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Developmental Validation of the Quantifiler™ Real-Time PCR Kits for the Quantification of Human Nuclear DNA Samples*

ABSTRACT: The Quantifiler™ Human DNA Quantification Kit and the Quantifiler™ Y Human Male DNA Quantification Kit were designed for the quantification of human genomic DNA in forensic samples. The kits use a real-time PCR-based process to quantify, respectively, total human DNA or human male DNA only. We report the results of a developmental validation study that we performed with the Quantifiler Kits, following the official SWGDAM guidelines. The Quantifiler Kits were tested for performance criteria such as species specificity, sensitivity, stability, precision and accuracy, and in addition, were tested with forensic case-type samples and mixed (male:female) DNA samples. The Quantifiler Kit methods were highly specific for human DNA, and could detect as little as 32 picograms of DNA using 2 µL of sample per assay. The accuracy and precision of the Quantifiler Kit methods was comparable or superior to that of other quantification methods.

KEYWORDS: forensic science, DNA quantification, forensic DNA analysis, polymerase chain reaction

Molecular technologies for DNA analysis, exemplified by human genotyping techniques using short tandem repeat (STR) sequences, have brought about a revolution in forensic investigations. Using these new technologies, analysts are now able to routinely work near the ultimate limits of sensitivity and discriminatory power in genotyping forensic casework samples. The most powerful STR genotyping methods use the polymerase chain reaction (PCR) to amplify target loci, allowing accurate genotype information to be gathered from as little as picogram amounts of genomic DNA (1). Yet, ancillary methods for the quantification of forensic DNA samples, an official requirement for STR analysis of casework samples (2), have until recently relied on older technologies, such as slot-blot hybridization, which were unable to match the accuracy and sensitivity of the STR assays they were intended to precede. In addition to the limited accuracy and sensitivity of many such older DNA quantification methods, the methods also tend to be relatively labor-intensive and time-consuming. Often, the results of the assays are non-objective and require a significant degree of interpretive skill from the analyst. Although reliable and robust, most of the older DNA quantification methods are poorly suited to high-throughput or automated sample flow.

We report the results of developmental validation of two new commercial kits that use “real-time” quantitative PCR to detect and quantify human nuclear DNA, designed for use with forensic samples. The Quantifiler™ Human and Quantifiler™ Y Human Male DNA Quantification Kits were intended to address some of the aforementioned limitations of older DNA quantification methods.

The high specificity of PCR amplification allows different choices in target DNA sequences to be made, according to the intended use of the assay. Examples of different strategies associ-

ated with assay target selection are: general human-specific DNA quantification using highly-repetitive *Alu* motif nuclear sequences (3), quantification with the assessment of the degradation state of DNA samples using the parallel amplification of differently sized fragments of mitochondrial DNA (4), or quantification with donor sex determination using the amplification of homologous but distinct regions of the X and Y chromosomes (4,5).

The Quantifiler kits were designed for the quantification of human nuclear DNA, and their genomic targets reflect the particular roles for which the kits were intended. The Quantifiler Human and Quantifiler Y Human Male kit assays detect, respectively, the human telomerase reverse transcriptase (hTERT) locus located on chromosome 5 (6–8), and the “sex reversal” (SRY) locus located on the Y chromosome (9,10). The Quantifiler Human kit assay detects total human genomic DNA (i.e., male and female), while the Quantifiler Y Human Male kit assay detects only male DNA. The Human kit assay is intended for general use as a preliminary DNA quantification step to performing routine STR analysis, while the Human Male kit assay is designed to be of use particularly in samples with mixed male-female DNAs, such as sexual assault evidence, where it may be useful to separately detect and quantify male DNA from a background of female DNA. Because of the kits’ intended use primarily as an adjunct to STR analysis, their respective genomic target loci were chosen to be single-copy, like the STR loci themselves, so that they would better predict the availability of amplifiable copies of the STR alleles in samples, and be subject to the same sort of stochastic sampling effects. In addition to the human DNA-specific elements of the Quantifiler kit reagents, an Internal PCR Control (IPC) system—identical between the two assays—is included as an internal quality control check confirming the function of all system components as well as the amplifiability of analysis samples.

The Quantifiler kit assays use “fluorogenic 5’ nuclease” real-time PCR technology for the detection of target sequence amplification (11). This system is based on a set of two oligonucleotide PCR primers specific for the target DNA sequence, or *amplicon*, plus

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*A portion of the data is presented in the Quantifiler™ Kits User’s Manual “Experiments and Results” section.

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a TaqMan[®] probe, labeled with a 5' fluorescent reporter dye and a 3' non-fluorescent quencher, that is homologous to the amplicon region between the PCR primers. The TaqMan probes used in the Quantifiler kits incorporate an additional chemical modification at their 3' ends, known as minor groove binder (MGB), to increase the melting temperature (T_m) and thereby allow the use of shorter, more highly sequence-specific probes (12,13). PCR amplification is performed using an all-in-one "master mix" reagent that includes a "hot-start" thermal-stable DNA polymerase, AmpliTaq Gold.[®]

The fluorogenic 5' nuclease assay process can be summarized as follows. At the start of PCR thermal cycling, all TaqMan probe molecules are intact, and the close physical proximity of the dye and quencher suppresses the fluorescence of the dye molecules by Förster-type energy transfer (14,15). During the course of synthesizing new DNA strands from the target template, the AmpliTaq Gold enzyme encounters the TaqMan probe annealed to the template DNA and hydrolyzes it with its 5' → 3' exonuclease activity. The hydrolysis of the probe separates the dye and quencher elements, which allows the dye molecules to fluoresce more intensely. The hydrolysis of probe molecules occurs in proportion to the amplification of the target sequence, and consequently, so does the resulting fluorescent signal (16,17). Eventually the fluorescent signal accumulates to the point where it can be detected over background fluorescence, and crosses a set fluorescence threshold. The cycle number at which the fluorescent signal crosses the threshold is defined as the "Threshold Cycle", or C_T . There is an exact inverse mathematical relationship between the starting copy number of target sequence molecules and the resulting C_T , which is the basis for quantitative PCR. In practice, the user makes a dilution series containing known quantities of a human genomic DNA concentration standard, and runs the dilutions along with samples to be quantified. A standard curve of C_T versus the concentration of the standard dilutions is automatically constructed by the analysis software, and samples are quantified automatically by mathematical comparison to the standard curve formula.

The Quantifiler kit reaction mix contains two independent sets of primers and TaqMan probes, with each set constituting a "detector." One detector uses a FAM[™] dye-labeled TaqMan probe and is specific for human genomic DNA (either the hTERT locus for the Quantifiler Human kit or the SRY locus for the Quantifiler Y Human Male kit). The other detector set uses a VIC[®] dye-labeled TaqMan probe and, along with a synthetic DNA template added at a fixed concentration, constitutes the IPC system. Both target and IPC detectors are designed to amplify in parallel in every reaction, and assessing the amplification of both systems together allows for the positive verification of human DNA-negative samples as well as an indication of PCR inhibitors that may be present. Figure 1 illustrates the interpretation of detector amplification. For example, in a sample with no human-specific amplification (negative FAM dye detector result), the positive amplification of the IPC system (positive VIC dye detector result) would verify that all system components were functioning normally; this allows for confirmation of true negative samples. In contrast, a sample that showed neither amplification of the human-specific nor IPC detectors would indicate the presence of PCR inhibitor in the sample, or a failure of instrument or reagent components; in either case the user is notified that human DNA may be present but not amplifiable.

The Quantifiler kits were designed and validated for use with the ABI PRISM[®] 7000 and ABI PRISM 7900HT Sequence Detection Systems (SDS) instruments, which includes the SDS software that controls the instruments and automatically performs data analysis.

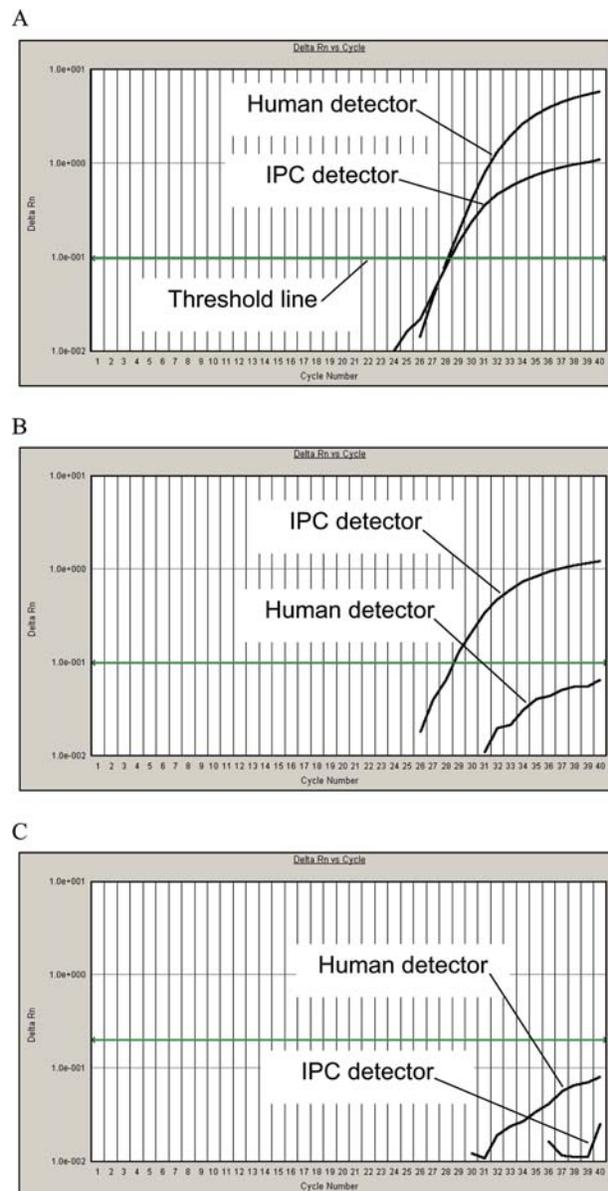


FIG. 1—Quantifiler Human kit assay amplification plots for positive, negative and inhibited reactions. Amplification plots for the human detector (FAM[™] reporter dye) and IPC detector (VIC[®] reporter dye) are indicated, showing reaction fluorescence (ΔRn) versus cycle number. A positive result is defined when a detector's amplification plot crosses the fluorescence threshold line, and the threshold cycle, or C_T , is set at that cycle number. Human and IPC detector results can be used to confirm a human-negative assay result or to detect assay failure due to the presence of PCR inhibitor in the analysis sample or faulty assay setup. A: Human DNA-positive reaction results, with positive amplification of both human and IPC detectors; B: Human DNA-negative reaction results, with negative amplification of the human detector but positive (confirmatory) amplification of the IPC detector; C: Assay results for a sample with total PCR inhibition, where both human and IPC detectors were negative.

Developmental validation of the Quantifiler kits was performed according to the guidelines of the Scientific Working Group on DNA Analysis Methods (SWGDM) (18). Although these guidelines were developed mainly with STR genotyping assays in mind, we sought to apply them as closely as possible to the Quantifiler kit validation since they are intended to be used as part of the same process flow that includes the STR assays themselves. However, certain of the guideline criteria, such as those concerning measurement

standards for alleles or the mode of inheritance of genetic markers, were not applicable to these quantification assays and were not tested.

Materials and Methods

Sources and Preparation of Genomic DNAs Used in Testing

Human genomic DNAs were obtained from different commercial suppliers or were extracted from single-source and casework samples by a forensic testing laboratory using an organic (phenol:chloroform) purification procedure (19). A set of 50 human genomic DNA samples, most from individuals of African-American ethnicity (approximately half male, half female), referred to in this paper as the “resolution panel,” were extracted from whole blood specimens by the supplier (SeraCare Life Sciences, Inc., Oceanside, CA). A set of 450 genomic DNAs known as the DNA Polymorphism Discovery Resource (referred to as the “Coriell panel” in this paper), was prepared by the provider (Coriell Cell Repositories, Camden, NJ, catalog number MPDR450). The Coriell panel, made up of approximately half male and half female individuals, represented multiple ethnicities and geographic regions of the United States; it consisted of the following number of individuals of different ethnic groups: 120 European-American, 120 African-American, 60 Mexican-American, 30 Native American and 120 Asian-American (20). Additional individual DNAs were obtained from the following suppliers: Raji cell line DNA (male), Lofstrand Labs Limited, Gaithersburg, MD; 9948 cell line DNA (male), Marligen Biosciences, Inc., Ijamsville, MD; “007” DNA extracted from whole blood (male), Serological Research Institute, Richmond, CA; K-562 cell line DNA (female), American Type Culture Collection, Rockville, MD. All human DNA samples were genotyped using the AmpF ℓ STR $^{\text{®}}$ Identifiler $^{\text{®}}$ PCR Amplification Kit (Applied Biosystems), which allowed independent verification of the sex of each donor by assessing the alleles of the amelogenin locus.

A panel of DNAs from primate species was obtained from BIOS Laboratories, Inc., New Haven, CT; the sex of the donor animals was not known for these samples. Additional DNAs from individual animals of known sex were prepared from whole blood specimens using an ABI PRISM $^{\text{TM}}$ 6100 Nucleic Acid PrepStation procedure, or were provided by the Oregon State Fish and Game Laboratory. Multiple genomic DNA samples of bacterial and yeast species were prepared from pure cultures using the IsoQuick $^{\text{TM}}$ Nucleic Acid Extraction Kit, Orca Research Inc., Bothell, WA; these purified DNA samples were combined in several pools, each containing DNA from five individual species.

DNA Quantification Using UV Absorbance, Dye Intercalation and Slot Blot Hybridization Methods

Different DNA quantification methods were used to assess the accuracy of the Quantifiler kit assays. Spectrophotometric absorbance at 260 nm wavelength (A_{260} method) was done with a Lambda 10 BIO spectrophotometer (PerkinElmer Instruments, Shelton, CT). The DNA concentrations of samples were then calculated using a standard conversion formula (21):

$$\text{Conc.}(\mu\text{g/mL}) = 50 \times A_{260}.$$

The PicoGreen $^{\text{®}}$ dsDNA Quantitation Kit (Molecular Probes, Eugene, OR), referred to herein as the dye intercalation assay, was performed according to the microplate protocol. Fluorescence emission was measured on an ABI PRISM 7000 Sequence Detection System instrument. After subtracting the background fluorescence

of a blank sample from all standards and unknown samples as directed in the kit protocol, the standard curve data points were plotted on a graph and a regression line with associated linear regression formula was determined using Microsoft Excel $^{\text{®}}$ 2000. Each sample was then quantified by entering its background-subtracted numerical fluorescence value into the regression formula and solving for DNA concentration.

Slot blot hybridization DNA quantification assays were performed using the QuantiBlot $^{\text{®}}$ Human DNA Quantitation Kit (Applied Biosystems) according to the protocol for chemiluminescent autoradiography. Quantification was done by visually comparing the blot intensity of samples against those of a two-fold dilution series of a human genomic DNA standard. In some instances, analysis samples were diluted so as not to exceed the maximum concentration range of the QuantiBlot kit assay.

Quantifiler Human and Quantifiler Y DNA Quantification Kit Assays

The Quantifiler kit assays were performed according to the protocol specified in the User’s Manual. Quantification standard dilutions were first made by serially diluting the 200 ng/ μL stock solution from the kits to the following concentrations: 50, 16.7, 5.56, 1.85, 0.62, 0.21, 0.068 and 0.023 ng/ μL in T $_{10}$ E $_{0.1}$ Buffer (10 mM Tris-Cl pH 8.0, 0.1 mM Na $_2$ EDTA) that contained 20 $\mu\text{g/mL}$ of molecular biology grade glycogen as a stabilizer (Roche Applied Science, Indianapolis, IN). Master mix was formulated by combining 10.5 μL of Primer Mix (specific for the Quantifiler Human or Quantifiler Y kits) and 12.5 μL of Quantifiler PCR Reaction Mix per reaction, multiplied by the number of reactions required, then dispensing the mix into an ABI PRISM $^{\text{TM}}$ 96-Well Optical Reaction Plate (Applied Biosystems) at 23 μL per reaction. 2.0 μL sample volume was added per reaction, with duplicate reactions of each quantification standard and single reactions of each analysis sample being run. After all reactions were set up, the optical plate was then covered with a clear plastic Optical Adhesive Cover (Applied Biosystems), pressed into place with a plastic applicator tool, and covered with a foam compression pad. The plate was then placed in the 96-well sample block of an ABI PRISM 7000 or ABI PRISM 7900HT Sequence Detection System (SDS) instrument. The SDS software controlling the 7000 or 7900HT instruments was programmed for the following thermal cycle:

$$95^{\circ}\text{C}/10 \text{ min hold} \rightarrow 40 \text{ cycles of}[95^{\circ}\text{C}/15 \text{ sec}, 60^{\circ}\text{C}/60 \text{ sec}]$$

After thermal cycling was completed (approximately 1:45 hours), data analysis was performed by the SDS software to generate standard curve data for quantification standards and quantification results for unknown samples.

AmpF ℓ STR Identifiler PCR Amplification Kit Genotyping Assays

STR analysis was performed using the standard protocol for the AmpF ℓ STR Identifiler PCR Amplification Kit assay. Identifiler kit reactions were analyzed on an ABI PRISM 3100 Genetic Analyzer with Data Collection Software v1.1. Electropherogram data was analyzed with GeneScan $^{\text{®}}$ Software v3.7.1 and Genotyper $^{\text{®}}$ Software v3.7 for use with the Windows $^{\text{®}}$ NT operating system.

Species Specificity Studies

The specificity of the Quantifiler Human and Quantifiler Y assays was assessed for a large number of human DNA samples and for panels of genomic DNA from non-human vertebrate species

and bacteria. The assay results were scored so that any sample with a human-specific detector (Human or Y) threshold cycle ($C_{T\text{FAM}}$) that was <40 (out of 40 cycles total) would be considered positive, while samples that had no detectable amplification of the human-specific detector at 40 cycles would be considered negative. The human DNAs were tested at approximately 20–40 ng per Quantifiler kit reaction. The sex of all human DNAs was independently verified by performing STR analysis with the AmpF ℓ STR Identifiler STR typing kit. The non-human species panels consisted of several species of higher vertebrates, mostly common domestic mammals such as dog, cat, pig, cow, horse, etc., but also containing several higher ape species like chimpanzee, gorilla, orangutan and macaque. For most of these samples, 0.25 to 1.0 ng of DNA was added per reaction, but a few samples were added at up to 40 ng per reaction. The sex of the donor was not known for most of the primate samples, complicating the interpretation of results from the Quantifiler Y assay. However, a sample of known male gorilla genomic DNA was obtained to help bolster information about assay specificity for non-human Y chromosomes. A bacterial species panel made up of several pools of genomic DNA, most containing five bacterial species each for a total of 54 species (including one yeast), was tested with both Quantifiler kit assays. These samples were tested with approximately 1×10^5 genome copies of each species per Quantifiler kit reaction. As with other specificity testing, the assay results were assessed on a plus/minus basis.

Sensitivity Studies

Serial five-fold dilutions of several human genomic DNAs were made in $T_{10}E_{0.1}$ Buffer containing 20 $\mu\text{g}/\text{mL}$ of glycogen as a stabilizer, to cover the range from 10 $\text{ng}/\mu\text{L}$ to 0.016 $\text{ng}/\mu\text{L}$ (16 $\text{pg}/\mu\text{L}$). Assay results with C_T that was <40 were considered to be positive, and as a further indication that the assay results were quantitatively accurate, they were plotted on a semi-logarithmic scale of C_T vs. Starting DNA Quantity ($\text{ng}/\mu\text{L}$). Quantitatively accurate results would be expected to fall along a straight line on such a semi-logarithmic plot, in accordance with the known mathematical relationship between C_T and starting DNA quantity (see explanation in the introductory section).

As a companion study to the sensitivity testing, the rate of false-positive Quantifiler kit assay results for human DNA-negative samples was also assessed. A set of 48 reactions of each Quantifiler kit assay (Human and Y) were prepared and dispensed as usual. Each reaction received 2 μL of TE Buffer as its sample volume. The assays were run as per the standard protocol except for one change: thermal cycling was extended from the usual 40 cycles to 50 cycles. The samples were analyzed with the SDS software after completion of the runs, and those with a C_T that was <50 would have been considered to be false-positive results.

The Hematin Model System for PCR Inhibition

To study the effects of PCR inhibition on the results of Quantifiler kit assays, a model PCR inhibition system was used. The model system employed the compound hematin, a known impurity that may be present in DNA extracted from bloodstains (22). Several preparations were made containing a human genomic DNA from the resolution panel at a fixed concentration of 1.0 ng per 2.0 μL , with concentrations of hematin added to 0, 10, 12, 14, 16, 18, 20 and 40 μM . These hematin concentration series samples were then analyzed by Quantifiler Human and Quantifiler Y kit assays, as

well as by the AmpF ℓ STR[®] Identifiler[®] PCR Amplification Kit. The hematin series samples were always added at 2.0 μL (1.0 ng of human DNA) per reaction, which meant that the final concentrations of DNA and hematin would be the same between corresponding Quantifiler kit and Identifiler kit reactions.

Preparation of Degraded Genomic DNA Samples

Environmental degradation of human genomic DNA was modeled using an exonuclease digestion system. DNA-free[™] DNase Treatment and Removal kit (Ambion, Austin, TX) reagents were used with human genomic DNA to create a timepoint series of samples with progressively greater levels of degradation. 20 μg of Raji DNA was mixed with kit buffer and water, and a “zero” timepoint sample (50 μL) was immediately collected. 2 units of the exonuclease DNase I was then added, and the reaction was incubated at ambient temperature (approx. 21°C). 50 μL samples were removed at regular intervals (2, 4, 8, 12, 20, 35 and 60 min) and immediately added to 5 μL of DNase Inactivation Reagent to terminate DNA digestion. Agarose gel electrophoresis was then performed by loading 2 μL of each timepoint sample on a 2% E-Gel (Invitrogen) and the extent of degradation of timepoint samples was assessed by inspection of the visualized genomic DNA bands relative to a ladder of DNA size standards.

To assess degradation at each timepoint, samples were analyzed by Quantifiler Human and Quantifiler Y kit assays, as well as by the AmpF ℓ STR[®] Identifiler[®] PCR Amplification Kit. The results of the Quantifiler Human kit assay for each timepoint sample was used to calculate an input quantity of 1.0 ng total of amplifiable DNA per Identifiler kit reaction. This meant that, with increasing DNA degradation in later timepoint samples, relatively more volume of the samples was added to the Identifiler kit reactions to compensate for the loss of amplifiable DNA.

Analysis of Case Type Samples

Casework-type samples were prepared from human genomic DNA deposited on different materials (primarily clothing fabric). DNA was extracted and purified from all samples using an organic solvent-based method (phenol:chloroform:isoamyl alcohol extraction). DNA quantification was done using the Quantifiler Human Kit assay. To provide additional verification of the Quantifiler kit assay results, Identifiler kit STR genotyping assays were set up to contain approximately 1 ng of human genomic DNA per reaction as determined by the Quantifiler kit assay; some samples had such low concentrations, however, that adding the maximum allowed volume of 10 μL to Identifiler kit reactions amounted to less than 1 ng of DNA.

DNA Mixture Studies

Single-source human genomic DNAs from Raji (male) and K-562 (female) cell lines were combined in a range of ratios from 1:1 to 1:1024 (male:female). The male DNA concentration was kept constant at 0.05 $\text{ng}/\mu\text{L}$, while the female DNA ranged from a low level of 0.05 $\text{ng}/\mu\text{L}$ in the 1:1 ratio sample, to a high level of 50 $\text{ng}/\mu\text{L}$ in the 1:1024 ratio sample. The DNA concentration in each mixture sample was determined separately using the Quantifiler Human and Quantifiler Y Kit assays. Three replicate assays of each sample were performed with each Quantifiler kit assay so that the standard deviations of the quantification results could be calculated.

Accuracy and Precision Studies

An assessment of the accuracy and precision of C_{TFAM} measurement was made by running replicate reactions of the eight quantification standard serial dilutions used in Quantifiler kit assays. Starting with the 200 ng/ μ L Quantification Standard stock reagent supplied with each Quantifiler Kit (the reagent is the same for both kits), a dilution series was made from a high of 50 ng/ μ L to a low of 0.023 ng/ μ L (i.e., 23 pg/ μ L) as specified in the standard protocol for the assays. Each of the dilutions was run in duplicate reactions with each of the Quantifiler kit assays per assay plate. This was repeated on three different ABI PRISM 7000 instruments on two different days to give a total of six runs of data (12 separate reactions per standard dilution.) The C_{TFAM} values for the eight quantification standard dilutions were tallied over all runs, and the mean and standard deviation of the C_T s were calculated.

Studies were done to compare Quantifiler kit assay results with those of different DNA quantification methods. In one experiment, six different purified human genomic DNA stocks were each diluted to three concentration levels, with approximate target concentrations of 2.0, 0.5, and 0.1 ng/ μ L. The diluted DNAs were then quantified with the Quantifiler Human and Quantifiler Y Kit assays, and with the A_{260} and dye intercalation methods. The DNA dilution levels were designed to be as much as possible within the effective working ranges of all methods, however, the lowest concentration samples (0.5 ng/ μ L and 0.1 ng/ μ L) were below the sensitivity limit for the A_{260} method and could not be measured directly. For the Quantifiler kit assays, each sample was run in three replicate reactions so that means and standard deviations for quantification results could be calculated. Another experiment was done to compare the Quantifiler kit assays, the A_{260} method and the QuantiBlot membrane hybridization assay kit. 50 human DNA samples from the “resolution panel” were tested by both of the Quantifiler kit assays, by the A_{260} method and by the QuantiBlot kit method using its chemiluminescent detection mode.

Method comparisons were done by calculating a “percent difference” between each sample’s Quantifiler Human or Quantifiler Y quantification result and corresponding comparison method results. This quantity was the absolute value of the method differences for each sample (e.g., its Quantifiler Human result minus its A_{260} result) expressed as a percentage of the Quantifiler kit assay result. Taking the average of all such individual percent difference values gave an overall measure of the magnitude of quantification result differences between the Quantifiler kit assays and comparison methods.

Results and Discussion

Specific validation experiments were patterned after criteria for developmental validation in the SWGDAM Revised Validation Guidelines (approved July, 2003) (18). The results are presented below to correspond with the outline of the SWGDAM guidelines, with the section numbers (2.1, 2.2, 2.3, etc.) referring to the specific SWGDAM Guideline sections where the validation specifications originated. This validation framework was used because the Quantifiler kit assays represent a “novel technology for forensic DNA analysis.” However, in some cases where noted, a literal interpretation of the guidelines applied poorly to validating a DNA quantification assay; in these cases, the specific guidelines were re-interpreted to be more relevant to performance testing of such a technology.

Characterization of Genetic Markers (SWGDAM 2.1)

The genetic markers, or target loci, for the Quantifiler Human and Quantifiler Y kit assays are, respectively, the hTERT and SRY

genes. hTERT is located at the distal end of chromosome 5 (map position 5p15.33), and SRY is centrally positioned on the human Y chromosome (map position Yp11.3). The genes are present at one copy per chromosome (6–10), which means that a normal female cell would contain two copies of hTERT and no copies of SRY, while a normal male cell would contain two copies of hTERT and one copy of SRY. The assay target amplicon DNA sequences are located within untranslated regions of their respective genes; the hTERT amplicon is in an intron region and the SRY amplicon is 5' to the coding sequence.

Although the hTERT gene is located near the telomere repeat sequences that define the terminal end of chromosome 5, and it is known that telomeres have a normal tendency to shorten with the age of the individual (23), the degree of shortening associated with *in vivo* aging would be unlikely to affect the hTERT gene itself. A mean lifetime loss of 1,500 base pairs (bp) of telomeric sequence was observed in one study of fibroblast cells from 43 individuals, ranging in age from infancy to 93 years, which represented approximately 30% of the telomeric region present at birth (23). To put this in perspective, while the hTERT gene is close to the end of chromosome 5 in relative terms, it is located approximately 1.3 million bp from the end of the chromosome (24), which should provide an ample buffer to any shortening process localized to the telomeric region itself.

Species Specificity (SWGDAM 2.2)

In validating the species specificity of the Quantifiler kit assays, genomic DNA samples from 500 humans (representing multiple racial/ethnic groups), several vertebrate species (including higher primates like chimpanzee, gorilla and orangutan), and 54 species of bacteria were tested.

Testing with human genomic DNAs gave the following results: the Quantifiler Human Kit assay detected all 240 males and all 260 females, while the Quantifiler Y Kit assay detected all 240 males and none of the 260 females (data not shown).

Specificity for higher vertebrate species (Table 1) showed no detection of non-primates of either sex by both Quantifiler kit assays.

TABLE 1—Specificity of quantifiler kit assays with non-human DNAs.

Organism*	Sex	Assay Results (Positive Or Negative)	
		Quantifiler Human	Quantifiler Y
Gorilla (2)	unkn.	+	–
Chimpanzee (2)	unkn.	+	+
Orangutan (2)	unkn.	+	–
Macaque (2)	unkn.	+/- [†]	–
Gorilla	M	+	–
Cat	M	–	–
Dog (2)	M	–	–
Mouse	M	–	–
Rabbit	M	–	–
Rat	M	–	–
Horse (2)	M	–	–
Bovine	M	–	–
Sheep	M	–	–
Pig	M	–	–
Deer	M	–	–
Chicken	M	–	–
Human (Control)	F	+	–
Human (Control)	M	+	+

* Numbers in parentheses indicate number of individuals tested.

[†] Weak amplification observed (unusually high C_T of 36–37 for the amount of DNA tested).

However, higher ape species (chimpanzee, gorilla, orangutan) were all detected by the Quantifiler Human kit assay, with apparent weak detection of another primate species, the macaque monkey. Weak detection was interpreted as a disproportionately high C_T result (the C_T of duplicate reactions was observed to be 36-37) in a reaction containing approximately 5 ng of macaque genomic DNA. Normal amplification efficiency in a sample containing a similar amount of human genomic DNA and run under similar conditions, for example, would have been expected to give a C_T in the range of 27. To put this C_T difference in perspective, 9 C_T units would normally correspond to a difference in DNA quantity (figuring for a doubling of DNA concentration with every thermal cycle) of 2^9 , or 512-fold. For the tests of ape and macaque DNAs with the Quantifiler Y Kit assay, detection was limited to just one individual chimpanzee; however, among this sample set, the sex of only one sample (male gorilla) was known, and it tested negative. A likely (but unproven) interpretation of this is that the one Quantifiler Y kit assay-positive chimpanzee was male, and the assay result indicates that male chimpanzee DNA is detected by the assay, while the negative assay result with known male gorilla DNA indicates that the Quantifiler Y kit assay does not detect this species.

All 54 of the bacterial DNAs and one yeast species' DNA were negative by both Quantifiler kit assays when tested in several pooled samples containing the DNAs of four to five isolates, each present at approximately 1×10^5 copies per assay (data not shown).

Sensitivity Studies (SWGDM 2.3)

We performed experiments to determine the range of concentrations of human genomic DNA within which positive detection and accurate quantification could be obtained using the Quantifiler kit assays. The results of sensitivity testing (Fig. 2) showed that, at all concentrations of each of the DNAs tested, the Quantifiler kit assays gave positive assay results, and that the resulting plots of C_T vs. DNA concentration showed all data points to be located along a straight line, indicating valid results. It provides a useful perspective on the sensitivity of detection to note that, on average, the lowest DNA concentrations tested (16 pg/ μ L or 32 pg per reaction) contained approximately 10 copies of the autosomal hTERT locus and 5 copies of the Y chromosome SRY locus, according to a standard conversion factor (1). In routine practice, the Quantifiler kit assays can detect even lower amounts of DNA than this, with positive results being frequently observed in samples containing as little as 6.0 pg per reaction (data not shown). It is likely that the assays approach single-copy sensitivity, but we omitted such low-copy samples from this study because the statistical uncertainties that complicate the interpretation of results obtained with them (i.e., stochastic sampling effects) seemed to add little to the goals of the study.

A corollary to the extremely high sensitivity of the Quantifiler kit assays is the confidence that low-level positive assay results (i.e., those with extremely low DNA concentrations and correspondingly high $C_{T\text{FAM}}$) reflect the actual presence of human DNA rather than false positive results in samples with no such DNA. As shown in the amplification plots in Fig. 3, 48 negative control reactions of each Quantifiler kit assay gave negative assay results at the end of PCR thermal cycling that had been extended beyond the usual limit to 50 cycles. Positive IPC amplification for all of the control reactions added additional confirmation of these results. The undetectably low false-positive rate for the Quantifiler kit assays in this study bolsters the confidence level with which any positive assay results could be interpreted as valid.

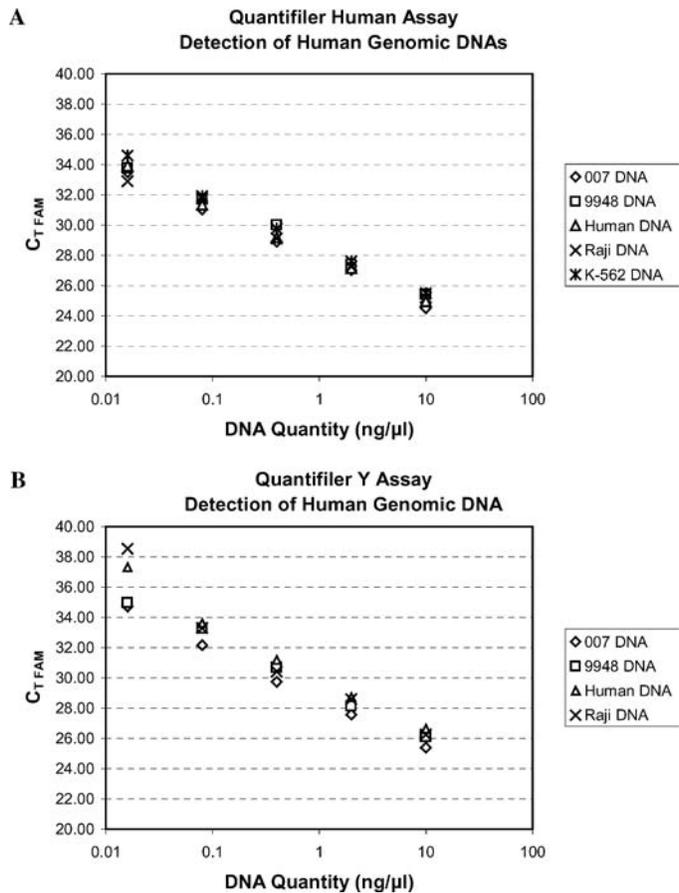


FIG. 2.—Sensitivity of Quantifiler kit assays. Five-fold serial dilutions were made of different human genomic DNA preparations to cover a range of concentrations from 10 ng/ μ L to 0.016 ng/ μ L (16 pg/ μ L). Quantifiler kit assays were performed using 2.0 μ L of each dilution per reaction. Plots of the resulting C_T s vs. DNA quantity showed the log-linear relationship expected for valid detection/amplification events. A. Plot of results for the Quantifiler Human assay; B. Plot of results for the Quantifiler Y assay. K-562 DNA was not detected by the Quantifiler Y assay because it is derived from a female source.

Stability Studies (SWGDM 2.4)

The SWGDAM Guidelines define stability as, “The ability to obtain results from DNA recovered from biological samples (a) deposited on various substrates, and (b) subjected to various environmental and chemical insults . . .” The validation of Quantifiler kit assay performance relating to the first part of this definition (a) is largely covered by a set of experiments presented later in this paper (*Case Type Samples*). The second part of the definition (b) was addressed by experiments using artificial, *in vitro* model systems to simulate DNA degradation and PCR inhibition.

Refer to Materials and Methods descriptions for “Preparation of Degraded Genomic DNA Samples and The Hematin Model System for PCR Inhibition.” These experiments were done to compare the results of Quantifiler Human assays with those of matched AmpF ℓ STR Identifier PCR Amplification Kit assays (“STR assays”) for samples that were treated to simulate PCR inhibition or environmental degradation. The intent of the studies was to show the limit of environmental compromise to forensic samples that would still allow relatively accurate quantification results to be obtained, as judged by the results of paired STR assays of the same samples.

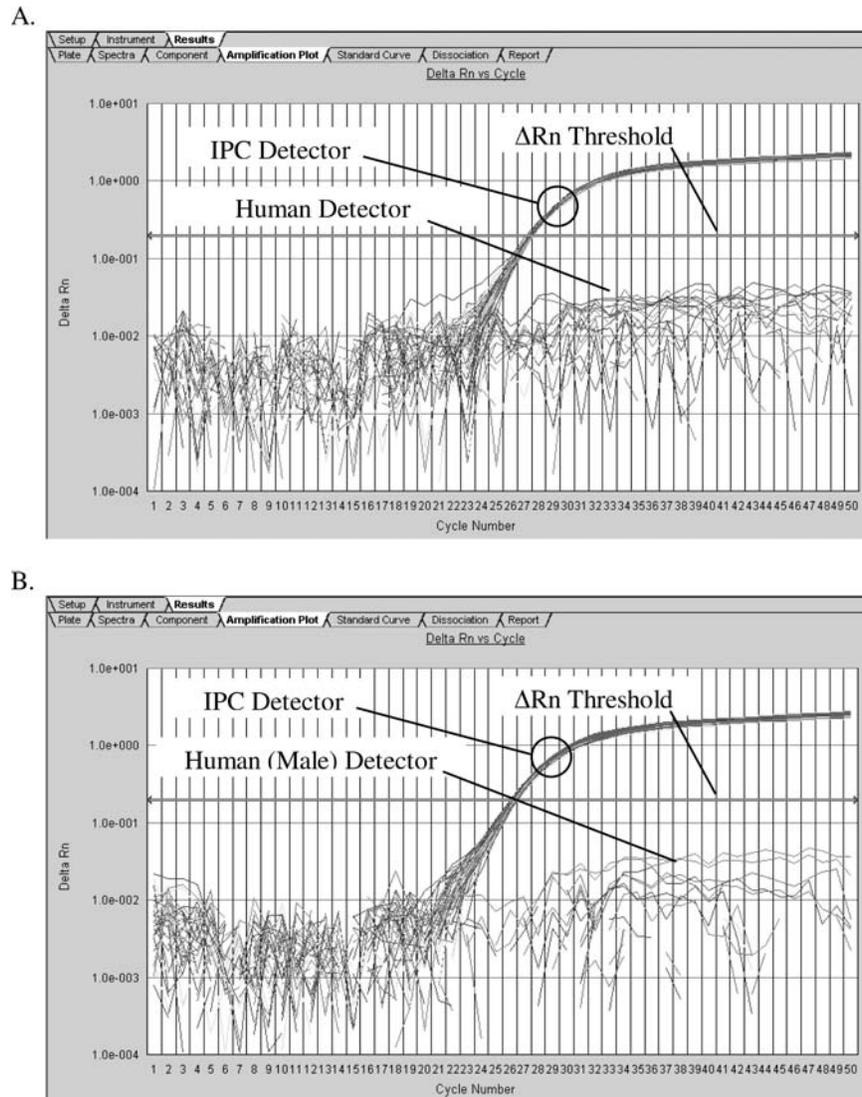


FIG. 3—Testing the Quantifiler Human and Quantifiler Y kit assay systems for false-positive background. Forty-eight reactions of each of the (A) Quantifiler Human and (B) Quantifiler Y kit assays were prepared, all configured as negative controls (i.e., the sample volume for each was plain TE buffer containing no human DNA.) The assays were prepared and run as normal, but thermal cycling was extended from the usual 40 cycles to 50 cycles. At the end of thermal cycling, none of the FAM (human DNA detector) amplification plots had crossed the 0.2 ΔRn threshold, so were called as negatives. Positive amplification of all assay IPCs confirmed the negative results as valid.

See results presented in Fig. 4. The range of hematin concentrations tested in the sample series (0 to 40 μM) caused progressive degrees of effect, from none to completely inhibited. The general effect of higher hematin concentration was to both raise the resulting assay C_T and lower the ΔRn fluorescent signal. The degree of the inhibitory effect was proportional to the hematin concentration, with the highest concentrations causing complete suppression of amplification of human target and IPC detectors. Designed to be a “canary in a coal mine,” the IPC detectors (which consist of exactly the same components in the Quantifiler Human and Quantifiler Y assays) were the most sensitive to hematin inhibition, with complete suppression of amplification at concentrations above 16 μM . Probably as a reflection of the two-fold difference in the relative concentration of their assay targets, the Quantifiler Human and Quantifiler Y assays showed some differences in their sensitivity to hematin given the same concentration of genomic DNA. The Quantifiler Human assay could amplify its target at hematin concentrations up to 20 μM , while concentrations over 16 μM caused

complete inhibition of amplification of the hemizygous Quantifiler Y assay target. The STR assays of the same set of samples showed that complete profiles could be obtained at hematin concentrations up to 20 μM .

The relationship between human target and IPC system results was designed so that, in most cases, any sample that allowed the amplification of the IPC system would allow human DNA to be detected if it were present. In other words, the IPC signal provides positive confirmation of the assay results of human DNA-negative samples. In cases that clearly show PCR inhibition, it would be strongly recommended to attempt to re-purify and re-run the sample to obtain more definitive results. The practical consequence of using Quantifiler kit assay results that had been compromised by marginally severe PCR inhibition would be to cause an underestimate of the affected sample’s DNA concentration. This could lead to the addition of too much DNA to a subsequent STR assay reaction, possibly causing off-scale electrophoresis peaks that required the STR assay to be re-run with a lower input amount of DNA. For those reasons,

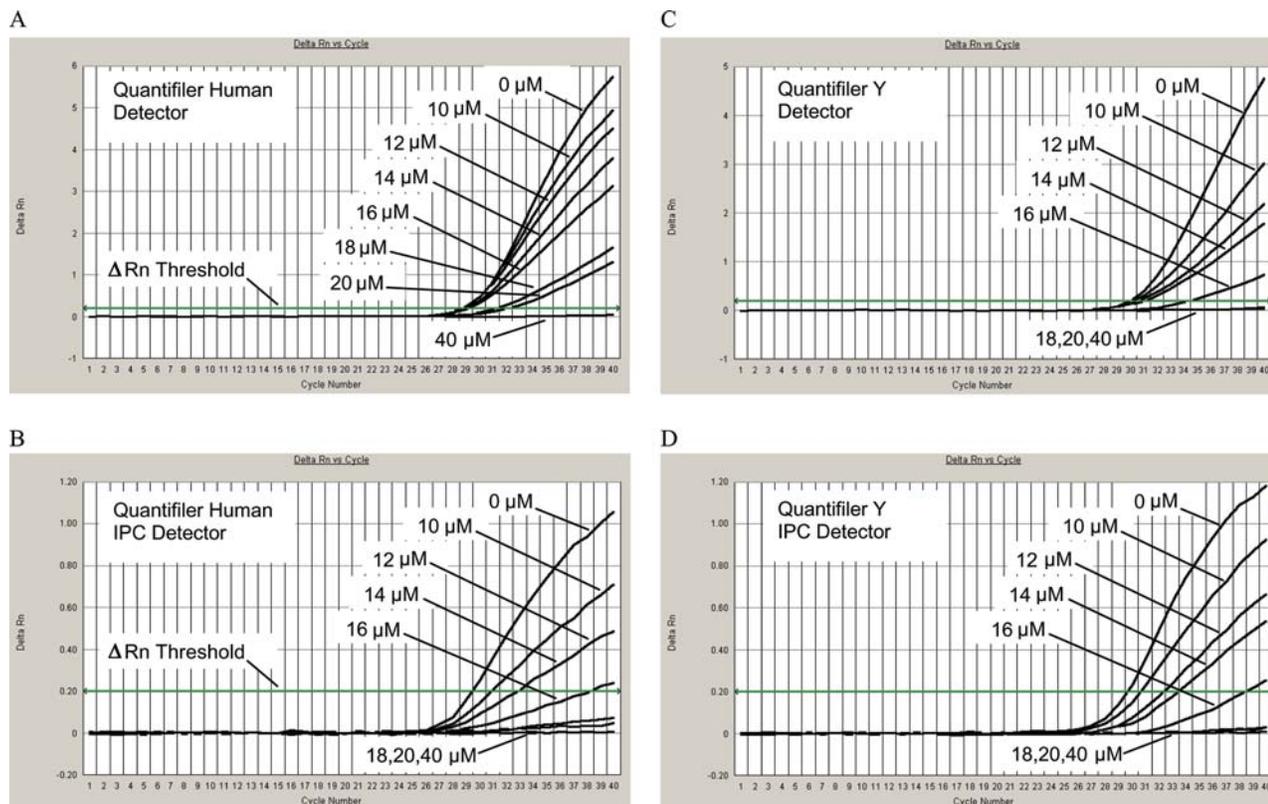


FIG. 4—Effect of hematin on *Quantifiler* kit assays and STR profiles. 1.0 ng samples of human genomic DNA containing from 0 μM to 40 μM hematin were assayed with the *Quantifiler Human* and *Quantifiler Y* assays and with the *AmpF ℓ STR ®* Identifier kit to evaluate the effect on PCR amplification. A and B: Human detector and IPC detector amplification plots for *Quantifiler Human* assay reactions, respectively. C and D: Y detector and IPC detector amplification plots for *Quantifiler Y* assay reactions, respectively. E: Identifier kit electropherogram profiles obtained from amplification of DNA samples with increasing hematin concentration.

the analyst would be recommended to re-purify and re-run any obviously inhibited samples rather than leave the results to chance.

A system for in vitro DNase I digestion of human genomic DNA was used to generate a series of time-point samples with progressively greater degrees of degradation. This was done to simulate the sort of environmental degradation to which forensic samples might be subjected, to study the effect on the results of *Quantifiler* Kit and STR analysis assays. Refer to results in Fig. 5. Identifier STR assays loaded at 1.0 ng of DNA per reaction as determined by the *Quantifiler* kit assay results produced full STR profiles up to the 5 min timepoint, even though the concentration of amplifiable DNA (according to the *Quantifiler* kit assay) had been reduced by 90% relative to the untreated control. Although the volumes of DNA samples added to STR amplification reactions were adjusted to compensate for the reduced DNA concentration due to DNase I digestion, a decrease in the RFU of resulting STR peaks was still evident with greater levels of degradation. The 15 min timepoint contained only 1% of the original amount of amplifiable DNA, and gave only a partial profile of mostly smaller molecular weight STR alleles. At 60 min, however, no amplifiable DNA was detected by either the *Quantifiler* kit assays or the Identifier kit assay (in this case, the Identifier kit assay was loaded with the maximum sample volume allowed per reaction: 10 μL).

The hematin inhibition and DNase I degradation experiments simulated the type of samples that may pose a challenge to DNA analysis methods. Since the *Quantifiler* kit assays are based on PCR amplification chemistry, they better match the performance characteristics of STR genotyping methods and provide a useful prediction

of whether or not analysis samples can be successfully amplified. The IPC system provides an additional measure of confidence in the quality of sample data, allowing confirmation of negative results as well as an indicator of samples that contain PCR inhibitor.

Case Type Samples (SWGAM 2.6)

Casework-type samples were prepared from human genomic DNA deposited on different materials, primarily clothing fabric. The primary success criterion for this study was for Identifier kit STR assays, set up with DNA input amounts determined by the results of *Quantifiler* kit assays, to provide complete profiles for all samples, with no off-scale or drop-out allele peaks that would have resulted from inaccurate DNA quantification. *Quantifiler Human* assay results showed that the range of DNA concentrations in the set of samples went from a low of 0.04 ng/ μL to a high of 1.89 ng/ μL . The results of the study are presented in Fig. 6. Identifier kit STR genotyping assays were all successfully genotyped with no offscale peaks or drop-out alleles. Typical peak heights for the known input of DNA were observed in most cases.

Mixture Studies (SWGAM 2.8)

The Mixture Study validation requirement required a somewhat different interpretation than that used for STR genotyping assays, which focuses on determining the proper genotypes of major and minor contributors. The *Quantifiler Human* and *Quantifiler Y*

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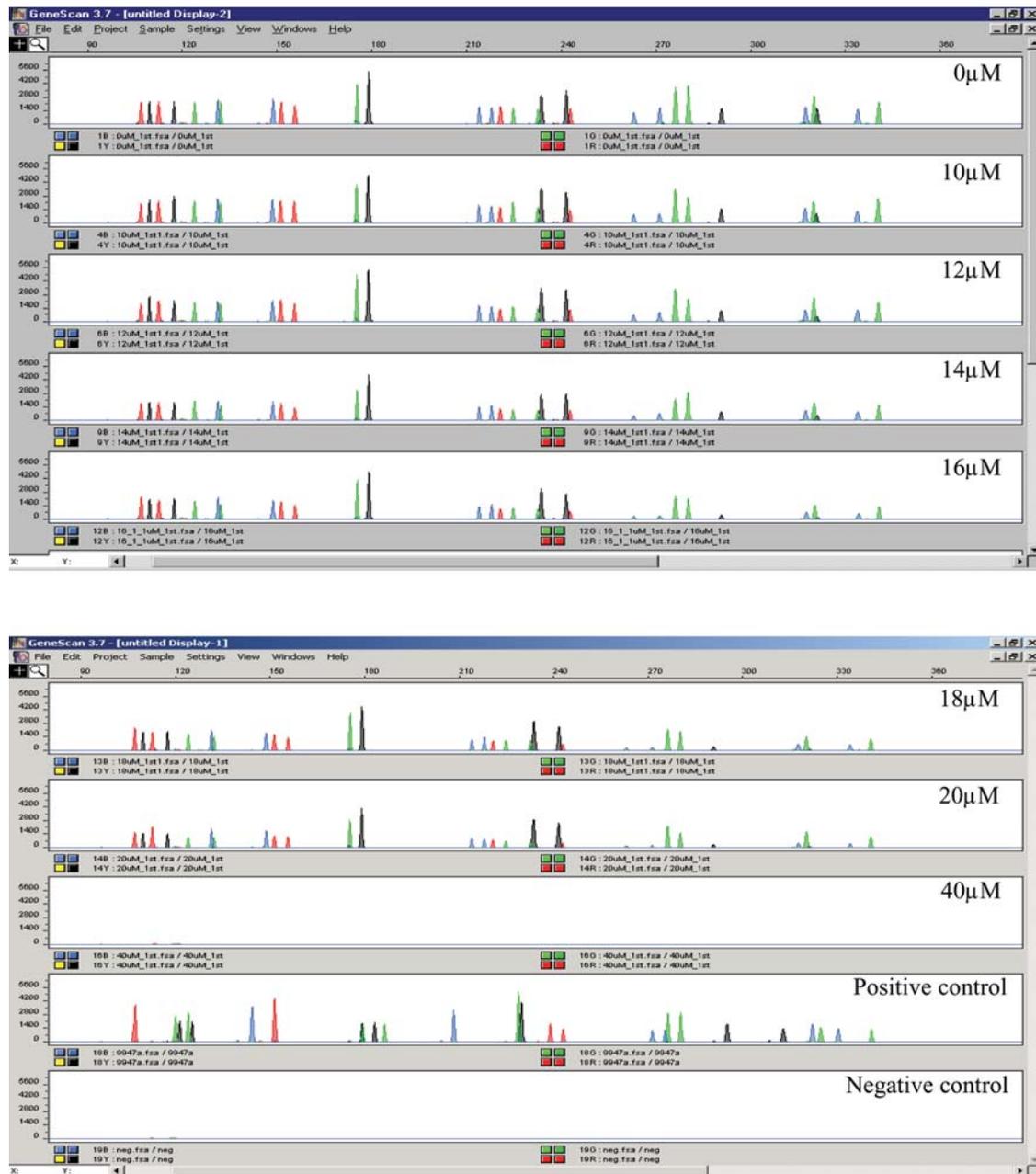


FIG. 4—Continued.

assays are separate DNA quantification assays that cannot resolve individual genotypes, but can be used together to determine the relative contributions of male and total human genomic DNA in samples. Such information could be very useful in analyzing certain types of evidence, particularly that resulting from sexual assaults where mixtures of male and female DNAs are typical.

As shown in Fig. 7, mixtures of purified male and female DNAs prepared in ratios from 1:1 to 1:1024 (male:female) were analyzed with the Quantifiler Human and Quantifiler Y assays. The Quantifiler Human assay results showed the large range of concentrations in the different samples, primarily reflecting the increasing concentrations of K-562 (female) DNA added to give increasing male:female DNA ratios. The Quantifiler Y assay, on the other hand, detected only the very low concentration of Raji

(male) DNA present at a constant level in all of the mixture samples. At the extremely low concentration of 0.05 ng/μL of male DNA, these samples would have contained, on average, less than 20 copies per reaction of the haploid Y-chromosome assay target gene contributed by the male DNA, yet they were accurately quantified even in the presence of a vastly larger proportion of female DNA.

Precision and Accuracy (SWGDM 2.9)

The SWGDAM Guidelines state, “The extent to which a given set of measurements of the same sample agree with their mean and the extent to which these measurements match the actual values

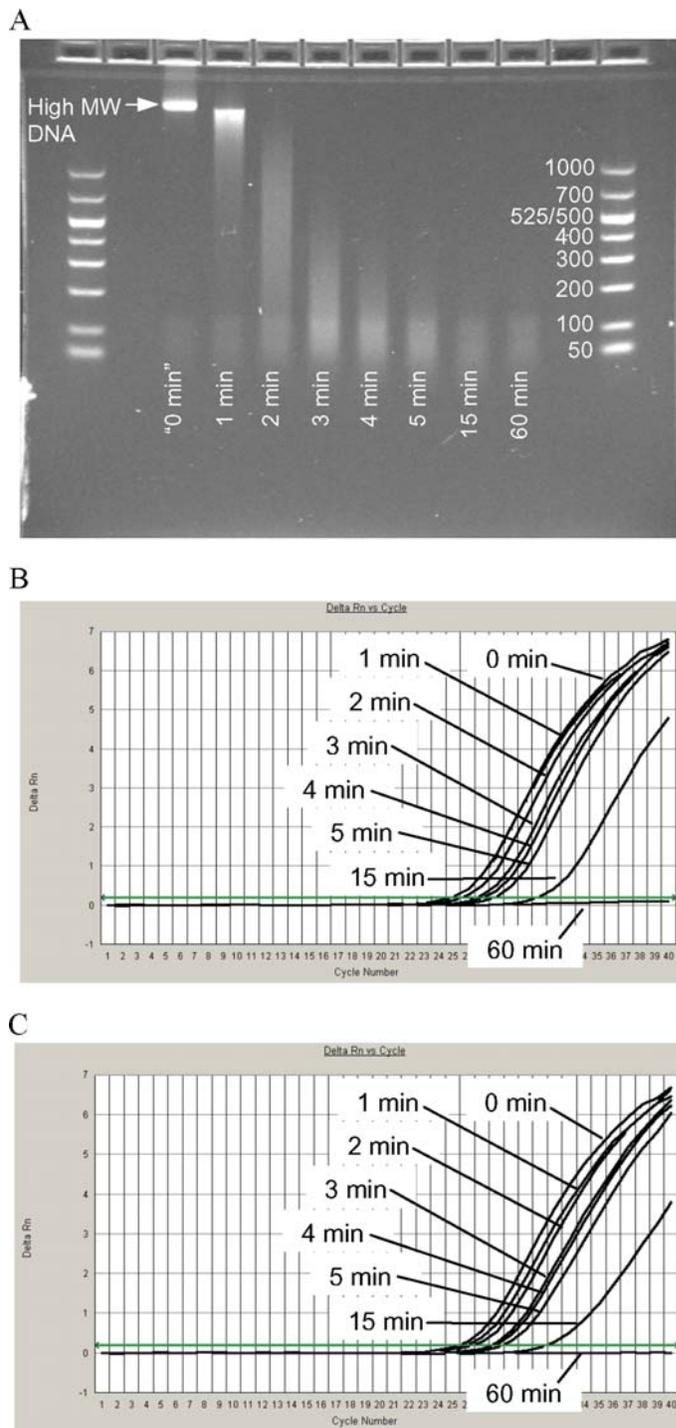


FIG. 5—Performance of *Quantifiler* kit assays and STR assays with degraded DNA. Intact Raji human genomic DNA was digested with exonuclease *DNase I* to simulate environmental degradation. Seven time point samples were collected to create a series of DNA samples with progressively greater levels of degradation. The samples were then quantified with the *Quantifiler Human* and *Quantifiler Y* assays. The quantity measurements for each sample were used to calculate the addition of 1.0 ng of DNA to *Identifiler STR* genotyping kit reactions. A: A 2% agarose gel (stained with ethidium bromide) illustrates the progression of DNA degradation at each time point. DNA size markers were included for reference. B and C: Amplification plots are shown for *Quantifiler Human* (B) and *Quantifiler Y* (C) assays of time point samples performed on an ABI PRISM 7000 Sequence Detection System to determine their DNA concentrations. D: Resulting electropherograms of STR profiles of the *DNase I* time point samples, run on an ABI PRISM 3100 Genetic Analyzer and analyzed with *GeneScan Software v3.7.1*.

D



FIG. 5—Continued.

being measured should be determined.” The first part of this guideline was easier to assess experimentally. Since there is not currently a standard reference material for human genomic DNA concentration, making a determination of the “actual value” of a human genomic DNA solution is not a trivial matter. Also, current DNA quantification methods are all subject to their own strengths and weaknesses, and none could be said to be a suitable “gold standard.” As such, we chose to approach these validation criteria by using three common methods for DNA quantification to produce a consensus against which the Quantifiler kit assays could be compared.

Beginning from the most basic level, the accuracy and precision of the Quantifiler kit assays is a function of the measurement of C_{TFAM} in amplification reactions, since that is the parameter from which all quantitative results are obtained. An assessment of the accuracy and precision of C_{TFAM} measurement was made by running replicate reactions of the quantification standard dilutions typically used in Quantifiler kit assays. Replicate assays were run in duplicate reaction wells on each of three different 7000 instruments

and repeated on two different days. The C_T results were assessed “as-is,” and were not translated into corresponding DNA concentration results. The reasons for this were twofold: no separate standard curves were run from which to determine DNA quantities for the dilution series reactions, and the intent of this portion of the study was to test the performance of the chemistry-instrument system in isolation from other factors that might add variability to this basic performance parameter. The accuracy and reproducibility of DNA quantification *per se* was addressed in other parts of the validation study.

The results of the C_T precision experiment are shown in Table 2. The mean C_{TFAM} results showed the normal inverse relationship with sample DNA concentration, going from the range of 23 to 24 for the highest standard (50 ng/ μ L) to 33 to 35 for the lowest standard (23 pg/ μ L). As a reflection of the two-fold difference in the copy number of their respective genomic DNA targets, the C_{TFAM} of the Quantifiler Human assay reactions were 1 C_T lower, on average, than those of the corresponding Quantifiler Y assay reactions. The average SD for the 12 replicate reactions of eight

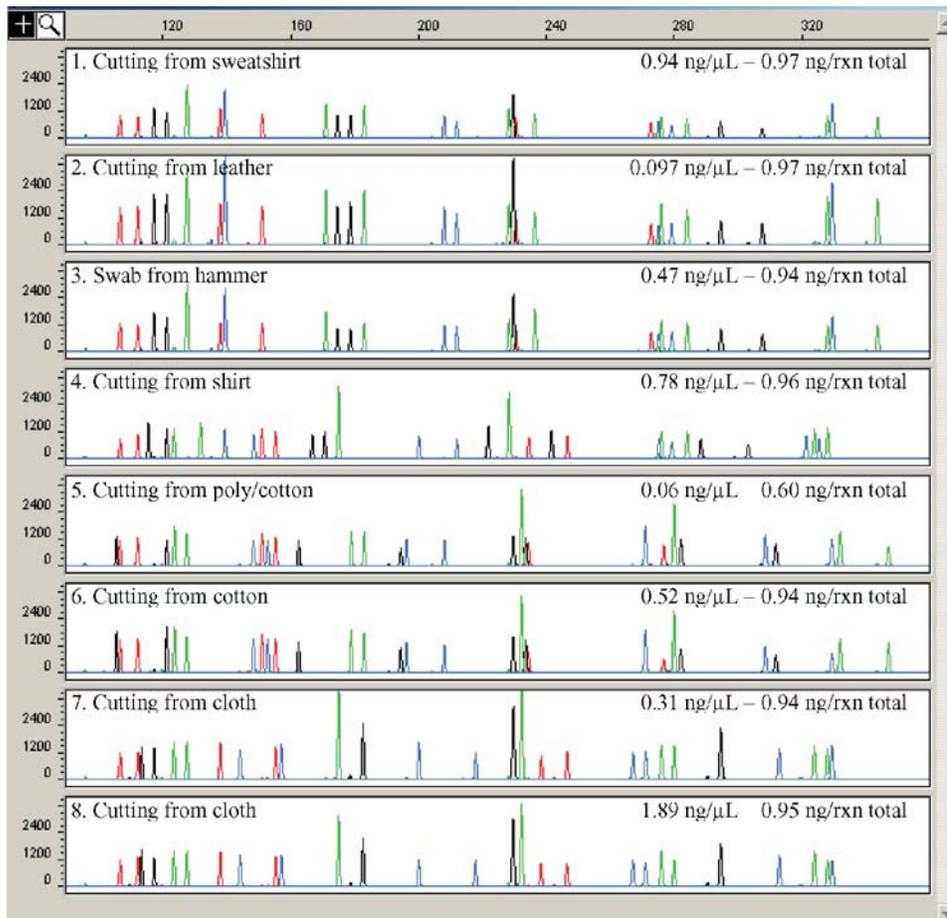


FIG. 6—Results of analyses of casework type samples. A set of forensic casework samples was obtained from a forensic laboratory. The samples were quantified using the Quantifiler Human kit assay run on the ABI PRISM 7000 Sequence Detection System. Quantification results were confirmed by running AmpF ℓ STR Identifiler STR genotyping assays. While the concentrations of samples ranged between 40 pg/ μ L and 1.89 ng/ μ L, sample additions to Identifiler reactions were adjusted to give a more consistent amount of 0.95 ng/ μ L to 1.05 pg/ μ L if the DNA concentration was high enough. Some samples (#5, #10 and #14) had very low DNA concentrations, so were added to the Identifiler kit reactions at the maximum allowed volume of 10 μ L. Identifiler kit assay results were then obtained by electrophoresis on the ABI PRISM 3100 genetic analyzer, and assessed according to the criterion that STR peaks should be within the range to generate successful genotype results (i.e., no drop-out or off-scale allele peaks). The figure shows the source material for samples, the Quantifiler kit quantification result and corresponding amount of DNA added (in total) to each Identifiler reaction. All samples generated full STR profiles.

TABLE 2—Quantifiler kit C_T run-to-run and instrument-to-instrument precision results.

DNA Conc. (ng/ μ L)*	Quantifiler Human Kit		Quantifiler Y Kit		N ‡
	Mean C_T	SD † C_T	Mean C_T	SD † C_T	
50	23.09	0.10	23.94	0.21	12
16.7	24.64	0.17	25.38	0.17	12
5.56	26.19	0.16	26.91	0.13	12
1.85	27.67	0.17	28.35	0.15	12
0.62	29.09	0.17	29.84	0.26	12
0.21	30.31	0.19	31.38	0.31	12
0.068	31.90	0.28	33.38	0.44	12
0.023	33.45	0.48	35.19	0.73	12

* Dilution series of Quantification Standard (human genomic DNA).

† SD = standard deviation.

‡ Replicates were run on three different ABI PRISM 7000 instruments on two different days (duplicate assays of each standard on 6 separate runs).

quantification standard dilutions was, respectively, 0.2 and 0.3 for the Human and Y kit assays, but in each case a consistent trend of increasing variability with decreasing copy number was seen. The

most likely explanation for this effect is the stochastic variability of sampling very low-copy DNA solutions, which increases as the copy number decreases. This is an unavoidable consequence of the principles of statistics and probability (25) rather than a function of the Quantifiler kit assays themselves. Overall, without attempting to interpret the exact mathematical consequences of C_T variability on final assay results, the study provided a useful indication of the stability in measuring this critical parameter, which was quite consistent on all levels from reaction-to-reaction, instrument-to-instrument and day-to-day.

Comparison studies were done with different DNA quantification methods to gain an overall consensus within which the accuracy and precision of the Quantifiler kit assays could be assessed. Of the comparison methods used, only one, the QuantiBlot Human DNA Quantitation Kit, is specific for human nuclear genomic DNA. The other two methods, based on A_{260} spectrophotometry and fluorescent dye intercalation, are not human DNA-specific nor frequently used in the forensic field, but are reliable and commonly used methods for general DNA quantification. The three comparison methods taken together were relatively consistent, and provided a useful basis for assessing the Quantifiler Kit assay results. In

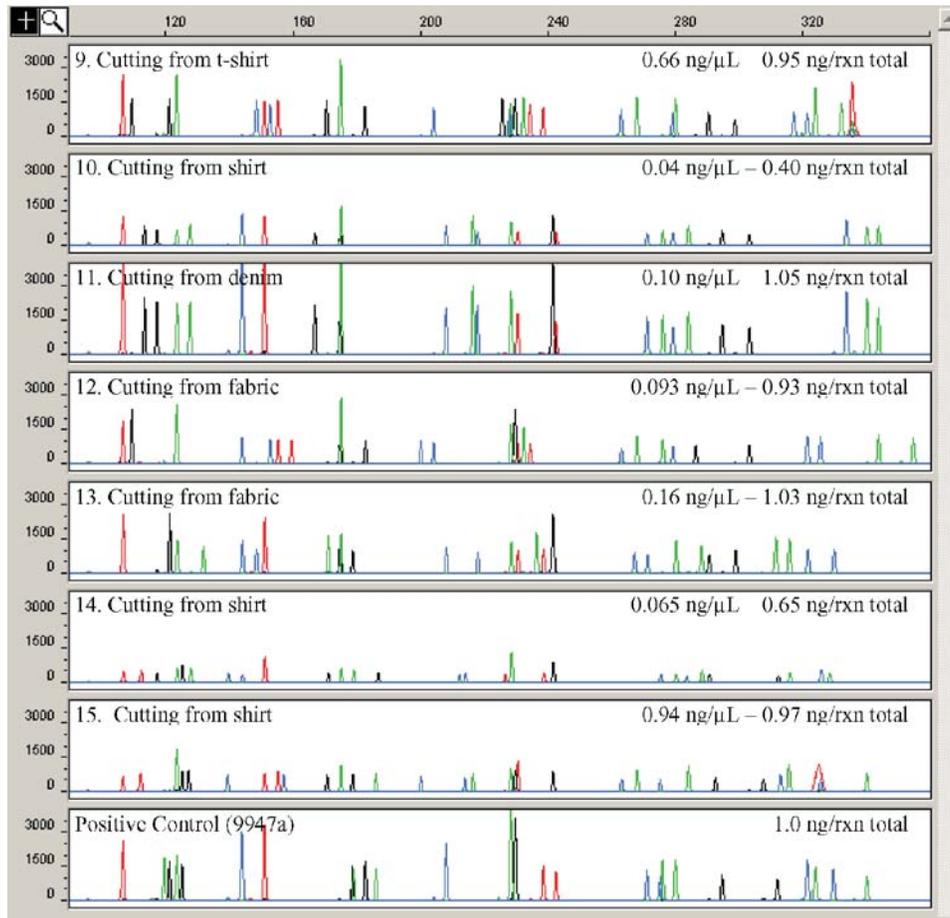


FIG. 6—Continued.

TABLE 3a—Reproducibility of quantifiler human assay quantification results.

Sample*	DNA Quantity (ng/μL) [†]			Mean Qty [‡]	SD Qty [‡]	CV (%) [§]
	Run 1	Run 2	Run 3			
007 A	2.580	2.830	2.900	2.770	0.168	6.07
007 B	0.894	0.779	0.892	0.855	0.066	7.70
007 C	0.216	0.160	0.192	0.189	0.028	14.84
9948 A	2.300	2.240	2.210	2.250	0.046	2.04
9948 B	0.504	0.481	0.573	0.519	0.048	9.22
9948 C	0.123	0.132	0.132	0.129	0.005	4.03
HG DNA A	1.810	1.790	2.240	1.947	0.254	13.06
HG DNA B	0.495	0.468	0.504	0.489	0.019	3.83
HG DNA C	0.128	0.106	0.106	0.113	0.013	11.21
K-562 A	1.360	1.350	1.360	1.357	0.006	0.43
K-562 B	0.379	0.425	0.460	0.421	0.041	9.64
K-562 C	0.096	0.126	0.096	0.106	0.017	16.21
Raji-1 A	1.920	1.800	1.770	1.830	0.079	4.34
Raji-1 B	0.484	0.402	0.466	0.451	0.043	9.56
Raji-1 C	0.149	0.120	0.104	0.124	0.023	18.35
Raji-2 A	1.720	1.860	1.700	1.760	0.087	4.95
Raji-2 B	0.419	0.407	0.408	0.411	0.007	1.62
Raji-2 C	0.113	0.088	0.061	0.087	0.026	29.75

Overall CV (%) = 9.3

* Three dilutions (A, B and C) were made of each of six human genomic DNAs.

[†] Determined by single-reaction Quantifiler kit assays on three separate runs.

[‡] Mean and standard deviation (SD) of each sample's replicate Quantifiler kit assay results.

[§] Coefficient of Variation, CV (%) = 100 × (SD ÷ Mean). Overall CV was calculated as the mean of individual CVs for the 18 samples.

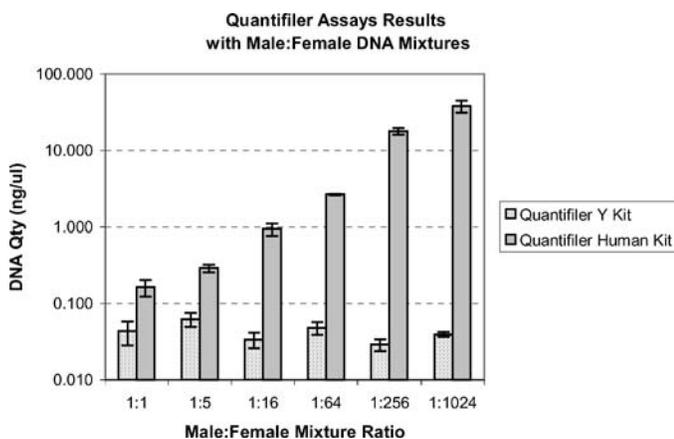


FIG. 7—Male DNA and total human genomic DNA can be independently quantified using the Quantifiler Y and Quantifiler Human Kit assays. Mixtures of Raji (male) and K-562 (female) human cell line genomic DNAs were combined in mixtures of 1:1, 1:5, 1:16, 1:64, 1:256 and 1:1024 (male:female ratio) by keeping the Raji DNA level constant at 50 pg/μL while adding increasing amounts of K-562 DNA. Each sample was analyzed in three replicate reactions each with the Quantifiler Y and Quantifiler Human Kit assays. The results of the Quantifiler Human assays showed the expected increase in total human genomic DNA, primarily as a function of the added major fraction of K-562 DNA. In contrast, the Quantifiler Y assay results were unaffected by the added female DNA, detecting only the constant level of Raji male DNA. Note that the vertical axis (DNA concentration) is plotted on a logarithmic scale.

TABLE 3b—Reproducibility of Quantifiler Y Kit Assay quantification results.

Sample*	DNA Quantity (ng/μL) [†]			Mean Qty [‡]	SD Qty [‡]	CV (%) [§]
	Run 1	Run 2	Run 3			
007 A	3.760	3.600	3.840	3.733	0.122	3.27
007 B	1.180	0.898	1.040	1.039	0.141	13.57
007 C	0.238	0.185	0.172	0.198	0.035	17.63
9948 A	2.590	2.540	2.670	2.600	0.066	2.52
9948 B	0.810	0.612	0.709	0.710	0.099	13.94
9948 C	0.146	0.130	0.151	0.142	0.011	7.71
HG DNA A	2.010	1.770	1.760	1.847	0.142	7.66
HG DNA B	0.577	0.462	0.591	0.543	0.071	13.03
HG DNA C	0.081	0.053	0.052	0.062	0.017	27.02
K-562 A [¶]	neg	neg	neg	n.d.	n.d.	n.d.
K-562 B [¶]	neg	neg	neg	n.d.	n.d.	n.d.
K-562 C [¶]	neg	neg	neg	n.d.	n.d.	n.d.
Raji-1 A	2.500	2.090	2.400	2.330	0.214	9.17
Raji-1 B	0.679	0.481	0.565	0.575	0.099	17.28
Raji-1 C	0.123	0.096	0.148	0.122	0.026	21.40
Raji-2 A	2.630	2.050	2.190	2.290	0.303	13.22
Raji-2 B	0.574	0.536	0.612	0.574	0.038	6.62
Raji-2 C	0.091	0.123	0.160	0.125	0.034	27.51
Overall CV (%) = 13.4						

* Three dilutions (A, B and C) were made of each of six human genomic DNAs.

[†] Determined by single-reaction Quantifiler kit assays on three separate runs.

[‡] Mean and standard deviation (SD) of each sample's replicate Quantifiler kit assay results.

[§] Coefficient of Variation, CV (%) = 100 × (SD ÷ Mean). Overall CV was calculated as the mean of individual CVs for the 18 samples.

[¶] K-562 is a human female cell line, so its DNA was not detected.

TABLE 4a—Comparison of results of Quantifiler Human Kit, A₂₆₀ and dye intercalation methods with human genomic DNA samples.

Sample*	Quant. Method Results (ng/μL) [†]			% Difference in Quant. Results [‡]	
	A ₂₆₀	DI	Qf	Qf vs. A ₂₆₀	Qf vs. DI
007 A	2.74	2.502	2.770	1.1	10.7
007 B	0.68 [§]	0.756	0.855	24.8	13.1
007 C	0.14 [§]	0.176	0.189	38.2	7.5
9948 A	1.90	2.286	2.250	18.4	1.6
9948 B	0.47 [§]	0.496	0.519	9.3	4.6
9948 C	0.09 [§]	0.103	0.129	35.8	25.2
HG DNA A	2.20	2.270	1.947	11.5	14.3
HG DNA B	0.55 [§]	0.584	0.489	11.1	16.2
HG DNA C	0.11 [§]	0.134	0.113	3.0	15.7
K-562 A	2.76	1.317	1.357	50.8	3.0
K-562 B	0.69 [§]	0.365	0.421	38.9	15.5
K-562 C	0.14 [§]	0.104	0.106	23.1	1.7
Raji-1 A	2.00	1.271	1.830	8.5	44.0
Raji-1 B	0.50 [§]	0.351	0.451	9.9	28.6
Raji-1 C	0.10 [§]	0.085	0.124	24.3	47.0
Raji-2 A	1.98	1.262	1.760	11.1	39.5
Raji-2 B	0.49 [§]	0.357	0.411	16.9	15.2
Raji-2 C	0.10 [§]	0.110	0.087	11.7	20.7
Overall % Difference = 19.4					

* Three dilutions (A, B and C) were made of each of six human genomic DNAs.

[†] DNA concentration measurements of each sample by A₂₆₀, dye intercalation (DI) and Quantifiler kit (Qf) methods. Quantifiler kit results were the mean of three assay runs.

[‡] “% Difference” is the absolute value of the difference between corresponding Quantifiler kit assay and comparison method results, expressed as a percentage of the Quantifiler kit result. Overall % Differences were the averages for all 18 samples.

[§] Below limit of detection for A₂₆₀ method, so extrapolated for the sake of comparison using dilution “A” A₂₆₀ measurements and the known dilution factors.

TABLE 4b—Comparison of results of Quantifiler Y Kit, A₂₆₀ and dye intercalation methods with human genomic DNA samples.

Sample*	Quant. Method Results (ng/μL) [†]			% Difference in Quant. Results [‡]	
	A ₂₆₀	DI	Qf	Qf vs. A ₂₆₀	Qf vs. DI
007 A	2.74	2.502	3.733	36.3	49.2
007 B	0.68 [§]	0.756	1.039	51.7	37.5
007 C	0.14 [§]	0.176	0.198	44.8	12.6
9948 A	1.90	2.286	2.600	36.8	13.7
9948 B	0.47 [§]	0.496	0.710	49.5	43.1
9948 C	0.09 [§]	0.103	0.142	49.8	38.2
HG DNA A	2.20	2.270	1.847	16.1	18.7
HG DNA B	0.55 [§]	0.584	0.543	1.2	6.9
HG DNA C	0.11 [§]	0.134	0.062	43.7	54.0
K-562 A	2.76	1.317	negative	n.d.	n.d.
K-562 B	0.69 [§]	0.365	negative	n.d.	n.d.
K-562 C	0.14 [§]	0.104	negative	n.d.	n.d.
Raji-1 A	2.00	1.271	2.330	16.5	83.3
Raji-1 B	0.50 [§]	0.351	0.575	15.0	64.0
Raji-1 C	0.10 [§]	0.085	0.122	22.2	44.5
Raji-2 A	1.98	1.262	2.290	15.7	81.5
Raji-2 B	0.49 [§]	0.357	0.574	16.0	60.7
Raji-2 C	0.10 [§]	0.110	0.125	26.1	13.2
Overall % Difference = 29.4					

* Three dilutions (A, B and C) were made of each of six human genomic DNAs.

[†] DNA concentration measurements of each sample by A₂₆₀, dye intercalation (DI) and Quantifiler kit (Qf) methods. Quantifiler kit results were the mean of three assay runs.

[‡] “% Difference” is the absolute value of the difference between corresponding Quantifiler kit assay and comparison method results, expressed as a percentage of the Quantifiler kit result. Overall % Differences were the averages for all 18 samples.

[§] Below limit of detection for A₂₆₀ method, so extrapolated for the sake of comparison using dilution “A” A₂₆₀ measurements and the known dilution factors.

one experiment, six different purified human genomic DNA stocks were each diluted to three concentration levels (approximately 2.0, 0.5, and 0.1 ng/μL), then quantified with the Quantifiler Human and Quantifiler Y Kit assays, with UV absorbance at 260 nm (“A₂₆₀ method”), and with the dye intercalation method. The results of replicate Quantifiler kit assays on the set of samples allowed a determination of the variability of assay results. See results in Table 3. Quantification result means and standard deviations (SD) were determined for the replicate assays, and the coefficient of variation (CV) was calculated for the sample replicate group. An overall (average) CV was then calculated as the mean the CVs of the different sample replicate groups. The overall CVs were: Quantifiler Human assay, 9.3%; Quantifiler Y assay, 13.4%.

Percent difference calculations were made (as described in Materials and Methods) between different quantification method results for each sample, and the average of these individual values was figured to provide an overall measure of the relative differences between the Quantifiler kit assays and each comparison method. The results of this phase of the study are shown in Table 4. The overall % differences were as follows: 19.4% for Quantifiler Human vs. A₂₆₀; 18.0% for Quantifiler Human vs. dye intercalation; 29.4% for Quantifiler Y vs. A₂₆₀; 41.4% for Quantifiler Y vs. dye intercalation.

Another study compared results from the Quantifiler kit assays, the A₂₆₀ method and the QuantiBlot membrane hybridization assay, using a similar approach to calculate % differences between methods. 50 DNA samples from the human resolution panel were tested by the different methods, and the results of this comparison are

TABLE 5—Comparison of results of Quantifiler Kit, A_{260} and QuantiBlot Kit methods.

Sample	Sex	A_{260} (ng/ μ L)	QB* (ng/ μ L)	Quantifiler Human Kit Results % Difference [†] from			Quantifiler Y Kit Results % Difference [†] from		
				ng/ μ L	A_{260}	QB*	ng/ μ L	A_{260}	QB*
1	M	17.5	20	6.69	61.7	66.6	10.13	41.9	49.4
2	M	15.4	20	14.30	7.1	28.5	16.78	9.0	16.1
3	M	13.9	30	15.48	11.4	48.4	14.30	2.9	52.3
4	M	11.4	20	12.44	9.6	37.8	12.45	9.7	37.8
5	M	10.3	20	12.69	23.2	36.6	11.00	6.8	45.0
6	M	13.9	20	12.54	9.8	37.3	13.56	2.4	32.2
7	M	11.5	40	13.78	20.1	65.6	12.28	7.1	69.3
8	M	11.2	20	13.51	21.2	32.5	11.77	5.6	41.2
9	M	9.8	20	15.09	54.0	24.6	13.06	33.3	34.7
10	M	9.7	20	13.98	44.1	30.1	12.29	26.7	38.6
11	M	13.0	20	11.27	13.3	43.7	12.85	1.2	35.8
12	M	13.3	30	9.92	25.1	66.9	11.59	12.5	61.4
13	M	n.d.	14	13.90	n.d.	0.7	11.31	n.d.	19.2
14	F	12.8	16	13.90	9.0	13.1	neg	n.d.	n.d.
15	M	15.7	16	12.62	19.4	21.1	13.89	11.2	13.2
16	M	12.1	24	13.09	8.2	45.5	10.78	10.9	55.1
17	M	n.d.	20	12.81	n.d.	36.0	14.36	n.d.	28.2
18	M	13.5	24	8.18	39.4	65.9	10.25	24.1	57.3
19	M	13.2	20	10.37	21.4	48.2	13.12	0.6	34.4
20	M	12.9	16	12.69	1.2	20.7	12.36	3.8	22.8
21	M	11.0	14	13.48	22.9	3.7	13.00	18.5	7.1
22	M	11.5	24	12.23	6.6	49.0	12.85	12.0	46.5
23	M	10.9	14	10.91	0.6	22.1	11.73	8.1	16.2
24	M	12.4	20	15.19	22.8	24.1	14.38	16.2	28.1
25	M	10.8	20	15.21	41.5	24.0	18.07	68.1	9.7
26	F	13.9	20	14.00	1.1	30.0	neg	n.d.	n.d.
27	F	11.5	32	13.16	14.4	58.9	neg	n.d.	n.d.
28	F	11.5	40	10.51	8.6	73.7	neg	n.d.	n.d.
29	F	11.2	20	10.45	6.3	47.8	neg	n.d.	n.d.
30	F	16.0	20	12.56	21.5	37.2	neg	n.d.	n.d.
31	F	10.9	20	12.12	11.7	39.4	neg	n.d.	n.d.
32	F	10.9	40	9.42	13.6	76.5	neg	n.d.	n.d.
33	F	11.5	20	13.95	21.3	30.3	neg	n.d.	n.d.
34	F	10.4	20	12.14	16.7	39.3	neg	n.d.	n.d.
35	F	11.1	40	12.38	11.3	69.1	neg	n.d.	n.d.
36	F	10.5	20	13.38	28.0	33.1	neg	n.d.	n.d.
37	F	12.0	24	12.50	4.2	47.9	neg	n.d.	n.d.
38	F	10.8	20	9.59	11.0	52.1	neg	n.d.	n.d.
39	F	11.4	16	10.42	8.8	34.9	neg	n.d.	n.d.
40	F	10.4	40	11.16	7.3	72.1	neg	n.d.	n.d.
41	F	12.6	20	12.49	0.9	37.6	neg	n.d.	n.d.
42	F	12.5	28	8.68	30.3	69.0	neg	n.d.	n.d.
43	F	12.2	20	13.57	11.5	32.2	neg	n.d.	n.d.
44	F	9.8	16	9.42	3.9	41.1	neg	n.d.	n.d.
45	F	12.4	16	10.96	11.6	31.5	neg	n.d.	n.d.
46	F	12.2	16	11.49	5.4	28.2	neg	n.d.	n.d.
47	F	10.4	40	12.93	24.1	67.7	neg	n.d.	n.d.
48	F	12.3	20	12.23	0.6	38.9	neg	n.d.	n.d.
49	F	10.7	40	15.02	40.4	62.5	neg	n.d.	n.d.
50	F	12.8	32	13.50	5.5	57.8	neg	n.d.	n.d.
				Overall Average % Difference = 16.9		42.0		15.1	35.5

* QB: QuantiBlot kit assay, determined for 10-fold dilutions, converted here to original conc.

[†] “% Difference” is the absolute value of the difference between corresponding Quantifiler kit assay and comparison method results, expressed as a percentage of the Quantifiler kit result. Overall % Differences were the averages for all samples.

shown in Table 5. The average percent differences between methods were as follows: 16.9% for Quantifiler Human kit vs. A_{260} ; 42.0% for Quantifiler Human kit vs. QuantiBlot kit; 15.1% for Quantifiler Y kit vs. A_{260} ; 35.5% for Quantifiler Y kit vs. QuantiBlot kit.

Despite the limitations of trying to determine the Quantifiler kit assays’ accuracy with neither a certified reference standard for human genomic DNA concentration nor a gold standard method for DNA quantification, these experiments showed that: (a) the Quantifiler kit assays were able to produce consistent quantification results with CVs in the range of 10% to 13%, and (b) the Quantifiler kit assays were in general agreement with three other common methods for DNA quantification.

Validation Experiments on the ABI PRISM 7900HT SDS

Separate validation experiments were done to test the performance of the Quantifiler kit assays on the ABI PRISM 7900HT SDS instrument. These experiments followed similar outlines to those described above for the ABI PRISM 7000 SDS, but focused on the most critical parameters of assay performance like precision and accuracy. All aspects of assay setup and thermal cycling were done identically to those run on the 7000 instrument.

The precision of $C_{T\text{FAM}}$ measurement on the 7900HT SDS was tested as described above using serial dilutions of the Quantifiler Human DNA Standard. A dilution series of the kit DNA Standard

was prepared to cover the range of concentrations from 50 ng/μL to 0.023 ng/μL in eight three-fold dilution steps. Duplicate Quantifiler Human and Quantifiler Y assays were then performed for each of the eight dilutions on each of three repeat runs performed on one 7900HT instrument. The C_TFAM assay results were then assessed, with mean and standard deviation calculated for the data from each of the standard dilutions collected over the three runs. The C_TFAM values for the standards were very similar to those collected for the ABI PRISM 7000 instrument, both in terms of their mean values as well as in their calculated SD (data not shown). Based on the results, assay precision would be expected to be comparable between the 7000 and 7900HT instruments.

The accuracy of Quantifiler kit assays performed on the 7900HT SDS was assessed by generating a consensus of quantification results for a set of samples using different comparison methods, as described earlier for testing with the ABI Prism 7000 instrument. The same set of DNAs and the corresponding results of comparison quantification methods were used. The % difference factor between the Quantifiler kit assay and comparison method results was determined as described earlier for the 7000 instrument. The overall % difference values were: 34.8% and 29.8%, respectively, for the Quantifiler Human kit vs. A₂₆₀ and Quantifiler Human kit vs. dye intercalation comparisons; 23.5% and 34.9%, respectively, for the Quantifiler Y kit vs. A₂₆₀ and Quantifiler Y kit vs. dye intercalation comparisons. Overall, these results indicate general agreement in quantification using the different methods.

Conclusion

Accurate and consistent quantification of DNA in forensic samples has been shown to be an important contributing factor to successful STR genotyping (26,27). The Quantifiler Human and Quantifiler Y Human Male DNA Quantification Kits were designed to specifically address some of the limitations of older quantification methods and allow better integration into the work flow environment of modern forensic laboratories.

These validation experiments showed the assays to be reliable and robust, and to produce quantification results consistent with other representative DNA quantification methods. The use of the Quantifiler kit assays as a preliminary procedure was shown to allow a high success rate for subsequent sample analysis with an STR genotyping method. The assays also possess certain unique characteristics that are likely to be of benefit in forensic DNA analysis, such as the ability to separately detect male DNA in mixed samples and to provide more information about the presence of PCR inhibitors in analysis samples. The assays are very frugal with analysis samples since they require the expenditure of only 2.0 μL of sample volume per assay.

In an era of continually increasing case loads, the use of this system should reduce the time needed for the mundane task of DNA quantification, while allowing additional time for more analysis-intensive activities. The combination of the Quantifiler Kit assays' performance and capabilities with their relative ease of use should offer a significant contribution to the overall efficiency and throughput of modern forensic laboratories.

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