Validation of a Multiplexed System for Quantification of Human DNA and Human Male DNA and Detection of PCR Inhibitors in Biological Samples

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Abstract

Forensic analysts routinely encounter samples containing mixtures of DNA from male and female contributors. In order to obtain optimal STR analysis results and select the appropriate STR analysis methodology for such samples, 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 analysis. This presentation will describe a single reaction multiplex assay for the Applied Biosystems 7500 Real Time PCR System designed for simultaneous quantification of human and human male DNA using the ribonuclease P RNA component H1 (RPPH1, VIC® labeled probe) human target and the sex determining region Y (SRY, FAM™ labeled probe) male-specific target. A synthetic oligonucleotide sequence was co-amplified as an internal PCR control (IPC, NED™ labeled probe). Standard curves for both assays were generated using human male genomic DNA. The validation studies were performed according to the DNA Advisory Board's (DAB) Quality Assurance Standards. The SRY and RPPH1 assays demonstrated human specificity with minimal cross-reactivity to DNA from other species. Reproducible DNA concentrations were obtained within a dynamic range of 0.023 to 50 ng/µl. In addition, the multiplex assay was highly sensitive to human male DNA in the presence of high amounts of female DNA, detecting as little as 25 pg/µl of human male DNA in the presence of a thousand-fold excess of human female DNA (25 ng/ul). The ability of the assay to predict inhibition of PCR was demonstrated by a shift of the IPC Ct values in the presence of increasing quantities of hematin and humic acid, common inhibitors of PCR. Experiments that were performed to demonstrate the correlation between the quantification results using the multiplex assay and the strength of STR profiles generated using the AmpFℓSTR® Identifiler®, Yfiler® and MiniFiler™ PCR Amplification Kits will also be discussed.

Keywords: Human DNA quantification, real-time PCR, PCR inhibitors, STR analysis for human identification

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Introduction

Accurate quantification of human DNA in forensic samples is essential for defining the input DNA needed for obtaining interpretable Short Tandem Repeat (STR) profiles. Real-time PCR assays like Quantifiler® Human DNA Quantification kit and Quantifiler ® Y Human Male DNA Quantification kit have proved very useful in STR profiling. Real-time quantification assays provide certain advantages over the traditional hybridization based assays: Greater dynamic range, more rapid, increased limit of detection, ability to predict the presence of PCR inhibitors and ability to automate (1-4). We describe Quantifiler® Duo DNA Quantification Kit that the forensic scientist can use as a tool for quantitative and qualitative assessment of total human and human male DNA in forensic type biological samples. Quantifiler® Duo DNA Quantification Kit is designed to quantify total human DNA and human male DNA simultaneously, determine the ratio of human male and female DNA,

detect PCR inhibitors, allow selection of the appropriate STR amplification kit, and predict success with downstream STR amplification.

Materials and methods

A multiplexed TaqMan ® was assembled that amplifies SRY (FAM™-labeled probe), RPPH1 (VIC®-labeled probe) and an Internal Positive Control-IPC (NED™-labeled probe). Assays were designed using the TaqMan® Gene Expression (5) assay (www.allgenes.com) design pipelines targeting genomic DNA. Multiplex reactions were optimized using factorial designs to optimize responses. Amplification reactions were performed on a 7500 Real-Time PCR System and the data were analyzed with the 7500 System SDS software v1.2.3 (Applied Biosystems, Foster City, CA). Assay species specificity was tested using a panel of mammalian and microbial DNAs. Non-human samples were obtained as purified DNA from BIOS Laboratories, Inc., New Haven, CT and ATCC, Manassas, VA. Human genomic DNA used was from a pool of male donors (EMD Chemicals Inc., Madison, WI). GeneAmp® PCR System 9700 and the ABI PRISM® 3100 Genetic Analyzer were used as described in the instruction manual.

Results and Discussion

A multiplex real-time PCR assay was assembled that amplifies sex determining region Y (SRY), ribonuclease P RNA component H1 (RPPH1) and a synthetic oligonucleotide sequence that served as an Internal Positive Control (IPC) (Fig 1). Amplification reactions were performed on a 7500 Real-Time PCR System and the data were analyzed with the 7500 System SDS software v1.2.3 (Applied Biosystems, Foster City, CA).

DNA from several mammalian and microbial sources was tested to determine species specificity (Fig 2). The two human control samples showed expected results. Only chimpanzee DNA was detected by the SRY assay. The assays didn't detect DNA from the remaining species included in the specificity panel (5 ng of DNA/rxn).

Human genomic DNA from a pool of male donors was used to generate in a single reaction two standard curves for the human and the human male-specific targets with the DNA concentration ranging from 50 ng/µl to 23 pg/µl in three-fold increments (Fig 3). The eight concentration points are 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068 and 0.023 ng/µl and 2.0 µl of each sample were tested with the multiplex assay. IPC artificial template was included in each reaction to obtain the C_T value of about 30 across the whole standard curve. The multiplexed assay performed well across a large dynamic range and is well suited for quantification of human samples.

Five different human DNA samples were diluted to approximately 20, 10, 0.1 and 0.05 ng/µl 1 ng/µl and tested for reproducibility of the quantification results in three successive runs. One sample was from a female individual, the other four samples were from male sources. Averages and standard deviations for all experiments were within acceptable limits and the results from the human and the human male specific assay are in concordance (Fig 4).

Purified genomic DNA from a male and a female individual were combined according to various ratios to mimic sexual assault evidence samples (Fig 5 and 6). The mixtures were tested with the multiplex assay to determine the concentration of total and male DNA. The multiplex assay can quantify 25 pg/µl of male DNA in the presence of up to approximately 25 ng/µl of female DNA (1:1000 ratio). Electropherograms obtained by using the

AmpFℓSTR® Yfiler® PCR Amplification kit for the 1:0, 1:800 and 1:1000 ratio samples are shown in Fig 7; the male STR profile is conclusive up to the 1:1000 mixture ratio.

Human male genomic DNA extracted from whole blood was mixed with varying final concentrations of hematin: 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, and 40 µM. 2.0 µl of each DNA/hematin mix, containing 1.0 ng of DNA, was quantified using the multiplex assay. The IPC C_T values were monitored (Fig 8). Partial inhibition was detected between 10 and 12.5 µM hematin; total inhibition at 15 µM hematin. 2.0 µl of each DNA/hematin mix was also added to the AmpFℓSTR® Identifiler® kit reactions (Fig 8). The results from the quantification assay provided reasonable predictions of samples that would fail STR analysis because of the presence of the PCR inhibitor (e.g. sample containing 15 µM hematin). 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. Similar results were obtained with samples containing increasing quantities of humic acid (data not shown).

Conclusions.

Quantification of human DNA in forensic samples is essential for defining input DNA needed for obtaining interpretable STR profiles. The most accurate method of choice for forensic DNA quantification is real-time PCR. We have developed a multiplex real-time PCR assay for the simultaneous quantification of human and human male DNA with IPC in forensic samples. The assay is efficient, specific, sensitive and robust. The results correlate well with the AmpFℓSTR® Identifiler® and Yfiler® kit performance in terms of predicting the generation of interpretable STR profiles for inhibited DNA samples and male/female DNA mixtures. The Quantifiler® Duo DNA Quantification Kit is a useful tool for the quantitative and qualitative assessment of DNA in forensic type biological samples.

References

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Target	Marker	Size	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™

Fig 1. Configuration of the Quantifiler® Duo Kit

Tested Species	RPPH1 Average Ct	SRY Average Ct
Orangutan	40.0	40.0
Chimpanzee A	40.0	**32.3**
Chimpanzee B	40.0	**31.1**
Gorilla A	40.0	40.0
Gorilla B	40.0	40.0
Macaque	40.0	40.0
Dog	40.0	40.0
Cow	40.0	40.0
Pig	40.0	40.0
Cat	40.0	40.0
Horse	40.0	40.0
Sheep	40.0	40.0
Chicken	40.0	40.0
Fish	40.0	40.0
Rabbit	40.0	40.0
Mouse	40.0	40.0
Rat	40.0	40.0
Hamster	40.0	40.0
Human Male	**27.0**	**27.9**
Human Female	**27.5**	40.0
E. coli	40.0	40.0
Pseudomonas	40.0	40.0
Neisseria	40.0	40.0
Staphylococcus	40.0	40.0
Saccharomyces	40.0	40.0
Candida	40.0	40.0

Fig 2. Species specificity of the Quantifiler® Duo Kit. A and B indicate DNA samples from two separate animals.

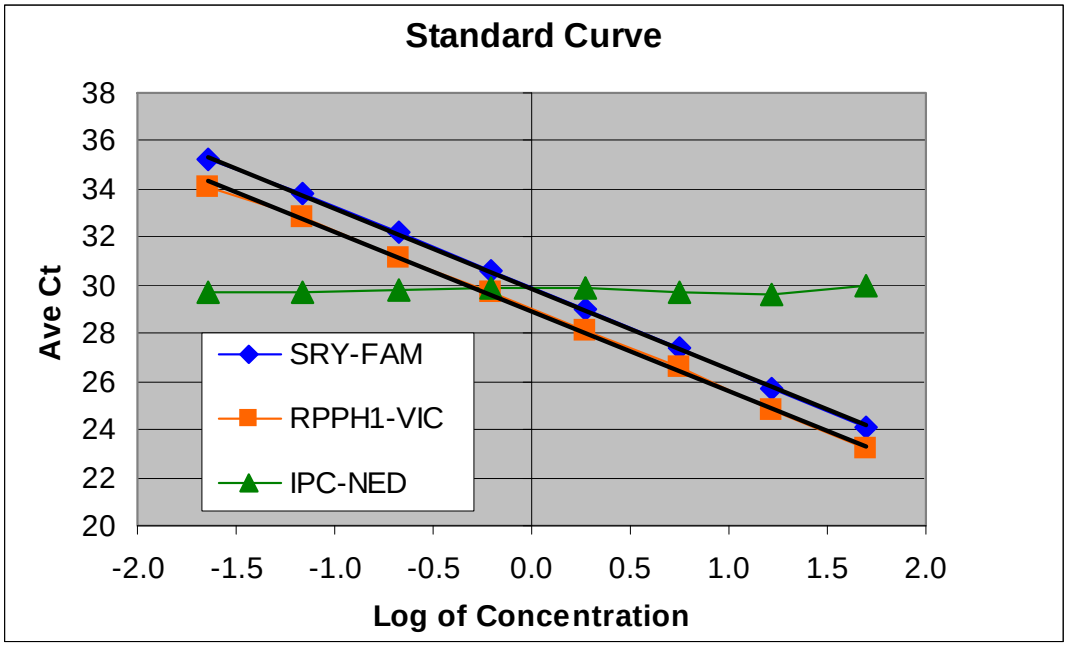

Fig 3. Standard curves and IPC C_T values for the Quantifiler® Duo Kit

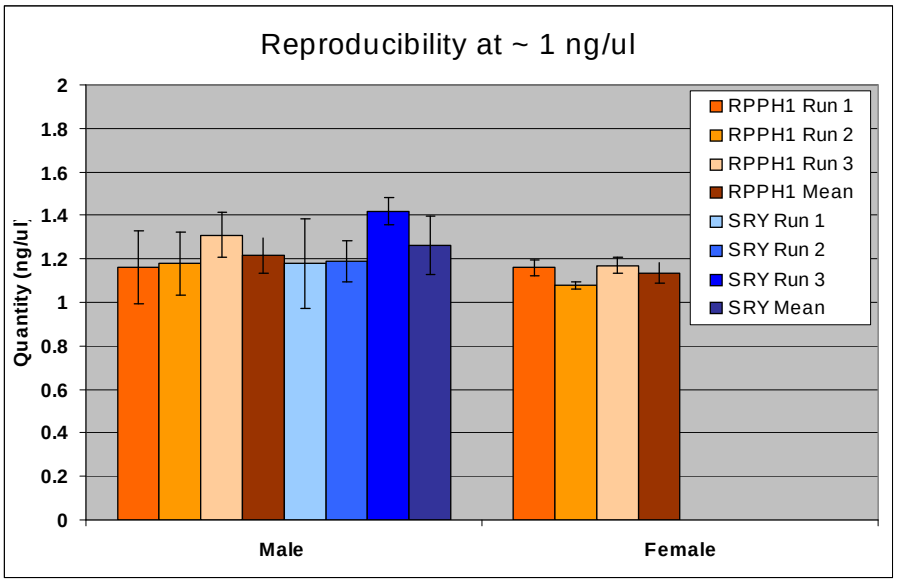

Fig 4. Reproducibility study for the Quantifiler® Duo Kit

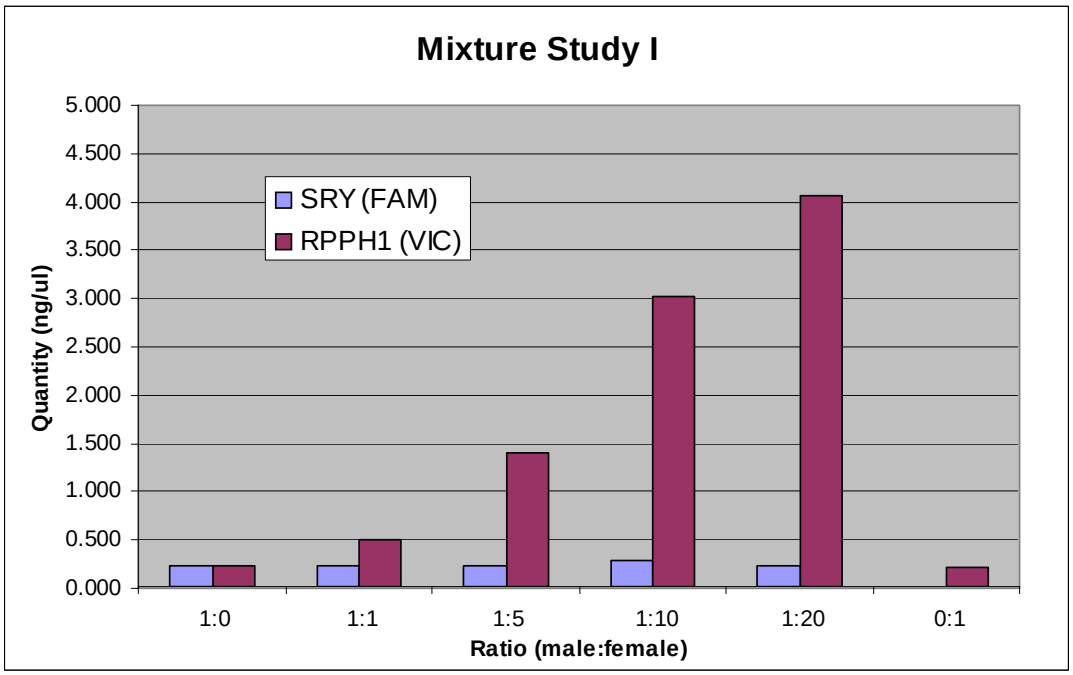

Fig 5. Mixture study for the Quantifiler® Duo Kit. Male DNA added at a constant concentration of 0.2 ng/µl

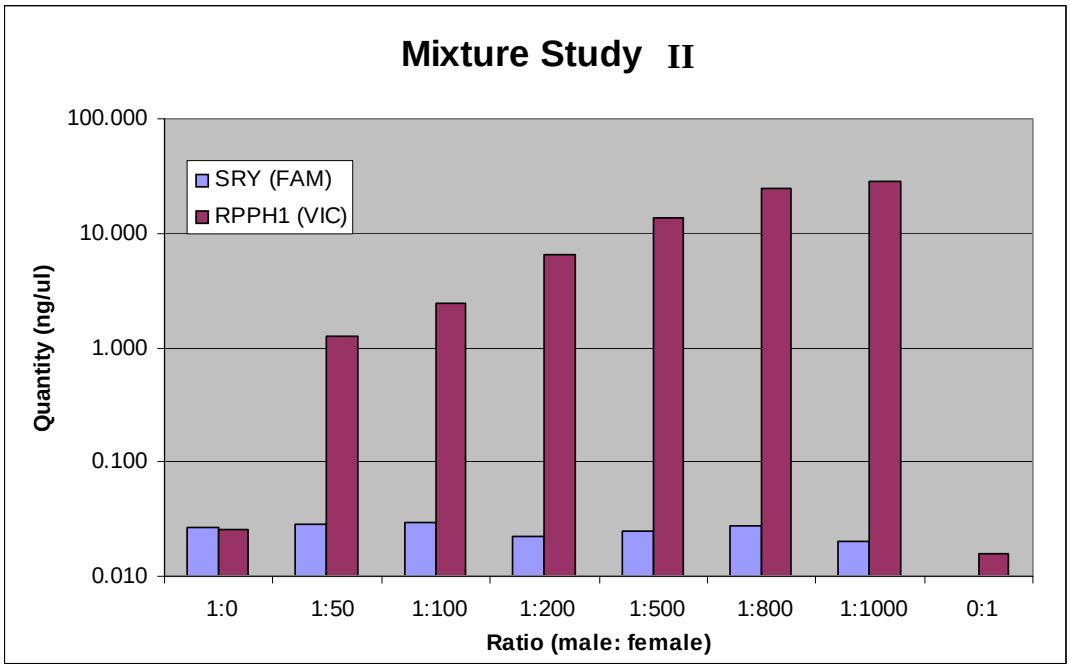

Fig 6. Mixture study for the Quantifiler® Duo Kit. Male DNA added at a constant concentration of 0.025 ng/µl

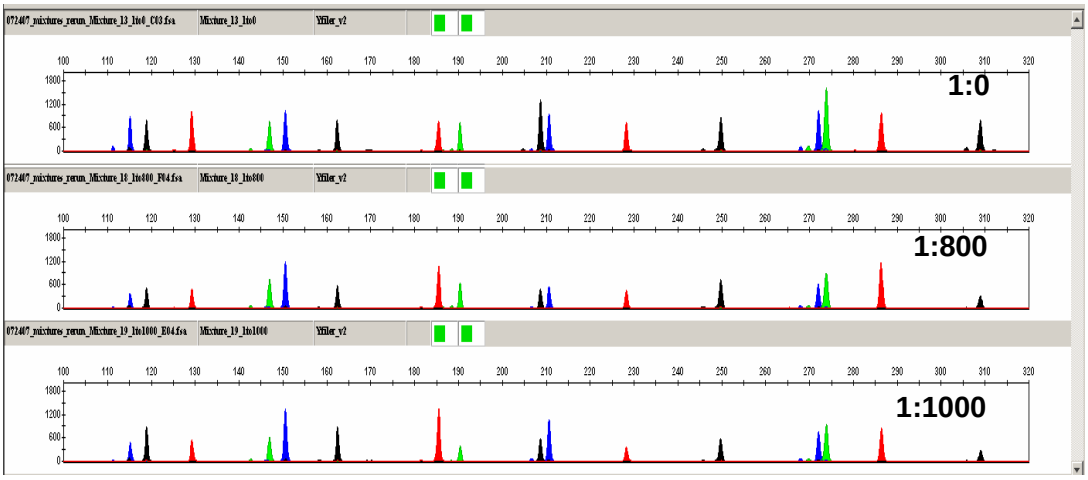

Fig 7. Yfiler® profile of the mixtures samples amplified using ~0.25 ng DNA/rxn

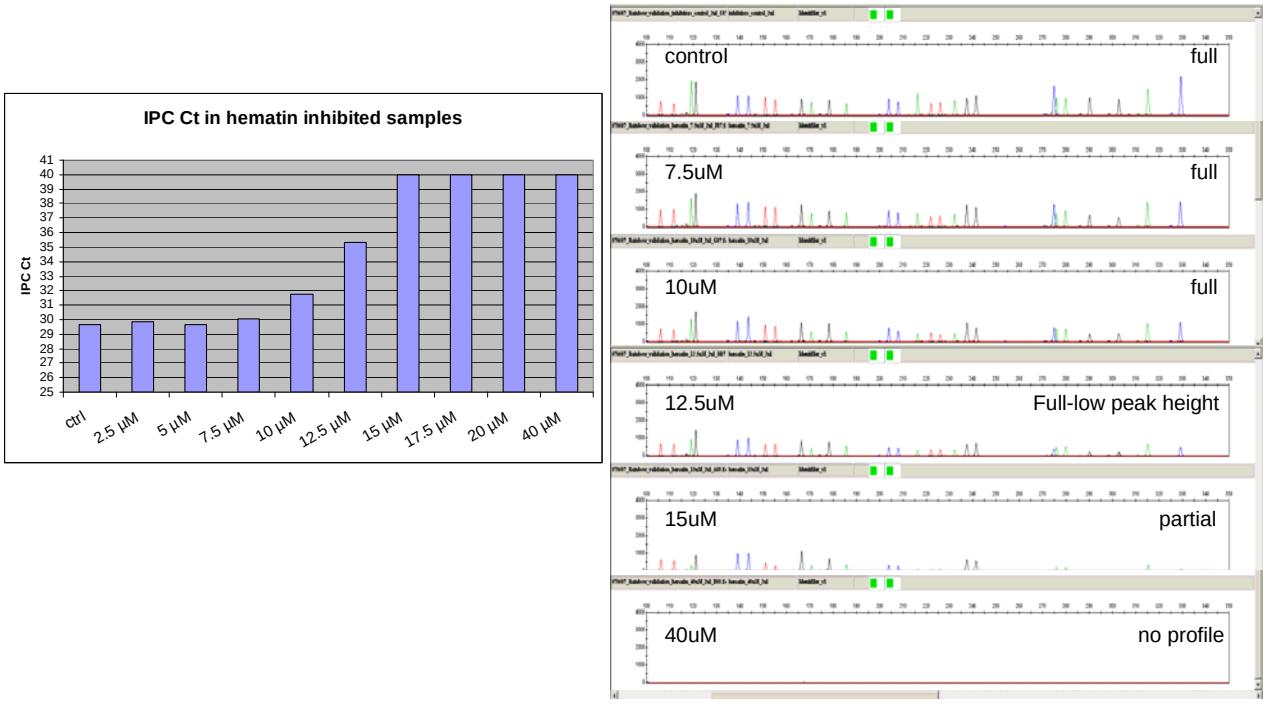

Fig 8. IPC C_T values and Identifiler® profiles for the hematin inhibited samples