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PAPER

CRIMINALISTICS

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Quantification of Human Mitochondrial DNA Using Synthesized DNA Standards*

ABSTRACT: Successful mitochondrial DNA (mtDNA) forensic analysis depends on sufficient quantity and quality of mtDNA. A real-time quantitative PCR assay was developed to assess such characteristics in a DNA sample, which utilizes a duplex, synthetic DNA to ensure optimal quality assurance and quality control. The assay's 105-base pair target sequence facilitates amplification of degraded DNA and is minimally homologous to nonhuman mtDNA. The primers and probe hybridize to a region that has relatively few sequence polymorphisms. The assay can also identify the presence of PCR inhibitors and thus indicate the need for sample repurification. The results show that the assay provides information down to 10 copies and provides a dynamic range spanning seven orders of magnitude. Additional experiments demonstrated that as few as 300 mtDNA copies resulted in successful hypervariable region amplification, information that permits sample conservation and optimized downstream PCR testing. The assay described is rapid, reliable, and robust.

KEYWORDS: forensic science, mitochondrial DNA, quantitative PCR, DNA quantification, assay, DNA quality, synthetic DNA standards

Sequence analysis of human mitochondrial DNA (mtDNA) has become a valuable forensic tool for specimens, such as hair and calcified tissue samples, in which nuclear DNA analysis may fail because of DNA degradation or insufficient template (1–4). Analysis of mtDNA may be possible in such cases because human cells contain hundreds to perhaps thousands of mtDNA copies (5). In addition, mtDNA is circular and more resistant to exonuclease digestion. Even though abundant on a cell basis, mtDNA quantities may be nonetheless limited. Thus, judicious use of the sample is important, so that sample consumption is minimized and optimized levels of template are amplified.

Another important consideration in forensic mtDNA analysis is the inhibition of amplification. Indeed, hair and calcified tissue may contain PCR inhibitors, such as melanin (6) and humic acid (7), respectively, at levels which could interfere with PCR and subsequent analysis. However, internal positive controls (IPCs) may be used to distinguish whether a DNA sample contains PCR inhibitors or insufficient template (8).

Real-time quantitative PCR (qPCR), a highly specific, sensitive, and reproducible method for quantifying nucleic acids (9), represents an attractive approach to quantifying mtDNA. Using a DNA standard, absolute copy numbers of a target sequence in a sample may be determined via qPCR. Optimally, the standard should contain a DNA sequence that is different from, but linked to, the sequence to be analyzed to eliminate the risk of contamination. Human mtDNA forensic analysis involves the noncoding hypervariable regions (HVRs) where most variation in mtDNA is found (10). However, the mtDNA coding region also contains sequence variation (11–13), which may someday be used in forensic analyses; therefore, it is virtually impossible to identify an mtDNA standard sequence that lacks sequence variation.

Several qPCR assays for the quantification of human mtDNA have been reported (14–25), and a number have been specifically developed for forensic purposes (14,20,21,23–25). Many, although not all, of these utilize highly specific fluorogenic probes and mtDNA-specific standard curves, which enable the absolute quantification of mtDNA down to 10 copies of mtDNA or less (14,17,25). Other notable features include high specificity for human DNA (21,23,24) and incorporation of an IPC for the detection of PCR inhibitors, which serves to highlight those samples that may require additional purification (21).

The qPCR assay described herein combines many of these features as well as quantification of degraded DNA, high reproducibility, and a wide dynamic range to enable quantification of low copy number samples, such as hair shafts and bone, as well as high copy number samples, such as blood and buccal swabs. In addition, the assay also utilizes a unique DNA standard that provides certain measures of quality control and contamination control and that lacks topological constraint, thereby allowing for greater accuracy of quantification. Finally, the minimum number of mtDNA copies required for forensic analysis is presented, thus permitting the assay to be used to direct the degree of sample dilution to minimize consumption while ensuring success.

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Materials and Methods

DNA Samples

Human and other vertebrate DNA samples were purchased from Zyagen Laboratories (San Diego, CA). Bacterial DNA and fungal DNA were purchased from the American Type Culture Collection (Manassas, VA).

Buccal swab DNA was extracted using an organic extraction method. Briefly, a half buccal swab was incubated at 56°C for 2 h in a buffer containing 10 mM Tris, 100 mM NaCl, 39 mM dithiothreitol, 10 mM EDTA, 2% SDS, and 1.2 units of proteinase K. The swab was removed, and the solution was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v). The aqueous layer was purified using a Microcon 100 centrifugal device (Millipore, Billerica, MA). DNA was recovered from the device with 60 μL of water and stored at −20°C. The DNA extracts were subsequently diluted with water and stored at 4°C during the assay period. The buccal swab was obtained in accordance with FBI Institutional Review Board guidelines and following informed consent.

qPCR Standard

The dsT8sig qPCR standard consisted of two complementary, PAGE-purified synthetic oligonucleotides (Ultramers™; Integrated DNA Technologies, Coralville, IA) shown in Table 1 that corresponds to positions 13,283–13,397 of the mtDNA revised Cambridge Reference Sequence (CRS; gi_115315570), that is, the target sequence plus five additional base pairs at both the 5′ and 3′ ends. For quality control, the standard also contained a signature or marker sequence, TAACGA, at positions 13,345–13,350. A similar standard, dsT8, utilized for assay specificity experiments was identical to dsT8sig except for the signature sequence and additional end base pairs.

Paired forward and reverse oligonucleotides were separately reconstituted in tris EDTA (TE) buffer (10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA), quantified by absorbance at 260 nm, and adjusted to 2 μM using the extinction coefficients 1,082,000 and 1,138,100 L/(mole·cm), respectively. The adjusted oligonucleotides were mixed in equal proportions to generate a 1 μM double-stranded, primary standard stock. The primary stock was diluted with TE buffer to generate a secondary standard stock containing 10^{10} copies of mtDNA per 2 μL, and the secondary stock was diluted serially with TE buffer to generate the following assay dilution series: 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 copies per 2 μL. Primary and secondary standards were aliquoted and stored at −80°C. The assay dilution series was prepared fresh daily.

TABLE 1—*Sequences of DNA standard, primers, and probe.*

qPCR standard (forward strand)
Tfor8sig : 5′-CAA TCG GCA TCA ACC AAC CAC ACC TAG CAT TCC TGC ACA TCT GTA CCC ACG CCT TCT TCA AAT AAC GAC TAT TTA TGT GCT CCG GGT CCA TCA TCC ACA ACC TTA ACA ATG AAC A-3′
qPCR standard (reverse strand)
Trev8sig : 5′-TGT TCA TTG TTA AGG TTG TGG ATG ATG GAC CCG GAG CAC ATA AAT AGT CGT TAT TTG AAG AAG GCG TGG GTA CAG ATG TGC AGG AAT GCT AGG TGT GGT TGG TTG ATG CCG ATT G-3′
Forward primer
Qfor8 : GGC ATC AAC CAA CCA CAC CTA
Reverse primer
Qrev8 : ATT GTT AAG GTT GTG GAT GAT GGA
Probe
QRL8 : 6FAM CAT TCC TGC ACA TCT G MGBNFQ

qPCR, quantitative PCR; MGBNFQ, minor groove binder nonfluorescent quencher.

Real-time qPCR

The mtDNA region amplified, or target sequence, corresponds to positions 13,288–12,392 of the revised CRS. qPCR experiments were conducted with 2 μL of sample DNA, standard DNA, or control reagents (described below) in a 25-μl duplex reaction containing 12.5 μL of TaqMan 2× Fast Universal Master Mix, no UNG (Applied Biosystems, Foster City, CA), 900 nM HPLC-purified forward and reverse primers (Integrated DNA Technologies), and 250 nM HPLC-purified 5′ 6FAM-labeled probe containing a 3′ minor groove binder nonfluorescent quencher (Applied Biosystems). The qPCR reaction also included the TaqMan Exogenous Internal Positive Control Reagents (Applied Biosystems) according to the manufacturer's recommendations, except for the blocking reagent which in control wells contained the Exogenous IPC Blocking Reagent at a final concentration of 0.8× instead of the recommended 1×. Additional control reactions consisted of water no-template controls (NTCs) and TE NTCs. Primer and probe sequences are shown in Table 1.

Reactions were amplified in duplicate, unless otherwise noted, on a 7900HT FAST Sequence Detection System (Applied Biosystems) in "Fast" mode: 20 sec at 95°C followed by 40 cycles of 1 sec at 95°C and 20 sec at 60°C. Data were analyzed for both the mtDNA- and IPC-specific probes with Sequence Detection Software (SDS) version 2.2.2 (Applied Biosystems). Cycle threshold (Ct) values were determined at 0.2 ΔRn using the automatic baseline algorithm. qPCR amplification efficiencies were calculated using the slope of the standard plot regression line: efficiency = $[10^{(-1/\text{slope})}] - 1$.

Exogenous IPC Analysis

Analysis of qPCR inhibition was qualitative. Uninhibited samples were defined as those which had IPC Ct values comparable to that of the NTCs within a given assay whereas highly inhibited samples had "undetectable" Cts. Partially inhibited samples were defined as those having Cts higher than the NTCs. A controlled inhibition experiment was conducted using twofold serial dilutions of EDTA (Sigma-Aldrich, Inc., St. Louis, MO) varying in final concentration from 10 to 0.078 mM per well.

mtDNA Hypervariable Region PCR

Tenfold serial dilutions of human DNA (Zyagen Laboratories) and HL60 DNA (ATCC) were subjected to HVR amplification in duplicate as follows. HV1 amplification: 10 μL of DNA in a reaction mixture containing 5 units AmpliTaq Gold DNA polymerase (Applied Biosystems), 1× GeneAmp PCR buffer (Applied Biosystems), 160 ng/μL BSA (#A3550; Sigma), dNTP mixture (dATP, dCTP, dGTP, and dTTP, 250 μM each), 600 nM forward primer (CAC CAT TAG CAC CCA AAG CT), and 600 nM reverse primer (GAG GAT GGT GGT CAA GGG AC). HV2 amplification reaction mixtures were performed similarly except for the forward primer, CTC ACG GGA GCT CTC CAT GC, and the reverse primer, CTG TTA AAA GTG CAT ACC GCC. Reaction mixtures were amplified using a 95°C hold for 9 min; 32 cycles consisting of 95°C for 10 sec, 60°C for 30 sec, and 72°C for 30 sec; and a 4°C hold. Amplicons were quantified with the Agilent DNA 1000 kit (Agilent Technologies, Inc., Waldbronn, Germany).

Results

Assay Sensitivity and Reproducibility

Data generated for the highly purified synthetic standard demonstrated consistently high assay sensitivity and reproducibility. Among 15 individual assays, 100,000,000 (10^8) copies of the highly purified standard per well exhibited an average Ct of 13.2 (range 12.5–13.7) (Table 2). The subsequent 10-fold dilution (10^7/well) exhibited a Ct value of 16.6 (range 15.9–17.0). The difference in Ct values between each successive dilution of the standard also averaged about 3.4 cycles. Ten copies of standard per well were always detected at an average Ct of 37.0 (range 35.9–38.2), well within the 40 cycles prescribed for the assay.

High consistency of results and assay reproducibility were also demonstrated by compiling the standard curve results from 15 separate assays (Fig. 1). Furthermore, these assays were conducted over a 7-month period, thereby demonstrating high stability of the qPCR standard when stored as aliquots at −80°C. In addition, these assays were performed by two different analysts on separate instruments, demonstrating that the assay is also highly robust. Table 3 reveals the linear regression data for each of the 15 assays. Of note, mtDNA quantities calculated for a theoretical sample using these regression data sets also reveals high reproducibility of quantification, yielding a coefficient of variation (CV) of 17% among the 15 different assays. These results may be attributable to consistently high amplification efficiencies that ranged from 94.5% to 100.2%.

To examine whether similar assay results would be obtained using different lots of the qPCR standard, three additional sets of the synthetic oligos were obtained and a new standard lot was prepared for each set. The four lots of qPCR standard, which included the original lot, were subjected to qPCR, and a standard curve plot that consisted of all four lots combined was constructed (Fig. 2). These results revealed consistent Ct values among all lots, thereby demonstrating the reliability of the qPCR standard preparations.

TABLE 2—*Standard Ct data among 15 separate quantitative PCR assays.*

Copies/well	Average	Minimum	Maximum	Range
10^8	13.2	12.5	13.7	1.2
10^7	16.6	15.9	17.0	1.1
10^6	20.0	19.3	20.4	1.1
10^5	23.5	22.9	23.9	0.9
10^4	26.9	26.1	27.3	1.2
10^3	30.4	29.5	30.9	1.5
10^2	33.6	32.4	34.1	1.7
10^1	37.0	35.9	38.2	2.3
10^0*	40.5	39.5	41.0	1.5

*Represents Y-intercept data. Ct, cycle threshold.

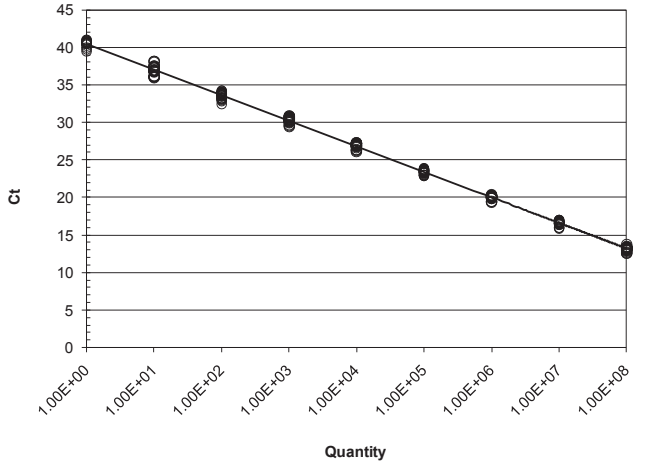

FIG. 1—*Reproducibility of standard curve. The plot represents a compilation of cycle threshold (Ct) data from 15 separate assays in which each standard was assayed in duplicate or more over a 7-month period. Nine of the assays were conducted by one analyst and six of the assays were conducted by a second analyst using a different instrument. The trend line representing the average Ct values among all assays is shown (R^2 = 0.9999). Overall efficiency was calculated to be 96.5% using the slope of this line. The ranges for individual standard quantities varied from 0.9 Ct (10^5 copies) to 2.3 Ct (10^1 copies). Standard quantities of 10^0 represent* Y*-intercepts of the trend lines for each of the 15 assays.*

TABLE 3—*Linear regression data among 15 separate quantitative PCR assays.*

Run	Slope	*Y*-intercept	R^2	Efficiency	Calc. Qty.*
1	−3.35	39.49	1.000	98.7%	6.49E+05
2	−3.41	39.98	1.000	96.4%	7.25E+05
3	−3.43	40.58	0.999	95.9%	1.02E+06
4	−3.40	40.33	0.999	96.9%	9.65E+05
5	−3.42	40.48	1.000	96.0%	9.70E+05
6	−3.32	39.77	0.999	100.2%	9.13E+05
7	−3.43	40.78	0.999	95.5%	1.13E+06
8	−3.39	40.54	0.997	97.2%	1.15E+06
9	−3.36	40.61	1.000	98.3%	1.34E+06
10	−3.44	40.79	0.999	95.1%	1.08E+06
11	−3.45	40.73	0.998	94.7%	9.99E+05
12	−3.41	40.68	1.000	96.3%	1.14E+06
13	−3.39	40.58	0.998	97.3%	1.19E+06
14	−3.46	40.92	1.000	94.7%	1.13E+06
15	−3.46	40.99	1.000	94.5%	1.15E+06
Average	−3.41	40.48	0.999	96.5%	1.0E+06
Standard deviation	0.04	0.42	0.001	1.6%	1.8E+05
Coefficient of variation	0.012	0.010	0.001	0.017	0.171
Minimum	−3.46	39.49	0.997	94.5%	6.49E+05
Maximum	−3.32	40.99	1.000	100.2%	1.34E+06
Range	0.14	1.50	0.003	5.7%	6.87E+05

*mtDNA quantity calcluated for a theoretical sample exhibiting a cycle threshold (Ct) value of 20.0, which also corresponds to the average Ct for 10^6 copies of standard.

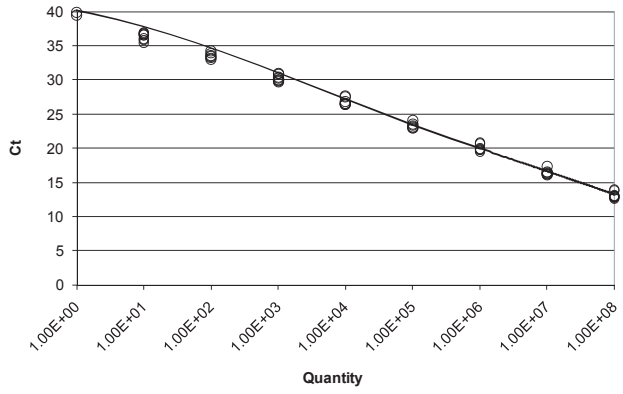

FIG. 2—*Consistency among different quantitative PCR (qPCR) standard lots. Four different lots of synthetic standard were prepared and subjected to qPCR in duplicate, and a standard curve plot consisting of all lots was generated (R^2 = 0.9960, efficiency = 98.6%). The ranges for individual standards varied from 1.2 Ct (10^3 copies) to 1.5 Ct (10^1 copies). Standard quantities of 10^0 represent the* Y*-intercepts of the trend lines for each of the four standards.*

Finally, assay reproducibility was also verified for an evidentiary-type specimen. Various dilutions of a buccal swab DNA extract were assayed five times over a 13-day period, and the results demonstrated high consistency of data, both within assays and between assays, for most dilutions (Fig. 3). Only those dilutions that initially quantified below 1000 copies displayed higher variability among replicates suggesting mtDNA instability following refrigerated storage of low copy sample. The results obtained for this buccal swab extract indicate an mtDNA yield of 2×10^8 mtDNA copies per swab. However, this may not be representative for all buccal swabs because variation among individuals and differences swab collection technique will likely affect yield.

IPC Analysis

The described mtDNA-specific qPCR assay also involves the amplification of an IPC to determine whether PCR inhibitors may be present within a DNA sample. This control includes IPC-specific primers and probe in the qPCR mixture as well as IPC DNA, which should always amplify when PCR inhibitors are absent. The highest level of IPC amplification, and therefore the lowest IPC Ct values, will be observed for the NTC controls that lack PCR inhibitors. In contrast, in the presence of inhibitors, IPC Ct values will increase relative to that for the NTCs or be "undetermined" depending on the level of inhibitor. An analysis with EDTA as inhibitor (Fig. 4) revealed that 0.078 mM EDTA yields a Ct value that is comparable to the Ct value for the NTC (0 mM EDTA) indicating a lack of inhibition. In contrast, a slight increase in Ct (e.g., 0.31 mM EDTA) indicated low-level inhibition, a marked increase in Ct (e.g., 1.25 mM EDTA) indicated a moderate level of inhibition, and Cts that were "undetermined" (e.g., ≥2.5 mM EDTA) suggested a potentially high level of inhibition. A similar response was also observed using the exogenous IPC blocking reagent as inhibitor (data not shown).

IPC Ct values can also increase in the absence of inhibitors when high levels of sample mtDNA compete for polymerase and dNTPs during amplification, termed "inhibition by competition." This phenomenon is readily observed with the inhibitor-free standards where the IPC Ct is slightly increased with 10^4 mtDNA standard copies per well (Fig. 5). At 10^5 copies of standard, the IPC Ct becomes "undetermined," and at 10^6 copies, IPC amplification appears indistinguishable from background. Inhibition by competition is an unavoidable consequence of the relatively low quantities of IPC DNA within the qPCR mixture. Nonetheless, IPC analysis is informative and remains valid for any sample that contains $\leq 10^4$ copies of mtDNA per well. Detection of inhibition in such samples would suggest that further sample purification might be warranted. For samples that contain $>10^4$ copies of mtDNA per well, no determination may be made regarding the presence of PCR inhibitors; however, given the high level of mtDNA in such samples, successful analysis is probable without repurification.

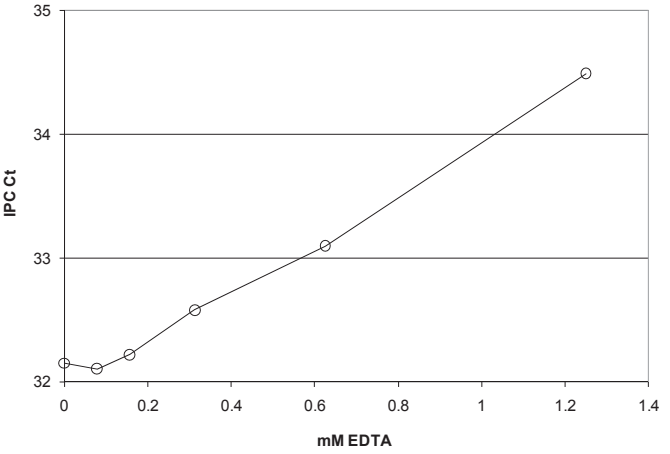

FIG. 4—*Inhibition of internal positive control (IPC) signal by EDTA. A stock EDTA solution was twofold serially diluted and added to wells in quadruplicate to yield final concentrations ranging from 10 to 0.078 mM. Average cycle threshold (Ct) values are shown, and Cts for final EDTA concentrations ≥2.5 mM were undetermined.*

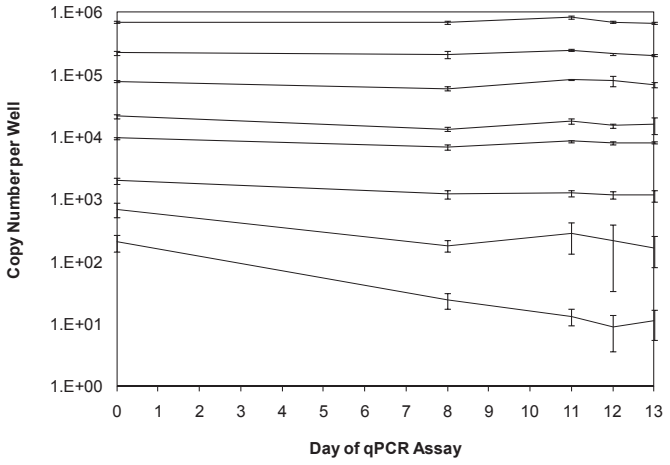

FIG. 3—*mtDNA quantification of buccal swab DNA extracts. Eight different dilutions (1/2.5–1/13,000) of a buccal swab extract were subjected to quantitative PCR (qPCR) (day 0). The dilutions were assayed again after 8, 11, 12, and 13 days of refrigerated storage. Each line represents the results for a single dilution; top to bottom represents the smallest to highest dilution. Error bars represent the standard deviation of eight replicates.*

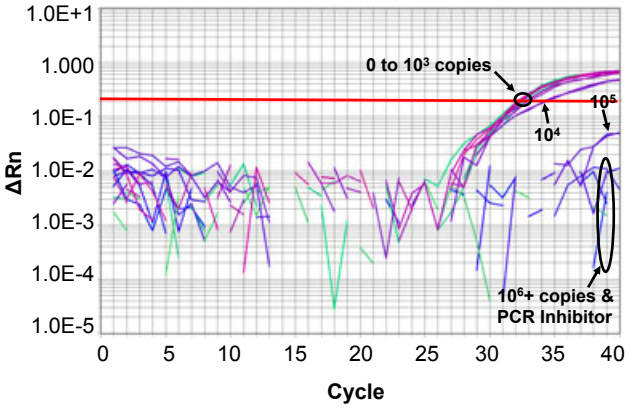

FIG. 5—*Internal positive control (IPC). A typical amplification plot for the exogenous IPC is shown for the quantitative PCR (qPCR) standard and controls. The plot illustrates that the lowest IPC cycle threshold (Ct) values are obtained from zero copies, that is, "no-template" controls, to 10^3 copies of standard per well. However, IPC amplification is completely inhibited in the presence of PCR inhibitor (e.g., IPC blocking reagent) or when $\geq 10^5$ copies of qPCR standard are present in a well, the latter representing "inhibition by competition." 10^4 Copies of the standard also inhibit IPC amplification by this mechanism, though only slightly. The red horizontal line depicts the 0.2 ΔRn threshold.*

Species Specificity

By nature, forensic DNA evidence may contain a mixture of human DNA and contaminating DNA from nonhuman sources. Furthermore, it can be difficult to determine whether small pieces of recovered skeletal remains are from a human or animal source. The qPCR primers and probe can potentially bind to and amplify nonhuman mtDNA, thus resulting in inaccurate yield determinations that may impact downstream analysis. Therefore, one key criterion in selecting a qPCR target sequence was low homology with nonhuman mammalian DNA. Several potential qPCR sequence target sites in the human mtDNA genome were compared with the mtDNA genomes of 16 domesticated and wild mammalian species, which could pose as environmental contaminants and were evaluated for sequence homology (26). A sequence within the NADH dehydrogenase subunit 5 gene exhibited relatively low homology and was selected as the qPCR target site (data not shown).

Specificity for this target site was demonstrated by assaying 100 ng of DNA from 19 mammalian species as well as DNA from nonmammalian vertebrates, bacteria, and fungi. The results for the nonhuman species were compared with that obtained for 100 ng of commercially prepared human DNA (Table 4). This approach assumed that all nonhuman DNA was free of human DNA and vice versa, and that the ratio of mtDNA to total DNA was equivalent for all species. Slight cross-reactivity was apparent for 19 of the 30 species examined with the greatest for cynomolgus monkey DNA that was quantified at a level representing 1% that of human DNA. At this level, an improbable level of contaminating DNA, 100-fold more, would be required to attain a signal equivalent to human DNA. Two additional primate species DNA, rhesus monkey and baboon, exhibited cross-reactivity approximately 10-fold lower than the cynomolgus monkey. Domesticated and wildlife species, often encountered with recovered human remains, did not show appreciable reactivity in the assay and virtually no cross-reactivity was observed among the bacterial and fungal species examined. Nonetheless, it is important to note that the assay does not distinguish between quantities of human mtDNA and quantities of nonhuman mtDNA within a DNA sample. However, given the high specificity of the assay, human mtDNA quantification should be virtually unaffected by the presence of nonhuman DNA contamination.

mtDNA Copy Number Required for Forensic mtDNA Sequence Analysis

The principal application of the mtDNA-specific qPCR assay is the conservation of forensic DNA samples to allow for additional analysis or reanalysis. In this respect, the optimal number of mtDNA copies needed for HVR amplification was determined.

TABLE 4—*Quantitative PCR cross-reactivity with nonhuman DNA.*

DNA Source	mtDNA Quantified per 100 ng of DNA*	% of Human
Human	1,900,000	100
Cynomolgus monkey	18,000	0.9
Cow	7800	0.4
Guinea pig	1200	0.1
Rhesus monkey	490	0.03
Baboon	140	0.01

*The following organisms were quantified at 35 copies or less per 100 ng DNA (<0.002% of human): chicken, deer, dog, horse, ferret, fish, gerbil, hamster, mouse, rabbit, rat, sheep, turkey, and *Candida albicans*. mtDNA was not detected in DNA from cat, donkey, goat, pig, *Aspergillus oryzae*, *Bacillus subtilis*, *Clostridium perfringens*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus epidermis*, and *Streptococcus pneumonia*.

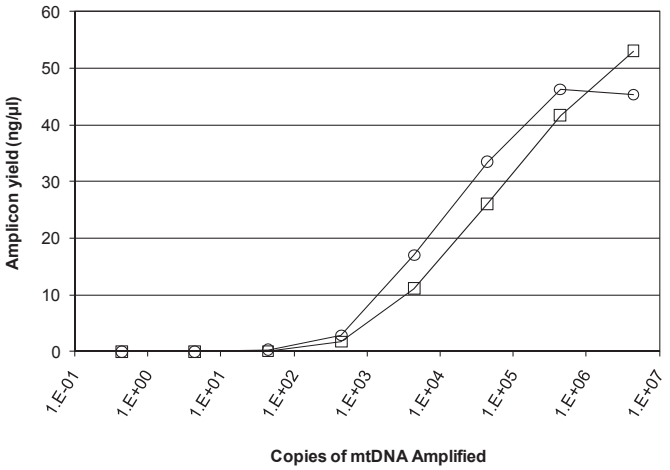

FIG. 6—*Hypervariable region amplicon yield from mtDNA. A commercially available human genomic DNA preparation was quantified using the quantitative PCR assay, and dilutions of the preparation were subjected to HV1 (circles) and HV2 (squares) amplification in duplicate. Duplicate values were consistent with each other and average values were plotted.*

Commercially available human DNA purified from normal cells was subjected to HV1 and HV2 amplification and to mtDNA-specific qPCR. The results revealed that 10 pg of human genomic DNA (440 mtDNA copies) subjected to HV1 and HV2 amplification yielded 3 and 2 ng/µL of amplicon, respectively, concentrations that are sufficient for sequence analysis (Fig. 6). These results were corroborated using HL60 cell line DNA in which 180 copies (1 pg) yielded 2 and 1 ng/µL of amplicon, respectively (data not shown). Based on these data, *c*. 300 copies of mtDNA may be sufficient for HV1 and HV2 amplification and subsequent sequence analysis. This amount of mtDNA also approximates the number of mtDNA copies present within a single human cell (5), suggesting that forensic mtDNA analysis may be possible on the total DNA from a single cell, assuming 100% yield, no DNA degradation, and the absence of PCR inhibitors. That is, the number of mtDNA copies required for analysis may be substantially higher for DNA isolated from suboptimal forensic specimens. Nonetheless, the described results support a rational dilution scheme for forensic mtDNA and demonstrate the utility of mtDNA-specific qPCR for sample conservation.

Discussion

Knowledge of the quantity and quality of mtDNA for a given DNA sample prior to commencement of forensic mtDNA analyses could increase the likelihood of successful analysis, improve the efficiency of analysis by indicating the need for sample repurification, and help conserve sample for potential additional analyses. The described qPCR assay was developed to quantify mtDNA with a high degree of sensitivity and specificity.

Current forensic human mtDNA sequence analysis types the HVRs located in the mtDNA control region. To preclude the possibility of the qPCR standard becoming a laboratory contaminant for these analyses, the region targeted for real-time amplification was within the NADH dehydrogenase subunit 5 gene that resides in the mtDNA coding region. However, future mtDNA analyses may expand to include sequence variation in such coding regions in an effort to increase the discriminatory power of forensic analysis. The NADH-5 region which corresponds to the qPCR standard contains 17 known polymorphisms to date (11,12). Any of these

polymorphisms could be used in future forensic mtDNA analyses, in which case the qPCR standard would be a potential contaminant. To address this possibility, the qPCR standard was designed with a "signature" or marker sequence, which, if translated, would encode a stop codon and a Met → Arg substitution, an unlikely occurrence in human mtDNA. This marker sequence would readily be identified upon sequencing or via the recently described mass spectrometry base composition method (27,28). Additionally, the marker sequence is located between the probe- and reverse primer–binding sites and does not affect qPCR amplification efficiency (data not shown). The qPCR target region also possesses minimal sequence homology to the mtDNA of other forensically relevant species, and the small amplicon size (105 base pairs) facilitates amplification even for degraded samples. Finally, the primers and probe hybridize to a region that has, to date, relatively few sequence polymorphisms (11,12), thus maximizing the sequence types which can be amplified.

The qPCR standard includes the target sequence amplified and consists of a mixture of two synthetic oligos that are readily available from a commercial source. Although not unique (29,30), this type of standard offers high yield, is easily obtained, and is cost-effective compared with the construction and production of traditional plasmid-based DNA standards. More importantly, a synthetic standard offers high quality assurance/quality control (QA/QC) value because it is highly pure and free of contaminating DNA, RNA, and protein, which could affect its absorbance at 260 nm and result in inaccurate quantification of the standard itself. Additional QA/QC measures include a signature sequence for ready identification as well as five additional mtDNA base pairs beyond the primer-binding sites to eliminate qPCR quantification inaccuracies, which may arise because of residual n-1, n-2, etc., oligo-products. In contrast to a synthetic standard, experiments involving a plasmid-based standard suggested that DNA supercoiling may result in inefficient amplification and significantly reduced Ct values for the plasmid standards, resulting in a *c*. 50-fold overestimation of copy numbers for samples that are not supercoiled (data not shown). This effect could be due to ineffective annealing of primer and probe because of topological constraints (22,31). While this effect may be overcome by nicking or linearization of the plasmid standard followed by repurification, such steps are not required for linear synthetic standards.

Exogenous IPC analysis can provide helpful information regarding the presence of PCR inhibitors in a DNA sample, which can suggest the need for further sample purification. However, care should be exercised when interpreting the IPC results because the described qPCR assay represents a duplex assay and involves the amplification of both mtDNA and IPC DNA. In this regard, mtDNA amplification is optimized to attain a wide dynamic range of quantification for both low and high copy samples whereas IPC amplification is not. Accordingly, samples containing a large number of mtDNA copies can readily deplete the polymerase and dNTPs and thus adversely inhibit IPC amplification. Therefore, an interpretation that suggests the presence of inhibitors could be erroneous, because of pseudo-inhibition or "inhibition by competition." Indeed, this phenomenon is always observed for this assay when assaying large quantities of the highly purified and inhibitor-free qPCR standards: beginning with 10^4 copies of standard per well, IPC Cts are slightly elevated; however, with 10^5 copies, IPC Cts become undetectable (Fig. 5). Therefore, among DNA samples that are assayed to contain $>10^4$ copies per well, no interpretation may be made regarding the presence or absence of inhibitors. However, at such quantities of mtDNA, knowledge of inhibitor status is of lesser importance because diluting a DNA sample by 100-fold or more to achieve conservation will also likely dilute inhibitors, if present, to subinhibitory levels. While others have suggested a quantitative approach to IPC analysis (32), the interpretation algorithm suggested here, namely consideration of repurification for samples that indicate inhibition and quantify at $<10^4$ copies of mtDNA per well, should be adequate for avoiding failed analyses while conserving sample, labor, and time.

The assay presented herein was found to exhibit consistently high amplification efficiencies as well as high reproducibility over time (Table 3). Linear regression data for each of 15 independent assays were used to generate quantification results for a theoretical sample. The calculated values were found to be highly consistent, exhibiting a CV of 17% (Table 3). This value was comparable to, or more favorable than, interassay CVs reported for alternative qPCR assays described elsewhere (16,18,23). One alternative assay yielded lower CV values (19), however, these were calculated among five independent assays.

Whereas the qPCR assay is described for a specific instrument, it may be nonetheless easily adapted for any real-time qPCR system which can detect the 6FAM, VIC, TAMRA, and ROX dyes. Furthermore, the 6FAM reporter dye may also be substituted by a dye that is compatible with the other dyes. The assay also utilizes a "fast" qPCR amplification protocol, which results in a rapid run time of 40 min; however, simple modifications would permit "standard" amplification in instruments that are not equipped for fast qPCR. Additional assay features include minimal sample consumption (2 μL) and an IPC to detect PCR inhibitors. In conclusion, the qPCR assay described is suitable for accurate and precise mtDNA quantification in DNA samples prior to forensic analysis and is reliable, robust, and highly sensitive. In addition, the assay may also be utilized in biomedical applications that demand similar characteristics.

Note Added Following Acceptance

Since completion of the manuscript, subsequent standard Ct values have averaged 1 cycle less than the average Ct values reported in Table 2. In addition, the Y-intercept value has averaged 1.6 cycles less than that reported in Table 3. Applied Biosystems (Foster City, CA), manufacturer of the probe and other qPCR reagents, indicated that the cause of these shifts was likely due to a brighter probe reporter dye acquired from a new supplier of amidite dyes. The manufacturer also reported that other users of their probes have experienced similar shifts in Ct values subsequent to the amidite supplier change.

In contrast to the average Ct and Y-intercept shifts, recent average slope, R^2, and efficiency values were virtually identical to the respective averages reported in Table 3. This observation suggests that the quality, *vis a vis* sensitivity and reproducibility, of the recent standard curves was unaffected by the observed Ct shifts. Further evaluation of our recently acquired assay data supports this assertion.

As demonstrated by our latest results, Ct values can readily be affected by assay reagents as well as by an instrument's optical system (33). In this regard, it would be prudent to document reagent lots as well as instrument maintenance events for each assay to aid in evaluating any significant or substantial assay-to-assay deviations in slope, R^2, efficiency, Y-intercept, Ct values, and Ct ranges.

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