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Developmental Validation of the Quantifiler® Duo DNA Quantification Kit for Simultaneous Quantification of Total Human and Human Male DNA and Detection of PCR Inhibitors in Biological Samples*

ABSTRACT: The Quantifiler® Duo DNA Quantification kit enables simultaneous quantification of human DNA and human male DNA as well as detection of inhibitors of PCR in a single real-time PCR well. Pooled human male genomic DNA is used to generate standard curves for both human (ribonuclease P RNA component H1) and human male (sex determining region Y) specific targets. A shift in the cycle threshold (C_T) values for the internal positive control monitors the presence of PCR inhibitors in a sample. The assay is human specific and exhibits a high dynamic range from 0.023 to 50 ng/μL. In addition, the multiplex assay can detect as little as 25 pg/μL of human male DNA in the presence of a 1000-fold excess of human female DNA. The multiplex assay provides assessment of the DNA extract and guidance for the selection of the appropriate AmpFℓSTR® Amplification Kit to obtain interpretable short tandem repeat profiles.

KEYWORDS: forensic science, DNA quantification, real-time PCR, DNA analysis, ribonuclease P RNA component H1, sex determining region Y, human DNA, human male DNA, DNA typing

Forensic DNA analysis is targeted to obtain a short tandem repeat (STR) profile from an evidence sample, which is then compared with the STR profiles from reference samples collected from the victim and the suspect to determine the contribution to the evidence sample (1,2). STR genotyping systems such as Identifiler®, Profiler Plus®, COfiler®, SGM Plus®, MiniFiler™, and Yfiler® kits are commercially available. The genotyping protocol, in general, involves extraction of DNA from the biological sample, quantification of the DNA, amplification for STR loci, and fragment analysis on a Genetic Analyzer. Quantification of human DNA in a forensic sample, which often contains nonhuman DNA, is an important step during STR profiling because the STR genotyping systems, unlike detection or single nucleotide polymorphism (SNP) assays, are sensitive to the quantity of DNA used in the PCR reaction: too little DNA may produce partial profiles whereas too much may produce off-scale data. For the forensic analyst, it is imperative to obtain an interpretable STR profile from forensic evidence samples, which often are in limited amount, and therefore, a reliable quantification method is vital. Hybridization-based quantification methods, e.g., Quantiblot®, that are traditionally used for the quantification of DNA in forensic samples are generally considered time-consuming, labor-intensive, and not suitable for automation (3). Further, it is difficult to predict the amplitude of the STR profile because of the difference in the sensitivity of quantification methods and STR genotyping systems. Real-time PCR assays like the Quantifiler® Human DNA Quantification Kit and Quantifiler® Y Human Male DNA Quantification Kit have proved very useful (4). Real-time PCR assays for quantification of human DNA offer several advantages over the traditional hybridization assays such as: (i) specificity for a certain target in the genome because of the careful assay design; (ii) ability to detect few copies of target DNA; (iii) quantitative relationship between the amount of target template and the amount of PCR product accumulated at any given cycle prior to reaching saturation; (iv) greater dynamic range; (v) multiplexing capabilities; (vi) easy to adopt; and (vii) automatable for high throughput.

A forensic evidence sample is often a mixture of human male and female DNA. Further, a forensic biological sample may be exposed to different environmental insults leading to DNA degradation and contamination with compounds that inhibit the PCR. Thus, it is desirable for the forensic analyst to have useful information about the forensic evidence sample prior to amplification for STRs. Real-time quantification assays can provide: (i) mixture ratio of human and human male DNA for choosing between autosomal and Y STR profiling based on the extent of the mixture ratio; (ii) presence of PCR inhibitors so that DNA extracts containing PCR inhibitors may be repurified prior to STR profiling; and (iii) quantification of human female and human male DNA useful for determining the quantity of extract to be used for amplification for different STR multiplex systems.

To obtain the quantity of human and human male DNA in a sample using Quantifiler® Human and Y kits, it is necessary to run two separate quantification assays. This approach may consume a considerable amount of sample, which is often available in limited quantities, as well as time and reagents. To overcome these hurdles,

multiplex real-time quantification assays have been described in recent years (5–8). The assays described by Walker et al. (5), Horsman et al. (6), and Nicklas and Buel (7) enable an estimation of the mixture ratio of human male and female DNA but were not designed to detect the inhibitors of PCR in the sample. The assay described by Swango et al. (8) enables forensic analysts to obtain the mixture ratio as well as the detection of the inhibitors of PCR. However, the human nuclear DNA amplification target THO1 (human tyrosine hydroxylase gene on chromosome 11) spans the polymorphic STR region (8). THO1 is a commonly used locus for human identification in forensic laboratories (2). First, the incorporation of such polymorphic STR target in the quantification assay is discouraged to avoid possible contamination incidences in the laboratory. Second, the length of the amplicon would vary by 44 nucleotides since alleles ranging from 3 to 14 repeat units have been characterized in different human population groups (for references see Short Tandem Repeat DNA Internet DataBase compiled by NIST available at http://www.cstl.nist.gov/biotech/strbase/str_TH01.htm). Though the detection probe is designed outside the polymorphic region, the possibility of variation in the efficiency of the assay due to the variation in the length of the amplicon can not be ruled out.

We describe a multiplex TaqMan® real-time PCR assay, the Quantifiler® Duo DNA Quantification Kit, for simultaneous quantification of human nuclear and human male DNA as well as detection of the presence of PCR inhibitors in a biological sample. The developed assay enables the assessment of the biological samples for downstream STR profiling.

Materials and Methods

Pooled human male genomic DNA used for generation of standard curves was obtained from EMD Biosciences Inc. (San Diego, CA). Genomic DNA from unknown individuals was obtained from Biochain (Hayward, CA), Sigma Chemical Company (St. Louis, MO), Promega (Madison, WI), or Serological Research Institute (Richmond, CA). Nonhuman samples were obtained as purified DNA from BIOS Laboratories, Inc. (New Haven, CT), Pel-Freez Biologicals (Rogers, AR), and American Type Culture Collection (Manassas, VA). Oligonucleotides, TaqMan® probes, Quantifiler® human DNA quantification kit, Quantifiler® Y male DNA quantification kit, AmpFℓSTR kits, 7500 Real-time PCR System, 3130 Genetic Analyzers and associated software were from Applied Biosystems (Foster City, CA). All other chemicals used in this study were of analytical grade.

Extraction and Quantitation of DNA

The DNA from anonymous donor samples (blood, saliva and semen, either liquid or stains, and buccal swabs) was extracted by using standard phenol–chloroform (9), or BloodPrep® DNA Chemistry and the ABI PRISM® 6100 Nucleic Acid PrepStation (Applied Biosystems) procedures. The quantity of DNA was determined by Quantifiler® Duo, Quantifiler® Human, and Quantifiler® Y Human Male DNA Quantification Kits (Applied Biosystems).

Real-Time PCR Amplification

Real-time PCR amplification reactions contained 10.5 μL of Primer-Probe Mix, 12.5 μL of Master Mix, and 2.0 μL of DNA sample. The Primer-Probe Mix contained forward and reverse primers and TaqMan® probes for ribonuclease P RNA component H1 (RPPH1), sex determining region Y (SRY), and internal PCR control (IPC) targets. The IPC template, a synthetic polynucleotide, was cloned into a plasmid. The Master Mix contained Reference dye, dNTPs, dUTP, MgCl₂, AmpliTaq® Gold DNA polymerase and preservatives in Tris-HCl, pH 8.0. Pooled human male genomic DNA at eight different concentrations (50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068, and 0.023 ng/μL) was amplified on each quantification run plate for generation of standard curves for RPPH1 and SRY targets. Amplification reactions were performed in a 7500 Real-Time PCR System (Applied Biosystems) following the manufacturer's instruction with conditions as follows: 50°C, 2 min; 95°C, 10 min; 40 cycles of 95°C, 15 sec and 60°C, 1.0 min. The data were analyzed using 7500 System sds Software v1.2.3 (Applied Biosystems) with a threshold value of 0.2.

STR Analysis

The samples were amplified with Identifiler®, Yfiler®, and MiniFiler™ kits using the procedure described in the User's Manual for the respective kit. The amplified products were analyzed on a 3130xl Genetic Analyzer (Applied Biosystems) with GeneMapper® ID Software v3.2.1 (Applied Biosystems).

Sensitivity Study

Two human male genomic DNA samples, one pooled and the other single source, obtained from commercial sources were diluted to obtain concentrations of 20.0, 5.0, 1.0, 0.1, 0.05, 0.04, 0.03, 0.023, 0.0115, 0.00575, 0.002875, and 0.00144 ng/μL in 10 mM Tris buffer, pH 8.0 containing 0.1 mM ethylene diamine tetraacetic acid (EDTA). Each dilution was quantified in triplicate using the Quantifiler® Duo DNA Quantification Kit.

Species Specificity

The DNA from nonhuman biological species was either obtained commercially or purified in the laboratory. For some of these DNA samples, the sex of the donor animal was unknown. For some species, multiple donor animals were tested. Most of the reactions utilized 5.0 ng of input DNA. For a few reactions, 10 ng of input DNA was used.

Precision and Accuracy

One set of eight serial dilutions was prepared containing 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068, and 0.023 ng/μL of the human male DNA standard present in the Quantifiler® Duo DNA Quantification Kit. Six reaction plates were set up and each of them contained 10 replicates of the eight dilutions. Two plates per instrument were run on three different 7500 Real-time PCR System instruments. The two runs were performed on two different days, using the same three 7500 Real-time PCR System instruments. For each dilution, the C_T values for RPPH1, SRY, and IPC signals were recorded for all 60 reactions.

Reproducibility

Four male and one female genomic DNA samples were diluted from initial estimated concentrations to 20.0, 10.0, 1.0, 0.1, and 0.05 ng/μL. All dilutions were made in 10 mM Tris buffer, pH 8.0 containing 0.1 mM EDTA. All samples and dilutions were run in triplicate using the Quantifiler® Duo Kit. Three runs were performed on different days. For each sample reaction, the C_T values were obtained and the DNA quantities calculated.

Mixture Study

Mixture samples containing 0.2 ng/µL of human male DNA and varying amounts of female DNA were prepared. The ratio of male to female DNA in these samples was 1:0, 1:1, 1:5, 1:10, 1:20, and 0:1. The mixture samples were processed in triplicate using the Quantifiler® Duo DNA Quantification Kit to determine the concentration of total human DNA (RPPH1 target) and male DNA (SRY target). Using the quantification results from the RPPH1 human target, *c*. 1.0 ng of human genomic DNA from each sample was added to an Identifiler® kit reaction. Similarly, using the results from the SRY male target, *c*. 1.0 ng of human genomic DNA from each sample was added to a Yfiler® kit reaction.

Another set of mixture samples containing 25 pg/µL of male DNA and increasing quantities of female DNA was prepared to obtain male to female DNA ratios of 1:0, 1:50, 1:100, 1:200, 1:500, 1:800, 1:1000, and 0:1. The samples were processed in triplicate using the Quantifiler® Duo Kit to determine the concentration of total human genomic DNA (RPPH1 target) and male DNA (SRY target). In addition, based on the results from the SRY male target, *c*. 1.0 ng of human genomic DNA from each sample was added to the Yfiler® reaction.

Calculation of Male to Female DNA Ratio

The Quantifiler® Duo kit provides the quantity of human and human male DNA in biological samples. From these values, one can calculate the ratio of male and female DNA using the following equation:

$$\text{Male DNA:Female DNA Ratio}$$
$$= \frac{\text{Male DNA}}{\text{Male DNA}} : \frac{(\text{Human DNA} - \text{Male DNA})}{\text{Male DNA}}$$

or

$$\text{Male DNA:Female DNA Ratio}$$
$$= 1 : (\text{Human DNA} - \text{Male DNA})/\text{Male DNA}$$

All quantities in the above equations are ng/µL. This ratio determines the extent of the mixture, which is useful for making the choice of STR analysis method: autosomal STRs or Y-STRs.

Inhibited Samples

Human male genomic DNA was mixed with hematin to obtain final concentrations of 0, 2.5, 5.0, 7.5, 10, 12.5, 15, 17.5, 20, and 40 µM in the 25-µL quantification PCR. A second set of inhibited samples was prepared by addition of humic acid to obtain final concentrations of 0, 1.0, 2.0, 3.0, 3.75, 7.5, 11.25, 15, and 30 ng/µL in the 25-µL quantification PCR. The concentrations described here were final concentrations of respective inhibitor in 25-µL PCR when 2 µL of sample is added. The concentration of the inhibitor in the sample was, therefore, 12.5 times higher. Since the final concentration in the PCR was the contributing factor for the inhibition, the samples were named accordingly and the same nomenclature was used for both the quantification and the STR reactions for simplicity. Two microliters of each sample, containing *c*. 1.0 ng of DNA, was quantified in triplicate using the Quantifiler® Duo DNA Quantification Kit. Results obtained using the RPPH1 human target of the Quantifiler® Duo kit were used to calculate DNA input for STR analysis using Identifiler® and MiniFiler™ kits.

Degraded DNA

One microgram of DNA (100 µL reaction at 10 ng/µL concentration) was treated for 20 min using varying quantities of the DNase I enzyme; 0.002, 0.01, 0.02, 0.05, 0.1, and 0.2 units. Samples were run on a 4% agarose gel for 25 min and visualized by staining with ethidium bromide to monitor the extent of degradation. The degraded DNA samples were processed with the Quantifiler® Duo kit to determine the quantity of amplifiable DNA at each level of degradation. Results obtained using the RPPH1 human target of the Quantifiler® Duo kit were used to calculate DNA input for STR analysis using Identifiler® and MiniFiler™ kits.

Case-Type Samples

Saliva and blood samples were collected from four human male donors (donors a and b for saliva, donors c and d for blood). Semen was from one human male donor (donor e). Forensic-type samples were prepared by loading 50 µL of saliva on cotton swab, 5 µL of blood on fabric, 5 µL of blood on denim, 5 µL of blood on filter paper, 5 µL of blood spiked with inhibitors on fabric and 1 µL of semen on fabric (samples 1–8). The DNA was isolated by a phenol:chloroform extraction method. Extracted DNA was quantified in triplicate using the Quantifiler® Duo Kit.

Results and Discussion

Availability of STR profiling kits with targeted capabilities such as the Yfiler® kit for Y-STRs (10) and the MiniFiler™ kit for compromised samples (11) made it essential to assess the quality of a DNA extract in addition to the DNA quantification. The developed triplex assay comprises coamplification of the ribonuclease P RNA component H1 or RPPH1 gene (Gene ID 85495) for quantification of total human DNA, the sex determining region Y or SRY gene (Gene ID 6736) for quantification of human male DNA, and a synthetic nucleotide template sequence as an IPC in a single reaction. The human target RPPH1 is also known as H1 RNA or H1RNA and is located on chromosome 14 (location 14q11.2). The male target SRY is also referred as TDF or TDY and is located on chromosome Y (location Yp11.3). The genes are present at one copy per chromosome. Thus, two copies of RPPH1 and one copy of SRY are amplified during the quantification assay. The TaqMan® probes for the measurement of the human male, human, and IPC targets were labeled with FAM™, VIC®, and NED™ dyes, respectively. The PCR mixture comprises two oligonucleotide primers and a TaqMan® probe specific for each target. The principle and mechanism of the real-time PCR quantification assay employing TaqMan® probes is described earlier (4). Briefly, the TaqMan® probes are labeled with a fluorescent reporter dye at the 5′ end and a nonfluorescent quencher along with a minor groove binder (MGB) moiety at the 3′ end. The extent of amplification for each target is determined by measuring the respective fluorescent dye released from the probe by 5′ nuclease activity of the DNA polymerase during the extension phase of the PCR (12). A threshold for the fluorescence is set at the beginning of the exponential phase based on the initial cycles when little change in the fluorescence occurs. Cycle threshold (C_T) value is the cycle at which the fluorescence signal crosses the threshold value. Thus, the lower the C_T value, the higher the quantity of DNA. Real-time PCR assays can quantify the DNA present in a given well by measuring the C_T value and comparing it with the standard curve C_T values. The multiplex was optimized *in silico* to avoid interactions between the oligonucleotides and minimize the formation of primer-dimers. This was confirmed by laboratory testing. The primer and probe concentrations

were optimized to ensure that the human male DNA was detected and quantified accurately in the presence of a large quantity of female DNA.

The primers were selected and designed based on the published sequences to obtain 140, 130, and 130-bp fragments for the RPPH1, SRY, and IPC targets, respectively. The size of the amplicons in the Quantifiler® Duo kit is greater than the length of the hTERT (62 bp) and SRY (64 bp) targets in the Quantifiler® Human and Quantifiler® Y Human Male Quantification Kits, respectively (4). The size of the amplicons was increased in the Quantifiler® Duo kit to avoid incidences of over estimation of the quantity of DNA and to obtain a better correlation between the DNA quantification values and predictability of the STR profiles as described in this paper.

Although the Quantifiler® Duo DNA Quantification Kit is not a DNA genotyping assay, it is intended for use before performing genotyping assays using AmpFℓSTR® PCR Amplification kits. The developmental validation studies were performed following the revised validation guidelines provided by the Scientific Working Group on DNA Analysis Methods (SWGDAM) Guidelines (13). By testing the procedure with samples commonly encountered in forensic and parentage laboratories, the validation process clarifies attributes and limitations that are critical for sound data interpretation in casework.

Standard Curves

Linearity of quantification of the triplex assay was determined from the standard curves for human and human male targets generated by amplification of pooled human male genomic DNA at concentrations ranging from 0.023 to 50.0 ng/µL (Fig. 1). As expected, the C_T value increased progressively with a decrease in the amount of human DNA. The C_T values for the IPC increased by about 0.5–1.0 at higher concentrations of human DNA because of a slight PCR competition. A linear relationship between the C_T values and the quantity of DNA template investigated was observed for both human and human male DNA. In general, the C_T values for the SRY target were higher than those for the RPPH1 target. This observation is in concordance with the haploid nature of the SRY target and the diploid nature of the RPPH1 target in the human male sample. The C_T values for the human target are higher than those for the male target in the multiplex assay reported by Horsman et al. (6); this could be attributed to lower amplification efficiency of the human target.
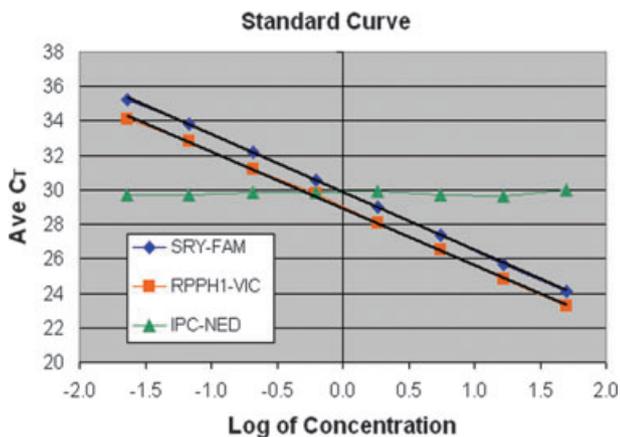
FIG. 1—*Example of a typical standard curve: cycle threshold (C_T) values for ribonuclease P RNA component H1 (RPPH1), sex determining region Y (SRY), and internal positive control (IPC) targets across the eight standard DNA concentrations (50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068, and 0.023 ng/µL).*

TABLE 1—*Sensitivity study: measured and expected quantities for two samples.*

Expected Quantity (ng/µL)	Measured Quantity (ng/µL)			
	Sample 1		Sample 2	
	RPPH1	SRY	RPPH1	SRY
20	18.500	19.540	20.910	20.383
5	4.000	4.330	4.943	4.800
1	0.832	0.909	0.802	0.751
0.1	0.099	0.111	0.096	0.108
0.05	0.050	0.048	0.056	0.058
0.04	0.039	0.053	0.038	0.039
0.03	0.026	0.033	0.038	0.031
0.023	0.020	0.022	0.022	0.033
0.01150	0.014	0.009	0.015	0.016
0.00575	0.010	0.007	0.010	–
0.00288	–	0.000	0.006	0.007
0.00144	–	0.006	–	–

SRY, sex determining region Y; RPPH1, ribonuclease P RNA component H1.

About 45 standard curves were generated on multiple instruments. The slope values for both standard curves ranged between −3.0 and −3.6 indicating amplification efficiency of 100 ± 10% for both the targets. Similarly, the R^2 values remained >98.0% allowing accurate quantification of each target simultaneously.

Sensitivity Study

Sensitivity studies were performed to determine the range of DNA concentrations that are able to produce reliable quantification results and to determine the limit of detection. The quantities of human and human male DNA obtained from the Quantifiler® Duo kit for the two samples were very similar to the expected quantities across a range of concentrations from 20 ng/µL to 23 pg/µL (Table 1). Furthermore, quantities as low as 11.5 pg/µL of human DNA were reproducibly detected across all replicates. At concentrations of 5.75 pg/µL and below, human DNA was not reproducibly detected across all replicates because of the stochastic variation in the amplification efficiency at low DNA input amounts. As expected, samples containing lower quantities of DNA exhibited greater variation in the quantification results because of stochastic effects. The limit of detection of human and human male DNA by the Quantifiler® Duo kit is similar to other real-time PCR-based assays for quantification of human and/or human DNA reported in the literature (4–8,14,15). Some of these reported assays have demonstrated a lower limit of detection than the Quantifiler® Duo kit. However, the ultimate goal of human and human male DNA quantification is to determine the volume of extract to be used as template for amplification using STR genotyping kits. The amount of DNA recommended for STR typing ranges from 0.5 to 2.0 ng for different kits as described in the User's Manuals for AmpFℓSTR® kits. In general, for samples containing DNA at concentrations of 0.1 ng/µL or less, it is necessary to add the maximum volume of DNA extract to the AmpFℓSTR® kit amplification reaction. Therefore, quantification values of <0.1 ng/µL do not affect the downstream STR reaction set-up. The limit of quantification and detection of human and human male DNA may be determined in each laboratory.

Species Specificity

The Quantifiler® Duo Kit measures the quantity of human and human male DNA in forensic-type samples. Since the forensic-type

samples may be contaminated with nonhuman DNA, specificity measurements of primers and probes in the Quantifiler® Duo DNA Quantification Kit are crucial. Cross-reactivity of primers and probes in the Quantifiler® Duo Kit was examined by testing DNA from various nonhuman species. Species specificity of the Quantifiler® Duo kit was investigated by amplification of DNA from orangutan, chimpanzee, gorilla, macaque, dog, cow, pig, cat, horse, sheep, chicken, fish (salmon), rabbit, mouse, rat, hamster, *Escherichia coli*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, and *Candida albicans*. None of the species investigated exhibited amplification signal for RPPH1, SRY, and IPC targets except for chimpanzee. DNA from two chimp samples exhibited C_T values of 32.3 and 31.1 for the SRY target compared with 27.9 C_T for a human male sample. Thus, the primers and probes in the triplex assay are specific for human DNA and some higher primates. Cross-reactivity of the primers and/or probes utilized in the PCR-based assays for quantification of human DNA towards higher primates is reported by other investigators: primers and/or probes for *Alu*Yb8 target exhibited cross-reactivity for orangutan and gorilla in the H-Quant (14) and some nonhuman species (5); male Y assay for primate species (5); and hTERT target for higher primates (4).

Precision and Accuracy

During a real-time PCR quantification, the C_T values are recorded. The C_T value for a sample is then translated into the quantity of DNA by comparing it with a linear plot generated from the C_T values of a series of standards containing known quantities of DNA. The precision of a real-time PCR assay is thus determined by the variation of C_T values. The precision of the Quantifiler® Duo kit was examined by performing two runs on different days on three different instruments using eight different concentrations

TABLE 2—*Precision and accuracy study: mean and standard deviations (SD) of the C_T values calculated for each dilution across all six plates.*

Quantification Standard Dilution (ng/µL)	C_T					
	RPPH1		SRY		IPC	
	Mean	SD	Mean	SD	Mean	SD
50	23.36	0.26	23.92	0.19	29.80	0.35
16.7	24.98	0.23	25.55	0.16	29.61	0.18
5.56	26.62	0.28	27.22	0.15	29.56	0.17
1.85	28.26	0.23	28.88	0.18	29.57	0.19
0.62	29.79	0.29	30.44	0.19	29.64	0.19
0.21	31.32	0.34	32.01	0.28	29.66	0.21
0.068	32.83	0.32	33.61	0.40	29.62	0.19
0.023	34.48	0.58	35.33	0.63	29.55	0.18

IPC, internal positive control; SRY, sex determining region Y; RPPH1, ribonuclease P RNA component H1.

of the standard DNA sample provided in the kit. Each run plate contained 10 replicates of the eight different concentrations. The mean C_T values and standard deviations (SD) ($n = 60$ for each concentration of DNA) for the RPPH1, SRY, and IPC targets are summarized in Table 2. The SD values ranged between 0.23 and 0.58, 0.15 and 0.63, and 0.17 and 0.35 for the RPPH1, SRY, and IPC targets, respectively. In general, the SD values for the RPPH1 and SRY targets were higher at lower concentrations of DNA.

Reproducibility

Four male and one female genomic DNA samples at five concentrations were tested on three different days to assess the reproducibility of the quantification results. The mean quantity, SD, and 95% confidence interval (95% CI) values are summarized in

TABLE 3—*Reproducibility study: measured and expected quantities, SD and 95% CI for four male and one female DNA samples across three consecutive runs.*

Sample	Sample Dilution (ng/µL)	SRY			RPPH1			IPC		
		Mean Quantity (ng/µL)	SD	95% CI (±percent)	Mean Quantity (ng/µL)	SD	95% CI (±percent)	C_T	SD	95% CI (±percent)
A	20	20.10	1.051	10.46	21.15	0.804	7.60	29.70	0.037	0.25
	10	8.98	0.400	13.36	9.11	0.341	11.49	29.73	0.034	0.23
	1	0.85	0.109	17.84	0.87	0.051	8.67	29.92	0.034	0.23
	0.10	0.08	0.007	25.83	0.09	0.014	17.86	29.97	0.039	0.26
	0.05	0.05	0.028	34.00	0.04	0.002	63.43	30.00	0.032	0.21
B	20	23.09	2.219	19.23	24.36	1.656	13.59	29.79	0.038	0.25
	10	11.22	0.485	1.45	11.49	0.529	15.11	29.77	0.046	0.31
	1	1.15	0.142	20.76	1.14	0.083	29.23	29.89	0.030	0.20
	0.10	0.11	0.013	42.93	0.10	0.008	42.32	29.98	0.040	0.27
	0.05	0.05	0.015	16.92	0.06	0.007	43.10	29.98	0.012	0.08
C	20	23.11	0.821	7.10	22.51	0.294	2.61	29.62	0.055	0.37
	10	9.25	0.601	35.03	8.72	0.562	32.37	29.67	0.053	0.36
	1	0.89	0.039	10.40	0.82	0.027	5.40	29.81	0.057	0.38
	0.10	0.11	0.027	10.00	0.10	0.008	23.96	29.89	0.053	0.36
	0.05	0.04	0.020	68.07	0.04	0.008	90.52	29.81	0.047	0.32
D	20	26.49	2.116	15.98	27.28	1.835	13.45	29.90	0.106	0.71
	10	13.09	0.596	12.20	13.26	0.261	9.44	29.87	0.028	0.19
	1	1.26	0.136	8.54	1.22	0.081	16.18	29.76	0.055	0.37
	0.10	0.12	0.032	43.59	0.12	0.008	28.35	30.02	0.034	0.22
	0.05	0.07	0.021	14.12	0.06	0.005	22.12	29.97	0.042	0.28
E	20	Female	–	–	24.91	0.586	4.70	29.97	0.023	0.16
	10	Female	–	–	12.11	0.486	8.94	29.87	0.007	0.04
	1	Female	–	–	1.14	0.049	6.55	29.77	0.018	0.12
	0.10	Female	–	–	0.12	0.016	21.07	29.73	0.089	0.60
	0.05	Female	–	–	0.06	0.008	67.71	29.71	0.047	0.32

IPC, internal positive control; SRY, sex determining region Y; RPPH1, ribonuclease P RNA component H1.

TABLE 4—*Mixture study: measured and expected male:female ratios and quantities for mock mixture samples.*

| Experiment* | Expected male: female DNA ratio | Quantity (ng/μL) | | Measured male: female DNA ratio |
		SRY	RPPH1	
Mixture study I	1:0	0.228	0.236	1:0.04
	1:1	0.229	0.507	1:1.21
	1:5	0.240	1.410	1:4.88
	1:10	0.280	3.030	1:9.82
	1:20	0.235	4.070	1:16.32
	0:1	Female	0.217	–
Mixture study II	1:0	0.027	0.026	1:0.04
	1:50	0.029	1.260	1:42.45
	1:100	0.029	2.460	1:83.25
	1:200	0.022	6.405	1:288.16
	1:500	0.025	13.770	1:545.43
	1:800	0.027	24.410	1:896.43
	1:1000	0.020	28.210	1:1388.66
	0:1	Female	0.016	–

*Mixture samples contained 0.2 ng/μL (Mixture study I) and 0.025 ng/μL (Mixture study II) of human male DNA and varying amounts of human female DNA.

Table 3. The 95% CI values were calculated as the mean of the DNA quantity, ±2 SD units for each sample, and expressed as a percentage of the mean quantification result. In general, the human and human male DNA quantities determined for all samples at all concentrations were close to the expected values. The quantification results obtained by the Quantifiler® Duo kit were highly reproducible; the SD values for the quantification of the SRY and RPPH1 targets ranged from 0.007 to 2.219 and 0.002 to 1.835, respectively. Similarly, the SD values for the C_T of the IPC target ranged from 0.04 to 0.60. The average 95% CI is ±24.2% and ±21.4% for the human and the male target, respectively.

Mixture Study

The ability of the Quantifiler® Duo kit to quantify human male DNA in the presence of human female DNA was investigated by using male-female mixture samples prepared by combining human male DNA with human female DNA preparations at varying ratios. The mixture studies were designed to simulate circumstances in which a small component of male DNA must be discerned from a high background of female DNA. Quantification results for the mixture samples are summarized in Table 4. It is evident that the mixture ratio measured using the Quantifiler® Duo kit was very close, within the variations of PCR, to the expected ratio in the mixture samples containing 0.2 ng/μL of male DNA (up to 1:20). In a separate experiment, the limit of detection of male DNA in the presence of large excesses of female DNA was investigated by combining 0.025 ng/μL of male DNA and corresponding quantities of female DNA to obtain mixture ratios up to 1:1000. The Quantifiler® Duo kit successfully detected male DNA in mixture samples containing as high as 1000-fold excess female DNA (Table 4). These results demonstrate the robustness and specificity of the Quantifiler® Duo kit. The measured mixture ratio for samples containing 0.025 ng/μL of male DNA and excess quantity of female DNA was between 10% and 40% higher than the expected mixture ratio. This variation could be due to stochastic effects during the quantitation of male DNA at such low concentration.

STR profiles for the mixture samples up to a 1:20 ratio were generated using the Identifiler® kit and 1.0 ng of human DNA as determined by the RPPH1 target (Fig. 2). The amplitude (rfus) of male alleles decreased with increasing ratios of male and female DNA. Interpretation of the minor male profile in such mixture samples was challenging because of the occurrence of shared alleles, minor-male alleles at stutter positions of female alleles, and dropout of minor alleles. Alleles from the minor male contributor were interpretable in the mixture samples having 1:1, 1:5, and 1:10 ratios of male:female DNA (one allele is indicated by the rectangles in Fig. 2 as an example). Y-STR profiles for male DNA in all mixture samples generated using the Yfiler® kit and 1.0 ng of human male DNA as determined by the SRY target were complete, conclusive, and consistent (data not shown). Figure 3 represents profiles from mixture samples at high mixture ratios and analyzed with the Yfiler® kit. The results demonstrate the utility of the Quantifiler® Duo kit in the analysis of mixture
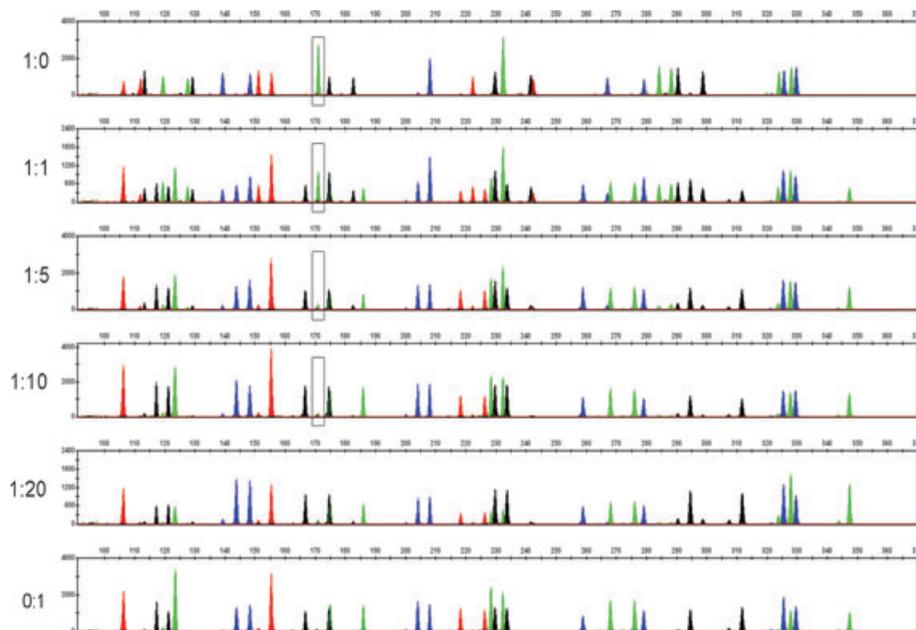
FIG. 2—*Mixture study I: short tandem repeat (STR) analysis using the Identifiler® kit of mixtures containing 0.2 ng/μL of male DNA and increasing amounts of female DNA according to the following male to female DNA ratios: 1:0, 1:1, 1:5, 1:10, 1:20, and 0:1. The rectangles indicate a peak belonging to the male minor component of the mixtures.*
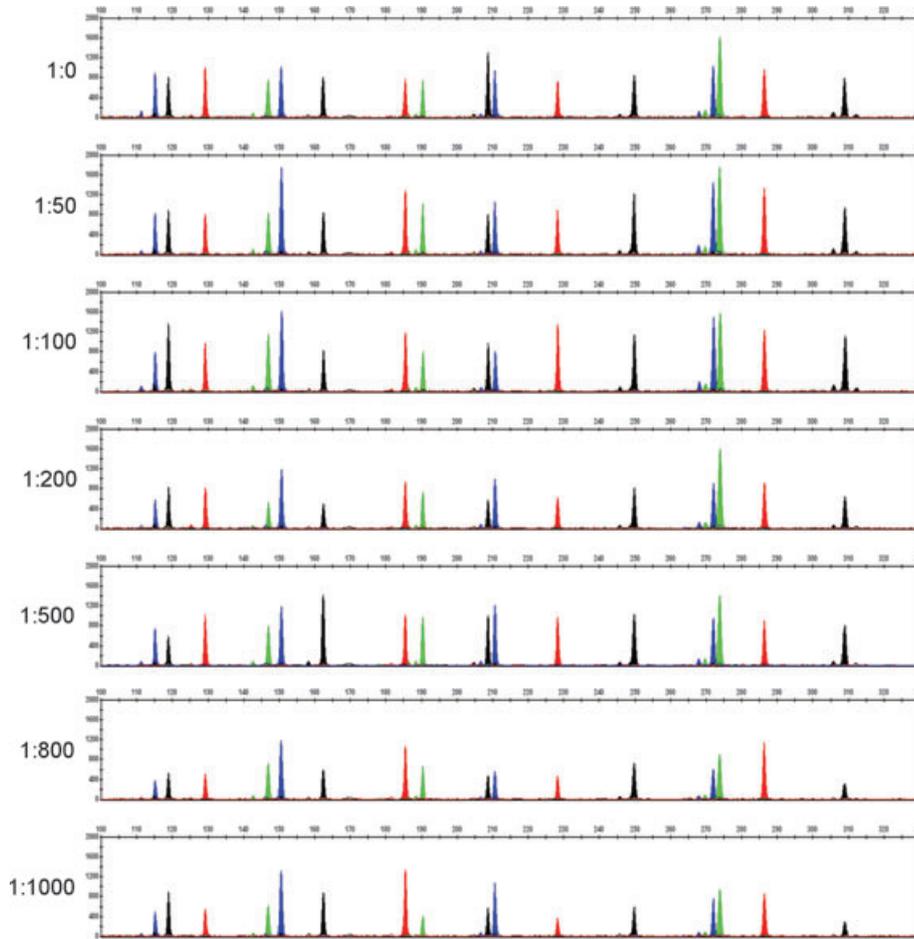
FIG. 3—*Mixture study II: STR analysis using the Yfiler® kit of heavy mixtures containing 0.025 ng/μL of male DNA and increasing amounts of female DNA according to the following male to female DNA ratios: 1:0, 1:50, 1:100, 1:200, 1:500, 1:800, 1:1000, and 0:1.*

TABLE 5—*Population study: measured quantities of human and human male DNA in samples that exhibit >25% difference in the quantities of human and human male DNA.*

Sample No.	Population	Duo Human (ng/μL)	Duo Male (ng/μL)	IPC C_T	Male-human, % difference
417	Caucasian	0.688	1.300	29.837	88.953
34	African-American	0.244	0.475	29.957	94.672
64	African-American	0.230	0.458	29.733	99.130
129	African-American	0.253	0.444	29.847	75.494
183	African-American	0.329	0.542	29.803	64.742

IPC, internal positive control.

FIG. 4—*Inhibitor study: C_T values for RPPH1, SRY, and IPC targets for inhibited samples containing 0.5 ng/μL DNA and humic acid at final concentrations of 0, 1.0, 2.0, 3.0, 3.75, 7.5, 11.25, 15, and 30 ng/μL in the qPCR.*

samples. Thus, using the results generated from the Quantifiler® Duo kit, it is possible to estimate which AmpFℓSTR® kit will be likely more successful and therefore make an educated decision to choose between autosomal STR and Y-STR analysis for genotyping male DNA in a mixture sample.

Population Study

Genomic DNA samples from 534 individual donors of Caucasian (130 male and 60 female), African-American (116 male and 24 female), and Hispanic (129 male and 75 female) population groups were analyzed using the Quantifiler® Duo DNA Quantification Kit. First, the kit detected and quantified DNA in all 534

human DNA samples (data not shown). All male samples exhibited the SRY signal. Second, the SRY signal was not detected for any of the female samples tested. Of the 375 male samples, for 370 the quantity values for human male DNA were within ±25% of the total human quantity. This range is normal and it is determined by the inherent variations in the PCR and liquid handling (pipetting). For the five remaining male samples, the kit provided male DNA quantity values that deviated from the human DNA quantity more than ±25%. The results are

TABLE 6—*Stability study: measured quantities of human and human male DNA in samples spiked with the inhibitors.*

		SRY		RPPH1		IPC	
Inhibitor	Sample Name (concentration in qPCR)	Quantity (ng/µL)	SD	Quantity (ng/µL)	SD	C_T	SD
Hematin	0 µM	0.411	0.033	0.397	0.045	29.647	0.112
	2.5 µM	0.406	0.099	0.399	0.029	29.833	0.078
	5 µM	0.393	0.041	0.358	0.045	29.677	0.123
	7.5 µM	0.371	0.035	0.253	0.030	30.050	0.122
	10 µM	0.092	0.013	0.024	0.010	31.753	0.377
	12.5 µM	0.007	0.004	0.000	–	35.347	1.048
	15 µM	0.000	0.000	0.000	–	40.000	–
	17.5 µM	0.000	0.000	0.000	–	40.000	–
	20 µM	0.000	0.000	0.000	–	40.000	–
	40 µM	0.000	0.000	0.000	–	40.000	–
Humic acid	0 ng/µL	0.411	0.033	0.397	0.045	29.647	0.112
	1 ng/µL	0.381	0.077	0.359	0.034	29.783	0.054
	2 ng/µL	0.370	0.060	0.244	0.029	29.813	0.071
	3 ng/µL	0.275	0.058	0.105	0.023	30.263	0.142
	3.75 ng/µL	0.155	0.041	0.055	0.016	30.807	0.411
	7.5 ng/µL	0.006	0.005	0.000	–	35.207	1.540
	11.25 ng/µL	0.000	0.000	0.000	–	40.000	–
	15 ng/µL	0.000	0.000	0.000	–	40.000	–
	30 ng/µL	0.000	0.000	0.000	–	40.000	–

IPC, internal positive control; SRY, sex determining region Y; RPPH1, ribonuclease P RNA component H1.
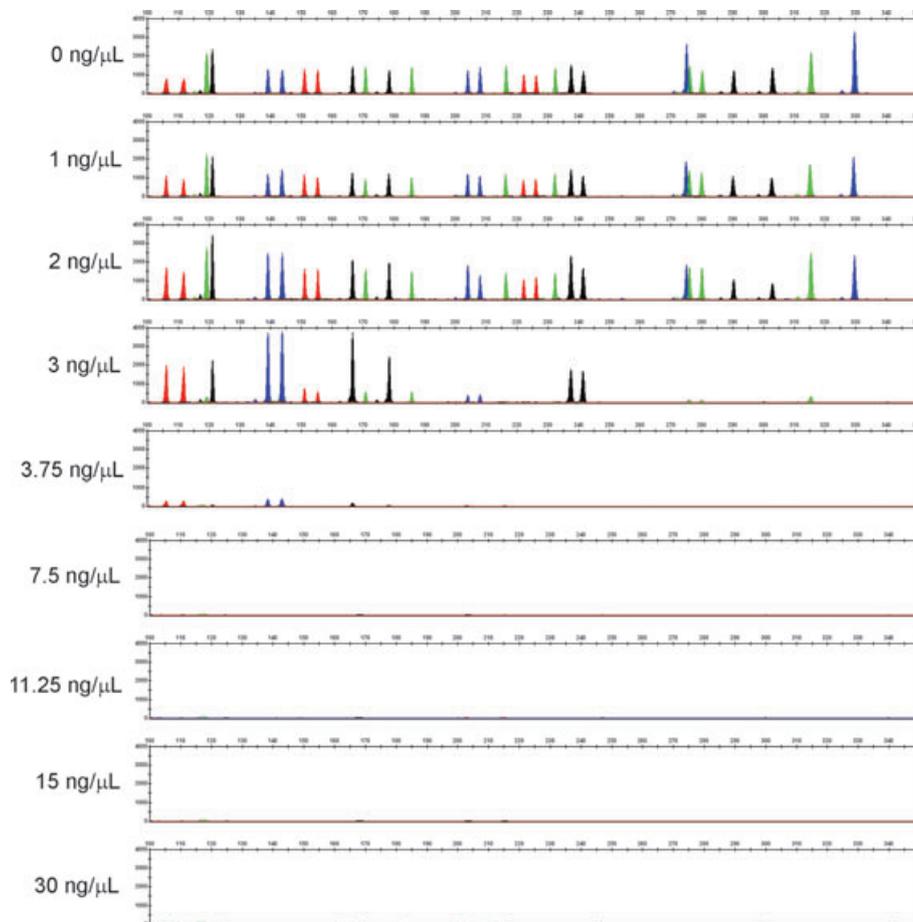
FIG. 5—*Inhibitor study: STR analysis of humic acid-inhibited samples using the Identifiler® kit. The sample name in the panel corresponds to the nomenclature in Table 6. The final concentration of the inhibitor in the PCR for STR varied and was determined by the volume of extract used. One nanogram of human DNA template (based on the quantification results summarized in Table 6) was used for amplification.*

summarized in Table 5. Higher measured quantities of human male DNA for these samples were probably because of duplication of the SRY gene. Similar findings were previously described and the ratio of human male DNA:human DNA (IDYZ5:*Alu*Ya5) measured for 54 males from different population groups ranged between 0.53 and 1.23 (7).

Inhibited Samples

Forensic DNA extracts may contain compounds which inhibit the amplification of nucleic acids if not removed during the extraction procedures. These PCR inhibitors can interfere with the reaction and cause varying levels of reduced PCR efficiency, including complete inhibition of PCR. An IPC template is co-amplified with the RPPH1 and SRY targets in the Quantifiler® Duo kit to monitor the presence of PCR inhibitors. Increased C_T values for the IPC target indicate the extent of the presence of PCR inhibitor(s). The ability of the Quantifiler® Duo assay to monitor the presence of PCR inhibitors in a given sample was investigated by adding to a DNA solution hematin and humic acid, inhibitors commonly found in blood and soiled samples, respectively. The studies were conducted to determine the limit of PCR inhibitors concentration in a forensic sample that would allow relatively accurate quantification results for obtaining an interpretable STR profile. Figure 4 summarizes the C_T values at different concentrations of humic acid. It is evident that the C_T values were relatively stable up to 3.0 ng/μL humic acid. The PCR efficiency in the Quantifiler® Duo kit decreased as the concentrations of humic acid increased. Complete inhibition of the amplification occurred at 11.25 ng/μL and higher concentration of humic acid. In general, the C_T values for all targets (human,

human male, and IPC) were affected similarly at a given concentration of inhibitor (Fig. 4). Similar results were obtained for hematin; C_T values were relatively stable up to 7.5 μM hematin and increased with increasing concentrations of the inhibitor. Complete inhibition was observed at concentrations higher than 15.0 μM hematin (data not shown).

The quantification results obtained from samples spiked with the inhibitors are shown in Table 6. The presence of hematin and humic acid resulted in the inhibition of PCR and adversely affected the quantification of DNA in a sample. Thus, the quantity of DNA was underestimated when the concentrations of hematin and humic acid were increased. Hematin and humic acid at concentrations higher than 12.5 μM and 7.5 ng/μL, respectively, completely inhibited the PCR so that no DNA was detectable in these samples. Using the quantification results from the RPPH1 target (Table 6), 1.0 ng of template DNA was introduced in the AmpF/STR® Identifiler® kit PCR amplification reaction; the results for samples spiked with humic acid are presented in Fig. 5. An interpretable, complete profile was obtained for the control sample and the samples labeled as 1 and 2 ng/μL; the sample labeled as 3 ng/μL exhibited partial profiles, and all other samples did not provide any STR profiles. The results demonstrate that an upward shift in the IPC C_T value with the Quantifiler® Duo kit can be used to predict the
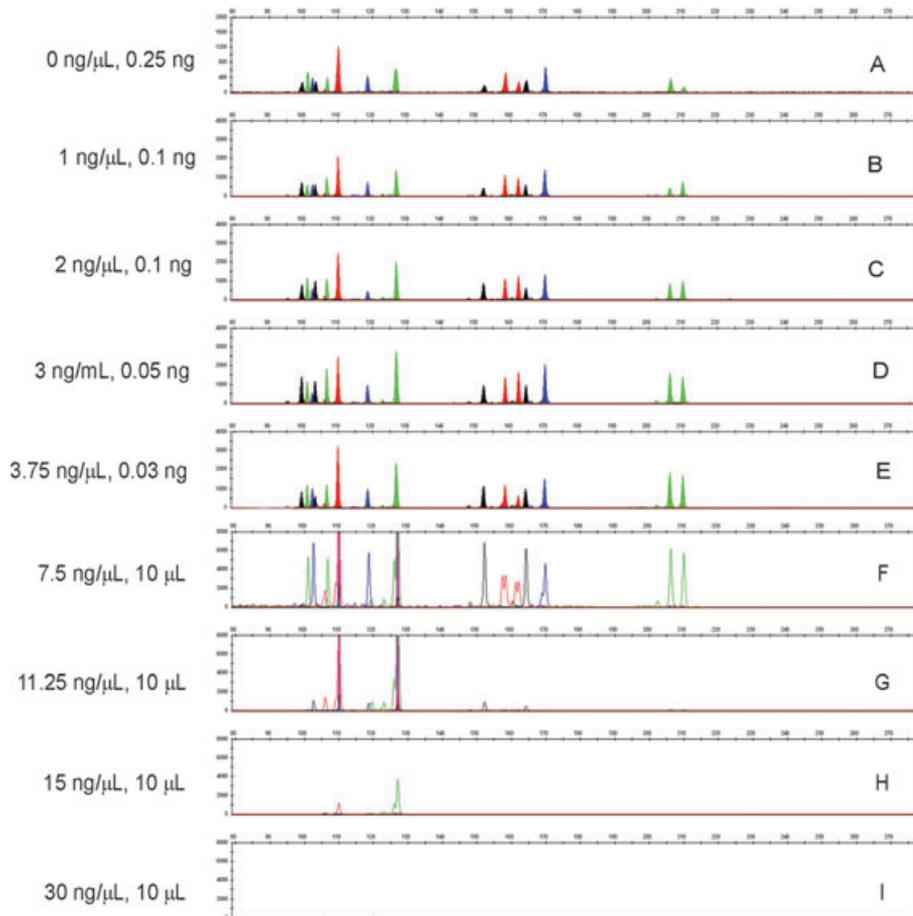
FIG. 6—*Inhibitor study: STR analysis of humic acid-inhibited samples before dilution of the highly inhibited samples using the MiniFiler^TM kit. 0.25 ng of human DNA is used for amplification of the sample in panel (A); 0.1 ng in panels (B) and (C); 0.05 ng in panel (D); 0.03 ng in panel (E) (based on the quantification results summarized in Table 6). Ten microliter of the extract is used for amplification of the samples in panels (F–I). Few alleles in panels (F) and (G) provided off-scale data as indicated by the off-scale Peak Indicator. Sample names in the panels correspond to the nomenclature in Table 6. The final concentration of the inhibitor in the MiniFiler^TM reaction varied and was determined by the volume of the extract used.*
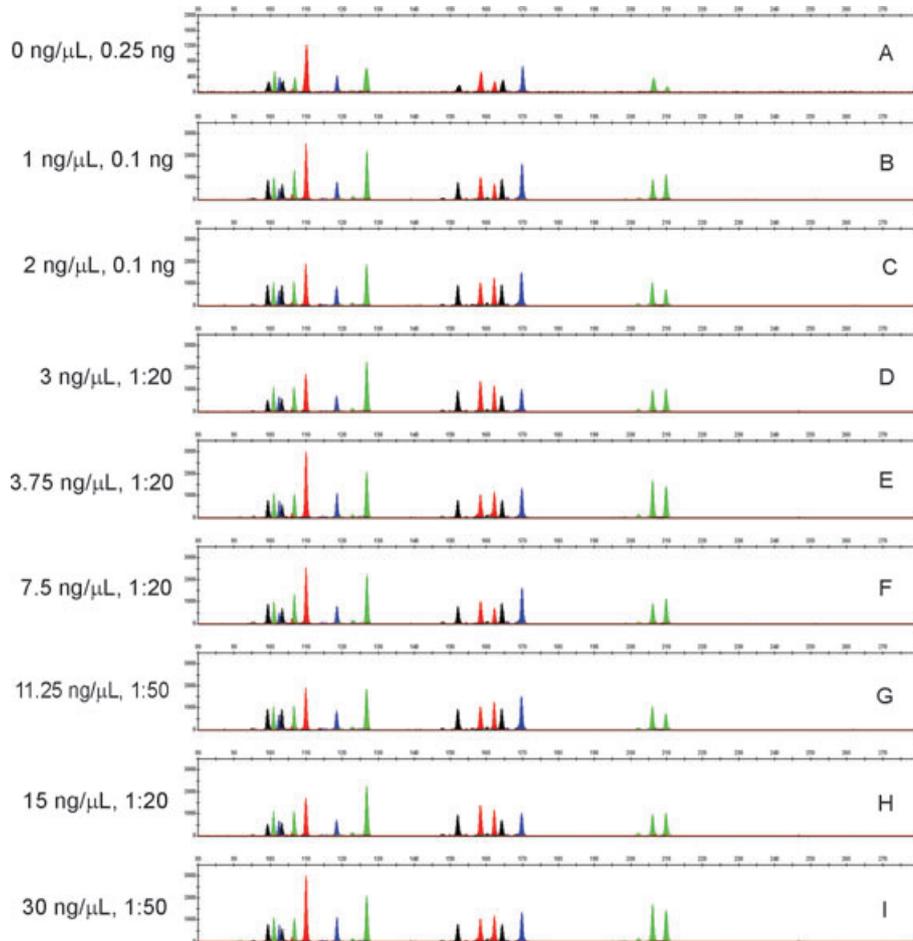
FIG. 7—*Inhibitor study: STR analysis of humic acid-inhibited samples after dilution of the highly inhibited samples using the MiniFiler*^{TM} *kit. A total of 0.25 ng of human DNA is used for amplification of the sample in panel (A); 0.1 ng in panels (B) and (C) (based on the quantification results summarized in Table 6). Ten microliters of the diluted extract, as indicated, is used for amplification of the samples in panels (D–I). The final concentration of the inhibitor in the MiniFiler*^{TM} *reaction varied and was determined by the volume of the extract used and the dilution.*

nature of the STR profile that will be generated (Figs. 4 and 5). Similar results were obtained for the samples spiked with hematin: full STR profiles were obtained for the control sample and samples labeled as 2.5 μM and 5 μM, a partial profile was obtained for the sample labeled as 7.5 μM, and no profile was obtained for all the other samples (data not shown).

The MiniFiler™ kit enables the recovery of STR profiles from compromised samples such as those which may be inhibited and/or

TABLE 7—*Stability study: measured quantities of human and human male DNA in degraded samples.*

Sample Name	DNase I (units)	SRY Quantity (ng/μL)	SD	RPPH1 Quantity (ng/μL)	SD	IPC C_T	SD
1	0	7.07	0.08	7.69	0.64	29.61	0.24
2	0.002	5.92	0.20	6.51	0.39	29.60	0.13
3	0.01	4.77	0.06	5.11	0.13	29.67	0.11
4	0.02	3.23	0.10	3.43	0.33	29.75	0.08
5	0.05	0.50	0.04	0.57	0.13	29.77	0.07
6	0.1	0.10	0.01	0.08	0.01	29.90	0.11
7	0.2	0.02	0.01	0.03	0.01	29.83	0.05

IPC, internal positive control; SRY, sex determining region Y; RPPH1, ribonuclease P RNA component H1.

degraded because of its design characterized by smaller amplicon lengths and improved PCR conditions (11). Figure 6 summarizes the results obtained with the MiniFiler™ kit for the samples spiked with humic acid. The control sample that did not contain any inhibitor generated a complete profiles when using 0.25 ng of template DNA for amplification. The samples labeled as 1 and 2 ng/μL provided complete profiles using 0.1 ng of DNA template. The samples labeled as 3 and 3.75 ng/μL provided complete profiles using 0.05 and 0.03 ng of DNA template, respectively. All of the other samples provided either partial or uninterpretable profiles using 10 μL of samples (low or no quantification results were obtained because of inhibition, Table 6). These results are consistent with observations of an IPC C_T shift during quantification (Fig. 4). Every PCR system, e.g., Quantifiler® Duo, Identifiler®, and MiniFiler™ kits, has a unique reagent formulation that provides a different response to samples containing inhibitors. When samples containing higher concentrations of humic acid were diluted (see dilution factor in Fig. 7) and 10 μL was amplified, conclusive interpretable profiles were obtained using the MiniFiler™ kit (Fig. 7). Similar results were obtained for the samples spiked with different concentrations of hematin (data not shown). Swango et al. (8) have calculated the extent of the reduction in the quantification of DNA based on the shift in the IPC C_T value. This type of prediction is applicable provided only one inhibitor is present and correlation of the IPC C_T values to the concentration of the

FIG. 8—*Degraded samples study: STR analysis of artificially degraded DNA samples using the Identifiler® kit. The DNA was treated with increasing concentrations of DNase I: (1) 0; (2) 0.002; (3) 0.01; (4) 0.02; (5) 0.05; (6) 0.1; and (7) 0.2 DNase I Units. The rfu scale varies per panel and ranges from 900 to 4000 rfu.*
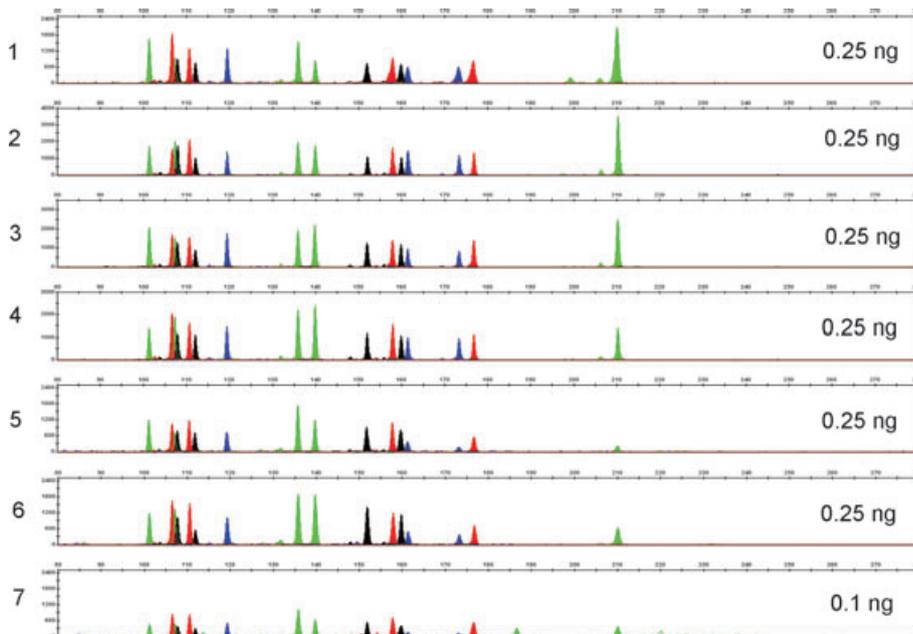
FIG. 9—*Degraded samples study: STR analysis of artificially degraded DNA samples using the MiniFiler™ kit. The DNA was treated with increasing concentrations of DNase I. (1) 0; (2) 0.002; (3) 0.01; (4) 0.02; (5) 0.05; (6) 0.1; and (7) 0.2 DNase I Units. A total of 0.25 ng of human DNA was amplified for samples 1–6; 0.1 ng for sample 7.*

inhibitor is established, as elaborated in the study (8). In our opinion, the shift in the IPC C_T provides an indication of the presence of PCR inhibitors and its correlation with the extent of underestimation of DNA quantification in forensic-type samples can become very difficult because of one or more of the following reasons: (i) it is difficult to identify the inhibitor present in the forensic sample; (ii) multiple inhibitors may be present in the sample; (iii) the inhibitor may be complexed with other compounds; and (iv) the extent of inhibition of

human, human male, and IPC targets can be affected to different extents by an inhibitor.

Degraded DNA

A common observation in forensic evidence samples is the fragmentation of full-length DNA molecules and the reduction of overall concentration of amplifiable DNA because of exposure to

TABLE 8—*Correlation study: measured and expected quantities for four male and one female DNA samples using Quantifiler® Duo, Human, and Y kits.*

Sample Name	Expected Quantity (ng/μL)	Mean Quantity Human			Mean Quantity Male		
		Quantifiler® Duo (ng/μL)	Quantifiler® Human (ng/μL)	Mean % difference*	Quantifiler® Duo (ng/μL)	Quantifiler® Y (ng/μL)	Mean % difference*
A	20.000	21.153	21.160	−0.033	20.103	16.910	18.882
	10.000	9.110	10.440	−12.739	8.983	9.020	−0.410
	1.000	0.869	0.831	4.573	0.854	1.120	−23.750
	0.100	0.091	0.071	28.169	0.083	0.113	−26.549
	0.050	0.043	0.044	−2.273	0.046	0.060	−23.333
B	20.000	24.363	27.690	−12.015	23.087	20.380	13.283
	10.000	11.493	12.290	−6.485	11.220	10.650	5.352
	1.000	1.143	1.080	5.833	1.147	1.310	−12.443
	0.100	0.104	0.098	6.122	0.110	0.160	−31.250
	0.050	0.061	0.044	38.636	0.049	0.082	−40.244
C	20.000	22.513	24.740	−9.002	23.112	20.270	14.021
	10.000	8.720	11.110	−21.512	9.250	9.220	0.325
	1.000	0.822	1.010	−18.614	0.894	1.110	−19.459
	0.100	0.098	0.129	−24.031	0.108	0.099	9.091
	0.050	0.044	0.056	−21.429	0.044	0.046	−4.348
D	20.000	27.283	16.730	63.078	26.487	22.800	16.171
	10.000	13.263	10.610	25.005	13.090	11.740	11.499
	1.000	1.217	1.470	−17.211	1.263	1.500	−15.800
	0.100	0.118	0.100	18.000	0.121	0.145	−16.552
	0.050	0.059	0.066	−10.606	0.073	0.074	−1.351
E	20.000	24.910	27.880	−10.653	Female	female	female
	10.000	12.107	13.270	−8.764	Female	female	female
	1.000	1.137	1.300	−12.538	Female	female	female
	0.100	0.116	0.162	−28.395	Female	female	female
	0.050	0.056	0.060	−6.667	Female	female	female

*Mean % difference was calculated as described in the text.

TABLE 9—*Case-type sample study: measured quantities in case-type samples.*

Sample No.	Sample Description	Quantity (ng/μL)		IPC C_T	SRY–RPPH1 (% difference)
		SRY	RPPH1		
1	Saliva on cotton swab	2.06	2.01	29.74	2.5
2	Saliva on cotton swab	11.00	11.35	29.77	−3.1
3	Blood stain on fabric	0.82	0.91	29.84	−9.7
4	Blood stain on fabric	2.09	2.07	29.68	1.0
5	Blood stain on denim	1.37	0.76	**32.54**	**81.0**
6	Blood stain on filter paper	1.35	1.34	29.48	0.7
7	Blood spiked with inhibitors and stained on fabric	1.84	1.72	29.69	7.0
8	Semen stain on fabric	1.78	1.82	29.47	−2.2

IPC, internal positive control; SRY, sex determining region Y; RPPH1, ribonuclease P RNA component H1.

environmental insults. A sample of high-molecular weight human-genomic DNA was used to generate a series of samples with varying levels of degradation. The quantity of DNA obtained by using the Quantifiler® Duo kit for the control and the samples at varying degrees of degradation is summarized in Table 7. Lower amounts of amplifiable DNA were obtained for the samples degraded with higher amounts of DNase I; the amount of human DNA decreased from about 7.69 to 3.43 ng/μL when 0.02 units of DNase I were used, and to 0.03 ng/μL when 0.2 units of DNase I were used according to the results provided by the human target RPPH1. The values obtained from the SRY target assay were very similar.

The correlation between the DNA quantity in the degraded samples and STR profiles generated for Identifiler® and MiniFiler™ was investigated. Using the RPPH1 target quantification results, 1.0 ng of DNA or 10 μL of the extract (for samples that exhibited <0.1 ng/μL of human DNA) were amplified using the Identifiler® kit. The peak heights (rfu values) of the alleles for STR loci with longer amplicons decreased for those degraded samples generated

with 0.01 and higher units of DNase I (Fig. 8, samples 3–7). However, complete interpretable STR profiles were obtained for samples generated with up to 0.02 units of DNase I (samples 1–4) and partial STR profiles were obtained for those samples generated with 0.05 and higher units of DNase I (Fig. 8, samples 5–7).

Amplification for MiniFiler™ was performed using 0.25 ng of DNA (based on the RPPH1 target quantification results) for each sample. Conclusive and complete STR profiles were obtained for samples that were generated using up to 0.02 units of DNase I (Fig. 9, samples 1–4). The samples that were generated using up to 0.05 and 0.1 units of DNase I provided interpretable profiles (samples 5 and 6); however, the amplitude (rfus) of the alleles for STR loci with longer amplicons decreased (Fig. 9). Samples that were generated using 0.2 units of DNase I provided interpretable profiles with low amplitude (rfus) for all loci when amplified using 0.1 ng of template DNA (Fig. 9, sample 7). The results indicate that interpretable profiles can be recovered from all the degraded samples generated in this study when using the Minifiler™ kit. The size
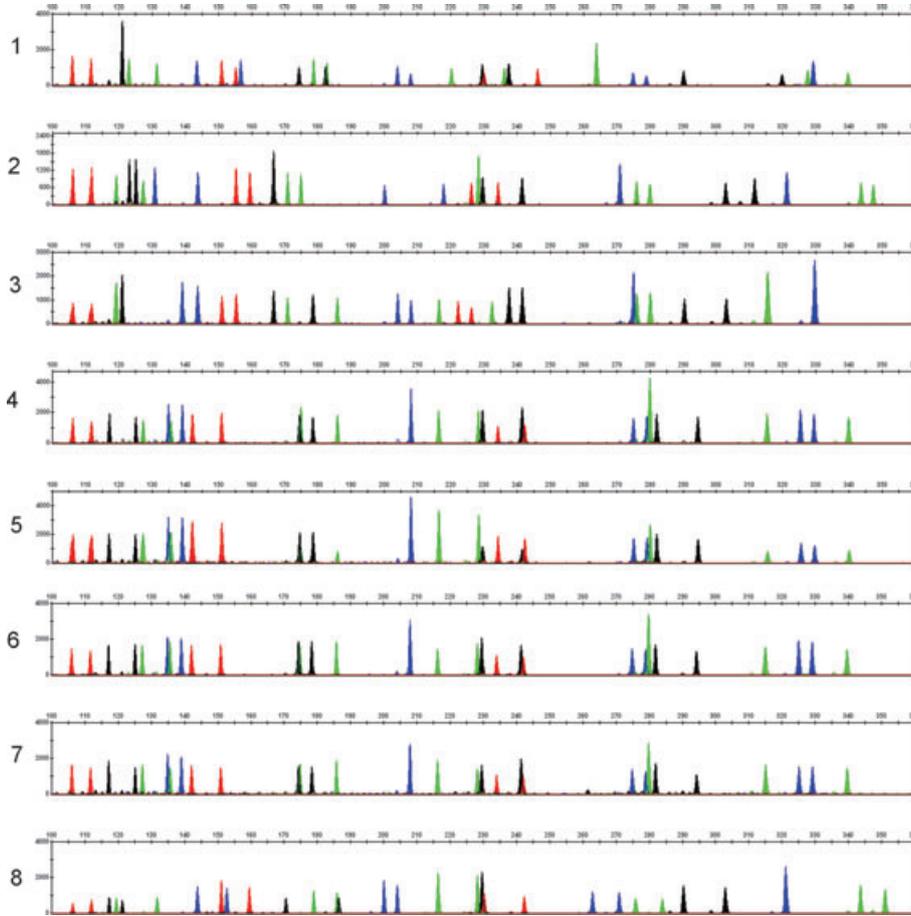
FIG. 10—*Case-type samples study: STR analysis using the Identifiler® kit of phenol-extracted DNA from the following samples: (1) saliva swab from donor a; (2) saliva swab from donor b; (3) blood stain on fabric from donor c; (4) blood stain on fabric from donor d; (5) blood stain on denim from donor d; (6) blood stain on filter paper from donor d; (7) blood stain spiked with inhibitors on fabric from donor d; and (8) semen stain on fabric from donor e.*

range of the amplicons generated using the MiniFiler™ kit is relatively smaller (70–283 bases) than those generated using the Identifiler® kit (102–359 bases) and therefore the success rate for obtaining STR profiles was higher.

Correlation Studies

Four male genomic DNA samples and one female genomic DNA sample at 20.0, 10.0, 1.0, 0.1, and 0.05 ng/μL were quantified in triplicate using the Quantifiler® Duo, Quantifiler® Human and Quantifiler® Y Human Male Quantification Kits. The quantification results from these three kits are summarized in Table 8. The mean percentage difference was calculated using the formulas:

%Difference Human
$$= \frac{(\text{Mean Quantity Q-Duo RPPH1 - Mean Quantity Q-H}) \times 100}{\text{Mean Quantity Q-H}}$$

%Difference Male
$$= \frac{(\text{Mean Quantity Q-Duo SRY - Mean Quantity Q-Y}) \times 100}{\text{Mean Quantity Q-Y}}$$

where Q-Duo is Quantifiler® Duo DNA Quantification Kit, Q-H is Quantifiler® Human DNA Quantification Kit, and Q-Y is Quantifiler® Y Human Male DNA Quantification Kit.

All male samples provided quantification results for both RPPH1 and SRY targets using these kits. No detectable SRY target signal was obtained for the female DNA sample, at any concentration, using the Quantifiler® Duo (male target) and Quantifiler® Y Human Male DNA Quantification Kits. The quantities of human and male DNA obtained from the Quantifiler® Duo DNA Quantification Kit were similar to the quantities obtained using either the Quantifiler® Human or Quantifiler® Y Human Male DNA Quantification Kit, respectively. The differences in the quantities of DNA obtained by the three kits in the present study may be due to one or more of the following: (i) the difference in the amplification targets used for quantification of human DNA: RPPH1 in the Quantifiler® Duo DNA Quantification Kit and hTERT in the Quantifiler® Human DNA Quantification Kit; (ii) the difference in the sizes of the human DNA targets: 140 bases in the Quantifiler® Duo DNA Quantification Kit and 62 bases in the Quantifiler® Human DNA Quantification Kit; (iii) the difference in the sizes of the male DNA targets: 130 bases in the Quantifiler® Duo kit and 64 bases in the Quantifiler® Y Human Male DNA Quantification Kit though in both kits the male target is SRY gene; (iv) the differences in assay complexity: the Quantifiler® Duo DNA Quantification Kit is a triplex PCR assay and the Quantifiler® Human and Quantifiler® Y Human Male DNA Quantification Kits are duplex PCR assays; (v) differences in the quantification standards used in the respective kits: human male genomic DNA in the Quantifiler® Duo DNA Quantification Kit and a cell line DNA in the Quantifiler®
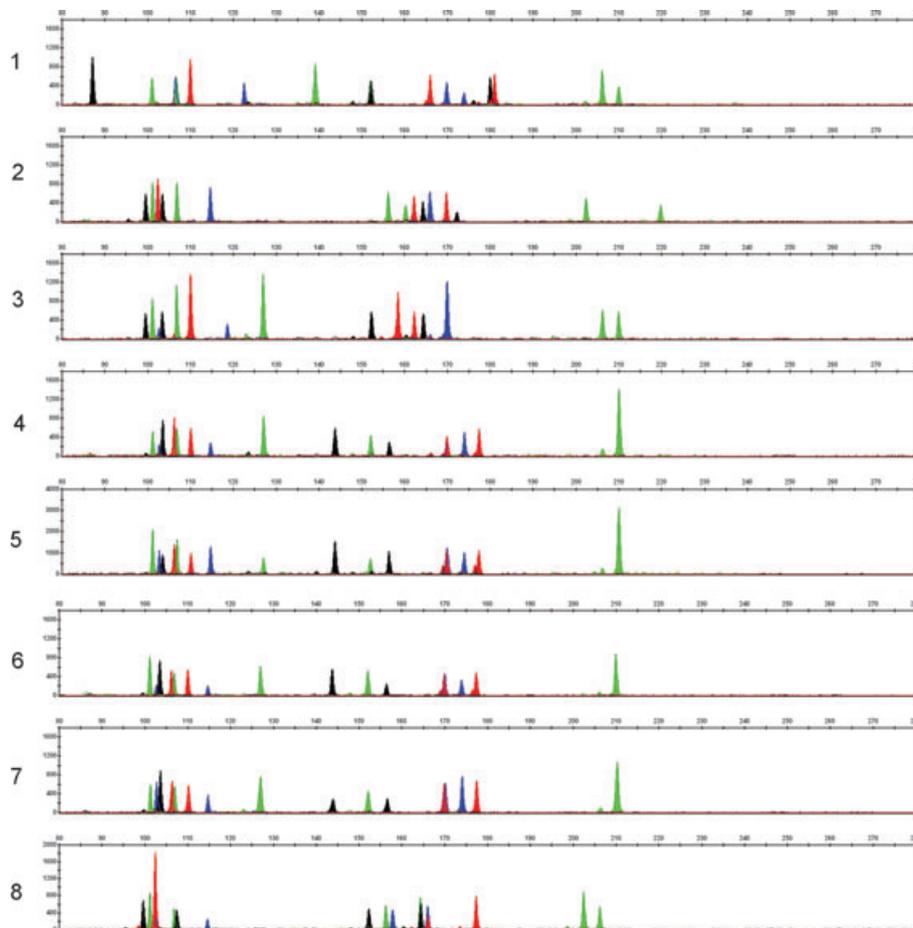
FIG. 11—*Case-type samples study: STR analysis using the MiniFiler^{TM} kit of phenol-extracted DNA from the following samples: (1) saliva swab from donor a; (2) saliva swab from donor b; (3) blood stain on fabric from donor c; (4) blood stain on fabric from donor d; (5) blood stain on denim from donor d; (6) blood stain on filter paper from donor d; (7) blood stain spiked with inhibitors on fabric from donor d; and (8) semen stain on fabric from donor e.*

Human and Quantifiler® Y Human Male DNA Quantification Kits; and (vi) differences in the optimized reaction mix: each PCR reaction mix is optimized to deliver the expected performance for a given kit.

The DNA quantification is performed to determine the volume of the DNA extract to be amplified to produce high-quality STR genotyping results. Thus, determining the optimum amount of input DNA required for each STR genotyping system based on the quantification values obtained using the Quantifiler® Duo kit may be required in each laboratory.

Case-Type Samples

Forensic-type samples were prepared using saliva, blood, and semen (samples 1–8) obtained from multiple male individual donors (donors a to e) on different substrates. The DNA was extracted by the phenol:chloroform extraction method and quantified in triplicate using the Quantifiler® Duo DNA Quantification Kit (Table 9). The yields of DNA ranged from 0.76 to 11.35 ng/μL for the RPPH1 target and 0.82 to 11.0 ng/μL for the SRY target. DNA quantities obtained for the human and male targets for all samples were similar except for the blood stain on denim (Table 9). For this sample, the quantity of male DNA was 81% higher than the quantity obtained from the human target. The blood stain on denim is a challenging sample type because of the combination of inhibitors present on the denim substrate and in

the blood. The lower quantity of total human DNA may result from inhibition of the amplification of the RPPH1 target, which is consistent with the greater C_T value obtained for the IPC indicating the presence of PCR inhibitors (numbers are marked in bold in Table 9). Complete and interpretable STR profiles were obtained for all the samples analyzed with peak heights between 500 and 4000 rfu for the Identifiler® using 1.0 ng of human DNA and 200–2000 rfu for the MiniFiler^{TM} using 0.1 ng of human DNA (Figs. 10 and 11).

Conclusions

The Quantifiler® Duo DNA Quantification Kit enables quantification of total human DNA and human male DNA and detection of the presence of PCR inhibitors in biological samples in a single amplification reaction. In addition to the quantification of DNA, the assay provides a quality assessment of forensic evidence samples in terms of determining male to female DNA mixture ratios and estimating the extent of PCR inhibition. The information is useful in selecting the appropriate AmpFℓSTR® amplification chemistry for obtaining an interpretable STR profile in the first attempt. The developed assay consumes minimal quantity of forensic sample. The Quantifiler® Duo DNA Quantification Kit is validated following the SWGDAM guidelines and its utility in forensic DNA analysis is demonstrated using forensic-type samples including mixtures, inhibited, and degraded samples. We developed a

reliable and robust assay for obtaining quantification results and assessment of the DNA extract for subsequent STR profiling. Based on the mixture ratio of human and human male DNA and the extent of PCR inhibition, it is possible to choose between autosomal and Y-STR profiling or to select a robust STR profiling kit such as the MiniFiler™ kit rather than repurifying a certain sample. The quantification results can then be utilized to determine the volume of DNA extracted to be amplified to obtain an interpretable STR profile.

In conclusion, the results obtained using the Quantifiler® Duo kit can aid in determining (i) if the sample contains sufficient human DNA and/or human male DNA to proceed with STR analysis, (ii) the optimal amount of sample to use in the various STR analysis applications, (iii) the relative quantities of human male and female DNA in a sample that can assist in the selection of the applicable STR chemistry, and (iv) if PCR inhibitors are present in a sample that may require additional purification before proceeding to STR analysis. The Quantifiler® Duo kit, therefore, is a useful tool that provides guidance for the selection of the appropriate AmpFℓSTR® amplification kit in order to increase the success of STR profiling in the first attempt. This approach will reduce the number of samples that need reprocessing thereby decreasing the turnaround time in a forensic laboratory.

Acknowledgments

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