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ORIGINAL ARTICLE

Optimization of Illumina® Nextera™ XT library preparation for whole mitochondrial genome sequencing and confirmatory Sanger sequencing

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ABSTRACT

Aim Due to the increasing use of mitochondrial DNA (mtDNA) sequencing in both forensic practice and clinical disease research, the aim of this study was to investigate the optimization of the next-generation sequencing (NGS) method for whole mitochondrial genome analysis on the Illumina MiSeq platform.
Methods Initial attempts using pre-made commercial primers were unsuccessful, leading to the design of novel custom-designed primers in our laboratory and optimization of sequencing chemistry and protocols. A comprehensive protocol was developed, involving long-range amplification, enzymatic fragmentation, and the use of IDT® for Illumina DNA/RNA UD Indexes and MiSeq Reagent Nano Kit v2 (300 cycles), whereby DNA extraction, quantification, and library preparation were all performed according to optimized protocols.
Results Successful amplification was confirmed using gel electrophoresis and Agilent Bioanalyzer, with optimized conditions yielding clear, specific amplicons 9.8 and 8.5 kb in length. Sequencing results demonstrated high-quality reads with an average coverage depth of 742x and a GC content of 43-45%. The study highlights the efficiency of custom primers and individual library normalization for reliable mtDNA sequencing.
Conclusion These findings advance the application of NGS in forensic and clinical settings by enhancing the detection of rare mutations and mitochondrial heteroplasmy, paving the way for routine mtDNA analysis using NGS technology.
Keywords: mitochondrial genome, mutations, next-generation sequencing

INTRODUCTION

Mitochondrial DNA (mtDNA) has been of utmost importance in forensic practice, as well as in clinical disease research (1). Next-generation sequencing (NGS) has proven to be a revolutionary method for the large-scale analysis of genomes and transcripts and is applicable in multiple research areas (2,3). There has been an increasing interest in using NGS in forensic science, including mtDNA analysis, because of its potential to capture variants across the whole mitochondrial genome and to detect heteroplasmy at low levels (4). In NGS analysis of mitogenome has overcome time and cost issues of Sanger sequencing (5). Still, validation of NGS results using the confirmatory Sanger sequencing of mitogenome is essential for reliable mtDNA analysis (6).

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The critical steps for NGS library preparation include primer selection, initial enrichment of the target molecule, index PCR, and normalization of the libraries prior to sequencing (7). Preparation of mitogenome libraries with Nextera™ XT kit has been described as rapid and able to accommodate large number of samples for simultaneous processing (8).
In this paper, we present the development and optimization of novel, custom-designed primers specifically created to amplify the entire mitochondrial genome with high efficiency and specificity. The primary objective of this research was to establish an effective methodology for whole mitogenome sequencing, with a focus on primer design to ensure high coverage, accuracy, and reproducibility. Furthermore, to validate the accuracy of the sequencing results, we employed confirmatory Sanger sequencing of the mitochondrial genome as a reference standard. This integrated approach not only improves the reliability of mitogenome sequencing but also provides a detailed framework for future studies aiming to analyse mitochondrial DNA with enhanced precision and efficiency. This approach also has the potential to facilitate broader applications, includ-

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ing clinical diagnostic, population genetics, and evolutionary biology by delivering more accurate and reliable sequencing data. By focusing on the design of custom primers and the optimization of the sequencing workflow, the aim of this study was to provide a robust and reproducible method for whole mitogenome analysis.

PARTICIPANTS AND METHODS

Participants and study design

Buccal swab samples were randomly selected and collected from 73 healthy adult individuals from Bosnia and Herzegovina during the period between November and December 2023. The participants were invited via an online invitation form distributed through the social media profiles of partner institutions. Selection criteria used for the participants were age, health status and non-relatedness with other participants in this study. All participants were confirmed to be unrelated, which is a critical consideration for the design and validity of future population-based studies. Participants with any known conditions associated with mtDNA mutations or disorders such as Leber hereditary optic neuropathy (LHON), Leigh syndrome, Mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), and similar diseases were excluded from the study. This measure was taken to focus on optimizing the methodology in a healthy population, thereby minimizing potential interfering effects related to mtDNA-associated pathologies.

In this study, whole mitochondrial genomes were sequenced using Next Generation Sequencing technology and research was performed in ALEA Genetic Centre in Sarajevo.

All participants gave their informed consents.

This study was approved by the Ethics Committee of the Department of Genetics and Bioengineering, International Burch University, decision number 04-179/23 of 30 November 2023.

Methods

Genomic DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Extracted DNA samples were quantified on Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA) using Qubit™ dsDNA High Sensitivity (HS) Kit (Thermo Fisher Scientific, Waltham, MA). Library preparation for mitogenome using Nextera™ XT protocol entails first amplification, DNA quantification, tagmentation of input DNA, second amplification - indexing, DNA clean-up and library normalization prior to sequencing. First amplification was done using Takara LA Taq® DNA polymerase (Takara Bio, Kusatsu, Shiga, Japan) and PCR primers MTL-F1, MTL-R1, MTL-F2 and MTL-R2 diluted according to manufacturer's instructions to 10 μM (9). Initial volume of DNA input for each sample was 1 ng. Expected fragment sizes were 9.1 kb and 11.2 kb.

The amplicon quality was first assessed by examining the band appearance on the agarose gel. After the first amplification, gel electrophoresis was performed for 30 minutes at 120 V. The exact size of the amplified fragments was determined using automated electrophoresis by the Agilent Technologies Bioanalyzer instrument (Agilent Technologies Inc., Santa Clara, CA). Protocol optimization was performed by designing new sets of primers (Table 1). Negative controls were introduced at three stages of the workflow, namely during DNA extraction (NC-EX), long-range PCR mtDNA enrichment (NC-PCR), and library preparation (NC-LIB). Annealing temperature for the initial amplification was optimized at 64℃ according to the new primer sets, while DNA input was 1 ng and amplification cycles were set to 30. Agilent Technologies Bioanalyzer instrument (Agilent Technologies Inc., Santa Clara, CA) and Agilent High Sensitivity DNA Kit (Agilent Technologies Inc., Santa Clara, CA) were used as a quantity and quality validation, and library normalization method.

Table 1. The sequences of newly designed primer sets

Primer label	Starting position	Sequence
MT1F	4580	5' – TCTTTGCAGGCACACTAC – 3'
MT1R	14470	5' – GGGGGAGGTTATATGGGTTT – 3'
MT2F	12858	5' – ATGATACGCCCGAGCAGA – 3'
MT2R	4793	5' – CAACCGCATCCATAATCCTT – 3'

Index PCR was performed using IDT® for Illumina DNA/RNA UD Indexes (Illumina Inc. San Diego, CA). Libraries were purified with Agencourt AMPure XP (Beckman Coulter, Brea, CA) magnetic beads and ratio between library and beads was 1:1.

Normalized libraries were pooled, denatured, and diluted as described in manufacturer's instructions. Paired-end sequencing was performed on Illumina® MiSeq (Illumina Inc. San Diego, CA) instrument using MiSeq Nano Reagent Kit v2, 300 cycles (2x151 bp) (Illumina Inc. San Diego, CA). In order to confirm NGS data, Sanger sequencing was performed using 32 primer pairs covering an entire mitochondrial genome (11). DreamTaq™ Hot Start PCR Master Mix (Thermo Fisher Scientific, Waltham, MA) was used. Primers were diluted to 10 μM, DNA input was 10 ng and PCR products were evaluated using gel electrophoresis. PCR products were enzymatically purified using 2 μL of ExoSAP-IT™ reagent (Thermo Fisher Scientific, Waltham, MA). BrilliantDye™ Terminator v3.1. Cycle Sequencing Kit (NimaGen, Nijmegen, The Netherlands) was used for the termination-dye-based reaction. Final sample purification was performed using NucleoSEQ™ kit (Macherey-Nagel Biotechnology, Düren, Germany) for dye terminator removal. Sanger sequencing was performed on SeqStudio™ Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA).

Bioinformatic analysis. While Illumina's Local Run Manager produces FASTQ files from pair-end reads, the Seqfu program was used to generate an interleaved FASTQ file from two separate files. Bowtie2 software mapped the short reads against the revised Cambridge Reference Sequence (rCRS) (10), followed by the use of SAMtools to sort the SAM files generated after the mapping pipeline and perform sequence pileup. BCFtools was then used to generate base calls and VCF files. Quality control of the reads was conducted using FastQC software, and MultiQC was utilized to generate a unified report of the completed sequencing data. For Sanger sequencing data analysis, newly in-house designed and developed software Mitowizz was used (22).

RESULTS

The electropherogram readings that were evidence of successfully amplified fragments were monitored, including a clearly defined smallest and largest fragment size marker, a clear ap-

pearance of the amplified fragment, and a distinct size (bp) of the amplified fragment. No results were obtained during the initial phase of amplification of mtDNA fragments using the primer sets provided by the manufacturer.

Optimal PCR amplification results were achieved after the introduction of two new sets of specifically designed primer pairs. Fragment sizes were approximately 9.8 kb and 8.5 kb. Input concentrations of 5 ng and 10 ng produced exceptionally strong fluorescent signals during gel and automated electrophoresis (Figure 1).

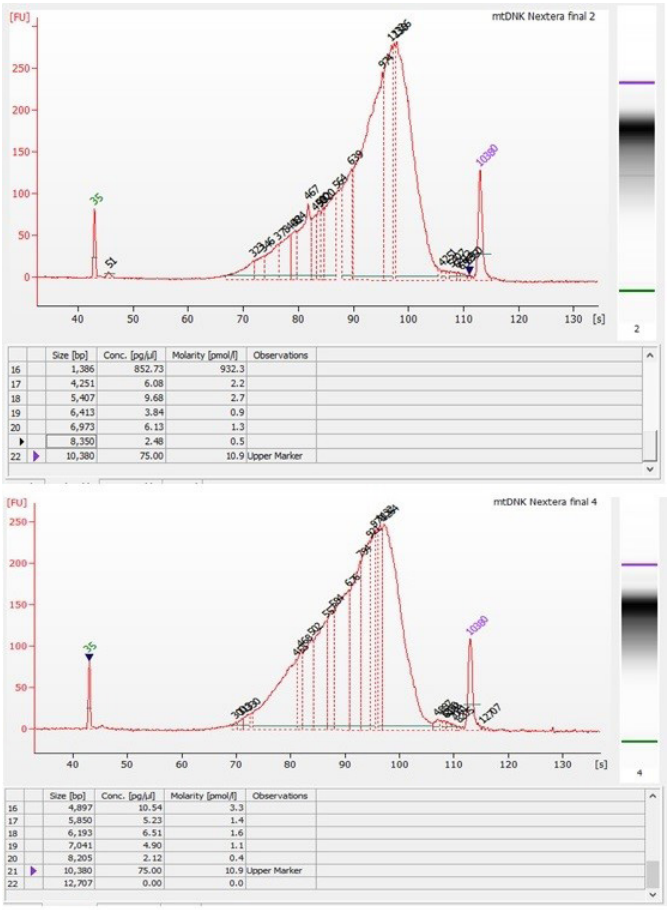

Figure 1. Results from Bioanalyzer instrument (Agilent Technologies Inc., Santa Clara, CA). Successful amplification of M1 primer set (upper panel); successful amplification of M2 primer set (lower panel). Both amplicon lengths are approximately 8 kb.

The electropherogram of the successfully amplified library revealed the lowest and largest size markers, as well as an amplification peak with an average fragment size of over 959 bp. The average concentration of all amplified libraries was 4,651.71 pg/µL, with a median molarity of 10,226.2 pmol/L. Insufficiently amplified libraries showed an electropherogram with the lowest and highest size markers, but with the absence of an amplification peak that would indicate the presence of the library.

All samples were sequenced with high base coverage depth averaging 742x, whereby all reads were above Phred 35, which are highest quality sequencing reads. Average value of the total sequenced reads per sample was 81,376. This indicates that complete process of NGS was successful. Our study showed that human mitochondrial genome consists of average 43-45% GC content.

Utilizing the confirmatory Sanger sequencing method, we validated the oligonucleotide primer binding sites at the anticipated locations for which they were originally designed.

DISCUSSION

Sequencing of mtDNA has been a research focus in the last decade with a number of sequencing and genotyping strategies being recommended. Different methods are selected based on the length of sequenced region and application, and they differ in terms of cost, accuracy, labour and time intensity (12). Our group has already published research on mtDNA sequencing using single-base extension (SBE) chemistry, which proved accurate, sensitive and especially powerful in mixture analysis, meaning that it is highly applicable for forensic use. A major drawback of the method is that it provides results on only at target positions, thus offering limited information with a low degree of robustness (13,14).

NGS methods are undoubtedly becoming increasingly important in mtDNA analysis, considering the information content and sequencing depth it offers, thus enabling even low-level heteroplasmy detection. Apart from Illumina platform used in the present study, semiconductor multiparallel sequencing (15-17) and Oxford Nanopore Technologies MinION™ (18) have also been previously described. NGS in mitogenome analysis is applicable to clinical practice too, apart from forensic or population genetics studies (19), especially due to its capability of detecting changes other than single nucleotide polymorphisms (SNPs), such as copy-number variations (CNVs). Although computational intensity and data analysis methods are still somewhat limiting the availability of NGS in mtDNA analysis (20,21), such challenges are getting solved and dominance of these methods on the market is expected to happen soon. Various optimizations have been published, but to the best of our knowledge, none have been done on custom designed primers for mitogenome sequencing (7,8).

Long-range DNA polymerase Takara LA Taq® DNA polymerase showed the best performance in terms of specificity and yield for mtDNA enrichment from DNA samples. By cross-referencing the Sanger sequencing results with the NGS data, we ensured that the designed primers performed as expected, thereby strengthening the reliability of the methodology and the integrity of the study's findings. This study, however, only provides a starting point for developing experimental models for more specific and easier detection of rare mutations on the mitochondrial genome, as well as enhanced identification of mitochondrial heteroplasmy. With optimized workflows, low-input DNA can still yield high-quality sequencing data, enabling the detection of rare variants or low-frequency mutations within the mitochondrial genome. Comprehensive mtDNA analysis is crucial for detecting mutations associated with mtDNA- related disorders including MELAS syndrome, LHON syndrome, and various neurodegenerative diseases) (20,21). Furthermore, it gives a more straightforward practical application of an optimized technique for sequencing the entire human mitogenome using the NGS technique, which is beneficial in everyday laboratory practice.

In conclusion, we have successfully optimized the workflow of custom-made primer pairs for the amplification of the complete human mitogenome. Currently, sequencing of whole mitochondrial genome is not routinely used. However, this research gives the guidelines for utilization of optimized protocol for both diagnostic and forensic purposes.

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TRANSPARENCY DECLARATION

Conflicts of interest: None to declare.

REFERENCES

1. Obal M, Zupanc T and Zupanič Pajnič I. Comparison of the optimal and suboptimal quantity of mitotype libraries using next-generation sequencing. Int J Legal Med 2024; 138(2):395-400.
2. Bruijns B, Tiggelaar R and Gardeniers H. Massively parallel sequencing techniques for forensics: A review. Electrophoresis 2018; 39(21):2642-54.
3. Misyura M, Zhang T, Sukhai MA, Thomas M, Garg S, Kamel- Reid S, et al. Comparison of Next-Generation Sequencing Panels and Platforms for Detection and Verification of Somatic Tumor Variants for Clinical Diagnostics. J Mol Diagn 2016; 18(6):842-50.
4. González MDM, Ramos A, Aluja MP, Santos C. Sensitivity of mitochondrial DNA heteroplasmy detection using Next Generation Sequencing. Mitochondrion 2020; 50:88-93.
5. Brandhagen MD, Just RS, Irwin JA. Validation of NGS for mitochondrial DNA casework at the FBI Laboratory. Forensic Sci Int Genet 2020; 44:102151.
6. Peck MA, Sturk-Andreaggi K, Thomas JT, Oliver RS, Barritt-Ross S, Marshall C. Developmental validation of a Nextera XT mitogenome Illumina MiSeq sequencing method for high-quality samples. Forensic Sci Int Genet 2018; 34:25-36.
7. Sukser V, Korolija M, Račić I, Rožić S, Barbarić L. Human whole mitochondrial genome sequencing and analysis: optimization of the experimental workflow. Croat Med J 2022; 63(3):224-30.
8. Ring JD, Sturk-Andreaggi K, Peck MA, Marshall C. A performance evaluation of Nextera XT and KAPA HyperPlus for rapid Illumina library preparation of long-range mitogenome amplicons. Forensic Sci Int Genet 2017; 29:174-80.
9. Illumina. Protocol: Human mtDNA Genome. Published 2016. San Diego, CA: Illumina; 2016.
10. Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat Genet 1999; 23(2):147.
11. Fendt L, Zimmermann B, Daniaux M, Parson W. Sequencing strategy for the whole mitochondrial genome resulting in high quality sequences. BMC Genomics 2009; 10:139.
12. Cooley AM. Mitochondrial DNA Analysis. Methods Mol Biol 2023; 2685:331-49.
13. Konjhodžić R, Salihefendić L, Čeko I, Kandić E, Ašić A, Kubat M. Detection of 13 Hypervariable Region 1 (HV1) SNPs using single-base extension (SBE) primers in parallel with Sanger sequencing. Gene 2023; 872:147438.
14. Konjhodžić R, Salihefendić L, Čeko I, Bubalo P, Škavić P, Ašić A, et al. Sanger sequencing of mitochondrial HV1 region: Population data for Bosnia and Herzegovina. Arch Legal Med 2024; 15(1):100449.
15. Cheng F, Zhang QX, Chen CJ, Li WT, Zhang JR, Zhang GQ, et al. Mitochondrial DNA Heteroplasmy of Hair Shaft Using HID Ion GeneStudioTM S5 Sequencing System. Fa Yi Xue Za Zhi 2021; 37(1):21-5.
16. Churchill JD, Stoljarova M, King JL, Budowle B. Massively parallel sequencing-enabled mixture analysis of mitochondrial DNA samples. Int J Legal Med 2018; 132(5):1263-72.
17. Cuenca D, Battaglia J, Halsing M, Sheehan S. Mitochondrial Sequencing of Missing Persons DNA Casework by Implementing Thermo Fisher's Precision ID mtDNA Whole Genome Assay. Genes 2020; 11(11):1303.
18. Zascavage RR, Thorson K, Planz JV. Nanopore sequencing: An enrichment-free alternative to mitochondrial DNA sequencing. Electrophoresis 2019; 40(2):272-80.
19. Palculict ME, Zhang VW, Wong LJ, Wang J. Comprehensive Mitochondrial Genome Analysis by Massively Parallel Sequencing. Methods Mol Biol 2016; 1351:3-17.
20. Mahmud S, Biswas S, Afrose S, Mita MA, Hasan MR, Shimu MSS, et al. Use of Next-Generation Sequencing for Identifying Mitochondrial Disorders. Curr Issues Mol Biol 2022; 44(3):1127-48.
21. Wang J, Balciuniene J, Diaz-Miranda MA, McCormick EM, Aref-Eshghi E, Muir AM, et al. Advanced approach for comprehensive mtDNA genome testing in mitochondrial disease. Mol Genet Metab 2022; 135(1):93-101.
22. Handžić N, Pećar D, Durgut S, Salihefendić L, Konjhodžić R. Development of MitoWizz: a Bioinformatics Solution for Mitochondrial DNA Analysis in Both Forensic and Clinical Applications. Acta Inform Med 2024; 32(3-4):221-4.

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