

TECHNICAL NOTE

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Human Genomic DNA Quantitation System, H-Quant: Development and Validation for Use in Forensic Casework

ABSTRACT: The human DNA quantitation (H-Quant) system, developed for use in human identification, enables quantitation of human genomic DNA in biological samples. The assay is based on real-time amplification of *Alu*Yb8 insertions in hominoid primates. The relatively high copy number of subfamily-specific *Alu* repeats in the human genome enables quantitation of very small amounts of human DNA. The oligonucleotide primers present in H-Quant are specific for human DNA and closely related great apes. During the real-time PCR, the SYBR[®] Green I dye binds to the DNA that is synthesized by the human-specific *Alu*Yb8 oligonucleotide primers. The fluorescence of the bound SYBR[®] Green I dye is measured at the end of each PCR cycle. The cycle at which the fluorescence crosses the chosen threshold correlates to the quantity of amplifiable DNA in that sample. The minimal sensitivity of the H-Quant system is 7.6 pg/μL of human DNA. The amplicon generated in the H-Quant assay is 216 bp, which is within the same range of the common amplifiable short tandem repeat (STR) amplicons. This size amplicon enables quantitation of amplifiable DNA as opposed to a quantitation of degraded or nonamplifiable DNA of smaller sizes. Development and validation studies were performed on the 7500 real-time PCR system following the Quality Assurance Standards for Forensic DNA Testing Laboratories.

KEYWORDS: forensic science, real-time PCR, human identification, polymerase chain reaction, DNA typing, DNA quantitation, *Alu* repeats, *Alu*Yb8, SYBR[®] green I, human DNA

Short tandem repeat (STR) loci distributed on autosomal and Y-chromosomes are now routinely used in forensic laboratories and court systems (1–4). In general, the STR profile from an evidence sample is compared with the profiles from known individuals to determine the contribution of a known individual's DNA to the evidence sample. DNA analysis of forensic samples typically involves isolation of the DNA, quantitation for human DNA, amplification of highly polymorphic STRs, separation of the amplified fragments, genotyping, and result interpretation. Within this process, the quantitation of human-specific DNA in the extract obtained from the biological sample is a crucial step. The intention of human DNA quantitation for forensic samples is to determine the amount of extracted DNA required for multiplex STR systems to obtain an interpretable STR profile with allele peak heights within the resolution range for unambiguous interpretation. Quantitation of human DNA is also useful in making decisions for using the low copy number DNA analysis strategies in order to obtain an interpretable STR profile (5). Forensic evidence samples frequently undergo environmental insults and are often contaminated with nonhuman biological material; thus, re-

agents like PicoGreen[®] are inappropriate for the quantitation of DNA in forensic evidence samples. The accuracy of the quantitation system is, therefore, important in forensic DNA analysis.

Quantiblot[®], a system based on hybridization of a biotinylated probe to DNA samples immobilized on a nylon membrane, has routinely been used for a long time in forensic laboratories (6). AluQuant[®], yet another commercially available system, is based on the hybridization of a proprietary probe to highly repetitive sequences on human chromosomes (7). The detection limit for these assay methods is about 0.1–0.15 ng of human DNA. DNA templates of less than 100 pg may yield an interpretable STR profile, either complete, or partial. Thus, the possibility of losing valuable information exists due to the decision not to amplify the samples that do not exhibit quantitation values for human DNA by such methods. Highly sensitive methods for quantitation of human DNA based on the amplification of *Alu* repeats have previously been reported (8,9). These methods are based on the measurement of amplification products after a specific number of cycles because after a certain number of cycles, product production may near the saturation point (e.g., fluorescence curve ceases to be exponential), causing an inaccurate quantity of DNA to be assumed.

In recent years, real-time PCR assays for quantitation of nucleic acids have proved to be very useful. This is because a quantitative relationship exists, in theory, between the amount of target sequence at the start and the amount of PCR product at any given cycle before reaching saturation. The advantages of real-time quantitation assays include a greater dynamic quantitation range,

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sensitivity, rapidity, automated analysis, measurement of amplifiable DNA, and cost-effectiveness. Several real-time PCR methods have been reported in the literature for quantitation of human DNA (10–15). Quantifiler™ systems for the quantitation of human and human male DNA are commercially available from Applied Biosystems (14). These systems are supported on the 7000, 7900 HT, and 7500 Sequence Detection Systems (Applied Biosystems) for forensic applications.

We have reported a method for quantitation of human DNA in biological materials using inter-*Alu* and intra-*Alu* PCR (15). Certain *Alu* repeats are stable insertions and hence unique nuclear markers that provide increased species specificity, even among higher primates, and are ideally suited for human identity testing. The *Alu* family of interspersed repeats is the most successful of the mobile genetic elements in terms of integration and copy number within primate genomes. Assay methods targeting certain *Alu* element subfamilies provide high sensitivity because of their high copy numbers in the human genome (16). *Alu* subfamilies that are present in the human genome but are essentially absent from nonhuman primates are now known (16). Here, we report an improved assay method called human DNA quantification (H-Quant) that can be used for quantitation of human DNA in forensic samples using the 7500 real-time PCR System (Applied Biosystems, Foster City, CA). The H-Quant assay method is based on the amplification of *Alu*Yb8 elements (17).

Materials and Methods

Oligonucleotide primers were synthesized and obtained from Operon (Alameda, CA). Ten times PCR buffer, AmpliTaq Gold™ DNA polymerase, and AmpFISTR® COfiler™ were obtained from Applied Biosystems (Foster City, CA). SYBR® Green I nucleic acid gel stain dye was obtained from Molecular Probes (Eugene, OR). Reference dye for quantitative PCR and bovine serum albumin (BSA) fraction V were obtained from Sigma Chemical Co. (St. Louis, MO). Human male genomic DNA was obtained from Novagen (Madison, WI). All other chemicals used in this study were of analytical grade.

Extraction and Quantitation of DNA

The DNA from anonymous donor samples was obtained from blood drawn in EDTA vacutainer tubes or buccal swabs and was extracted either by Chelex® (18), BioRobot EZ1 (Qiagen, Valencia, CA), or QIAamp® MiniKit (Qiagen) procedures. The quantity of human DNA was determined by the Quantifiler™ human DNA quantitation kit (Applied Biosystems). DNA in the samples used for sensitivity studies was prepared by serial dilutions using 10 mM Tris-HCl, pH 8.0, containing 0.01 mM EDTA.

Real-Time PCR Amplification

Amplification reactions contained 5.0 µL of H-Quant primer mix, 17.8 µL of H-Quant reaction mix, 0.2 µL of AmpliTaq Gold™ (5 U/µL), and 2.0 µL of DNA sample. The volume of the DNA template was kept constant at 2.0 µL. This is to ensure compatibility with the real-time instrument software (7500 System SDS Software v1.2.3f2, Applied Biosystems). The software provides the results as a concentration of DNA/µL of the template solution. The amount of template DNA, therefore, is expressed as a concentration rather than a total input quantity in the text. The H-Quant primer mix was prepared by combining the forward and reverse primers (6.0 µM) in 100 mM Tris-HCl, pH 8.3, containing 500 mM KCl, 15 mM MgCl₂, and 0.01% gelatin. The primers were

selected and designed based on the published sequences to obtain a 216 bp fragment (15). The H-Quant reaction mix contained SYBR® Green I dye (× 0.35), reference dye (× 0.55), BSA (7 µg/mL), dNTPs (1.12 mM) in 10 mM Tris-HCl, pH 8.3 containing 50 mM KCl, 1.5 mM MgCl₂, and 0.001% gelatin. The final concentrations of the reagents in the reaction were: buffer (× 1), primers (1.2 µM), dNTPs (0.8 mM), MgCl₂ (3.0 mM), BSA (0.25 µg), SYBR® Green I dye (× 0.25), and reference dye (× 0.2). Amplification reactions were performed in a 7500 real-time PCR system (Applied Biosystems) following the manufacturer's instruction under the following: 95°C, 10 min; 35 cycles of 95°C, 15 sec, and 72°C, 1 min. A standard curve was constructed by using serial dilutions of human male genomic DNA (Novagen) ranging from 7.6 pg/µL to 50 ng/µL. The standards were included with each batch of quantitation reaction. The data were analyzed using 7500 System SDS Software v1.2.3f2 (Applied Biosystems).

Amplification for STRs

The amplifications using the AmpFISTR® COfiler™ PCR amplification kit were performed as recommended by the manufacturer using GeneAmp® PCR System 9700 (3). The amplified products were analyzed on a 3100 Genetic Analyzer as described in the instruction manual. The data were analyzed using Genotyper® software (Applied Biosystems).

Results and Discussion

During real-time PCR, accumulation of the PCR product is monitored with each cycle. In the H-Quant assay, accumulated PCR product is measured by the fluorescence resulting from the binding of SYBR® Green I dye to the amplified products. Very little change in the fluorescence is expected at the initial cycles of PCR and is used to determine the baseline for the amplification plot. The threshold fluorescence signal is the level of fluorescence intensity above background. In general, the threshold is set in the exponential phase of the amplification for accurate quantitation results. Thus, the samples with low amounts of DNA template would cross the threshold at a higher cycle number. The cycle at which the fluorescence crosses the threshold value is termed as cycle threshold or C_T . The higher the C_T value, the lesser the quantity of template DNA. The efficiency of the H-Quant assay was measured in terms of the C_T value. In the H-Quant system, a high C_T value can be measured without interference from the background, thus yielding a sensitivity as low as 7.6 pg/µL of DNA.

The Quality Assurance Standards for Forensic DNA Laboratories were followed for conducting validation studies (19). The experiments are described in a sequential order and the values established in the previous experiment are maintained throughout the remaining experiments.

Optimization of PCR

In a real-time PCR that is targeted to detect the products using intercalating dyes like SYBR® Green I, any "primer-dimer," or primer adducts contribute to the observed fluorescence. This phenomenon is commonly observed when high amounts of primers are incorporated. Using lower amounts of primers decreases the sensitivity. The quantity of primers, therefore, is a critical parameter in optimizing the real-time PCR assay. The contribution of dimer and primer adducts toward the fluorescence is determined by the amount of fluorescence provided by a no template DNA control (NTC). NTC or amplification of a negative control was

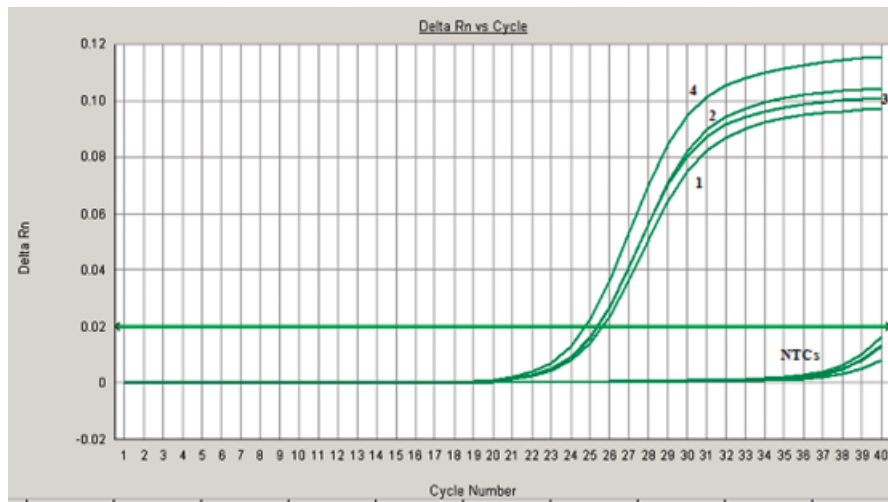


FIG. 1—Two microliters of human DNA (200 pg/μL) was amplified using 0.6 (line 1), 0.8 (line 2), 1.0 (line 3), and 1.2 μM primers (line 4) in the human DNA quantification (H-Quant) assay. NTCs indicate no template controls. The threshold value is indicated by a solid horizontal line with arrows.

incorporated into each experiment. The primer pair titration was tested at 0.6, 0.8, 1.0, and 1.2 μM (final concentration) using a DNA template ranging from 20.0 pg/μL to 2.0 ng/μL from a human DNA sample. The primer concentration did not influence the assay significantly at any concentration of template DNA. The results from 200 pg/μL of human DNA are presented in Fig. 1 as representative data from the experimental set. An increase in the primer concentration from 0.6 to 1.2 μM decreased the C_T value from 25.7 to 24.9. Primers at 1.2 μM were used in the H-Quant assay.

The concentration of MgCl₂ was varied from 1.5 to 6.0 mM. Five human DNA samples with concentrations ranging from 7.6 pg/μL to 50.0 ng/μL were used. The results from 50 ng/μL of template DNA are summarized in Fig. 2 as representative data from the experimental set. The efficiency of the assay was increased when the MgCl₂ concentration increased from 1.5 mM to 3.0 mM; the C_T value decreased from 16.7 to 15.2. However, the sensitivity decreased slightly when the concentration of the MgCl₂ was higher than 3.0 mM. Similar results were obtained for the five

human DNA samples and all template DNA concentrations. MgCl₂ at 3.0 mM was used in the H-Quant assay.

Higher concentrations of dNTPs are precursors for the synthesis of DNA and can inhibit the PCR either by entrapment of Mg²⁺ ions or substrate inhibition of DNA polymerase (20,21). The optimal concentration of dNTPs in the H-Quant assay was determined by amplification of five human DNA samples (7.67 pg/μL to 50.0 ng/μL) at 0.4, 0.8, 1.2, and 1.6 mM of dNTPs. The 0.4 and 0.8 mM tests gave very similar C_T values; however, the NTC gave a slightly higher background reading with 0.4 mM dNTPs (data not shown). Increasing the dNTPs to 1.2 and 1.6 mM exhibited an increased C_T value, indicating an inhibitory effect on the assay; the C_T values observed for 620 pg/μL of human DNA using 0.4, 0.8, 1.2, and 1.6 mM dNTPs were 20.9, 20.8, 21.4, and 23.5, respectively. dNTPs at 0.8 mM were used in the H-Quant assay.

In the H-Quant assay, SYBR[®] Green I is the reporter dye. In addition, a reference dye (proprietary dye obtained from Sigma) is incorporated into the assay. The reference dye enables normalization of the fluorescence signal generated by the reporter dye.

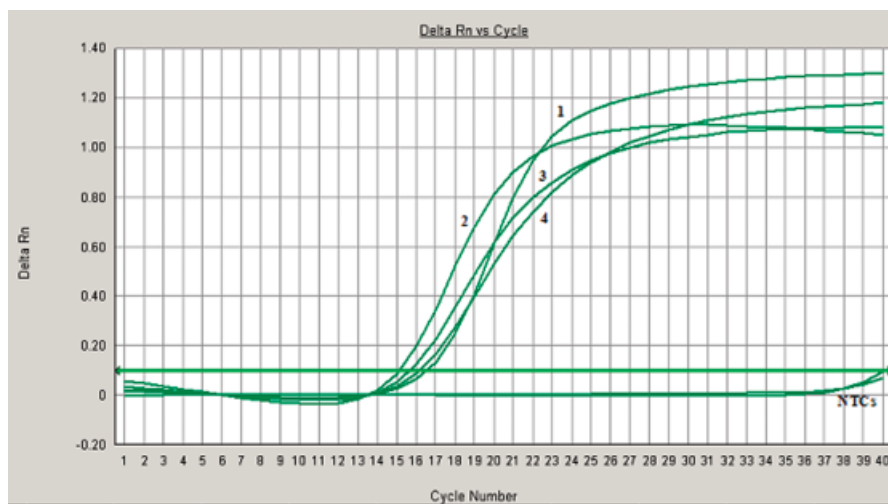


FIG. 2—Effect of MgCl₂ on the quantitation of DNA using the human DNA quantification (H-Quant) assay. 2.0 μL of human DNA (50 ng/μL) was amplified with 1.5 (line 1), 3.0 (line 2), 4.5 (line 3), and 6.0 mM MgCl₂ (line 4). NTCs indicate no template controls. The threshold value is indicated by a solid horizontal line with arrows.

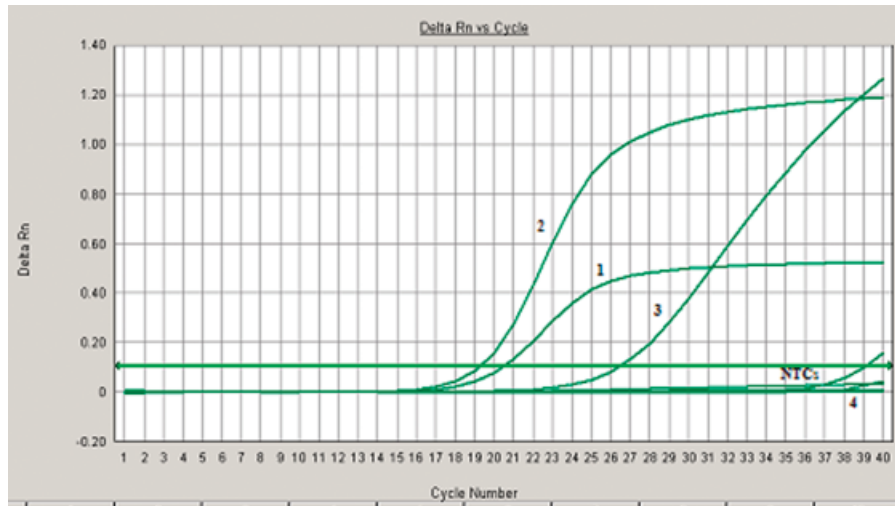


FIG. 3—Two microliters of human DNA (620 pg/ μ L) was amplified using 0.125 (line 1), 0.25 (line 2), 0.5 (line 3), and $\times 1.0$ concentration (line 4) of the SYBR[®] Green I dye in the human DNA quantification (H-Quant) assay. The concentration of the reference dye in the assay was $\times 0.2$. NTCs indicate no template controls. The threshold value is indicated by a solid horizontal line with arrows.

The concentrations of these dyes would, therefore, contribute to the sensitivity of the H-Quant assay. SYBR[®] Green I dye is obtained as 10,000 \times in dimethyl sulfoxide (DMSO), whereas the reference dye is provided as a 100 \times solution. Five human DNA samples of concentrations ranging from 7.6 pg/ μ L to 50.0 ng/ μ L were amplified at different concentrations of SYBR[®] Green I and the reference dye. The concentration of SYBR[®] Green I was investigated at $\times 0.125$, $\times 0.25$, $\times 0.5$, and $\times 1$ and the reference dye at $\times 0.2$, $\times 0.5$, and $\times 1$ (Figs. 3 and 4). Both the sensitivity and magnitude of the exponential phase increased when the concentration of SYBR[®] Green I was increased from $\times 0.125$ to $\times 0.25$ (Fig. 3). Further increase to $\times 0.5$ inhibited the PCR reaction significantly; complete inhibition was observed at $\times 1.0$ concentration. Decreasing the amount of reference dye from $\times 1$ to $\times 0.5$ and $\times 0.2$ increased both the sensitivity and geometric phase of the PCR curve (Fig. 4). Thus, a combination of $\times 0.25$ SYBR[®] Green I dye and $\times 0.2$ reference dye gave the

best results as judged by the C_T values and geometric phase and was used in the H-Quant assay.

BSA is generally incorporated into the PCR reaction mixture as an additive to aid in the amplification of samples containing small amounts of DNA or possibly degraded DNA samples. The concentration of BSA to add to the reaction mixture was determined by a series of experiments that included 0.0, 0.125, 0.25, 0.5, and 0.75 μ g of BSA fraction V and using DNA template ranging from 7.6 pg to 50.0 ng. Incorporation of BSA up to 0.25 μ g exhibited a slight increase in sensitivity; the C_T value decreased by 0.7 cycle for 620 pg/ μ L of template DNA. Higher amounts of BSA, however, showed a steady increase in C_T (data not shown). 0.25 μ g of BSA was incorporated into the H-Quant assay.

The AmpliTaq[™]Gold DNA Polymerase was incorporated at 0.5, 1.0, and 1.5 U and using DNA template ranging from 200 pg to 50 ng. One unit of the DNA polymerase showed a lower C_T value, indicating greater sensitivity compared with those samples

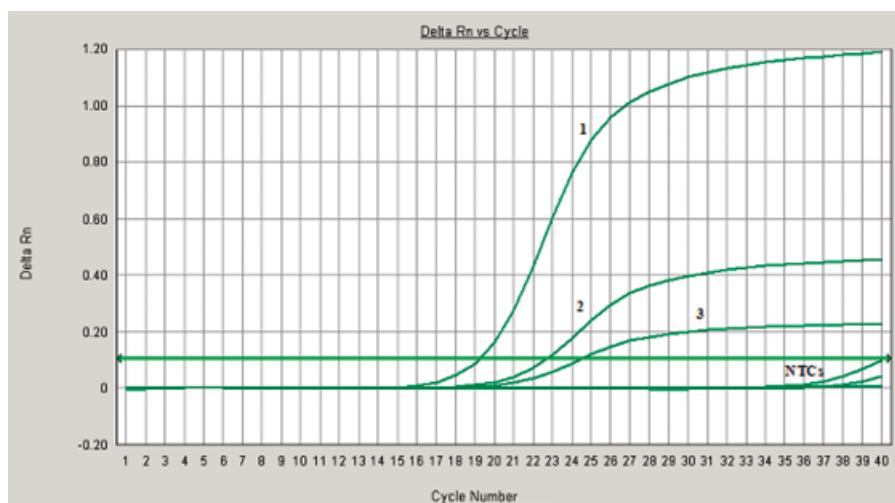


FIG. 4—Two microliters of human DNA (620 pg/ μ L) was amplified using 0.2 (line 1), 0.5 (line 2), and $\times 1.0$ concentration (line 3) of the reference dye in the human DNA quantification (H-Quant) assay. The concentration of the SYBR[®] Green I dye in the assay was $\times 0.5$. NTCs indicate no template controls. The threshold value is indicated by a solid horizontal line with arrows.

tested with 0.5 U of the DNA polymerase (data not shown). When 1.5 U of the polymerase were used, the fluorescence signal from the NTC increased significantly, although a slight increase in sensitivity in the detection of samples was observed (data not shown). A balance between the sensitivity of the assay and minimal background reading was observed using 1.0 U of AmpliTaqTMGold DNA Polymerase.

Nonhuman Species

Several different nonhuman species were tested using 2–4 ng of DNA template with the H-Quant assay in order to determine the human specificity of the *AluYb8* primers. The different specimens included DNA from cat, dog, sheep, orangutan, gorilla, *Escherichia coli* (ATCC# 700927D), *Staphylococcus aureus* subsp. *aureus* (ATCC# 35556D), *Neisseria gonorrhoeae* (ATCC# 700825D), *Candida albicans* (ATCC 14053D), and hepatitis B virus (ADW) (ATCC 45020D). The DNA from cat, dog, orangutan, gorilla, *E. coli*, *S. aureus* subsp. *aureus*, *N. gonorrhoea*, *C. albicans*, and Hepatitis B virus (ADW) did not yield any products. Of the two sheep samples tested, sheep-1 did not yield an amplified product; however, sheep-2 gave a C_T value such that the quantitative value was 0.45 pg/ μ L. The sheep-2 sample yielded a concentration of 4.3 ng/ μ L of DNA when quantitated using the Pico Green[®] reagent and a Victor3 fluorescence reader (Perkin Elmer, Wellesley, MA), a quantitation method that is not human specific (22). Thus, the H-Quant assay for the sheep-2 sample yielded results that were 9555 times less than the nonspecific quantitation method. An extremely low level of cross-amplification has been observed for some mobile element-based genetic systems because of the ubiquitous nature of 7SL- and tRNA-related SINE families and this factor typically does not interfere with the assay used for human DNA (15). The nonhuman primate species investigated in the present study provided some results (orangutan, 0.28 pg/ μ L, and gorilla, 34.3 pg/ μ L) using the H-Quant assay. Thus, the *AluYb8* primers in the H-Quant assay are specific for human DNA and some great apes. The human DNA-specific AluQuantTM Probe Mix (Promega Corporation, Madison, WI) exhibited a low level of amplification with the DNA from mouse, yeast, *E. coli* (strain B), and *C. perfringens* (7). A low level of amplification up

to 0.07% for some animal species is observed using the Ya5 sub-family Alu (plasmid pPD39)-specific primers (10).

Sensitivity Study

The sensitivity of the H-Quant assay system was investigated by quantitation of human DNA samples with quantities of DNA ranging from 5.0 pg/ μ L to 24.4 ng/ μ L (Fig. 5). As expected, the C_T value increased progressively with a decrease in the amount of human DNA. In a separate experiment, the quantity of DNA was further decreased to 2.0 pg/ μ L (data not shown). The results indicate that the H-Quant assay is highly sensitive and exhibits a very dynamic range. Although it was possible to detect and quantitate 2.0 pg/ μ L of DNA, a greater margin of error existed at lower concentrations of DNA. The sensitivity of the H-Quant assay was determined at 7.6 pg/ μ L.

Precision and Reproducibility Study

A total of 20 runs were performed at different times and on different days using the human DNA sample at concentrations ranging from 8 pg/ μ L to 50 ng/ μ L. A calibration curve was generated for each experiment. The precision of the H-Quant assay was evaluated by the variation in the values of slope for the calibration curve. The slope values ranged between -4.15 and -3.30 . For these set of runs, the average slope value was -3.722 and the standard deviation was 0.288. Besides the variability inherent in pipetting, the constitutions of PCR reagents contribute to the slope value of the calibration curve. It is, therefore, recommended to derive an acceptable range for the slope value for each batch of primer mix and reaction mix.

Concordance Study

Sixteen different human samples were quantified using the H-Quant assay and the QuantifilerTM H-Quant kit. The results of these sixteen samples from the two different assays were compared. The results obtained from the H-Quant human DNA quantitation assay and the QuantifilerTM Human DNA Quantitation kits are summarized in Table 1. The quantitation values obtained from the two systems were comparable. However, the Quantifiler

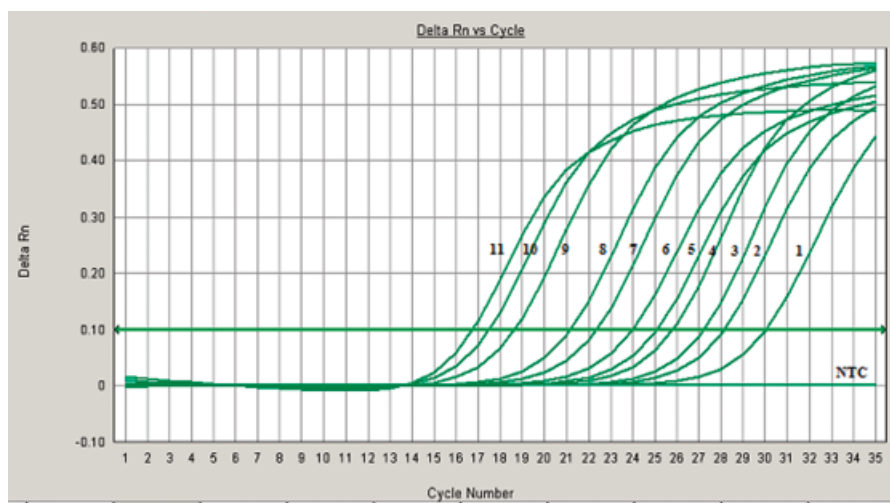


FIG. 5—Sensitivity of the human DNA quantification (H-Quant) assay. Two microliters of human DNA containing 5 pg/ μ L (line 1), 10 pg/ μ L (line 2), 20 pg/ μ L (line 3), 50 pg/ μ L (line 4), 0.1 ng/ μ L (line 5), 0.2 ng/ μ L (line 6), 0.5 ng/ μ L (line 7), 1.0 ng/ μ L (line 8), 5.0 ng/ μ L (line 9), 10.0 ng/ μ L (line 10), and 24.4 ng/ μ L (line 11) were amplified using the H-Quant assay reagents. NTC indicates no template control. The threshold value is indicated by a solid horizontal line with arrows.

TABLE 1—Concordance study between the developed H-Quant assay and the Quantifiler™ human DNA quantitation kit.

Sample	H-Quant (ng/μl)	Quantifiler™ Human (ng/μl)
1	3.35	4.76
2	8.10	9.79
3	3.07	4.11
4	3.59	5.03
5	2.74	4.04
6	2.34	3.58
7	3.16	3.95
8	3.78	6.13
9	3.33	4.27
10	1.73	2.09
11	18.35	25.48
12	19.75	30.46
13	24.21	28.57
14	0.922	0.172
15	0.935	0.170
16	1.15	0.203

results were consistently higher than those run with the H-Quant assay, except for the samples containing 1.15 ng/μL or lower quantity of human DNA. The sizes of amplicons obtained from the H-Quant and Quantifiler™ human DNA quantitation system were 216 and 62 bases, respectively. Because the Quantifiler™ human DNA quantitation system detects a smaller amplicon, it is possible that this assay detects additional degraded DNA compared with the H-Quant assay that contributes to the higher values.

Correlation with the Amplification of STRs

Quantitation of human DNA in the biological samples is performed to determine the volume of extract for amplification of STR loci using multiplex genotyping systems. In general, 0.5–2.0 ng of DNA provides an STR profile with allele peak heights

within the fractionation range and devoid of artifacts. The values of DNA quantitated by the H-Quant were correlated with the STR profile obtained using the AmpFISTR® COfiler™ amplification kit. The profile obtained by amplification of 0.5 ng of template DNA is presented in Fig. 6.

Conclusions

The quantity of human DNA in biological samples can be determined using the H-Quant system. The methods explore the real-time PCR principles for the quantitation of human DNA in unknown biological samples. The system's sensitivity was sufficient for quantification of as little as 7.6 pg/μL of human DNA. These data demonstrate that the H-Quant system is valid and reliable for quantitation of human DNA in forensic DNA analysis.

Acknowledgments

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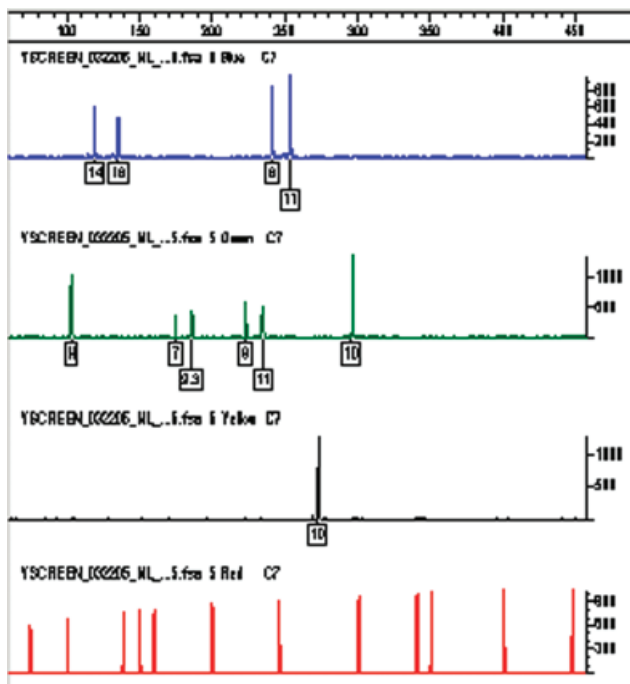


FIG. 6—Short tandem repeat (STR) profile for the 0.5 ng of human DNA amplified with the AmpFISTR® COfiler™ and analyzed on a 3100 Genetic Analyzer. The quantity of DNA in the extract was determined by using the developed human DNA quantification (H-Quant) system.

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