Real-Time DNA Quantification of Nuclear and Mitochondrial DNA in Forensic Analysis

BioTechniques 33:402-411 (August 2002)

Hanna Andréasson, Ulf Gyllensten, and Marie Allen
Uppsala University, Rudbeck Laboratory, Uppsala, Sweden

ABSTRACT

The rapid development of molecular genetic analysis tools has made it possible to analyze most biological material found at the scene of a crime. Evidence materials containing DNA quantities too low to be analyzed using nuclear markers can be analyzed using the highly abundant mtDNA. However, there is a shortage of sensitive nDNA and mtDNA quantification assays. In this study, an assay for the quantification of very small amounts of DNA, based on the real-time Taq-Man® assay, has been developed. This analysis will provide an estimate of the total number of nDNA copies and the total number of mtDNA molecules in a particular evidence material. The quantification is easy to perform, fast, and requires a minimum of the valuable DNA extracted from the evidence material. The results will aid in the evaluation of whether the specific sample is suitable for nDNA or mtDNA analysis. Furthermore, the optimal amount of DNA to be used in further analysis can be estimated, ensuring that the analysis is successful and that the DNA is retained for future independent analysis. This assay has significant advantages over existing techniques because of its high sensitivity, accuracy, and the combined analysis of

nDNA and mtDNA. Moreover, it has the potential to provide additional information about the presence of inhibitors in forensic samples. Subsequent mitochondrial and nuclear analysis of quantified samples illustrated the potential to predict the number of DNA copies required for a successful analysis in a certain typing assay.

INTRODUCTION

Forensic DNA identification systems allow routine DNA typing of minute amounts of DNA, such as those found in small bloodstains, shed hairs, semen stains, and saliva stains (2,25). Developments in the sensitivity of routine analysis methods have broadened the spectrum of biological materials that can be analyzed in a criminal investigation. Teeth, dandruff, epidermal cells, and fingerprints on various items are now commonly used as biological evidence materials (12,21,29). Moreover, very old and degraded DNA materials such as skeletal remains can also be analyzed (10,14,27).

When the DNA found in an evidence sample is not too scarce, fragment size analysis of multiplex short tandem repeat loci is used in routine forensic DNA analysis. The short tandem repeat markers are very polymorphic, and a multiplex analysis of 10–16 loci has a very high discrimination power (25). Although the sensitivity of nDNA analysis has increased in recent years, there are several types of evi-

dence materials in which the DNA content is too limited to perform an nDNA analysis. To overcome the limited DNA amounts, the analysis of mtDNA has frequently been used because of the high copy number of mtDNA molecules per cell (6). An mtDNA analysis is very sensitive, and amounts as small as 30 fg DNA (corresponding to 10 mtDNA molecules) can be analyzed, although larger DNA quantities should be used when analyzing forensic materials as a safeguard (1). However, since the discrimination power of mtDNA analysis is substantially lower in comparison with a multi-loci nuclear analysis, it is most useful in cases where additional circumstantial evidence exists and for exclusions.

It is of great importance in forensic DNA analysis to ensure that the limited amount of DNA found in a particular evidence material is not wasted: first, in an analysis that is not sensitive enough, and second, by using more DNA in each reaction than is required for a successful, reliable analysis. It is also important to guarantee that the more discriminating nDNA analysis is performed on a material whenever possible. Thus, to ensure the optimal use of the valuable DNA material in an evidence material, a highly sensitive quantification assay for nDNA and mtDNA is a useful tool.

The routine quantification of forensic samples is done, in many forensic laboratories, by hybridization to the human-specific D17Z1 probe. Initially,

Table 1. Primer and Probe Sequences for Singleplex and Multiplex Quantification Analysis

Name	Specificity	5' Sequence
mt-8294F	mtDNA	CCACTGTAAAGCTAACTTAGCATTAACC
mt-8436R	mtDNA	GTGATGAGGAATAGTGAAGGAGTATGG
mt-8345	mtDNA	VIC CCAACACCTCTTTACAGTGAATGCCCA
RB1-2672F	nDNA	CCAGAAAATAAATCAGATGGTATGTAACA
RB1-2750R	nDNA	TGGTTTAGGAGGGTTGCTTCC
RB1-2727	nDNA	FAM CAGCACTTCTTTTGGACACACGGTCG

The fluorescent label of the probe is shown in 5' of the sequence.

this method required the use of radioactivity (33). Later, biotinylated oligonucleotide probes replaced the radioactively labeled probes (32). A less common method for quantification is competitive PCR, which requires the analysis of multiple reactions as well as an internal standard for each sample to be quantified (8). A more sensitive fluorescence-based system for routine DNA quantification has been developed using the intercalating dye PicoGreen® (16). Recently, several assays and detection systems suitable for DNA quantification have been developed. The AluQuant™ system (Promega, Madison, WI, USA), based on a luciferase reaction read in a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, USA), detects nDNA in the range of 0.1–50 ng (22). Pyrosequencing™ (Pyrosequencing AB, Uppsala, Sweden) has also been used to quantify small DNA amounts in the presence of a competitor (26). While there are several quantification assays, these are limited to nDNA quantification and will not quantify mtDNA. A recent study has demonstrated the use of real-time PCR for the detection and quantification of mtDNA (24). However, this system is designed to detect a 4977-bp deletion in human tissue for age determination rather than determination of mtDNA copy numbers in forensic samples.

DNA quantification based on the real-time 5' exonuclease detection assay (TaqMan®), using the ABI PRISM® 7700 instrument (both from Applied Biosystems, Foster City, CA, USA), has gained widespread use in many areas for low-copy DNA quantification (15,17,20). Real-time detection is a very fast and accurate technology that

does not require post-PCR processing since the detection is done during each PCR cycle. The TaqMan assay is based on the cleavage of a target-specific probe by the 5'→3' exonuclease activity of *Taq* DNA polymerase, resulting in an increase in reporter emission intensity. The threshold cycle (C_T) is inversely proportional to the target sequence concentration. Furthermore, a multiplex analysis of multiple targets is possible using dyes with a large difference in emission wavelength [e.g., 6-carboxyfluorescein (FAM) and VIC™; ABI PRISM7700 User's Manual].

In this study, a DNA quantification system for both nDNA and mtDNA, using the TaqMan assay, is described. The quantification assay has been designed as a multiplex analysis of mtDNA and nDNA simultaneously, limiting the DNA amounts required for the quantification of forensic casework samples. Thus, a simultaneous estimation of the total number of nDNA copies and the total number of mtDNA molecules in a particular evidence sample is obtained. The nuclear target in this multiplex quantification system is the human retinoblastoma susceptibility gene (*RB1*), a nuclear-encoded single copy gene located on chromosome 13 (23). No variations have been reported in the region from nucleotides 1311 to 3083 of the *RB1* gene, and the target in this quantification system is located between nucleotides 2672 and 2750 (National Center for Biotechnology Information, Bethesda, MD, USA). The mitochondrial target spans over the genes for tRNA lysine and ATP synthase 8 in the coding region of the mitochondrial genome. Here the targeted region, from nucleotides 8294 to 8436, has a few rare polymorphic sites reported (Mitomap;

<http://infinity.gen.emory.edu/mitomap.html>) (18). The use of this assay allows the optimal analysis method to be selected and the optimal amounts of DNA to be used, which conserves valuable DNA material. Moreover, the analysis can give an indication of the presence of inhibitors in the sample.

MATERIALS AND METHODS

DNA Extraction

Human control DNA from the Burkitt lymphoma human cell line (Applied Biosystems) was used, and the DNA concentrations were converted to number of DNA copies, assuming that 1 ng DNA corresponds to 333 genome equivalents. Plasmid clones contained a 613-nucleotide-long mtDNA fragment (8049–8661). Copy numbers for this control plasmid were determined by limited dilution PCR analysis of reactions containing one mtDNA copy per tube. The expected frequency of positive reactions was calculated according to a Poisson distribution. Quantifications were also done by spectrophotometric measurements (7). The forensic evidence material was purified by one of three methods. (i) The Wizard® Genomic DNA Extraction Kit (Promega) was used to extract DNA from evidence material collected on cotton swabs; (ii) Chelex® 100 (Bio-Rad Laboratories, Hercules, CA, USA) was used to extract DNA from bloodstains (31); and (iii) hair was extracted using proteinase K and DTT (1,30). All DNA extractions were done in, or resuspended to, a total volume of 100 µL.

DNA Quantification

Primers and probes were designed using the Primer Express™ software (Applied Biosystems). The probes were labeled at the 5'-end with FAM for nDNA detection and with VIC (fluorescein-based) for mtDNA detection. A quencher dye, 6-carboxytetramethylrhodamine (TAMRA), was linked to the 3'-end of both probes (Applied Biosystems).

The quantification assay was performed in a total volume of 25 µL containing 5 µL DNA extract, 1× TaqMan

Research Report

Universal PCR Master Mixture, 400 nM each primer (mt-8294F and mt-8436R or RB1-2672F and RB1-2750R), and 200 nM probe (mt-8345 or RB1-2727) (Table 1). To enhance the PCR efficiency in samples containing inhibitors, 0.16 mg/mL non-acetylated BSA (Sigma, St. Louis, MO, USA) and 3.75 U extra AmpliTaq Gold® DNA polymerase (Applied Biosystems) were included in the reactions.

The amplification and detection were performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems). The PCR was initiated with 2 min at 50°C for the activation of the AmpErase® UNG (Applied Biosystems), followed by 10 min at 95°C. This was followed by 50 cycles of 15 s at 95°C and 1 min at 50°C. Reactions without templates served as negative controls. The calculation of C_t was performed using the Sequence Detection System software, version 1.6.3 (Applied Biosystems), and plotting against the log of the copy number-generated standard curves. The C_t value was set to 0.10 for all reactions, and the copy number value for unknown samples was inferred from the regression line of the standard curves.

To perform two independent reactions in the same tube, the primer concentrations were adjusted. A multiplex optimization experiment was set up using 50, 300, and 900 nM each of mitochondrial:RB1 primers (i.e., mitochondrial:RB1 primer concentration ratios of 50:50, 50:300, 50:900, 300:50, 300:300, 300:900, 900:50, 900:300, and

900:900) for the amplification of both mtDNA and nDNA. Further optimization by limiting the mtDNA primer concentration (using 40, 60, 80, 100, 120, 140 nM each of mitochondrial primers), while the RB1 primers were kept at a constant concentration of 300 nM, was done to achieve equally efficient amplifications. The optimization experiments were performed on samples containing total DNA amounts that corresponded to 10 and 100 mtDNA or nDNA molecules in addition to the standard curve dilutions. The probe concentration was kept constant at 200 nM in all experiments. The fluorescence intensities of the reporter dyes (FAM and VIC) and quencher dye (TAMRA) were recorded throughout the amplification process, as for a singleplex TaqMan, and standard curves were generated for mtDNA and nDNA respectively.

HLA-DRB1 Analysis and mtDNA Sequencing

The HVII region of the mtDNA D-loop was amplified in 12 latent fingerprints using the primer pair L00029 and H00408 (28). Sequencing was performed according to the manufacturer's protocol (<http://docs.appliedbiosystems.com/search.taf>), using the ABI PRISM BigDye™ Primer Cycle Sequencing Ready Reaction Kit and an ABI PRISM 377 instrument (both from Applied Biosystems). The nuclear marker HLA-DRB1 was analyzed in 18 swab-collected samples derived from various accessories (glasses, earrings, rings, and

watches). The typing was done using a RELI™ SSO typing kit (DynaL Biotech Ltd., Bromborough, UK), according to the manufacturer's recommendations (<http://www.tissue-typing.com/ssso.html>) (5). Both targets (mtDNA and DRB1) were amplified for 38 PCR cycles.

RESULTS

The TaqMan assay used in this study is designed to amplify both a nuclear and an mtDNA target as a tool in routine forensic DNA analysis. Standard curves, of serial dilutions of genomic and cloned mtDNA with known concentrations, were generated to estimate the mtDNA and nDNA copy numbers in a specific sample. The dilution series were analyzed using the primer/probe set for the nuclear target (RB1-2672F, RB1-2750R, and RB1-2727) and the mitochondrial target (mt-8294F, mt-8436R, and mt-8345) separately (Figure 1, A and B). Decreasing amounts of input DNA revealed consistently higher C_t numbers for both the mitochondrial and nuclear target. Moreover, in most experiments, both targets could be detected down to single copies in the dilution series for the standard curve, which indicated that the system is highly sensitive. In addition, the linear relationship between the log of the input DNA copy number and concordant C_t values when analyzed in five separate experiments clearly demonstrates the usefulness, sensitivity, and reproducibility of the assay for the quantifi-

Figure 1. Amplification of mtDNA and nDNA. The y-axis is shown as a logarithmic scale. (A) mtDNA, 10^5 (red)– 10^1 (pink) starting copies detected using the mt-8345 probe. (B) nDNA, 10^5 (red)– 10^0 (pink) starting copies. The reaction was performed using the RB1-2727 probe.

Table 2. Summary of TaqMan Quantification Results of Diverse Forensic Samples

Sample Type	mtDNA Copy Number/Sample					nDNA Copy Number/Sample				
	0– 1000	1000– 10 000	10 000– 100 000	>100 000	Total	0– 100	100– 1000	1000– 10 000	>10 000	Total
Hair	2	3	6	15	26	10	5	3	6	24
Bloodstains	–	5	3	14	22	–	3	2	14	19
Fingerprints	7	8	17	2	34	6	15	4	–	25
Skin debris ^a	6	8	32	20	66	5	20	22	5	52
Saliva stains ^b	3	3	2	1	9	–	1	–	–	1
Other ^c	25	35	14	5	79	12	15	4	2	33
Total	43	62	74	57	236	33	59	35	27	154
%	18	26	31	24		21	38	23	18	

The table contains the results of all analyzed samples such as skin cells, saliva stains, hair, and bloodstains, including negative samples.

^aClothes, jewelry, cell phones, etc.

^bStamps, robber hoods, etc.

^cBlood, skin debris, or saliva.

cation of limited amounts of DNA (Figure 1, A and B, and Figure 2A). Samples diluted to 0.1 DNA copy were analyzed with an amplification success rate of 44% (data not shown). The deviation from the expected frequency of 10% could be due to contamination, stochastic effects of a deviation of the copy numbers in the dilution series. Nevertheless, the presence of negative reactions without templates and the fact that a majority of the samples were negative

indicated that the dilutions of the standard curve samples had DNA copy numbers within the expected range.

The assay was tested on a large number of different evidence materials that had been mtDNA sequence analyzed previously (Table 2). The quantification of these samples was performed using the singleplex system. Approximately half of the samples contained less than 10 000 mtDNA copies or 1000 nDNA copies (44% and 59%, respectively). A

majority of the samples were sent to the laboratory because the DNA amounts were too low to perform nDNA analysis. Thus, the results show the average DNA copy numbers typical of samples containing limited DNA rather than average DNA amounts in forensic samples in general. The nDNA quantification was performed only on samples with more than 2000 mtDNA copies. The quantification results were divided into three groups containing low, inter-

Figure 2. Comparison of the standard curves for nDNA and mtDNA. Each estimate is based on five replicates, and the x-axis is shown as a logarithmic scale. (A) Standard curves based on the singleplex reactions of a mitochondrial clone and genomic DNA. Blue ■, mtDNA; green ◆, nDNA. (B) Standard curves based on the analysis of genomic DNA. Blue ■, mitochondrial target, multiplex; green ◆, nuclear target, multiplex; red ●, mitochondrial target, singleplex control; and yellow ▲, nuclear target, singleplex control.

Research Report

mediate, and high DNA amounts to study the DNA content in different sample categories. Noteworthy, skin samples (skin debris and fingerprints) appear to contain substantial amounts of DNA. Duplicate analysis within experiments and repeated analysis of the same samples in different tests showed reproducible results.

Interestingly, some samples showed an amplification curve with a slightly different shape in the TaqMan assay, which indicated lower amplification efficiency. Forensic evidence materials and ancient DNA often contain inhibitors, even after applying extraction and purification procedures, which lower the efficiency of the reaction. The addition of BSA and high polymerase concentrations to the reaction has proven efficient in overcoming inhibitors (13,27). Several of the samples were quantified, both with and without the addition of BSA and extra AmpliTaq Gold DNA polymerase. The analysis of the samples with additives produced a curve shape consistent with regained amplification efficiency in comparison with the analysis without the additives (Figure 3). Furthermore, the analysis of saliva stains on stamps and envelopes taken from 40 different letters from 1952 all showed altered curves without the BSA addition. The quantification assay with the addition of BSA resulted in copy numbers ranging from 50 to 500 mtDNA copies per microliter of extract for the 40 samples.

The subsequent mtDNA sequence analysis (HVII) was successful in 95% of the samples when BSA was used, in contrast to a success rate of 50% for the same analysis when the samples were amplified without BSA. This presents the possibility of detecting the presence of inhibitors in forensic samples by the altered shape of the curve. In the analysis of the forensic materials presented in Table 2, approximately 10% of the samples showed an altered curve shape. There is a risk of underestimating the copy numbers in samples with inhibitors, as illustrated by the analysis of sample 1 with and without additives, which resulted in a large C_t value difference (Figure 3).

To evaluate the success rate in subsequent typing of forensic materials quantified using the TaqMan system, mtDNA sequencing of the HVII region in the D-loop and analysis of the nuclear marker HLA-DRB1 were performed (Table 3). The quantification results shown in Table 3 reflect the total DNA copy numbers in the samples. One-tenth of the extract (10 μ L) was used in the PCR to amplify the 381-bp-long mtDNA target and the 272-bp DRB1 target. The results indicate that unambiguous mtDNA sequence results can be obtained when at least 80 mtDNA copies are used and, similarly, that DRB1 typing will require at least 200–300 nDNA copies.

To evaluate the system's performance when using multiple probes

Table 3. mtDNA and DRB1 Analysis of Forensic Samples Quantified for Their Total mtDNA or nDNA Content

Latent Fingerprints ^a			
No.	mtDNA ^c	PCR	HVII Sequence
1	0	+	-
2	100	-	-
3	100	+	-
4	200	+	-
5	300	-	-
6	800	+	+
7	2000	+	+
8	2800	+	+
9	8200	+	+
10	9900	+	+
11	19 000	+	+
12	30 500	+	+
Skin Debris ^{a,b}			
No.	nDNA ^c	PCR	DRB1
13	0	-	-
14	400	-	-
15	400	+	-
16	700	-	-
17	1100	+	-
18	1600	+	-
19	2100	+	-
20	2100	+	+
21	3100	+	-
22	3300	+	+
23	3400	+	+
24	3500	+	+
25	4700	+	+
26	6600	+	+
27	7600	+	+
28	15 500	+	+
29	56 600	+	+
30	106 500	+	+

^aThe fingerprints were analyzed by HVII mtDNA sequencing, and the skin samples were analyzed by DRB1 typing.

^bSkin samples derived from various accessories (glasses, earrings, rings, and watches).

^cQuantification figures were set to the closest hundred.

+, a positive reaction, readable sequence, or interpretable DRB1 typing. -, a negative or ambiguous result.

Figure 3. Quantification of mtDNA in two saliva stains from 1952. Blue ■, sample 1 with the addition of BSA and extra enzyme; green ■, sample 1, no additives; yellow ■, sample 2 with the addition of BSA and extra enzyme; and red ■, sample 2, no additives.

with different dyes in a single reaction, limiting primer concentrations were tested on dilution series from genomic DNA. Several different primer concentration combinations for the two targets were examined. The C_t values for both targets were found to be identical to singleplex reactions only when the mtDNA primer concentration was limited. A concentration of 300 nM for the RB1 primers and an mtDNA primer concentration of 140 nM were found to reveal comparable results for singleplex and multiplex reactions (Figure 2B). To compare the singleplex and multiplex systems, a total of 28 samples were analyzed using both systems (data not shown). The results showed that the quantification data from the singleplex and multiplex reactions for both the nuclear target and mitochondrial target were similar and well within the range of variation.

DISCUSSION

Forensic DNA analysis of biological evidence materials containing very small amounts of DNA is becoming widely used in criminal investigations. Therefore, a reliable and highly sensitive DNA quantification system is necessary to make optimum use of the limited material available. This quantification system has been proven to be sensitive, reliable, reproducible, and very useful in routine forensic DNA analysis. The system can be used to pre-screen evidence material before further analysis to select the correct analysis method (nuclear or mitochondrial markers; single or multiplex PCR). The standard curves for the singleplex and multiplex reactions were linear with similar C_t values for both the nuclear and mitochondrial targets, indicating similar amplification effi-

ciency for both targets.

To obtain quantification from low starting copy numbers (less than 100), 50 PCR cycles were performed. Thus, all possible precautions to avoid contamination, such as physical separation, tip barriers, and chemical treatment, are necessary. The use of the TaqMan Universal PCR Master Mix is preferred since AmpErase UNG is included to prevent contamination from previous PCR products (19).

A recommendation in the design of the quantification assay is to keep the targets short because the assay will perform optimally using short products (application note 117MI02-02; <http://docs.appliedbiosystems.com/search.taf>). Most targets used in forensic identification are larger in size than the TaqMan product, and the DNA is often degraded. Using longer products would give a better estimate of the number of

Research Report

available high molecular weight targets, but it would also result in a failure to detect short fragments present in a degraded sample.

According to manufacturers of forensic typing kits, short tandem repeats can be used when at least 170–330 DNA copies are present in the sample. The sensitivity of the AmpFISTR® Profiler Plus™ kit (Applied Biosystems) is 1–2.5 ng, and the sensitivity of the PowerPlex™16 System kit (Promega) is 0.5–1 ng nDNA. However, interpretable results can be obtained using less than 0.5 ng DNA. Moreover, by using additional PCR cycles, the sensitivity of the STR analysis can be further increased (9,11). In materials where there are fewer cells than required for a nuclear analysis, mtDNA analysis is an alternative. However, mtDNA analysis is prone to contamination because of the high DNA copy number per cell. The occurrence of contamination can be minimized by following guidelines and recommendations for mtDNA analysis. This includes, among other

precautions, two independent PCR and sequence analyses of each sample and the analysis of an independent extract where possible (3).

In comparison with many other quantification assays used in forensic DNA analysis, quantification using the TaqMan assay is faster, easier, and more sensitive. The early methods for quantification of human genomic DNA, based on the hybridization of probes to DNA immobilized on nylon membranes, had a detection limit of about 500 copies (1.6 ng nDNA) (33). The use of biotinylated oligonucleotide probes has increased this sensitivity to a detection limit of about 50 copies (150 pg) (32). The fluorescence-based assay for DNA quantification by Hopwood et al. (16) is capable of detecting 80 copies (250 pg/mL) (16). The Alu-Quant system can quantify as few as 30 copies (0.1 ng), and 1–30 samples can be analyzed in 1–2 h (22). TaqMan is a very rapid and robust assay with high-throughput capacity. The quantification of one full plate will, in only 2 h, reveal the total number of mtDNA and nDNA molecules down to a few copies in 80 forensic samples (16 wells are used for standard curves and reactions without templates).

An interesting observation is that inhibitors present in the samples give rise to an altered curve shape in the quantification analysis. The amplification of samples containing inhibitors showed increased amplification efficiency and a normal curve shape after the addition of either BSA or extra enzyme. However, this is an initial observation that should be further evaluated by the titration of known inhibitors and BSA in the quantification analysis.

Typing of the HLA-DRB1 gene in samples quantified for their nDNA content showed that at least 200 nDNA copies are required for an interpretable DRB1 typing of this 272-bp target. However, the HLA-DRB1 amplification and typing were done according to the standard protocol, without other optimizations for limited forensic materials than addition of BSA. Consequently, optimizing this nDNA analysis or using other nDNA typing systems can substantially increase the sensitivity. Similarly, the mtDNA sequence analysis of the HVII region of mtDNA-quantified

samples showed that when at least 80 mtDNA copies were used in the PCR, the analysis was successful.

There are several areas in forensic analysis in which the TaqMan assay can be useful in addition to DNA copy number determination in a particular evidence sample. First, the assay can be used to determine the average DNA copy number in a particular type of forensic sample, such as fingerprints, dandruff, hairs, or other materials. Furthermore, the effects on DNA content of different treatments of forensic materials, such as the chemical visualization of fingerprints, can be evaluated. The assay can be used to determine the amplification efficiency and yield of separate templates when optimizing multiplex PCR-typing systems by using probes labeled with different dyes. In addition, the ratio of mtDNA molecules per cell can be evaluated in different tissues. This is useful in both forensic evidence evaluation and in studies of diseases associated with mtDNA mutations. Moreover, DNA extraction procedures are inefficient and associated with a substantial loss of valuable DNA in the forensic sample. Therefore, different DNA extraction and purification procedures can be evaluated and compared using this quantification system with regards to DNA recovery and removal of inhibitors. A major problem in the forensic analysis of minute amounts of DNA is to determine the tissue source of the sample (e.g., whether the sample is derived from skin cells, saliva, or blood). An RT-PCR of tissue-specific mRNA markers using the real-time quantification assay can be useful in these cases. Although it will be a challenge to recover RNA from forensic samples due to limited amounts and degradation, it has been shown that epithelial-specific mRNA can be detected in six-month-old bloodstains (4).

This study presents a highly sensitive quantification assay based on the TaqMan real-time fluorescent 5' exonuclease assay. It is a rapid and reliable test with high throughput because of its 96-well plate format. A major advantage over the existing quantification assays is high sensitivity and the real-time format, which eliminates the need for further analysis after the amplification step. The system quantifies both

mtDNA and nDNA in various forensic evidence materials, and the assay can be performed as a multiplex in which mitochondrial and nuclear markers are detected simultaneously to save valuable DNA, time, and cost. Thus, an estimation of the optimal DNA target, mtDNA or nDNA, is possible, in order to obtain a discrimination power as high as possible for that particular sample. Moreover, the assay will ensure that a minimum amount of DNA is used for a successful amplification and that DNA is retained for repeated analysis.

ACKNOWLEDGMENTS

This work was supported by grants from the Beijer Foundation and the Swedish Research Council for Medical Sciences (MFR grant nos. K2002-31P-12577-05C and K2002-31X-13095-04B) to M.A. Dr. Lucia Cavelier kindly provided the mtDNA clone.

REFERENCES

- Allen, M., A.S. Engstrom, S. Meyers, O. Handt, T. Saldeen, A. von Haeseler, S. Paabo, and U. Gyllensten. 1998. Mitochondrial DNA sequencing of shed hairs and saliva on robbery caps: sensitivity and matching probabilities. *J. Forensic Sci.* 43:453-464.
- Allen, M., T. Saldeen, U. Pettersson, and U. Gyllensten. 1993. Genetic typing of HLA class II genes in Swedish populations: application to forensic analysis. *J. Forensic Sci.* 38:554-570.
- Bar, W., B. Brinkmann, B. Budowle, A. Carracedo, P. Gill, M. Holland, P.J. Lincoln, W. Mayr, et al. 2000. DNA Commission of the international society for forensic genetics: guidelines for mitochondrial DNA typing. *Int. J. Legal Med.* 113:193-196.
- Bauer, M., A. Kraus, and D. Patzelt. 1999. Detection of epithelial cells in dried blood stains by reverse transcriptase-polymerase chain reaction. *J. Forensic Sci.* 44:1232-1236.
- Begovich, A.B. and H.A. Erlich. 1995. HLA typing for bone marrow transplantation. New polymerase chain reaction-based methods. *JAMA* 273:586-591.
- Bogenhagen, D. and D.A. Clayton. 1974. The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells. Quantitative isolation of mitochondrial deoxyribonucleic acid. *J. Biol. Chem.* 249:7991-7995.
- Cavelier, L., A. Johannisson, and U. Gyllensten. 2000. Analysis of mtDNA copy number and composition of single mitochondrial particles using flow cytometry and PCR. *Exp. Cell Res.* 259:79-85.
- Diviacco, S., P. Norio, L. Zentilin, S. Menzo, M. Clementi, G. Biamonti, S. Riva, A. Falaschi, et al. 1992. A novel procedure for quantitative polymerase chain reaction by coamplification of competitive templates. *Gene* 122:313-320.
- Gill, P. 2002. Role of short tandem repeat DNA in forensic casework in the UK—past, present, and future perspectives. *BioTechniques* 32:366-372.
- Gill, P., P.L. Ivanov, C. Kimpton, R. Piercy, N. Benson, G. Tully, I. Evett, E. Hagelberg, et al. 1994. Identification of the remains of the Romanov family by DNA analysis. *Nat. Genet.* 6:130-135.
- Gill, P., J. Whitaker, C. Flaxman, N. Brown, and J. Buckleton. 2000. An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. *Forensic Sci. Int.* 112:17-40.
- Ginther, C., L. Issel-Tarver, and M.C. King. 1992. Identifying individuals by sequencing mitochondrial DNA from teeth. *Nat. Genet.* 2:135-138.
- Hagelberg, E., B. Sykes, and R. Hedges. 1989. Ancient bone DNA amplified. *Nature* 342:485.
- Handt, O., M. Hoss, M. Krings, and S. Paabo. 1994. Ancient DNA: methodological challenges. *Experientia* 50:524-529.
- Holland, P.M., R.D. Abramson, R. Watson, and D.H. Gelfand. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. USA* 88:7276-7280.
- Hopwood, A., N. Oldroyd, S. Fellows, R. Ward, S.A. Owen, and K. Sullivan. 1997. Rapid quantification of DNA samples extracted from buccal scrapes prior to DNA profiling. *BioTechniques* 23:18-20.
- Josefsson, A., K. Livak, and U. Gyllensten. 1999. Detection and quantitation of human papillomavirus by using the fluorescent 5' exonuclease assay. *J. Clin. Microbiol.* 37:490-496.
- Kogelnik, A.M., M.T. Lott, M.D. Brown, S.B. Navathe, and D.C. Wallace. 1997. MITOMAP: an update on the status of the human mitochondrial genome database. *Nucleic Acids Res.* 25:196-199.
- Kwok, S. and R. Higuchi. 1989. Avoiding false positives with PCR. *Nature* 339:237-238.
- Livak, K.J., S.J. Flood, J. Marmaro, W. Giusti, and K. Deetz. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl.* 4:357-362.
- Lorente, M., C. Entrala, J.A. Lorente, J.C. Alvarez, E. Villanueva, and B. Budowle. 1998. Dandruff as a potential source of DNA in forensic casework. *J. Forensic Sci.* 43:901-902.
- Mandrekhar, M.N., A.M. Erickson, K. Kopp, B.E. Krenke, P.V. Mandrekhar, R. Nelson, K. Peterson, J. Shultz, et al. 2001. Development of a human DNA quantitation system. *Croat. Med. J.* 42:336-339.
- McGee, T.L., D.W. Yandell, and T.P. Dryja. 1989. Structure and partial genomic sequence of the human retinoblastoma susceptibility gene. *Gene* 80:119-128.
- Meissner, C., S.A. Mohamed, H. Klueter, K. Hamann, N. von Wurmb, and M. Oehmichen. 2000. Quantification of mitochondrial DNA in human blood cells using an automated detection system. *Forensic Sci. Int.* 113:109-112.
- Moretti, T.R., A.L. Baumstark, D.A. Defenbaugh, K.M. Keys, J.B. Smerick, and B. Budowle. 2001. Validation of short tandem repeats (STRs) for forensic usage: performance testing of fluorescent multiplex STR systems and analysis of authentic and simulated forensic samples. *J. Forensic Sci.* 46:647-660.
- Nygren, M., M. Ronaghi, P. Nyren, J. Albert, and J. Lundeberg. 2001. Quantification of HIV-1 using multiple quantitative polymerase chain reaction standards and bioluminescent detection. *Anal. Biochem.* 288:28-38.
- Paabo, S., J.A. Gifford, and A.C. Wilson. 1988. Mitochondrial DNA sequences from a 7000-year old brain. *Nucleic Acids Res.* 16:9775-9787.
- Sullivan, K.M., R. Hopgood, and P. Gill. 1992. Identification of human remains by amplification and automated sequencing of mitochondrial DNA. *Int. J. Legal Med.* 105:83-86.
- van Oorschot, R.A. and M.K. Jones. 1997. DNA fingerprints from fingerprints. *Nature* 387:767.
- Vigilant, L. 1999. An evaluation of techniques for the extraction and amplification of DNA from naturally shed hairs. *Biol. Chem.* 380:1329-1331.
- Walsh, P.S., D.A. Metzger, and R. Higuchi. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques* 10:506-513.
- Walsh, P.S., J. Varlaro, and R. Reynolds. 1992. A rapid chemiluminescent method for quantitation of human DNA. *Nucleic Acids Res.* 20:5061-5065.
- Waye, J.S., L.A. Presley, B. Budowle, G.G. Shutler, and R.M. Fournery. 1989. A simple and sensitive method for quantifying human genomic DNA in forensic specimen extracts. *BioTechniques* 7:852-855.

Received 19 December 2001; accepted 5 April 2002.

Address correspondence to:

Dr. Marie Allen
Department of Genetics and Pathology
Rudbeck Laboratory
751 85 Uppsala, Sweden
e-mail: marie.allen@genpat.uu.se

For reprints of this or
any other article, contact
Reprints@BioTechniques.com