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A Duplex Real-Time qPCR Assay for the Quantification of Human Nuclear and Mitochondrial DNA in Forensic Samples: Implications for Quantifying DNA in Degraded Samples*

ABSTRACT: A duplex real-time qPCR assay was developed for quantifying human nuclear and mitochondrial DNA in forensic samples. The nuclear portion of the assay utilized amplification of a ~170–190 bp target sequence that spans the repeat region of the TH01 STR locus, and the mitochondrial portion of the assay utilized amplification of a 69 bp target sequence in the *ND1* region. Validation studies, performed on an ABI 7000 SDS instrument using TaqMan® detection, demonstrated that both portions of the duplex assay provide suitable quantification sensitivity and precision down to 10–15 copies of each genome of interest and that neither portion shows cross-reactivity to commonly encountered non-human genomes. As part of the validation studies, a series of DNase-degraded samples were quantified using three different methods: the duplex nuclear-mitochondrial qPCR assay, the ABI Quantifiler™ Human DNA Quantification Kit qPCR assay, which amplifies and detects a 62 bp nuclear target sequence, and slot blot hybridization. For non-degraded and moderately degraded samples in the series, all three methods were suitably accurate for quantifying nuclear DNA to achieve successful STR amplifications to yield complete profiles using the ABI AmpF/STR® Identifiler™ kit. However, for highly degraded samples, the duplex qPCR assay provided better estimates of nuclear template for STR amplification than did either the commercial qPCR assay, which overestimated the quantity of STR-sized DNA fragments, leading to an increased proportion of undetected alleles at the larger STR loci, or slot blot hybridization, which underestimated the quantity of nuclear DNA, leading to an increased proportion of STR amplification artifacts due to amplification of excess template.

KEYWORDS: forensic sciences, DNA quantitation, quantitative polymerase chain reaction, degraded DNA, TH01, ND1, melt transition

The need to increase throughput in forensic DNA genotyping has led to a growing interest in developing new DNA quantification methods that are more efficient than the slot blot hybridization method currently used in many forensic DNA labs (1). The slot blot method, though it offers excellent specificity and good sensitivity (2,3), relies on a protocol that is time-consuming, labor-intensive, and not readily transferable to automation. In response, there have been a number of recent publications that describe alternative approaches for quantifying DNA in forensic samples, including a liquid hybridization assay (4,5) and several end-point PCR assays (6–8). Another approach that is proving to be useful for forensic DNA quantifications is real-time quantitative PCR (qPCR), a method widely used in biomedical research and molecular diagnostics (9–13). Quantitative PCR assays have been developed for various forensic applications, including the quantification of human nuclear DNA (14–16), human mitochondrial DNA (17–19), and human Y-chromosomal DNA (16,19). These assays exploit features of qPCR that make the technique particularly attractive for forensic applications: (i) qPCR assays can be designed to quantify specific genomes of interest; (ii) the assays can be sensitive enough to detect only a few copies or even a single copy of target DNA; (iii) qPCR dynamic detection ranges readily span the roughly three orders of magnitude (e.g., 30 pg to 30 ng of nuclear DNA) needed for most forensic applications; and (iv) the experimental protocols for real-time qPCR quantitations are straightforward, labor-saving, and amenable to automation. Moreover, the use of target-specific detection chemistries (e.g., TaqMan® (20) or Molecular Beacon (21) probes) makes it possible to design multiplex, real-time qPCR assays that can simultaneously quantify more than one target in a sample, offering the possibility for saving time, labor, and extracted DNA.

In this report, we describe a duplex real-time qPCR assay for the simultaneous quantification of human nuclear and mitochondrial DNA in forensic samples. This assay was designed to be of general utility for forensic DNA quantifications, but to be particularly useful for the post-extraction analysis of samples that contain highly degraded DNA. Such samples, though not uncommon in standard casework, are more often encountered in instances of mass disasters, mass graves, and missing persons' cases (22,23). Short tandem repeat (STR) genotyping, due to its high power of discrimination for human identification, is typically the analytical method of first choice. However, the quality and/or quantity of extracted nuclear DNA in these degraded samples often preclude successful STR genotyping, resulting in partial or no STR profiles. Such samples can then be analyzed by less discriminatory typing methods based

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on polymorphisms in hypervariable subregions I and II (HVI and HVII) of the human mitochondrial control region. Presently, the initial decision as to how to proceed with analysis, either by nuclear STR or mitochondrial typing, is commonly based on a slot blot quantification approach that: (i) has been reported to underestimate the quantity of nuclear DNA in degraded samples (24) and in samples that contain high levels of microbial contamination (23); (ii) provides no information about the quality (fragment length) of the quantified nuclear DNA; (iii) provides no direct information about the quantity of human mitochondrial DNA in the sample; and (iv) has no predictive information on the existence of inhibitors that might interfere with the PCR. Due to these quantification deficiencies, the actual forensic analysis of challenging samples often begins by obtaining inadequate STR typing results, and then proceeds by using any remaining extracted DNA to attempt mitochondrial typing. It has been noted previously (17) that the efficiency and quality of this analysis procedure could be improved substantially by obtaining reliable estimates of the amounts of human nuclear and mitochondrial DNA in these samples prior to beginning analysis. Based on such estimates, an optimal analytical approach could be selected at the outset, leading directly to optimal genotyping or haplotyping results and to a concomitant savings in time, labor, reagent/kit costs and extracted DNA.

Several recent publications have addressed exactly these issues for forensic samples, particularly for degraded samples. For example, von Wurmb-Schwark et al. (25) have developed a duplex endpoint PCR assay to detect a nuclear target (164 bp amplicon at betaglobin) and a mitochondrial target (260 bp at *ND1*). In their assay, the endpoint PCR products were resolved and detected by capillary electrophoresis (CE)/fluorescence and, though the results were not claimed to be quantitative, the intensities of the nuclear and mitochondrial signals were shown to be useful for selecting the appropriate forensic analysis tool for the typing of anthropological bone samples. Alonso et al. (19,26) have developed several qPCR assays for degraded and challenging samples, including a duplex assay that simultaneously quantifies human X- and Y-chromosomes (106 bp and 112 bp targets at the amelogenin gene). They have also developed two singleplex qPCR assays for quantifying the human mitochondrial genome, one with a 113 bp HVI target and the other with a 287 bp HVI target. Due to the size difference between the two target sequences, they demonstrated that a comparison of the quantification results from these mitochondrial qPCR assays could provide information about the degree of DNA degradation. Lastly, Andreasson et al. (17) have described a nuclear-mitochondrial duplex qPCR assay. The nuclear portion of this assay quantifies a 79 bp target at the retinoblastoma (*RB1*) gene, and the mitochondrial portion quantifies a 135 bp target spanning the junction of the $tRNA^{lys}$ and *ATP8* genes. Their results demonstrated that the duplex qPCR approach provides useful and sensitive DNA quantifications while saving analyst time and often-limited DNA. It has been noted elsewhere (1), however, that the choice of *RB1* as a qPCR target sequence may not be ideal because cross-species sequence homology investigations indicate that the nuclear *RB1* target sequence is relatively conserved. Consequently, though this assay can be expected to provide accurate results for forensic samples of known human origin, it has not been demonstrated to be sufficiently primate-specific to be of general forensic utility.

We describe here a new nuclear-mitochondrial duplex qPCR assay developed following the same general approach of Andreasson et al. (17), except that we have chosen alternative nuclear and mitochondrial target sequences for amplification and quantification. For the nuclear portion of the qPCR assay, we have chosen a target sequence that spans the repeat region of the primate-specific TH01 STR locus, a locus that has been used widely for forensic applications. This target sequence is of direct interest for quantification, considering that the primary reason for quantifying human nuclear DNA in forensic samples is to determine the amount of extract to amplify with a commercial multiplex STR PCR kit (8). Our results indicate that for degraded samples our choice of the relatively long TH01 target sequence (~170–190 bp) leads to improved STR typing results, compared to results based on quantification of a short target sequence (e.g., 62 bp in the Applied Biosystems Quantifiler™ qPCR kit (16)) or via slot blot hybridization. For the mitochondrial portion the assay, we selected a relatively short target sequence (69 bp) in the mitochondrial *ND1* gene. This selection provides a sensitive means for determining the presence of human mitochondrial DNA, degraded or not, in forensic samples. In addition to describing results on quantifying DNA in degraded samples, we also cover aspects of development of the duplex qPCR assay, and details from forensic validation studies, including studies of precision, reproducibility, sensitivity, species specificity, and applications to casework-type samples.

Materials and Methods

Standards and Samples

Pre-quantified, high molecular weight, human genomic DNA extracts obtained from Promega (Female-#G1521) and from Applied Biosystems (TaqMan® Control DNA) were typically used as qPCR quantification standards. Pre-quantified DNA extracts obtained from Applied Biosystems (genomic DNA standards from the Quantifiler™ and QuantiBlot™ kits), Promega (K562 and Male-#G1471), and ATCC (HL60) were also used as control samples in several studies.

Unless otherwise indicated, DNA extracts from reference, nonprobative and simulated casework samples were obtained using the California Department of Justice casework organic extraction protocol (ProK/SDS digestion, phenol/chloroform extraction, then Centricon100 (Millipore) concentration into Tris-EDTA buffer (TE^{-4})). For simulated sexual assault samples, differential extractions of sperm and epithelial fractions were performed using a DTT-based digestion protocol (27). For non-root hair shafts (2 cm portions), extractions were performed by either (i) a magnetic-bead-based protocol (28) or (ii) a protocol in which a tissue grinder was first used to homogenize the hair shaft with 150 μL TE^{-4}, after which the homogenate was extracted into 50 μL of boiling 20% Chelex (Bio-Rad). DNA extracts were stored at −20°C, except for Chelex-extracted samples, which were stored at +4°C.

For degradation studies, DNA samples were fragmented by treatment with DNase I (Invitrogen). Separate tubes of high molecular weight HL60 DNA, each tube containing 1.4 μg of DNA in 5 μL TE^{-4}, were treated with 0.5 U of DNase I, 10x DNase I Reaction Buffer, and sterile water to bring the reaction volume to 10 μL. Increasing degrees of fragmentation were achieved by allowing the tubes to digest at room temperature for periods of 1, 2, 3, 4, 5, 15, 30, 45, 60 min and overnight. At the end of each digestion period, DNase activity was quenched by adding 2 μL of 25 mM EDTA and heating each tube at 65°C for 15 min. The samples were not further purified or concentrated. A "0 min" sample of intact DNA which contained all reaction components for digestion except DNase I was treated identically to the samples in the degradation series. The degree of DNA fragmentation was assessed by gel electrophoresis (2% agarose, ethidium bromide detection) using HyperLadder I (Bioline) and Ready-Load λ-DNA/Hind III (Invitrogen) size markers.

Non-human DNA samples were purchased as pre-quantified genomic DNA extracts from several vendors: *E. coli*, *C. perfringens* (Sigma), *B. subtilis*, *S. epidermidis*, *C. albicans* (ATCC), mouse (Promega), and cat, chicken, cow, dog, fish, horse, monkey, pig, rat (Zyagen Labs, San Diego, CA).

STR Genotyping

The AmpF*l*STR® Identifiler™ PCR Amplification kit (Applied Biosystems) was used for STR genotyping. PCR amplifications of 1 ng of nuclear DNA in a 25 μL reaction volume were performed according to vendor instructions on a GeneAmp® 9700 PCR thermocycler (Applied Biosystems). STRs were resolved and detected on a Prism® 3100 Genetic Analyzer (Applied Biosystems) according to vendor instructions, except that the electrokinetic sample injection time was dropped from the default of 10 seconds to 5 seconds, and the data were analyzed with a baseline of 35 rather than the default of 51. Alleles were identified at a minimum threshold of 100 RFU using GeneScan® (v.3.7.1/NT) and Genotyper® (v.3.7/NT) (Applied Biosystems) for data analysis.

Mitochondrial HVI/HVII PCR

The HVI and HVII portions of the mitochondrial DNA control region were amplified in a duplex PCR using reagents from the LINEAR ARRAY™ Mitochondrial DNA HVI/HVII Region-Sequence Typing Kit (Roche Applied Science) (29,30). This kit produces nominal 444 bp (HVI) and 416 bp (HVII) amplicons. Template quantities for PCR were determined either from our nuTH01 duplex qPCR assay (using 100 pg of nuclear DNA per duplex HVI/HVII amplification) or from our mtND1 duplex qPCR assay to quantify the mitochondrial genome (using ~14,000 mitochondrial copies per duplex HVI/HVII amplification). Post-amplification yields and purities of the HVI and HVII PCR products were assessed by gel electrophoresis (4% NuSieve 3:1 (FMC) agarose gel, ethidium bromide staining) of 5 μL of PCR product using Low DNA Mass Ladder (Invitrogen) as a size and quantity marker.

Slot Blot Quantification

The QuantiBlot™ Human DNA Quantification Kit (Applied Biosystems) was used according to vendor instructions. Hybridized probes were detected by chemiluminescence (SuperSignal West Femto (Pierce)) using a CCD camera system (CCDBio 16SC (Hitachi/MiraiBio)). CCD data were analyzed semi-automatically using SlotQuant software running under the GeneTools (SynGene) analysis package. Quantification standards (DNA Standard A from the kit) ranged from 20 ng to 25 pg for each run.

Quantifiler™ qPCR Quantification

The Quantifiler™ Human DNA Quantification Kit (Applied Biosystems), hereafter referred to as "Quantifiler™ qPCR," was used according to vendor instructions for data collection on an Applied Biosystems 7000 Prism® SDS qPCR instrument.

qPCR Assay Design

Primers and probes (Table 1) for the nuTH01 TaqMan® and mtND1 TaqManMGB® (MGB = Minor Groove Binder) singleplex qPCR assays were designed using Applied Biosystems' PrimerExpress™ v2.0 software. In general, design guidelines were followed as recommended by Applied Biosystems (31), although the software settings in PrimerExpress™ were frequently relaxed to allow amplicon lengths to exceed the recommended maximum length of 150 bp. DNA sequences for design work were downloaded from the GenBank resource at the National Center for Biotechnology Information (NCBI) website (32). Sequence information was also obtained from the STRBase (33) and MitoMap (34) websites.

TABLE 1—*Oligonucleotide sequences for nuTH01 primers and probe, and for mtND1 primers, probe, and mitochondrial copy number standard.*

Oligonucleotide	Sequence {5′ → 3′}
nuTH01-F	AGG GTA TCT GGG CTC TGG
nuTH01-R	GGC TGA AAA GCT CCC GAT TAT
nuTH01-probe	*FAM*-ATT CCC ATT GGC CTG TTC CTC CCT T-*BHQ*
mtND1-F	CCC TAA AAC CCG CCA CAT CT
mtND1-R	GAG CGA TGG TGA GAG CTA AGG T
mtND1-probe	*VIC*-CCA TCA CCC TCT ACA TC-*MGB-NFQ*
mtND1-standard	GAG CGA TGG TGA GAG CTA AGG TCG GGG CGG TGA TGT AGA GGG TGA TGG TAG ATG TGG CGG GTT TTA GGG

The nuclear qPCR assay (nuTH01) was designed to span the STR sequence at the human tyrosine hydroxylase (*TH01*) gene on chromosome 11 (11p15.5) using sequence from GenBank locus AF536811. In this design work, we configured PrimerExpress™ to fix the positions of the 3′ ends of the qPCR primers so as to match known or deduced primers from commercial STR amplification kits (35,36). This was done so that the qPCR amplifications might more accurately predict quantifications for STR genotyping. The positions of the fluorogenic probe and the 5′ ends of the primers were typically unconstrained for optimization by PrimerExpress™. The mitochondrial qPCR assay (mtND1) was designed in the *ND1* gene of the human mitochondrial genome using sequence from Genbank locus HUMMTCG (37). The *ND1* gene expresses subunit 1 of the mitochondrial NADH dehydrogenase protein.

For both the nuclear and mitochondrial assays, prior to ordering any synthetic oligonucleotides for experimental work, potential primer and probe sequences were compared to DNA sequences available through the NCBI website by using the Basic Local Alignment Search Tool (BLAST*n*) (38). The purpose of these comparisons was to identify and avoid inadvertent homologies of primers and/or probes to non-target genomes that might lead to undesirable cross-species reactivities. In addition, attempts were made to avoid known single nucleotide polymorphisms (SNPs) in the primer and/or probe sequences. SNP information was obtained from several sources: the NCBI SNPdB website (39), the On-Line Mendelian Inheritance in Man (OMIM) website (40), and, for mitochondrial sequences, the MitoMap website (34). Once developed, singleplex and duplex qPCR assays were optimized based on published recommendations (41,42).

nuTH01-mtND1 qPCR Quantifications

Primer and probe sequences for the nuTH01 and mtND1 qPCR assays are provided in Table 1. For nuTH01-mtND1 duplex qPCR amplifications, each assay was run as a 20 μL amplification that included 10 μL of TaqMan® Universal Master Mix 2X, no UNG (Applied Biosystems), 4 μL of sample, with the remaining 6 μL composed to give final concentrations of: 0.16 μg/μL non-acetylated

BSA (Sigma); 600 nM in each nuTH01 primer; 200 nM in nuTH01-probe; 50 nM in each mtND1 primer; 100 nM in mtND1-probe. Primers (Qiagen-Operon (Alameda, CA)) were diluted in sterile, de-ionized water. Probes (Qiagen-Operon for nuTH01, Applied Biosystems for mtND1) were diluted in TE^{-4}.

For nuTH01 and mtND1 singleplex TaqMan®/TaqManMGB® qPCR amplifications, assays were run using the same reagents as described for the duplex qPCR assay, except to replace the non-desired primer/probe combination with sterile water or TE^{-4}. Singleplex SYBR® Green I assays were typically run as 20 μL amplifications that included 10 μL of 2X SYBR® Green Master Mix (Applied Biosystems) or 10 μL of 2X Brilliant® QPCR Master Mix (Stratagene), 4 μL of sample, with the remaining 6 μL composed to give final concentrations of 300 nM in each appropriate primer.

Real-time qPCR data were collected on an Applied Biosystems Prism® 7000 SDS instrument controlled by a computer running version 1.0 of the 7000 SDS Collection software. The instrument was typically configured for the following run conditions: 20 μL sample volumes; 9600 emulation mode; one 10 min 95°C polymerase activation step, followed by 45 cycles of 2-step qPCR (15 s of 95°C denaturation, 60 s of 60°C combined anneal/extension). Well-to-well variations in background fluorescence were corrected for by use of a ROX-labeled passive reference, included as part of the Applied Biosystems qPCR Master Mix for each sample. For runs that used SYBR® Green detection, a melt curve was collected after the final cycle of PCR extension by configuring the SDS Collection software to monitor SYBR® Green fluorescence as the temperature was increased (~1.8°C /min) from 60°C to 95°C.

Amplification curves were analyzed by using empirically established cycle threshold and baseline settings for each type of assay (for nuTH01, threshold = 0.15, baseline 6–18 cycles; for mtND1, threshold = 0.06, baseline 3–13 cycles). For each qPCR run, the SDS Collection software generated a linear calibration plot of CT (cycle threshold) vs. log C_0 (initial standard DNA concentration) by using amplification results from a freshly prepared dilution series of pre-quantified high molecular weight human genomic DNA standard (Promega Female or Applied Biosystems TaqMan®). DNA quantifications for unknown samples were interpolated from the resulting linear calibration curve. These calibration and interpolation steps are semi-automatic features of the SDS Collection software. For the nuclear qPCR assay, calibration plots were constructed using data from standard DNA dilutions containing 25, 5, 1, 0.5, 0.1, and 0.05 (in duplicate) ng of total DNA per sample. For the mitochondrial qPCR assay, the calibration plots were extended to lower quantities of template per sample (0.010, 0.001, and 0.0001 (in duplicate) ng). At least one negative control, 4 μL of TE^{-4} or sterile water, was included in each run.

Nuclear DNA copy numbers were estimated using the ratio of one haploid nuclear copy per 3.3 pg genomic DNA (43). Mitochondrial copy numbers were estimated using a ratio of 400 mitochondrial copies per 3.3 pg of Promega genomic standard DNA or 450 mitochondrial copies per 3.3 pg of HL60 genomic standard DNA. These mitochondrial copy number ratios were empirically estimated by running the Promega and HL60 standard DNA samples against a dilution series of quantified (UV-vis absorption) mtND1 synthetic oligonucleotide standard (see Table 1 for oligonucleotide sequence of the mitochondrial copy number standard). For each qPCR run, we used the high molecular weight genomic DNA dilution series to generate two linear calibration plots, one for the nuclear portion and one for the mitochondrial portion of the duplex assay.

Where appropriate, qPCR amplification efficiencies were determined from the slopes of the linear calibration curves (% PCR efficiency = $100[(10^{(-1/\text{slope})} - 1)]$ (44).

Oligonucleotide Melting Profile Calculations

Melting profiles for selected TH01 alleles were calculated using MELT94, a DOS-based program available on-line (45). This program uses the theory and equations of Poland (46) and of Fixman and Freire (47) to calculate variations in thermal stability along the sequence of a DNA fragment (48). Stacked melting profiles were constructed by exporting MELT94 output data (temperatures for 50% helical:50% melted states vs. sequence number) into an Excel® spreadsheet.

Results and Discussion

Design and Development of the nuTH01-mtND1 Duplex qPCR Assay

In the preceding Materials and Methods section of this report we included a brief outline of the procedures used to design our singleplex nuclear (nuTH01) and mitochondrial (mtND1) qPCR assays. In this section, we provide a more detailed discussion of selected aspects of assay design and development, including: (i) choices of target DNA sequences; (ii) experimental results to establish that the singleplex nuTH01 and mtND1 qPCR assays work successfully in a duplex amplification; and (iii) the observation of unusual, but predictable, SYBR® Green melt curves for the TH01 qPCR target.

Selection and Design of the Nuclear TH01 Amplification Target

Because the primary reason for quantifying nuclear DNA in forensic samples is to determine the amount of template to use as input for STR genotyping, we anticipated that the quantification of an STR target would be directly predictive of success for STR genotyping. Furthermore, STR loci have been well characterized with respect to cross-species reactivity (49,50) and DNA mutation rates (51) because of their widespread use in forensic DNA analysis and in paternity investigations. Although initially we did not focus solely on the TH01 locus for assay development, an examination of the DNA sequences at each of the CODIS STR loci, in conjunction with assay design work using PrimerExpress™, quickly identified the TH01 locus as a promising target. The TH01 locus contains suitable sequence in both STR flanking regions for design and placement of a TaqMan® detection probe. The TH01 STR target was also attractive because the amplicons are of sufficient lengths to place them roughly in the middle of the size range produced by the commercial STR kits, but are not long enough to overly compromise the TaqMan® qPCR efficiency. During development, we designed and tested a number of potential qPCR assays at the TH01 STR locus. These assays were evaluated by comparing their qPCR properties (e.g., PCR efficiency (>90 %), sensitivity (low CT), and precision (low variance in CT, especially at low template quantities)) in order to identify an optimal assay, designated here as nuTH01. The target sequence for the nuTH01 qPCR assay is shown in Fig. 1, which displays the relative positions of the PCR primers, the STR repeat region, and the 5′-FAM/BHQ fluorogenic probe.

Selection and Design of the Mitochondrial ND1 Amplification Target

For design of the mitochondrial qPCR assay, we selected a region of the *ND1* (NADH dehydrogenase subunit 1) gene corresponding to bases 3485–3553 of the Cambridge Reference Sequence (CRS) (52). This target was chosen because previous cross-species

FIG. 1—*Sequence information for the nuTH01 portion of the duplex qPCR assay showing relative positions of the forward and reverse primers (horizontal arrows), the TaqMan® detection probe (underlined on top strand), and the $(CATT)_7$ STR region (underlined on bottom strand). The small, vertical arrow marks the sequence position that approximately separates the nuTH01 amplicon into low and high melting temperature domains. The position of this arrow corresponds to Sequence Number 96 in Fig. 5.*

sequence homology studies (53, and personal communication with Cummings MP) had indicated this sub-region of the *ND1* gene to be significantly non-conserved, a promising feature for developing a species-specific assay, and because this region of *ND1* has few known SNPs, especially when compared to the HVI/HVII control sub-regions. Using a number of web-based searching tools and avoiding any well-known disease-associated SNPs such as the LHON-associated SNP at CRS 3460, we designed and evaluated several assays at the *ND1* target. Using the same criteria as for the nuTH01 qPCR assay, we selected an optimal mitochondrial qPCR assay, designated mtND1 (Table 1).

The mtND1 assay quantifies a relatively short (69 bp) target sequence, one strand of which is shown in Table 1 as the sequence for our mtND1 copy number standard. One advantage to selecting an assay with a short target sequence was that it allowed purchase of a synthetic oligonucleotide which could then be used as a copy number standard for the mitochondrial assay, rather than preparing a standard by cloning or PCR product purification. Also, we anticipated that a short qPCR assay would detect more sensitively the presence of human mitochondrial DNA, even in degraded DNA samples.

Development of the nuTH01-mtND1 Duplex qPCR Assay

In order to develop a successful duplex qPCR assay, it is necessary to identify reaction conditions that effectively allow two amplifications to occur independently in the same tube. The goal is to avoid the predicament in which one of the amplifications reduces the PCR efficiency of the second amplification. Such a situation can lead to a delayed amplification for the second assay, an artificially large CT value, and a corresponding underestimation of the quantity of target DNA measured by that assay. One strategy for avoiding this situation is to develop duplexed qPCR assays to run under conditions that limit PCR amplification of the more abundant target sequence, for example by limiting the primer and/or probe concentrations for this amplification (42).

For the nuTH01-mtND1 duplex qPCR assay, we expected that the ratio of mitochondrial genome copies to haploid nuclear genome copies would normally exceed 100 for relevant forensic samples. This expectation was based on literature reports of mitochondrial-to-nuclear copy number ratios for various tissue types (54,55). Consequently, our approach for developing a duplex assay relied on finding appropriate limiting conditions for the mtND1 qPCR portion of the assay, while running the nuTH01 portion under optimized conditions. The development was accomplished in several steps (42). The nuTH01 singleplex qPCR assay was first examined at a range of primer (50–900 nM) and probe (50–300 nM) concentrations to determine optimal conditions for the assay. These experiments indicated that optimal sensitivity (low CT) and precision (low standard deviation of CT) were obtained by running the nuTH01 assay with 600 nM primer and 200 nM TaqMan® probe concentrations. We similarly examined the mtND1 singleplex qPCR assay at a range of primer (25–900 nM) and probe (25–200 nM) concentrations to determine that adequate sensitivity and precision could be obtained by running this assay with 50 nM primer and 100 nM TaqManMGB® probe concentrations. Our goal of running the mtND1 assay at limiting conditions is evident from the very different primer concentrations identified for the two singleplex qPCR assays. In order to determine if these reaction conditions were suitable for duplex qPCR amplifications, we compared results obtained by quantifying identical samples in both singleplex and duplex qPCR formats (Fig. 2). This comparison indicated that the qPCR assays, whether run in singleplex or in duplex, gave nearly

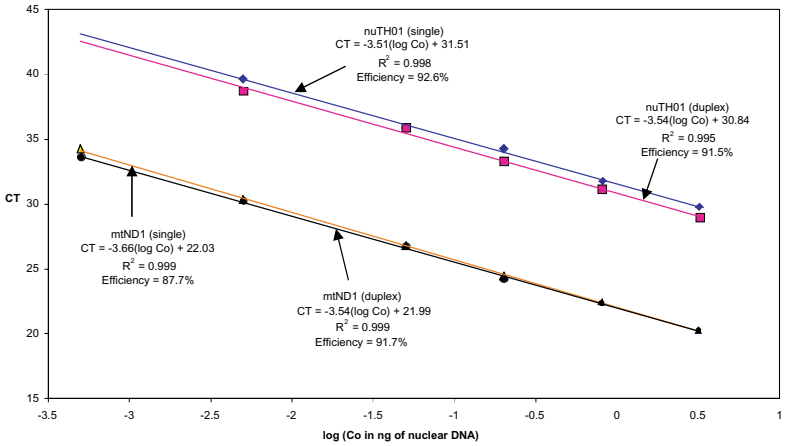

FIG. 2—*Standard curves for nuTH01 and mtND1 qPCR assays run in singleplex and in duplex modes. For the nuTH01 qPCR assays, the data points shown are averages of duplicate measurements on samples containing 3.2, 0.8, 0.2, 0.05, and 0.005 ng of Promega genomic DNA (female). For the mtND1 qPCR assays, duplicate measurements on samples containing 0.0005 ng of DNA were also included. The PCR efficiency for each assay was calculated using the slope of the CT v. log C_0 standard curve.*

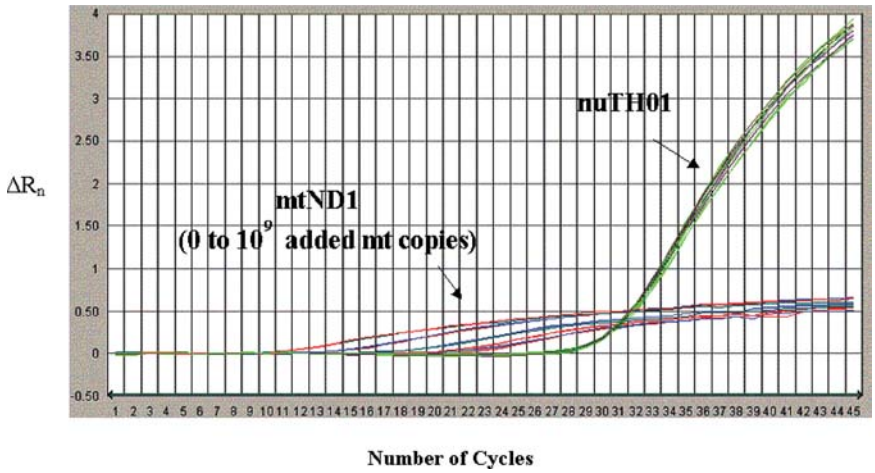

FIG. 3—*qPCR amplification curves for nuTH01-mtND1 duplex assays with 3 ng of control DNA plus 0, 10^6, 10^7, 10^8, and 10^9 excess copies of single-stranded mtND1-standard. Assays were run in duplicate.*

identical quantifications and efficiencies. The nuTH01 qPCR assay neither lost efficiency nor showed a delayed CT when run in duplex vs. singleplex. (Due to a small amount of "leakage" of the VIC fluorescence signal into the FAM detection channel, the nuTH01 qPCR assay actually appeared to amplify with a slightly lower CT when run in duplex than when run in singleplex format.) As a final developmental step, we challenged the duplex assay by spiking a 3 ng control sample of DNA with increasing amounts of synthetic oligonucleotide mtND1 copy number standard, adding up to a one-billion-fold excess number of effective mitochondrial copies to the original DNA sample. As shown in Fig. 3, the nuTH01 amplification curves and the corresponding CT values did not change significantly, even in the presence of an unrealistic excess of mitochondrial copies.

Because we designed the duplex qPCR assay to limit the mtND1 amplification conditions while running the nuTH01 amplification under robust conditions, the duplex assay was not optimized for samples in which the number of nuclear genome copies is nearly equivalent to or greater than the number of mitochondrial genome copies. For such samples, the more robust nuTH01 amplification can lower the efficiency of the mtND1 amplification, resulting in an underestimation of the quantity of mitochondrial DNA. So far, we have encountered only one type of forensic sample—the largely tail-less sperm cells from differential extraction pellets—in which the ratio of mitochondrial genome copies to nuclear genome copies is not large. Although such samples are not commonly used for mitochondrial haplotyping, this limitation of the assay should be kept in mind when interpreting mtND1 quantification data.

TH01 SYBR® Green Melt Curves

The selection of a qPCR target sequence that spans the repeat region of an STR locus introduces the possibility that the resulting assay will depend in some way upon the STR genotype, for example, that the nuTH01 assay will give measurably different results for a 6,6 TH01 genotype than for a 9,9.3 TH01 genotype. We have not, within the precision and accuracy of our quantification experiments, seen any such effects for our nuTH01 qPCR assay. The only allele-dependent effect that we have seen was the observation of unusual SYBR® Green melt curves. While this effect has no detrimental impact on the quality of TH01 qPCR assay, it is novel and requires an explanation.

In initial developmental experiments, each potential assay was evaluated by performing qPCR runs using SYBR® Green I detection. This detection method allows for early identification of sub-optimal primer combinations, avoiding use of the more expensive, dye-labeled TaqMan® detection probes. As part of these initial assessments, we evaluated the specificity of the PCR amplifications by gel electrophoresis (4% NuSieve 3:1 agarose gel with EtBr staining) of the post-run qPCR reaction mixtures and by using the qPCR instrument to generate SYBR® Green melt curves. During this developmental work, for each of our potential TH01-based qPCR assays, we observed unusual SYBR® Green melt curves (Fig. 4).

For qPCR assays detected with SYBR® Green, a melt (or dissociation) curve can be generated at the end of the final PCR cycle by configuring the qPCR instrument to monitor the SYBR® Green fluorescence of each sample as the temperature is slowly increased from ~60°C (the extension temperature of the final PCR cycle) to ~95°C. At temperatures corresponding to the melting of PCR products there will be concomitant decreases in SYBR® Green fluorescence due to de-intercalation of the reporter dye. In order to easily visualize these changes in fluorescence, melt curves are typically plotted as the negative first derivative of the change in fluorescence (−dF/dT) so that each "melt transition" will appear to be a single peak in the plot. A well-designed qPCR assay is expected to produce a single amplicon and, typically, a single transition in the SYBR® Green melt curve.

Figure 4, which shows the SYBR® Green melt curves collected for several different samples using the TH01 qPCR assay, indicates that each sample shows not one, but two melt transitions—a high melting temperature major peak ($T_m \sim 81°C$) and a lower melting temperature "shoulder" ($T_m \sim 78$–$79°C$). This observation of two melt transitions was not anticipated because the TH01 qPCR assay was otherwise unremarkable; it showed evidence for only one PCR product in post-amplification yield gels, and there was no evidence for spurious melt transitions in any negative controls. The two-step appearance of the melt transitions suggests the presence of two sequence-dependent melting domains in each TH01 amplicon. This suggestion is supported by calculations of allele-dependent melting profiles for the amplicons performed with a software package, MELT94, which uses sequence information to estimate the melting temperature of a DNA fragment along its double-stranded length (Fig. 5). Such calculations separate the TH01 amplicon into two distinct melting domains—a low melting temperature

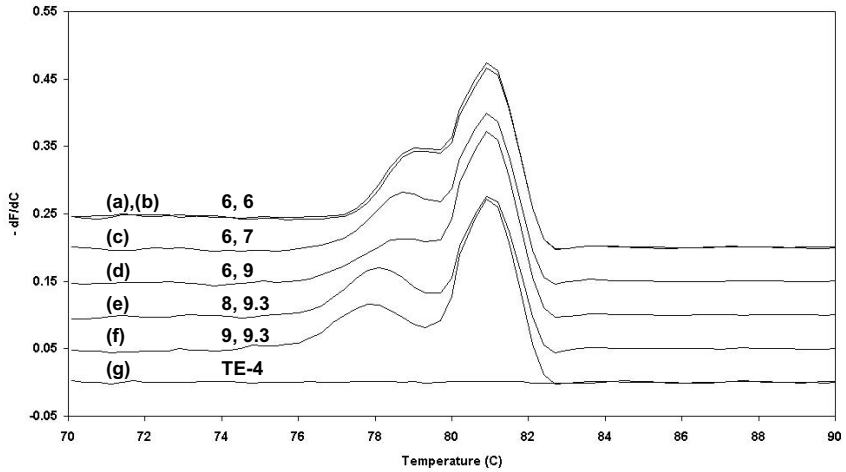

FIG. 4—*SYBR® Green melting curves obtained for DNA extracts from six different individuals and a negative control sample using the TH01 qPCR assay. The TH01 genotype for each sample is indicated. Traces (a) and (b) are from two different individuals, both genotype 6, 6 at TH01.*

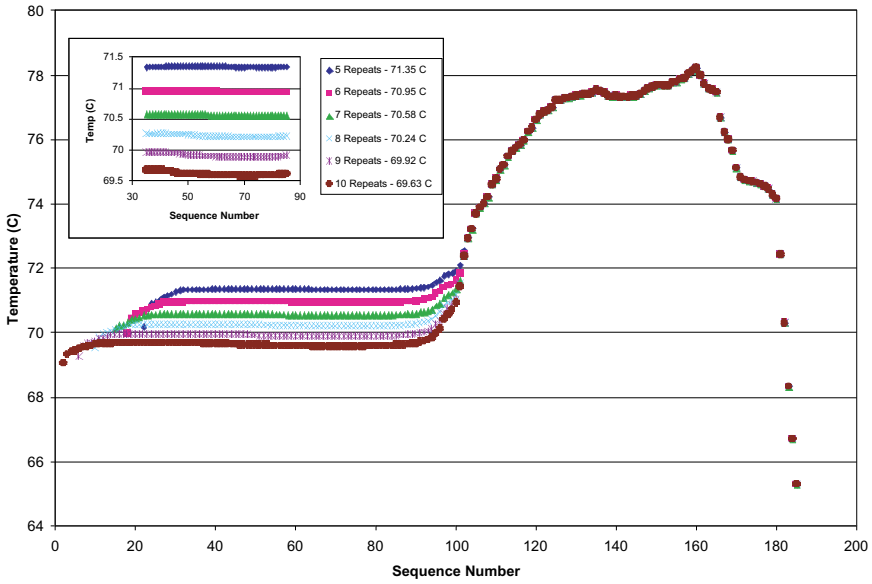

FIG. 5—*Calculated melting profiles for TH01 amplicons (STR alleles 5–10) as generated by MELT94 software. Sequence Number 96 corresponds to the position of the small, vertical arrow in Fig. 1. The inset figure shows an expansion of the melting profiles in the STR region.*

domain that includes the STR region (Sequence Number 0–~96) and a high melting temperature domain (Sequence Number ~96–~180). These calculations predict that the melting temperature of the low-melt domain will decrease as the number of STRs increases (Fig. 5, inset), a prediction that is in qualitative agreement with the trend seen for the experimental melting curves shown in Fig. 4. This trend is due to an increase in the AT-percentage of the low-melting domain sequence as the number of CATT repeats increases. The melting profile of the high-melt domain is predicted to be independent of the TH01 genotype (Fig. 5), consistent with the allele-independent, overlapping melt transitions ($T_m \sim 81°C$) shown in Fig. 4.

The stepwise melting of small DNA fragments due to sequence-dependent melting domains has been reported (56), and similar sequence-dependent denaturation phenomena form the basis for separations by such techniques as denaturing gel gradient electrophoresis (DGGE) (57). To our knowledge, however, genotype-dependent stepwise melt transitions have not been previously reported for STR amplicons. It is intriguing that the SYBR® Green melt curves for the TH01 qPCR assay might form the basis for a fairly simple and rapid, albeit low-resolution, means for STR genotyping at this locus, as well as at other STR loci that show similar allele-dependent melt curves.

nuTH01-mtND1 Duplex qPCR Validation: Precision, Sensitivity, and Reproducibility

Precision of the nuTH01-mtND1 duplex qPCR assay was assessed by running 96 identical assays in a single wellplate using 4 ng of pre-quantified standard DNA (Promega Human Genomic Female DNA) per quantification. For the nuTH01 portion of the duplex assay, we measured an average CT of 28.94 cycles with a standard deviation (SD) of 0.13 cycles and a CT range of 0.59 cycles. For the mtND1 portion of the assay, we measured an average CT of 22.63 cycles with a SD of 0.21 cycles and a CT range of 0.98 cycles. No systematic deviations in CT across rows or columns of the 96-well plate for either portion of the duplex assay were observed. Although we did not include a standard DNA dilution series in this

TABLE 2—*Intra-run sensitivity and precision results for the nuTH01-mtND1 duplex qPCR assay. Results are based on five replicate quantifications of serially diluted Promega Female Genomic DNA standards. Quantities are per 4 μL of sample. Standard deviations are in parentheses.*

	nuTH01 qPCR			mtND1 qPCR	
Input DNA Quantity (ng)	Average Quantity (ng)	RSD (%)	Approx. Input Quantity (mt copies)	Average Quantity (mt copies)	RSD (%)
100	95.8 (7.7)	8.0	1.2×10^7	$8.6\ (0.97) \times 10^6$	11
10	10.7 (0.90)	8.4	1.2×10^6	$1.3\ (0.15) \times 10^6$	12
5	4.7 (0.088)	1.9	6.1×10^5	$7.6\ (0.95) \times 10^5$	12
1	1.2 (0.034)	3.0	1.2×10^5	$1.3\ (0.24) \times 10^5$	18
0.5	0.48 (0.057)	12	6.1×10^4	$7.1\ (1.4) \times 10^4$	20
0.1	0.10 (0.021)	21	1.2×10^4	$1.0\ (0.20) \times 10^4$	20
0.05	0.051 (0.013)	26	6.1×10^3	$7.2\ (1.5) \times 10^3$	21
0.01	0.0057* (0.0017)	30*	1.2×10^3	$1.3\ (0.21) \times 10^3$	17
0.005	—	—	6.1×10^2	$7.1\ (2.0) \times 10^2$	28
0.001	—	—	1.2×10^2	$1.1\ (0.39) \times 10^2$	35
0.0001	—	—	1.2×10^1	$1.1\ (0.41) \times 10^1$	39

* Based on four replicates, because one quantification dropped out at this template quantity.

run, the standard deviation of CT can be used to estimate the relative standard deviation (RSD or %CV) of genome copy number as $100(2^{(\text{SD of CT})}-1)$; this estimation assumes 100% PCR efficiency for the assay. By this means, we estimate the intra-plate RSD of genome copy number to be ~10% for the nuTH01 assay and ~16% for the mtND1 assay.

Sensitivity of the duplex qPCR assay was assessed by quantifying Promega Human Genomic Female standard DNA using template quantities ranging from 100 ng to 1 fg. All quantifications were performed in replicates of five in order to estimate the minimum level of input DNA at which stochastic effects (e.g., qPCR signal dropout and reduced quantification precision) can be expected to become significant. Results from these runs are summarized in Table 2. As expected, these results indicate that as the number of template copies diminishes, there is a general decrease in precision for both the nuTH01 and mtND1 portions of the duplex assay. For the nuTH01 portion of the qPCR assay, adequate levels of precision (<30% RSD) are seen down to approximately 50 pg of template DNA (~15 haploid nuclear copies), and we have generally seen similar levels of precision down to approximately 32 pg or ~10 copies (data not shown). At lower quantities of nuclear template, loss of precision, as well as an increase in the proportion of "dropped out" amplification curves occurred (e.g., 20% of the nuTH01 qPCR assays dropped out and were undetected at 10 pg of template, 30% at 5 pg, 80% at 1 pg, and 100% at <1 pg). This is probably due to stochastic effects in sampling and in amplification at low levels of template. For the mtND1 portion of the qPCR assay, adequate levels of quantification precision (<40% RSD) were seen down to at least 100 fg of nuclear template, which corresponds to ~12 mitochondrial template copies for the Promega genomic female standard DNA. All mtND1 qPCR amplification curves dropped out at 10 fg of nuclear template.

Reproducibility of the duplex assay was assessed by comparing quantifications of six different high molecular weight control DNA samples obtained from three independent qPCR runs as performed by two different analysts on two different days. Samples were quantified as single replicates in each run. The results are summarized in Table 3, where we estimate "reproducibility" as the effective RSD for each sample by pooling the quantification results from the three runs. These inter-plate RSD values are, on average, in reasonable agreement with the intra-plate RSD values reported in our discussion of assay precision and sensitivity. A notable exception is the single large RSD (62%) observed for the mtND1 quantification of sample A.

TABLE 3—*Inter-run reproducibility results for nuTH01-mtND1 duplex qPCR quantifications of six commercial high molecular weight genomic DNA standards (samples A–F). Slot blot quantities are based on single replicate measurements for each sample. qPCR quantities are reported as averages, based on pooling the results from the three single-replicate qPCR runs. Standard deviations are in parentheses. Promega Genomic Female DNA was used as the quantification standard for all three qPCR runs.*

		nuTH01 qPCR		mtND1 qPCR	
Sample	Slot Blot Quantity (ng/μL)	Average Quantity (ng/μL)	RSD (%)	Average Quantity (1000 mt copies/μL)	RSD (%)
A	0.85	0.64 (0.08)	12	130 (79)	62
B	0.80	0.96 (0.10)	10	880 (150)	17
C	0.34	0.53 (0.10)	20	310 (35)	11
D	1.31	1.58 (0.31)	19	430 (31)	7
E	0.97	1.51 (0.17)	11	790 (170)	21
F	0.40*	0.45 (0.05)	12	360 (73)	21

* Sample F represents the calibration standard used for the slot blot quantifications; the value for sample F (0.40 ng/μL) is a defined, rather than a measured quantity.

nuTH01-mtND1 Duplex qPCR Validation: Species Specificity

To assess species specificity of the nuTH01 and mtND1 qPCR amplifications, the duplex assay was run using template DNA from each of fifteen different non-human species. Duplicate assays, each containing 1 ng of template, were used for each species. No cross-reactivity to non-human DNA was observed for either the nuTH01 or mtND1 portions of the duplex qPCR assay. We further challenged the duplex assay by amplifying 100 ng portions of each of the microbial DNA samples, and we observed no amplifications above the level of background in either the nuTH01 or mtND1 quantifications. These results indicate that the nuTH01-mtND1 duplex qPCR assay is sufficiently specific for forensic applications. Although we did not test the assay with DNA from higher primates, based on previous work with the TH01 STR locus (49,50) and on sequence homology investigations, we

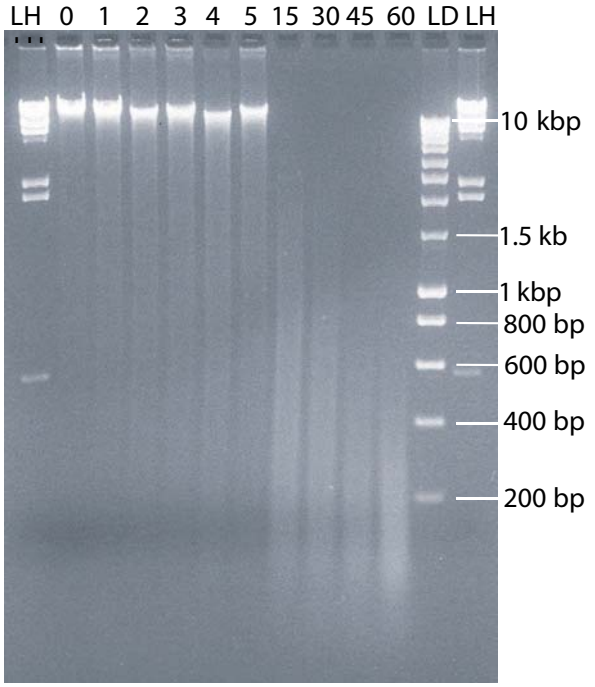

FIG. 6—*HL60 DNase degradation series (EtBr-stained). Lanes labeled 0 to 60 indicate DNase treatment time in minutes. Lanes labeled LH and LD represent λ-HindIII and low-mass DNA ladder, respectively.*

anticipate that the nuTH01 qPCR assay would amplify and detect such samples.

nuTH01-mtND1 Duplex qPCR Validation: Degraded DNA

Forensic evidence samples often contain DNA that has been degraded by environmental and/or microbial exposures. To assess the ability of the nuTH01-mtND1 duplex qPCR to quantify DNA in such samples, a DNA degradation series was prepared by treating aliquots of high molecular weight genomic DNA (HL60) with DNase I for increasing periods of time ranging from one minute to overnight. Figure 6 shows a gel illustrating the degree of DNA fragmentation for the DNase-treated samples. An increase in the degree of DNA fragmentation during the first 5 min of DNase treatment is evident from increased intensity of the low molecular weight "smear" in lanes 3 to 7 of the gel. During this time, the extent of degradation is best described as "moderate" given the consistent appearance of a fairly intense high molecular weight band in these lanes. For samples with at least 15 min of DNase treatment, however, there is no evidence for the high molecular weight band. For the purpose of this discussion, we describe these samples as "highly degraded."

Samples from the DNA degradation series were quantified by three different methods: slot blot hybridization, nuTH01-mtND1 duplex qPCR, and the Applied Biosystems Quantifiler™ Human DNA Quantification kit. Because the nuTH01 assay amplifies a ~170–190 bp target sequence, while the Quantifiler™ kit amplifies a 62 bp target sequence, we included the Quantifiler™ assay in our comparison as a means to evaluate the significance of amplicon size for quantifying degraded DNA. For the qPCR assays, 2 μL of diluted sample (1:20 in TE^{-4}) were quantified in duplicate for each point in the degradation series. For the slot blot assays, single quantifications were obtained on 4 μL of the same diluted samples. Quantification results (nuclear quantifications only) for the

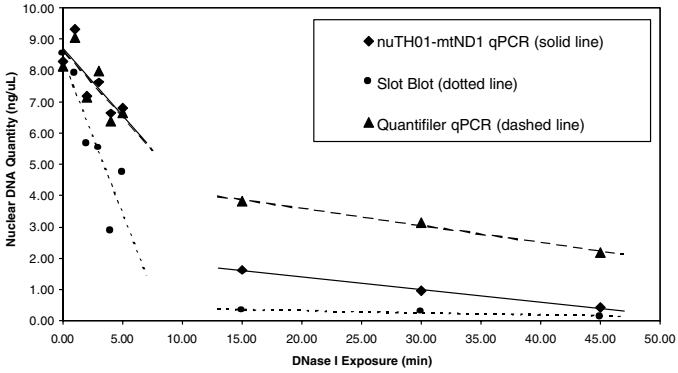

FIG. 7—*Nuclear quantifications of DNase-degraded HL60 samples by nuTH01-mtND1 duplex qPCR, Quantifiler™ qPCR, and slot blot hybridization. For the qPCR assays, each point represents the average of duplicate quantifications. For slot blot, each point represents a single quantification. Lines represent linear least-squares fits to early (0–5 min) and late (15–45 min) time points in the degradation series.*

three methods are summarized graphically in Fig. 7, from which a number of conclusions can be drawn. First, for undigested, high molecular weight DNA (0 min sample), the three methods provide quantifications that are in good agreement with each other, as well as in reasonable agreement with the concentration (~6 ng/uL) estimated for a 1:20 dilution of the DNA stock. Second, for the moderately degraded samples (1–5 min of DNase treatment), the two qPCR-based quantification methods are in good agreement, but both give quantification values significantly higher than those obtained from the slot blot method. Third, for highly degraded samples (>15 min of DNase treatment), none of the quantification methods agree. Relative to both qPCR-based methods, the slot blot method detects significantly less DNA. Even the nuTH01-mtND1 duplex and Quantifiler™ qPCR assays disagree, the latter assay detecting significantly more DNA than the former.

These results, which indicate that the quantity of DNA measured in degraded samples depends upon the quantification method used, were not entirely unexpected. There have been previous reports that the slot blot method underestimates the quantity of nuclear DNA in degraded or compromised samples (14,23,24), and it has been suggested that this effect is due to a lowered binding efficiency of the degraded DNA fragments to the slot blot membrane (14). Some degree of difference in quantifying degraded DNA by qPCR was also to be expected, considering the size difference between the target sequences for the nuTH01 and Quantifiler™ assays. The Quantifiler™ qPCR assay effectively measures the concentration of nuclear DNA fragments at least 62bp long, whereas the nuTH01 qPCR assay measures the concentration of fragments at least 170–190 bp long. For high molecular weight DNA samples these two concentrations are practically the same, but for highly degraded DNA samples there is a greater concentration of smaller than larger fragments. This concentration difference is indicated by the yield gel shown in Fig. 6 and is reflected in the divergent DNA quantities measured by the Quantifiler™ and nuTH01 qPCR assays.

The primary reason for accurately quantifying nuclear DNA in forensic samples is to ensure the correct amount of template is used for STR amplification. When too little template DNA is amplified, incomplete STR profiles can result, while amplification of excess template can cause such problems as poor inter-locus balance, increased stutter peak intensities, incomplete non-template-directed nucleotide addition, off-scale signals, and signals due to cross-dye "pull-up," each of which can complicate or preclude accurate STR genotyping. For degraded samples, as we have seen, different

quantification methods can provide very different estimates of DNA quantity. To investigate the implications of these differences, we attempted to determine which of the three quantification methods would provide the most suitable estimates of nuclear DNA quantity for STR typing of degraded samples. To this end, we used the quantification results shown in Fig. 7 to prepare nominal "1ng" portions of nuclear DNA for each time-point in the DNase-degradation series based on each of the three quantification methods. These "1ng" portions were then amplified and genotyped using the AmpF*l*STR® Identifiler™ STR kit.

Figure 8 provides a qualitative, graphical overview of the STR genotyping results for the entire set of DNase-degraded samples. Panel (A) in this figure suggests that for untreated and moderately degraded DNA samples (0–5 min DNase treatment), all three methods were suitable for quantifying nuclear DNA for STR genotyping. At these moderate levels of degradation, regardless of the method of quantification, all STRs were detected with signals greater than 490 RFU per locus, and there was no evidence of artifacts that would complicate or preclude accurate genotyping. For the more highly degraded DNA samples (15–60 min DNase treatment), however, success rates for STR genotyping were seen to depend upon the method of DNA quantification.

Panel (C) of Fig. 8 shows that for amplifications based on Quantifiler™ quantifications, a relatively large proportion of alleles were undetected for all of the AmpF*l*STR® Identifiler™ reporter dyes. As the extent of DNA fragmentation increased, the larger STR alleles for each reporter dye were the first that failed to reach the 100 RFU analytical threshold. The implications for using a short qPCR target sequence are evident. In these highly degraded samples, there were more 62 bp than longer STR-sized DNA fragments. As a consequence, the Quantifiler™ assay overestimated the quantity of longer template fragments, leading to under-amplification of the longer STR alleles and, ultimately, to inadequate quantities of PCR product for detection. With this quantification method, at very high levels of degradation (45–60 min of DNase treatment) less than half of the Identifiler™ STR alleles were detected, and the overestimation of DNA quantity was so pronounced that even the shorter STR alleles lost significant intensity due to under-amplification (Figs. 8*C* and 9*B*, and Table 4).

By contrast, quantification of these same samples using the nuTH01-mtND1 duplex qPCR method led to an improvement in the success rate for STR genotyping (Fig. 8). Even for the most highly degraded samples (60 min of DNase treatment), more than 75% of the HL60 alleles were detected. For the purpose of STR genotyping highly degraded samples, these results indicate that the ~180 bp nuTH01 qPCR target is more appropriate for quantifying DNA than is the shorter 62 bp target. Data provided in Table 4 show that by basing DNA quantification on the medium-sized nuTH01 target, the intensities of the detected TH01 STRs were maintained at a fairly consistent level (~1800–3100 RFU/locus) despite the varying extent of DNA fragmentation. Even for highly degraded samples, the short (~121 bp) D19 STRs were not over-amplified to the extent that they resulted in off-scale peaks, nor were the long (~293 bp) D18 STRs under-amplified to the extent that they went undetected.

STR amplifications based on slot blot quantifications resulted in very few undetected alleles, even for the most highly degraded samples (Fig. 8). However, the electropherograms shown in panels (C) and (D) of Fig. 9 indicate the presence of several artifacts due to the over-amplification of excess template DNA. These artifacts include shoulder peaks due to incomplete non-template-directed nucleotide addition, cross-dye "pull-up" signals due to off-scale peaks in adjacent detector channels, and stutter peaks above 100 RFU. For some slot-blot quantified samples in the DNA degradation series, off-scale peaks led to pull-up signals as intense as 2700 RFU (data not shown). Off-scale peaks are evident even for samples exposed to DNase for only 30 minutes (Table 4). Inter-locus balance, as represented by the D19/D18 ratios, was significantly worse for the slot-blot quantified samples than for the qPCR-quantified samples (Table 4). Although some of these artifacts (e.g., off-scale peaks, "pull-up" signals) are possibly remedied by reducing CE sample injection times, other artifacts (e.g., shoulders due to incomplete nucleotide addition, poor inter-locus balance) are repairable only by re-amplification.

Highly degraded samples present analytical challenges for DNA quantification and for subsequent STR genotyping. The quantity and quality of DNA in such samples, which are typically comprised of fragments with a wide range of sizes, cannot be represented by any single qPCR assay, which is fundamentally biased to detect and quantify the subpopulation of fragments that are at least as long as the specific target sequence being amplified. In principle, the most accurate estimate of *overall* DNA quantity in degraded samples

TABLE 4—*Selected AmpF*l*STR® Identifiler™ STR intensities for DNase-degraded HL60 samples. All amplifications used nominal 1 ng nuclear template amounts as quantified by nuTH01-mtND1 duplex qPCR, Quantifiler™ qPCR, and slot blot hydridization. STR intensities are provided as RFU per locus. For samples exposed to DNase for 0–5 min, the qPCR-based STR intensities are identical because only one STR amplification was performed based on nearly identical quantifications of these samples by the nuTH01 and Quantifiler™ assays (see Fig. 7). Off-scale peaks, as identified by GeneScan® software, are indicated in bold. For HL60, the average Identifiler™ STR allele sizes for TH01, D19, and D18 are 177 bp, 121 bp, and 293 bp, respectively (60).*

DNase Exposure (min)	nuTH01-mtND1 Duplex qPCR				Quantifiler™ qPCR				Slot Blot Hybridization			
	TH01 (RFU)	D19 (RFU)	D18 (RFU)	D19/D18	TH01 (RFU)	D19 (RFU)	D18 (RFU)	D19/D18	TH01 (RFU)	D19 (RFU)	D18 (RFU)	D19/D18
0	1824	2809	3677	0.76	1824	2809	3677	0.76	2724	2671	4780	0.56
1	1998	1851	2730	0.68	1998	1851	2730	0.68	2861	2360	3747	0.63
2	2883	2489	3253	0.77	2883	2489	3253	0.77	3636	2789	3849	0.72
3	2697	2400	3210	0.75	2697	2400	3210	0.75	3122	2465	3183	0.77
4	2417	2043	2519	0.81	2417	2043	2519	0.81	3791	2880	2637	1.09
5	2469	2119	3133	0.68	2469	2119	3133	0.68	2894	2469	3332	0.74
15	2393	3434	1537	2.23	955	1472	584	2.52	6389	7053	1566	4.50
30	3108	5156	1329	3.88	1036	1786	543	3.29	7797	**9103**	1766	5.15
45	2540	5408	738	7.33	534	1153	115*	···	6563	**9011**	903	9.98
60	1941	5249	473	11.10	349	825	···	···	5916	**8845**	543	16.29

* Only one STR allele of heterozygous pair detected.

	DNase Treatment	Percent of STR Alleles Detected	Number of STR Artifacts*	Blue (FAM) STRs								Green (VIC) STRs							Yellow (NED) STRs						Red (PET) STRs			
				D8		D21		D7		CSF		D3	THO1		D13		D16	D2	D19	vWA	TPOX		D18		AML	D5	FGA	
A)	All Assays			12	13	29	30	11	12	13	14	16	7	8	8	11	11	17	14	16	8	11	14	15	X	12	22	24
	0 min	100	- none -																									
	1 min	100	- none -																									
	2 min	100	- none -																									
	3 min	100	- none -																									
	4 min	100	- none -																									
	5 min	100	- none -																									
B)	nuTH01-mtND1 qPCR																											
	15 min	100	- none -																									
	30 min	96	- none -																									
	45 min	84	2 P																									
	60 min	76	- none -																									
C)	Quantifiler qPCR																											
	15 min	84	- none -																									
	30 min	76	- none -																									
	45 min	48	- none -																									
	60 min	28	- none -																									
D)	Slot Blot Hybridization																											
	15 min	100	1 O, 2 P, 2 S, 3 A																									
	30 min	100	1 O, 3 P, 2 S, 3 A																									
	45 min	92	2 O, 3 P, 1 S, 3 A																									
	60 min	92	2 O, 3 P, 3 S, 4 A																									

* O = off-scale peak; P = detected pull-up signal; S = detected stutter signal; A = detected shoulder due to incomplete A addition

FIG. 8—*Overview of AmpFlSTR® Identifiler™ STR results obtained for DNase-degraded HL60 DNA samples. Each shaded rectangle indicates the detection of an STR allele with intensity at least 100 RFU. Un-shaded rectangles indicate alleles that were undetected at the 100 RFU analytical threshold. For each reporter dye, the Identifiler™ alleles are presented left to right in order of increasing size. STR amplifications used nominal 1 ng input levels of nuclear DNA, as quantified by the nuTH01-mtND1 duplex qPCR assay (panels A and B), by the Quantifiler™ assay (panels A and C), or by slot blot hybridization (panels A and D).*

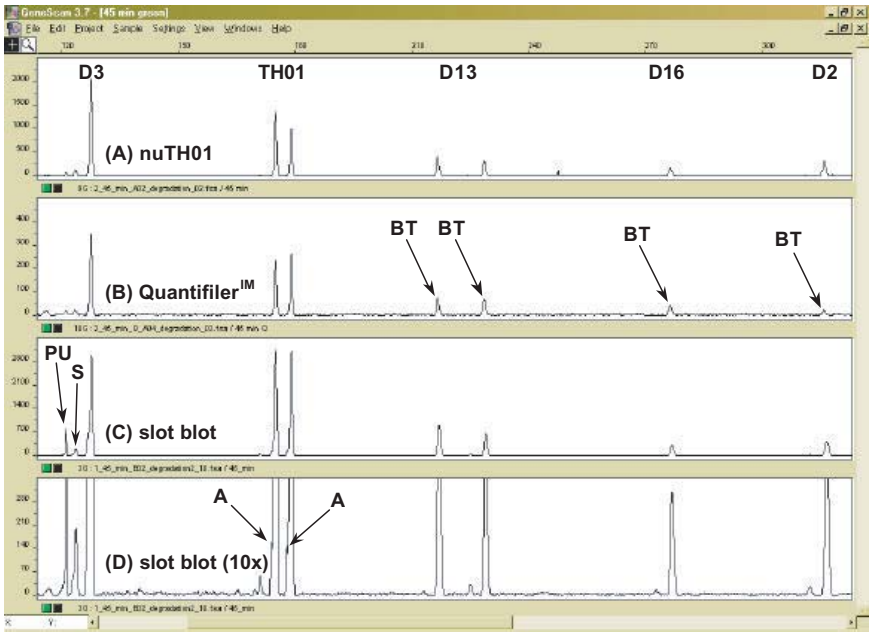

FIG. 9—*AmpFlSTR® Identifiler™ VIC-labeled STRs from DNase-degraded (45 min) HL60 samples. Panels show STR alleles for amplifications of "1 ng" amounts of nuclear template as determined by: (A) nuTH01-mtND1 duplex qPCR, (B) Quantifiler™ qPCR, and (C) slot blot hybridization. Panel (D) is a 10-fold vertical scale expansion of panel (C). Detection and/or amplification artifacts are indicated as: BT = below detection threshold; PU = "pull-up" signal from adjacent detection channel; S = detected stutter signal; A = detected shoulder due to incomplete non-template nucleotide addition. Full vertical-scale RFU values for the panels are: (A) 2500 RFU; (B) 500 RFU; (C) 3500 RFU; (D) 350 RFU.*

should be provided by qPCR assays that use very short target sequences. However, for the specific purpose of STR genotyping, the *overall* DNA quantity in a degraded sample is not necessarily the measurement of interest. Our results indicate that if qPCR methods are to be used to quantify degraded DNA for STR genotyping, there are advantages in selecting a qPCR target sequence that is of appropriate length to detect those DNA fragments that are most relevant to the method of analysis. This is particularly true for the highly multiplexed commercial STR genotyping kits that are used in most forensic DNA labs, kits that recommend the use of a fairly narrow range of template DNA to give optimal success rates for STR amplification and detection. For the AmpF*l*STR® Identifiler™ STR kit, which amplifies STRs ranging from ~100 bp to ~400 bp, our genotyping results indicate that "1 ng" quantities of highly degraded DNA are better estimated by using a ~180 bp nuclear qPCR target sequence, as in the nuTH01-mtND1 duplex assay, than by using a 62 bp qPCR target sequence, as in the Quantifiler™ qPCR assay. Although this discussion is based on our experiments with a specific set of DNase-degraded samples, the general conclusion that the target length of a qPCR assay is an important consideration for quantifying DNA in degraded samples is sound. We are aware, however, that the degree of advantage gained by using a long target sequence for qPCR will likely depend on precisely how the sample is extracted and purified.

In addition to quantifying the amount of nuclear DNA, we also used the mtND1 portion of our duplex qPCR assay to estimate the quantity of mitochondrial genome copies in this same set of DNase-degraded samples. These mtND1 quantification results are provided graphically in Fig. 10. In this figure, the mitochondrial quantification results shown at each time-point in the degradation series are normalized relative to the quantification result for the untreated sample (1.3 million mt copies/μL). For comparison, the figure also shows normalized nuclear quantifications, as estimated by the nuTH01 and Quantifiler™ qPCR assays for the same set of DNase-degraded samples. Notice that the mtND1 and Quantifiler™ assays generally measured higher normalized quantities of DNA than did the nuTH01 assay. For example, both the mtND1 and Quantifiler™ assays indicated that ~25% of the initial DNA remained after 45 minutes of DNase exposure, while the nuTH01 assay detected only ~5% of the initial DNA concentration. It is likely that because the mtND1 qPCR assay amplifies a relatively short target sequence (69 bp), the normalized quantifications for the mtND1 and Quantifiler™ assays were very similar, despite quantifying entirely different genomes. Because we have already seen that the selection of a short nuclear target has implications for STR genotyping, we were motivated to learn if the selection of a short mitochondrial quantification target would have a similar detrimental impact on HVI/HVII amplification success rates.

To address this issue, we performed two separate sets of HVI/HVII duplex PCR amplifications on samples from the DNA degradation series. In the first set, for each time-point in the series we amplified 100 pg of nuclear DNA, as measured by the nuTH01 portion of the duplex qPCR assay. This approach, to use the quantity of nuclear DNA to indirectly estimate the amount of template for HVI/HVII PCR amplifications, is commonly used in the forensic analysis of mitochondrial DNA, if there is sufficient nuclear DNA to be quantified (30,58). In the second set, for each time point we amplified ~14,000 mitochondrial genome copies, as estimated directly by the mtND1 portion of the duplex qPCR assay. (For pristine samples of HL60, we had previously determined that 100 pg of nuclear DNA represented ~14,000 mitochondrial genome copies.) The post-amplification yield gel results for both sets of HVI/HVII duplex PCR amplifications are shown in Fig. 11.

A "successful" HVI/HVII duplex PCR amplification was considered to produce sufficient quantities of the HVI and HVII PCR products for successful cycle sequencing. For cycle sequencing, our laboratory has validated a protocol that uses the Applied Biosystems Terminator BigDye® v1.1 Cycle Sequencing System

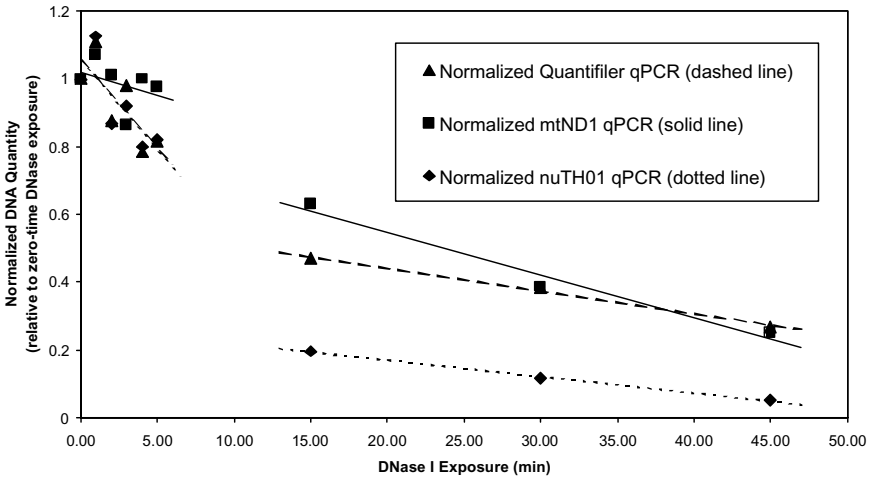

FIG. 10—*Normalized quantifications of DNase-degraded HL60 samples by mtND1 (duplex) qPCR, Quantifiler™ qPCR, and nuTH01 (duplex) qPCR. Each point represents the average of duplicate quantifications. For each assay, the DNA quantities are normalized relative to the measured quantity at 0 min of DNase-treatment, i.e., to 8.1 ng/μL for the Quantifiler™ assay, to 8.3 ng/μL for the nuTH01 assay, and to 1.3 × 10^6 mt copies/μL for the mtND1 qPCR assay. Lines represent linear least-squares fits to early (0–5 min) and late (15–45 min) time points in the degradation series.*

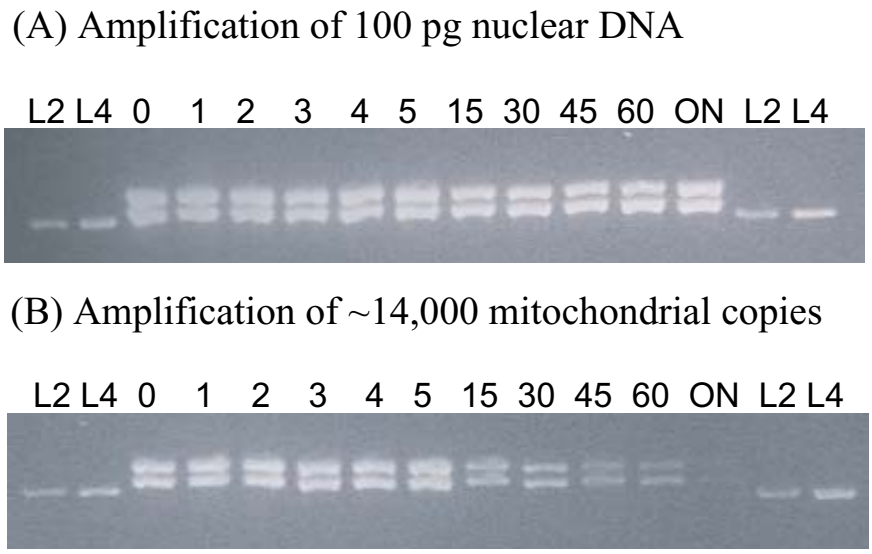

FIG. 11—*HVI/HVII post-amplification yield gel results for DNase-degraded HL60 samples. Gel is EtBr stained. Each pair of bands represents the HVI (~444 bp) and HVII (~416 bp) PCR products from 34 cycles of the duplex amplification. Numerical lane designations (0–60) represent DNase digestion times in minutes; ON represents overnight DNase digestion; L2 and L4 represent, respectively, 20 ng and 40 ng ladder bands (400 bp). In panel (A), amplifications used 100 pg of nuclear DNA as estimated by the nuTH01 portion of the duplex qPCR assay, except for the ON sample, which used 20 μL of digest. In panel (B), amplifications used 14,000 mitochondrial genome copies as estimated by mtND1 portion of the duplex qPCR assay.*

(30). Although the manufacturer recommends using 3–10 ng/20 μL of PCR product in each 50 μL cycle-sequencing reaction (59), local validation studies indicate that successful sequencing can be routinely achieved even with as little as 250 pg of PCR product per reaction. Even using the more conservative recommendation of 3–10 ng/20 μL, we can deduce that a "successful" HVI/HVII amplification is one that produces at least 0.15 ng/μL of each PCR product. This concentration is well below the visual detection limit of our ethidium bromide-stained gel. Consequently, our criterion for a successful HVI/HVII amplification is simply that the post-amplification yield gel shows visible evidence, even weak visible evidence, for two appropriately sized product bands. Referring again to Fig. 11, the visual appearance of two such bands for all of the amplifications indicates that both targets of the duplex qPCR assay successfully quantified mitochondrial DNA for HVI/HVII duplex PCR and that all amplifications produced more than enough of each PCR product for successful cycle sequencing. It is, however, evident that there were implications for selecting a short target sequence for the mtND1 qPCR assay. In particular, Fig. 11 (panel (B)) shows that for highly degraded samples (15 min—overnight DNase treatment) increasingly weak HVI/HVII bands are seen in the post-amp yield gel. This trend indicates that the "short" mtND1 qPCR assay overestimated the number of ~400 bp mitochondrial genome copies, just as the Quantifiler™ qPCR assay overestimated the quantity of 100–400 bp nuclear genome copies in these same samples. However, because the mitochondrial cycle sequencing protocol is capable of obtaining successful results over a very wide input range of PCR product (250 pg–10 ng), these overestimations of the quantity of mitochondrial DNA by the mtND1 qPCR assay did not lead to any failed HVI/HVII amplifications.

TABLE 5—*Summary of nuTH01-mtND1 qPCR quantification results for selected reference and casework-type samples. Where indicated, NSF and SF represent, respectively, the non-sperm fraction and sperm fraction from a differential extraction protocol. All quantification results, qPCR and slot blot, are from single replicate experiments. IS indicates that insufficient sample was available for amplification.*

Sample	Number and Description	nuTH01 qPCR (ng/4 μL)	Slot Blot (ng/4 μL)	mtND1 qPCR (1000 copies/4 μL)	Genome Copy Ratio—Mitochondrial: Nuclear	Mitochondrial HVI/HVII Post-Amplification Yield Gel	Identifiler™ STR Data (RFUs per locus)				Approximate Mitochondrial Copies Amp'ed for Limited Samples
							TH01	D19	D18	D19/D18	
1	liquid blood	0.42	0.60	200	1600	±	1213	5199	255	20.4	
2	liquid blood	1.22	0.95	380	1000	+	1349	1065	980	1.1	
3	dried blood on black sateen	19.0	11.6	1400	240	+	1814	1444	1403	1.0	
4	dried blood in potting mix	3.86	9.11	480	410	+	7829	6079	1033	5.9	
5	dried blood on newspaper	0.51	0.45	60	390	+	2522	1586	1363	1.2	
6	dried blood on denim	0.12	0.10	37	1000	+	4486	3562	775	4.6	
7	dried blood on wool	2.25	1.50	300	440	+	2096	1335	1230	1.1	
8	dried blood on carpet	0.46	0.39	86	620	+	2747	2204	1429	1.5	
9	dried blood on leather	0.15	0.10	18	400	+	2562	1468	1292	1.1	
10	dried blood on toothpick	1.48	0.66	290	650	+	2466	1399	1145	1.2	
11	femur (powdered)	0.24	0.05	170	2300	+	2412	3231	378	8.6	
12	non-root hair shaft (Chelex)	0	0	9.1	—	+	IS	IS	IS	IS	
13	non-root hair shaft (DNA-IQ)	0	0	2.0	—	±	IS	IS	IS	IS	5000
14	toenail	4.83	0.88	9700	6600	+	2319	3038	459	6.6	
15	toenail	1.06	0.22	1400	4400	+	1810	1523	333	4.6	
16	semen on denim (NSF)	0.53	0.13	50	320	+	3459	2335	1323	1.8	
17	semen on boxer shorts (NSF)	0.23	0.09	990	14000	±	2867	4702	456	10.3	
18	vaginal swab (NSF)	32.1	19.9	880	90	+	3046	1869	927	2.0	
19	electric razor shavings	1.83	0.80	2900	5200	±	1661	1920	484	4.0	
20	swab of razor	0.42	0.07	210	1700	+	1218	915	534	1.7	
21	bloody bandage	52.4	10.1	1700	110	+	1747	1319	1055	1.3	
21	saliva from cigarette butt	0.77	0.29	470	2000	+	1814	1553	671	2.3	
22	saliva from envelope	0.52	0.35	160	1000	+	1890	1308	847	1.5	
24	toothbrush	1.85	1.06	390	700	+	1608	1314	749	1.8	
25	saliva from coffee cup	0.84	0.29	270	1100	+	3017	2051	936	2.2	
26	buccal swab	16.0	5.10	4800	990	+	1677	1182	1130	1.0	
27	buccal swab	17.8	6.70	4900	910	+	1722	1476	937	1.6	
28	buccal swab	5.49	2.13	2400	1400	+	2059	1335	1134	1.2	
29	saliva from envelope	0.04	0	3.4	280	±	IS	IS	IS	IS	1300
30	vaginal swab (SF)	0.04	0	0.036	2.7	0	1204	1417	1005	1.4	300
31	vaginal swab (SF)	4.68	2.99	0.71	0.5	+	2464	1555	1101	1.4	
32	semen on denim (SF)	2.03	1.61	0.014	0.02	+	1580	1067	1038	1.0	700
33	semen on leather (SF)	0.27	0.09	0	0	+	3579	2635	1888	1.4	(20 μL)
34	semen on cotton fabric (SF)	39.4	28.5	0.16	0.01	+	2164	1592	1272	1.3	7000
35	semen/blood on swab (SF)	0.74	1.06	0.046	0.2	+	1857	1832	1304	1.4	2300
36	semen on boxer shorts (SF)	0.40	0.15	38	310	±	3963	3068	552	5.6	
37	muscle tissue	133	31.4	20000	500	+	1323	620	696	0.9	
38	baby tooth	0.26	0.23	38	480	±	2017	1846	1354	1.4	
39	baby tooth	10.6	2.19	800	250	+	2432	1780	1052	1.7	
40	tooth	53.1	34.1	5300	330	+	1723	1197	1198	1.0	

nuTH01-mtND1 Duplex qPCR Validation: Reference and Casework-type Samples

In order to further evaluate the nuTH01-mtND1 duplex qPCR assay, we quantified DNA extracts from a variety of reference and simulated casework samples for subsequent STR genotyping and mitochondrial HVI/HVII amplifications. A summary of results for a subset of 40 samples from this study is presented in Table 5. For STR genotyping, each amplification used 1 ng of nuclear template DNA as determined by the nuTH01 portion of the duplex qPCR assay. STR results are represented in the table by the RFU values per locus for three of the 15 AmpF*l*STR® Identifiler™ STR loci, TH01(163–202 bp), D19(102–136 bp), and D18(262–346 bp) (60). Data for these particular loci were selected because their amplicons represent intermediate, short, and long STR alleles, respectively, in the genotyping kit. The D19/D18 RFU ratios are tabulated to provide a simple STR-based metric to indicate DNA degradation. For HVI/HVII amplifications, ~14,000 mitochondrial copies were used per reaction, as quantified by the mtND1 portion of the duplex qPCR assay. For some samples, limited quantities required amplification of fewer than 14,000 copies (last column in the table). HVI/HVII amplifications were evaluated by post-amplification gel electrophoresis, where the gel results are represented by either a "+" (to indicate two visible HVI/HVII product bands with expected intensities), a "±" (to indicate two visible product bands with lower than expected intensities), or a "0" (to indicate that no product bands were seen). Both "+" and "±" results indicate that the amplifications provided more than enough HVI/HVII product for cycle sequencing.

For nearly all of these casework-type samples, quantifications provided by the nuTH01-mtND1 duplex qPCR assay led to successful STR and HVI/HVII amplifications. With one exception, each sample that contained a sufficient quantity of nuclear DNA was amplified to give a full STR profile. For the single exception, sample #1, only the CSF alleles were undetected, a result consistent with the very high degree of DNA degradation evident in this sample (D19/D18 > 20). Sample #4 was inhibited, as indicated by nuTH01 and mtND1 qPCR amplification curves with shallow slopes and reduced final plateau values (data not shown). Due to the presence of co-extracted inhibitors in this sample, the nuTH01 qPCR assay underestimated the quantity of nuclear DNA, leading to STR amplification of excess template and to unusually intense TH01 and D19 allele signals. Sample #30 gave an unsuccessful HVI/HVII duplex amplification; this sample was extracted from a sperm cell fraction that contained very little nuclear or mitochondrial DNA. Several other sperm cell extracts (samples 31–35) gave successful HVI/HVII duplex amplifications despite having very low or even undetected quantities of mitochondrial DNA, indicating that the mtND1 portion of the assay significantly underestimated the mitochondrial quantity in these particular samples. As discussed earlier in this paper, such underestimations of DNA by the mtND1 qPCR assay are expected when the ratio of mitochondrial to nuclear genome copies is low, as is the case for these sperm fraction samples. For non-sperm-cell-fraction samples, the empirical ratios of mitochondrial to nuclear genome copies (mt#:nu#) were seen to range from 90 to 14,000. The breadth of this range is likely due, in part, to intrinsic differences in the mt#:nu# for the various tissue types and, in part, due to differences in the extent of DNA fragmentation in these samples, as the short mitochondrial ND1 qPCR target sequence detects smaller DNA fragments than does the longer nuclear TH01 target sequence. It is worth noting that for any sample exhibiting an empirical mt#:nu# greater than 2000, there was corresponding evidence for DNA degradation in the STR intensity ratios (D19/D18 > ~4), suggesting that genome copy number ratios estimated from the duplex qPCR assay can provide useful information about DNA fragmentation.

Conclusions

We have developed a duplex qPCR assay for the specific quantification of human nuclear and mitochondrial genomes in a variety of forensic-type samples. The nuclear portion of the assay (nuTH01) quantifies DNA in samples with concentrations ranging from ~10 pg/μL to at least 25 ng/μL, while the mitochondrial portion of the assay (mtND1) provides quantifications over the range ~4 copies/μL to ~2 million copies/μL. The quantifications were suitably accurate and precise for determining template quantities for nuclear STR genotyping and mitochondrial HVI/HVII sequencing applications.

For quantifying DNA in highly degraded samples, our results indicate that the length of the qPCR target sequence is an important consideration when selecting an assay for forensic use. For the purpose of STR genotyping, the nuTH01 portion of the duplex qPCR assay, which uses a ~170–190 bp target sequence, was found to be well suited for estimating nuclear DNA quantities in highly degraded samples. Because this assay detects and quantifies DNA fragments that are of the same general length as the STR alleles amplified in commercial genotyping kits, it provided an optimal estimate of the quantity of nuclear template to amplify for successful STR genotyping. By contrast, less than optimal results were obtained when these same highly degraded samples were quantified using a nuclear qPCR assay with a much shorter target sequence (62 bp). This assay, by detecting shorter DNA fragments, overestimated the quantity of STR-sized fragments, resulting in a relatively high proportion of under-amplified and undetected STR alleles. Slot blot quantifications of the same highly degraded samples resulted in underestimated quantities of nuclear DNA, leading to over-amplification of excess template, off-scale STR peaks, and undesired artifact signals.

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