Forensic Sciences Research, 2025, Vol. 10, Issue 1
https://doi.org/10.1093/fsr/owae018
Research Article

Quantitation of human mitochondrial DNA and whole mtGenomes sequencing of fingernail/hair shaft samples

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Abstract

The analysis of mitochondrial DNA (mtDNA) is suitable for fingernail/hair shaft samples in forensic science. A successful mtDNA forensic analysis depends on the quantity and quality of the mtDNA. The application of massively parallel sequence techniques to the analysis of mtDNA has the potential to improve the recovery of genetic information from difficult forensic specimens and to increase the discrimination potential of mtDNA by capturing and comparing full mtGenomes. In this study, we constructed mtDNA-specific standard curves using real-time PCR. The 105-base pair target sequence facilitates the amplification of degraded DNA and is minimally homologous to non-human mtDNA. The results indicated that the assay enables the absolute quantification of down to 10 copies of mtDNA and provides a dynamic range of eight orders of magnitude. Whole mtGenome sequencing experiments demonstrated that as few as 2 000 mtDNA copies resulted in a successful full region amplification and sequencing. Moreover, the frequency of point heteroplasmy from one donor showed that hairs from the same donor have been found to differ within and among themselves and from other tissues, which could impact the interpretation of the results obtained in a forensic investigation.

Keywords: mitochondrial DNA; real-time PCR; massively parallel sequencing; forensic genetics

Introduction

Most eukaryotic cells have a form of double-membrane organelle called a mitochondrion, which can produce energy through processes called oxidative phosphorylation that take place in the mitochondrial matrix [1, 2]. Additionally, mitochondria are crucial for cell growth and division, cellular differentiation, and death [1, 3]. In a circular molecule of 16 569 bp, human mitochondrial DNA (mtDNA) contains 37 genes, including two rRNA-encoding areas (12S rRNA and 16S rRNA), 22 genes encoding tRNAs, and a non-coding region known as the D-loop [4, 5]. Depending on the tissue's energy requirements, each cell can contain 100–10 000 mitochondria, and each mitochondrion carries up to 11 copies of the mtDNA [3, 6]. Three distinct features of mtDNA distinguishing it from nuclear DNA include its high mutation rate and uniparental, non-recombining mode of inheritance [7, 8].

The analysis of mtDNA has been considered a valuable resource in forensic science and population genetics [8–10]. Due to higher copy numbers than nuclear DNA, mtDNA is particularly useful for ancient DNA investigations and forensic casework, in which nuclear DNA here is so degraded that the intact DNA fragments are too short for conventional STR analysis [8, 11–13]. The analysis of mtDNA is especially suitable for challenging samples, such as hair shafts without roots or fingernails. Moreover, all individuals that are maternally linked have the same mtDNA haplotype, barring mutations, due to the maternal mode of inheritance and lack of recombination of mtDNA [8, 14]. This character can be applied in informative and distinctive kinship analyses. Sanger sequencing of hypervariable regions I (16 024–16 365) and II (73–340) (HVI and HVII) is the conventional approach for forensic mtDNA testing. Frequently found haplotypes have been divided into smaller groupings using single nucleotide polymorphisms in hypervariable regions to offer lineage information [15–17]. In recent years, massively parallel sequencing (MPS) has made it possible to quickly and affordably sequence the whole mitochondrial genome (mtGenome), enabling the collection of more mtDNA genetic information and the accurate detection of intraindividual mtDNA variants [18–20]. Recent research has indicated that for some haplogroups, more than 70% of mtDNA variants can be observed outside of HVI/II [10]. As a result, whole mtGenome sequence can offer significantly more discrimination power for forensic genetics than conventional Sanger method [21].

Received: August 3, 2023. **Accepted:** February 20, 2024

In this study, an MPS-based mtDNA testing process for fingernail/hair shaft (with/without root) samples was established, which consisted of mtDNA quantification and whole mtGenome sequencing. For mtDNA quantification, we designed the primers and TaqMan probe to construct mtDNA-specific standard curves by using real-time PCR, because mtDNA commercial standard substance was rare (NIST has recently made a Human DNA Quantitation Standard with mtDNA quantity as a non-certified value, presented as a ratio of mtDNA to nuclear DNA quantity [22, 23]). For whole mtGenome sequencing, MPS was conducted on the Ion Torrent Personal Genome Machine system (PGM; Thermo Fisher Scientific, Foster City, CA, USA). Considering the low concentration and short fragments of fingernail/hair shaft samples, the Precision ID mtDNA panel (Thermo Fisher Scientific) was selected for this work. The multiplex PCR reaction comprised 162 amplicons in two multiplex pools with an average targeted fragment size of 163 bp using the Ion AmpliSeq technology (Thermo Fisher Scientific). The purpose of this study was to establish this reliable mtDNA profile method for fingernail/hair shaft samples and evaluate its forensic application value.

Materials and methods

Samples and DNA extraction

The fingernail/hair shaft samples involving in this study were provided by four donors with fully written informed consent. The samples were placed in a tube containing 5% Terg-a-Zyme™ and placed in a sonicating water bath at room temperature (25°C) for 20 min. Samples were then washed thrice with 100% ethanol and ultrapure water. Each hair sample was 2-cm long and each fingernail sample weighed 10 mg. These were extracted using the DNA IQ™ Tissue and Hair Extraction Kit (Promega, Madison, WI, USA). Samples were added to 100 μL of 1 mol/L DTT and 75 μL of the stock Proteinase K solution and incubated at 56°C for 2 h (hair shaft) or overnight (fingernail). All the samples were covered. Two volumes of lysis buffer and 7 μL of resuspended DNA IQ™ were added to the lysates, vortexing for 3 s at high speed, and incubating for 5 min at room temperature. The tube was placed in the MangeSphere Technology Magnetic Separation Stand and all solutions were removed without disturbing the resin pellet on the side of the tube. The DNA was eluted in 60 μL of low Tris-EDTA buffer (10 mmol/L Tris pH 8.0, 0.1 mmol/L EDTA) at 65°C after being washed three times with beads complexed with DNA. Control DNA 9947A (Promega) was used for construction of mtDNA-specific standard curves.

Construction of qPCR standards

The mtDNA qPCR standard substance consisted of two complementary fragments. The qPCR standard backbone pMD18-T vector was purchased from TIAGEN (Beijing, China). The insertion fragment of mtDNA (2 202 –2 878) was amplified from Control DNA 9947A *via* PCR using a primer set that consisted of a sense primer: 5′-CGCTTTGACTGGTGAAGTCTTAGC-3′ and an anti-sense primer: 5′-CGTTCAAGCTCAACACCCACTAC-3′. Amplification was performed for 30 cycles using Taq DNA polymerase (TOYOBO, Osaka, Japan), with following condition: 95°C for 5 min, 94°C for 30 s, 60°C for 30 s, 72°C for 45 s, and 72°C for 10 min. The PCR amplicons were verified using 1.5% agarose gel electrophoresis, and the target fragments were retrieved and purified using the Agencourt AMPure XP PCR Purification System (Beckman Coulter, Fullerton, CA, USA), as recommended by the manufacturer. The target fragments were then ligated into the pMD18-T vector using the DNA ligation kit (TIAGEN). The recombinant vectors of pMD18-T-mtDNA were transformed into *Escherichia coli* DH5α competent cells for amplification. Recombinant vectors were isolated from *E. coli* DH5α cells using the plasmid extraction kit (TIAGEN), and the vectors were sequenced using Sanger sequencing. The concentration of the constructed vector was quantified using the Qubit 2.0 Fluorometer and the Qubit® dsDNA BR Assay Kit (Thermo Fisher Scientific). The copy number of pMD18-T-mtDNA was calculated with an online tool (http://cels.uri.edu/gsc/cnDNA.html). The following assay dilution series was created by serially diluting the sample with TE buffer: 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 copies per 2 μL. The 10^8 concentration standards were aliquoted and stored at −80°C for the daily new preparation of the assay dilution series.

mtDNA quantification

The mtDNA quantification corresponding to positions 2 774–2 878 of the revised Cambridge Reference Sequence (CRS) was done by using the forward primer: 5′-CGCTTTGACTGGTGAAGTCTTAG-3′ and reverse primer: 5′-CAGGTCCTAAACTACCAAACCTG-3′; the TaqMan probe sequence used was 5′-FAM-GACCTCGGAGCAGAACCCAACCTCCGAGC-BQ1–3′. Quantitative real-time PCR was performed using 25-μL duplex reaction consisting of 2-μL DNA template (standard/sample), 900-nm PAGE-purified forward and reverse primers, 250-nm PAGE-purified TaqMan probe, and 12.5 μL of TaqMan 2 × Quantifiler™ PCR Reaction Mix. The reaction condition was as follows: 94°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 60 s. The experiments were performed in a LightCycler 96 PCR System (Roche, Rotkreuz, Switzerland) and results were analyzed using the LightCycler software 3.3 (Roche).

Library preparation and whole mtGenome sequencing

A total of 44 fingernail/hair shaft samples were sequenced using the Ion PGM™ System, including eight fingernails, three plucked hairs, and three shed hairs. Each hair was cut into fragments of six different lengths (0–2, 2–4, 4–6, 6–8, 8–10, and 10–12 cm). The library preparation was performed using the Precision ID mtDNA Whole Genome Panel and the Precision ID Library Kit (Thermo Fisher Scientific). The mtDNA was amplified using the "2-in-1" method, following the manufacturer's recommendation. The master mix and primer pool were contained in 20-μL reaction volumes. After amplification, 10 μL of each reaction were pooled in a single tube and subsequently used for library preparation. The mixed PCR amplicons were digested with 2 μL of FuPa reagent and were ligated with adapters and barcodes. Library purification was performed using the Agencourt AMPure XP PCR Purification System (Beckman Coulter). Libraries were assessed using the Agilent High Sensitivity DNA Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and were quantified using the Ion Library TaqMan Quantitation Kit (Thermo Fisher Scientific). In accordance

with the manufacturer's protocols, libraries were normalized and pooled to equimolar concentrations and emulsion PCR (emPCR) was performed on the Ion OneTouch™ 2 instrument (Thermo Fisher Scientific). Template-positive ISPs were loaded onto Ion 318 v2 Chips (Thermo Fisher Scientific) and sequencing was performed on the Ion PGM™ System by using Ion PGM™ Hi-Q™ View Sequencing Kit (Thermo Fisher Scientific).

Data analysis

The mtGenome sequencing data were analyzed using the VariantCaller (v 5.0) plugin of the Ion Torrent Software Suite (v 5.0.1). The HID Genotyper v2.01 plugin was used with the default parameters to perform read mapping against the modified CRS (rCRS +80 bp) in accordance with the phylogenetic alignment guidelines. The polymorphisms reported by the VariantCaller (v 5.0) plugin were manually verified using the Integrative Genomic Viewer (IGV) programme on Binary Alignment Map files. According to the updated guidelines for mtDNA, all variants were recognized when the read depth was $\geq 30\times$, the quality score was ≥ 20, and the heteroplasmy detection threshold was set to 10%.

Results and discussion

Vector construction and qPCR assay sensitivity

The gene segment of human mtDNA (677 bp) was obtained using PCR, and then ligated to the pMD18-T vector. The new pMD18-T-mtDNA was identified *via* restriction enzyme digestion and nucleotide sequencing. The full sequencing of pMD18-T-mtDNA was shown in Supplementary material S1. A 105-bp target region for mtDNA quantity and minimally homologous to non-human mtDNA was selected in order to ascertain the number of amplifiable template molecules with a minimum length necessary for downstream genotyping.

In this study, a series of 10-fold dilutions (10^8–10^1 copies) were performed and each concentration was diluted to 10 replicates of the same DNA standard to assess the repeatability. As Table 1 shown, average Ct and standard deviation (SD) for each concentration were calculated. The 10^8 copies of the highly purified standard per well showed a Ct value of 10.49 (range, 10.20–10.62), while the 10-fold dilution (10^7 copies) displayed an average Ct of 13.73 (range, 13.65–13.78). A total of 1 000 copies of pMD18-T-mtDNA were quantified in 27.11 cycles (SD 0.16 cycles). For the repeatable assay, most dilutions showed low variability with no significant difference, only when dilutions that were initially quantified as 10 copies displayed a higher variability among replicates, which may be caused by qPCR amplification efficiency [24]. In the exponential phase of qPCR, the replication of the DNA template is measured by the amplification efficiency. Quantitation for mtDNA can increase the effectiveness of the analysis and save priceless sample extracts. The assay sensitivity was 10 copies of mtDNA, and 10 copies of standard per well were consistently detected at an average Ct of 33.70 (range, 32.25–35.65), which was within the assay's recommended 40 cycles (Figure 1). Additionally, the average number of cycles that the Ct values varied between each consecutive dilution of the standard was 3.31. The assay demonstrated a high sensitivity in quantifying the pMD18-T-mtDNA standard copies, even at low concentrations. Several qPCR assays have been published for the quantification of human mtDNA, and some of them were especially for forensic application [25–30]. Hereby, we established mtDNA-specific standard curves and highly specific fluorogenic probes, allowing the absolute quantification of mtDNA down to 10 copies for the following fingernail/hair shaft samples quantification and sequencing.

Mitochondrial DNA quantification

The fingernail/hair shaft samples (with/without root) involving in this study were quantified using qPCR, as mentioned above, and all of the samples were examined in triplicate, and the positive results ranged from fewer than 250 mtDNA copies/μL to 5×10^6 mtDNA copies/μL. mtDNA quantity in hair varied greatly between individuals and was dependent on the growth phase at the time of shedding or plucking. It was demonstrated that the first two centimetres (including the hair root) of six shed hairs contained an average of 17 270 mtGE/cm copies (SD = 5 891 mtGE/cm). The next five adjacent segments (2–4, 4–6, 6–8, 8–10, and 10–12 cm) contained, on average, 4 373, 4 232, 2 972, 2 772, and 2 295 mtGE/cm copies per centimetre (SD = 803, 1 252, 822, 1 244, and 1 283), respectively (Figure 2). In contrast, the six plucked head hairs revealed an average of 1 205 312 mtDNA copies (SD = 377 498) in the first two centimetres (including the hair root). The next five adjacent segments (2–4, 4–6, 6–8, 8–10, and 10–12 cm) contained, on average, 8 972, 8 476, 6 509, 7 287, and 5 181 copies per centimetre (SD = 686, 3 035, 586, 1 353, and 573), respectively (Figure 2, Supplementary Table S1). As reported, both when comparing hair fragments and whole hairs, the mtDNA content in plucked head hairs was obviously higher than that in shed hairs [11, 12, 31]. In addition, hair roots contained more mtDNA than hair shafts in both plucked and shed hairs. The results also showed that as one moved out from the root to the end, the mean mtDNA content per fragment declined. Besides, mtDNA

Table 1. Standard cycle threshold data among 10 separate quantitative PCR assays.

Copies/well	Average	Minimum	Maximum	Range	SD
10^8	10.49	10.20	10.62	0.42	0.12
10^7	13.73	13.65	13.78	0.13	0.05
10^6	17.34	16.98	17.48	0.50	0.14
10^5	20.99	20.64	21.10	0.46	0.12
10^4	23.66	23.62	23.72	0.10	0.03
10^3	27.11	26.82	27.46	0.64	0.16
10^2	30.19	29.54	31.03	1.49	0.48
10^1	33.70	32.25	35.65	3.40	1.13

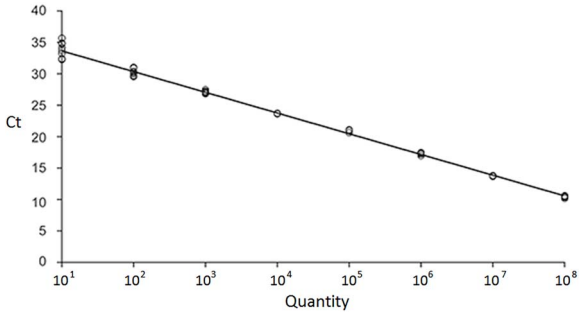

Figure 1 Reproducibility of standard curve. The plot represents a compilation of cycle threshold data from 10 separate assays.

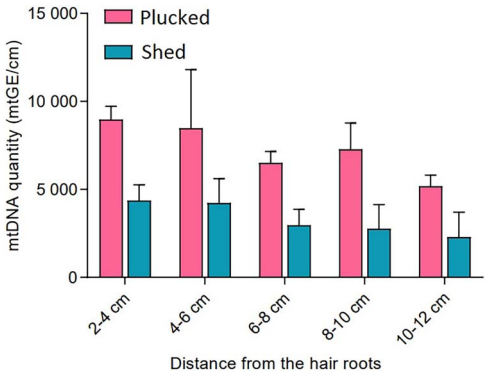

Figure 2 The mtDNA quantification of six plucked head hairs and shed head hairs in different fragments.

quantity in hair varied strongly between different individuals. As a common skin appendage, hairs are frequently found at crime scenes as biological evidences. Due to the loss of nuclear DNA during the normal process of hair growth, these hair shafts (without roots) typically do not exhibit detectable nuclear DNA under normal circumstances. In comparison, mitochondria are preserved inside the keratin matrix of the hair shaft and mtDNA can be detected [25, 32, 33].

Nails are another kind of challenging biological evidence in criminal scenes and can be used for genotyping and the identification of individuals in forensic science [25, 33]. In terms of the effectiveness of nail DNA extraction, a thorough protein digestion is a crucial step for getting better-quality DNA in greater quantities. In our study, a total of eight fingernail samples (10 mg each) were analyzed, and the yield of fingernail mtDNA varied from 4.71×10^5 to 1.15×10^6 copies/μL (Figure 3), which were much higher than that of hair shaft samples. The qPCR test mentioned here could quantify mtDNA with a high level of sensitivity and specificity. Thus, the front-end amplification reaction will always contain an exact and precise amount of mtDNA template thanks to the use of quantification estimations to standardize samples. Prior to beginning forensic mtDNA analyses, knowledge regarding the quantity and quality of biological evidences can increase the likelihood of successful profiles, increase efficiency, and preserve samples for potential future analyses [11, 25].

Whole mtGenome sequencing of fingernail/hair shaft samples

The Precision ID mtDNA Whole Genome Panel was used for mtGenome amplification, which included a two-primer pool multiplex PCR assay. Ion AmpliSeq technique produces PCR

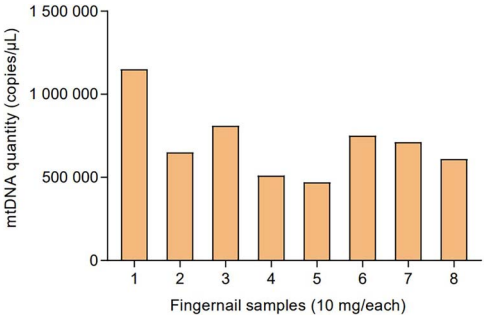

Figure 3 The mtDNA quantification of eight fingernails.

amplicons with an average size of 163 bp from each pool's 81 primer pairs (https://www.ampliseq.com/) and this panel was evaluated by Pereira et al. [21]. In a previous work, this panel was compared with the traditional Sanger sequencing on testing the extremely degraded sample materials, and significant advantages were shown [34]. We chose the "2-in-1" method for entire mtGenome amplification, which was recommended for low-copy number samples (particularly suitable for the challenging samples). This method is considered to be cost-effective and can provide higher read depths, even when highly degraded mtDNA is present. According to the quantitative results, the initial amount of mtDNA template amplified for each sample was ~2 000 copies. Each PCR amplicon was ligated with adaptor and barcode; then, emulsion PCR and sequencing were performed. Although the average coverage gave an indication of the data quality, the read coverage at any particular position determined how reliable a base call was. The mtGenome sequence could be determined with sufficient data, and all samples had comparable coverage. The average mapped reads were 174 765 per sample, and the mean read depth was $1\,249 \pm 438$ (mean ± SD) per amplicon. The Ion Torrent VariantCaller (v 5.0) plugin was used to evaluate the mtGenome sequencing data, and IGV was used to manually confirm the results. A total of 1 326 variations were found relative to the rCRS in the 44 mtGenomes sequenced, and they were distributed in 91 base positions of the mtGenome. On the basis of PhyloTree build 17 (http://phylotree.org) [35] and with reference to additional guidelines, the haplogroup assignments were carried out using MitoTool (http://mitotool.org) [36] and EMMA (http://empop.online) [37].

A point heteroplasmy was observed in 14 samples belonging to one individual, including two fingernails and two hairs (each hair was cut into six fragments). As was previously indicated, the bottleneck that occurs in the mitochondria during oogenesis is thought to affect the frequency of alleles at heteroplasmic locations in related tissues, even in the same tissues [38, 39]. We calculated the heteroplasmy frequency between different hair samples and finger nails. The difference in the heteroplasmy frequency of hair 1 ranged from 0.45 to 0.83, and the difference in hair 2 ranged from 0.07 to 0.64 (Table 2). The differences in mitochondrial heterogeneity between different segments of each hair varied greatly, and there was heterogeneity from hair to hair, but the difference in the frequency of the two fingernail samples was not significant. Whole mtGenome sequencing experiments demonstrated that over 2 000 mtDNA copies could accomplish the successful full region amplification and sequencing. Moreover, the frequency of point heteroplasmy from one donor showed that hairs from the same donor vary among themselves, within

Table 2. The heteroplasmy frequency between different hair samples and fingernails.

Sample ID	Sample type	Coverage	Reference	Frequency	Mutation	Frequency
ZXY-H 1–1	Hair1 0–2 cm	675	A	0.58	G	0.42
ZXY-H 1–2	Hair1 2–4 cm	2 372	A	0.83	G	0.17
ZXY-H 1–3	Hair1 4–6 cm	1 130	A	0.76	G	0.23
ZXY-H 1–4	Hair1 6–8 cm	934	A	0.74	G	0.26
ZXY-H 1–5	Hair1 8–10 cm	1 249	A	0.45	G	0.55
ZXY-H 1–6	Hair1 10–12 cm	839	A	0.46	G	0.54
ZXY-H 2–1	Hair2 0–2 cm	643	A	0.64	G	0.36
ZXY-H 2–2	Hair2 2–4 cm	937	A	0.24	G	0.76
ZXY-H 2–3	Hair2 4–6 cm	951	A	0.07	G	0.93
ZXY-H 2–4	Hair2 6–8 cm	1 277	A	0.13	G	0.87
ZXY-H 2–5	Hair2 8–10 cm	786	A	0.31	G	0.69
ZXY-H 2–6	Hair2 10–12 cm	1 033	A	0.21	G	0.79
ZXY F-1	Fingernail	481	A	0.56	G	0.44
ZXY-F-2	Fingernail	487	A	0.55	G	0.45

themselves, and from different tissues [38, 40]. These findings imply that the allele frequency at heteroplasmic locations fluctuates in various ways as a result of transmission events or environmental factors, which has an impact on how the findings from forensic investigations should be interpreted.

Conclusions

In forensic practice, mitochondrial DNA is often analyzed when a sample lacks intact nuclear DNA, such as hair shafts or nails, which are common biological pieces of evidence at crime scenes. In our study, we established the mitochondrial DNA testing process of fingernail/hair shaft samples, which consisted of mtDNA quantification and whole mtGenome sequencing. We constructed mtDNA-specific standard curves by using real-time PCR, which allowed for absolute mtDNA quantification down to 10 copies. Hair roots have larger levels of mtDNA than hair shafts, and fingernails have many more copies of mtDNA than hair shafts do. For mtDNA whole mtGenome sequencing, we demonstrated that it was feasible to recover the complete mtDNA sequence information from fingernail/hair shaft samples (without root) by using MPS in forensic caseworks. Besides, we also showed that the frequency of point heteroplasmy varies significantly between tissues (hair and fingernail) or in the same tissues (hairs) from one individual.

Acknowledgements

The authors wish to thank all the volunteers who provided their samples used in this study.

Authors' contributions

Hui Li and Yu Cao conducted the experiments and wrote the manuscript. Chengtao Li and Shiquan Liu designed the study and corrected the manuscript. Fan Yang and Xiling Liu helped to draft the manuscript. Ruiyang Tao, Ruocheng Xia, Ruxin Zhu, and Lei Jiang participated in the experiments and data analyses. All authors contributed to the final manuscript and approved it.

Compliance with ethical standards

Ethical review for recruitment and analysis was provided by the Ethics Committee at the Academy of Forensic Science, Ministry of Justice, China. Written informed content was obtained from the donors for the fingernail/hair samples to be used in this study and data were appropriated stored.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was supported by grants from the National Natural Science Foundation of China [grant numbers 81930056 and 82293654] and the Shanghai Scientific and Technological Innovation Project [grant number 21ZR1481700].

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Forensic Sciences Research, 2025, Vol. 10, Issue 1
https://doi.org/10.1093/fsr/owae018
Research Article