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Quantifiler® Duo DNA Quantification Kit: A Guiding Tool for Short Tandem Repeat Genotyping of Forensic Samples

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Abstract

Forensic analysts routinely encounter samples containing mixtures of DNA from male and female contributors and PCR inhibitors due to exposure to environmental insults. In order to select the appropriate STR analysis methodology for such samples and obtain optimal results at first pass, it is desirable to determine the relative quantities of male and female DNA and to detect the presence of PCR inhibitors at an early stage in the sample processing workflow. Here we describe a multiplex real-time PCR assay that can provide the desired information in a single reaction. Briefly, the simultaneous quantification of human and human male DNA is achieved by measuring the RPPH1 human target and the SRY male-specific target. At the same time a synthetic sequence is co-amplified as an Internal PCR Control (IPC) to detect the presence of PCR inhibitors. The assay has a good dynamic range (0.023–50 ng/µL) and can detect 25 pg/µL of human male DNA in the presence of ten thousand-fold excess of human female DNA. In addition, the ability of the assay to predict PCR inhibition was demonstrated by shifted IPC C_T values in the presence of increasing quantities of hematin. All the real-time PCR results showed a good correlation with the downstream STR profiles obtained from a large set of various sample types therefore demonstrating that this assay can be considered a guiding tool to predict the performance of the STR genotyping kits with forensic samples.

Keywords: DNA quantification; Real-time PCR; DNA analysis; Human DNA; Human male DNA; DNA typing

Abbreviations: DNA: Deoxyribo Nucleic Acid; IPC: Internal PCR Control; LOD: Limit of Detection; LOQ: Limit of Quantification; PCR: Polymerase Chain Reaction; RPPH1: Ribonuclease P RNA Component H1; SRY: Sex Determining Region Y; STR: Short Tandem Repeat; Y-STR: Y-chromosome STR

Introduction

Quantification of human DNA plays a central role in forensic short tandem repeat (STR) profiling for many reasons. Forensic evidence samples routinely contain DNA from non-human species, are contaminated with compounds that inhibit PCR, are mixtures from female and male contributors in various proportions, and have been exposed to environmental insults, leading to DNA degradation. All these factors make STR analyses more challenging. In addition, several autosomal STR and Y-chromosome STR (Y-STR) genotyping kits are now available commercially and mtDNA analysis is available from regional centers. Thus, to select the appropriate STR analysis methodology and obtain interpretable STR profiles, it is desirable to determine the quantity of human DNA, estimate relative quantities of female and male DNA, and detect PCR inhibitors. Detection of the minor male component in sexual assault samples during the quantification step can be valuable in determining the need to follow-up with Y-STR analysis. Detection of both total human and human male components in a single reaction reduces sample consumption. Further, detection of potential PCR inhibitors is useful in directing further sample analysis towards re-extraction of nucleic acid or the use of more robust kits like AmpF*l*STR® MiniFiler™ or Identifiler® Plus PCR DNA Amplification kit.

The Quantifiler® Duo DNA quantification kit was designed to address all these needs and in particular for processing sexual assault cases. The multiplex assay can quantify total human DNA and human male DNA simultaneously, determine the male to female DNA ratio, and detect PCR inhibitors in a single TaqMan® assay reaction. The Quantifiler® Duo Kit, therefore, provides an assessment of the DNA extract obtained from the forensic sample and generates information to select an appropriate STR profiling system. This tool would enable acquisition of interpretable STR profiles on the first attempt using a minimal quantity of evidence samples thereby saving reagents, reducing the reprocessing rate and the time to result for forensic analysts.

The amplicon sizes of the targets in the multiplex were selected to ensure equivalent amplification efficiencies and quantification values that maximize the success rate for obtaining interpretable STR profiles even when the DNA is degraded. We tested a very broad range of amplicon sizes and determined which one was the most appropriate to provide the best success rates across multiple STR genotyping kits. Lastly, we checked the copy number variation databases (http://www.sanger.ac.uk/humgen/cnv/) to ensure that the human gene targets selected exhibited minimal copy number variation across the major human populations.

In this article, we describe the features of the multiplex assay we developed with respect to its high sensitivity, precision and accuracy, reproducibility, robustness, ability to detect inhibitors, and ability to predict the generation of interpretable STR profiles even with compromised and degraded DNA samples. In addition, we report some challenges we encountered during the development and optimization of the multiplex assay.

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Materials and Methods

Real-time PCR amplification setup

The principles and mechanism of absolute quantification using a standard curve and a TaqMan® assay reaction were described earlier [1, 2]. Briefly, in order to obtain quantification results from an unknown DNA sample, its C_T value must be interpolated into a standard curve generated in the same experiment. Thus, pooled human male genomic DNA (EMD Biosciences Inc. Madison, WI) at eight different concentrations (50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068 and 0.023 ng/µL) must be amplified in duplicate on each quantification run plate to compute standard curves for RPPH1 and SRY targets (Figure 1).

Real-Time PCR amplification was carried out in 25 µL reactions containing 10.5 µL of Primer Mix, 12.5 µL of PCR Reaction Mix, and 2.0 µL of DNA sample. The Primer Mix contained forward and reverse primers and TaqMan® probes (Applied Biosystems, Foster City, CA) 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 PCR Reaction Mix contained ROX™ reference dye , dNTPs, dUTP, $MgCl_2$, AmpliTaq Gold® DNA polymerase and preservatives in Tris-HCl, pH 8.0 (Applied Biosystems, Foster City, CA). Genomic DNA from unknown individuals was obtained from Biochain (Hayward, CA), or Serological Research Institute (Richmond, CA). Non-human 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). The DNA from anonymous donor samples (blood, saliva and semen) was extracted by using standard phenol-chloroform method [3].

Amplification reactions were performed in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) 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, Foster City, CA) with a baseline of 3 to 15 cycles and a threshold value of 0.2. The 7500 SDS software provides an easy to follow wizard for creating and setting up a plate document representing the arrangement of samples (standards and unknowns) and reagents on the reaction plate.

A new version of the instrument software, HID Real-Time PCR Analysis Software v1.0, was launched to include features that will make even easier to set up and analyze quantification runs: pre-packaged templates with pre-assigned target detectors and pre-set standard curve parameters; quality flags to ease data analysis and provide guidance for downstream STR analysis; STR reaction setup calculations; and creation of a final report.

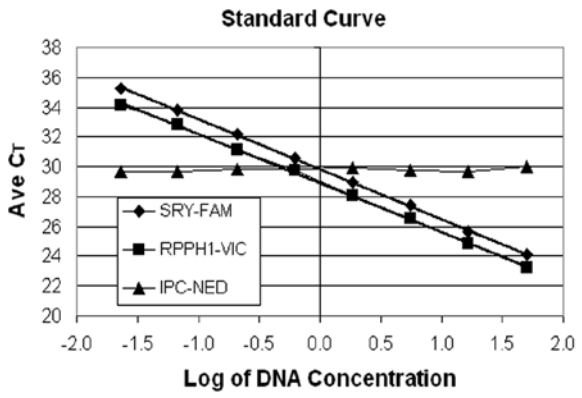

Figure 1: Example of a typical standard curve: C_T values for RPPH1, SRY and IPC targets across the eight standard DNA concentrations (50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068 and 0.023 ng/µL). Reproduced with permission from Reference 12.

STR analysis

The samples were amplified with different AmpFℓSTR® PCR Amplification Kits including Identifiler®, Yfiler® and MiniFiler™ kits (Applied Biosystems, Foster City, CA) using the procedure described in the User's Manual for each respective kit. The amplified products were subjected to capillary electrophoresis on a 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA) and the data analyzed using GeneMapper® ID Software v3.2.1 (Applied Biosystems, Foster City, CA).

Results

Assay description and development

We developed a multiplex assay for simultaneous amplification of the human autosomal target ribonuclease P RNA component H1 (RPPH1; Chromosome: 14; Location: 14q11.2; GeneID: 85495), the human male-specific Y-chromosome target sex determining region Y (SRY; Chromosome: Y; Location: Yp11.3; GeneID: 6736), and a synthetic oligonucleotide sequence embedded in a plasmid as an internal PCR control (IPC). The three TaqMan® probes were labelled with VIC®, FAM™ and NED™ dyes, respectively (Table 1). Measurement of the extent of amplification of RPPH1, SRY, and IPC enables the quantification of total human DNA, quantification of human male DNA and indication of the presence of inhibitors of PCR, respectively.

Particular attention was paid to the target gene choice and to the assay design. We carefully chose target genes that were not related to human diseases, that were not affected by copy number variation or the presence of known Single Nucleotide Polymorphisms (SNPs) and that were human/non-human primate specific. To accomplish these goals, the potential target genes were evaluated by screening the NCBI database for cancer and other disease-related targets and then the assays were designed using the proprietary TaqMan® Gene Expression assay design pipelines [4] (www.allgenes.com) targeting genomic DNA and avoiding gene regions where known SNPs were present.

Furthermore, we checked the copy number variation database (http://www.sanger.ac.uk/humgen/cnv/) to ensure that the selected human gene targets exhibited minimal copy number variation across the main human populations. Often multicopy genes may be selected as targets of interest for quantification assays because their nature can boost the overall sensitivity of the assay, especially at low DNA concentrations where stochastic effects may affect the intensity of the fluorescence signal. The risk is that such genes may be repeated a different number of times within the genome across different human populations therefore under-mining the accuracy of the measurement. For this reason, we avoided targeting genes like *TSPY* for quantification because the copy number of this gene varies from few copies to 41, with a mode of about 25, in the human male population [5]. Instead, we used single copy targets to better correlate quantification results to the performance of the downstream STR chemistries that utilize single copy targets for genotyping purposes. Furthermore, the DNA included in the kit and used to generate the standard curve is human genomic DNA pooled from multiple donors (traditional Quantifiler® kits used DNA extracted from a mammalian cell line) to avoid any copy number

discrepancy between the standard itself and the unknown sample. In addition, using the proprietary assay design pipeline allowed us to create a very specific assay with minimal cross-reactivity to non-human DNA. Species specificity of the primers present in our multiplex assay was demonstrated using the DNA from several mammalian and microbial sources. The two human control samples, one male and one female, showed expected results. None of the other species investigated exhibited amplification signal except for chimpanzee, suggesting that the developed triplex assay is specific for human DNA and some higher primates. Cross reactivity of the human DNA quantification assays as well as STR profiling systems to higher primates is reported by many investigators [6-11].

A challenge encountered during the development of the Quantifiler® Duo assay was selecting the amplicon size of the two target genes. The objective was to improve and expand upon the previous Quantifiler® Human DNA Quantification Kit (which has been widely adopted in forensic laboratories) to provide a more reliable estimate of the human DNA present not only in pristine samples, but also in compromised samples. Such samples may be exposed to environmental conditions that degrade DNA molecules and reduce amplification efficiency in PCR reactions. Exposure to environmental conditions may cause fragmentation of full length DNA molecules and can reduce the overall concentration of amplifiable DNA. If the amplicon size of the quantification assay is too small, the results may overestimate the amount of DNA that can be amplified by the downstream STR chemistries (except for mini STR analysis) leading to incomplete or uninterpretable genotyping profiles.

We tested a very broad range of amplicon sizes from 60 bp to as large as 338 bp and determined that an amplicon size of about 140 bp provided success across multiple STR genotyping kits even when the DNA quality is compromised. The RPPH1 target amplicon size is 140 bp and the SRY amplicon size is 130 bp (Table 1). These amplicon sizes estimate the quantity of DNA required for amplification for the majority of the loci in the AmpFℓSTR® PCR Amplification Kits: 13 STR loci in Identifiler® kit (except D3 and D19), 5 STR loci in MiniFiler™ kit (except D13, D16 and CSF1PO) and 14 STR loci in Yfiler® kit (except DYS393 and DYS456). To ensure similar amplification efficiency across all three targets of the multiplex, we had to redesign the IPC template so that the IPC amplicon size would match that of the other two targets. We selected an amplicon size of 130 bp, but nonetheless the IPC assay exhibited preferential amplification. We realized that not only the amplicon size is important, but also the complexity and the accessibility of the target sequence. The IPC artificial template was a single stranded DNA sequence, easily accessible to the polymerase whereas the RPPH1 and SRY genes were "hidden" in the complex human genome. Thus, we inserted the IPC synthetic template in a plasmid achieving similar extent of amplification among all three targets not only in clean samples, but especially in samples containing PCR inhibitors.

Lastly, the whole multiplex assay was optimized using multifactorial designs (Design Of Experiment studies) to unveil interdependencies among the considered variables (primer concentration, probe concentration, master mix components concentration, etc.) and optimize responses.

Assay performance

The assay performance was studied by measuring sensitivity, precision and accuracy, and reproducibility.

Real time PCR quantification assays are designed for estimation of the quantity of DNA in a sample within the linearity range of the

Target	Marker	Amplicon Size	Reporter Dye
Human DNA	RPPH1 (Ribonuclease P RNA component H1)	140 bp	VIC®
Human Male DNA	SRY (Sex determining Region Y)	130 bp	FAM™
IPC	Artificial Template	130 bp	NED™

Table 1: Configuration of the Quantifiler® Duo DNA quantification kit.

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	----	---	0.006	0.007
0.00144	----	0.006	----	----

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Table 2: Sensitivity study: measured and expected quantities for two samples.

amplification of the respective targets. However, forensic scientists tend to extend the use of the assay to screening applications to detect the presence of DNA, particularly male, below the quantification range. Thus, it is important to distinguish between the limit of quantification (LOQ) and limit of detection (LOD). Sensitivity studies were performed to determine the range of DNA concentrations that generate reliable quantification (LOQ) and detection (LOD). A series of dilutions ranging from 20.0 to 0.00144 ng/µL were prepared for two human male genomic DNA samples (one pooled and the other single source). The DNA preparations obtained from commercial sources were diluted in 10 mM Tris buffer, pH 8.0 containing 0.1 mM EDTA and each dilution was quantified in triplicate using the Quantifiler® Duo DNA Quantification Kit. The linearity of quantification using the Quantifiler® Duo assay ranged from 50 ng/µL to 23 pg/µL as represented by the linearity of the standard curve in this range. The quantities of human and human male DNA obtained from the Quantifiler® Duo kit for the two samples investigated were similar to the expected quantities across a range of concentrations from 20 ng/µL to 23 pg/µL, the latter being the LOQ of the assay (Table 2). It was possible to detect quantities as low as 5.75 pg/µL for the human assay (LOD) with some variation for the male specific assay, due to its haploid nature (Y chromosome loci are haploid). Lower DNA concentrations could not be reproducibly detected across all replicates due to stochastic variation in sample input. Thus, greater variation in the quantification results was observed for the samples containing lower quantities of DNA. The Quantifiler® Duo Kit assay can accurately quantify 23 pg/µL of human genomic DNA in a sample. When 2.0 µL of a sample at this concentration is loaded in a reaction, the well contains only approximately 7 diploid human genome equivalents. These equivalents correspond to approximately 14 copies of the RPPH1 target and approximately 7 copies of the SRY target.

The major objective of estimation of human or male DNA is to determine the volume of extract to be used for amplification of

Sample	Sample Dilution (ng/µL)	SRY			RPPH1			IPC		
		Mean Quantity (ng/µL)	Std Dev	95% Confidence (± percent)	Mean Quantity (ng/µL)	Std Dev	95% Confidence (± percent)	C_T	Std Dev	95% Confidence (± 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	8.91	9.11	0.341	7.49	29.73	0.034	0.23
	1	0.85	0.109	25.65	0.87	0.051	11.72	29.92	0.034	0.23
	0.10	0.08	0.007	17.50	0.09	0.014	31.11	29.97	0.039	0.26
	0.05	0.05	0.028	112.00	0.04	0.002	10.00	30.00	0.032	0.21
B	20	23.09	2.219	19.22	24.36	1.656	13.60	29.79	0.038	0.26
	10	11.22	0.485	8.65	11.49	0.529	9.21	29.77	0.046	0.31
	1	1.15	0.142	24.70	1.14	0.083	14.56	29.89	0.030	0.20
	0.10	0.11	0.013	23.64	0.10	0.008	16.00	29.98	0.040	0.27
	0.05	0.05	0.015	60.00	0.06	0.007	23.33	29.98	0.012	0.08
C	20	23.11	0.821	7.11	22.51	0.294	2.61	29.62	0.055	0.37
	10	9.25	0.601	12.99	8.72	0.562	12.89	29.67	0.053	0.36
	1	0.89	0.039	8.76	0.82	0.027	6.59	29.81	0.057	0.38
	0.10	0.11	0.027	49.09	0.10	0.008	16.00	29.89	0.053	0.35
	0.05	0.04	0.020	100.00	0.04	0.008	40.00	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	9.11	13.26	0.261	3.94	29.87	0.028	0.19
	1	1.26	0.136	21.59	1.22	0.081	13.28	29.76	0.055	0.37
	0.10	0.12	0.032	53.33	0.12	0.008	13.33	30.02	0.034	0.23
	0.05	0.07	0.021	60.00	0.06	0.005	16.67	29.97	0.042	0.28
E	20	female	----	----	24.91	0.586	4.70	29.97	0.023	0.15
	10	female	----	----	12.11	0.486	8.03	29.87	0.007	0.05
	1	female	----	----	1.14	0.049	8.60	29.77	0.018	0.12
	0.10	female	----	----	0.12	0.016	26.67	29.73	0.089	0.60
	0.05	female	----	----	0.06	0.008	26.67	29.71	0.047	0.32

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Table 3: Reproducibility study: measured and expected quantities, standard deviations and 95% confidence intervals for four male and one female DNA samples across three consecutive runs.

STR multiplex systems. The maximum sample volume that can be incorporated in most of the STR multiplex systems is 10 µL. Thus, 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, the lack of accuracy in quantifying samples containing less than 0.1 ng/µL of DNA doesn't affect the downstream STR reaction setup.

The precision of the Quantifiler® Duo Kit was tested by performing two runs on different days (one run per day) on three different instruments. The pooled human male DNA standard present in the Quantifiler® Duo Kit was diluted to obtain solutions containing 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068 and 0.023 ng/µL of DNA. Using this set of DNA dilutions, six reaction plates were set up and each of them contained 10 replicates of the 8 dilutions. Two plates per instrument were run on three different 7500 Real-Time PCR System instruments. The C_T values for RPPH1, SRY and IPC targets were recorded for all 60 reactions of each dilution. The standard deviation values for the RPPH1, SRY, and IPC targets across all 6 plates ranged from 0.23 to 0.58, 0.15 to 0.63, and 0.17 to 0.35, respectively (data not shown). The data showed that at lower DNA concentrations, the standard deviations increased, most likely due to stochastic effects [12].

Replicate analysis of human DNA samples was performed using the Quantifiler® Duo Kit to assess the reproducibility of the quantification results obtained.

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 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.

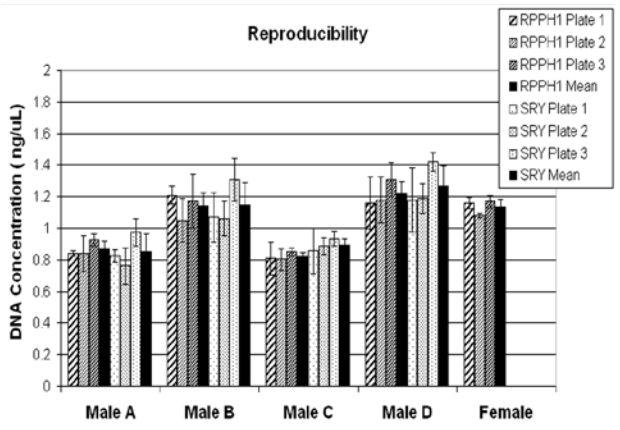

Figure 2: Reproducibility of the Quantifiler® Duo DNA quantification kit. Five samples diluted to various concentrations of DNA were quantified in triplicates. Results for all samples at 1 ng/µL of human DNA are shown as an example.

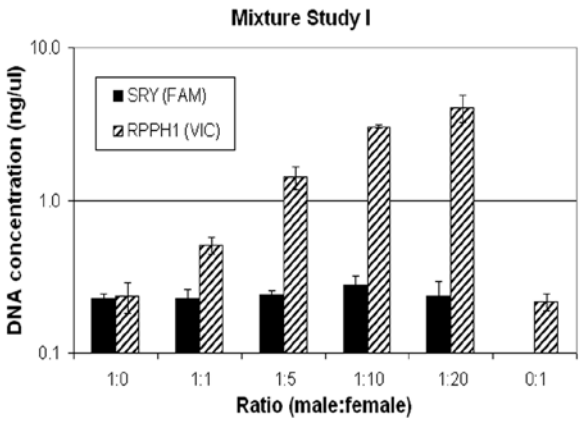

Figure 3: Mixture Study I: Quantification values obtained for a constant amount of male DNA (0.2 ng/µL) in the presence of increasing amounts of female DNA.

Three runs were performed on different days. For each sample reaction, the C_T values were obtained and the DNA quantities calculated. The quantification results obtained were highly reproducible; the standard deviation values for the quantification of the SRY and RPPH1 targets ranged from 0.007 to 2.219 and 0.002 to 1.835, respectively (Table 3). Similarly, the standard deviation values for the C_T of the IPC target ranged from 0.007 to 0.106. Figure 2 shows graphically the reproducibility results for the five samples at 1 ng/µL. At this concentration, the range of standard deviations for each target is 0.027 to 0.083 for the human target and 0.039 to 0.142 for the male target.

Analysis of forensic-type samples

Mixture study: Interpretation of mixture sample STR profiles from sexual assault cases creates different types of challenges for forensic scientists. The DNA from the perpetrator is often present in small quantity compared to the DNA from the victim. If the victim DNA is present in large quantity, the amplification of the minor contributor DNA may get suppressed or provide uninterpretable results using autosomal STR analysis because of the endpoint nature of this analysis. In the real-time PCR assays, unlike amplification of autosomal STRs, it is possible to detect the presence of a male minor contributor in the presence of excess female DNA since measurement of amplification is recorded at each cycle during all phases of amplification curve. The information about the mixture sample enables the forensic analyst to make a decision regarding the use of autosomal STR or Y-STR profiling, an important decision that may determine the success of the analysis. The mixture studies we performed were designed to simulate circumstances in which a small component of male DNA must be discerned from a high background of female DNA. 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 (0.2 ng/µL) with different quantities of human female DNA. The ratio of male to female

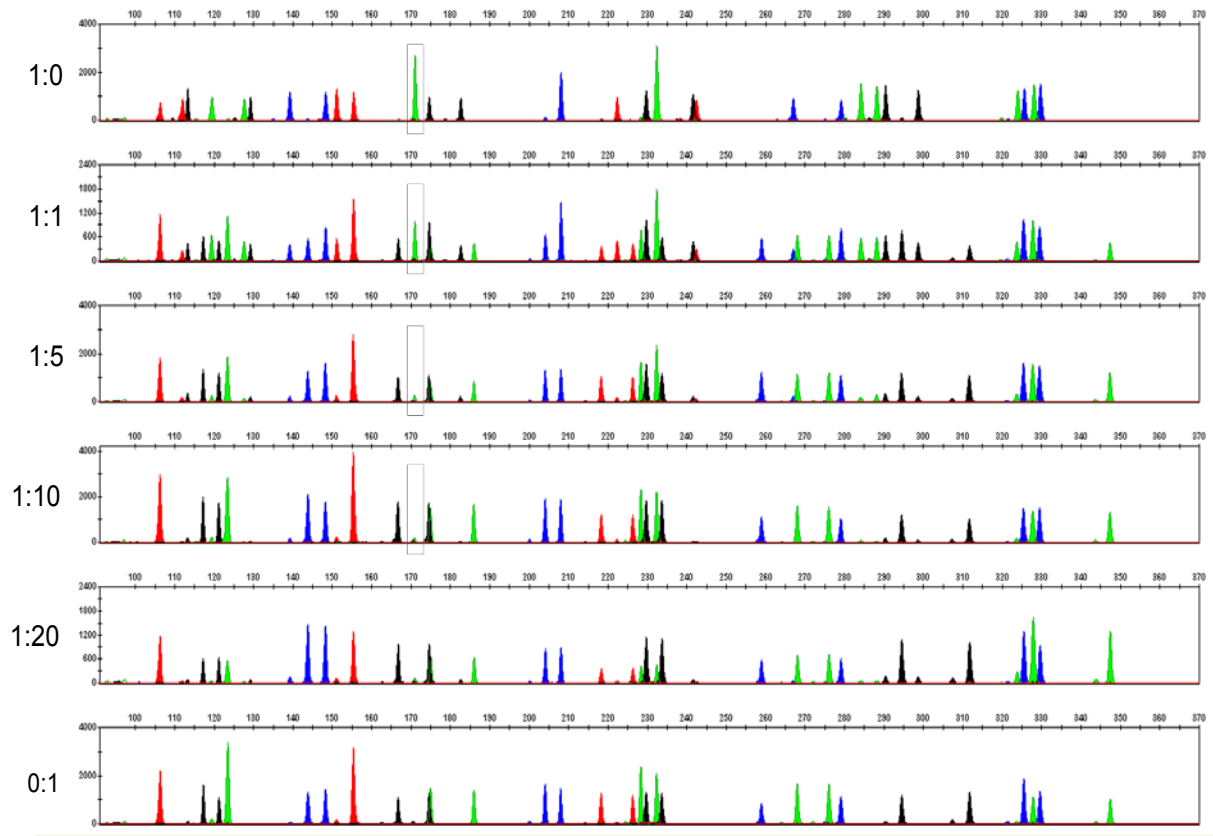

Figure 4: Mixture study I: Identifiler® profiles of mixtures containing 0.2 ng/µL of male DNA and increasing amounts of female DNA according to the following male to female DNA ratios: 1:0, 1:1, 1: 5, 1:10, 1:20 and 0:1. The rectangles indicate a peak belonging to the male minor component of the mixtures. Reproduced with permission from Reference 12.

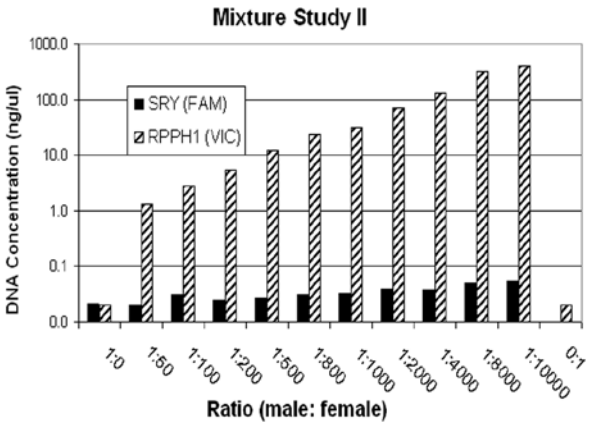

Figure 5: Mixture Study II: Quantification values obtained for a constant amount of male DNA (0.025 ng/µL) in the presence of increasing amounts of female DNA.

DNA in the mock mixture samples was 1:0, 1:1, 1:5, 1:10, 1:20 and 0:1. Quantification results for the mixture samples are graphed in Figure 3. For all samples, the male DNA produced consistent quantification values at all excess quantities of female DNA present. Thus, the ability to quantify the male DNA was not adversely affected by the presence of the quantities of female DNA investigated. Based on the results from the RPPH1 target, approximately 1.0 ng of human genomic DNA from each sample was analyzed using the Identifiler® Kit. As expected, the peak height of male alleles decreased with increases in the ratio of female to male DNA (Figure 4). Interpretation of the minor male profile in such mixture samples was challenging due to 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 unshared allele is indicated by the rectangles in Figure 4 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).

The ability of the Quantifiler® Duo assay to detect male DNA in extreme cases of mixture samples was demonstrated in a separate experiment. The limit of detection of male DNA in the presence of large excess 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 1:10,000. The Quantifiler® Duo kit successfully detected male DNA in mixture samples containing as high as 10,000 fold excess female DNA (Figure 5). 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 approximately between 27% lower and 29% higher than the expected mixture ratio (data not shown). This variation could be due to stochastic effects during the quantitation of male DNA at such low concentration. Based on the results from the

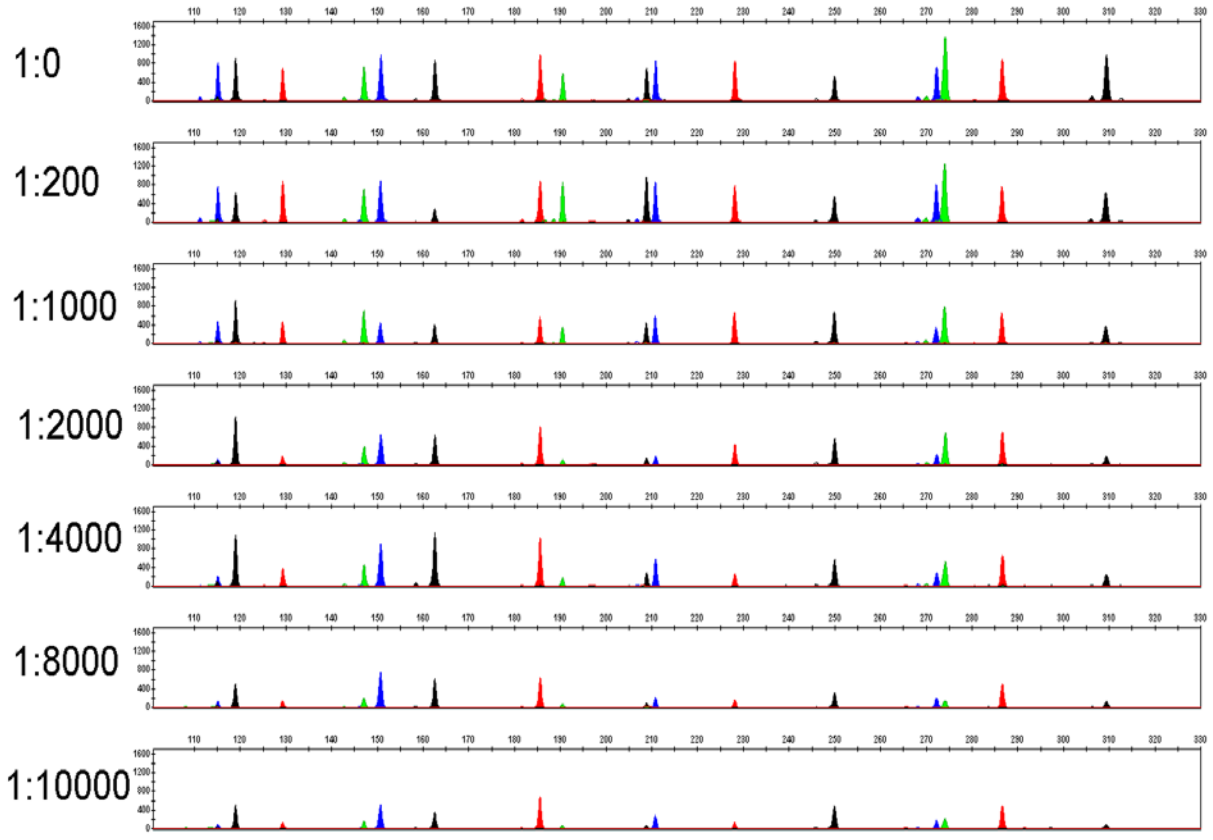

Figure 6: Mixture study II: Yfiler® profiles of mixtures containing 0.025 ng/µL of male DNA and increasing amounts of female DNA according to the shown male to female DNA ratios.

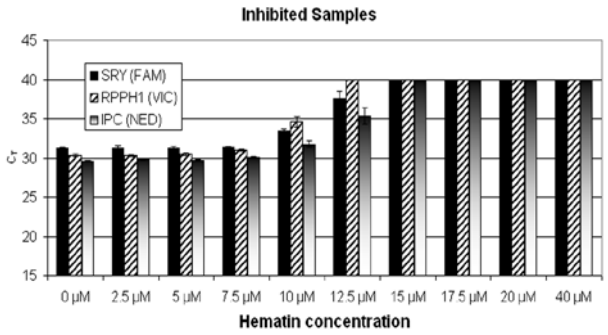

Figure 7: C_T values for hematin-inhibited samples obtained by using the Quantifiler® Duo DNA Quantification Kit.

SRY target, approximately 0.25 ng of human genomic DNA from each sample was analyzed using the Yfiler® Kit. Figure 6 represents a subset of 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 samples. Thus, using the results generated from the Quantifiler® Duo kit, it is possible to estimate which AmpFℓSTR® kit will likely be more successful and therefore make an educated decision to choose between autosomal STR and the Y-STR analysis for genotyping male DNA in a mixture sample.

Inhibited samples: Human genomic DNA was mixed with varying concentrations of hematin to assess the impact of inhibitors on both the Quantifiler® Duo reactions and the subsequent STR reactions performed using the Identifiler® Kit. An IPC template is co-amplified with the RPPH1 and SRY targets in the Quantifiler® Duo kit to monitor

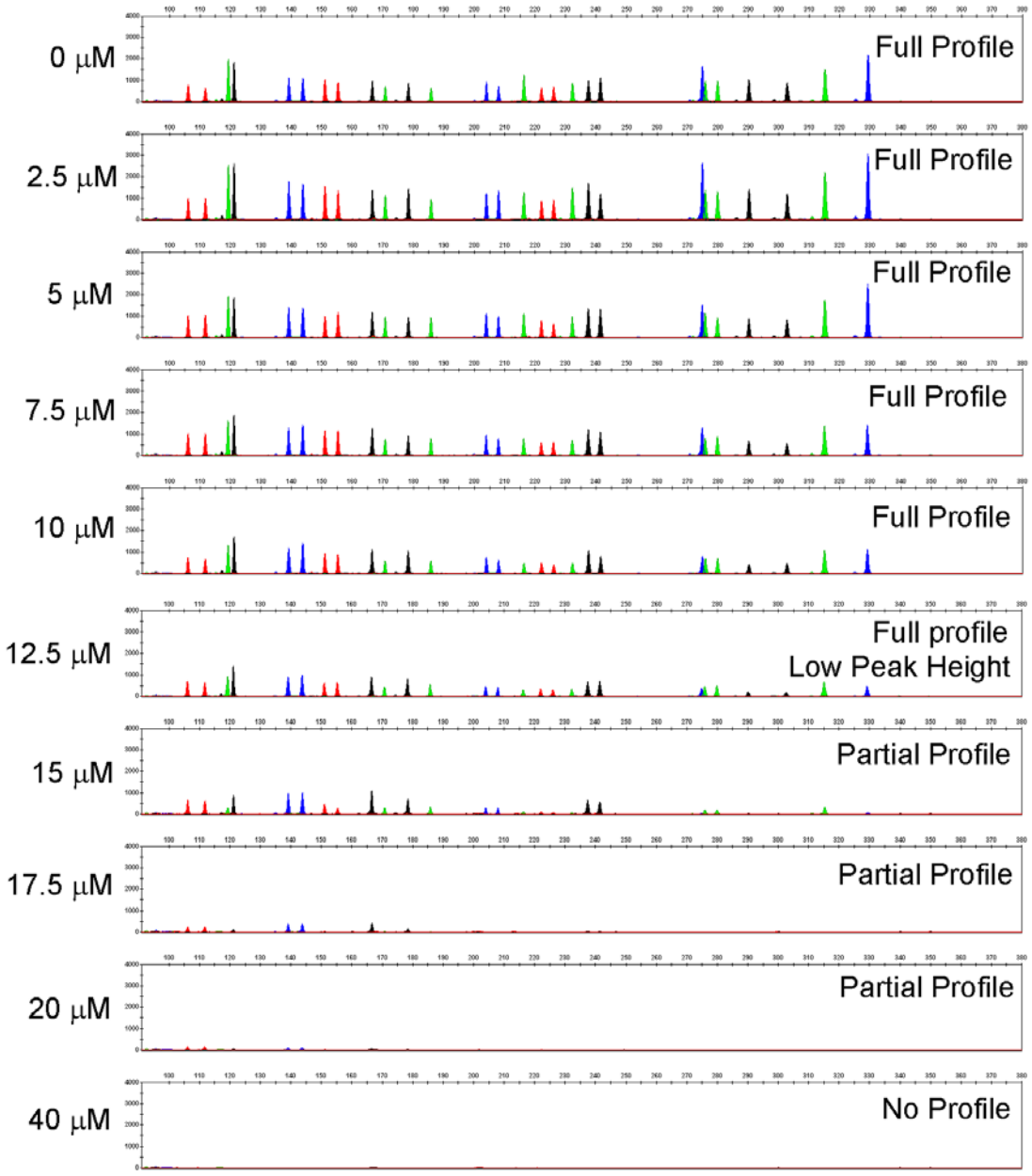

Figure 8: Identifiler® profiles of samples containing a range of hematin concentrations.

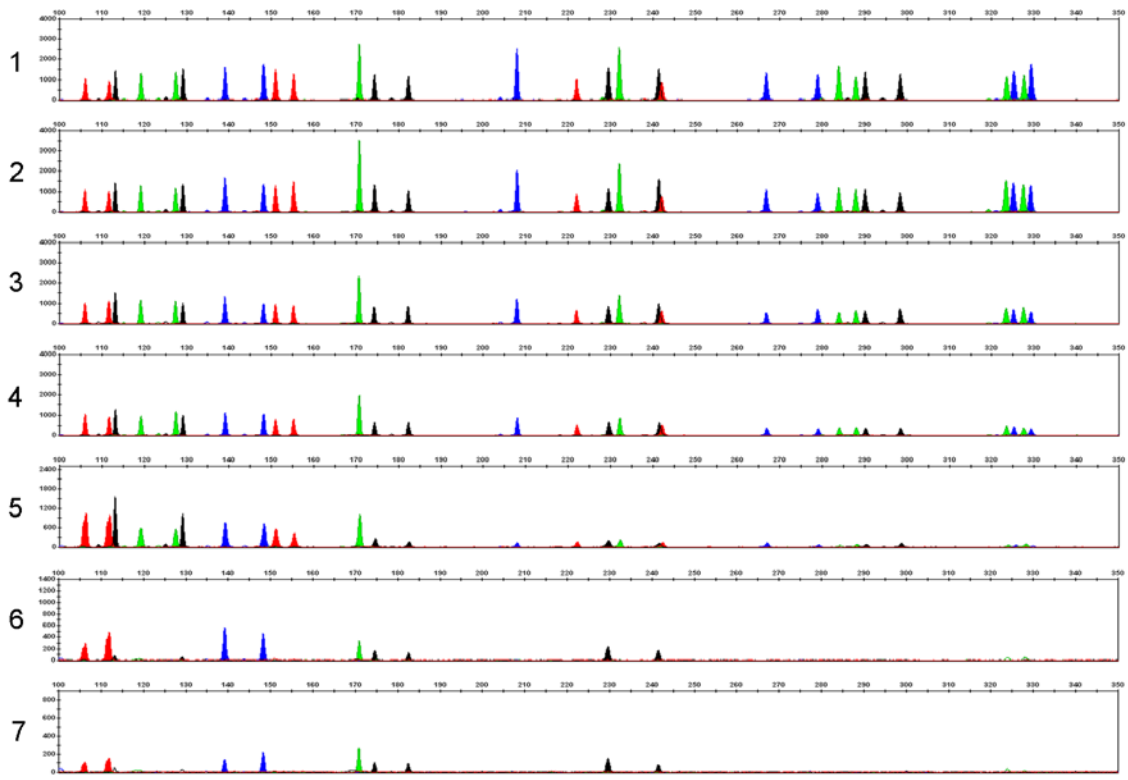

Figure 9: Degraded samples study: Identifiler® profiles of artificially degraded DNA samples. The DNA was treated with increasing concentrations of DNase I. (1) 0 DNase I Units; (2) 0.002 DNase I Units; (3) 0.01 DNase I Units; (4) 0.02 DNase I Units; (5) 0.05 DNase I Units; (6) 0.1 DNase I Units; (7) 0.2 DNase I Units. The rfu scale varies per panel and ranges from 900 to 4000 rfu. Reproduced with permission from Reference 12.

the presence of PCR inhibitors. Increased C_T values for the IPC target indicate the extent of the presence of PCR inhibitor(s). The results from the samples containing humic acid are described earlier [12].

Human male genomic DNA was mixed with varying final concentrations of hematin: 0, 2.5, 5.0, 7.5, 10, 12.5, 15, 17.5, 20, and 40 μM; these are the concentrations of hematin in 25 μL PCR when 2 μL of sample spiked with hematin is added. 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. 2 μL of each sample, containing approximately 1.0 ng of DNA, was quantified in triplicate using the Quantifiler® Duo DNA Quantification Kit. 2 μL of each DNA/hematin mix, containing approximately 1.0 ng of DNA, was also added to the Identifiler® Kit reactions in order to have the same final concentration of inhibitor in both the quantification and the STR reaction. The bar graph (Figure 7) illustrates higher C_T values as the concentrations of hematin increased. C_T values were relatively stable up to 7.5 μM hematin, with results displaying more pronounced C_T shifts at higher concentrations. Complete inhibition of the amplification occurred at 15.0 μM hematin.

In general, at a given concentration of inhibitor, the C_T values for all targets (human, human male and IPC) were affected similarly. The Identifiler® Kit results (Figure 8) were consistent with the quantification results: as the concentration of hematin increased (indicated by the increasing IPC C_T values), the overall STR peak heights decreased. Complete STR profiles were obtained at 10 μM hematin, complete but with much lower peak height at 12.5 μM hematin and partial profiles at 15 μM hematin. The STR amplification reaction was completely inhibited at 40 μM hematin. Thus, the results from the Quantifiler® Duo DNA Quantification Kit provided reasonable predictions of samples that would produce lower quality STR profiles due to the presence of a PCR inhibitor.

Degraded DNA: Forensic samples may be exposed to environmental conditions that degrade DNA causing fragmentation of full length molecules. Therefore, artificially degraded samples were tested with the Quantifiler® Duo assay to determine the quantity of amplifiable DNA at increasing levels of degradation. High-molecular weight human genomic DNA was used to generate a series of samples with varying levels of degradation. 1 μg of DNA (100 μL reaction at 10 ng/μL concentration) was treated for 20 minutes using increasing quantities of the DNase I enzyme: 0.002, 0.01, 0.02, 0.05, 0.1 and 0.2 units. For simplicity these samples will be called 1, 2, 3, 4, 5, 6, and 7. Samples were run on a 4% agarose gel for 25 minutes and visualized by staining with ethidium bromide to monitor the extent of degradation (data not shown). The degraded DNA samples generated at the seven DNase I concentrations were processed with the Quantifiler® Duo kit and the results obtained using the RPPH1 human target were the following: 7.69, 6.51, 5.11, 3.43, 0.57, 0.08 and 0.03 ng/μL as the quantity of the DNase I enzyme increased. These quantities were used to calculate DNA input for STR analysis using Identifiler® and MiniFiler™ kits.

For Identifiler® kit reactions, 1.0 ng of DNA was used whenever possible. 10 μL of sample was added to the Identifiler® Kit reactions for those samples containing 0.1 ng/μL DNA or less. Figure 9 illustrates the profiles obtained with the Identifiler® Kit. As expected, the peak heights (rfu values) of the alleles for STR loci with longer amplicons decreased as the degradation state increased. As a consequence of the increasing

degradation level, the concentration of amplifiable DNA decreased, as reported by the quantification results, resulting in the need to add a higher volume of sample to the subsequent PCR reaction. However, complete interpretable STR profiles were obtained for samples generated with up to 0.02 units of DNase I (samples 1 to 4). Partial STR profiles were obtained for those samples generated with 0.05 and higher units of DNase I (samples 5 to 7). These samples are highly degraded and allele dropouts were expected.

For MiniFiler™ kit reactions, 0.25 ng of DNA was used for most of the samples; 0.1 ng of template DNA was used for sample 7 (highest degree of degradation). As shown in Figure 10, conclusive and complete STR profiles were obtained for samples that were generated using up to 0.1 units of DNase I (samples 1 to 6), even though the amplitude (rfus) of the alleles for STR loci with longer amplicons decreased for sample 5 and 6. The sample that was generated using 0.2 units of DNase I (sample 7) provided interpretable profiles with low amplitude (rfus) for all loci. The results indicate that interpretable profiles can be recovered from all the degraded samples generated in this study when using the Minifiler™ Kit and input DNA determined using the Quantifiler® Duo kit.

Case-type samples: Different sample types that are commonly processed in a forensic laboratory were also evaluated. A variety of forensic-type samples were prepared using semen, saliva and blood obtained from multiple individual male donors.

Forensic type samples were prepared by loading 50 µL of saliva on a cotton swab (samples 1 and 2), 5 µL of blood on fabric (samples 3 and 4), 5 µL of blood on denim (sample 5), 5 µL of blood on filter paper (sample 6), 5 µL of blood spiked with inhibitors on fabric (sample 7) and 1 µL of semen on fabric (sample 8). The DNA was isolated by a phenol extraction method [3] and the extracted DNA was quantified in triplicate using the Quantifiler® Duo Kit. 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 (data not shown) with very similar human and male DNA quantities for each individual sample.

Based on the results from the Quantifiler® Duo kit (RPPH1 human target), approximately 1.0 ng of human genomic DNA was added to each Identifiler® kit reaction. Figures 11 and 12 show examples of STR profiles obtained from a saliva swab (sample 2) and from a blood stain on fabric (sample 3), respectively. Complete and interpretable STR profiles were obtained for all samples analyzed with peak heights between 500 and 4000 rfu (data not shown).

Conclusions

The Quantifiler® Duo DNA Quantification Kit provides quantitative results for a variety of biological sample types with good sensitivity, precision and reproducibility. Its ability to discern a minor male contributor in the presence of large excess of female DNA in mixture samples makes it a useful tool for the analysis of sexual assault cases. In addition, it is not only a quantification assay, but also a means to assess the quality of a DNA sample providing an indication of the potential presence of PCR inhibitors. All this information is crucial for the forensic analyst to decide which STR technology is the most appropriate for the downstream genotyping analysis. In summary, this assay can be considered a guiding tool that can reasonably predict the performance of the genotyping kits thereby increasing the success rate of the STR analysis at first pass.

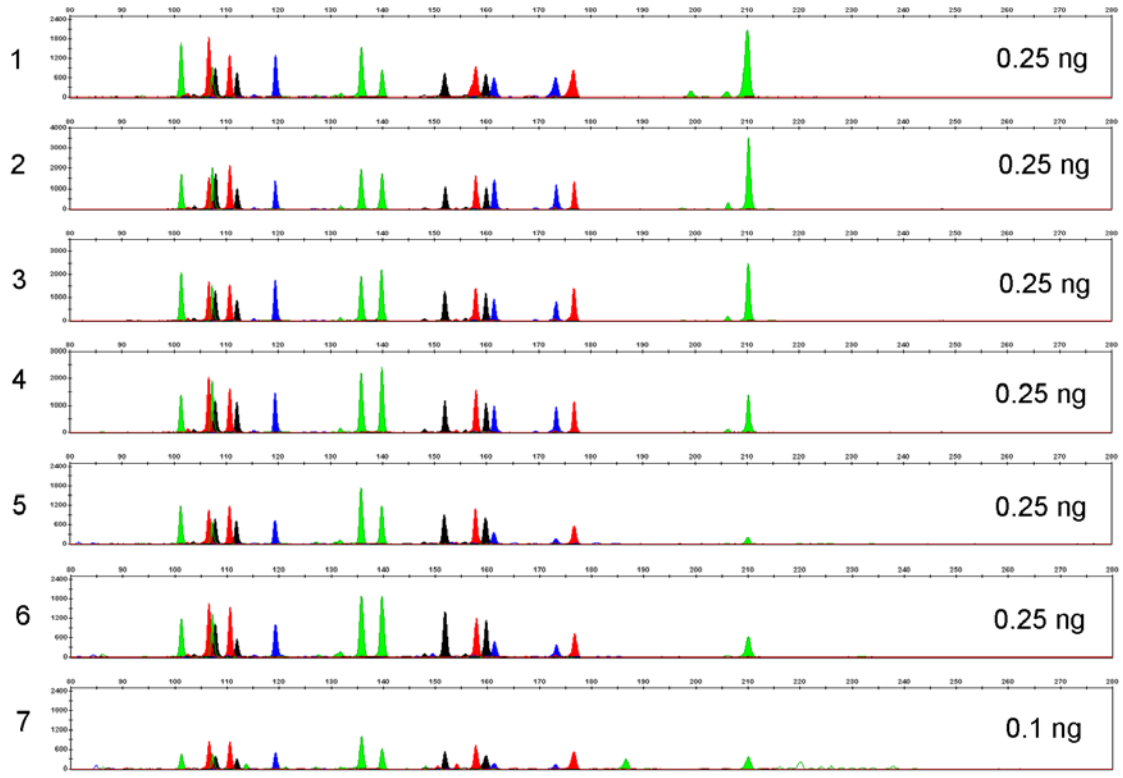

Figure 10: Degraded samples study: MiniFiler™ profiles of artificially degraded DNA samples. The DNA was treated with increasing concentrations of DNase I. (1) 0 DNase I Units; (2) 0.002 DNase I Units; (3) 0.01 DNase I Units; (4) 0.02 DNase I Units; (5) 0.05 DNase I Units; (6) 0.1 DNase I Units; (7) 0.2 DNase I Units. 0.25 ng of human DNA was amplified for samples 1 through 6; 0.1 ng for sample 7. Reproduced with permission from Reference 12.

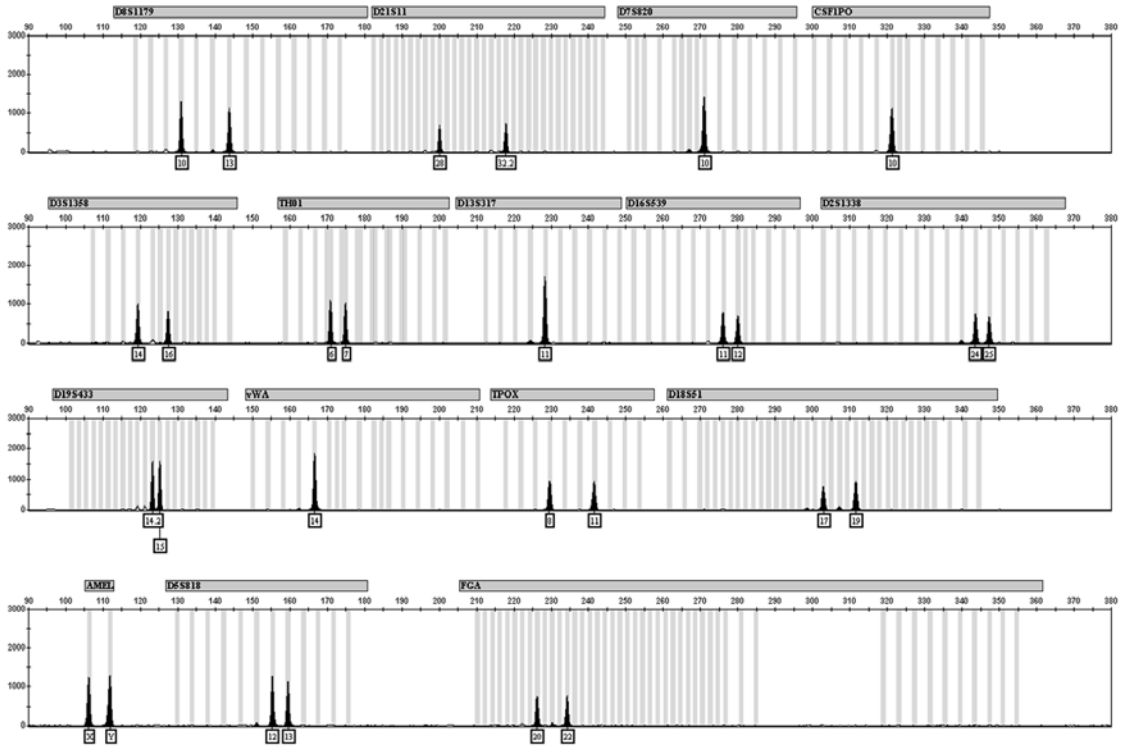

Figure 11: Case-type samples study: Identifiler® profile of phenol extracted DNA from a saliva swab (sample 2). The DNA extract contained 11.0 and 11.35 ng/µL of human male and human DNA, respectively.

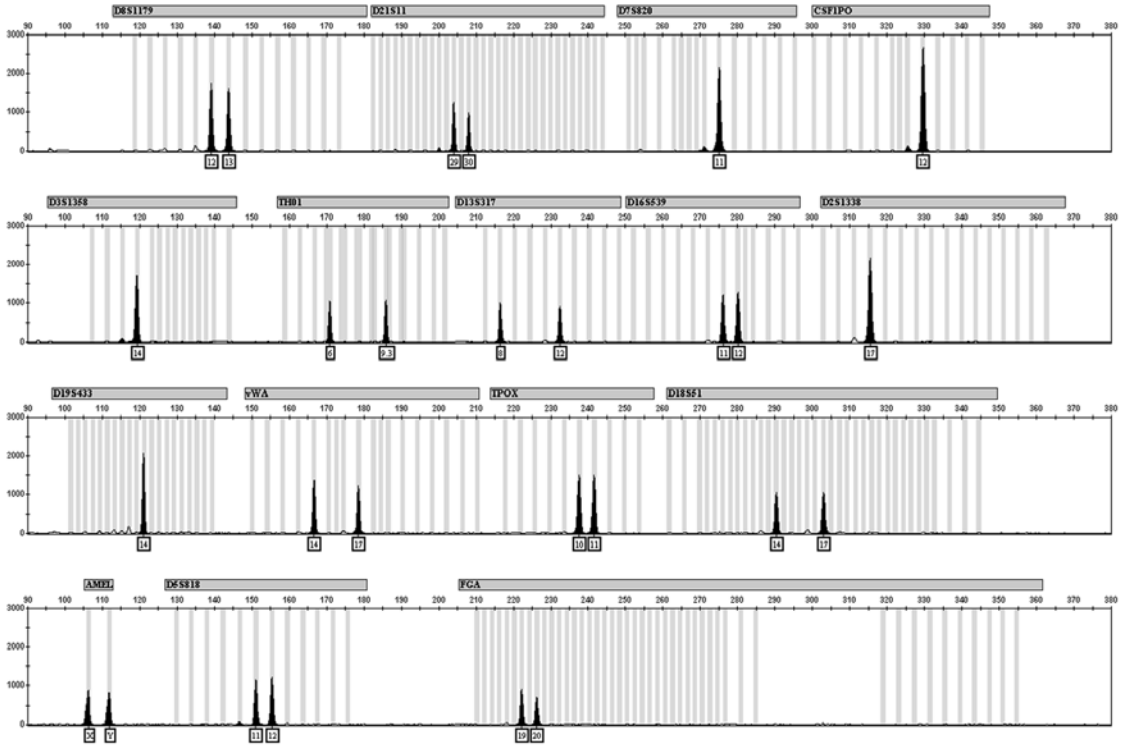

Figure 12: Case-type samples study: Identifiler® profile of phenol extracted DNA from a blood stain on fabric (sample 3). The DNA extract contained 0.82 and 0.91 ng/µL of human male and human DNA, respectively.

Acknowledgements

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