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Abstract

Quantification of human DNA is key in forensic genetics. A more accurate estimate of the amount of DNA is essential for planning and optimising genotyping assays, as is evaluating the presence of PCR inhibitory substances and DNA degradation status. Multiplex qPCR assays are helpful in forensics because they can quantify different targets simultaneously, thus saving valuable samples, time, and labour. The aim of this study was to highlight the challenges in the developmental validation of a multiplex real-time PCR assay and the drawbacks encountered in translating a previously described and validated assay (SD quants) to a different technology by modifying the dye probes and reagent mix to be used in a different instrument. We developed a TaqMan probe-based multiplex qPCR using reagents and fluorescent probes adapted for the Rotor-Gene 6000 instrument (QIAGEN, Hilden, Germany). The initial assay combined two mitochondrial DNA (mtDNA) and two nuclear DNA (nDNA) targets, with amplification products of different sizes (mtDNA = 69 and 143 bp; nDNA = 71 and 181 bp), to estimate the DNA degradation status and an internal positive control (IPC) to detect potential inhibitors. During the initial testing of the assay, we observed an interaction between the 69 bp mtDNA target and the 71 bp nDNA target probe, and experiments were conducted to resolve this issue without success. We removed the small nDNA target (71 bp) and changed from a 5-plex to a 4-plex qPCR assay (qMIND). The final tetraplex assay was tested on 105 forensic samples and/or small amounts of degraded DNA, such as bones, teeth, fingernails, formalin-fixed paraffin-embedded tissues (FFPE), and hair shaft samples. The quantification results were compared with data acquired from the same samples using another commercially available quantification system commonly used in forensic laboratories. In addition, the short tandem repeat (STR) profiles were investigated to determine their correlation with the quantitative values obtained. Overall, the qPCR assay was robust and reliable for DNA quantification in samples commonly used in forensic practice.

Keywords: Forensic DNA quantification, Multiplex TaqMan qPCR assay, Degraded DNA, Mitochondrial DNA, Nuclear DNA

1. Introduction

In forensics, the ideal scenario is represented by obtaining a full and good-quality genetic profile from evidence present at the crime scene; however, the geneticist is confronted with challenging samples containing poor quality and/or quantity of DNA in many cases. Determination of DNA quantity is essential for successful PCR analysis, for which a precise amount of DNA input is required. Correct quantification of DNA saves time and preserves the extracted DNA. This is particularly important for forensic samples where the state of preservation and amount of genetic material present are not known a priori. Moreover, with particular relevance to highly compromised specimens, PCR may fail because of insufficient DNA quantity and because of the presence of co-extracted inhibitors and degraded DNA.

Currently, real-time quantitative PCR (qPCR) is the technique of choice for quantification in the forensic field because it is highly sensitive, specific, and reproducible. Another advantage is that it enables the simultaneous quantification of several targets, thereby saving valuable samples, time, and labour. Several commercially available qPCR assays are used to evaluate the quantity and/or quality of nuclear DNA (nDNA) [1-5]. However, highly compromised samples, such as ancient DNA specimens or highly degraded samples, often fail to produce results using the nDNA system. The use of mitochondrial DNA (mtDNA) significantly increases the chances of obtaining interpretable results for these samples, given the greater number of copies in human cells. However, unlike nDNA, there are few commercially available assays for mtDNA quantification, the only two solutions are the NovaQUANT Human Mitochondrial to Nuclear DNA Ratio Kit (Merck KGaA, Darmstadt, Germany) [6] and Human Mitochondrial DNA Copy Number Quantification qPCR Assay Kit (ScienCell Research Laboratories, Inc., Carlsbad, CA, USA). However, neither of these solutions enables evaluating mtDNA degradation. Therefore, several research groups have studied specific qPCR methods to quantify and/or evaluate the degradation of mtDNA in the last two decades [7-20], some of which have described the simultaneous quantification of mtDNA and nDNA [7-11, 13-14, 19]. Among these, a method that meets the characteristics required for an ideal qPCR assay, such as target specificity, high sensitivity, and multiplex compatibility between mtDNA and nDNA, was published by Xavier et al. [19]. They described a multiplex qPCR assay (SD quants system) performed on an ABI Prism 7500 instrument (Applied Biosystems, Foster City, CA, USA), which simultaneously quantified mtDNA and nDNA, provided information on mtDNA degradation, and detected the presence of inhibitors using an internal positive control (IPC). Compared to similar assays, this test enabled direct and absolute quantification using a synthetic oligonucleotide to calculate the number of mtDNA copies in the genomic DNA standard. However, the SD quants assay is not compatible with all qPCR instruments because it is specifically designed to run on Applied

Biosystems/Thermo Fisher Scientific real-time PCR platforms. Therefore, changes that mainly concern the fluorescent dyes used are required to adapt the SD quants assay to other platforms.

In this study, we described a TaqMan probe-based multiplex qPCR assay based on the SD quants system [19], but suitable for the Rotor-Gene 6000 instrument (QIAGEN, Hilden, Germany). Our initial assay combined five targets: two mtDNA targets, two nDNA targets, and one IPC control. The mitochondrial and nuclear amplified products were of different sizes, 69 bp and 143 bp for mtDNA and 71 bp and 181 bp for nDNA. The targets selected can provide information on the degradation status of the samples, and IPC enables the detection of potential inhibitors. The novel aspects of our multiplex assay were (1) adaptation of the SD quants system to the Rotor-Gene 6000 instrument with reagents and fluorescent probes optimised for this instrument, (2) addition of a large nDNA target (181 bp) and (3) a newly redesigned small nDNA target (71 bp). However, during the qPCR assay, we observed an interaction between the 69 bp mtDNA target and 71 bp nDNA target probe. Despite the many experiments conducted, we could not resolve this interaction; therefore, we removed the 71 bp small nDNA target, and the qPCR assay was changed from a 5-plex to a 4-plex. The final tetraplex assay enabled the assessment of the quantity of both genomes and the degradation status of mtDNA. This assay helps increase the success rate of DNA typing and the use of the optimal amount of DNA for subsequent processes to preserve the DNA extract and is undoubtedly a valuable tool in forensic genetic practice.

The aims of this study were to (1) underline the challenges and drawbacks encountered in adapting an already validated qPCR assay to a different technology, and (2) describe a multiplex qPCR assay, which is very useful for forensic samples with small and/or degraded DNA routinely encountered in forensic practice.

The tetraplex assay was performed on 105 challenging forensic samples comprising bones, teeth, fingernails, formalin-fixed paraffin-embedded tissues (FFPE) and hair shaft samples. Human head hair is among the most commonly encountered crime scene evidence types, but it is the most limited in terms of DNA quantity and quality. These limitations can be overcome with whole mitochondrial genome (mtGenome) sequencing using massive parallel sequencing (MPS) technology. MPS provides an increase in information and discriminatory power and enables better detection of heteroplasmy than conventional Sanger sequencing, even from samples with only a minute amount of mtDNA, such as hair shaft samples [21-26]. Concerning mtDNA, it is very important to know the concentration in forensic samples because too little or too much DNA added in an amplification reaction would yield suboptimal sequencing results. For instance, adding too much DNA to the MPS library preparation process can produce undesirable outcomes due to several factors, including the detection of nuclear mitochondrial DNA segments (NUMTs)[22, 27-28]. NUMTs can complicate mtDNA sequence analysis by producing a mixture of sequences that may be misinterpreted as point heteroplasmy (PHP), potentially resulting in an incorrectly mtDNA haplotype. The quantification and degradation results acquired from these real casework samples were verified by comparing amplification products obtained using different amplification methods commonly used in forensic practice, such as PowerPlex ESX 17 and ESI

17 Fast Systems (Promega, Madison, WI, USA), PowerPlex Fusion 6C System (Promega) for nDNA, and sequencing of the control region (CR) or the entire mtGenome.

2. Material and methods

2.1. Multiplex TaqMan qPCR design

The qPCR primers and probes for mtDNA and IPC were obtained from the literature [19]. Two nuclear targets were designed ex novo and integrated into a previously developed qPCR assay to evaluate nDNA quantification and degradation. The sequence of the RNU2 multi-copy gene (GenBank accession number L37793.1) was used to design these two nDNA targets (nuRNU2_181bp and nuRNU2_71bp). Primers and probes for this target were designed using the Primer3plus v.2.4.2 software (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) [29]. The human specificity of the primer and probe sets was tested using NCBI's Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Nucleotide Basic Local Alignment Search (BLASTn) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) tools. We aligned the nucleotide sequences of the GRCh37 genome assembly using the BLAT tool available in the UCSC browser (https://genome.ucsc.edu/cgi-bin/hgBlat) to evaluate the number of potential target sequences for each of the five targets. Dimer and hairpin evaluations were performed using both AutoDimer software (https://www-s.nist.gov/dnaAnalysis/primerToolsPage.do) [30] and the Multiple Primer Analyzer software package (https://www.thermofisher.com/it/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html). A pentaplex real-time PCR assay was developed by combining two mtDNA and two nDNA targets with amplification products of different sizes (Table 1) to estimate the DNA degradation status, plus an IPC to detect potential inhibitors. All primer and probe sequences, together with the final concentrations used, are listed in Table 1.

All primers, small nDNA target, and IPC probes were synthesised by Microsynth AG (Balgach, Switzerland), while mitochondrial and large nDNA target probes were synthesised by Thermo Fisher Scientific (Waltham, Massachusetts, USA) and Aurogene s.r.l. (Rome, Italy), respectively.

All quantification targets were first tested in singleplex reactions to determine whether they worked. We maintained the concentrations reported in [19] and shown in Table 1 for the final primer/probe concentration used in the multiplex PCR.

Target name	5-plex	4-plex	Primer and probe sequences (5'-3')	Final concentration	Amplicon size (bp)	Reference
mtND1	Yes	Yes	**F**_CCC TAA AAC CCG CCA CAT CT	300 nM	69 bp	[19]
			R_ GAG CGA TGG TGA GAG CTA AGG T			
			Probe _ VIC-CCA TCA CCC TCT ACA TC-**MGBNFQ**	100 nM		
mt143bp	Yes	Yes	**F**_CCA CTG TAA AGC TAA CTT AGC ATT AAC C	300 nM	143 bp	[19]
			R_ GTG ATG AGG AAT AGT GTA AGG AGT ATG G			
			Probe _ FAM-CCA ACA CCT CTT TAC AGT GAA-**MGBNFQ**	100 nM		
nuRNU2_181bp	Yes	Yes	**F_** GTG TGG ACT CTG GTG ACC TG	900 nM	181 bp	NA
			R_ CTC AGC TAT CAC CTC TGC CG			
			Probe _Quasar 705-TCC CAG GGC CAC CCG TAA CT-**BHQ-2**	500 nM		
nuRNU2_71bp	Yes	No	**F**_TCT CGC TGT TCA ACT CCC AC	900 nM	71 bp	NA
			R_ GCA AAC TGA CAC AGG AAG CG			
			Probe _ ROX-AGG AGC GAG AAC ATG CGG TGT-**BHQ-2**	500 nM		
IPC	Yes	Yes	**F**_ATC AGC TTA GCG TGC AGT CA	100 nM	70 bp	[19]
			R_ TCT TCG TCG TAA CGG TGA GC			
			Probe _ Cy5-GTT GCA CTA CTT CAG CGT CCC A-**BHQ-2**	100 nM		
OLIGO IPC	Yes	Yes	ATC AGC TTA GCG TGC AGT CAG ATA ATG TTG CAC TAC TTC AGC GTC CCA AGC TCA CCG TTA CGA CGA AGA G	0.001 nM	-	[19]

Table 1. Description of primers and probes used in the qPCR assay and final 20 µL reaction concentrations.

2.2. qPCR assay validation

All reactions were performed in a final volume of 20 µL , containing 5 µL of 4x QuantiNova Multiplex PCR Master Mix (QIAGEN) [31], 2 µL of the sample, the respective primer and probe concentration (Table 1) and nuclease-free water up to the final volume. Amplification and fluorescence detection were performed using a Rotor-Gene 6000 instrument with the following PCR conditions: 2 min at 95 °C for denaturation and enzyme activation and 40 cycles at 95 °C for 3 s and 60 °C for 30 s during the combined step of annealing and extension. Cycle threshold (C_t) calculations, standard curves, and quantity calculations were performed using the Rotor-Gene Q software v.2.1.0 (QIAGEN). We investigated the linear dynamic range (R^2), PCR efficiency, and slope parameters for all runs according to the MIQE guidelines [32]. Occasionally, one or two points were removed if a particular DNA dilution significantly deviated from the trend line produced by the remaining data.

Both the nDNA standard and the mitochondrial genome equivalent (mtGE) calculation used in this study were performed as previously described [19]. Briefly, for the nuclear targets, an eight-point standard curve was performed from a starting concentration of 62.5 ng/µL (listed on the tube label) down to 0.0008 ng/µL

of a pre-quantified human genomic DNA-G1521 (Promega). A short double-stranded mtDNA sequence was used in five serial dilutions to estimate the number of mtGE in the human genomic DNA-G1521 standard.
As described in the MIQE guidelines [32], eight serial dilutions of the nDNA standard were analysed in triplicate in every developmental run. A no-template control (NTC) was used to test for reagent contamination in each run.

2.2. DNA samples and amplification of forensic markers

In total, 105 forensic samples were subjected to the finalized qPCR assay to evaluate its performance. More specifically, 77 of the 105 samples were hair shaft samples, and the remaining were forensic casework samples of different matrices, such as bones (n = 11), FFPE tissue (n = 4), fingernails (n = 5), and teeth (n = 8). Written informed consent was obtained from all living donors, and the study was reviewed by the Comitato Etico per la Ricerca di Ateneo of the Marche Polytechnic University of Ancona, Italy (Prot.n.0212568 of 28/11/2022).
Forensic samples were extracted using the DNA IQ Casework Pro Kit for the Maxwell 16 instrument (Promega), as described in the manufacturer's manual [33]. Hair samples were extracted following a protocol described in [25] and skeletal remains (bone and teeth) were extracted using the Bone DNA Extraction kit (Promega) protocol [34] together with the Maxwell 16 instrument. The DNA extract was stored at −20 °C until quantification.
Except for the hair shaft samples, the samples selected for this study had previously been quantified using the Plexor HY System kit (Promega), according to the manufacturer's instructions [35], and the runs were performed in the Rotor-Gene 6000 instrument. In detail, the Plexor HY System kit quantifies the concentration of total human DNA and male human DNA simultaneously. Moreover, this kit contains an IPC to test for the presence of PCR inhibitors.
Autosomal short tandem repeat (STR) analyses were performed using one of the following commercial kits: PowerPlex ESX 17 and ESI 17 Fast Systems and PowerPlex Fusion 6C System kit on a GeneAmp 9700 PCR thermocycler (Life Technologies, Carlsbad, CA, USA). The amplified product was separated on the ABI Prism 3500 Genetic Analyzer instrument (Life Technologies) and analysed with GeneMapper ID-X v1.4 software using the panel and bins provided by the manufacturers [36-38].
Seven of these (M5, 6864-3, 5898, 5899-1, 5899-2, 5900-1, 5900-2) were subjected to Sanger sequencing of the mtDNA CR (16024-576). PCR amplification and sequencing of the entire CR were performed according to [39- 41]. Briefly, the CR was amplified with ten different primer sets in two independent multiples to generate overlapping amplicons covering the entire CR of the mtDNA. Sanger sequencing data were generated using a 3130 Genetic Analyzer (Life Technologies). All sequences were imported into SeqScape v2.5 (Life Technologies) and aligned according to the revised Cambridge Reference Sequence for human mtDNA (rCRS) [42].

The entire mtGenome was sequenced using MPS and the Applied Biosystems Precision ID mtDNA Whole Genome Panel (Thermo Fisher Scientific) for 47 samples. The libraries were manually prepared using the Precision ID Library kit (Thermo Fisher Scientific) with the "2 in 1 protocol" [43]; the maximum amount of DNA (6 µL) and 26 cycles were used for the amplification phase. Each library was quantified in duplicate using the Ion Library TaqMan Quantitation Kit (Thermo Fisher Scientific), normalised to a final concentration of 40 pM, and run on the Ion GeneStudio S5 System (Thermo Fisher Scientific) using three different chips. Bioinformatics data analysis was performed using Torrent Suite v. 5.10.1 and the Coverage Analysis v. 5.12.0.0 plugin, and secondary analysis was performed using Integrative Genomic Viewer (IGV)[44]. The sequencing results of these samples are part of a larger, ongoing collaborative study; therefore, we will not discuss the final haplotypes obtained for these samples in this paper. 23 hair shaft samples were not subjected to mtDNA sequencing based on the qPCR assay results.

3. Results and discussion

3.1. Assessment of designed qPCR assay

As described above, this assay was based on the SD quants system [19], a TaqMan probe-based multiplex qPCR assay that uses reagents and fluorescent probes suitable for the ABI Prism 7500 instrument. However, the SD quants system is incompatible with the real-time instrument in our laboratory; for example, NED dye, which is related to a large mtDNA target in [19], is not detectable in any of the five channels available in the Rotor-Gene 6000 instrument. Therefore, it was necessary to redesign the qPCR assay by modifying the dye probes and reagent mixture to adapt it to our instrument. The use of Rotor-Gene 6000 compared to the ABI Prism 7500 instrument offers some advantages related to its unique centrifugal rotary design. This rotary format ensures optimal thermal and optical uniformity among the samples, which is critical for a precise and reliable analysis. In addition, the continuous rotation of the samples during the run prevented condensation and the formation of air bubbles.

3.1.1. Target and reagent mix selection

Based on the literature [7, 11, 13, 19], we selected mtDNA targets proven to be highly specific with few polymorphic sites to enable stable hybridisation between the primer/probe oligonucleotide and the target sequence. The large mtDNA target (mt143bp) spans the tRNA lysine and ATP synthase 8 genes from bases 8249 to 8436 in the coding region of mtDNA [7, 13, 19], whereas the small mtDNA target (mtND1) spans the ND1 gene (NADH Dehydrogenase Subunit I) from bases 3485 to 3553 in the coding region [11, 19].

Regarding nDNA targets, choosing a multi-copy gene is ideal because it provides better sensitivity and accuracy [45]. The selected nuRNU2 locus encodes a small nuclear RNA involved in pre-mRNA processing, which is conserved among primates and is organised as a tandemly repeated motif on chromosome 17 [1, 46], making it useful for this purpose. The ability of a qPCR assay to detect genetic information from non-

target species should be determined. Several analyses with Primer-BLAST were performed by querying the "Nucleotide nr/nr" database to verify the species specificity for all primer and probe sets. The mtDNA targets yielded only sequences on the human mtGenome, and no other PCR products were obtained using the synthetic IPC target. However, different results were obtained for the nuRNU2 target, which showed hits with primate genomes. In general, detecting genetic information from non-human species does not necessarily invalidate the use of the assay but may help define the limits of the assay. Therefore, we recommend carefully considering the results obtained for the nuRNU2 target when they are discordant with those obtained for the mtDNA targets. Analysis using the BLAT tool (https://genome.ucsc.edu/cgi-bin/hgBlat) did not identify target sequences for the nuRNU2 templates that matched perfectly. The only sequences found contained four to seven mismatches, most of which were located at the primer- and probe-binding sites. Consequently, these mismatches can influence oligonucleotide hybridisation or primer extension. The lack of dimers and hairpin structures was verified using both AutoDimer [30] and the Multiple Primer Analyzer software.

The QuantiNova Multiplex PCR kit was selected as the reagent mix for our qPCR assay because it does not contain ROX dye, which enabled us to use all five available channels to simultaneously quantify the five targets. Given the fixed optical path and centrifugal system, the Rotor-Gene instrument does not require the use of ROX or Texas probes as passive internal reference dyes, ensuring consistent excitation from sample to sample.

3.1.2. Assay optimisation

To develop a successful multiplex qPCR assay, it is necessary to identify the best reaction conditions to enable independent amplification of each target and avoid preferential amplification of certain targets. Preferential amplification increases when one of the two targets investigated is more abundant than the other, as is the case with nDNA and mtDNA in human cells. In these situations, the limitation of the primer of the most abundant target can rebalance the final amplification products; therefore, the concentrations of primers and probes of mitochondrial targets in this qPCR assay were limited compared to those of nuclear targets. The optimisation of this qPCR assay consisted of several steps.

First, we tested all the targets in singleplex reactions to evaluate the performance of each primer pair and probe. Subsequently, we performed the pentaplex reaction using the same concentrations of each primer and probe reported in Table 1. However, we observed poor values in terms of R^2 (0.793) and PCR efficiency (1285%) for the small mtDNA target (mtND1) during the reaction. The high PCR efficiency value obtained may have been due to the occurrence of primer dimers or primer–probe interactions. However, these were excluded from the previously performed in silico prediction. Moreover, the presence of dimers was excluded using capillary and agarose electrophoresis of multiplex PCR products.

An accurate inspection of the shape of the standard curve (Figure 1) revealed an unexpected trend starting from Standard 4 (0.5 ng/µL) to Standard 8 (0.008 ng/µL). More precisely, the C_t points of Standards 4 –8 were very similar, almost overlapping, and were not separated or well-spaced, as expected. Therefore, we considered the possibility of non-specific hybridisation of the probe, especially in the presence of low concentrations of input DNA.

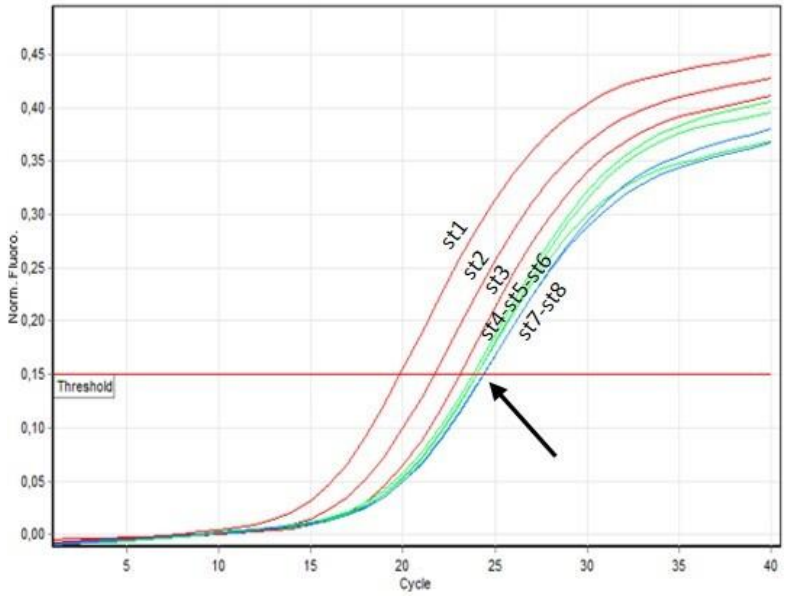

Figure 1. Standard curve for small mtDNA target (mtND1) resulting after the pentaplex qPCR reaction. The arrow points the overlapped C_t values of the lower standards.

We performed several tests (n = 14; Supplementary Table 1) to understand the cause of the low performance observed for the mtND1 target by assessing different combinations of targets and primer/probe concentrations. Detailed information about all the experiments is provided in the supplemental material. Test no. 4 identified an interaction between the mtND1 target and the nuRNU2_71bp target; therefore, we focused on the small nDNA target to verify whether the interaction was due to the primers or probes used. Two tests (Test nos. 10 and 11; Supplementary Table 1) were performed to confirm this hypothesis. In Test no. 10, a new PCR primer pair (nuRNU2_75bp) and the nuRNU2_71bp probe were used for the small nDNA target. However, the resulting qPCR curves showed the same shape again, suggesting that the problem could be due to the probe being used for the small nDNA target. Test no. 11, where we kept the same conditions as Test no. 10, but without the nuRNU2_71bp probe, confirmed this hypothesis and resulted in the expected standard curve for the mtND1 target (Supplementary Table 1). Many experiments were carried out after proving the existence of an interaction between the mtND1 target and the nuRNU2_71bp probe to avoid this interaction, but without success (Tests no. 12–14, Supplementary Table 1). Therefore, the small nDNA target was removed from the qPCR assay by changing from the pentaplex to the tetraplex assay, which was named mitochondrial-inhibition-nuclear-DNA (qMIND).

The tetraplex reaction was tested according to the concentrations reported in Table 1. The final volume of 20 µL was reached by increasing the amount of nuclease-free water required in the qPCR reaction to compensate for the volume of the primers and probes of the eliminated target. Table 2 shows the R^2, PCR efficiency, and slope of the final tetraplex reaction. Both PCR efficiency and R^2 fulfilled the interval established as the optimum according to MIQE guidelines [32] with 90 < PCR Efficiency< 110 and $R^2 \geq 0.980$, indicating the robustness of the assay.

	Target	R^2	PCR efficiency	Slope
qMIND Tetraplex	Small mtDNA (mtND1)	0.983	1.12	-3.066
	Large mtDNA (mt143bp)	0.995	0.99	-3.348
	Large nDNA (nuRNU2_181bp)	0.980	1.00	-3.556

Table 2. Linear dynamic range (R^2), PCR Efficiency and slope values obtained using the qMIND tetraplex reaction divided by the target.

3.1.3. qMIND tetraplex assay validation

The qMIND assay performance was evaluated through the triplicate intra and inter-assay analyses of DNA standard curves, amplified with a starting concentration of 62.5 to 0.0008 ng/µL. Overall, the results showed very good R^2 (≥ 0.980), PCR efficiency (82-109%), and slope ($-3.1 \leq x \leq -3.8$) values, suggesting that the proposed assay is reliable. More specifically, the PCR efficiency, R^2 and slope values for each target in the five independent multiplex runs were evaluated and are reported in Supplementary Table 2. Referring to the MIQE guidelines, all multiplex runs had R^2 values > 0.980, while the PCR efficiencies of all targets were within the range established in the guidelines (90 < PCR Efficiency % < 110), except for slightly lower values obtained from the mtND1 target in multiplex runs 3 and 5. These suboptimal values may have been due to operator errors during manual pipetting. Nevertheless, other targets fulfilled the criteria reported in the MIQE guidelines, indicating the robustness of the qPCR assay.

The qMIND tetraplex assay was mainly designed to assess the mtGenome quantity and quality in forensic samples. Therefore, a very important point in the development of the assay was the determination of the quantity of mtGenome in the human genomic DNA, i.e. G1521 used as a standard, to generate a unique DNA calibration curve for the simultaneous quantification of both genomes. Therefore, a double-stranded mitochondrial oligonucleotide of known concentration [19] was used to estimate the mtGE copies in eight dilutions of the genomic standard. Five dilutions of the mitochondrial oligonucleotide were tested in triplicate. The resulting standard curve yielded an R^2 of 0.998 and a PCR efficiency of 89%, confirming the accuracy of the results (Supplementary Table 3). Supplementary Table 4 reports the values of mtGE copies found in dilutions of human genomic DNA and the C_t values. The reported values represent the average of two replicates for each standard dilution.

3.2. Forensic samples

All forensic samples evaluated in this study were quantified in five qPCR runs, in which each standard was tested in triplicate and each sample was tested in duplicate, as described in the MIQE guidelines [32]. The performance of the qMIND tetraplex assay was tested using forensically relevant tissues, such as skeletal remains, FFPE tissues, fingernails, and hair shaft samples, which are known to contain highly fragmented DNA. In these types of samples, it is often impossible to obtain useful information from nDNA, although there are more chances of obtaining successful results with mtDNA. In fact, this assay was designed considering these particular samples that can be encountered in routine forensics, and it includes two differently sized mtDNA targets to evaluate mtGenome degradation, as well as one nDNA target. Moreover, the assay was suitable in a forensic context in terms of time and quantity of starting material, as it works with only 2 µL of extract and in less than 2 hours.

3.2.1. Mitochondrial target in forensic samples

The quantification values for mtDNA in teeth, FFPE tissue, bone, and fingernail samples are reported in Supplementary Table 5 and Supplementary Figure 1. As expected, the mtDNA quantification values were appreciable for samples with low amounts of nDNA. The quantification values of the mtND1 target for these forensic sample types ranged from 842.920 to 42,901,496.120 mtGE/µL, while the values for the mt143bp target ranged from 406.680 to 19,109,967.630 mtGE/µL.

The two differently sized mtDNA targets available in this qPCR assay enabled the evaluation of the degradation status of these samples, given the ratio of smaller to larger DNA fragments (degradation index (DI)). A value of DI ≤ 1 indicates a sample with intact DNA (concentration of small and large fragments are approximately equal); conversely, a value of DI > 1 indicates a sample with slightly or moderately degraded DNA according to how many more small fragments are present than large ones. The DI of the casework samples varied from 0.764 to 3.954, indicating that the mtDNA of these samples was not degraded (22% of the total samples) or slightly degraded (76% of the total samples). Only one sample (6950 F) showed a DI > 10, indicating high mtDNA degradation. Autosomal STR typing, which resulted in a partial profile, supported the degradation status assumed for the mtGenome. Unfortunately, mtDNA typing was not performed for this sample. Information on the state of mtDNA degradation is useful, especially for highly compromised samples, and directs forensic analysts towards the most appropriate genotyping strategies.

Hair is one of the most commonly found traces at crime scenes [12, 47-49]. However, most of this evidence consists of hair fragments without roots and hair shaft samples, in which nDNA quantity or quality is insufficient to generate standard STR profiles; thus, mtDNA testing is the method of choice. The qMIND tetraplex assay was used to quantify 77 hair shaft samples from 13 individuals (Supplementary Table 6, Supplementary Figures 1 and 2). In total, 68 hair shaft samples were collected and processed as described previously [25]. The quantification values of the small mtDNA target, mtND1, ranged from 55.900 to

3,543.710 mtGE/µL; conversely, the values for the large mtDNA target ranged from 0.400 to 2,642.920 mtGE/µL. The DI of hair shaft samples varied from 1.078 to 585.361, with the highest DI value detected for the last fragment, i.e., that closest to the tip.

Our quantification results confirmed earlier observations reported in other studies [26, 50-51] i.e., i) mtDNA quantity and quality decrease along the length of the shafts, and ii) mtDNA quantity and quality vary considerably within individuals. This study revealed high intra-inter individual differences in mtDNA content among and along hair fragments.

Sanger sequencing of the CR was previously performed on 7 (M5, 6864-3, 5898, 5899-1, 5899-2, 5900-1, and 5900-2) of the 77 hair shaft samples quantified in the study. We observed a correlation between the quantitative and qualitative results obtained using the qMIND tetraplex reaction and mtDNA sequencing for all these samples. More precisely, we expected successful mtDNA sequencing given the very low DI values identified, for six of the seven hair shaft samples; conversely, we expected a partial profile of sample 5899-2 that showed a high degradation value.

In total, 47 hair shaft samples were subjected to the whole mitogenome sequencing using MPS. The correlation between MPS sequencing results and the mtDNA quality/quantity used for MPS library preparation is shown in Supplementary Figure 2. Based on the DI values described above, the plot reports the number of mtGE copies used for library amplification in two different sample groups. The first group, named 1 < DI < 10, included 40 samples showing slight or moderate DNA degradation, whereas the second group, named DI > 10, included 7 samples with degraded DNA. As shown in the plot, the number of mtGE copies decreased with increasing DI. Overall only four samples had more than 2900 mtGE copies as DNA input for PCR library preparation, as required in the “Application guide” [43]. The MPS mean depth values were similar in both degradation groups despite the DNA input. This outcome was expected because all samples were normalised at the same concentration (40 pM) before the templating phase. Full mtDNA profiles were obtained from samples with a DI < 10, and, at most, only 5% of the amplified amplicons for the MPS library were lost in samples with a higher DI. In total, 23 hair shaft samples were not subjected to mtDNA sequencing because of the very low amount of mtDNA (< 10 mtGE copies). Therefore, knowledge of mtDNA quantity enables the selection of samples for processing and optimisation of library preparation by choosing different PCR conditions; that is, cycle numbers can be increased if the quantity of input DNA is low [43].

3.2.2. Nuclear target in forensic samples

The quantification values of the nDNA target of the qMIND tetraplex in forensic casework samples are reported in Supplementary Table 7. These were compared with the quantification values of the Plexor HY System kit. We observed differences in terms of quantification values between the average quantification values of all forensic samples in both methods and these differences were found to be significant after applying a *t*-test (*p*-value < 0.05).

However, these differences did not exist in all samples analysed. The size difference that existed within the nuclear targets used for quantification could explain this outcome. In fact, the Plexor HY amplified a 99 bp region of the nDNA target while the qMIND amplified an 181 bp fragment. Therefore, different results can be expected in the case of DNA degradation.

Previous STR typing performed on the same DNA samples suggested a general DNA degradation status.

Eight samples showed concordant results in both qPCR methods tested (Figure 2), and all samples showed complete STR profiles. Overall, the results suggested that the genetic material was not degraded in these samples.

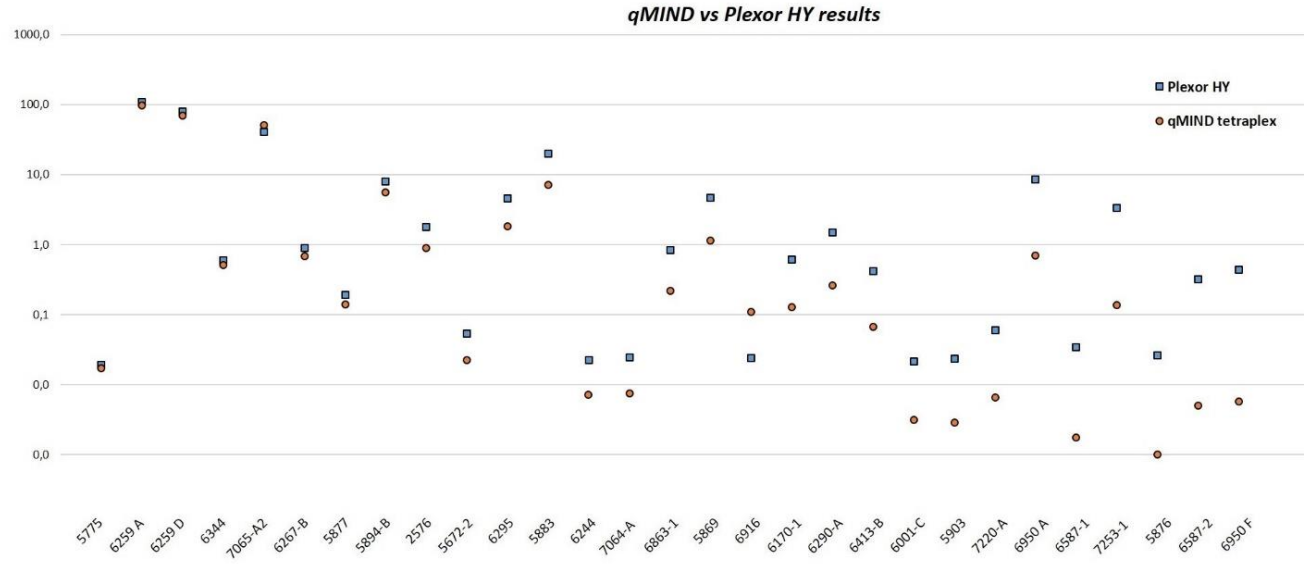

Figure 2. Comparison of casework forensic samples quantification results obtained using qMIND assay and Plexor HY system. In the Y-axis is reported the DNA concentration values (ng/μL) in logarithmic scale.

Indeed, the majority of forensic samples tested showed different quantification values for the autosomal target between the two qPCR assays (Figure 2), with the quantification results of the qMIND target being lower than those of the nuclear target of the Plexor HY kit. The patterns of the STR profiles can explain this difference. In fact, all these samples showed a partial STR profile with a moderate downward slope, suggesting a degradation status of approximately 150 bp. Therefore, DNA degradation explained the differences observed in the quantification of autosomal targets, except for two samples where complete STR profiles were obtained and discordant quantification results remained unexplained.

No results were reported for hair shaft samples for the nDNA target (Supplementary Table 7). These results suggested that the decontamination method used to eliminate exogenous DNA present on the external surface of the shaft was effective. Thus, this qPCR test can be a valuable way to assess whether hair decontamination has been fully achieved, offering a decision point for stopping further analysis of a particular hair segment.

3.2.3 Inhibitor control

The qMIND assay was designed to contain an IPC to identify the presence of inhibitors that could limit PCR efficiency or even prevent PCR amplification. This control included the IPC-specific primer and probe, as well as Oligo IPC, an artificial oligonucleotide designed as previously described [19]. This standard was diluted in TE buffer to achieve serial dilution from 6.0×10^{19} to 600,000 copies/µL and should always amplify when PCR inhibitors are absent. None of the samples in any of the five qPCR runs contained inhibitors.

4. Conclusion

We presented a TaqMan probe-based multiplex qPCR, developed and validated following the MIQE guidelines, which simultaneously quantified nDNA and mtDNA in challenging forensic samples in this study. Initially, our assay combined two differently sized mtDNA (69 and 143 bp) and two nDNA (71 and 181 bp) targets to estimate DNA quantity and DNA degradation status, plus an IPC to detect potential inhibitors. Although this assay is based on an already validated qPCR system, we encountered several obstacles in its development. During the first pentaplex reaction, unsatisfactory results were observed in terms of R^2, PCR efficiency, and slope for the mtND1 target. A series of tests enabled us to identify the interaction between the mtND1 target and nuRNU2_71bp target probe. However, despite the many experiments conducted, we could not resolve this interaction. Therefore, we decided to remove the small nDNA target from the initial 5-plex assay. Overall, our tests emphasised the difficulty of translating an already validated test into a different technology, modifying dye probes, and using a mixture of reagents for adaptation to different instruments.

The qMIND tetraplex assay, tested on 105 challenging forensic samples, was used to identify the amount of nDNA, the quality/quantity of mtDNA and the presence of inhibitors simultaneously using only 2 µL of DNA extract in less than 2 hours all at once. Quantification of the standard curve of human genomic DNA- G1521 ranging from 0.0008 to 62.5 ng/µL with R^2 values > 0.98 was possible. This assay enabled direct and absolute quantification of mtDNA using a synthetic oligonucleotide to calculate the number of mtDNA copies in the human genomic DNA standard.

The results obtained from the qMIND assay for the nuclear genome were compared with the quantification values obtained previously with the Plexor HY System kit and autosomal STR profiles. The greatest discrepancies were found in older or highly degraded samples. However, this was predictable considering the different sizes of the nuclear targets in the two assays examined.

A positive correlation was observed between the quantity and quality of mtDNA copies and the mtDNA profiles. Note that this multiplex nDNA-mtDNA assay is very useful for hair shaft samples because it enables optimisation of MPS library preparation and provides an early assessment of the possible contributions of exogenous DNA, which is critical for the subsequent success of hair genetic typing.

In conclusion, this DNA quantification assay was robust, sensitive, accurate, and reproducible for each forensic matrix tested. We recommend this assay for screening forensically relevant samples with limited DNA quantity and/or quality to identify the optimal downstream analysis for successful DNA profiling.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] B. E. Krenke, N. Nassif, C.J. Sprecher, C. Knox, M. Schwandt, D. R. Storts, Developmental validation of a real-time PCR assay for the simultaneous quantification of total human and male DNA, Forensic Sci. Int. Genet. 3(1) (2008) 14–21, https://doi.org/10.1016/j.fsigen.2008.07.004.

[2] M. M. Ewing, J. M. Thompson, R. S. McLaren, V. M. Purpero, K. J. Thomas, P.A. Dobrowski, G. A. DeGroot, E.L. Romsos, D.R. Storts, Human DNA quantification and sample quality assessment: Developmental validation of the PowerQuant® system, Forensic Sci. Int. Genet. 23(2016) 166-177, https://doi.org/10.1016/j.fsigen.2016.04.007.

[3] A. Holt, S. C. Wootton, J. J. Mulero, P. M. Brzoska, E. Langit, R. L. Green, Developmental validation of the Quantifiler® HP and Trio Kits for human DNA quantification in forensic samples, Forensic Sci. Int. Genet. 21 (2016) 145-157, https://doi.org/10.1016/j.fsigen.2015.12.007.

[4] M. Vraneš, M. Scherer, K. Elliott, Development and validation of the Investigator® Quantiplex Pro Kit for qPCR-based examination of the quantity and quality of human DNA in forensic samples, Forensic Sci. Int. Genet. Suppl. Ser. 6 (2017) e518-e519, https://doi.org/10.1016/j.fsigss.2017.09.207.

[5] J. Kim, J.Y. Jung, S.Y. Kwon, P. Kang, H. Park, K.M. Seong, T.U. Kim, H.K. Yoon, S.K. Lim, Development and forensic validation of human genomic DNA quantification kit, Int. J. Legal Med. 134(3) (2020) 963–975, https://doi.org/10.1007/s00414-019-02131-z.

[6] NovaQUANT™ Human Mitochondrial to Nuclear DNA Ratio Kit, 2011, EMD Millipore Corporation, https://www.merckmillipore.com/IT/it/product/NovaQUANT-Human-Mitochondrial-to-Nuclear-DNA-Ratio-Kit,EMD_BIO-72620.

[7] H. Andréasson, U. Gyllensten, M. Allen, Real-time DNA quantification of nuclear and mitochondrial DNA in forensic analysis, Biotechniques 33(2) (2002) 402-404, 407-411, doi:10.2144/02332rr07.

[8] A. Alonso, P. Martín, C. Albarrán, P. García, O. García, L.F. de Simón, J. García-Hirschfeld, M. Sancho, C. de La Rúa, J. Fernández-Piqueras, Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies, Forensic Sci. Int. 139 (2-3) (2004), 141–149, https://doi.org/10.1016/j.forsciint.2003.10.008.

[9] N. von Wurmb-Schwark, T. Schwark, M. Harbeck, M. Oehmichen, A simple Duplex-PCR to evaluate the DNA quality of anthropological and forensic samples prior short tandem repeat typing, Leg. Med. (Tokyo) 6(2) (2004) 80–88, https://doi.org/10.1016/j.legalmed.2003.10.002.

[10] J.A. Walker, D.J. Hedges, B.P. Perodeau, K.E. Landry, N. Stoilova, M.E. Laborde, J. Shewale, S.K. Sinha, M.A. Batzer, Multiplex polymerase chain reaction for simultaneous quantitation of human nuclear, mitochondrial, and male Y-chromosome DNA: application in human identification, Anal. Biochem. 337(1) (2005) 89–97, https://doi.org/10.1016/j.ab.2004.09.036.

[11] M.D. Timken, K.L. Swango, C. Orrego, M.R. Buoncristiani, 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, J. Forensic Sci. 50(5) (2005) 1044–1060, doi:10.1520/JFS2004423.

[12] H. Andréasson, M. Nilsson, B. Budowle, H. Lundberg, M. Allen, Nuclear and mitochondrial DNA quantification of various forensic materials, Forensic Sci. Int. 164(1) (2006) 56–64, https://doi.org/10.1016/j.forsciint.2005.11.024.

[13] H. Niederstätter, S. Köchl, P. Grubwieser, M. Pavlic, M. Steinlechner, W. Parson, A modular real-time PCR concept for determining the quantity and quality of human nuclear and mitochondrial DNA, Forensic Sci. Int. Genet. 1(1) (2007) 29–34, https://doi.org/10.1016/j.fsigen.2006.10.007.

[14] R. Fregel, M. Almeida, E. Betancor, N.M. Suárez, J. Pestano, Reliable nuclear and mitochondrial DNA quantification for low copy number and degraded forensic samples, Forensic Sci. Int. Genet. Suppl. Ser. 3(1) (2011) e303-e304, https://doi.org/10.1016/j.fsigss.2011.09.014.

[15] M.F. Kavlick, H.S. Lawrence, R.T. Merritt, C. Fisher, A. Isenberg, J.M. Robertson, B. Budowle, Quantification of human mitochondrial DNA using synthesized DNA standards, J. Forensic Sci. 56(6) (2011) 1457–1463, https://doi.org/10.1111/j.1556-4029.2011.01871.x.

[16] M.L. Sprouse, N.R. Phillips, M.F. Kavlick, R.K. Roby, Internal validation of human mitochondrial DNA quantification using real-time PCR, J. Forensic Sci. 59(4) (2014) 1049–1056, https://doi.org/10.1111/1556-4029.12477.

[17] C. Goodwin, D. Higgins, S.S. Tobe, J. Austin, A. Wotherspoon, M.E. Gahan, D. McNevin, Singleplex quantitative real-time PCR for the assessment of human mitochondrial DNA quantity and quality, Forensic Sci. Med. Pathol. 14(1) (2018) 70–75, https://doi.org/10.1007/s12024-017-9944-8.

[18] M.F. Kavlick, Development of a triplex mtDNA qPCR assay to assess quantification, degradation, inhibition, and amplification target copy numbers, Mitochondrion 46 (2019) 41–50, https://doi.org/10.1016/j.mito.2018.09.007.

[19] C. Xavier, M. Eduardoff, C. Strobl, W. Parson, SD quants-Sensitive detection tetraplex-system for nuclear and mitochondrial DNA quantification and degradation inference, Forensic Sci. Int. Genet. 42 (2019) 39-44, https://doi.org/10.1016/j.fsigen.2019.06.004.

[20] K.T. Wai, P. Gunn, M. Barash, Development of the MitoQ assay as a real-time quantification of mitochondrial DNA in degraded samples, Int. J. Legal Med. 133(2) (2019) 411–417, https://doi.org/10.1007/s00414-018-1956-8.

[21] W. Parson, G. Huber, L. Moreno, M.B. Madel, M.D. Brandhagen, S. Nagl, C. Xavier, M. Eduardoff, T.C. Callaghan, J.A. Irwin, Massively parallel sequencing of complete mitochondrial genomes from hair shaft samples, Forensic Sci. Int. Genet. 15 (2015) 8–15, doi: 10.1016/j.fsigen.2014.11.009.

[22] V. Pereira, A. Longobardi, C. Børsting, Sequencing of mitochondrial genomes using the Precision ID mtDNA Whole Genome Panel, Electrophoresis 39(21) (2018) 2766–2775, https://doi.org/10.1002/elps.201800088

[23] J.M. Gallimore, J.A. McElhoe, M.M. Holland, Assessing heteroplasmic variant drift in the mtDNA control region of human hairs using an MPS approach, Forensic Sci. Int. Genet. 32 (2018) 7-17, http://dx.doi.org/10.1016/j.fsigen.2017.09.013.

[24] C. Strobl, M. Eduardoff, M.M. Bus, M. Allen, W. Parson, Evaluation of the precision ID whole MtDNA genome panel for forensic analyses, Forensic Sci. Int. Genet. 35 (2018) 21–25, https://doi.org/10.1016/j.fsigen.2018.03.013.

[25] C. Turchi, F. Melchionda, F. Alessandrini, V. Onofri, M. Pesaresi, L. Buscemi, A. Tagliabracci, Pitfalls, challenges and caveats in whole mitochondrial genome sequencing from hair shafts by MPS: Where, when and how to address them, Forensic Sci. Int. Genet. Suppl. Ser. 8 (2022) 219-221, https://doi.org/10.1016/j.fsigss.2022.10.041.

[26] L.C. Canale, J.A. McElhoe, G. Dimick, K.M. DeHeer, J. Beckert, M.M. Holland, Routine Mitogenome MPS Analysis from 1 and 5 mm of Rootless Human Hair, Genes (Basel) 13(11) (2022) 2144, https://doi.org/10.3390/genes13112144.

[27] G. Dayama, S.B. Emery, J.M. Kidd, R.E. Mills, The genomic landscape of polymorphic human nuclear mitochondrial insertions, Nucleic Acids Res. 42(20) (2014) 12640-12649, https://doi: 10.1093/nar/gku1038.

[28] J.C. Cihlar, C. Amory, R. Lagacé, C. Roth, W. Parson, B. Budowle, Developmental Validation of a MPS Workflow with a PCR-Based Short Amplicon Whole Mitochondrial Genome Panel, Genes 11 (2020) 1345. https://doi.org/10.3390/genes11111345.

[29] A. Untergasser, I. Cutcutache, T. Koressaar, J. Ye, B.C. Faircloth, M. Remm, S.G. Rozen, Primer3--new capabilities and interfaces, Nucleic Acids Res. 40 (15) (2012) e115; doi: 10.1093/nar/gks596.

[30] P.M. Vallone, J.M. Butler; AutoDimer: a screening tool for primer-dimer and hairpin structures. Biotechniques 37(2) (2004) 226-231; doi: 10.2144/04372ST03.

[31] QuantiNova Multiplex PCR Kit 06/2016, QIAGEN, QuantiNova Multiplex PCR Kit Handbook: www.qiagen.com/HB-2105.

[32] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55(4) (2009) 611–622, https://doi.org/10.1373/clinchem.2008.112797.

[33] DNA IQ™ Casework Pro Kit for Maxwell® 16, Promega, Technical manual TM332, Revised 12/16.

[34] Bone DNA extraction kit, Promega, Technical manual TM691, Revised 8/22.

[35] Plexor® HY System for Applied Biosystems 7500 and 7500 fast Real-Time PCR systems technical manual, Promega, Technical manual TM293, Revised 9/17.

[36] PowerPlex® ESX 17 Fast System for Use on the Applied Biosystems® Genetic Analyzers, Promega, Technical manual TMD040, Revised 1/21.

[37] PowerPlex® ESI 17 Fast System for Use on the Applied Biosystems® Genetic Analyzers, Promega, Technical manual TMD041, Revised 2/21.

[38] PowerPlex® Fusion 6C System for Use on the Applied Biosystems® Genetic Analyzers, Promega, Technical manual TMD045, Revised 12/18.

[39] C. Eichmann, W. Parson, 'Mitominis': multiplex PCR analysis of reduced size amplicons for compound sequence analysis of the entire mtDNA control region in highly degraded samples, Int. J. Legal Med. 122(5) (2008) 385-388, doi: 10.1007/s00414-008-0227-5.

[40] C. Berger, W. Parson, Mini-midi-mito: adapting the amplification and sequencing strategy of mtDNA to the degradation state of crime scene samples, Forensic Sci. Int. Genet. 3(3) (2009) 149-153, doi: 10.1016/j.fsigen.2009.01.011.

[41] A. Brandstätter, H. Niederstätter, M. Pavlic, P. Grubwieser, W. Parson, Generating population data for the EMPOP database - an overview of the mtDNA sequencing and data evaluation processes considering 273 Austrian control region sequences as example, Forensic Sci. Int. 166 (2-3) (2007) 164-175, doi: 10.1016/j.forsciint.2006.05.006.

[42] R.M. Andrews, I. Kubacka, P.F. Chinnery, R.N. Lightowlers, D.M. Turnbull, N. Howell, Reanalysis and revision of the Cambridge reference sequence for Human mitochondrial DNA, Nature Genetics 23 (1999) 147; https://doi.org/10.1038/13779.

[43] Precision ID mtDNA Panels with the HID Ion S5™/HID Ion GeneStudio™ S5 System Application guide, MAN0017770, rev C.0.

[44] H. Thorvaldsdottir, J.T. Robinson, J.P. Mesirov, Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration, Brief. Bioinform. 14(2) (2013) 178-92, https://doi.org/10.1093/bib/bbs017.

[45] H.E. LaSalle, G. Duncan, B. McCord, An analysis of single and multi-copy methods for DNA quantitation by real-time polymerase chain reaction, Forensic Sci. Int. Genet. 5(3) (2011) 185–193, https://doi.org/10.1016/j.fsigen.2010.03.002.

[46] J.A. Nicklas, E. Buel, Quantification of DNA in forensic samples, Anal. Bioanal. Chem. 376(8) (2003) 1160–1167, https://doi.org/10.1007/s00216-003-1924-z.

[47] T. Melton, G. Dimick, B. Higgins, L. Lindstrom, K. Nelson, Forensic mitochondrial DNA analysis of 691 casework hairs, J. Forensic Sci. 50 (2005) 73–80.

[48] M. Nilsson, S. Norlin, M. Allen, Sequencing of mtDNA in shed hairs: A retrospective analysis of material from forensic cases and a pre-screening method, The Open Forensic Science Journal 5 (2012) 13–22, doi:10.2174/1874402801205010013.

[49] D. Cuenca, J. Battaglia, M. Halsing, S. Sheehan, Mitochondrial sequencing of missing persons DNA casework by implementing Thermo Fisher's precision ID mtDNA whole genome assay, Genes (Basel) 11 (11) (2020) 1303, https://doi.org/10.3390/genes11111303.

[50] S. Desmyter, M. Bodner, G. Huber, S. Dognaux, C. Berger, F. Noël, W. Parson, Hairy matters: MtDNA quantity and sequence variation along and among human head hairs, Forensic Sci. Int. Genet. 25 (2016) 1–9, https://doi.org/10.1016/j.fsigen.2016.07.012.

[51] M.D. Brandhagen, O. Loreille, J.A. Irwin, Fragmented Nuclear DNA is the Predominant Genetic Material in Human Hair Shafts, Genes (Basel) 9(12) (2018) 640, https://doi.org/10.3390/genes9120640.