Quantitation of DNA for Forensic DNA Typing by qPCR (quantitative PCR): Singleplex and Multiplex Modes for Nuclear and Mitochondrial Genomes, and the Y Chromosome.

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Abstract

Several new qPCR assays have been developed in single and multiplex modes for quantifying DNA in forensic samples. These assays, listed below, were developed to improve the accuracy and precision of DNA quantifications, as well as to provide tools to increase the efficiency and throughput of the analysis of complex forensic samples:

(i) a duplex assay that quantifies simultaneously the human nuclear and mitochondrial genomes. This assay was developed to quantify DNA in the degraded samples that are often encountered in missing person's cases. It has been optimized and validated for forensic casework.

(ii) a triplex assay that quantifies two human nuclear target sequences, one target that is ~170-190 basepairs (bp) in length and the other 67 bp in length, and that also includes an internal positive control (IPC) assay. By comparing quantifications from the two differently sized nuclear target sequences, the assay effectively estimates the extent of DNA degradation in a sample. Moreover, the IPC portion of the assay provides a means for detecting for the presence of co-extracted PCR inhibitors. In addition to the triplex assay, a duplex assay, which quantifies the long nuclear target sequence and detects the IPC, has also been developed.

(iii) several singleplex assays to quantify specifically the human Y-chromosome.

All assays (i-iii) were developed utilizing 5'-hydrolysis ("TaqMan®") detection chemistries for data collection on the ABI 7000 instrument. Most features should be portable to other real-time qPCR instruments capable of multiplex detection and so should be of general utility to the forensic community. Two of the assays ((i) and (ii)) have been described in manuscripts accepted for publication. We have also initialized formalized training in the application of qPCR to forensic samples through a course that is offered through the California Criminalistics Institute. This course includes discussion of the theory of qPCR, as well as hands-on laboratory and data analysis experiences with real-time qPCR assays developed here and elsewhere.

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Executive Summary:

The quantitation of DNA plays a central role in all areas and applications of forensic DNA analysis. The careful evaluation of the quantity of DNA extracted from biological samples is an imperative for DNA typing via the Polymerase Chain Reaction (PCR). For example, very discrete windows of input DNA concentration are allowed for balanced amplification of the 13 CODIS STR loci with the commercial kits that are commonly used for forensic casework and databank genotyping. Stochastic effects peculiar to the PCR take over above and below a narrow range of DNA optima for the typing assays. In addition, in the forensic arena, quantitation is often crucial as samples may be limited. Surprisingly, the DNA quantitation methods commonly employed in forensics are often less than accurate, are time consuming, and require the consumption of a substantial percentage of what may be a precious sample. In general, forensic analysts have learned to work with less than ideal quantitation methods, which are thought by many to be "good enough."

In order to improve the accuracy and precision of DNA quantifications, as well as to provide tools to increase the efficiency and throughput of the analysis of complex forensic samples, we have developed several new quantitation assays based on real-time quantitative PCR (qPCR) methods. These assays include:

(i) a duplex qPCR assay that quantifies simultaneously the human nuclear and mitochondrial genomes;

(ii) a triplex assay for assessing the quantity and quality (DNA fragmentation and presence of PCR inhibitors) of human nuclear DNA;

(iii) several singleplex assays to quantify human male (Y-chromosomal) DNA.

The nuclear-mitochondrial duplex qPCR assay (i) was developed primarily for the type of degraded and compromised samples that are commonly encountered in missing persons' evidence. For such evidence, the extracted DNA can be so degraded or so limited in quantity that attempts to genotype using the standard autosomal STR analysis methods are often unsuccessful. Under these circumstances, a common approach is to turn to the large number of mitochondrial copies (initially present in 100s of copies per cell) that might remain intact and available for analysis, for example by DNA sequencing. For such samples, accurate and

sensitive assessments of the quantities of both the nuclear and mitochondrial genomes are very valuable in guiding the DNA analyst to the most effective and efficient genotyping approach, and it is for this purpose that the nuclear-mitochondrial duplex qPCR assay was developed and validated. For this assay, the nuclear portion 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 included evaluations of species specificity, sensitivity, precision, and reproducibility, as well as applications to various casework-type samples. In addition, a series of DNase-degraded samples were quantified using three methods: the nuclear-mitochondrial duplex qPCR assay; a qPCR assay that uses a much shorter (62 bp) target sequence; and slot blot hybridization. For non-degraded and moderately degraded samples in the series, all three methods were suitably accurate for estimating the quantity of nuclear DNA template to achieve successful STR amplification and genotyping. However, for highly degraded samples, the duplex qPCR assay provided better estimates of nuclear template than did either the 62 bp qPCR assay, which overestimated the quantity of STR-sized DNA fragments, or slot blot hybridization, which underestimated the quantity of nuclear DNA. The nuclear-mitochondrial duplex qPCR assay has been validated for casework in the California DOJ Missing Persons DNA Program and is anticipated to go “on-line” by May 2005.

In the course of developing the nuclear-mitochondrial duplex qPCR assay, it became clear that an assay that could simultaneously assess DNA quantity and quality (i.e., degree of fragmentation, presence of inhibitors) would be very useful for highly compromised samples. To this end, we developed a multiplex quantitative PCR assay (ii) to amplify target sequences of different length, which allows for the assessment of DNA degradation in samples of forensic interest. The targets were chosen to provide quantification and fragment length information relevant to the STR amplification targets commonly used for forensic genotyping. The longer target (nuTH01, 170-190 bp) spans the TH01 STR locus. Although not one of the longest loci used for STR genotyping, it was chosen as a good compromise given the target length limitations on qPCR efficiency with TaqMan® detection. The shorter target (nuCSF, 67 bp) was designed in the upstream flanking region of the CSF1PO STR locus. In addition to these human nuclear targets, the assay

includes an internal PCR control (IPC) target sequence to allow for an assessment of PCR inhibition. The assay was rigorously tested on samples with varying amounts of degradation, and the ratio of nuCSF:nuTH01 quantifications was shown to provide a good estimation of the degree of degradation present in a sample. This estimate, along with the internal control for PCR inhibition, provides a valuable tool for post-extraction sample assessment. In addition, this triplex assay has been evaluated for species specificity, precision, and sensitivity to PCR inhibition.

The development of a qPCR assay specific for the human Y-chromosome was motivated primarily by its potential utility for the analysis of sexual assault casework samples. Such an assay would allow the analyst to differentiate those samples that contain sufficient male DNA for genotyping from those that contain so little male DNA that further analyses, at least by the STR methods currently in common use, would be uninformative. Moreover, the *combined* quantitation information from a Y-specific qPCR assay and an autosome-specific qPCR assay could provide an estimate of the *relative* amounts of human male and female DNA in an extract. This information could then be used to differentiate those samples that would benefit from "standard" autosomal STR genotyping approaches (i.e., those samples with maleDNA:femaleDNA ratios greater than ~1:10) from those samples that would be analyzed more productively using Y-specific amplification methods (e.g., Y-STRs). To this end, we developed several Y-specific qPCR assays. These assays have been evaluated for their specificity to the Y-chromosome, as well as for sensitivity and precision. The development of an autosomal-Y duplex qPCR assay is currently ongoing.

We have presented our qPCR results at several forensic venues (the 4th and 5th Annual DNA Grantees' Meetings in Washington (DC), the 14th Promega International Symposium on Human Identification in Phoenix (AZ)), and two manuscripts have been accepted for publication. In addition, we offer training in forensic aspects of qPCR through a California Criminalistics Institute course. This course includes a lecture component, as well as hands-on laboratory and computer data analysis experience with various forensic qPCR assays.

Introduction

The quantitation of DNA plays a central role in all areas and applications of forensic DNA analysis. The careful evaluation of the quantity of DNA extracted from biological samples is an imperative for DNA typing via PCR. For example, very discrete windows of input DNA concentration are allowed for balanced amplification of the 13 CODIS STR loci with the commercial kits that are commonly used for forensic casework and databank genotyping. Stochastic effects peculiar to the PCR take over above and below a narrow range of DNA optima for the typing assays. In addition, in the forensic arena, quantitation is often crucial as samples may be limited. Surprisingly, the DNA quantitation methods commonly employed in forensics are often less than accurate, are time consuming, and require the consumption of a substantial percentage of what may be a precious sample. In general, forensic analysts have learned to work with less than ideal quantitation methods, which are thought by many to be "good enough."

Yield gel procedures using ethidium bromide fluorescence provide information about the quantity and quality (in terms of overall molecular weight). This procedure, however, may require the consumption of a significant amount of sample, due to its poor sensitivity, and does not result in a quantitation that is specific to human DNA. In addition, quantitation results obtained by this method are only relatively accurate. Other quantitation approaches based on fluorescent detection, such as those using PicoGreen™ (1) or SYBR™ Green (2), also suffer from lack of human specificity since they are intercalating dyes that detect for the presence of any sources of double-stranded DNA. QuantiBlot™, another commonly employed technique in the forensic laboratory (3), is more sensitive than gel electrophoresis and utilizes a human (primate) specific probe. The QuantiBlot™ method is, however, labor intensive to perform and suffers from a lack of accuracy. More recently, an ingenious method, the AluQuant™ Human DNA Quantitation System (4), has been developed for human-specific quantitation of forensic samples without requiring immobilization of the sample as QuantiBlot™ does. This procedure employs a series of enzymatic reactions to produce a luminescent signal proportional to the quantity of human DNA present.

The intensification of large scale suspect-less casework programs and, more recently, the implementation of teams devoted to the identification of human remains by means of DNA typing

suggest that DNA quantitation, which has been done so far by labor-intensive techniques and likely not susceptible by their very nature to great productivity gains, would benefit from fresh approaches. Clearly, the efficiency of forensic DNA analysis would benefit from any new mode of quantitation that would be more accurate, more susceptible to automation, and more sensitive for estimating quantities in low-copy number samples (Table 1).

TABLE 1 - *A comparison of forensic DNA quantification methods.*

Method	Human Specific/ Sample Investment	Sensitivity (pg*)/Accuracy	Instrumentation Requirements	Time to Completion (hrs)	Samples per Quantitation Event	Personnel Time Required	Potential Automation/Multiplex
Ethidium Bromide	no/high	1000/low	low	2	24	high	no/no
PicoGreen™	no/low	50/high	high	1	86	low	yes/no
QuantiBlot™	yes/low	25-50/low	low	4-6	32	high	no/no
AluQuant™	yes/low	100/high	medium	4	90	low	yes/no
qPCR	yes/low	~30/high	highest	2	84	lowest	yes /yes

* assuming ~3.3 pg per haploid human nuclear genome

Objectives

In our NIJ grant proposal, we proposed to use real-time quantitative PCR (qPCR), a method commonly employed for a variety of molecular diagnostic and biotechnology procedures, for the quantification of DNA in forensic samples. In particular, we were interested in developing qPCR assays to quantify the human nuclear genome, the human mitochondrial genome, and the human Y-chromosome, and, as appropriate, to multiplex these assays in order to quantify more than one genome (or chromosome) in a single reaction. For example, we anticipated that a human-specific nuclear/mitochondrial duplex assay would be an especially useful tool for quantifying DNA in the highly compromised samples that are commonly encountered in missing person's cases. Such an assay would allow the analyst to quickly differentiate those samples that contain enough nuclear DNA for successful STR genotyping from samples that contain such small quantities of DNA that they would only benefit from mitochondrial haplotyping or from those that are essentially bereft of any human DNA. In addition, we expected that a human-specific nuclear/Y-chromosome duplex assay would be especially useful for increasing the throughput of analysis for

the large number of suspect-less sexual-assault evidence kits that are being processed, allowing the analyst to efficiently identify samples that contain sufficient quantities of male DNA for genotyping. Using such newly developed tools, we anticipated that increases in the efficiency of DNA analysis would lead to improved genotyping results at lower costs. As described in our proposal, for each assay, our objectives were to develop, optimize, and validate according to SWGDAM guidelines, and also to make the assays available to the forensic community through presentations, publications, and training.

Progress Toward Meeting Objectives

As we will describe in the remainder of this final report, we were successful in meeting the majority of our objectives. We have developed a number of qPCR assays that are useful for quantifying DNA in forensic samples: a nuclear/mitochondrial duplex assay and several Y-specific qPCR assays. The nuclear/mitochondrial duplex assay has undergone extensive on-site validation, a standard operating protocol has been written for this assay, and it is expected to go "on-line" in the California DOJ Missing Persons DNA Program (MPDP) by May 2005. Much of the developmental work surrounding these assays has been presented at forensic meetings as oral or poster presentations (5-8), and two manuscripts have been accepted for publication (9-10). In the area of training, we have offered and will continue to offer, as demand requires, a one-week course that includes theory, hands-on laboratory, and computer instruction in forensic qPCR. The first offering of this course, "Advanced DNA Extraction and Quantification with Real Time qPCR" (R220), was offered through the California Criminalistics Institute and was attended by a total of 12 forensic DNA analysts, seven from the California DOJ Missing Persons DNA Program and five from non-DOJ agencies in California (the LA County Sheriff Dept, the Fresno County Sheriff Dept, the Orange County Sherriff Dept, the San Diego Police Dept, and the San Diego County Sheriff Dept).

Throughout the development and validation of the aforementioned qPCR assays, we gained experience in collecting data on various types of samples, and we began to identify critical issues for obtaining accurate qPCR quantifications. In particular, as part of the validation of the duplex nuclear/mitochondrial assay, experiments with samples containing highly degraded DNA indicated that the

length of the qPCR-amplified target sequence was one of the most important issues for obtaining accurate quantifications for STR genotyping applications. This observation motivated us to develop a duplex qPCR assay that could simultaneously amplify and quantify two nuclear target sequences of different length, allowing for an assessment of the extent of DNA degradation in samples. This duplex "degradation qPCR assay" was subsequently augmented to include an Internal PCR Control (IPC) amplification. The resulting triplex qPCR assay, which is described in some detail later in this report and in a submitted manuscript (10), provides an estimation of both quantity and quality (degree of degradation and presence of co-extracted PCR inhibitors) of the extracted DNA. It should be very useful as a diagnostic tool for analyzing DNA in highly compromised casework samples.

qPCR Fundamentals

The quantification of DNA via the real-time detection of PCR-amplified target sequences was first described by Higuch et al. in a 1993 report (11) that outlined the basic principles of what the authors called "kinetic PCR" but what is now more commonly referred to as qPCR. Although these early experiments used home-built instrumentation, the utility of qPCR for quantifying polynucleic acids was quickly recognized and a commercial instrument was on the market by 1996 (the Perkin-Elmer ABI 7700 Sequence Detection System). The increasing use and acceptance of qPCR in biomedical research and in molecular diagnostics is evident from both the rapid growth in peer-reviewed papers that use the method (8 publications in 1998, >500 in 2002) (12) and the current availability of real-time qPCR instruments from a variety of vendors (e.g., ABI, Roche, BioRad, Stratagene, Corbett). As mentioned in a recent review article (13), qPCR is "a technique whose time has come."

The quantification of DNA by qPCR relies on the detection of amplified product ("amplicon") at each cycle of the PCR. Detection of the PCR product is accomplished with the use of thermal cyclers that are capable of measuring real-time fluorescence changes due to amplicon production. The result is an "amplification curve" (Figure 1) for each sample. As indicated in this figure, the amplification curve can be separated into at least three phases: (i) the "lag" phase, during which the PCR is occurring efficiently

(exponential growth), but the amplicon-induced fluorescence is at or below the background level of detection; (ii) the "exponential" phase, during which the PCR is occurring efficiently and the signal is above background; and (iii) the "plateau" phase, which occurs during later cycles of the PCR, at which point amplification efficiencies are reduced by such factors as polymerase degradation, reactant consumption, and amplicon re-annealing (in competition with binding of the PCR primers).

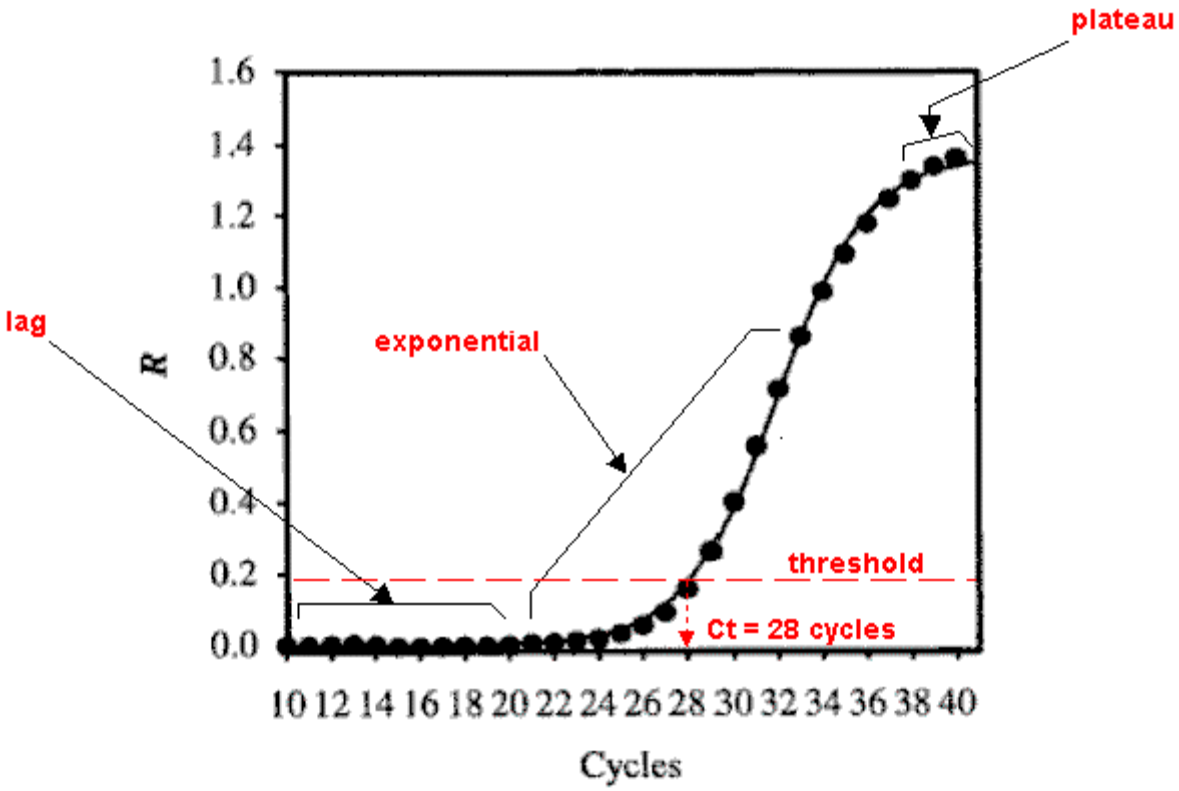
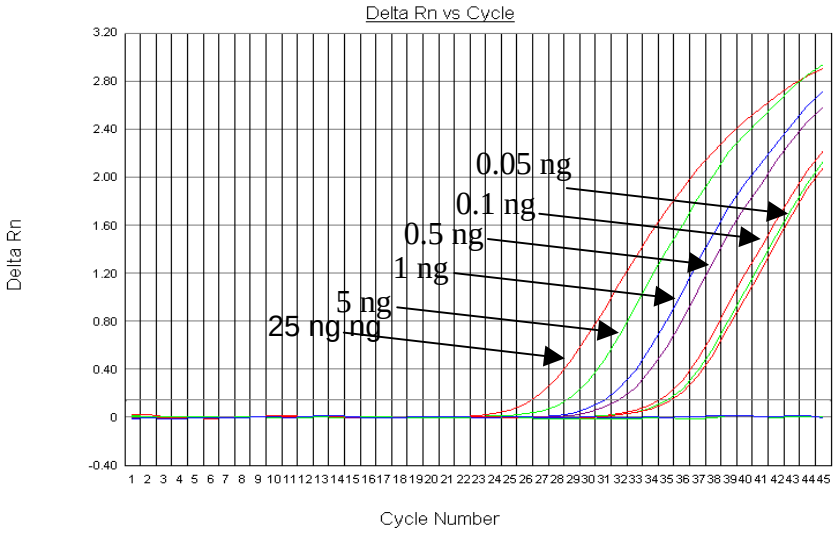


FIG. 1 - *qPCR Amplification Curve. R is the fluorescence signal at each cycle due to amplicon production. Locations of lag, exponential, and plateau phases of the curve are indicated. The Ct (or C_T) value for the curve is the cycle at which the amplification curve crosses the user-selected threshold.*

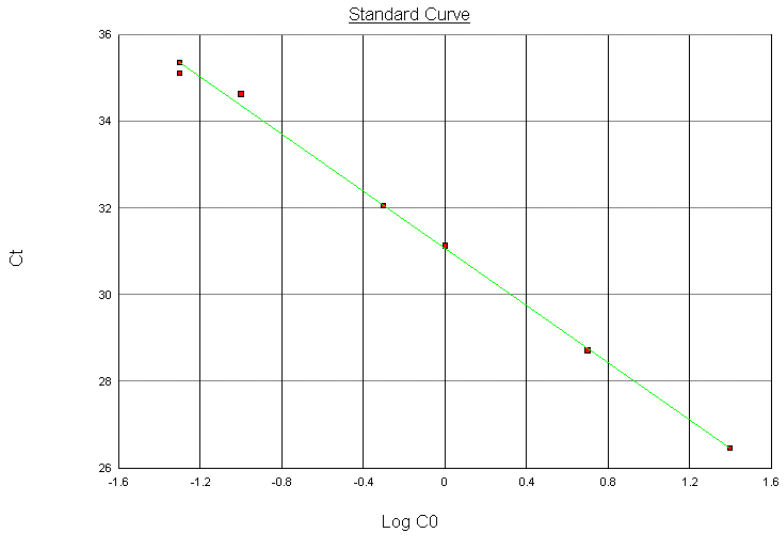
In qPCR, the accurate quantification of DNA relies on using data from the *exponential* phase of the amplification curve. In particular, for each amplification curve, a "cycle threshold" (Ct or C_T) is defined as the cycle number at which the curve crosses a fluorescence threshold that has been established for the assay (Figure 1). This threshold is selected to be high enough to avoid background noise in the fluorescence signal, but low enough to remain in the exponential portion of the amplification curve, thereby avoiding confounding effects due to the lowered PCR efficiencies encountered in the plateau phase. Qualitatively, a

low C_T indicates the presence of more initial template DNA than does a higher C_T, because the lower value indicates that fewer cycles are needed to produce sufficient amplicon for fluorescence detection. (Ideally, for a qPCR assay operating at 100% efficiency, a single cycle delay in C_T represents a 2-fold decrease in the initial quantity of template DNA in the sample.) Quantitatively, the initial amount of template DNA in an unknown sample is determined from C_T by use of a standard curve derived from qPCR data collected for a dilution series of a known DNA quantitation standard (Figure 2). Due to the exponential nature of qPCR, a plot of the C_T vs. the logarithm of the initial concentration in each standard dilution leads to a straight line for the standard curve. The quantity of DNA in each unknown sample can then be deduced from its C_T by interpolation from this line. Fortunately, these post-run manipulations of the data (e.g., determination of C_T for each sample, development of the standard curve, and interpolation to estimate DNA quantity) are semi-automated features on all commercial qPCR systems.



(a) *Amplification curves for a dilution series of standard, quantified DNA. Quantities shown are in ng of genomic DNA. Delta Rn is the normalized, baseline-corrected fluorescence signal for each sample.*

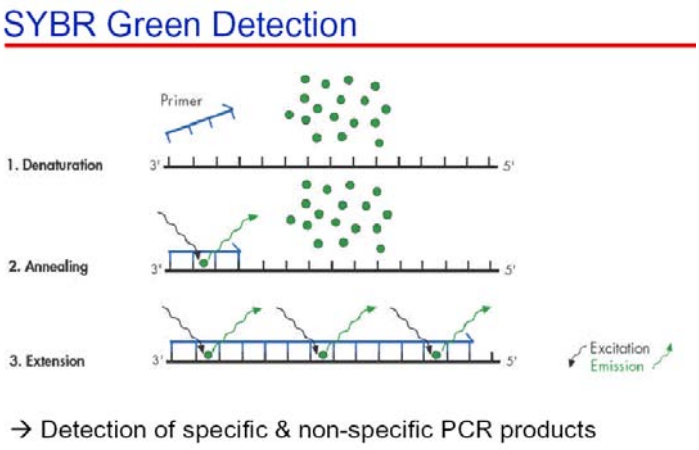
FIG. 2 - *Development of a qPCR Standard Curve. (figure continued on following page)*



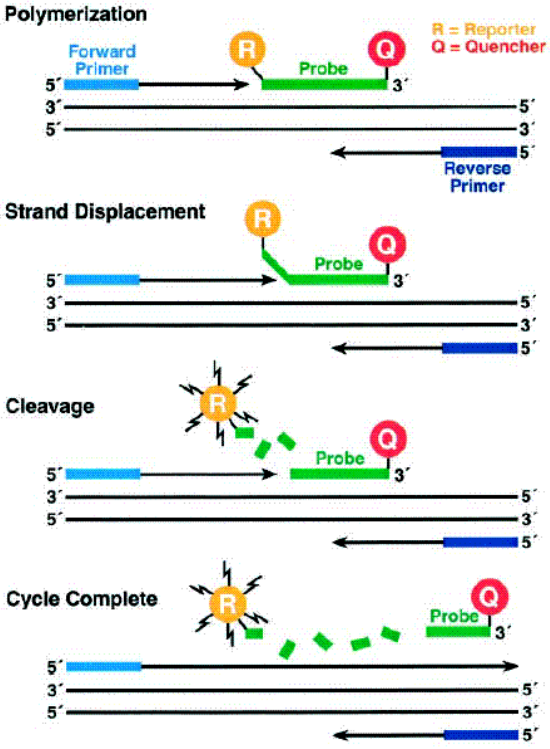
(b) *Standard curve based on data from (a). Ct is the cycle threshold as determined from the amplification curve plot. C0 is the known quantity of input template (in ng).*

FIG. 2 - *Development of a qPCR Standard Curve.(continued)*

Although a wide variety of fluorescence-based detection chemistries have been developed for real-time qPCR applications (14), many assays currently use (i) SYBR™ Green or (ii) 5'-hydrolysis ("TaqMan™") chemistry for amplicon detection. These methods are briefly presented in Figure 3. The SYBR™ Green method, which relies on the large increase in fluorescence signal when the dye binds to double-stranded DNA (i.e., to the qPCR amplicon), is typically the simpler method to implement, but is limited to singleplex quantifications. In addition, SYBR™ Green will detect non-specific amplification products, for example, due to "primer dimers" or spurious cross-reactivity to non-target sequences. TaqMan™ assays, which utilize a sequence-specific dye-labeled oligonucleotide (a "TaqMan™ probe"), are often more difficult to design and develop, but can be more specific than SYBR™ Green-detected assays. More significantly, by using differently labeled TaqMan probes for different target sequences (e.g., FAM-label for a nuclear target sequence and VIC-label for a mitochondrial target sequence), the TaqMan™ approach allows for the possibility of multiplexing two or more different assays in a single tube (17).



(a) SYBR™ Green detection (15). Upon extension, the SYBR™ Green reporter dye binds to the double-stranded amplicon as it forms. Upon binding, the emission intensity of the dye increases by many fold.



(b) TaqMan™ Detection (16). The TaqMan™ probe is complimentary to sequence of one amplicon strand between the forward and reverse primers. Each probe is dual-labeled with a reporter dye (R, e.g., FAM) on the 5' end and a fluorescence quencher (Q) on the 3' end. Upon extension, the 5'nuclease activity of Taq polymerase cleaves the TaqMan™ probe so that for each cycle of PCR an additional fluorophore is released per amplicon.

FIG. 3 - *Selected real-time qPCR detection chemistries.*

There are a number of experimental factors that influence the accuracy of qPCR quantifications. One of these is the presence of co-extracted PCR inhibitors, which can lead to underestimations of DNA quantities due to amplifications curves with delayed C_T's and shallow slopes. If a sample is sufficiently inhibited, the result can be the complete absence of detectable PCR product (a null amplification). Although the presence of PCR inhibition can sometimes be deduced from the shape of the amplification curve, another approach has been to spike the quantification assay with an Internal PCR Control (IPC) assay for detecting the presence of inhibitors. (We will describe this approach in more detail later in this report as part of the discussion of our triplex degradation qPCR assay.)

Another factor that must be considered when assessing the accuracy of qPCR is the extent of fragmentation of the extracted DNA. DNA samples can be highly degraded due to environmental or microbial exposures. For such samples, there will be a greater number of smaller than larger DNA fragments, a circumstance that is readily seen in the STR intensity profiles of degraded samples (e.g., off-scale short Amelogenin alleles and undetected long CSF1PO STR alleles). A qPCR assay based on the amplification of a single target sequence will necessarily quantify those DNA fragments that are as long or longer than the target sequence. As a consequence, for highly degraded samples, the quantities of DNA measured by assays that measure short and long target sequences can differ significantly. As we will discuss later in this report, for the purpose of STR genotyping, there are advantages to choosing a qPCR target sequence that measures relatively long (>150 bp) target sequences.

One additional factor that influences the accuracy of the qPCR measurement is the accuracy of the quantitation of the "known" DNA that is being used to develop the standard curve. The qPCR assay can be no more accurate than the calibration standard. Although NIST is currently looking into the establishing a standard reference material for DNA quantifications, forensic labs often rely on the use of pre-quantified vendor-supplied DNA extracts that have been quantified by UV-vis spectroscopy.

Advantages of qPCR

qPCR has a number of features, summarized below, that make it particularly attractive for quantifying DNA in forensic samples:

(i) qPCR is based on the same principle (the polymerase chain reaction) as are the techniques commonly used for forensic genotyping (e.g., the PCR amplification of short tandem repeats in chromosomal DNA and of polymorphic regions in the mitochondrial genome). Consequently, qPCR provides particularly relevant information about the quantity of "amplifiable" DNA in sample. That is, the qPCR method is sensitive not only to the quantity of DNA, but also to the quality of DNA (e.g., degree of DNA degradation, amount of co-extracted PCR inhibitors). Techniques such as membrane hybridization (e.g., QuantiBlot™), solution hybridization (e.g., AluQuant™) and dye binding/intercalation (e.g., PicoGreen™) are not based on PCR and do not necessarily provide relevant quantifications of "amplifiable" DNA.

(ii) qPCR assays can be designed to be very specific to the DNA target sequence of interest. For example, primate-specific PCR primers can be used for quantifying nuclear DNA in forensic samples, and assays can be similarly designed to be highly specific for other forensically relevant chromosomes (e.g., to the Y-chromosome) or genomes (e.g., to the mitochondrial genome or to genomes of non-human organisms).

(iii) qPCR assays can be designed to amplify, detect and quantify more than one genome/chromosome of interest in a single tube. This ability to multiplex assays relies on the use of probes labeled with different fluorophores to detect different DNA target sequences (e.g., FAM for one target sequence, VIC for a different target sequence), as well as on the use of instruments capable of separately detecting the fluorescence emissions from the different probes. Although multiplex assays are more challenging to develop than singleplex assays, they offer the advantages of providing the maximum amount of information while consuming a minimal amount of extract, analyst time and labor.

(iv) Because of the exponential nature of PCR, qPCR assays have a very large dynamic range of detection. Assays can be sensitive enough to detect down to just a few copies of the target sequence of interest, although the accuracy and precision of qPCR assays typically decrease when fewer than ~10 copies of target sequence are present for detection. And, under most conditions, qPCR assays can accurately quantify tens of thousands of target sequences. Consequently, qPCR dynamic detection ranges readily span the roughly three orders of magnitude (~30 pg - ~30 ng of nuclear DNA) needed for most forensic applications.

(v) Compared to most of the commonly used quantification methods (the non-human-specific PicoGreen™ method being the exception), the experimental protocols for qPCR assays are straightforward, laborsaving, and amenable to automation. Moreover, qPCR data analysis is largely automated.

Cal-DOJ-Developed qPCR Assays

The remaining sections of this report describe selected aspects of the development and validation of the qPCR assays that comprised the bulk of work performed under the NIJ award. In the first section (i), we describe a duplex qPCR assay capable of simultaneously quantifying human nuclear and mitochondrial DNA. This assay, which was developed primarily for quantifying DNA in the type of highly compromised samples encountered in missing persons programs, was found to be particularly suited for quantifying highly degraded DNA for STR typing. In the second section (ii), we describe a triplex qPCR assay that was developed to assess DNA quantity and quality in forensic samples. In the third section (iii), several Y-chromosome-specific qPCR assays are described, which will be used in a duplex autosomal-Y qPCR assay that is currently under development.

(i) 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

Introduction

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 (18). The slot blot method, though it offers excellent specificity and good sensitivity (3,19), 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 (4,20) and several end-point PCR assays (21-23). Another approach that is proving to be useful for forensic DNA quantifications is real-time qPCR, a method widely used in biomedical research and molecular diagnostics

(24-27). Quantitative PCR assays have been developed for various forensic applications, including the quantification of human nuclear DNA (28-30), human mitochondrial DNA (31-33), and human Y-chromosomal DNA (30,33).

In this section, we describe a new 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 (34,35). 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 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 (36) and in samples that contain high levels of microbial contamination (35); (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 (31) 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. (37) 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. (33,38) 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. (31) 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 (18), 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. (31), 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. 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, non-probative 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 (39). For non-root hair shafts (2 cm portions), extractions were performed by either (i) a magnetic-bead-based protocol (40) 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 -20ºC, except for Chelex-extracted samples, which were stored at +4 ºC.

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 ºC 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 mitochondrial control regions of HVI and HVII were amplified in a duplex PCR using reagents from the LINEAR ARRAY™ Mitochondrial DNA HVI/HVII Region-Sequence Typing Kit (Roche Applied Science) (41,42). 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 2) 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 (43), 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 (44). Sequence information was also obtained from the STRBase (45) and MitoMap (46) websites.

TABLE 2 – *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 (47, 48). 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 (49). 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*) (50). 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 (51), the On-Line Mendelian Inheritance in Man (OMIM) website (52), and, for mitochondrial sequences, the MitoMap website (46). Once developed, singleplex and duplex qPCR assays were optimized based on published recommendations (53,54).

nuTH01-mtND1 qPCR Quantifications

Primer and probe sequences for the nuTH01 and mtND1 qPCR assays are provided in Table 2. 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 minute 95 ºC polymerase activation step, followed by 45 cycles of 2-step qPCR (15 s of 95 ºC denaturation, 60 s of 60 ºC 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 ºC /min) from 60 ºC to 95 ºC.

Amplification curves were analyzed by using empirically established cycle threshold and baseline settings for each type of assay (for nuTH01, cycle threshold = 0.15, baseline 6-18 cycles; for mtND1, cycle 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 (55). 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 2 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 = $100[(10^{(-1/slope)} - 1)]$) (56).

Oligonucleotide Melting Profile Calculations

Melting profiles for selected TH01 alleles were calculated using MELT94, a DOS-based program available on-line (57). This program uses the theory and equations of Poland (58) and of Fixman and Freire (59) to calculate variations in thermal stability along the sequence of a DNA fragment (60). 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 (61,62) and DNA mutation rates (63) 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 Figure 4, which displays the relative positions of the PCR primers, the STR repeat region, and the 5'-FAM/BHQ fluorogenic probe.

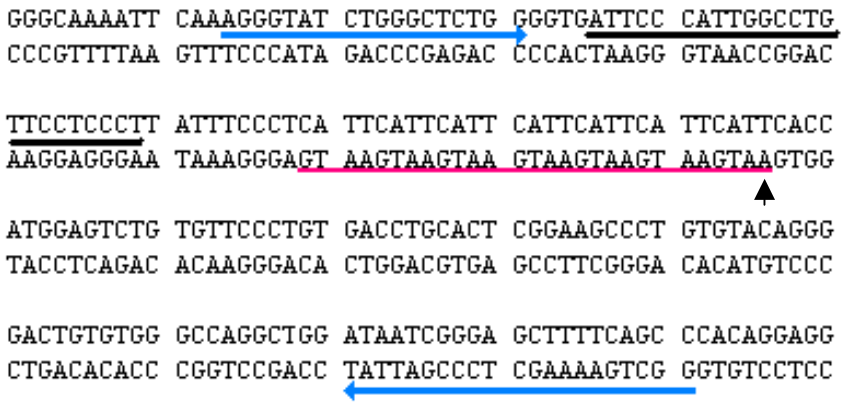


FIG. 4 - *Sequence information for the nuTH01 portion of the duplex qPCR assay showing relative positions of the forward and reverse primers (horizontal arrows), the TaqMan® detection probe (underlined on top strand), and the $(CATT)_7$ STR region (underlined on bottom strand). The small, vertical arrow 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 Figure 8.*

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) (64). This target was chosen because previous cross-species sequence homology studies (65, 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 2).

The mtND1 assay quantifies a relatively short (69 bp) target sequence, one strand of which is shown in Table 2 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 (54).

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 (66,67). 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 (54). 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 (Figure 5). This comparison indicated that the qPCR assays, whether run in singleplex or in duplex, gave nearly 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 Figure 6, the nuTH01 amplification curves and the

corresponding CT values did not change significantly, even in the presence of an unrealistic excess of mitochondrial copies.

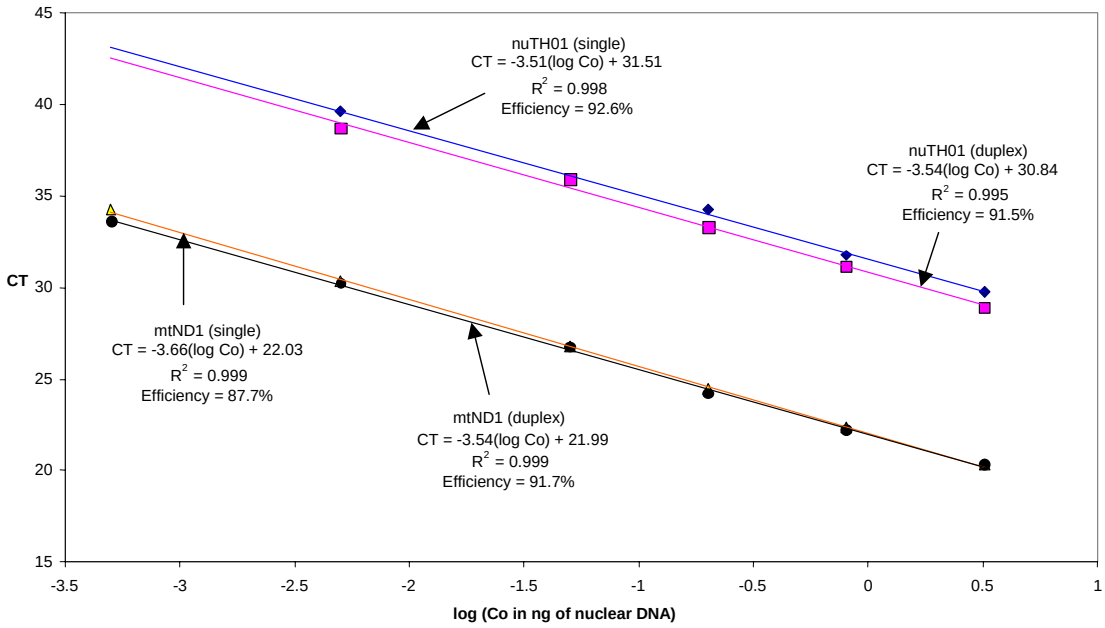


FIG. 5 - *Standard curves for nuTH01 and mtND1 qPCR assays run in singleplex and in duplex modes. For the nuTH01 qPCR assays, the data points shown are averages of duplicate measurements on samples containing 3.2, 0.8, 0.2, 0.05 and 0.005 ng of Promega genomic DNA (female). For the mtND1 qPCR assays, 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.*

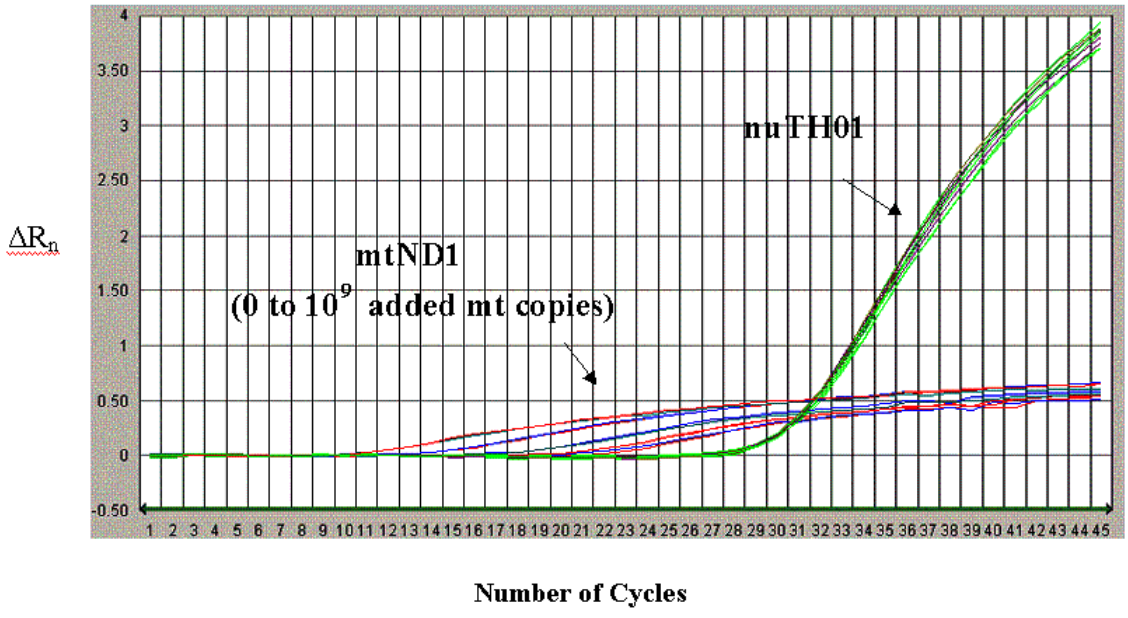


FIG. 6 - *qPCR amplification curves for nuTH01-mtND1 duplex assays with 3 ng of control DNA plus 0, 10^6, 10^7, 10^8, and 10^9 excess copies of single-stranded mtND1-standard. Assays were run in duplicate.*

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 (Figure 7).

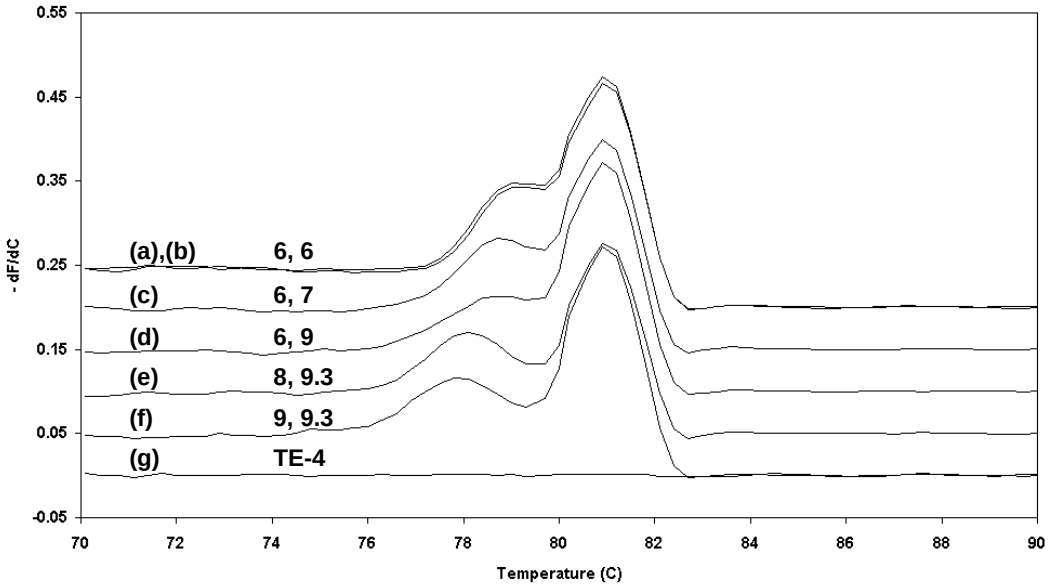


FIG. 7 - *SYBR® Green melting curves obtained for DNA extracts from six different individuals and a negative control sample using the TH01 qPCR assay. The TH01 genotype for each sample is indicated. Traces (a) and (b) are from two different individuals, both genotype 6,6 at TH01.*

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 ºC (the extension temperature of the final PCR cycle) to ~95 ºC. 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 7, 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 ~ 81 ºC) and a lower melting temperature "shoulder" (T_m ~ 78-79 ºC). 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. 8). Such calculations separate the TH01 amplicon into two distinct melting domains - a low melting temperature 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. 8, inset), a prediction that is in qualitative agreement with the trend seen for the experimental melting curves shown in Figure 7. 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. 8), consistent with the allele-independent, overlapping melt transitions (T_m ~81 ˚C) shown in Figure 7.

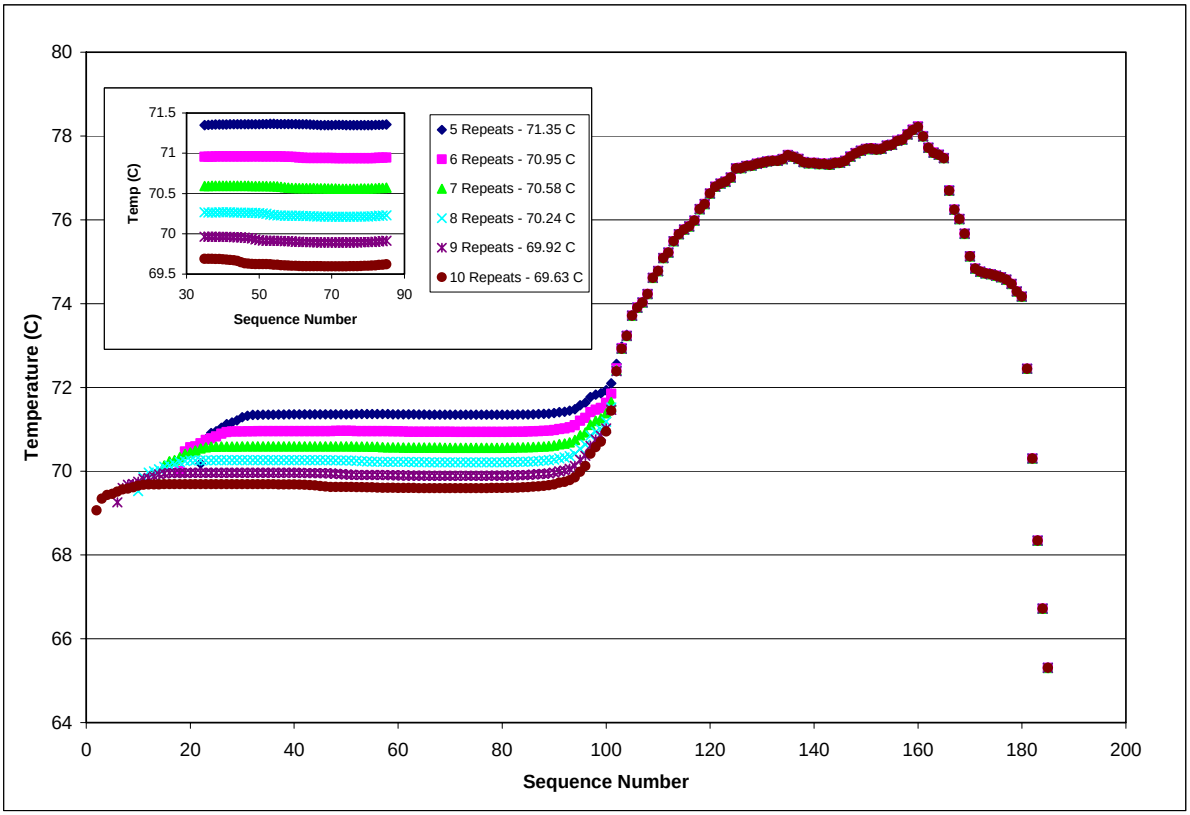


FIG. 8 - *Calculated melting profiles for TH01 amplicons (STR alleles 5-10) as generated by MELT94 software. Sequence Number 96 corresponds to the position of the small, vertical arrow in Figure 4. The inset figure shows an expansion of the melting profiles in the STR region.*

The stepwise melting of small DNA fragments due to sequence-dependent melting domains has been reported (68), and similar sequence-dependent denaturation phenomena form the basis for separations by such techniques as denaturing gel gradient electrophoresis (DGGE) (69). 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 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 3. 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.

TABLE 3 - *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.

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 4, 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 4 - *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 (61,62) and on sequence homology investigations, we 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 9 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 minutes 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 minutes 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."

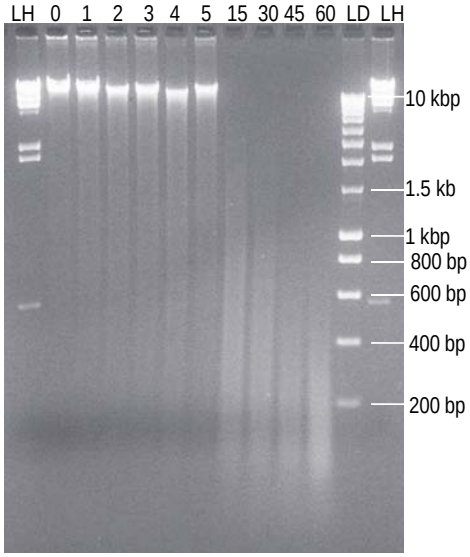


FIG. 9 - *HL60 DNase degradation series (EtBr-stained). Lanes labeled 0 to 60 indicate DNase treatment time in minutes. Lanes labeled LH and LD represent λ-HindIII and low-mass DNA ladder, respectively.*

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 three methods are summarized graphically in Figure 10, 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.

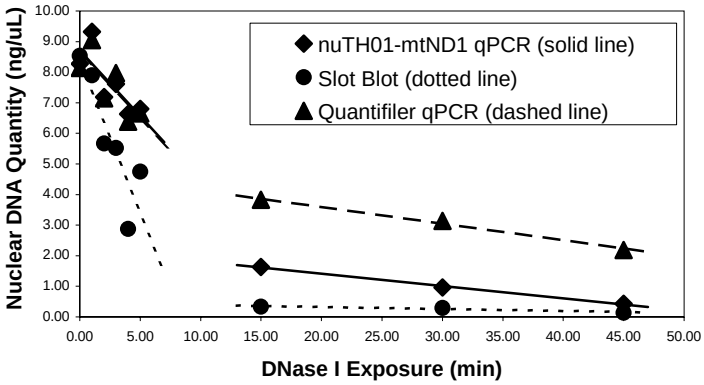


FIG. 10 - *Nuclear quantifications of DNase-degraded HL60 samples by nuTH01-mtND1 duplex qPCR, Quantifiler™ qPCR, and slot blot hybridization. For the qPCR assays, each point represents the average of duplicate quantifications. For slot blot, each point represents a single quantification. Lines represent linear least-squares fits to early (0-5 min) and late (15-45 min) time points in the degradation series.*

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 (28,35,36), 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 (28). 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 170-190bp 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 Figure 9 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 Figure 10 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 11 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

	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. 11 - *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).*

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 Figure 11 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 minutes 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. 11C and 12B, and Table 5).

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 (Figure 11). 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 5 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-

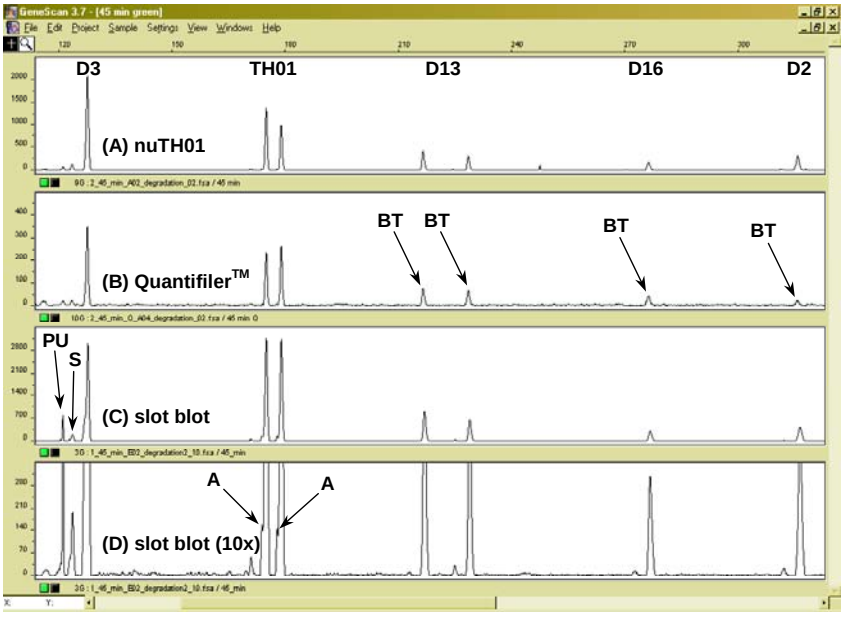
amplified to the extent that they resulted in off-scale peaks, nor were the long (~293 bp) D18 STRs under-amplified to the extent that they went undetected.

TABLE 5 - *Selected AmpFlSTR® 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 minutes, 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 Figure 10). 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 (72).*

	nuTH01-mtND1 Duplex qPCR				Quantifiler™ qPCR				Slot Blot Hybridization			
DNase Exposure (min)	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

FIG. 12 - *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.*



STR amplifications based on slot blot quantifications resulted in very few undetected alleles, even for the most highly degraded samples (Fig. 11). However, the electropherograms shown in panels (C) and (D) of Figure 12 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 5). 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 5). 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 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 Figure 13.

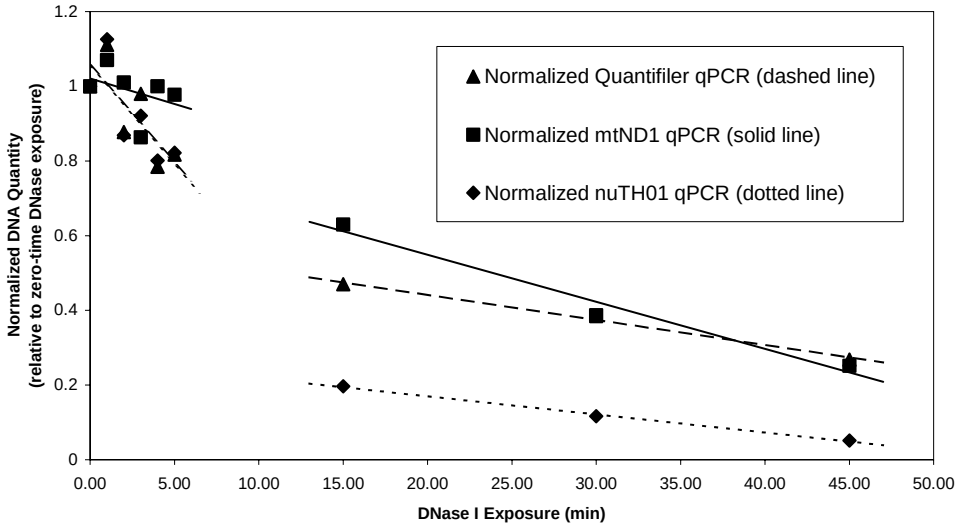


FIG. 13 - *Normalized quantifications of DNase-degraded HL60 samples by mtND1 (duplex) qPCR, Quantifiler™ qPCR, and nuTH01 (duplex) qPCR. Each point represents the average of duplicate quantifications. For each assay, the DNA quantities are normalized relative to the measured quantity at 0 minutes of 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 x 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.*

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 (42, 70). 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 Figure 14.

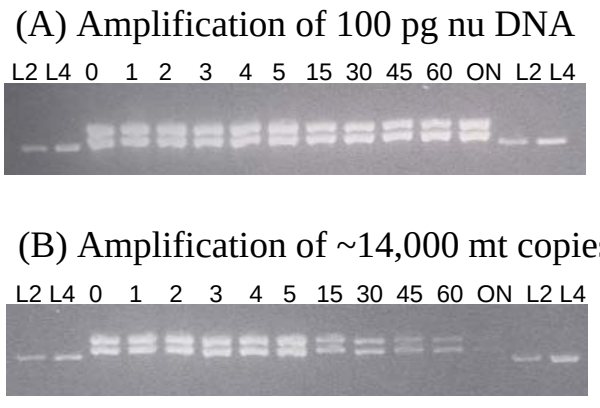


FIG. 14 - *HVI/HVII post-amplification yield gel results for DNase-degraded HL60 samples. Gel is EtBr stained. Each pair of bands represents the HVI (~444 bp) and HVII (~416 bp) PCR products from 34 cycles of the duplex amplification. Numerical lane designations (0 - 60) represent DNase digestion times 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.*

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 (42). Although the manufacturer recommends using 3-10 ng/20 µL of PCR product in each 50 µL cycle-sequencing reaction (71), 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 Figure 14, 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, Figure 14 (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 the 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.

For highly degraded DNA samples, it is clear that qPCR quantification results depend upon the length of the amplified target sequence. Although this dependence complicates quantification by qPCR, it also holds promise for providing information about the degree of DNA fragmentation in degraded samples. For example, in the nuTH01-mtND1 duplex qPCR assay, the relatively long nuclear target sequence makes

quantification of the nuclear genome more sensitive to the degree of fragmentation than quantification based on the shorter mitochondrial target sequence. This difference in sensitivity makes it possible to relate the degree of DNA fragmentation in a sample to the empirical ratio of mitochondrial genome copies to nuclear genome copies (mt#:nu#) as determined by the nuTH01-mtND1 duplex qPCR assay. Specific examples of this relationship are seen in the STR and qPCR data obtained from the HL60 DNase-degradation series. For the non-degraded HL60 sample, the mt#:nu# was ~530, as estimated from the duplex qPCR assay, and the degree of fragmentation was low, as expected and as deduced from the low D19/D18 STR intensity ratio of 0.76 (Table 5). For moderately degraded HL60 samples (1-5 min DNase treatment), the qPCR-based mt#:nu# ratios remained fairly constant, varying between ~390 to ~510, as did the corresponding D19/D18 STR intensity ratios, varying between 0.68 to 0.81 (Table 5). However, for the more highly degraded HL60 samples, the qPCR-based mt#:nu# ratios increased dramatically and systematically (1400 for 15 min, 1700 for 30 min, 2600 for 45 min, 3700 for 60 min DNase treatment) in direct correspondence with increasing D19/D18 STR intensity ratios (from 2.23 to 11.10, Table 5). These related trends suggest that if one were to determine an empirical mt#:nu# ratio for an HL60 sample of unknown quality, it would be possible to use this ratio to infer an approximate degree of DNA fragmentation in that sample. Of course, the extension of such an approach to estimate the degree of DNA fragmentation for the variety of sample and tissue types encountered in real forensic samples would require empirical knowledge of the mt#:nu# ratios for purified extracts from the corresponding non-degraded samples and tissues.

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 6. 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-

TABLE 6 - *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	

136 bp), and D18(262-346 bp) (72). 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. Finally, the empirical ratio of mitochondrial genome copies to nuclear copies (mt#:nu#) spanned a wide range (<1 to 14,000) for the various types of DNA extracts. Depending upon sample type, degraded STR patterns (D19/D18 > ~4) were seen at mt#:nu# ratios ranging from 310 to

14,000. It is significant that for any sample exhibiting a mt#:nu# ratio greater than 2000, there was corresponding evidence for DNA degradation in the STR intensity ratios. This correspondence is consistent with our previous discussion of mt#:nu# ratios for the DNase-degraded samples and suggests that the genome copy number ratios estimated from the duplex qPCR assay can provide useful information about DNA quality.

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 dramatically underestimated quantities of nuclear DNA, leading to over-amplification of excess template, off-scale STR peaks, and undesired artifact signals.

Because the nuTH01-mtND1 duplex qPCR assay utilizes a long target sequence for detection of the nuclear genome and a short (69 bp) target for mitochondrial detection, the empirical mitochondrial-to-nuclear genome copy number ratio was seen to depend upon the degree of DNA fragmentation of the sample. In a

controlled DNase-degradation series, this copy number ratio was seen to vary from ~530 for intact DNA to ~3700 for highly degraded DNA. For these samples and generally for casework-type samples, increased mitochondrial-to-nuclear genome copy number ratios were correlated with increased occurrences of degraded STR patterns (i.e., size-dependent imbalances in STR allele intensities).

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(ii) A qPCR Assay for the Assessment of DNA Degradation in Forensic Samples

Introduction

Short tandem repeat (STR) genotyping, due to its high power of discrimination for human identification, is typically the analytical method of first choice for forensic applications. The poor quality and/or quantity of extracted nuclear DNA in samples of forensic interest, however, often preclude successful STR genotyping, resulting in partial or unsuccessful STR profiles. The successful analysis of such samples could benefit from methods that would not only assess the quantity of DNA, but also the presence and degree of DNA degradation. Reynolds et al. (73), for example, have suggested that DNA degradation could be assessed by PCR amplification and detection of two DNA target sequences of different lengths. This approach has been used by Alonso et al. (38) who have developed two singleplex qPCR assays to separately quantify short and long mitochondrial target sequences to assess degradation in forensic samples. As part of our previous development and validation of a duplex qPCR assay for quantifying nuclear and mitochondrial DNA (9 and previous section in this report), we have also shown that for highly degraded samples the quantity of DNA depends significantly on the length of the target sequence that is amplified in the assay. For highly fragmented DNA samples, a qPCR assay that amplified a 62 bp target sequence detected more DNA than a qPCR assay that amplified a ~170-190 bp target, a result

consistent with the allele-length-dependent STR intensity profiles that are commonly seen for degraded DNA (34). In this report, we describe a real-time qPCR assay for the simultaneous quantification of two different target lengths of human nuclear DNA, an assay which determines if, and to what degree, the DNA is degraded. In addition, the inclusion of a third amplification target, a synthetic oligonucleotide, provides an internal PCR control (IPC) that allows for the assessment of PCR inhibition. 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 often encountered in instances of mass disasters, mass graves, and missing persons' cases (34,35).

Materials and Methods

qPCR Assay Design

Primers and probes for the nuTH01-nuCSF-IPC triplex TaqMan® qPCR assay (Table 7) were designed using Applied Biosystems' PrimerExpress™ v2.0 software with the exception of the nuCSF forward primer (nuCSF-F) which was designed with Primer3 (74). DNA sequences for design work were downloaded from the STRBase (45) website and the GenBank resource at the National Center for Biotechnology Information (NCBI) website (44).

TABLE 7 - *Oligonucleotide sequences for nuTH01 primers and probe, nuCSF primers and probe and IPC primers, probe, and single-stranded oligonucleotide template.*

Oligonucleotide	Sequence
nuTH01-F	5'- AGG GTA TCT GGG CTC TGG - 3'
nuTH01-R	5'- GGC TGA AAA GCT CCC GAT TAT - 3'
nuTH01-probe	5'- ***FAM***-ATT CCC ATT GGC CTG TTC CTC CCT T-***BHQ*** - 3'
nuCSF-F	5'- GGG CAG TGT TCC AAC CTG AG - 3'
nuCSF-R	5'- GAA AAC TGA GAC ACA GGG TGG TTA - 3'
nuCSF-probe	5'- ***VIC***-CAA CCT GCT AGT CCT T-***MGB-NFQ*** - 3'
IPC-F	5'- AAG CGT GAT ATT GCT CTT TCG TAT AG - 3'
IPC-R	5'- ACA TAG CGA CAG ATT ACA ACA TTA GTA TTG - 3'
IPC-probe	5'- ***NED***-TAC CAT GGC AAT GCT-***MGB-NFQ*** - 3'
IPC-oligo	5'- AAG CGT GAT ATT GCT CTT TCG TAT AGT TAC CAT GGC AAT GCT TAG AAC AAT ACT AAT GTT GTA ATC TGT CGC TAT GT - 3'

The nuTH01 assay is as previously described in the preceding section (i) of this report. In brief, it spans the STR sequence at the human tyrosine hydroxylase (*TH01*) gene on chromosome 11 (11p15.5), has an amplicon length of ~170-190 bp, and utilizes a FAM-labeled hydrolysis probe for detection. The nuCSF assay targets an intron region of the human *c-fms* proto-oncogene for the *CSF-1* receptor gene on chromosome 5 (5q33.3-34). This target, located in the flanking region of the CSF1PO STR locus, was selected for its specificity for higher primates as indicated by sequence homology investigations. The nuCSF assay produces a 67 bp amplicon and utilizes a VIC-labeled TaqManMGB® probe for detection. The IPC (internal PCR control) assay targets a 77 bp synthetic single-stranded oligonucleotide that was designed to avoid homologies to sequences found in the NCBI GenBank database. The IPC assay was designed with a NED-labeled TaqManMGB® probe for use in this triplex format.

Prior to ordering any synthetic oligonucleotides, the Basic Local Alignment Search Tool (BLAST*n*) (50) on the NCBI website was used to compare potential primer and probe sequences to all available DNA sequences to avoid inadvertent homology to non-target sequences and identify possible occurrences of cross-species reactivity. Known single nucleotide polymorphisms (SNPs) were also avoided by obtaining SNP information from the NCBI SNPdB website (51) and the On-Line Mendelian Inheritance in Man (OMIM) website (52). Once developed, singleplex, duplex and triplex qPCR assays were optimized based on published recommendations (53,54).

Standards and Samples

Pre-quantified, high molecular weight human genomic female DNA (#G1521) purchased from Promega (Madison, WI) was used as the quantification standard for all experiments. Human DNA extracts for these experiments consisted of pre-quantified HL60 DNA (ATCC, Manassas, VA) and TaqMan® Control Human Genomic DNA (Applied Biosystems, Foster City, CA) and various reference, non-probative and simulated casework sample extracts generated in our laboratory. All of the casework-type samples were extracted using an organic extraction protocol consisting of ProK/SDS digestion, phenol/chloroform extraction and Centricon100 (Millipore, Bedford, MA) concentration into Tris-EDTA buffer (TE^{-4}) (most

samples) or differential extractions of sperm and epithelial fractions using a DTT-based digestion protocol (39) (simulated sexual assault samples). Pre-quantified, non-human genomic DNA extracts were purchased from various vendors: *E. coli*, *C. perfringens* (Sigma, St. Louis, MO), *B. subtilis*, *S. epidermidis*, *C. albicans* (ATCC), mouse (Promega) and cat, chicken, cow, dog, fish, horse, monkey, pig, and rat (Zyagen Labs, San Diego, CA). All extracts were stored at -20 ºC.

To study the effects of inhibition on the nuTH01-nuCSF-IPC assay, porcine hematin from Sigma (#H3281) was chosen for use as an inhibitor. Twenty-five milligrams of solid hematin were dissolved in 1 mL of 1 N NaOH. The resulting solution was diluted to 39.5 mL with sterile water to produce a 1 mM hematin solution. Separate tubes, each containing 40 ng of TaqMan® Control Human Genomic DNA, were treated with increasing volumes of 1 mM hematin and brought to a total volume of 40 µL with sterile water. The final hematin concentrations ranged from 0 µM (0 µL of 1 mM hematin) to 800 µM (32 µL of 1 mM hematin). In order to control for the possible inhibitory effect of NaOH on the PCR reaction, a separate tube received 40 ng of DNA, 0 µL of hematin, 4 µL of 0.2 N NaOH and sterile water to a final volume of 40 µL. The final NaOH concentration in this tube was the same as in the tube with the greatest concentration of hematin (800 µM).

A degradation series was prepared by digesting high molecular weight HL60 DNA with DNase I (Invitrogen, Carlsbad, CA) for progressive lengths of time. A reaction containing 15.7 µg DNA, 10x DNase I Reaction Buffer and sterile water to bring the reaction volume to 110 µL was prepared, 10 µL was removed, and 2.5 µL DNase I (1 U/µL) was added. A degradation series was achieved by incubating the reaction at room temperature (24 ºC), removing 10 µL volumes at 2.5, 5, 10, 15, 20, 25, 40, 60, 90 and 180 minute time points, and stopping DNase activity by mixing the DNA with 2 µL of 25 mM EDTA and heating at 65 ºC for 15 min. Although the 0 min sample contained no DNase, this sample was “stopped” in the same manner as all other samples. DNA fragmentation was assessed by gel electrophoresis (2% agarose, ethidium bromide detection) using the DNA molecular weight size marker XIV (Roche Applied Science, Indianapolis, IN). Four microliters of a 1:120 dilution of each degraded sample was assayed via qPCR.

nuTH01-nuCSF-IPC Triplex qPCR Quantifications

Primer and probe sequences for the nuTH01-nuCSF-IPC triplex qPCR assay are displayed in Table 7. The optimized triplex assay was run in a 20 µL reaction volume that included 10 µL of 2X TaqMan® Universal Master Mix, no UNG (Applied Biosystems), 0.5 µL of 5 U/µL AmpliTaq Gold® enzyme, 1 µL of 3.2 µg/µL non-acetylated BSA (Sigma) and 4 µL of sample. Primers, probes and IPC oligo were added at the following concentrations: 600 nM each nuTH01 primer; 200 nM nuTH01-probe; 400 nM each nuCSF primer; 100 nM nuCSF-probe; 100 nM each IPC primer; 100 nM IPC-probe; 90,000 copies IPC-oligo. Primers and the probe for the nuTH01 assay and the IPC primers and oligo were purchased from Qiagen-Operon (Alameda, CA). The IPC and nuCSF probes and both nuCSF primers were purchased from Applied Biosystems. Primer stocks were made in sterile, de-ionized water while probe stocks were made in TE^{-4}. Developmental singleplex, duplex and triplex assays were run using the same reagents as described above, although concentrations were altered for optimization experiments and, unless otherwise indicated, additional AmpliTaq Gold® enzyme was not included in the reaction. In these cases, the remaining volume was made up with TE^{-4}.

An Applied Biosystems Prism® 7000 SDS instrument and ABI Prism® 7000 SDS Software version 1.1 were used to collect real-time qPCR data. The instrument was configured for the following run conditions: 20 µL sample volumes; 9600 emulation mode; one 10 minute 95 ºC polymerase activation step, followed by 45 cycles of 2-step qPCR (15 s of 95 ºC denaturation, 60 s of 60 ºC combined anneal/extension). A ROX-labeled passive reference oligonucleotide (included in the Applied Biosystems qPCR Master Mix) was employed to correct for well-to-well variations in background fluorescence.

Amplification curves were analyzed using the automatic baseline feature of the version 1.1 ABI Prism® 7000 SDS software (except the IPC portion of the nuTH01-nuCSF-IPC assay in the inhibition experiment which was analyzed using a manual baseline set between cycles 6 and 20) and a different empirically established threshold for each assay (nuTH01 threshold = 0.15; nuCSF threshold = 0.05; IPC threshold = 0.025). For some developmental optimization experiments an IPC cycle threshold of 0.05 was used. For each experiment, the 7000 SDS software used amplification results from a freshly prepared

dilution series of standard DNA to generate two calibration curves (one for nuTH01 and one for nuCSF) by plotting C_T (cycle threshold) vs. log C_0 (initial standard DNA concentration). DNA quantifications for unknown samples were interpolated from the resulting linear calibration curves. These calibration and interpolation steps are semi-automatic features of the 7000 SDS software. For both the nuTH01 and nuCSF assays, calibration plots were constructed using data from duplicate samples containing 32, 3.2 and 0.32 ng of Promega human genomic female standard DNA and triplicate samples containing 0.032 ng. One TE^{-4} negative control was included in each run. All efficiency calculations were determined using the slopes of the calibration curves in the following formula (56): % PCR efficiency = $(10^{(-1/slope)} - 1)*100$.

STR Genotyping

STR genotyping was performed using the AmpF*l*STR® Identifiler™ PCR Amplification kit from Applied Biosystems. Amplifications were typically performed using 1 ng of template DNA, as recommended by the manufacturer, although 2 and 4 ng of template were used for some studies. Twenty-five microliter amplifications were performed in an ABI GeneAmp® 9700 PCR thermocycler and STRs were resolved and detected on an ABI Prism® 3100 Genetic Analyzer, according to the manufacturer's instructions. Samples were injected for 3, 5 or 10 sec depending upon the goal of the experiment. Data were analyzed at a minimum threshold of 100 RFU and a baseline of 35 using GeneScan® (v.3.7.1/NT) and Genotyper® (v.3.7/NT) from Applied Biosystems.

Results and Discussion

Development of the nuTH01-nuCSF Duplex qPCR Assay

A multiplex qPCR assay to assess DNA degradation relies on the simultaneous quantification of long and short target sequences in the same genome. For the long target sequence, we selected the nuTH01 qPCR assay (170-190 bp) developed for a previously described nuclear-mitochondrial duplex qPCR assay (9). This target, though not as long as the largest STR amplicons produced by commercial genotyping kits, is roughly in the middle of the range of STR amplicons and was previously found to accurately predict DNA

quantifications for STR genotyping applications (9). Furthermore, the 170-190 bp length is not large enough to overly compromise the TaqMan® PCR efficiency. For the short target sequence (<70 bp), several candidate assays were designed and tested at primate-specific nuclear target sequences. One assay, designated here as nuCSF, possessed the best qPCR properties, e.g., lowest cycle threshold (C_T) values, lowest variance in C_T, optimal PCR efficiency, and optimal final fluorescence signals (ΔR_n, the normalized, baseline-corrected qPCR fluorescence). This assay, which amplifies a 67 bp target sequence located ~40 bp upstream of the $(AGAT)_n$ repeats at the CODIS CSF1PO STR locus, was selected for further work.

The nuTH01 and nuCSF qPCR assays were tested on both non-degraded and highly degraded DNA samples to ensure that equivalent results would be obtained in both singleplex and in duplex reactions. For these experiments, four HL60 samples from an existing DNaseI degradation series were used as template. These samples exhibited significantly different degrees of degradation because of varied exposure to DNase I (0 min, 5 min, 20 min, 60 min). Gel electrophoresis results (Figure 9) indicated that the 0 min sample consisted of high molecular weight DNA, while the average fragment length was >2000 bp for the 5 min sample, ~400 bp for the 20 min sample and ~100 bp for the 60 min sample. Given these differences in average DNA fragment size, we expected that the empirical ratio of the quantity of short nuCSF amplicons to the quantity of long nuTH01 amplicons would provide a useful estimation of the degree of DNA degradation present in each sample. Throughout the remainder of this paper, this ratio of nuCSF to nuTH01 quantification will be referred to as the "qPCR degradation ratio." An initial experiment showed that nearly equivalent quantifications could be obtained by running the nuTH01 and nuCSF qPCR assays in singleplex and duplex (Table 8 – Expt. 1). Although there was a slight decrease in efficiency in the duplex qPCR assays, the degradations ratios for the singleplex and duplex assays were in very good agreement.

TABLE 8 - *Summary of assay conditions and results from four key optimization experiments for the nuTH01-nuCSF-IPC triplex qPCR assay.*

		Forward Primer/Reverse Primer/Probe ** (nuTH01 assay, nuCSF assay, IPC assay)	Extra Taq?	nuTH01 Efficiency	nuCSF Efficiency	qPCR Degradation Ratio in Degraded Samples			
						0 min	5 min	20 min	60 min
Expt. 1	nuTH01 singleplex*	600/600/200	no	92%	-	1.0	1.5	4.0	17.0
	nuCSF singleplex*	400/400/100	no	-	93%				
	nuTH01-nuCSF duplex	600/600/200, 400/400/100	no	89%	88%	1.3	1.5	3.7	15.4
Expt. 2	nuTH01-nuCSF duplex	600/600/200, 400/400/100	no	94%	94%	0.9	1.4	3.9	21.3
	nuTH01-nuCSF-IPC triplex	600/600/200, 400/400/100, 50/50/200	no	93%	97%	1.1	1.9	4.4	54.9
Expt. 3	nuTH01-nuCSF duplex	500/500/200, 300/300/200	no	84%	83%	1.0	1.4	3.5	15.5
	nuTH01-nuCSF duplex	500/500/200, 300/300/200	2.5 U	86%	83%	1.0	1.6	3.2	10.3
	nuTH01-nuCSF-IPC triplex	500/500/200, 300/300/200, 100/100/100	no	70%	92%	1.0	1.2	3.1	27.1
	nuTH01-nuCSF-IPC triplex	500/500/200, 300/300/200, 100/100/100	2.5 U	91%	96%	1.2	1.4	3.4	10.4
Expt. 4	nuTH01-nuCSF duplex	600/600/200, 400/400/100	no	89%	88%	1.0	1.2	3.0	15.1
	nuTH01-nuCSF duplex	600/600/200, 400/400/100	2.5 U	87%	96%	1.1	1.2	3.2	13.4
	nuTH01-nuCSF-IPC triplex	600/600/200, 400/400/100, 100/100/100	no	87%	86%	0.8	1.6	4.4	243.4
	nuTH01-nuCSF-IPC triplex	600/600/200, 400/400/100, 100/100/100	2.5 U	95%	100%	0.9	1.3	3.0	21.8

* Singleplex qPCR degradation ratios were determined by combining the data from the nuCSF and nuTH01 singleplex assays.

** Values indicate nM concentrations of primers and probes for each of the three assays within the triplex.

Development and Optimization of the nuTH01-nuCSF-IPC Triplex qPCR Assay

As the next step, an IPC assay for detecting the presence of co-extracted PCR inhibitors was added to the duplex nuTH01-nuCSF assay. An IPC assay is especially useful for samples that are inhibited to such an extent that no amplification is detected above baseline. For such samples, an IPC assay can differentiate null amplifications due to inhibition from those simply due to insufficient quantities of template DNA. For our IPC assay, we designed a TaqManMGB® assay utilizing a 77 bp artificial target sequence (Table 7 – "IPC-oligo"). Using the BLAST*n* tool to search the GenBank database, this artificial target sequence was custom designed so that the primer binding regions would be specific to the target and would avoid spurious amplifications of human and non-human genomic DNA. Real-time qPCR experiments using the IPC primers (900 nM IPC-F and IPC-R) with SYBR Green detection gave null amplifications for up to 64 ng of human female DNA (results not shown). Based on these null amplifications and on the lack of significant homologies to GenBank sequences, the IPC was determined to be suitably specific for forensic use. For the purpose of developing a triplex qPCR assay, the TaqManMGB® probe for the IPC was labeled with NED. Although the NED detection channel is significantly less sensitive than either the FAM or VIC channels on the ABI Prism® 7000 SDS instrument, it was the sole channel available for triplex detection as the ROX channel was used for normalization to a passive reference dye. Consequently, the signal-to-noise ratios for the IPC assay were sub-optimal, a situation that would likely improve if the assay were run on an instrument optimized for NED detection (e.g., the ABI 7500 (75,76)) or if a detection channel capable of improved spectral separation (e.g., for Cy5) were available. A singleplex TaqManMGB® IPC assay performed on 1 ng of genomic DNA from human and 15 non-human species confirmed that the IPC assay did not amplify any of the examined species (results not shown).

An initial attempt at a triplex qPCR assay combined the IPC assay (including 90,000 copies of IPC-oligo template) with the initial nuTH01-nuCSF duplex (Table 8 – Expt. 2). Primer-limiting conditions were utilized for the IPC assay to minimize the effect of adding this third reaction to the PCR amplification. Most samples gave nearly equivalent degradation ratios in duplex and triplex. For the most highly degraded DNA sample, however, there was an artificially high degradation ratio (54.9 for the triplex assay vs. 21.3 for

duplex) due to a delayed C_T for the nuTH01 assay in triplex. The nuTH01 assay, which was the last to cross the amplification threshold in triplex, likely experienced a reduced PCR efficiency due to the earlier nuCSF and IPC amplifications. The signal strength (ΔR_n) of the IPC assay was also significantly reduced in the triplex assay (compare Figures 15a and 15b). Based on these results, it was apparent that it would be necessary to optimize PCR conditions for the triplex assay, both to ensure accurate nuTH01 quantifications for highly degraded samples and to improve the IPC amplification.

Several approaches were taken to optimize the triplex assay. First, in order to improve the IPC signal, we increased the primer concentrations and lowered the probe concentration relative to those initially used in the triplex assay. Second, the primer and probe concentrations for the nuTH01 and nuCSF assays were modified, mainly in an attempt to limit the nuCSF PCR so that it would not reduce the efficiency of the nuTH01 PCR in degraded samples. This modification alone was unsuccessful. In this triplex, for example, the PCR efficiency of the nuTH01 assay (70%) was significantly lower than that of the nuCSF assay (92%) (Table 8 – Expt. 3). As a final approach, additional AmpliTaq Gold® polymerase (2.5 U/20 µL reaction) was used to augment the ABI Universal Master Mix, a common tactic for increasing efficiencies in multiplex qPCR assays (77). The additional enzyme in the triplex reactions led to improved PCR efficiencies for both the nuCSF and nuTH01 assays as well as to more accurate degradation ratios for highly degraded DNA. The additional polymerase also improved the signal strength of the IPC assay (compare Figures 15c and 15d). Based on the slightly higher PCR efficiencies obtained in Experiment 4 relative to Experiment 3 (Table 8), the PCR conditions of Experiment 4 were selected for the triplex qPCR assay. As a final optimization step, we titrated the triplex assay with polymerase (0, 1, 2, and 2.5 additional U per 20 µL reaction) and found that at least 2 U of additional AmpliTaq Gold® polymerase were needed to achieve efficient PCR for all assays in the triplex (results not shown).

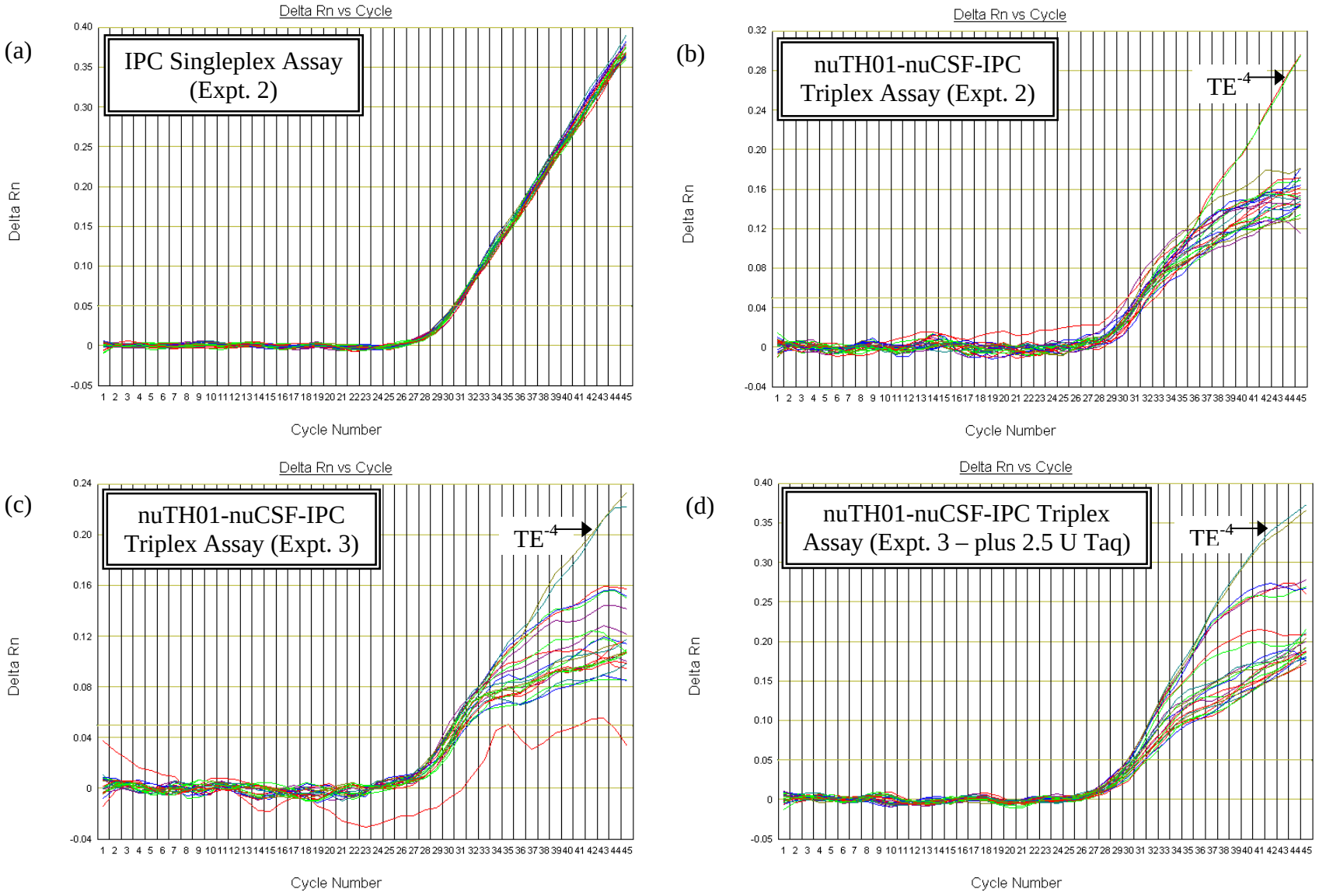


FIG 15 - *Developmental IPC amplification curves. Comparisons of (a) and (b) show that the signal strength (ΔR_n) was significantly reduced in the triplex assay and of c) and d) show that adding extra Taq enzyme improved signal strength.*

Species Specificity of the nuTH01-nuCSF-IPC Triplex qPCR Assay

Although no cross-reactivity to non-human DNA was observed for any of the singleplex assays individually, it was important to assess species specificity of the nuTH01-nuCSF-IPC triplex qPCR amplification. To do this, the triplex assay was run using 1 ng of template DNA in duplicate from each of fifteen different non-human species. No cross-reactivity to non-human DNA was observed. These results indicate that the nuTH01-nuCSF-IPC triplex qPCR assay is sufficiently specific for forensic applications. Although the tests did not include DNA from higher primates, based on previous work at the TH01 and CSF1PO STR loci (61,62) we anticipate that the nuTH01-nuCSF-IPC triplex qPCR assay would amplify and detect such samples. Sequence homology (NCBI GenBank BLAST*n*) investigations do not, however, indicate significant homologies to non-human DNA sequences for the nuCSF primer pair.

Detection of DNA Degradation with the nuTH01-nuCSF-IPC Triplex qPCR Assay

The premise for a degradation qPCR assay is that, due to the availability of fewer target sequences of sufficient length for amplification in degraded samples, an assay requiring a longer target template should be more sensitive to degradation than an assay requiring a shorter target template. In developing and optimizing the nuTH01-nuCSF-IPC triplex assay, we indeed demonstrated this difference in sensitivity. That is, for the small set of DNase-degraded samples that were used in development and optimization, the empirical ratio of shorter (67 bp nuCSF) to longer (170-190 bp nuTH01) target sequences was seen to be closely related to the extent of DNA degradation. Although this qPCR degradation ratio appeared to be a good empirical tool for diagnosing DNA fragmentation, it was also important to determine if this ratio would be useful for predicting the success of future STR analysis.

To assess the ability of the nuTH01-nuCSF-IPC triplex assay to quantify DNA in degraded samples, a 10-point degradation series was prepared by treating aliquots of high molecular weight genomic DNA (HL60) with DNase I for increasing periods of time ranging from 2.5 to 180 minutes. The result was a degradation series exhibiting incremental increases in the extent of DNA fragmentation (Figure 16).

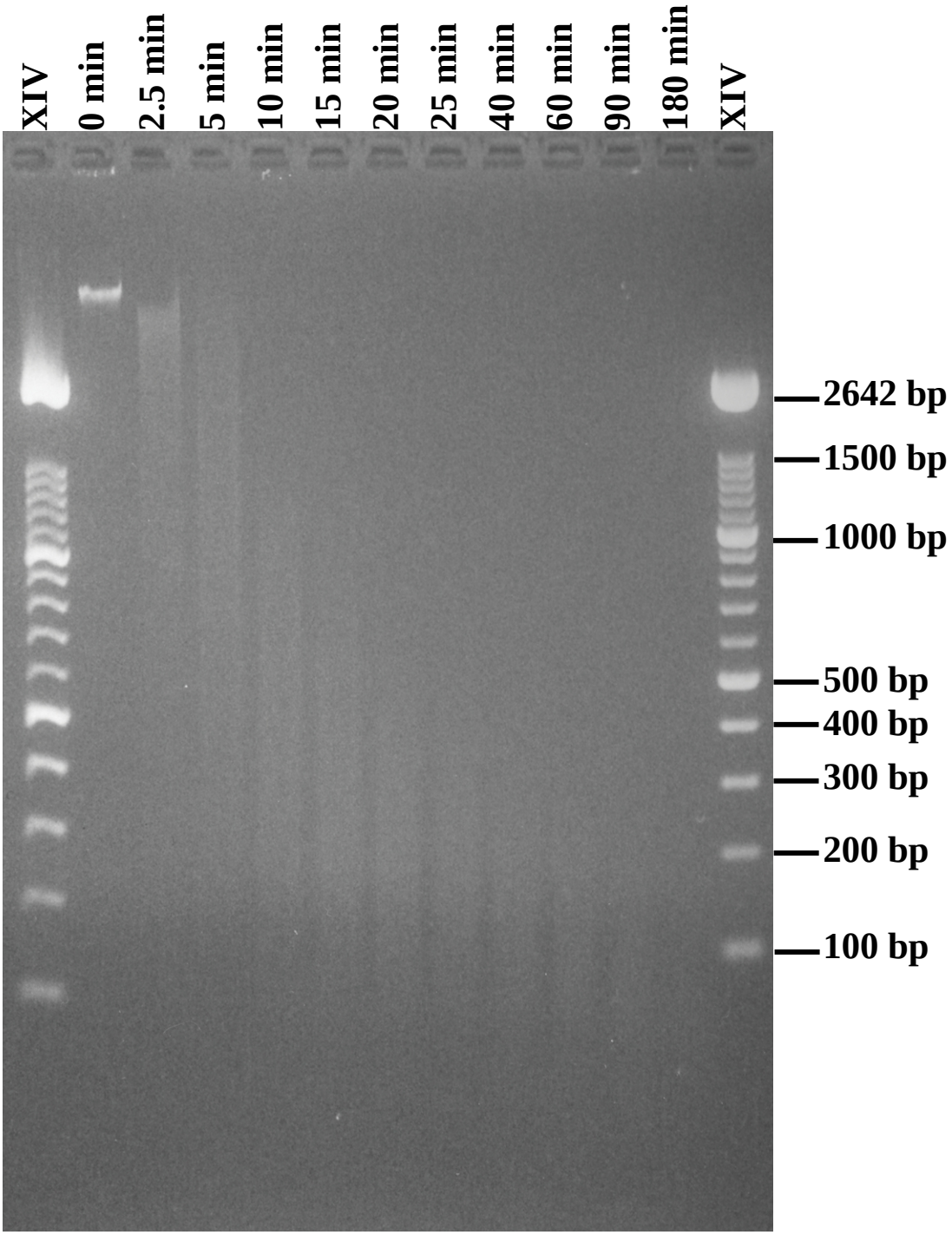


FIG. 16 - *HL60 DNase I degradation series (EtBr-stained). Times indicate lengths of DNase I treatment. Lanes labeled XIV contain DNA molecular weight marker XIV.*

Samples from the degradation series as well as an untreated sample (0 min) were assayed in triplicate with the nuTH01-nuCSF-IPC triplex qPCR assay. As expected, due to their quite dissimilar amplicon lengths, the nuTH01 and nuCSF portions of the assay experienced different sensitivities to the level of degradation in the DNase-treated samples (Figure 17, see below). Just as the agarose gel showed a gradual progression in degree of degradation in the samples, so did the qPCR-based degradation ratios display a similarly gradual progression (Table 9, see below).

The development of guidelines for the analysis of degraded samples required the testing of such samples under a variety of conditions. With this in mind, AmpF*l*STR® Identifiler™ PCR amplifications were performed for each sample in the DNase degradation series using DNA concentrations determined by the nuTH01 portion of the assay. (For the purpose of STR genotyping of degraded samples, we had previously determined that a large qPCR target sequence is more appropriate for DNA quantifications than is a short qPCR target (9).) Where possible, 1, 2, and 4 ng of template were amplified for each degradation timepoint. These different quantities were used to test the hypothesis that higher genotyping success rates might be achievable by increasing the overall template amount (from the manufacturer recommended template amount of 1 ng to 2 and 4 ng) so that the longer STR target sequences, which are present in relatively smaller concentrations in degraded samples, might amplify to give stronger signals after electrophoretic resolution and detection. For the three final timepoints the extremely low concentrations of DNA in these samples, coupled with the 10 µL maximum template input volume allowed in Identifiler™ reactions, made it possible to perform only 1 and 2 ng amplifications on the 60 min sample and 1 ng only on the 90 and 180 min samples. PCR product from each amplification was electrokinetically injected on the ABI Prism® 3100 Genetic Analyzer for 3, 5 and 10 seconds, in order to determine if any of the permutations of injection time and initial template quantity would lead to increased genotyping success rates for degraded samples. Results are summarized in Table 9, from which several conclusions can be drawn:

(a) Across the set of DNase-degraded samples, an increase in the qPCR degradation ratio was correlated with an increase in interlocus imbalance in the STR profiles due to DNA degradation. In Table

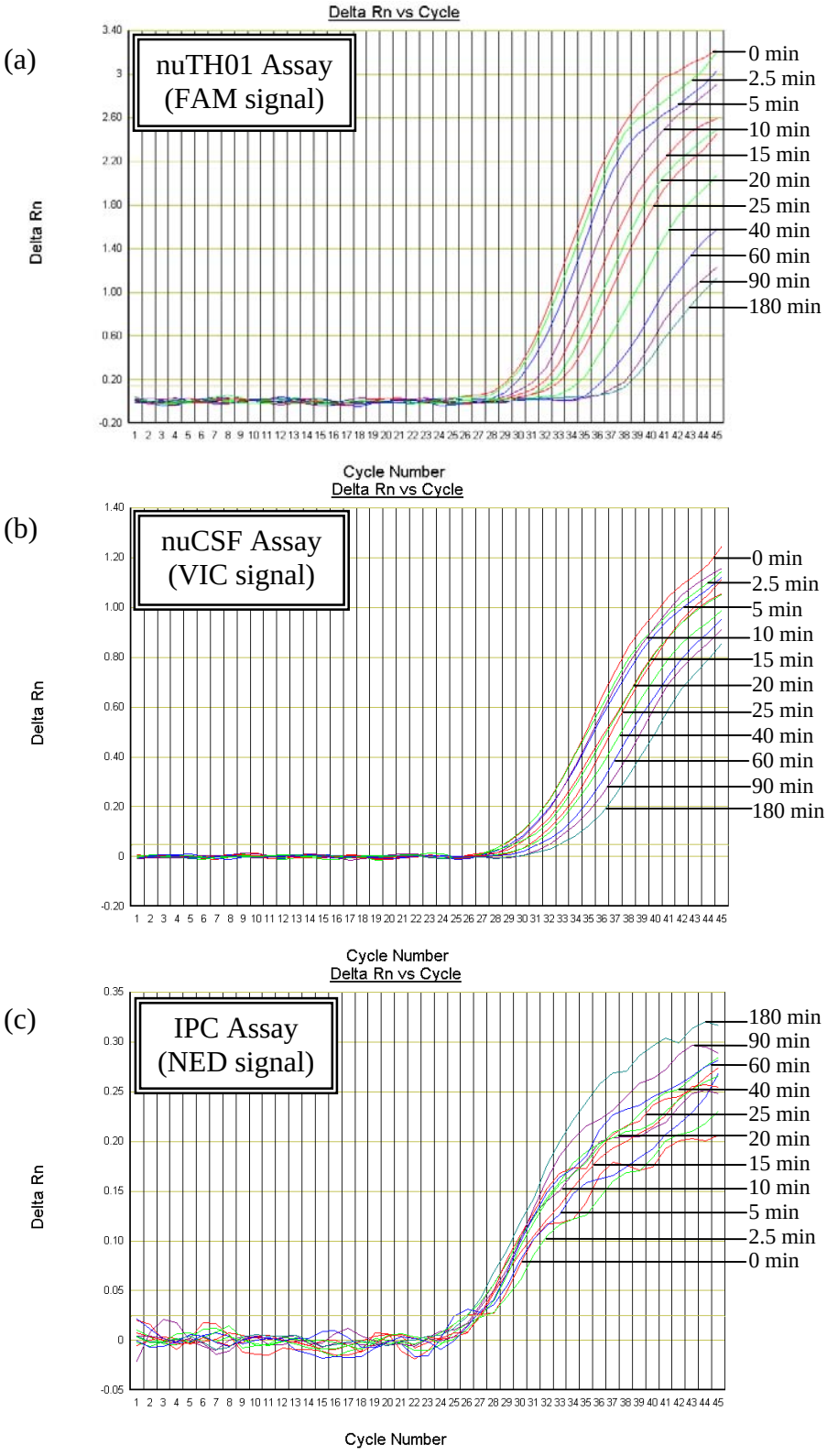


FIG. 17 - *Amplification curves for DNase I degraded samples. Although all samples were assayed in triplicate, for clarity only a single replicate is displayed for each. Times indicate length of DNase treatment for each sample.*

TABLE 9 - *Summary of AmpFlSTR® Identifiler™ STR results for DNase I degraded HL60 DNA. Detected stutter peaks, incidences of incomplete non-template nucleotide addition ("-A peaks") and off-scale peaks are indicated as "s", "a" and "o", respectively.*

		% Peaks Detected			D19/D18 STR Ratio			qPCR Degradation Ratio
Sample	Template	3 sec inj.	5 sec inj.	10 sec inj.	3 sec inj.	5 sec inj.	10 sec inj.	
0 min	1 ng	100	100	100 *1s, 1a*	0.6	0.7	0.7	1.6
	2 ng	100 *2s, 2a*	100 *2s, 2a*	100 *1s, 2a*	0.9	0.9	0.9	
	4 ng	100 *2s, 5a*	100 *2s, 5a*	100 *4s, 5a, 2o*	1.4	1.5	1.3	
2.5 min	1 ng	100	100	100	1.1	1.2	1.2	1.5
	2 ng	100	100 *1s, 1a*	100 *1s, 3a*	1.6	1.6	1.6	
	4 ng	100 *1s, 4a*	100 *2s, 4a*	100 *3s, 4a, 1o*	2.5	2.4	2.4	
5 min	1 ng	100	100	100	1.6	1.6	1.7	2.0
	2 ng	100 *1s, 1a*	100 *1s, 1a*	100 *2s, 3a, 1o*	2.4	2.3	2.3	
	4 ng	100 *2s, 6a*	100 *3s, 5a*	100 *4s, 5a, 2o*	3.6	3.5	2.5	
10 min	1 ng	96	100	100	2.6	2.5	2.6	2.9
	2 ng	92	100	100 *1s, 3a, 1o*	4.1	4.1	3.9	
	4 ng	100 *3s, 7a*	100 *3s, 6a, 1o*	100 *5s, 7a, 3o*	6.4	6.2	3.5	
15 min	1 ng	72	92	100	4.6	4.5	4.5	3.9
	2 ng	92 *1s, 1a*	92 *1s, 2a*	100 *2s, 4a, 1o*	8.2	7.9	6.1	
	4 ng	92 *1s, 7a*	92 *3s, 6a, 1o*	100 *5s, 7a, 2o*	10.3	10.4	6.1	
20 min	1 ng	72	92	92 *1o*	8.3	8.4	8.6	6.5
	2 ng	84 *1s, 2a*	92 *1s, 4a*	96 *3s, 5a, 2o*	13.3	13.2	9.2	
	4 ng	84 *1s, 8a*	92 *2s, 8a, 2o*	92 *5s, 7a, 2o*	19.1	17.1	8.7	
25 min	1 ng	72	88	92 *1o*	11.6	11.9	11.1	5.5
	2 ng	68 *2a*	84 *1s, 3a*	92 *1s, 4a, 2o*	35.0	19.6	14.5	
	4 ng	80 *7a*	84 *1s, 8a, 2o*	92 *4s, 7a, 2o*	27.4	26.4	13.3	
40 min	1 ng	60 *1a*	80 *1s, 1a*	88 *1s, 2a, 2o*	-	29.0	18.8	9.7
	2 ng	60 *3a*	72 *6a, 1o*	84 *3s, 7a, 2o*	-	83.9	25.3	
	4 ng	48 *7a*	52 *8a, 2o*	72 *1s, 9a, 2o*	-	-	-	
60 min	1 ng	48 *3a*	72 *6a, 1o*	80 *2s, 7a, 2o*	-	-	-	28.1
	2 ng	28 *4a*	28 *3a*	36 *5a, 1o*	-	-	-	
90 min	1 ng	4	8	12	-	-	-	43.6
180 min	1 ng	0	0	0	-	-	-	25.0

9, we represent the appearance of degradation in the STR profile by a single number, the ratio of the short (121 bp) D19 locus intensity to the long (average allele length 293 bp) D18 locus intensity, because we had previously found that this ratio was a simple STR-based metric for DNA degradation (9).

(b) For non-degraded and moderately degraded (2.5-10 min of DNase treatment) samples, 100% of the STR alleles were detected when using the nuTH01 qPCR quantification results in conjunction with our standard STR amplification (1 ng template) and electrophoresis (5 sec injection time) conditions. For these particular samples, as expected, an increase in the template quantity and/or injection time generally led to less than optimal genotyping results. For example, increasing the quantity of template generally increased the number of off-scale STR alleles, the number of alleles with shoulders due to incomplete non-template nucleotide addition ("-A peaks") and the number of alleles with stutter peaks above the analytical detection threshold. In addition, the use of increased template generally led to poorer interlocus balance, as reflected by increasing ratios of D19 to D18 STR signals. Increasing the electrophoresis injection time often led to increases in the number of off-scale, -A, and stutter peaks detected above the analysis threshold, although the injection time had no detrimental effect on interlocus balance.

(c) For more highly degraded samples (15-60 min of DNase treatment), the standard amplification and electrophoresis conditions did not lead to 100% success rates for genotyping, primarily due to allelic dropout at the larger STR loci. For these samples, moderate improvements in genotyping success rates could be obtained by increasing the injection time from 5 to 10 sec (e.g., an increased success rate of 80% to 88% of the alleles detected for the 40 min sample), although these improvements typically resulted in an increase in the number of STR artifacts (off-scale, stutter or -A) detected above threshold. An increase in the amount of template to 2 or 4 ng, however, did not generally lead to any improvements in genotyping success rates. Instead, the main effect of increasing the quantity of template for these samples was to increase the number of artifact signals and to decrease the success rate for genotyping. When using the Identifiler™ amplification kit, these results indicate that the detrimental effects of DNA degradation on STR genotyping cannot be overcome simply by adding more template, but are better addressed by using the recommended 1 ng of

template DNA as determined by the longer-target assay, then judiciously increasing the injection time to 10 seconds in order to detect some additional fraction of the larger alleles.

(d) For the most highly degraded samples (90-180 min of DNase treatment), very low genotyping success rates were seen. For these samples, the nuTH01 qPCR quantifications were 8.3(SD=1.3) pg and 5.9(4.4) pg per assay, respectively. These values, which are well below the lowest point (32 pg) in the qPCR standard curve for our triplex assay, correspond to average C_Ts greater than 37 cycles and to a range that is below the accurate quantification limit for the assay. It is likely that the low genotyping success rate seen for these highly degraded samples is simply due to limits in accuracy of the nuTH01 qPCR assay at these very low copy numbers. It is possible that these very highly degraded samples would have benefited from increasing the STR amplification input to 2 or 4 ng, though we did not test for this possibility.

Detection of PCR Inhibition with the nuTH01-nuCSF-IPC Triplex qPCR Assay

The IPC was specifically included in the triplex degradation assay to detect for the presence of PCR inhibition. To assess the sensitivity of the IPC to inhibition, a series of artificially inhibited DNA extracts were examined using the triplex qPCR assay. Twenty identical tubes of non-degraded DNA were treated with different amounts of hematin, a known PCR inhibitor (78), such that the final concentrations of hematin in the standard 20 µL qPCR assays ranged from 0 µM to 160 µM. Each sample was assayed in duplicate with the nuTH01-nuCSF-IPC triplex qPCR assay, and the results are summarized in Table 10. It is evident from these results that an increased concentration of hematin can lead to one or more changes in the qPCR signal, including: (i) the complete loss of an amplification curve (a "null" amplification); (ii) a delayed C_T; and/or (iii) a reduced final fluorescence signal (lower ΔR_n).

To determine how results from the IPC assay are predictive of success in STR genotyping, samples from the hematin-inhibition series were amplified and genotyped using the AmpF*l*STR® Identifiler™ kit. For these amplifications, 1 ng quantities of template DNA were used based on estimates by both the nuTH01 and nuCSF qPCR assays. If, based on the qPCR assays, the use of 1 ng of template was not possible, 10 µL of extract was used. The STR results are summarized in Table 10, which includes a comparison of the

TABLE 10 - *Summary of nuTH01-nuCSF-IPC triplex qPCR and AmpFlSTR® Identifiler™ STR results using TaqMan® Control Human Genomic DNA inhibited to varying degrees with hematin. Percentages of STR peaks detected are percentages obtained with 5 second injections only.*

	nuTH01-nuCSF-IPC qPCR Results		Identifiler™ STR Results			
	IPC Assay:		Using Input Quantity from: nuTH01 Assay		Using Input Quantity from: nuCSF Assay	
µM Hematin in qPCR Assay	Mean (StDev) C_T	Mean (StDev) ΔR_n Ratio	µM Hematin in STR Amp	% Peaks Detected	µM Hematin in STR Amp	% Peaks Detected
0 µM + NaOH	27.57 (1.03)	0.88 (0.161)	0	100	0	100
0 µM	27.43 (0.40)	0.93 (0.153)	0	100	0	100
5 µM	27.91 (0.16)	0.77 (0.135)	2	100	2	100
10 µM	27.74 (0.15)	0.96 (0.159)	3	100	4	100
15 µM	28.32 (0.38)	0.86 (0.141)	5	100	5	100
20 µM	28.42 (0.08)	0.69 (0.114)	7	100	9	100
25 µM	28.52 (0.18)	0.71 (0.131)	9	100	11	100
30 µM	28.05 (0.42)	0.69 (0.113)	13	100	15	100
35 µM	27.76 (0.48)	0.61 (0.113)	13	100	19	100
40 µM	28.03 (0.04)	0.54 (0.098)	26	61	36	0
45 µM	30.21 (0.02)	0.36 (0.061)	36	7	40	0
50 µM	30.65 (0.53)	0.36 (0.091)	61	0	45	0
55 µM	30.67 (0.26)	0.32 (0.069)	110	0	70	0
60 µM	31.35 (0.07)	0.29 (0.053)	120	0	112	0
65 µM	30.92 (0.56)	0.27 (0.079)	130	0	130	0
70 µM	31.28 (0.43)	0.41 (0.071)	140	0	140	0
75 µM	31.78 (2.18)	0.44 (0.144)	150	0	150	0
80 µM	31.20 (0.21)	0.48 (0.081)	160	0	160	0
120 µM	41.93 (*)	0.13 (0.060)	240	0	240	0
160 µM	-	-	320	0	320	0

* Only one replicate was detected above the analysis threshold.

genotyping success rate to the final hematin concentration in each Identifiler™ STR amplification. Depending on which quantitation assay was used, this comparison indicates that the STR amplification became partially inhibited at concentrations of 26-36 μM hematin and were completely inhibited starting at 36-61 μM. The IPC assay began to show an inhibition effect, as evidenced by a delay in the C_T value, at 45 μM and above that concentration continued to exhibit progressively greater delays in C_T (Table 10). There was, in addition, one aspect of the IPC portion of the triplex assay that correlated to reduced success rates for STR genotyping - that aspect was the hematin-induced reduction in the final ΔR_n value. These reductions in ΔR_n for the IPC assay are represented in Table 10 as the "ΔR_n ratio," which were calculated as the ratio of the final ΔR_n for the sample to the average final ΔR_n for the non-inhibited control samples. Based on these results, our current interpretation guidelines for using the IPC to positively indicate for inhibition are: (i) total absence of an IPC amplification curve; (ii) a C_T delay of $\geq$1 in the IPC relative to the mean C_T of the non-inhibited control samples used in that run; (iii) a ΔR_n ratio in the IPC that is $\leq$60%. The last indicator is the most sensitive for predicting inhibition using our IPC system.

Casework-type Samples with the nuTH01-nuCSF-IPC Triplex qPCR Assay

Twenty-three non-probative and simulated casework samples were quantified to assess the capability of the nuTH01-nuCSF-IPC triplex qPCR assay to accurately quantify DNA in forensic samples. It was also desirable to determine if evidence of DNA degradation in these samples, as detected by the qPCR assay, would be predictive of STR genotyping success. Each sample was amplified twice with the AmpF*l*STR® Identifiler™ PCR Amplification kit, once using 1 ng of template DNA based on qPCR quantification of a long target sequence (nuTH01) and a second time using 1 ng of template based on a short target (nuCSF). The purpose of the two amplifications was to confirm that for highly degraded DNA samples optimal STR genotyping success rates would be achievable using quantifications based on the longer qPCR assay (9). PCR product from each amplification was injected for 5 sec on the ABI Prism® 3100 Genetic Analyzer.

Results from this study are summarized in Table 11. Based on the STR genotyping success rate ("% Peaks Detected"), it is clear that the nuTH01 portion of the triplex qPCR assay provided accurate

TABLE 11 - *Summary of nuTH01-nuCSF-IPC qPCR quantification and AmpFlSTR® Identifiler™ STR results for selected casework-type samples. All quantification results are from single replicate experiments. Average peak height is the total RFU signal from detected peaks at all loci divided by the total number of detected peaks.*

		qPCR Degradation Ratio	% Peaks Detected		D19/D18 Ratio		Average Peak Height (RFU)	
Sample	Sample Type		nuTH01 Assay	nuCSF Assay	nuTH01 Assay	nuCSF Assay	nuTH01 Assay	nuCSF Assay
1	Blood	13.9	93	52	22.8	-	1319	122
2	Tooth	1.3	100	100	1.7	1.6	714	630
3	Blood	0.7	100	100	1.1	1.0	591	1039
4	Muscle	0.8	100	100	1.3	1.7	695	759
5	Toothbrush	1.3	100	100	1.7	1.3	785	666
6	Toothbrush	1.1	100	100	1.3	1.6	798	642
7	Razor Shavings	3.1	100	97	5.7	3.4	821	321
8	Toenails	2.6	97	89	9.8	5.8	828	343
9	Toenails	3.4	100	89	5.3	4.0	829	290
10	Femur	4.0	100	70	9.2	9.5	887	304
11	Blood on Redwood	1.1	100	100	1.3	1.2	998	1089
12	Blood in Soil	2.3	100	100	2.9	1.4	1478	480
13	Cigarette Butt	1.8	100	100	2.6	1.8	1022	503
14	Blood on Denim	1.2	100	100	2.4	2.1	984	1101
15	Semen on Wool	1.2	100	100	1.2	1.1	792	619
16	Semen on Cotton	1.0	100	100	1.2	1.7	1054	965
17	Semen on Wool	2.1	100	100	1.8	1.3	1312	619
18	Semen on Cotton	1.4	100	100	1.6	1.2	1105	615
19	Semen on Boxers	3.4	100	91	8.8	5.3	1045	371
20	Buccal Swab	1.8	100	100	2.6	2.3	911	437
21	Buccal Swab	2.0	100	100	2.1	2.4	644	312
22	Buccal Swab	1.3	100	100	1.2	1.0	664	1021
23	Control DNA	1.1	100	100	0.6	0.5	379	384

quantifications. Only two samples (1 and 8) yielded less than 100% genotyping success rates. These were the two most degraded samples based on the D19/D18 STR intensity ratios. There was also generally good concordance between the values of the qPCR-based degradation ratio and the D19/D18 STR intensity ratio. Based solely on this small set of casework-type samples, it appeared that when a degradation ratio was measured to be greater than ~2.5, then some degree of interlocus imbalance (D19/D18 > ~5) in the STR profile could be predicted. There were no amplification artifacts except in sample 1 which contained three stutter peaks, four -A peaks and one off-scale peak. For the entire set of samples, there were neither strong signs of inhibition indicated by the IPC portion of the triplex qPCR assay nor evidence of PCR inhibition suggested by the STR results. When quantifications from the short nuCSF qPCR assay were used to prepare

"1 ng" of template DNA for STR amplification, decreased genotyping success rates were observed for degraded samples (samples 1, 7-10, and 19). Due to the short qPCR target sequence, the nuCSF assay overestimated the quantity of amplifiable DNA. As a consequence, larger STR alleles were undetected and lower than optimal average STR peak heights were obtained.

Conclusions

We have previously described the importance of qPCR target sequence length when quantifying DNA for STR genotyping (9). Results using the nuTH01-nuCSF-IPC triplex assay confirm that, for highly degraded samples, improved genotyping success rates are obtained when an appropriately long qPCR target (the nuTH01 target) is used for determining the amount of template to be amplified for STR analysis.

We have also shown that a degradation ratio, calculated from quantitation results obtained with the nuTH01-nuCSF-IPC triplex qPCR assay, provides a good estimate of the quality of extracted DNA. In the past, information about DNA quality has been difficult to obtain and was not quantitative. Typically, the extent of DNA degradation could be assessed only by gel electrophoresis with ethidium bromide staining, a procedure that is laborious, not very sensitive and could consume much of a sometimes precious sample. If the sample quantity was very limited, generally no information about DNA quality could be obtained, leaving the analyst to make an educated guess based on the condition of the tissue from which it was extracted. As described here, the nuTH01-nuCSF-IPC triplex qPCR assay is a tool for simultaneously assessing the quantity and quality of DNA thereby sacrificing a minimal quantity of extract. Furthermore, the inclusion of an internal PCR control provides immediate feedback about the amplification "status" of the sample. If the IPC does not successfully amplify, steps can be taken to overcome PCR inhibition before STR genotyping is attempted. Given that the primary sample-related variables directly impacting the success of STR genotyping are quantity of DNA, quality of DNA, and purity of the DNA sample with regard to PCR inhibitors, and given that forensic samples often tend to be compromised in one or more of these regards, the triplex qPCR assay described here should be a valuable tool that will improve the first-pass success rate for STR genotyping, thus minimizing the consumption of extracted DNA and maximizing the throughput and

efficiency of DNA analysis. Finally, the nuTH01-nuCSF-IPC triplex assay should be useful for identifying those samples that would be analyzed more successfully with newly developed genotyping technologies (e.g., mini-STRs (79,80) or SNP genotyping (81)), rather than with the STR technologies currently in common use.

(iii) Design and Development of a Human Y-Chromosome-Specific qPCR Assay

Introduction

The development of a qPCR assay specific for the human Y-chromosome was motivated primarily by its potential utility for the analysis of sexual assault casework samples. Such an assay would allow the analyst to differentiate those samples that contain sufficient male DNA for genotyping from those that contain so little male DNA that further analyses, at least by the STR methods currently in common use, would be uninformative. Moreover, the *combined* quantitation information from a Y-specific qPCR assay and an autosome-specific qPCR assay could provide an estimate of the *relative* amounts of human male and female DNA in an extract. This information could then be used to differentiate those samples that would benefit from "standard" autosomal STR genotyping approaches (i.e., those samples with maleDNA:femaleDNA ratios greater than ~1:10) from those samples that would be analyzed more productively using Y-specific amplification methods (e.g., using Y-STRs (82)).

Given the potential utility of such an assay, it is not surprising that several Y-specific qPCR assays have been previously described, both for forensic (33,38,83-84) and clinical (85) applications, including a commercially available Y-chromosome-specific forensic assay (30). Our primary interest was in developing a Y-specific assay that would be not only useful in singleplex but that could be multiplexed with an autosomal qPCR assay in order to save DNA extract and to increase the efficiency of analysis. To this end, we designed and tested a number of singleplex Y-chromosome-specific qPCR assays, some of the more promising of which will be described here. Developmental work on the Y-autosome multiplex qPCR assay is preliminary and ongoing.

Methods and Materials

Pre-quantified, high molecular weight human genomic male DNA (#G1471), purchased from Promega (Madison, WI), was used as a quantification standard and positive control for the Y-specific assays. Pre-quantified, high molecular weight human genomic female DNA (#G1521), also from Promega, was used as a negative control for gender specificity.

Real-time qPCR assays were designed as described previously (9) using PrimerExpress™(v2.0) software defaulted to the Applied Biosystems Universal TaqMan™ assay conditions. Based on a previously reported Y-chromosome-specific qPCR assay for clinical applications (85), design work centered on an intronic region (Yp11.3) of the SRY gene, in particular using bases 1726-1965 from GenBank locus L10102 (HUMSRYB). Some efforts were also made to design assays at or near published Y-STR loci (82). These design efforts were unsuccessful (results not shown), mainly because sequences between the primers and the Y-STRs were not suitable (too short, or too AT-rich) for placement of a TaqMan™ detection probe. Prior to ordering 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*) (50). 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 or cross-chromosome reactivities. Primer and probe sequences for selected Y-specific qPCR assays are provided in Table 12. The primers labeled “MT1SRYF” and “MT1SRYR” had been used previously for a clinical assay (85). Otherwise, the primers and probes were designed as outlined above. The oligonucleotides listed in Table 12 were used in various combinations to construct potential Y-specific qPCR assays, as indicated by the assays listed in Table 13.

All qPCR assays were run in singleplex, using either SYBR™ Green or TaqMan™ detection. Assays using SYBR™ Green detection chemistry were run as 20 µL amplifications that included 10 µL of SYBR™ Green 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) and 300 nM in each primer. Assays using TaqMan® detection chemistry were run as 20 µL amplifications that included 10 µL of

TABLE 12 - *Primer and probe sequences used for selected Y-chromosome-specific qPCR assays. Oligonucleotides were obtained from Operon (Alameda, CA), unless otherwise indicated (from ABI).*

Oligonucleotide	Sequence (5' → 3')
SRYNEWF1	CGC ATT TTT CAG GAC AGC AGT A
SRYNEWF2	CGT TTT GTG ACA TAA AAG GTC AAT G
SRYNEWR1	AAA CAT GGT AAT TCA GTA ACG TTG ACT AC
MT1SRYF	TGG CGA TTA AGT CAA ATT CGC
MT1SRYR	CCC CCT AGT ACC CTG ACA ATG TAT T
SRYMODF1	AAA TTG GCG ATT AAG TCA AAT TCG
SRYMODR2	GTA TTC ATT CTC AAG CAA AAC ATG GTA A
SRYNEWTM1	***HEX***-TGC CCT GCT GAT CTG CCT CCC T-***BHQ***
YTMMGB1	***VIC***-CCC TGC TGA TCT GCC T-***MGBNFQ*** (ABI)
VICSRYaMGB1	***VIC***-ATT CTC AAG CAA AAC ATG-***MGBNFQ*** (ABI)

TABLE 13 - *Descriptions of and results for selected Y-chromosome-specific qPCR assays. Results are shown for the TaqMan™- (or TaqManMGB™-)detected assays only. Sequence information for the primers and probes is provided in Table 12.*

	Oligonucleotides Used in Assay			Sequence Parameters			Assay Fluorescence Signal Strength			Parameters Derived from Assay Standard Curve			Standard Deviation of CT at Various DNA Inputs		
Assay	F.Primer	R.Primer	Probe	Length of Amplicon (bp)	Length of Probe (nt)	3' Primer to 5' Probe (nt)	Probe Signal Before Hydrolysis (RFU)	Probe Signal After Hydrolysis (RFU)	Final Delta Rn Value	PCR Efficiency (%)	Y-Intercept (cycle)	R-Squared	at 6.4ng (cycle)	at 0.64ng (cycle)	at 0.064ng (cycle)
SRY-1	SRYNEWF2	SRYNEWR1	SRYNEWTM1	131	22	2	1200	2850	1.2	92.4	32.4	0.979	0.16	0.15	0.55
SRY-2	SRYNEWF1	SRYNEWR1	YTMMGB1	82	16	4	850	4000	2.1	79.0	32.0	0.956	1.12	0.02	0.73
SRY-3	SRYNEWF2	SRYNEWR1	YTMMGB1	131	16	4	800	4250	2.8	95.0	31.6	0.988	0.13	0.18	0.44
SRY-4	MT1SRYF	MT1SRYR	VICSRYaMBG1	137	18	2	1450	3900	1.6	90.7	32.7	0.971	0.30	0.15	0.74
SRY-5	MT1SRYF	MT1SRYR	YTMMGB1	137	16	45	800	4400	2.7	91.2	30.7	0.992	0.19	0.08	0.35
SRY-6	SRYMODF1	SRYMODR2	YTMMGB1	121	16	22	800	4100	2.3	97.2	31.1	0.993	0.02	0.42	0.12

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 primer, and 200 nM in TaqMan™ (or TaqManMGB™) probe.

Real-time qPCR data were collected on an Applied Biosystems Prism® 7000 SDS instrument controlled by version 1.0 or 1.1 of the 7000 SDS Collection software. The instrument was typicallyconfigured for the following run conditions: 20 µL sample volumes; 9600 emulation mode; one 10 minute 95 ºC polymerase activation step, followed by 45 cycles of 2-step qPCR (15 s of 95 ºC denaturation, 60 s of 60 ºC combined anneal/extension). Well-to-well variations in background fluorescence were corrected for by use of a ROX-labeled passive reference oligonucleotide, 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 ºC /min) from 60 ºC to 95 ºC. Amplification curves were analyzed by using empirically established threshold and baseline settings. For assays that used SYBR® Green detection, the threshold was set at 0.15; for assays that used TaqMan™ (or TaqManMGB™) detection, the threshold was set from 0.08 to 0.1, with the goal of keeping the threshold in the middle of the exponential growth portion of the amplification curves for each type of assay. For both types of detection, data from cycles 6 through 15 were used for baseline corrections.

For each qPCR run, the SDS Collection software generated a linear standard curve plot of C_T (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 standard DNA (male). Standard curves were constructed using data from standard DNA dilutions containing 6.4 ng (or 3.2 ng) of total DNA per sample (in duplicate), 0.64 ng (in triplicate) and 0.064 ng (in replicates of five). For negative controls, TE^{-4} (in duplicate), 0.32 ng female DNA (in duplicate), and 64 ng female DNA (in duplicate) were included for each assay. Nuclear DNA copy numbers were estimated using the ratio of one haploid autosomal copy per 3.3 pg genomic DNA or one copy of Y chromosome per 6.6 pg genomic DNA (55).

Where appropriate, qPCR amplification efficiencies were determined from the slopes of the linear calibration curves (% PCR efficiency = $100[(10^{(-1/slope)} - 1)]$) (56).

Results

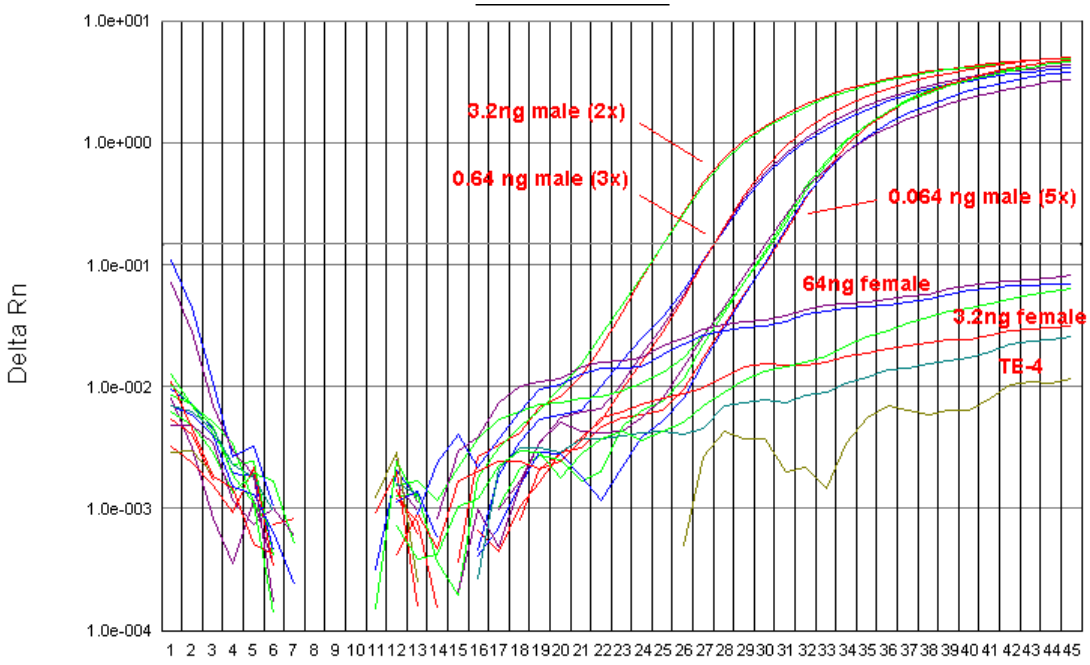
In developing a Y-chromosome-specific assay, we considered several important qPCR properties: (i) specificity for amplifying the Y-chromosome (i.e., no spurious cross-reactivity to human autosomes or to the X-chromosome); (ii) absence of amplicon in no-template control samples (TE^{-4}); (iii) PCR efficiency (>90%); (iv) fluorescence signal strength; (v) quality of standard curve (correlation coefficient); (vi) sensitivity of quantification; and (vii) precision of quantification (e.g., standard deviation of C_T), especially at low copies of template. A number of potential Y-specific qPCR assays were examined, and six promising TaqMan®-detected assays were identified based on the criteria just listed. Results using these assays are provided in Figure 18 (SYBR® Green-detected) and in Table 13 (TaqMan®-detected).

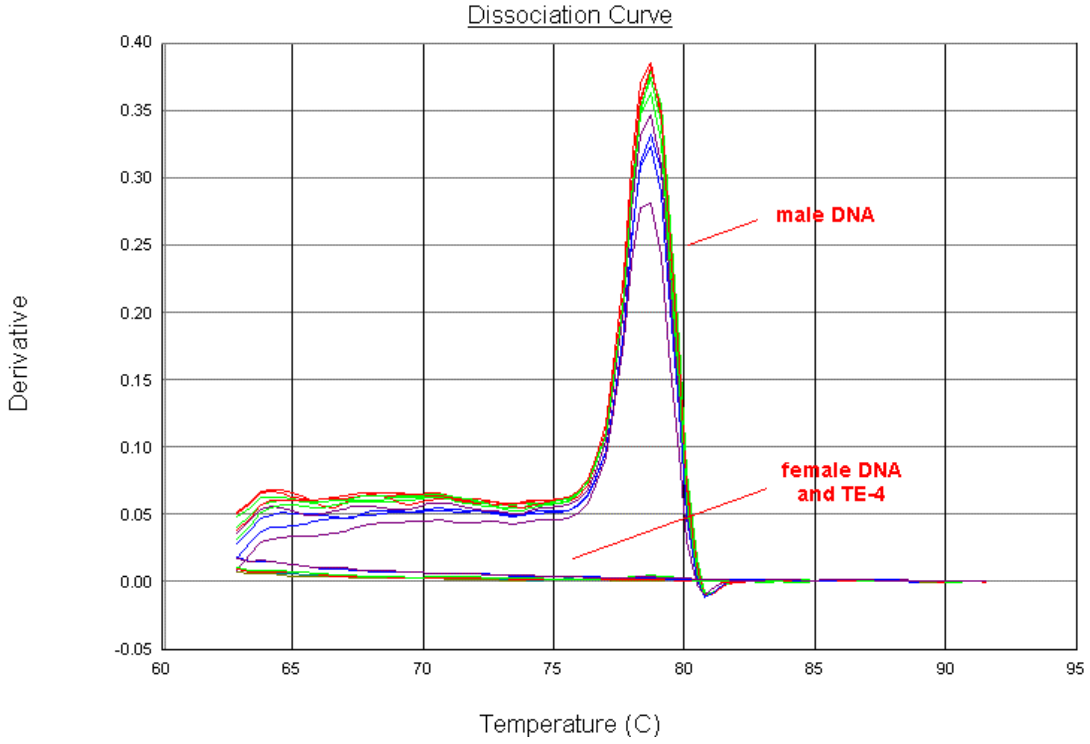
As indicated by the SYBR® Green-detected results shown in Figure 18, most primer combinations showed no evidence for any spurious amplification of either female DNA or TE^{-4}. The primers for assays SRY-1 and SRY-3 showed a very slight amplification of female DNA (Fig. 18(b)) in one of two replicates, though the lateness of C_T (41 cycles, corresponding to a deduced template amount of 0.17 pg) indicated that the spurious amplification was very inefficient. Neither assay SRY-1 or SRY-3 showed any amplification of female DNA when using TaqMan® detection (results not shown).

Table 13 summarizes the pertinent properties of the six TaqMan®-detected Y-specific assays. Based upon these properties, assays SRY-5, SRY-3, and SRY-6 appear to be the most promising candidates for further work. Assays SRY-1 and SRY-4 showed relatively poor final fluorescence signals (final delta Rn values less than 2), and assay SRY-2 exhibited relatively poor PCR efficiency (<80%) and poor C_T precision. Moreover, SRY-2 amplifies a short target sequence (82 bp), which may not be ideal for quantifying DNA in degraded samples.

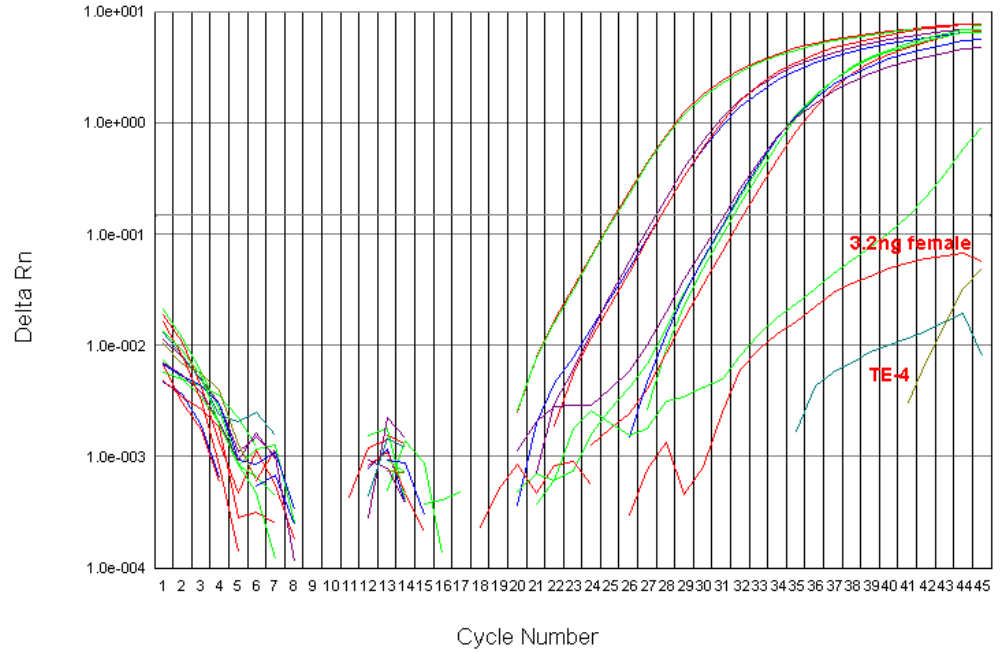
FIG. 18 - *SYBR™ Green qPCR results for selected Y-chromosome-specific qPCR assays. For each assay, the top panel shows amplification curves from a standard dilution series of male DNA; the bottom panel shows the corresponding melt (or dissociation) curves. Panel (a) provides the template quantities used for each standard dilution series.*

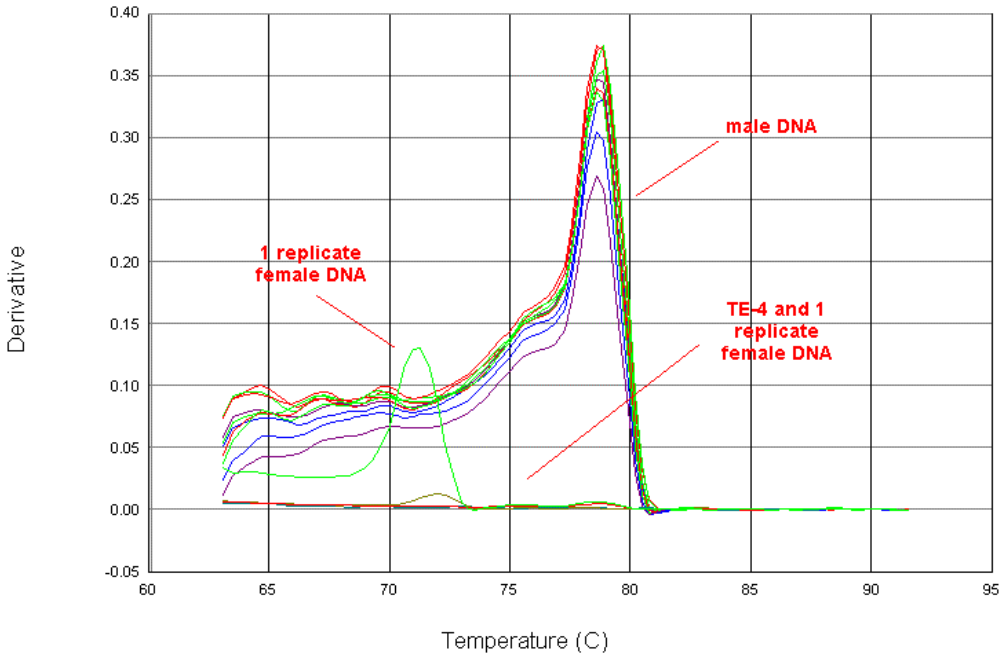
(a) SRY-4 and SRY-5 (forward primer = MT1SRYF, reverse primer = MT1SRYR)



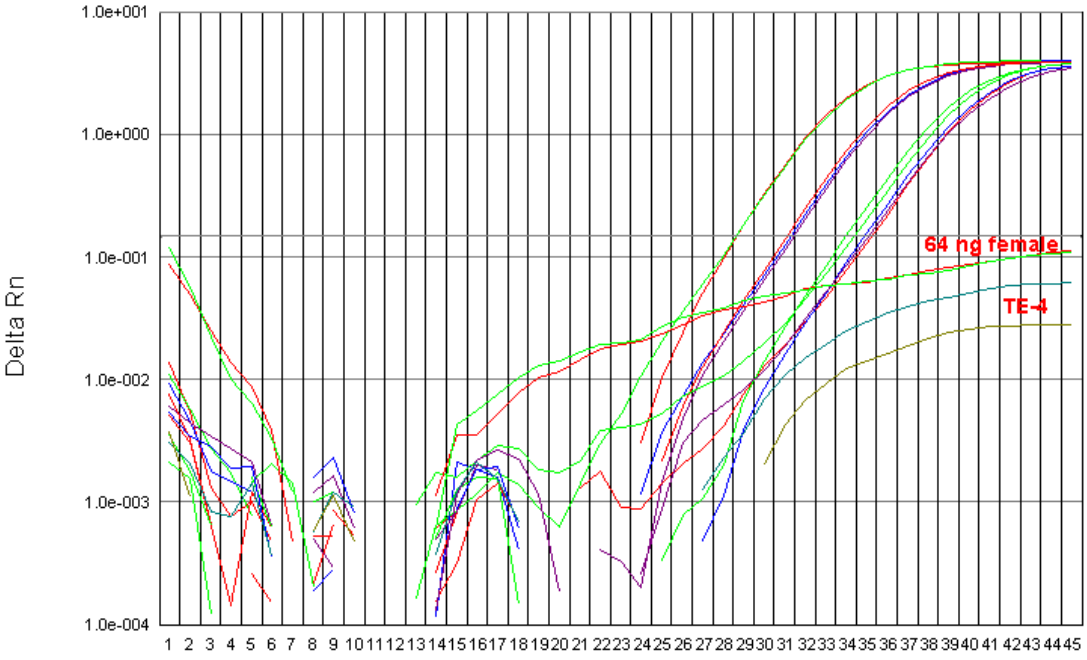


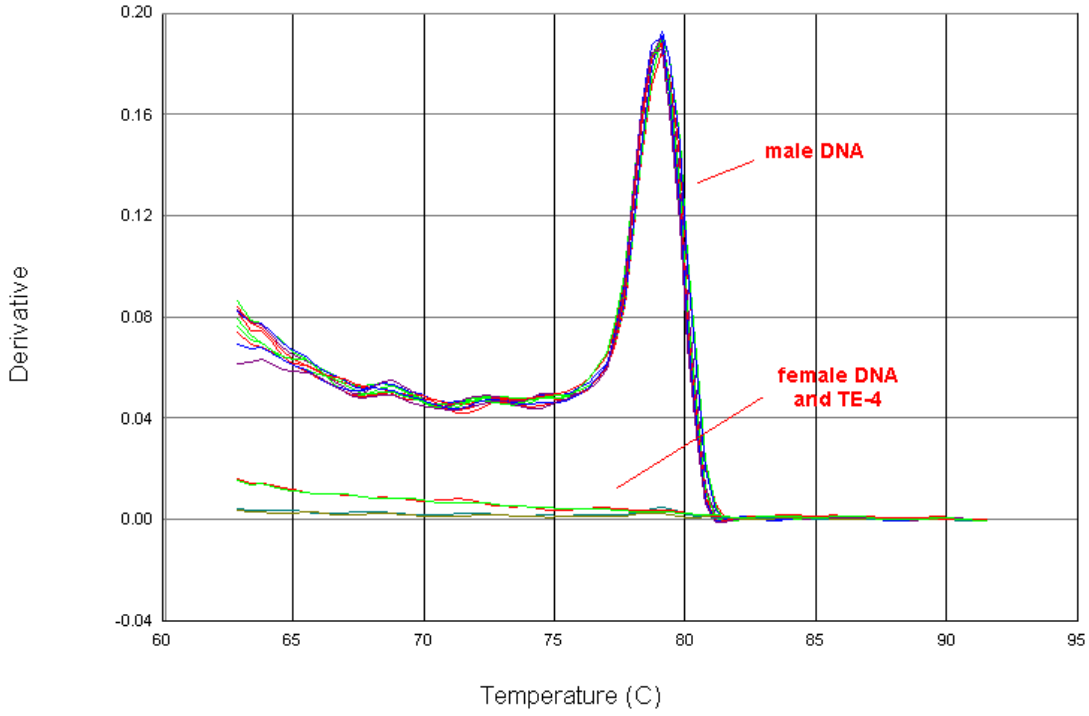
(b) SRY-1 and SRY-3 (forward primer = SRYNEWF2, reverse primer = SRYNEWR1)



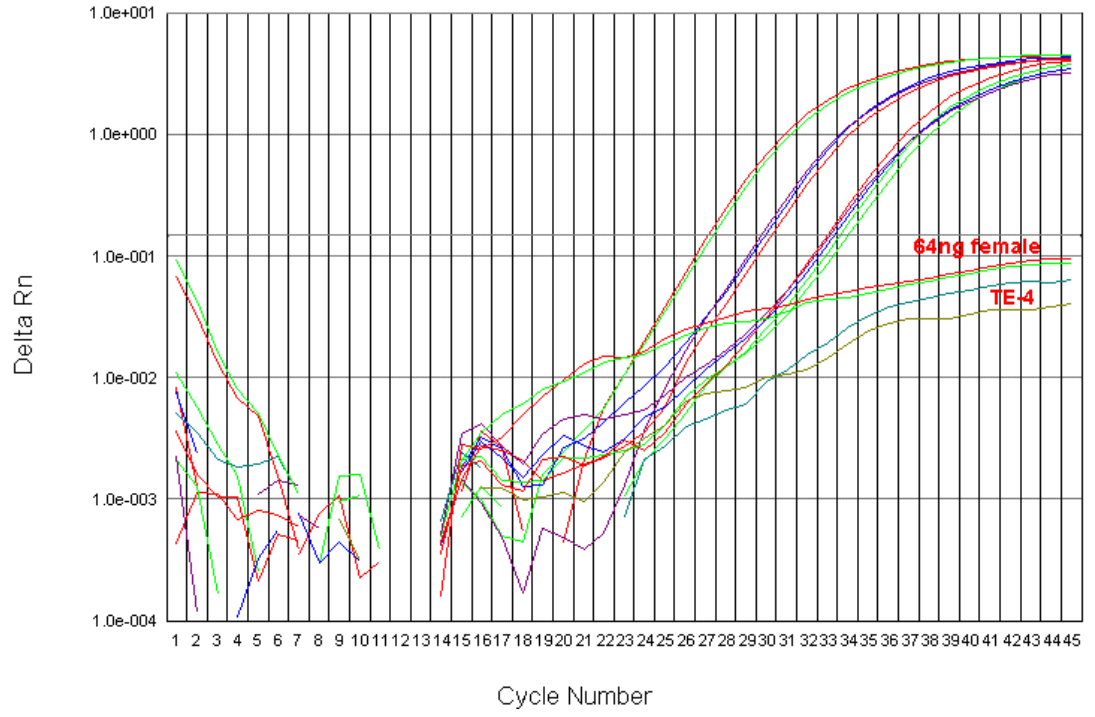


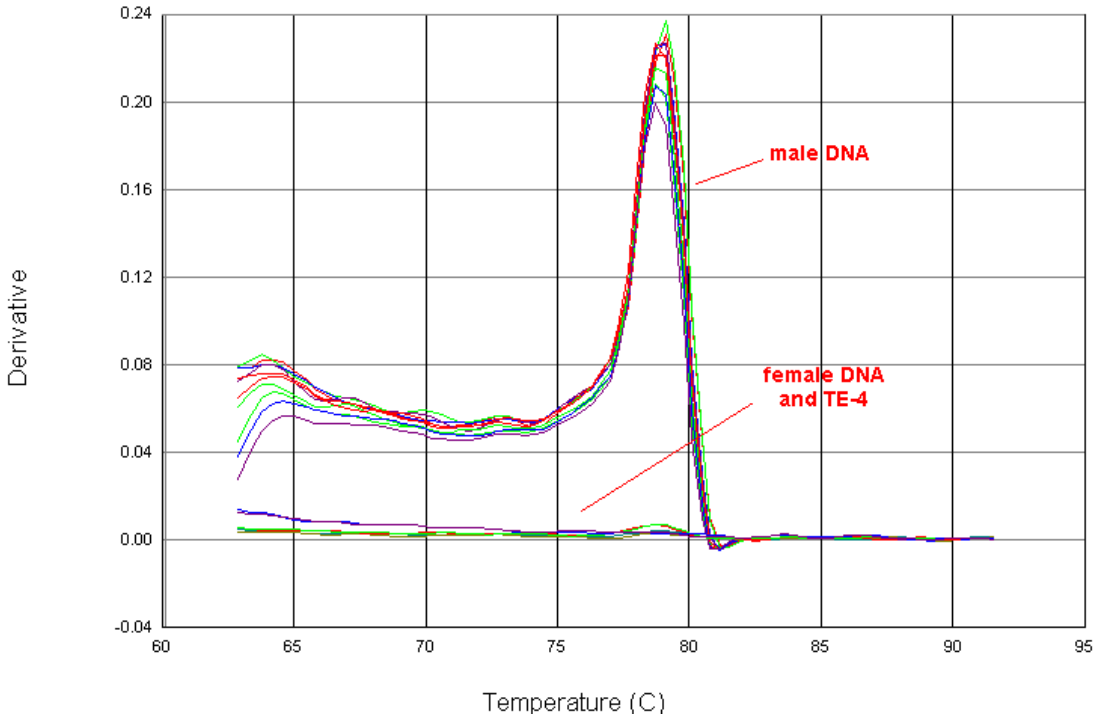
(c) SRY-2 (forward primer = SRYNEWF1, reverse primer = SRYNEWR1)





(d) SRY-6 (forward primer = SRYMODF1, reverse primer = SRYMODR2)





Discussion

Having identified at least three promising Y-chromosome-specific qPCR assays, the next steps will be to examine their abilities to provide accurate quantifications under less than ideal conditions, in particular, in mixtures with large excesses of human female DNA and in mixtures with excesses of non-human (microbial and non-microbial) DNAs. The assays will also be examined in duplex with autosomal qPCR assays and with an IPC assay. This work is ongoing.

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