/μL to undetectable) given the size of the stain that was extracted.

TABLE 3—*Quantification of aged postmortem blood samples using 10 μL reactions.*

| Samples | Quantity (ng/μL) | SD |
|---|---|---|
| Postmortem blood 1 | 0.237 | 0.049 |
| Postmortem blood 2 | 0.150 | 0.016 |
| Postmortem blood 3 | 0.019 | 0.011 |
| Postmortem blood 7 | 0.157 | 0.006 |
| Postmortem blood 10 | 0.194 | 0.020 |
| Postmortem blood 13 | 0.051 | 0.014 |
| Postmortem blood 15 | ND | ND |

### *Short Tandem Repeat Profiles*

The AmpFℓSTR® Profiler Plus® ID kit was used to test whether the DNA quantification results obtained using the reduced-scale Quantifiler™ assays could be used to accurately determine the amount of DNA extract needed to obtain full STR profiles. Based on the results of the reduced-scale reactions, all casework-type samples used in the current study were predicted to contain sufficient quantities of amplifiable DNA for successful STR profiling (i.e., 0.5 ng in a 10 μL Profiler Plus® reaction volume). For samples where reduced-scale Quantifiler™ assays indicated a DNA concentration of less than 0.132 ng/μL (e.g., glacial acetic acid-treated blood and some postmortem blood samples), the DNA extract was concentrated by vacuum centrifugation prior to STR amplification.

Figure 4 shows the Profiler Plus® STR profiles where the amount of input DNA was determined using reduced scale Quantifiler™ assays of samples of saliva; head hair with an attached root; blue denim stained with saliva, blood or semen; and cotton fabric stained with semen or blood. The resulting STR profiles were characterized by strong allelic peak heights that were well balanced at all nine of the Profiler Plus® loci with a slight but expected trend toward lower peak heights for alleles at the larger loci. In no case, however was there any allele drop out, significant peak imbalance, split peaks, enhanced stutter effects or other stochastic effects associated with having significantly over or under estimated the amount of DNA needed to generate a high quality STR profile.

Figure 5 shows the Profiler Plus® STR profiles corresponding to blood samples exposed to a wide range of environmental contaminants of the type that are encountered in a forensic context. Again, the amount of DNA extract needed for a total input of 0.5 ng to

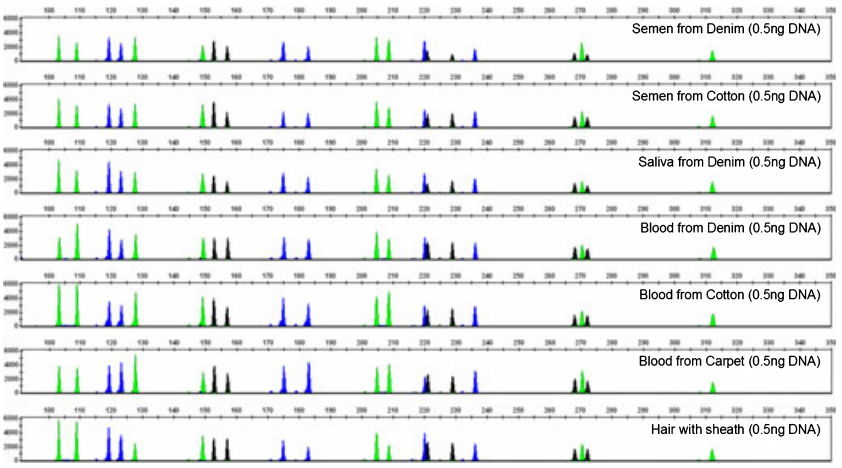


FIG. 4—*Profiler Plus® STR electropherograms for samples of saliva; head hair with an attached root; blue denim stained with saliva, blood or semen; and cotton fabric stained with semen or blood. The resulting STR profiles were characterized by strong allelic peak heights that were well balanced at all nine of the Profiler Plus® loci with a slight but expected trend toward lower peak heights for alleles at the larger loci. The amount of DNA extract required for 0.5 ng of input DNA for each sample was determined from reduced-scale (10 μL) Quantifiler™ assays.*

the STR amplification reaction was based on the results of the reduced-scale Quantifiler™ assays. Full STR profiles were generated for all but one sample without allele dropout, significant peak imbalance or other unacceptable stochastic effects. Peak heights were consistently strong across all nine loci for blood-stained swabs that had been contaminated with laundry detergent, gasoline, used motor-oil, and sodium hydroxide. The one exception was the profile from the blood-stained swabs that had been contaminated with glacial acetic acid where peak heights were generally lower across all nine loci but still above the minimum peak height threshold for allele calls. The STR profile for the acetic acid treated swab however, displayed significant peak imbalances at multiple loci and allele drop out at the D18S51 locus.

A Profiler Plus® STR profile was also generated for swab no. 3 of the 1 μL blood/top soil replicates. The IPC $C_{T\ VIC}$ value of 33.93 for the reduced-scale assay of this swab suggested significant PCR inhibition. As with all other samples, 0.5 ng of amplifiable DNA was added to the Profiler Plus® STR amplification reaction. The resulting STR profile (Fig. 6) was characterized by balanced allelic peak heights at individual loci but a steep inverse relationship between peak height and allele length. For example, the allele peak heights for the amelogenin locus exceeded 5400 RFU while

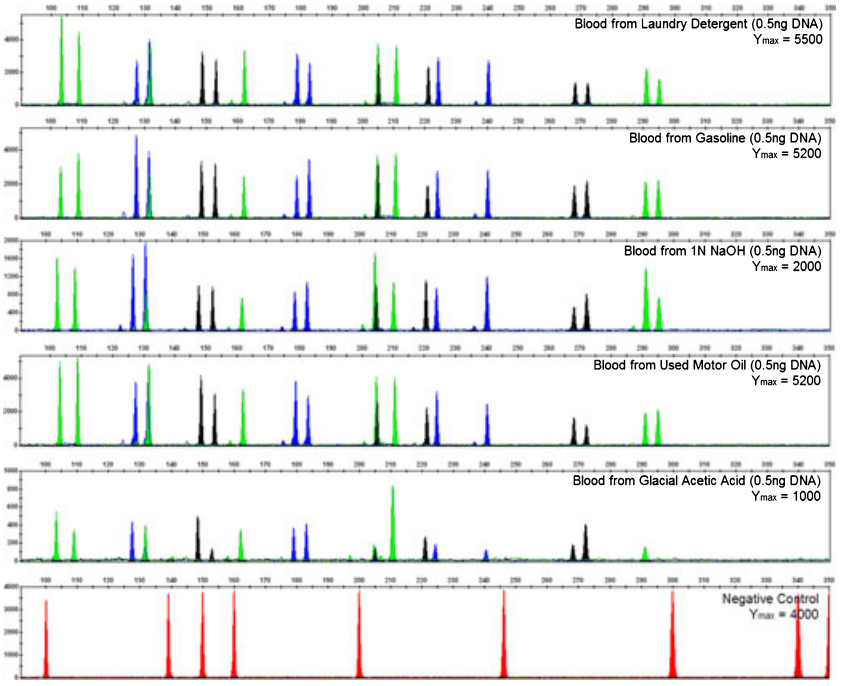


FIG. 5—*Profiler Plus® STR electropherograms for samples exposed to a variety of environmental contaminants. Full STR profiles were generated with strong allelic peak heights at all nine of the Profiler Plus® loci and without allele dropout, significant peak imbalance or other unacceptable stochastic effects for blood-stained swabs that had been contaminated with laundry detergent, gasoline, used motor-oil, and sodium hydroxide. Only the STR profile for the acetic acid treated swab, displayed significant peak imbalances at multiple loci and allele drop out at the D18S51 locus. The amount of DNA extract required for 0.5 ng of input DNA for each sample was determined from reduced-scale (10 μL) Quantifiler™ assays.*

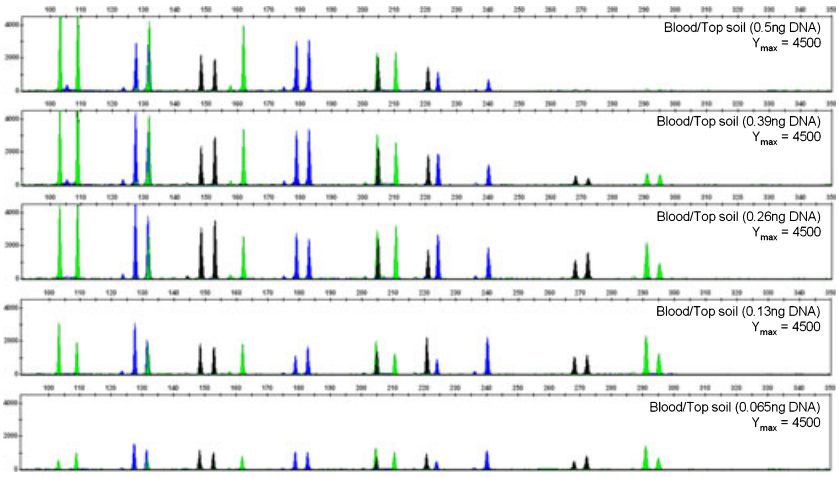


FIG. 6—*Profiler Plus® STR electropherograms for a series of dilutions of the DNA extract from a blood/soil mixture with evidence of PCR inhibition. At the highest quantities of input DNA, allelic peak heights were balanced at individual loci but there was a steep inverse relationship between peak height and allele length. At the lowest quantities of input DNA, there was significant reduction in total fluorescence signal with an increase in stochastic anomalies. The amount of DNA extract required for 0.5 ng of input DNA for each sample was determined from reduced-scale (10 μL) Quantifiler™ assays.*

those at the D18S51 (85-137 RFU) locus approached the allele call threshold used by many forensic labs. This progressive drop in allelic peak heights is consistent with PCR inhibition and was anticipated on the basis on the IPC results.

One common standard operating procedure designed to ameliorate the adverse effects of PCR inhibition is to reduce the amount of DNA extract used in the STR amplification reaction and thereby to dilute the inhibitor. Based on the quantification data from reduced-scale assays of swab no. 3 of the 1 μL blood/top soil replicates four additional Profiler Plus® STR amplification reactions were prepared in which the DNA extract was diluted such that 0.39, 0.26, 0.13 or 0.065 ng were added to the reaction. The resulting STR profiles showed improved amplification at the larger loci suggesting that the effect of the inhibitors was reduced. Below a DNA input quantity of 0.39 ng, however, significant allelic imbalance at some loci (e.g., FGA and D18S51) was observed. Consistent with the expected performance of this human ID kit, very low DNA input quantities were characterized by an overall reduction in fluorescence signal and an increase in stochastic anomalies.

Finally, STR profiles were generated from six of the seven postmortem blood samples (Fig. 7). Based on the results of reduced scale Quantifiler™ assays, 0.5 ng of amplifiable DNA was used for each Profiler Plus® STR amplification reaction. Complete profiles were obtained for postmortem samples 1 and 2 although peak imbalance of potential concern was noted at several loci. The remaining four postmortem blood samples yielded only partial

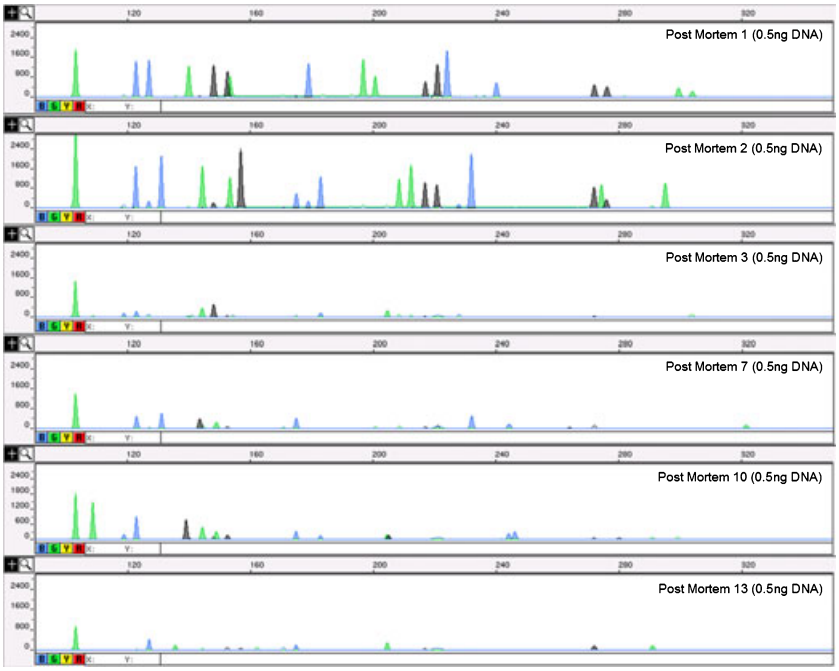


FIG. 7—*Profiler Plus® STR electropherograms for highly degraded postmortem blood samples. Complete profiles were obtained for postmortem samples 1 and 2 although peak imbalances were noted at several loci. The remaining four samples yielded partial profiles consistent with extensive DNA degradation. The amount of DNA extract required for 0.5 ng of input DNA for each sample was determined from reduced-scale (10 μL) Quantifiler™ assays.*

profiles with amplification limited primarily to the smallest loci in each channel. This was consistent with extensive DNA degradation during the storage of these samples and the fact that Quantifiler™ assays are not able to predict the exact degree of DNA degradation in a given sample but only the presence of the amplifiable 62 bp target amplicon used for quantification.

## Conclusion

Quantitative real-time PCR is now a widely used methodology for the quantification of human DNA by the forensic community. While it is possible to more accurately and precisely determine the amount of amplifiable DNA in a sample, the reagent costs associated with commercial kits such Quantifiler™ are significantly higher than earlier methods. One of the simplest ways to reduce these reagent costs would be to scale down the assay reaction volume. In theory, as long as the concentration of reagents is constant, it should be possible to reduce qRT-PCR reactions to sub-microliter volumes. This has been demonstrated on microfluidic platforms where qRT-PCR assays have been successfully performed in 300 nL droplets (29). Until such microfluidic devices are commonplace in forensic laboratories, efforts to use reduced-scale reactions will need to be tailored to more readily available platforms (e.g., the ABI Prism 7000, 7500, and 7900HT Sequence Detection Systems). On these instruments, automated Nanodrop™ dispensers (Innovadyne Technologies, Santa Rosa, CA) allow qRT-PCR reactions to be scaled down to a 1.25 μL volume in a 384-well format. While feasible, such high-density formats and small volumes are less amenable to forensic casework where liquid handling by human analysts is the norm.

In the current study, we evaluated the accuracy and reproducibility of reducing the reaction volume of the Quantifiler™ assay from the manufacturer recommended 25 μL to a 10 μL reaction volume which consumes only 0.8 μL of the typical DNA extract, a volume that can be readily handled with ease using a manual P2 micropipettor. The performance of 10 μL reactions was investigated for a wide range of sample types. The amplification efficiency and reproducibility of reduced-scale reactions were found to be virtually indistinguishable or to trend slightly better than the manufacturer's recommended protocol for amplification of a broad range of dilutions of the commercially prepared human DNA standard provided with the Quantifiler™ kit. The same equivalence in performance was seen with single source blood samples extracted from cotton swabs. With more challenging mixtures of blood and top soil the reduced scale reactions yielded equivalent to slightly more precise quantification results than full-scale assays of the same DNA extract. Moreover, the level of PCR inhibition in blood/soil mixtures, as indicated by an elevated $C_{T\ VIC}$ value in the IPC, was indistinguishable between reduced and full scale reactions. Finally, reduced-scale reactions were used to quantify the DNA content of a variety of simulated casework samples and post mortem blood samples containing highly degraded DNA. Subsequent STR profiling using the AmpFℓSTR® Profiler Plus® ID kit and DNA concentration data from 10 μL Quantifiler™ assays resulted in successful STR profiling free of the stochastic effects associated with significantly over or under estimating the amount of DNA in a sample.

*In toto,* the current study demonstrates the concordance of performance between the 25 μL and 10 μL Quantifiler™ reaction volumes. This reduction in reaction volume has the practical benefit of increasing the effective number of reactions per kit by 250%. This reduces the cost per assay by 60% while consuming less sample.

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