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Development of a Human-Specific Real-Time PCR Assay for the Simultaneous Quantitation of Total Genomic and Male DNA*

ABSTRACT: A duplex real-time quantitative PCR assay was developed for forensic DNA analysis, which provides simultaneous quantitation of total genomic human DNA and human male DNA. The assay utilizes two spectrally resolved fluorogenic probes in a 5′ nuclease (TaqMan™) assay. Within the range of organisms empirically tested and based upon theoretical specificity using National Center for Biotechnology Information GenBank sequences, primer and probe sequences were shown to be human specific, and the Y-chromosome probe, male-specific. A mixture-challenge study resulted in accurate quantitation of 25 pg male DNA in a mixture of up to 1:5000 (male:female DNA). Additional experimental results include comparisons with the slot blot method and commercial real-time PCR kits. The assay developed addresses the shortcomings of the traditional slot blot method as well as the commercial real-time PCR kits. This method is shown to be specific, relatively simple, rapid, has low limits of detection, and consumes limited sample in addition to reporting both the male and total genomic DNA concentrations present.

KEYWORDS: forensic science, real-time PCR, quantitative PCR, DNA quantitation

For a number of years, the slot blot method has been used to quantitate human DNA in forensic samples (1,2). However, the slot blot method is labor-intensive, subjective in interpretation, and not highly sensitive. Current research has focused on the use of real-time quantitative PCR (qPCR) as a more accurate, more sensitive, and less labor-intensive method. In addition, a PCR-based assay for human DNA quantitation, unlike the slot blot method, quantitates the PCR-amplifiable DNA, although it should be noted that significant sample degradation could result in quantitation but no short tandem repeat (STR) amplification, because of differing amplicon sizes.

Real-time PCR has the capability of quantitation based upon measurement of the increase in fluorescence signal with each cycle and comparison with a standard curve. A number of detection chemistries are currently available—the most popular being SYBR® Green detection (3), fluorogenic probes (4,5), and molecular beacon technology (6). Although relatively simple and cost-effective, SYBR® Green, an intercalating dye, was not amenable to the current work because the method is unable to differentiate between the two targets for simultaneous quantitation of human male and total human genomic DNA. Fluorogenic (TaqMan®) probe chemistry was utilized in this study with spectrally distinguishable reporter dyes for each target sequence. Probes are 5′-labeled with a fluorophore (e.g., VIC® or FAM™) and 3′-labeled with a quencher dye (e.g., TAMRA™). Therefore, the probe does not emit fluorescence because of the fluorescence resonance energy transfer (FRET) that occurs between the fluorophore and the quencher. However, upon annealing to the target site within the amplified region, the probe is degraded by the 5′ nuclease activity of the *Taq* DNA polymerase during the extension step of the PCR. This results in separation of the fluorophore from its quencher, preventing FRET, and allowing fluorescent emission by the fluorophore. Each subsequent thermocycle results in degradation of additional probe, generating a corresponding increase in fluorescence.

With the growing capabilities for discrimination based upon Y-chromosome STR typing (7–9), the need to quantitate the male contribution to a sample and male/female ratio is becoming increasingly evident. The most commonly utilized method for DNA quantitation in forensic laboratories, slot blot, does not specifically quantitate the male DNA component in a mixture of male and female DNA. Although a Y-chromosome probe could be designed for use in the slot blot system, it remains a laborious method with a poor limit of detection. An even more laborious method of sex determination of the sample was demonstrated by Andréasson and Allen (10). This method uses real-time PCR, but uses SYBR® Green and discrimination is based upon a melting curve analysis. This method does not result in separate quantitation results for each sex, but is more qualitative in nature.

Commercial kits (Applied Biosystems, Foster City, CA) for human DNA and Y-chromosome DNA quantitation using real-time qPCR are available in singleplex reactions. However, multiplex PCR has the advantage of consuming half as much DNA as compared with the commercial kits (two singleplex reactions) in addition to reducing the cost of the analysis. Fluorogenic probe-based multiplex real-time PCR assays have been reported, including the detection of potato pathogens (11), assessment of human

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DNA content in immune-deficient mice (12), and detection of bacterial pathogens causing louse-borne human diseases (13). Of interest to the forensic science community are fluorogenic probe-based duplex real-time PCR assays developed for the quantitation of nuclear and mitochondrial DNA (14,15).

This paper reports the development of a duplex real-time PCR assay for simultaneous quantitation of both the total human genomic DNA as well as total male DNA. The male DNA quantitation, improved limits of detection, and linear range combined with the limited sample consumption of the qPCR duplex assay make it an attractive alternative to the conventional slot blot DNA quantitation methods and commercially available qPCR kits.

Materials and Methods

Primers/Probes

Primer and TaqMan® (Roche Molecular Systems, Inc., Pleasanton, CA) probe sequences for both the autosomal and Y-chromosome locus were selected using Primer Express 2.0 software (PE Biosystems, Foster City, CA). The sequences chosen are listed in Table 1. Sequences of STR loci were obtained from GenBank accession M68651 (human tyrosine hydroxylase, TPOX) and accession NM_003140 (sex-determining region of the Y-chromosome, SRY). The amplicon for autosomal DNA was chosen to be near, but not within, the tetrameric repeat region of the TPOX locus.

For total human genomic DNA quantitation, the amplicon is a 63 bp fragment of the TPOX locus. The forward and reverse primers for the autosomal locus are 21 and 22 bases in length, have a GC content of 57% and 50%, and T_m of 59°C and 60°C, respectively. The TPOX probe (20 bases, $T_m = 68$°C) is covalently labeled on the 5′ terminus with a VIC™ fluorophore (Applied Biosystems) and at the 3′ terminus with a tetra-methylcarboxyrhodamine (TAMRA) quencher. The Y-chromosome amplicon is a 70 bp fragment of the SRY locus of the Y chromosome. The forward and reverse primers for the SRY locus are 22 and 19 bases in length, have a GC content of 45% and 58%, and T_m of 58°C and 59°C, respectively. The SRY probe (25 bases, $T_m = 70$°C) is labeled on the 5′ terminus with an FAM (6-carboxyfluorescein) dye and at the 3′ terminus with a TAMRA quencher. The primer and probe sequences are shown in Table 1. Both probes were synthesized by Applied Biosystems. Primers were synthesized by Invitrogen Corp. (Carlsbad, CA). A BLAST nucleotide search (National Center for Biotechnology Information, NCBI) indicated the primer and probe sequences did not show significant identity to nonhuman DNA (except higher primates). Hence, under the qPCR conditions, amplification product is not expected from nonhuman DNA. It would be extremely rare that any potential cross-reactivity to other higher primate DNA would become an issue in the analysis of a forensic case sample.

DNA Samples

High molecular weight male DNA (Promega, Madison, WI, G1471) and K562 (female) DNA (Promega, DD2011) were used for assay development and optimization studies. Male testis DNA (Biochain Institute Inc., Hayward, CA, D1234260) was used for the mixture analysis, where it was necessary to have DNA from a single source for subsequent calculations. The concentrations of both DNA samples were subject to quality control by their respective manufacturer and the authors, which utilized absorbance at 260 nm/280 nm to determine the purity and the accurate quantitation of the DNA sample. Species specificity of the assay was tested using *Escherichia coli* (Strain W3110, Sigma, St. Louis, MO, D0421), *Candida albicans* (ATCC, Manassas, VA, 10231D), Mouse (Promega, G3091), and *Saccharomyces cerevisiae* (Invitrogen, Frederick, MD).

Real-Time PCR Assay

PCR was performed using an Applied Biosystems Prism® 7000 Sequence Detection System in 96-well microtiter plates according to the manufacturer's specifications. The reaction mixtures were incubated at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of two-step amplification: 95°C for 15 sec (denaturation) and 60°C for 1 min (annealing/extension). PCR was performed under Perkin-Elmer 9600 Emulation conditions, which results in a total run time of 1 h, 48 min. For each microtiter plate, a standard curve, in duplicate, of male DNA diluted in Tris-EDTA buffer was run, ranging from 50 ng to 10 pg total DNA.

Reaction components consisted of 300 nM each primer (TPOX forward, TPOX reverse, SRY forward, SRY reverse), 250 nM each probe (TPOX and SRY), and 0.01 ng/μL BSA. Either 2X JumpStart Taq Ready Mix (Sigma, P/N D7440) or 2X TaqMan™ Universal PCR Master Mix (Applied Biosystems, P/N 4304437) was added to result in a final concentration of 1X. When using the Sigma Ready Mix, 1X ROX passive reference dye and an additional 2 mM $MgCl_2$ were added to the reaction. Unless otherwise indicated, the results presented were obtained using the ABI master mix. In contrast to the Quantifiler™ kits, no internal positive control (IPC) was utilized in the duplex assay. In all reactions, 2 μL of sample DNA was added. PCR was performed in a total reaction volume of 25 μL. After distribution of 23 μL of master mix and 2 μL of the appropriate DNA sample to each well, the microtiter plate was covered with an optical adhesive cover (Applied Biosystems), and centrifuged at 2500 r.p.m. for approximately 15 sec. A compression pad was placed on the microtiter plate, and the plate was placed in the instrument.

Slot Blot

DNA was also quantified using a modified slot blot method with chemiluminescence detection using a SuperSignal® West Femto

TABLE 1—*Primers and probes used for duplex real-time PCR amplification and detection.*

Target	Gene	Primer/Probe	Fluorophore-Nucleotide Sequence-Quencher (5′ → 3′)
Autosomal	TPOX	TPOX forward	CGGGAAGGGAACAGGAGTAAG
		TPOX reverse	CCAATCCCAGGTCTTCTGAACA
		TPOX probe	VIC-CCAGCGCACAGCCCGACTTG-TAMRA
Male	SRY	SRY forward	ACGAAAGCCACACACTCAAGAA
		SRY reverse	GTGAGCTGGCTGCGTTGAT
		SRY probe	FAM-AGCACCAGCTAGGCCACTTACCGCC-TAMRA

TPOX, human tyrosine hydroxylase; SRY, sex determining region of the Y chromosome.

reagent (Pierce Biotechnology, Rockford, IL, 34096) for increased sensitivity. The standard DNA ladder (156 pg–10 ng total DNA per well) was prepared using high molecular weight male DNA (Promega, G1471). Following hybridization, the image was analyzed by Bio Image® Intelligent Quantifiler software (Bio Image Systems Inc., Jackson, MI) for quantitation results.

Quantifiler™ Comparison

For comparisons with the commercial Quantifiler™ and Quantifiler Y™ kits (Applied Biosystems), qPCR reactions were prepared following the manufacturer's instructions. A standard curve of male DNA as prepared for the duplex assay was also utilized in these reactions.

Mixture Challenge

Mixtures of male and female DNA were amplified with qPCR to assess the ability of the assay to quantitate male DNA successfully in the presence of an abundance of female DNA. DNA mixtures from 1:1 to 1:5000 (male:female) were prepared by creating a dilution series of female DNA and then adding a fixed amount of male DNA to each female DNA sample of the dilution series. In this way, the amount of male DNA in the qPCR reaction remained constant in a wide range of amounts of female DNA. Five fixed amounts of male DNA were tested (12.5, 25, 50, 125, and 250 pg) in 1:1 to 1:5000 (male:female) ratios.

Autosomal STR analysis was performed using AmpF*l*STR® ProfilerPlus™ kits (Applied Biosystems) according to the manufacturer's specifications, with the exception that half-reactions (25 μL) were performed. Y-chromosome STR typing was performed using the PowerPlex® Y kit (Promega) following the manufacturer's directions. PCR products were separated on an Applied Biosystems 310 Genetic Analyzer under the following conditions: 5 sec electrokinetic injection (15 kV), 25 min 15 kV separation, and a 1X Genetic Analyzer Buffer separation buffer (Applied Biosystems). The capillary (50 μM ID, 36 cm effective length, 47 cm total length) was filled with a POP4 sieving polymer (Applied Biosystems) and maintained at 60°C for the duration of the separation. For all STR amplifications, qPCR results were used to calculate the volume of sample to add to the amplification, using 1 ng as a target. For autosomal STR reactions, 1 ng total DNA was targeted. For Y-STR reactions, 1 ng male DNA was targeted. If the sample amount was less than 200 pg (in 2 μL DNA added to the qPCR reaction), as determined by qPCR, 10 μL of sample was amplified.

Results and Discussion

Development of a new assay for quantitation of human genomic DNA in forensic samples requires that a number of criteria be met by the new assay. These include low limits of detection, limited sample consumption, high specificity to human DNA, and cost-effectiveness. These and other means of evaluating the qPCR assay are delineated in the appropriate subsections below.

Assay Optimization

Assay optimization experiments were performed including primer/probe concentration, primer-limiting conditions for the TPOX locus, and Mg^{2+} concentration optimization (data not shown). In addition, the use of two different commercial reaction master mixes was explored (Applied Biosystems Taqman™ Universal Master Mix and the Sigma JumpStart™ Taq ReadyMix™). Differences of note between the performance of the two master mixes are delineated in the appropriate subsections below. Overall, the two master mixes performed similarly; however, the cost of the duplex assay was significantly lower on using the Sigma master mix.

The performance of the assay was compared under singleplex and duplex conditions for each locus to ensure that significant negative effects were not observed as a result of integrating the two assays into one duplex assay. A comparison of the quantitation results under each of the two conditions is shown in Fig. 1. The expected slope of the curves is 1, if identical efficiencies for the quantitation of each component in both the duplex reaction and singleplex reactions is achieved. Therefore, a slope of 1 indicates no detrimental effects to the quantitation as a result of combining the two amplifications into one duplex assay. The experimentally observed slopes for the TPOX quantitation are 1.15 (full range) and 1.12 (low range inset). For the SRY quantitation, the observed slopes are 1.01 (full range) and 1.05 (low range inset). Therefore, it is evident that the efficiency of the reaction is only slightly negatively affected by simultaneously quantitating male and total human DNA in a single reaction. This slight reduction in efficiency is offset by the more conservative use of

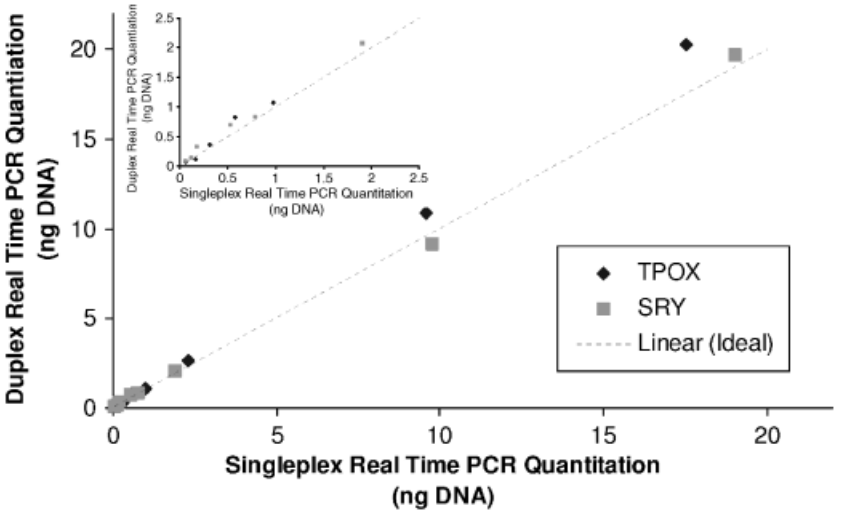

FIG. 1—*Comparison of quantitation results for the quantitative PCR in singleplex and duplex reactions. The slopes of the curves are indicated in the text.*

original sample DNA. That is, only half of the DNA is required, as only one reaction is necessary rather than two singleplex reactions.

Quantitation Limits

The limits of detection of the duplex assay were measured with a dilution series of male DNA. The limit of detection (data not shown) was determined to be 2 and 6 pg DNA, for the TPOX and SRY loci, respectively. Here, the limit of detection is defined as a detectable signal (above the baseline) in at least two of four replicate samples (16). For the autosomal and Y-chromosome loci, respectively, the limit of quantitation was determined to be 10 and 30 pg, where limit of quantitation is, analytically, defined as five times the limit of detection (16). This compares favorably with the lowest DNA quantitation standard recommended for the Quantifiler™ kits (46 pg) and the lowest DNA standard used in the slot blot assay (125 pg, according to standard protocol in this laboratory). These lower limits of quantitation (10 and 30 pg) resulted in reliable detection of the DNA and remained in the linear range of quantitation, as determined by the coefficient of correlation (r^2) repeatedly being greater than 0.99 (data not shown). The limit of quantitation is of little consequence here as any DNA amount lower than 150 pg will, typically, result in addition of the maximum amount of sample to the STR amplification in order to improve the likelihood that one will obtain a full STR profile. In common practice, the lowest point on the standard curve in our laboratory is 46 pg DNA. However, the limits of detection and quantitation should be independently evaluated in each laboratory. During validation, the *useful* lower limits of detection/quantitation should be defined. That is, each laboratory must assess their case management strategy in light of the determined limits of this quantitation assay. This is discussed below with regard to obtaining full and partial STR profiles based upon the quantitation result obtained by this assay. However, as with any forensic sample, it is not possible to predict with complete accuracy how any potentially compromised biological sample will behave.

The assay detection limits are below the DNA concentrations at which generation of a partial STR profile would be expected. This is a significant advantage over the slot blot method where, in some samples, one obtains an STR profile although no slot blot quantitation result was acquired. With these low limits of detection, it is anticipated that, with experience, a strong correlation can be made between the level of input DNA (to the PCR) required and successful generation of a full or partial STR profile. With this correlation, samples with DNA amounts too low to give interpretable results can be set aside for future testing. For example, in our laboratory, a full STR profile (Profiler Plus™) is typically obtained with 125 pg DNA. Less than 125 pg of DNA commonly results in loss of one or more loci. For PowerPlex® Y, a full STR profile is typically obtained for 250 pg male DNA, whereas less than 250 pg male DNA often results in loss of loci. In our experience with the qPCR assay, when no DNA is detected, a full STR profile is not obtained, although, very occasionally, a partial profile is obtained. Upon validation and casework experience, this type of data can be incorporated into a casework management strategy.

The quantitation assay was also evaluated up to 100 ng input DNA, which is a sufficient range for commonly encountered samples in a forensic laboratory. The duplex qPCR assay remained linear up to 100 ng DNA, which is equivalent to the upper limit of quantitation reported for the Quantifiler™ kits. Figure 2*A* shows a typical standard curve and associated amplification plot for the

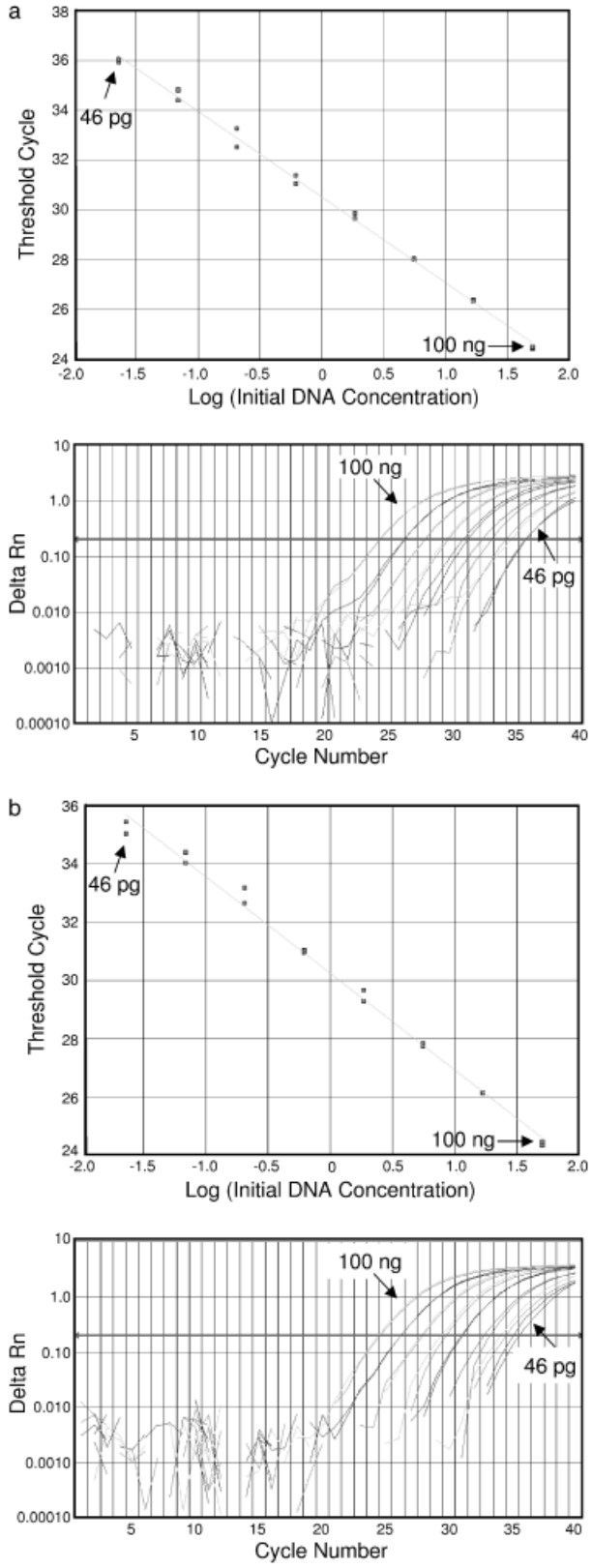

FIG. 2—*(A) Representative standard curve and amplification plot for the TPOX locus. Initial DNA amounts indicated are calculated based on 2 μL DNA per reaction. (B) Representative standard curve and amplification plots for the SRY locus. Initial DNA amounts indicated are calculated based on 2 μL DNA per reaction.*

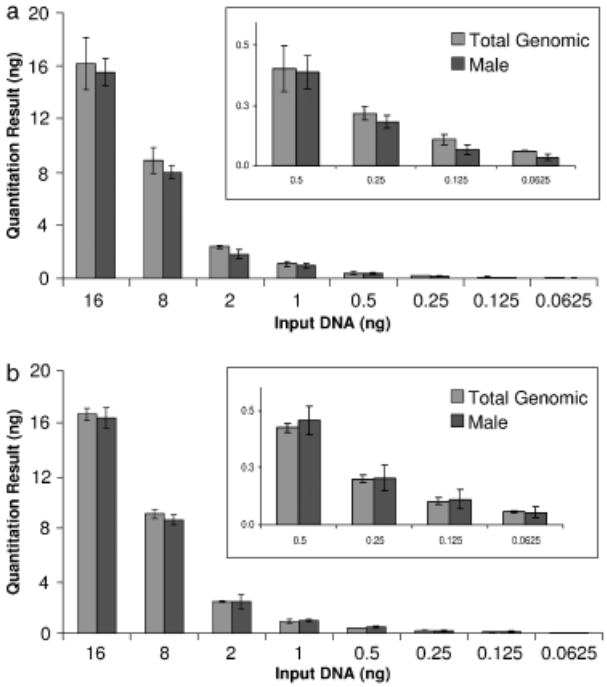

FIG. 3—*(A) Intraplate reproducibility of the duplex assay using the ABI Taqman Universal Master Mix. Error bars indicate the standard deviation of three replicate runs on a single microtiter plate. (B) Interplate reproducibility of the duplex assay using the ABI Taqman Universal Master Mix. Error bars indicate the standard deviation of three replicate runs on separate microtiter plates.*

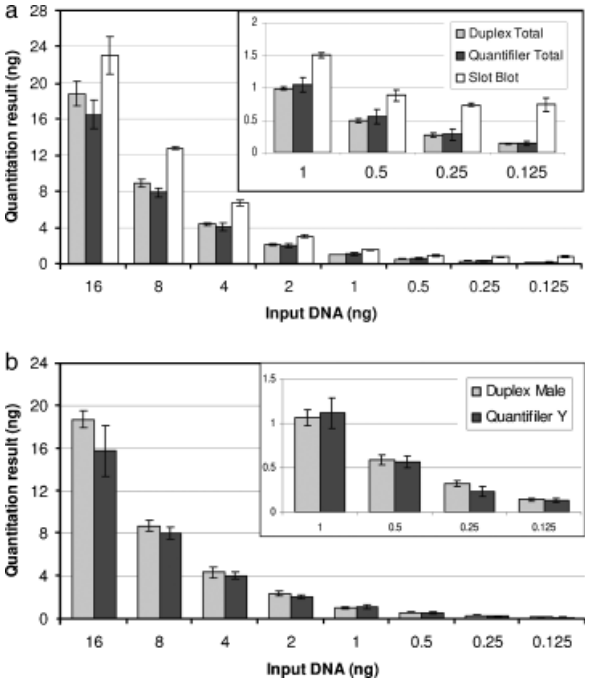

FIG. 4—*(A) Comparison of the total genomic DNA quantitation result from the duplex TPOX locus, Quantifiler™ Human, and slot blot. Values are reported with error bars indicating the standard deviation of the mean obtained in four replicate runs. (B) Comparison of the male DNA quantitation result from the duplex SRY locus and Quantifiler™ Y. Values are reported with error bars indicating the standard deviation of the mean obtained in four replicate runs.*

TPOX (total genomic) locus; Fig. 2*B* displays the same data for the SRY (male) locus.

Probe/Primer Specificity

The primers and probes designed in this study for detection of male and total genomic human DNA were BLAST searched against the NCBI database to ensure lack of homology to nonhuman DNA. In addition to the empirical comparison, the assay was tested experimentally against select common contaminants of forensic samples, including *E. coli* and *C. albicans*. The specificity of the Y-chromosome amplification was likewise tested with the addition of increasing amounts of female DNA. Both master mixes (Sigma JumpStart™ Quantitative PCR ReadyMix™ with additional $MgCl_2$ (3.5 mM final concentration) and Applied Biosystems TaqMan™ Universal Master Mix) were compared for their specificity. When using the Applied Biosystems master mix, no false-positive result was obtained upon replicate testing of 0.75 and 10 ng of *E. coli*, *C. albicans*, Mouse, or *S. cerevisiae* DNA. In addition, no positive result was obtained at the Y-chromosome locus by the addition of female human DNA (0.75, 10, and 100 ng). With the Sigma JumpStart™ ReadyMix™, occasionally, a result at either the TPOX or SRY locus was observed, but in all cases this was below the reported limit of quantitation. In no case was a result for nonhuman DNA obtained above the limit of quantitation. While nonhuman DNA was occasionally detected in the sample, the signal was below that quantitated in this assay and, hence, would not be reported (see preceding section). In addition, these results were not reproducibly obtained, which leads one to believe that it may be a result of random mispriming. No nonspecific increase in signal was obtained from the Y-chromosome locus when testing female DNA only. This is a similar level of nonspecific amplification as reported by other real-time PCR assays (17).

Precision

Intraplate and interplate reproducibility was tested using both the Applied Biosystems TaqMan™ Universal PCR Master Mix and the Sigma JumpStart™ Quantitative PCR ReadyMix™. Figure 3 shows the reproducibility obtained with the Applied Biosystems Master Mix. Relative standard deviations within a plate and between plates were typically $\leq 20\%$ of known input amount. As anticipated, relative standard deviations increased slightly (up to 30%) as the copy number decreased ($\leq$ 125 pg). These relative standard deviations are comparable with that reported by Applied Biosystems (18) and the results obtained experimentally in our laboratory (data not shown).

Accuracy

Side-by-side comparisons of quantitation by the developed duplex assay with the Quantifiler™ kits (Applied Biosystems) were performed. The results are shown in Fig. 4. The duplex assay reported here performed in a manner similar to the Quantifiler™ kits in terms of percent error based on the known amounts of DNA added to the reaction mixture over the linear range of the duplex assay.

The percent error, defined as

$$\text{percent error} = \frac{|\text{calculated} - \text{known}|}{\text{known}} \times 100 \qquad (1)$$

was calculated for eight DNA masses from 16 ng to 125 pg. The average percent error for the duplex assay was 8.0% (total

genomic, TPOX locus) and 15.0% (male, SRY locus) compared with 6.3% (Quantifiler™ Human) and 5.3% (Quantifiler™ Y) for the Quantifiler™ kits. The difference in percent error is not statistically significant for the total genomic DNA quantitation using the duplex assay and the Quantifiler™ Human kit. In comparison, the average percent error was 77.4% for the slot blot assay. Note that the slot blot results were calculated using the BioImage® software. More commonly, slot blot results are estimated to the value of the closest standard (of the standard curve). As a result, the average error may be greater for the slot blot method if visually compared in this manner.

Compatibility with various Extraction Methods

The compatibility of the real-time PCR duplex assay was assessed by amplifying DNA extracted using various extraction protocols, including phenol-chloroform, Chelex, and Qiagen methods (data not shown). Quantitation results correlated well with the results obtained by slot blot quantitation, indicating that there is no significant effect on quantitation based upon any typical level of reagent carryover from these methods.

Population Study

The developed real-time qPCR assay was used to quantitate DNA in approximately 158 Y-STR database samples. These samples constitute three races: Hispanic, Caucasian, and African American. All samples were detected with the duplex assay. Of those samples amplified with PowerPlex Y™ (85), amplification produced peak heights that correlated with the expected results based on the qPCR data. Therefore, it can be reasonably inferred that the amplicons in the duplex assay are in conserved regions of the loci, so as to be useful for most human DNA samples.

Mixture Analysis

As the duplex assay involves amplification of two fragments in competing reactions, unlike the singleplex counterparts, the SRY amplification could potentially be inhibited by the presence of overwhelming amounts of female DNA. To test the ability of the assay to detect small amounts of male DNA in a mixture of male and female DNA, mixtures of male and female DNA, ranging from 1:1 to 1:5000 ratio of male:female DNA, were prepared. Male DNA was detected in all mixtures of male and female DNA, where the amount of male DNA in the sample was constant at 25 pg. The only instance in which male DNA was not detected via qPCR was the 1:5000 (male:female) mixture, which contained only 12.5 pg of male DNA in 62.5 ng of female DNA. In this sample, a partial Y STR profile was obtained with PowerPlex Y®. As might be expected, no useful secondary profile was obtained when the sample was amplified with ProfilerPlus™.

For both PowerPlex Y® and Profiler Plus™, the amplifications were successful using the real-time qPCR data to calculate the sample volume to be added to the STR reactions. When run on the ABI Prism 310, the amplifications resulted in peak heights within the laboratory's analysis parameters. Additionally, the real-time data proved to be a good indicator of the ratio of male to female DNA. The Profiler Plus™ peak height ratios were reasonably consistent with the male to total DNA ratios calculated from the qPCR data and the known male/total ratios in the mixtures. As anticipated, at male:female ratios greater than 1:10, a full secondary Profiler Plus™ profile was not observed. Occasionally, a limited number of loci from the secondary contributor will be detected in samples with male:female ratios greater than 1:10. As anticipated, at male:female ratios of approximately 1:10, a useful secondary Profiler Plus™ profile was commonly observed. A limited number of loci from the secondary contributor may be detected in samples containing male:female ratios greater than 1:10. Because this duplex assay accurately detects male DNA in the presence of overwhelming amounts of female DNA, it is possible to determine the optimal amount of sample to add to a PowerPlex Y® or other Y-chromosome-specific amplification. In addition, as the duplex assay can reasonably determine the ratio of male to female DNA in a mixed sample, it is possible to determine which samples will result in a useful secondary profile when amplified with Profiler Plus™. Those samples that will not result in a useful secondary profile can be identified and amplified for Y STR profiling (rather than autosomal profiling), hence conserving sample and reagents.

Casework Mixture Example

The duplex qPCR assay has been utilized extensively in a wide variety of casework samples, using both the ABI and Sigma master mixes. Our experience has shown the Sigma JumpStart™ ReadyMix™ to be more robust (data not shown) and is, therefore, the master mix being used currently in our laboratory. Further, this assay has been used to identify probative evidence in sexual assault samples.

As an example of the use of the duplex qPCR assay in casework samples, the nonsperm fraction from a vaginal swab was tested, indicating 49% male DNA. Using the quantitation results to calculate the amount of sample to add to the STR amplification, a Profiler Plus™ STR profile was obtained, as shown, in Fig. 5. The ability of the duplex qPCR assay to determine accurately the ratio of male/total DNA present in a sample was assessed by comparing the qPCR result with that calculated from the STR profile. The percent male in the mixture was then calculated at each locus as follows:

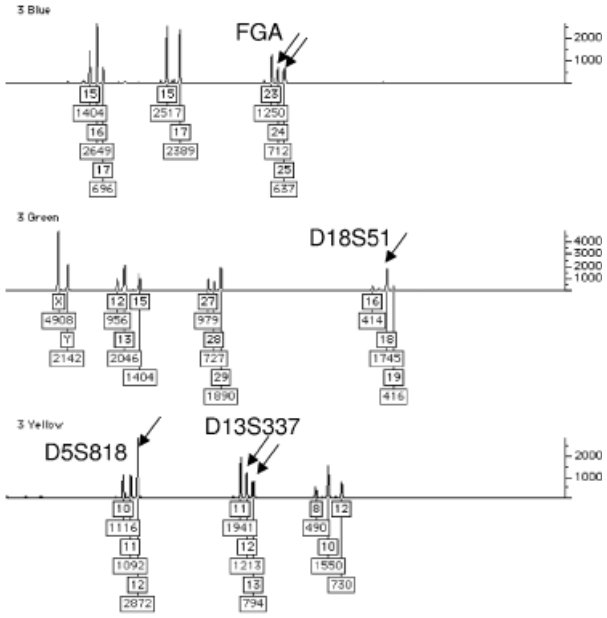

FIG. 5—*Profiler Plus™ amplification results obtained from nonsperm fraction of a casework example. The four indicated loci were used to calculate the percent male in the DNA mixture, as the male and female profiles did not share alleles in these loci. Alleles from the male are indicated by the arrows. Peak heights (RFUs) are indicated below the allele label in each locus.*

TABLE 2—*Calculation of the percent of male DNA in sperm fraction of a casework sample using the short tandem repeat profile.*

Locus	% Male
D13S337	50.8
D18S51	67.8
D5S8181	56.5
FGA	51.9
Average of the above loci	56.8

$$\text{percent male} = \frac{\text{sum of male peak heights}}{\text{sum of male and female peak heights}} \times 100 \quad (2)$$

Table 2 shows the percent male detected at each locus, with an average of 56.5% across all four loci for which the two individuals did not share an allele. This strong correlation between the qPCR and STR results demonstrates the utility of the simultaneous quantitation of male and female DNA in mixtures commonly encountered in a forensic laboratory.

Inhibition

The utility of an IPC for assessment of inhibition was evaluated. Owing to limitations of the ABI 7000 instrument, detection of an additional dye (for an IPC) was not possible. While the addition of an IPC is intended in the future, this will require slight alterations to the assay design. Specifically, the 5′ quenchers must be replaced with nonfluorescent quenchers to allow for detection of the reporting fluor of the IPC. Alternatively, one could utilize a real-time PCR instrument that is capable of detecting greater than four fluorophores simultaneously.

Even without the use of an IPC as in the Quantifiler™ kits, it was determined that inhibition is often discernable by observing the slope of the amplification plot. Extensive studies of inhibited samples have not been performed in this work. However, in casework use, a number of known inhibited samples were evaluated (data not shown)—from which it was determined that one could often establish whether a sample is inhibited based upon the slope of the amplification plot. While inhibited samples are less likely to affect DNA concentration determination via slot blot, in our experience, this is not always advantageous. That is, the examiner has no indication that the sample is inhibited prior to STR amplification. Therefore, the use of slot blot quantitation values may result in poor amplification. However, using the qPCR assay, the examiner can sometimes see that amplification is inhibited to some degree and, as a result, better judge the amount of DNA to add to the STR amplification.

Advantages of the Duplex qPCR Assay

The duplex real-time qPCR assay developed has numerous advantages over the conventional slot blot DNA quantitation method. The limit of detection is comparable to the commercially available Quantifiler™ kits and is a significant improvement over the lower limit of detection obtained using the slot blot method. The accuracy of the assay was shown to be better than the slot blot method and similar to the Quantifiler™ kits. The duplex qPCR assay also represents a significant timesavings over the slot blot method, as the slot blot method requires three or more hours of hands-on time, whereas the qPCR assay requires less than 1 h of manual setup, followed by an automated reaction of approximately 2 h. In addition, DNA quantitation from the qPCR is automatic, while slot blots require visual examination or transfer to an imager for DNA quantitation.

The simultaneous quantitation of male and total human genomic DNA affords the user several distinct advantages over singleplex reactions. Foremost, the analyst obtains information about the male component of a mixture, which is not provided in other assays, such as that of Richard et al. (19) and Nicklas and Buel (17) or the slot blot method. The ratio of male DNA to female DNA in a sample allows the analyst to best determine the downstream processing steps to pursue, thereby eliminating unnecessary use of sample in reactions that will not provide useful data, such as an uninterpretable secondary profile. In comparison with the Quantifiler™ singleplex kits, the duplex assay decreases sample use, as only 2 μL of sample is necessary to obtain both the total genomic and male DNA concentrations; this is particularly important where the sample is limited or DNA concentrations are low. The duplex qPCR assay is also significantly less costly than the Quantifiler™ kits; the Quantifiler™ kit (for both reactions) is 5.5 times the cost of the duplex assay using the Sigma master mix and 2.2 times the cost using the ABI master mix.

Conclusions

A robust qPCR assay for the simultaneous detection and quantification of total human genomic and male DNA for use in forensic DNA analysis has been developed. The precision, accuracy, specificity, and low limits of detection of the assay have been demonstrated. This DNA quantitation assay constitutes a superior alternative to the slot blot methods commonly used in forensic laboratories as well as other commercially available qPCR assays.

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