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Developmental Validation of a Real-Time Quantitative PCR Assay for Automated Quantification of Human DNA*

ABSTRACT: Our laboratory has developed an automated real-time quantitative PCR assay for detecting human DNA. The assay utilizes an in-house, custom-designed TaqMan®-MGB sequence-specific probe (CFS-HumRT) and the ABD 7900HT SDS platform. Developmental validation has followed TWGDAM (1) guidelines and demonstrates that the assay is primate specific, is highly sensitive, yields consistent results, and works with human DNA extracted from a variety of body fluid stains. When operating within the dynamic range of the system using high-quality DNA samples, the technique yields similar quantification results to our current QuantiBlot™ assay with the added benefit of time saving through automation. Furthermore, the QPCR assay identifies how much amplifiable DNA is in a sample and thus has the potential to predict PCR success in downstream applications such as STR analysis.

KEYWORDS: forensic science, genomic DNA, quantification, real-time PCR, ABI 7900 Sequence Detection System, automation

The process of forensic human identification involves sensitive polymerase chain reaction (PCR) multiplex assays. These tests perform optimally within a narrow range of template DNA, necessitating reliable quantification of human DNA. Furthermore, contaminating DNA from nonhuman sources (e.g., bacteria and fungi) often associated with forensic samples must not interfere with quantification.

The Perkin-Elmer QuantiBlot™ kit is currently used by the Centre of Forensic Sciences (CFS) to determine the quantity of DNA in samples (2). Results generated by this analysis enable us to amplify a desired target amount and identify samples that meet our minimum criteria for amplification (3,4).

We have used the slot blot method to quantify DNA in forensic casework samples for over ten years and have found it to be reliable and sensitive (lower limit of detection of 50 pg). Unfortunately the technique is time consuming, labor intensive, and the interpretation of a result is subjective. The availability of automated instrumentation offers the potential to eliminate labor-intensive steps while providing more objective measurements.

Real-time quantitative PCR (QPCR) is widely applied in diagnostic applications such as gene expression assays (5,6), mutation analysis (7,8), and quantification of residual disease (9,10). This "real-time" technology detects PCR products as they accumulate and relies upon the quantitative relationship between the amount of starting target sequence and amount of PCR product generated (11,12). PCR product detection is most often performed using a sequence specific probe that has a fluorescent reporter dye and quencher dye that are attached to the 5′ and 3′ ends, respectively (13). When the fluorogenic probe is intact, the reporter dye emission is quenched. During PCR, the probe anneals specifically between the forward and reverse primers. When the probe is cleaved by the 5′ nuclease activity of the DNA polymerase, the reporter dye is separated from the quencher and a sequence-specific signal is generated (14). In the early rounds of amplification, the change in fluorescent signal is negligible and beyond the sensitivity of the detector. This defines the baseline of the amplification plot. With each additional PCR cycle more product accumulates and thus more reporter molecules are cleaved. During the geometric phase of the amplification, the detection system registers a significant change in fluorescence, typically set at ten times standard deviation of baseline emissions. This point is referred to as the threshold cycle (C_T) and at any given cycle within the geometric phase of PCR is proportional to the log of the starting amount of nucleic acid (12). When samples of known starting amount are amplified simultaneously under the same reaction conditions as the unknowns, it is possible to construct a standard curve and from this standard curve determine the starting amount of DNA in each of the unknowns.

In this paper we report Part 1 of the developmental validation of a rapid and automated human specific DNA quantification assay using a TaqMan® fluorogenic probe (CFS-HumRT) and the ABI 7900HT Sequence Detection System (Applied Biosystems). Included is: primer and probe design, PCR optimization, species specificity, interference studies, population studies, reproducibility, and comparability with our current slot blot method for quantification. Part 2 of our validation studies will address issues such as: the effects of sample quality and purity on PCR efficiency, interpretation of assay results, the impact of DNA quantification using the CFS-HumRT assay on subsequent STR typing, and the development of laboratory specific protocols for deployment in forensic casework.

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Materials and Methods

Sample Source and DNA Extraction

Unless indicated, a modified SDS/Proteinase K lysis with organic extraction and microfiltration was used to isolate DNA from body fluid stains (15).

The source of DNA used in the assay optimization, interference, and replicate studies was buccal swabs taken from CFS staff. The nonhuman DNA samples utilized in the cross-reactivity studies were previously extracted and used in earlier validation studies (16). DNA from these samples was quantified on a 1% agarose gel prior to QPCR analysis. High-molecular-weight DNA from Mouse (Balb/C), Saccharomyces cerevisiae, and *E. coli* (strain W3110) were purchased from Sigma Chemical Co. (St. Louis, MO).

The initial human population study used DNA that had been recently extracted from 157 casework comparison bloodstain or buccal swab samples. The samples were from individuals of a variety of racial origins. Additional human population studies were performed using approximately 100 samples from each of our Caucasian, Black, East Asian, and Southeast Asian STR databases. These samples were previously extracted and had been stored at −20°C for approximately ten years.

High-quality DNA samples extracted from 109 body fluid stains were used to compare QPCR quantification with slot blot quantity estimates. The sample set consisted of 19 bloodstains, 37 buccal swabs, 24 vaginal epithelial fractions, 23 vaginal sperm fractions, and 6 genomic DNA samples received in an external proficiency test. The same volume of stock DNA and the same set of DNA quantification standards were used to compare methods.

Slot Blot Quantification

DNA samples quantified by the slot blot method employed the QuantiBlot™ Human DNA Quantitation Kit (Perkin-Elmer) with chemiluminescence detection. Following the manufacturer's instructions (17), unknown samples were quantified manually by comparing their signal intensity to that of a series of known DNA standards prepared by serial dilution and ranging from 50 pg to 100 ng. The "standard A" DNA provided in the QuantiBlot™ kit was the source of DNA for standards of less than 5 ng. For higher amounts, standards were prepared using K562 genomic DNA purchased from Promega.

Real-Time PCR Quantification

DNA samples quantified by QPCR used custom-made primers and probe (PE Biosystems). Unless indicated, 200 n*M* of CFS-HumRT probe and 750 n*M* of forward and reverse primer were used for PCR. In all instances, 5 μL of DNA extract was amplified using 10 μL of TaqMan® Universal PCR Master Mix (2X, PE Biosystems) for a total reaction volume of 20 μL. Amplifications were performed using the universal thermocycling parameters recommended for quantitative TaqMan® assays (18). In addition, included in each PCR was an initial 2-min AmpErase® UNG step and a 10-min AmpliTaq Gold® enzyme activation step. Amplification and target detection was performed on the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) utilizing 96-well or 384-well plates with optical covers supplied by PE Biosystems.

The creation of the standard curve and estimation of DNA quantity of an unknown was performed automatically by the SDS software. Unless indicated, at least one set of DNA standards was loaded on each PCR plate along with one no template control (NTC). TaqMan® Control Human Genomic DNA, purchased from PE Biosystems, was used to prepare the DNA standards for all QPCR analyses. The control DNA stock was quantified in house against the current QuantiBlot™ DNA standards prior to serial dilution and QPCR.

Results and Discussion

Identification of Primers and Probe

Three factors were considered necessary when designing the QPCR assay: (1) the probe should be specific for human DNA, (2) the assay should be reliable and perform the same regardless of the source of the sample, and (3) the sensitivity of the assay should be equivalent if not better than that of the QuantiBlot™ system currently employed to quantify human DNA. In order that these criteria be met, we designed a TaqMan®-MGB (minor groove binder) probe and primers from a single-copy, nonpolymorphic DNA sequence.

Using Primer Express® Software, version 2.0 (Applied Biosystems) and the recommended design parameters (19), a number of sequences from within reported STR sites, but outside the polymorphic region, were tested as possible candidates.

Identification of suitable primers and a sequence specific target for the assay were derived from the reported GenBank® Sequences for the HUMTH01 loci, Accession D00269 (Fig. 1). The amplicon is 31 bps downstream from the polymorphic repeat region. The designed probe has a GC content of 67%, *Tm* of 70°C, is 15 bps in length and labeled with the TaqMan® VIC reporter dye. Both forward and reverse primers are 21 bps in length and have a *GC* content of 43 and 57% and a *Tm* of 58 and 60°C, respectively. The total amplicon size is 62 bps.

A search of sequences contained in GenBank using the entire CFS-HumRT amplicon was conducted. As expected, the sequence for human tyrosine hydroxylase returned a 100% identity to the CFS-HumRT amplicon. The sequence for the tyrosine hydroxylase

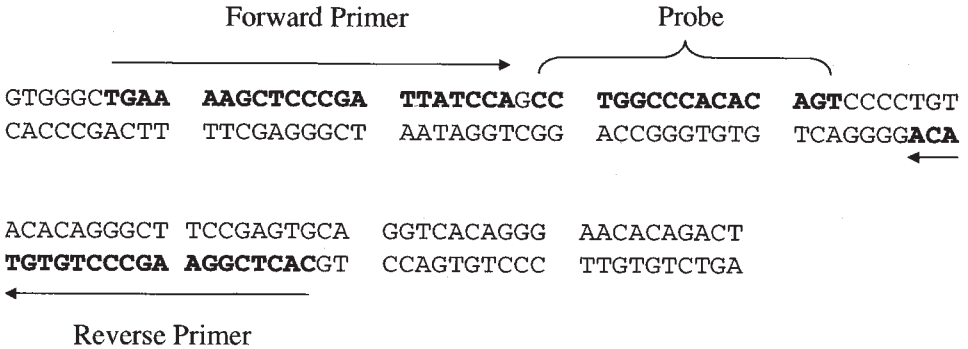

FIG. 1—*CFS-HumRT probe and primer sequences.*

TABLE 1—*Mean C_T values (±STD) detected for 1 ng of DNA amplified in triplicate using 25 different combinations of primer concentration.*

	Forward Primer, n*M*				
Reverse Primer n*M*	150	300	600	750	1000
150	29.047 13 (±0.269 24)	28.715 81 (±0.044 927)	28.485 91 (±0.120 035)	28.641 54 (±0.143 116)	29.026 25 (±0.065 087)
300	28.818 86 (±0.124 989)	28.642 54 (±0.366 5)	28.524 75 (±0.200 711)	28.737 39 (±0.137 666)	28.725 82 (±0.113 528)
600	28.655 14 (±0.104 752)	28.466 16 (±0.139 524)	28.803 12 (±0.086 291)	28.583 17 (±0.103 68)	28.781 19 (±0.209 868)
750	28.941 33 (±0.120 381)	28.765 8 (±0.173 327)	28.447 47 (±0.104 458)	28.551 24 (±0.086 697)	28.691 93 (±0.090 284)
1000	28.901 33 (±0.158 736)	28.535 71 (±0.190 96)	28.807 15 (±0.260 618)	28.98887 (±0.095391)	28.979 88 (±0.117 719)

gene of some other higher primates (e.g., orangutan and gorilla) also showed significant identity to the CFS-HumRT amplicon, with one or two mismatches.

No other sequences exhibited enough alignment with both the forward and reverse primers to yield any PCR product.

Assay Optimization

Forward and reverse primer concentrations were independently varied such that those amounts providing optimal assay performance could be identified. Approximately 1 ng of DNA was amplified in triplicate using 200 nM of probe for each of the 25 different primer concentrations (18). Results are shown in Table 1. Amplification using any of the 25 different combinations of forward and reverse primer concentration was successful. The factors considered when choosing the optimal primer concentrations were: earliest detection (lowest C_T), greatest change in normalized fluorescence (ΔR_n), and least variability among the replicates. Using these criteria, both the 600/750 nM and 750/750 nM forward and reverse primer combinations were selected for use in additional optimization studies.

Using the two combinations of forward/reverse primer that yielded similar C_T values in the previous study, a probe optimization matrix was performed. Three replicates of 1 ng of DNA were amplified for each of five different concentrations of probe. As shown in Table 2, C_T values decreased as the concentration of probe increased from 50 nM up to 200 nM. However, when the probe concentration was increased to 250 nM, little if any reduction in C_T was observed. Once again both combinations of forward and reverse primers examined yielded similar results. Because PCR master mix preparation is simplified when the same concentration of forward and reverse primers is used, 750 nM of both primers along with 200 nM of probe was chosen for all further validation studies utilizing the 96-well PCR block.

Initial validation studies on the instrument were performed using a 384-well PCR block that allowed for a maximum amplification volume of 20 μL. Amplification using larger reaction volumes was examined following installation of a 96-well block in the instrument. Approximately 1 ng of human DNA was amplified in triplicate in either a 20, 30, or 40-μL reaction volume along with one set of DNA standards for each PCR volume. As seen in Table 3, increasing the PCR reaction volume resulted in later detection of the sequence-specific target. The same trend was noted with the DNA standards. All additional validation studies were continued using the 20-μL reaction volume; this has the added advantage of conserving reagents while allowing us to detect amplification product sooner.

TABLE 2—*Mean C_T values (±STD) obtained from three replicate amplifications of 1 ng of DNA using five different probe concentrations.*

	Forward/Reverse Primer, n*M*	
Probe, n*M*	600/750	750/750
50	29.686 3 (±0.105 829 808)	29.848 36 (±0.158 951 274)
100	29.406 43 (±0.111 690 794)	29.373 35 (±0.011 129 951)
150	29.047 (±0.048 255 654)	29.052 75 (±0.005 432 507)
200	28.940 27 (±0.120 471 243)	28.923 94 (±0.188 291 51)
250	29.043 13 (±0.136 768 312)	28.902 78 (±0.089 043 364)

TABLE 3—*C_T values obtained from 1 ng of DNA amplified in different PCR volumes.*

Replicate	20 μL PCR	30 μL PCR	40 μL PCR
1	29.078 95	29.426 97	30.050 44
2	29.363 37	29.470 02	30.126 55
3	29.187 45	29.711 68	30.146 87

Relationship of Fluorescence Signal to DNA Concentration and PCR Efficiency

Assuming a PCR efficiency of 100%, a two-fold serial dilution should exhibit a corresponding decrease in C_T by one cycle (12). An example of an amplification plot generated by the CFS-HumRT QPCR assay for such a serial dilution is given in Fig. 2.

Performing numerous replicates of these standards across multiple PCRs, we were able to reliably detect from 25 ng (this is the highest amount of standard used) to a lower limit of 25 pg of human DNA. The QPCR assay is capable of detecting as little as 6 pg of DNA, although in the replicate studies performed, detection occurred about 50% of the time. This is a considerable improvement in sensitivity in comparison to QuantiBlot™ analysis, which is only able to detect 50 pg on a consistent basis. The system can also detect greater than 25 ng (up to 500 ng of K562 DNA tested). However, due to the stock concentration of the standard DNA, the stan-

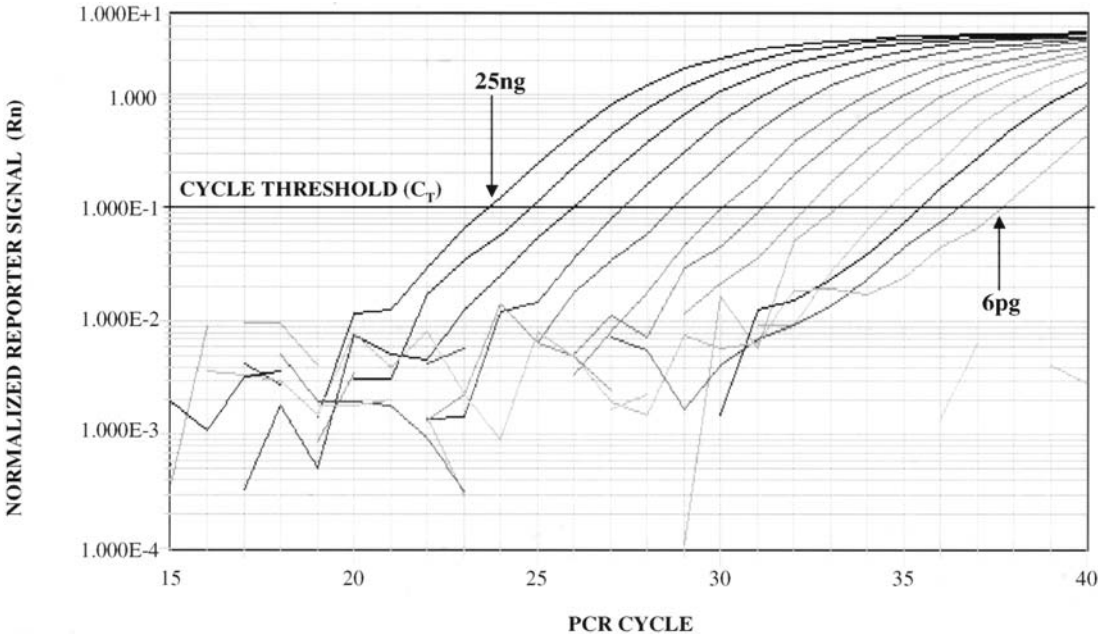

FIG. 2—*Amplification plot (log view) for a two-fold serial dilution of human genomic DNA.*

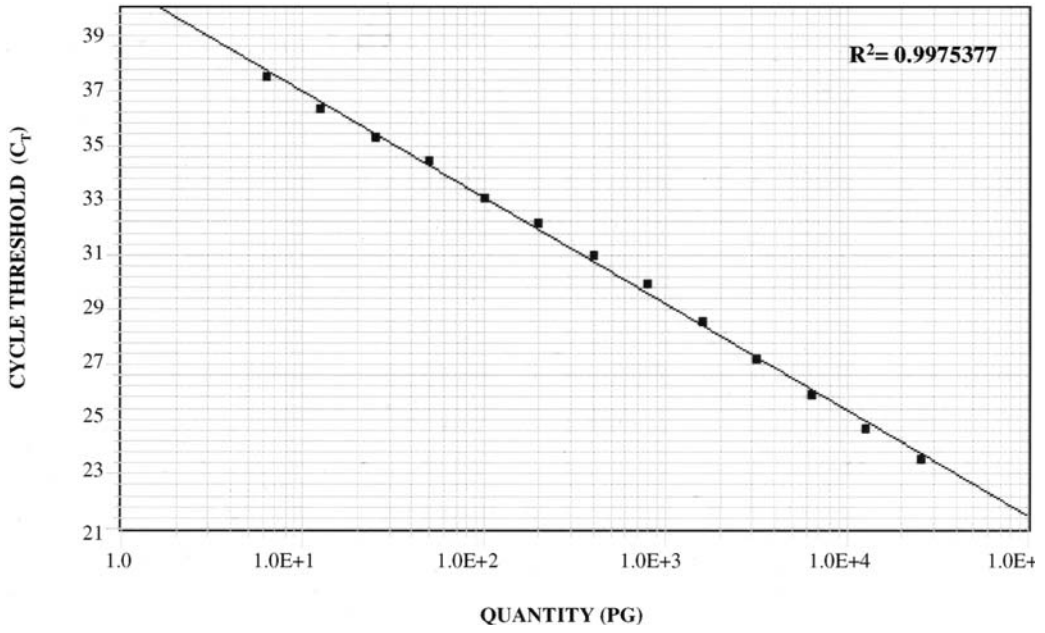

FIG. 3—*Standard curve showing C_T values plotted against the log of the initial amount of human genomic DNA.*

dard curve generated has an upper limit of 25 ng. CFS casework experience has shown most samples quantify at less than 25 ng/μL in an extraction volume of 15 μL.

When plotted on a log scale of DNA versus cycle number, the standard curve generated by the geometric phase should approximate a straight line (12). An example of a standard curve of fluorescence produced from human DNA using the CFS-HumRT QPCR assay is given in Fig. 3. As illustrated, the amount of fluorescence signal generated is proportional to the amount of human DNA in the sample over a wide range of DNA concentrations with a correlation co-efficient approaching 1 ($R^2 = 1$). In most instances, standard curves generated throughout this validation study have demonstrated correlation coefficients exceeding $R^2 = 0.98$ as determined by the instrument's software.

Cross-Reactivity with Nonhuman DNA

To evaluate the specificity of the probe, numerous species of potential concern in analysis of forensic samples were tested. The sequence-specific target was detected with some primate species, including chimpanzee and gorilla. However, no signal was detected when 5 ng of DNA from bird, dog, cat, fish, cow, goat, rabbit, pig, hen, sheep, snake, lion, moose, bear, or deer was added to the PCR reaction. Mouse, yeast, and *E. coli* DNA were tested at quantities as high as 100 ng and did not produce any signal. In addition to testing

the specificity of the probe, the possibility of interference by a contaminating nonhuman DNA source was also examined. Studies were performed with mixtures containing 100 ng of yeast DNA and as little as 100 pg of human DNA (Table 4). Even when the QPCR assay was performed with mixtures containing 1000 times more nonhuman than human DNA, the assay was successful at amplifying only the human template with no significant impact on accuracy.

Population Studies

Human population studies were initially done using 157 different casework comparison samples. Although these samples were of unknown racial origin, preliminary results indicated the CFS-HumRT sequence specific target is conserved in the human population. Additional population studies were performed using approximately 100 samples from each of our Caucasian, Black, East Asian, and Southeast Asian STR databases. All of the 395 samples tested yielded a QPCR result, indicating the target sequence is conserved within different racial classifications.

TABLE 4—*Mean quantity of DNA (±STD) detected by the CFS-HumRT assay in three different human DNA samples mixed with or without 100 ng of yeast DNA (N = 3).*

Sample	Human DNA, pg	Human + Yeast DNA, pg
1	5491.648 (±366.200 8)	5 399.471 (±346.346)
2	609.3253 (±53.987 63)	626.360 8 (±20.434 58)
3	61.766 45 (±2.705 884)	72.248 43 (±7.137 389)

TABLE 5—*Mean quantity (±STD) determined for two unknown DNA samples run in replicates of ten in each of four separate CFS-HumRT assays.*

	DNA Quantity, pg	
Replicate	Unknown 1	Unknown 2
1	1 228.687 4 (±104.394 554)	7 670.036 (±412.811)
2	1 524.2 (±248.978 58)	7 598.67 (±714.652)
3	1 298.060 3 (±87.808 6)	8 187.211 (±503.210 6)
4	1 321.882 7 (±72.124 29)	7 486.245 (±322.886 4)
All	1 343.208 (±179.445 4)	7 735.541 (±560.204 7)

Replicate DNA Quantification and Precision

Reproducibility, or precision, is important for all quantitative applications and can be measured through the use of replicates. Poor precision increases the risk that the experimental values will differ significantly from the actual values. Two different DNA samples were amplified in replicates of ten in each of four separate PCRs ($n = 40$). A standard curve generated from one set of DNA standards loaded on each QPCR plate was used to quantify the "unknown" DNA samples. Results are shown in Table 5. Quantification using this assay gave reproducible results. DNA replicates typically yielded quantities within 10% of their mean value. The range in DNA quantity reported for a given set of replicates was approximately 3 STDS (results not shown).

The standard curve and DNA quantities for unknowns are automatically generated by the system's software, and the values generated are to the fourth decimal place. The slot blot technique relies on a subjective assessment of band intensity using the human eye and therefore is less able to discriminate small differences. Real-time QPCR offers the advantage of automation with more consistent results because subjectivity due to human interpretation is eliminated. In addition, QPCR quantifies how much amplifiable DNA is present in a sample and thus has the potential to predict STR PCR success or failure.

Comparison of Quantification Techniques

Our laboratory has been performing STR analysis in casework for over eight years. During this time we have relied on slot blot analysis using the QuantiBlot™ kit to quantify all casework samples. In order for QPCR to be incorporated successfully into casework, the technique must meet or exceed the performance of the QuantiBlot™ method currently employed.

One hundred and nine high-quality DNA samples extracted from a variety of body fluid stains were quantified by the QuantiBlot™ system and using the CFS-HumRT QPCR assay.

The two quantification methods yielded similar results (correlation coefficient of 95%) regardless of sample type (Fig. 4). Results

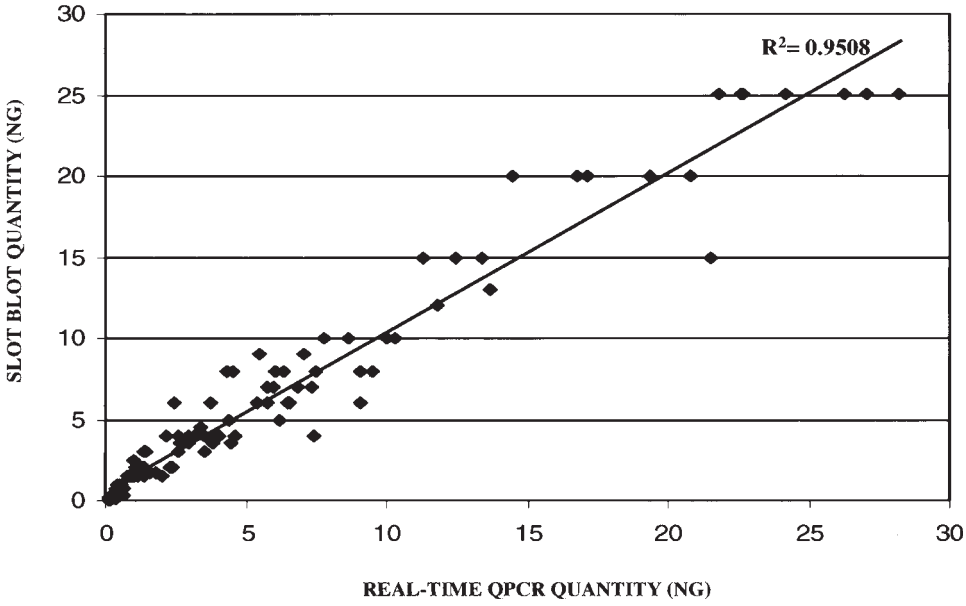

FIG. 4—*Comparison of the DNA quantity estimates for 109 samples as determined by slot blot analysis and by the CFS-HumRT QPCR assay.*

seem to demonstrate greater variability in the >10 ng range of the plot (although there are fewer data points). The differences observed might be due to inaccuracies in the slot blot analysis, since interpretation of bands by the human eye becomes more subjective as signal intensities increase. Studies examining the accuracy of the QPCR using neat and diluted DNA samples indicate the system performs similarly for a wide range of DNA concentrations (results not shown).

Conclusions

Part one of the developmental validations presented here demonstrates that CFS-HumRT QPCR is an efficient and reliable way to quantify high-quality human DNA extracted from a variety of body fluids. Part two will determine how the QPCR assay performs with DNA of poor quality. Forensic samples can be contaminated with PCR inhibitors that could alter quantification results; however, we consider the potential of the QPCR system to detect amplifiable DNA rather than total human DNA a huge advantage. Identifying problematic samples prior to STR amplification not only saves the laboratory time and money, but also offers an opportunity to consider how a challenging sample might best be treated in order to obtain a DNA profile.

An important advantage of the QPCR system over our current QuantiBlot™ procedure is the projected improvement in efficiency. First, less laboratory space is required to perform QPCR (approximately 40 ft^2 needed for PCR set-up and the SDS instrument) than is required for QuantiBlot™ analysis (approximately 150 ft^2 needed for slot blot/hybridization equipment and a separate dark room). Second, there is a significant reduction in the hands-on time required to process samples. The amount of operator intervention needed to analyze 96 samples by QPCR and by QuantiBlot™ is 0.75 h and 4.5 h, respectively. Thirdly, QPCR offers a huge gain in capacity; with the automation accessory this instrument offers a 24 h unattended operation capable of processing 4000 samples per day. Finally, all of the above is achieved with no significant increase in the costs of the reagents required to perform quantification (approximately $0.96 and $0.76 per sample for QPCR and QuantiBlot™, respectively). Therefore, a reduction in required laboratory space and the ongoing savings in staff time offset the cost of the SDS instrument.

On completion of the developmental validation, the CFS-HumRT QPCR assay with the use of the ABI PRISM® 7900HT Sequence Detector will provide the CFS with a fully automated and rapid process for quantifying human DNA in forensic casework.

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