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ORIGINAL ARTICLE

qPCR and mtDNA SNP analysis of experimentally degraded hair samples and its application in forensic casework

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Abstract A mitochondrial DNA (mtDNA) quantification PCR (qPCR) method was developed and applied in a study with experimentally degraded hair samples (first study) and in a criminal case (second study). In the first study, the amount of detectable mtDNA decreased drastically after an incubation time of 1 month on a moist tissue in a heating cabin at 37°C. In the second study, when the qPCR assay showed positive quantification results, further analysis of 32 mtDNA single-nucleotide polymorphisms (SNPs) via SNaPshot technique was always possible, indicating that successful mtDNA SNP analysis of forensic samples can be guaranteed by pre-screening samples with the qPCR described here.

Keywords qPCR · Forensic hair analysis · mtDNA · SNP

Introduction

The forensic analysis of nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) in human hair samples is a challenging task. Both nDNA and mtDNA are degraded in human hair and nDNA is virtually absent in hairs without root, but as mtDNA has a higher copy number per cell it can be detected more easily [1, 2]. The routine forensic analysis of mtDNA can be accomplished by Sanger sequencing by means of which mostly parts of the non-coding mitochondrial hypervariable region (HVR) are analyzed [3]. A more sensitive technique is the SNaPshot analysis [4] by means of which single-nucleotide polymorphisms (SNPs) of the mtDNA coding region or the HVR can be analyzed. Several studies have presented useful assays for mtDNA quantitative PCR (qPCR) [5, 6] or for mtDNA and nDNA [7, 8]. Nevertheless, there is no study that shows the application of an mtDNA qPCR assay to detect the amount of quantifiable mtDNAs in artificially degraded hair samples. Also, there is no study that shows the application of an mtDNA qPCR assay to optimize the routine application of 32 mtDNA SNPs [9] in forensic hair analysis [10, 11].

In this study, a qPCR assay was used to find out whether the amount of detectable mtDNA molecules is affected by the environmental factors of time, humidity, and warmth in human hairs. To minimize intra-individual differences, several telogen hairs of the same individuals were examined and analyzed with regard to length, diameter, color, and the existence of a medulla (first study). The assay was also applied to an analysis of 135 hairs, which were older than 14 years, to test their applicability for 32 mtDNA SNP analysis (second study).

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Materials and methods

Pre-studies

The mtDNA primers for qPCR were F16328 5′-GTAC ATAGCACATTACAGTCAAATC-3′ and R16440

5′-GCACTCTTGTGCGGGATATT-3′ according to the revised Cambridge Reference Sequence (GenBank accession no. NC_012920). The probe sequence was 5′-6-FAM-ATT TCACGGAGGATGGT-BHQ1-3′ with the underlining representing locked nucleic acid bases (Applied Biosystems, Foster City, CA, USA). The qPCR was accomplished by a 7000 Real-Time PCR System (Applied Biosystems) using the following parameters: 6.75 μL qPCR mastermix (Promega, Mannheim, Germany), 0.2 μL of each 50 pmol primer pair, 0.1 μL of 50 pmol probe, 1.25 μL bovine serum albumin, 2.0 μL distilled water, and 2.0 μL DNA. The cycling parameters were 2 min at 50°C, then denaturation for 10 min at 95°C and finally 50 steps of 95°C for 15 s and 60°C for 1 min. For the analysis of artificially degraded hairs, 2.0 μL of distilled water was replaced with 2.0 μL of DNA extract of wolf blood or goat blood.

A plasmid standard was cloned using common laboratory methods. Primers F16261 5′-CTCACCCAYTAGGACCA CCAACAAA-3′ and R16440 5′-GCACTCTTGTGCGGG ATATT-3′ were used to generate a PCR template. *Adenylation*: The amplification product was put on ice and 10 μL was mixed with 1 μL of 10 mM dATP and 1 μL Eurogentec Taq. Incubation was accomplished at 70°C for 30 min. *Cleaning*: Purification was done with the NucleoSpin Extract Kit (Macherey Nagel, Düren, Germany) according to the manufacturer's recommendations. *Ligation*: The pGEM-TEasy vector system (Promega) was used, mixing 5 μL ligation buffer, 1 μL vector, 3 μL of adenylated and cleaned PCR product, and 1 μL T4 DNA ligase (3 U/μL). *Transformation*: Competent cells of *Escherichia coli* JM 109 (Promega) were thawed on ice and 25 μL was inverted with 4 μL ligation solution on ice for 20 min. A heat shock was accomplished for 45 s at 42°C, and then the solution was put on ice again for 2 min. After that, the bacteria were put into 200 μL of a 2.5% (w/v) LB solution for 90 min at 37°C and 150 rpm. The bacteria were placed on LB–agar (2.5% w/v and 1.5% w/v, respectively) having a final concentration of 0.1% (w/v) ampicillin sodium (AMP), 0.1% (w/v) 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside and 0.1 (w/v) isopropyl-β-D-1-thiogalactopyranoside. Incubation was done at 37°C for 16–24 h. *Plasmid isolation*: White cloning colonies were picked and diluted in 1 mL of distilled water, mixed with 6 mL 0.1% (w/v) AMP–LB medium, and incubated for 16 h at 37°C at 225 rpm. Plasmid isolation was accomplished by the Gen Elute Plasmid Miniprep Kit (Sigma Aldrich, Hamburg, Germany) according to the manufacturer's recommendations. The sequence of the insert was verified by Sanger sequencing using the BigDye Terminator Cycle Kit on a 310 Genetic Analyzer (Applied Biosystems). UV–Vis photospectrometry was accomplished to analyze the DNA concentration. The copy number of plasmids in the solution was calculated using the following parameters: The size of an isolated plasmid was assumed to be 3,200 base pairs, with an average base pair weight of 660 g/mol. Dilutions of 10^8 down to 10^2 plasmid copies were used to calibrate the reference curve for qPCR.

Plasmids were visualized by scanning electron microscopy (SEM). For SEM analysis, a Stereoscan 180 (Cambridge Instruments, Dortmund, Germany) with a Centaurus backscattered electron detector (K.E. Developments Ltd, Cambridge, UK) was used. For sample preparation, 1 μL plasmid solution was spotted on an aluminum carrier, dried at room temperature, and sputtered with 5 nm gold via Cressington Carbon Coater 108 Auto (Cressington, North Shore City, New Zealand). Data was visualized with "analySIS" software.

The linearity and quality of the results of the assay was tested with standard samples of the human quantifiler (ABI) before testing the hair samples. The standard samples were diluted in one-third steps from 50 ng genomic DNA down to 0.0076 ng genomic DNA and analyzed twice per run. The inter-run variability was tested thrice with the female cell line DNA 9947A (Promega), the diluted saliva extract of a voluntary donor, and a pre-quantified DNA with an amount of 0.02 ng genomic DNA. The tests were run three to four times and in different batches.

First study

Hairs of 6 cm in length were taken from five female volunteers by combing the hair softly after washing. They were cut 2 cm distally from the root, cleaned with 70% ethanol, and examined using a microscope. The main characteristics of the hairs are demonstrated in Table 1. Two hairs from every volunteer were extracted immediately in particular according to [12] and analyzed by the qPCR assay. The mean value of detected mtDNA molecules was taken as mtDNA amount at t_0. For four different time intervals, two hairs each were then stored between tissues that were moistened with distilled water. The wet tissues were placed into sealable plastic bags to minimize evaporation and incubated at 37°C in a heating cabin for 1 week, 1 month, 3 months, and 6 months. At the end of each incubation time, the hair samples were removed and analyzed in particular by applying the qPCR assay. The mean value of the two hairs of each set was taken for further evaluation.

Second study

One hundred thirty five hairs that were older than 14 years and had been obtained from a criminal case were analyzed to check their applicability to molecular analysis via 32 mtDNA SNPs [9]. 63 of these hairs had a telogen root and

Table 1 Characteristics of the chosen hairs of five volunteers, first study

Sample name	Age of donor	Sex	Color	Coloring	Ethnicity	Diameter (mm)	Medulla	Hair care
H1	30	Female	Blond	No	European	0.025	No	Daily washing, daily conditioning, monthly deep conditioning
H2	29	Female	Brown	No	European	0.05	No	All 2 days washing, all 2–3 months deep conditioning
H3	47	Female	Blond	No	European	0.05	No	All 2–3 days washing, all 4 days conditioning, monthly deep conditioning
H4	50	Female	Black	Yes	European	0.08	Yes	All 2–3 days washing and conditioning
H5	22	Female	Brown	Yes	European	0.06	No	Daily washing, daily conditioning, weekly deep conditioning

The hairs were examined microscopically and only very similar hairs were taken in accordance with the mentioned parameters of color, diameter, and medulla

75 were without root (for the characteristics of the hairs, see Table 2 and ESM 1) because the roots of the hairs were cut off and used for previous nDNA analysis. Extraction of DNA was performed according to [12] and the analysis of 32 mtDNA SNPs was accomplished according to [9] (see "quality values" in ESM 1).

Results and discussion

Pre-studies

By analyzing the amount of mtDNA copies of the standard samples of the human quantifiler, the best-fit straight line had an R^2 value ranging from 0.97 to 0.99; the weakest correlation was achieved the more the samples had been diluted, which can be attributed to stochastic effects. The inter-run variability of four measures of a 0.01-ng dilution of cell line DNA 9947A was 9.80% (standard deviation divided by mean value, multiplied with 100%). The inter-run variability for the diluted saliva extract was 10.73% for four measures and the inter-run variability of a pre-quantified DNA with an amount of 0.02 ng genomic DNA was 30.75%.

After evaluation of the analysis parameters, the R^2 values for qPCR standard curve using plasmid DNA ranged from 0.95 to 0.99 (Fig. 1). The slope ranged from −3.8 to −2.0. The reason for this variance was due to missing data points or outliers which occurred at a range of less than 10^5 mtDNA copies. There was an uneven distribution of plasmids in the stock solution as well as in the qPCR mix. Via SEM, it was possible to visualize that the plasmids were aggregated. We tried to disperse the plasmids by adding polyoxyethylene(20)-sorbitan-monolaurate and sodium dodecyl sulfate to the solution, but it had no effect on the plasmid aggregation. Of course, the effect of aggregation was affecting the standard dilutions and the R^2 value of the standard calibration curve. The outliers or quantification dropouts became more frequent the stronger the dilution of the plasmids was. In Fig. 2, a SEM photograph (secondary image; 15 kV) of a dried plasmid solution that was spotted on an aluminum surface can be seen. To minimize the effect of aggregation, inert wolf DNA was added to the dilution series (the same effect was achieved with some other inert animal DNA) due to the simple assumption that the solvation of a DNA molecule increases the more free water is in a solution. This leads to a worse annealing of primers, so inert DNA will reduce the amount of free water. After

Table 2 Hair groups according to color, second study

Hair color	SNP electropherogram quality				Total no.	Average length (cm)	Median length (cm)
	0	1	2	3			
Blond	1	1	8	12	22	2.6	2.0
Light brown	4	7	3	8	22	2.7	2.0
Brown	1	4	4	24	33	2.6	2.5
Dark brown	5	6	6	27	46	2.8	2.0
Gray	0	3	2	2	7	2.3	2.0
Red-brown	0	2	0	3	5	3.4	3.0

The average length and the median are given to indicate that there were only a few long hairs that shifted the average length to the right. The SNP electropherogram quality values indicate the results where no SNPs ("0"), sporadic SNPs ("1"), a partial profile with haplogroup ("2"), and a full profile ("3") were obtained

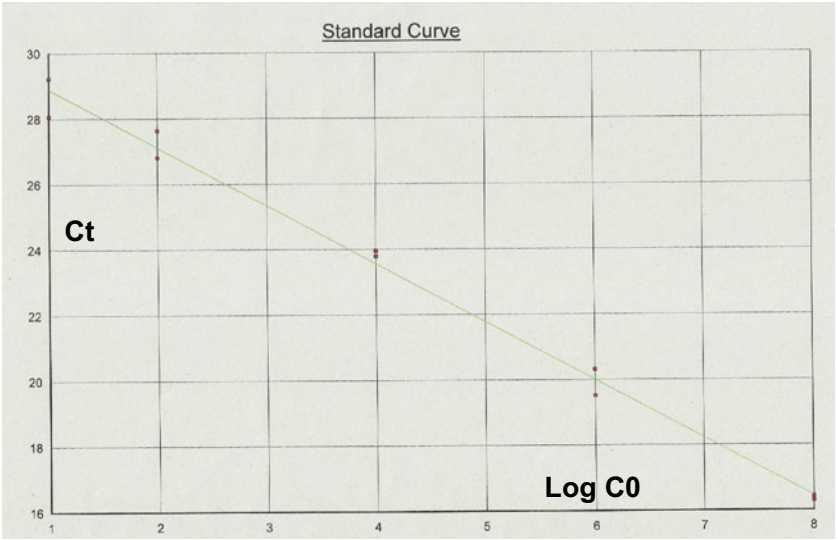

Fig. 1 Standard curve of the qPCR assay. The R^2 value was 0.99 and the slope was −2.0

adding wolf DNA to the reaction mix, the effect of outliers and dropouts decreased. Further information is given in ESM 2. As 10^4 copies of plasmid make up only 35 fg (0.35 pg) DNA, which is about 5% of the weight of the genome of a single cell (6.6 pg), adding inert DNA (i.e., DNA which is not homologous to the qPCR primers and the qPCR probe) should result in a more equal distribution of the plasmids. The plasmids will still aggregate but now they will be more equally distributed in the solution due to the amount, and intermolecular force of the inert DNA. Besides the effect of aggregation, there is also the possibility that our circular plasmid is inhibiting primer annealing in the first stages of PCR. This could end up in slight quantification differences [13]. A linearized plasmid was not used due to the circular nature of mtDNA and the easy-to-use application of circular plasmids.

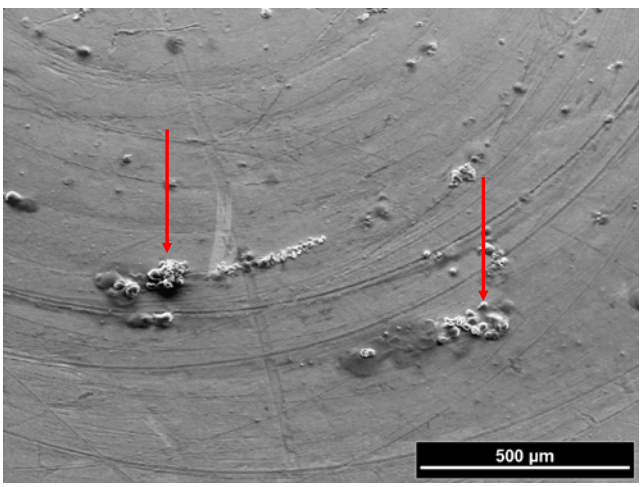

Fig. 2 Cutout of 1 µL plasmid solution (10^3 copies/µL) spotted on an aluminum surface. *Red arrows* indicate aggregated plasmids

To obtain good quality results, it is recommended to analyze all samples for one study in one batch. Equal analysis results were obtained for reference specimen in one batch, but it appeared that in some other analysis a few different results were obtained compared to the first-batch analysis. This may be due to the minor quality of the DNA and the standard plasmids after thawing and freezing, so the best results will be obtained if there are aliquots of the plasmids. Variance may also be due to slightly different amounts and qualities of chemicals in the new mastermixes and due to different human physical and mental states and methods of operation. Without absolute automation techniques, it is impossible to rule out variances in time of incubation and in time of non-treatment.

First study

The amount of detectable mtDNA molecules decreased drastically over 1 month of wet incubation at 37°C except for one hair (Fig. 3). This hair (H1) had the smallest diameter and had the highest treatment of washing and conditioning. The blond color was light and the cuticle seemed to be less intact than the cuticle of the other hairs. The detectable amount of mtDNA molecules was very low right from the start. The other hairs had a high t_0 value of nearly 500,000 mtDNA molecules or more that became less than 200,000 after 1 month of wet incubation at 37°C. In the following months, this value was quite stable, with a tendency to further decrease detectable mtDNAs. As these hairs were analyzed in different batches, the small changes may be due to different batch analyses. A full or partial profile of 32 mtDNA SNPs [9, 14] was found in all hair extracts with positive qPCR. The fast degradation at the start of the experiment, corresponding to the detectable mtDNA molecules in qPCR, agrees with the DNA stability

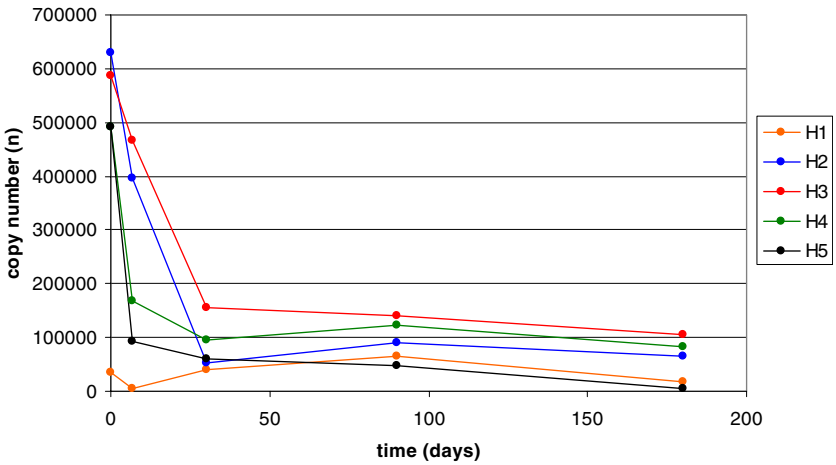

Fig. 3 Over-time degradation of human hairs. Data points were taken at the beginning (t_0), after 1 week (7 days), 1 month (30 days), 3 months (90 days), and 6 months (180 days). The *legend* on the *right side* indicates the five different hair donors

studies of [15], indicating an over-time loss of detectable mtDNA in the presence of warmth, humidity, and oxygen. Obviously, this process is not linear.

Second study

The hair samples were analyzed by qPCR and 110 of them had a positive result ranging from less than 100 mtDNA copies up to 5,000,000 mtDNA copies. All hair samples were analyzed two to three times by 32 mtDNA SNPs, and in 123 hairs at least single SNPs were found. A quality criterion was established for the SNP results to disburden the following analyses: A "3" was assigned when a full profile was obtained, a "2" was assigned when a partial profile and a haplogroup were obtained [see 14], a "1" was assigned when sporadic SNPs were detected, and a "0" was assigned if no reproducible SNPs were found. For haplogroup analysis in forensic samples, only SNP profiles with quality criteria "2" or "3" are usable.

In 13 hairs with a negative qPCR, no SNPs ("0") were found; in ten hairs with a negative qPCR, sporadic SNPs ("1") were found; in four of them, a partial profile ("2") was found. There were no qPCR with a positive result and a negative 32 mtDNA SNP analysis, i.e., 110 samples had a positive qPCR and positive mtDNA analysis with 32 SNPs.

Due to microscopic examination, the hair samples were divided into six groups according to their color (Table 2). The average length of the hairs was similar in the four major groups, i.e., blond, light brown, brown, and dark brown. There were only few gray and red-brown hairs; therefore, further conclusions could not be drawn. The best mtDNA SNP typing results were found for the brown hairs where 72% had the quality criteria "2" or "3". The worst results were found for the light brown hairs (36%). This indicates that there is a better typing result for stronger pigmented hairs, which corresponds with the results of a previous publication [11].

Only 63 of the 135 hairs had a telogen root; 72 were hair fragments without a root. Moreover, 82.5% of the hairs with a telogen root had a full ("3") or partial ("2") mtDNA SNP profile; hairs without a root only had a full or partial profile of 67%. The average amount of mtDNA copies for hairs with a root and positive qPCR was 443,000; for hairs without a root, it was 249,000. There was a tendency of longer hairs to have better typing results than short ones.

An optimization for mtDNA SNP analysis was accomplished. If the qPCR showed results of more than 10^5 copies of mtDNA, only 1 µL DNA extract was used for 32 mtDNA SNP typing. If there were less than 10^5 copies but more than 10^4 copies, 2 µL DNA extract was used. If there were less than 10^4 copies, 3 µL DNA extract was used. If no mtDNA is detected, it is possible to use an alternate PCR mastermix according to [9], in which 1 µL of water has to be subtracted per sample. This 1 µL of missing water is filled up by 1 µL more DNA extract.

Conclusion

The results demonstrate that the qPCR and the subsequent 32 mtDNA SNP analyses are suitable for forensic application in casework samples. The findings correspond to previous publications. The qPCR is well suited for a quality check of mtDNA extracts which have to be further examined by means of mtDNA SNP analysis. There have been no negative results for 32 mtDNA SNPs if the qPCR was positive. In cases with many samples, this will reduce the working time and laboratory expenses. With 91% of hairs showing positive results for mtDNA SNPs (quality criteria "1", "2", or "3"), we had gained a very similar value as [16]. Nevertheless, the qPCR analysis and the subsequent analysis for 32 mtDNA SNPs according to [9], in combination with the recommendations described in this manuscript, take a maximum of 6 µL DNA extract (2 µL for qPCR and at most 4 µL for mtDNA SNP analysis),

whereas the protocol of [16] used 10–15 μL without knowing the mtDNA copy number.

This study indicates that the detectable mtDNA amount decreases drastically over a time period of 1 month in most of the human hair samples if these are incubated in a warm and wet place. However, this decrease becomes less strong within a testing period of 6 months. The results suggest that hairs should be stored in airtight, dry, and not-too-warm places. In one hair, the amount of mtDNA molecules was very low right from the start, which can be due to the characteristics and/or treatment of the hair.

Small amounts of plasmid for standard curve calibration are challenging. The aggregation effect, which may result in dropouts and outliers, can be overcome by adding inert DNA to the dilution series or the reaction mix.

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