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Research Article

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Construction and optimization of human (*Homo sapiens*) mitochondrial DNA primers in the *D-loop hypervariable segment II* region in forensic biology

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ABSTRACT

DNA analysis is fundamental in forensic identification; however, nuclear DNA profiling often fails when samples are degraded. Mitochondrial DNA (mtDNA), which is maternally inherited and present in high copy numbers, provides a reliable alternative, particularly through analysis of the D-loop hypervariable segment II (HVS II). This study aimed to design and optimize human mtDNA HVS II–specific primers for forensic applications.An experimental approach combining in silico and in vitro methods was employed. Primer design and evaluation were conducted using the NCBI database, Primer3Plus, and NetPrimer Biosoft. Buccal swab samples from seven individuals were extracted using the PrepFiler™ Forensic DNA Extraction Kit, and DNA quality was assessed using a NanoVue spectrophotometer. Primer optimization was performed using gradient PCR, and sequencing was carried out using the Sanger method.Two primer pairs successfully amplified the HVS II region, generating fragments of 433–513 bp at an optimal annealing temperature of 56.5 °C. Sequence analysis revealed heteroplasmy and identified haplogroups N21 and L4b within the same maternal lineage. Both in silico and in vitro results confirmed that the designed primers specifically and reliably amplified human mtDNA.In conclusion, the optimized HVS II primers demonstrate strong potential for forensic casework involving degraded or limited biological samples and support improved resolution in population-level mtDNA analyses.

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INTRODUCTION

According to the Global Organized Crime Index in 2023, a total of 193 countries recorded in the international database experienced an increase in crime rates with a total global score of 5.03 (Ocindex,

2023). This condition can be overcome through a forensic science approach. Forensic science is of high urgency in handling global criminal cases because it plays a role in revolutionizing the resolution of past, present, and future cases by overcoming the limitations of conventional short tandem repeat (STR) profiling methods. The integration of innovations, such as ancient DNA analysis, enables forensic science to provide scientific certainty and support the realization of justice for victims and their families accurately and efficiently (BCC Research, 2025; Budowle et al., 2025). Forensic science is the application of science and scientific methodology in solving criminal cases on the basis of law (Shen & Vieira, 2016). One branch of forensic science that deals with DNA genetic material is Forensic Biology (Chango et al., 2024). In Forensic Biology, DNA analysis based on individual unique nucleotide threads for forensic purposes can be done through DNA profiling (Halim et al., 2022). DNA profiling in modern forensics is of utmost importance because it enables precise and scientifically accountable identification of individuals in criminal investigations and trials. Strengthening and developing the concept of DNA profiling in modern forensics, including the integration of digital DNA data, is crucial to ensuring the reliability of evidence, legal certainty, and the effectiveness of justice enforcement (Sahoo & Mohapatra, 2025). The success of individual DNA profiling is highly dependent on the condition of the biological samples found at the crime scene (Kuś et al., 2016).

Since 1996, genetic identification of missing persons using biological traces has been conducted using autosomal DNA or nuclear DNA (nDNA) profiles (Cavalcanti et al., 2024). DNA isolated from old or damaged samples is often found to be degraded or not available at all (Amorim et al., 2019). Factors affecting nDNA degradation generally come from external factors that impact the quality of biological samples, such as substrate-sample conditions, crime scene conditions, and sampling time (Buś et al., 2016). Degraded biological samples, such as bone skeletal remains (Inkret & Pajnič, 2025), dental skeletal remains (Victoria, 2024), hair shaft remains (Zhou et al., 2025), and decayed fingernails (Peksak & Şensöz, 2025) generally contain very limited amounts of nDNA (Bhoyar et al., 2024; Szargut et al., 2025). As an alternative to overcome the limitations in using nDNA, mitochondrial DNA (mtDNA)-based genetic profiling methods were introduced in forensic biology analysis (Connell et al., 2023; Panneerchelvam & Nor, 2023). Conventional nDNA profiling is often ineffective for such samples due to low copy number, linear structure, and susceptibility to degradation, a limitation that has not been fully addressed in forensic investigations in Indonesia. This highlights the urgent need for alternative approaches, such as targeting the mitochondrial HVS II region, to enable reliable genetic analysis in challenging forensic contexts. Furthermore, mtDNA research and primer development still face limitations in the availability of primers that are truly specific, robust, and standardized for various sample conditions, especially degraded samples in a forensic context. Primer validation is often not conducted comprehensively and in an integrated manner, either through in silico or in vitro approaches across populations, thereby potentially affecting the accuracy and reproducibility of results. In addition, understanding of heteroplasmy, population variation, and the influence of environmental and epigenetic factors on mtDNA primer performance has not been fully integrated into the development of existing methodologies (Ferreira & Rodriguez, 2024).

MtDNA is genetic material located in the mitochondria, which are membrane cell organelles located in the cytoplasm (Court, 2021). In forensic biology, mitochondrial DNA is often used to analyze paternity and kinship cases, especially in tracing maternal lineage. Unlike nDNA, which is inherited from both parents, mtDNA is only passed down through the maternal line (Amorim et al., 2019). In addition, mtDNA is also often used in ancient DNA analysis to triage the identification of disaster victims (Court, 2021). Some of the advantages of using mtDNA include a high amount of copy number in one cell, resistance to

stress and degradation, and small genome size (Kowalczyk et al., 2021). Mitochondrial DNA is circular and contains control regions that play a role in the replication and transcription process (Amorim et al., 2019). This control region or non-coding region is known as the displacement loop (D-Loop) of mtDNA with three hypervariable segments, namely segments I, II, and III (HVS I, II, and III) (Harino et al., 2024; Vallbona-Garcia et al., 2024). The mtDNA genetic profile analysis is based on the condition of single nucleotide polymorphism (SNPs) variation through sequencing at the two most variable regions in the genome, namely HVS I (nucleotides 16,024-16,365) and HVS II (nucleotides 73-340) (Court, 2021). Information on the variation of SNPs in hypervariable regions is used to form individual haplotypes which are then classified into haplogroups based on maternal lineage similarity (Li et al., 2024; Rakha et al., 2016). Analysis of nucleotide sequence variation in the D-Loop region of mtDNA allows analysis of individuals on a population scale. Analysis of nucleotide sequence variation in the mtDNA D-Loop region facilitates population-level characterization of maternal lineages while enabling discrimination of maternal haplotypes, thereby supporting individual identification and comparative analysis of biological samples in forensic investigations, particularly when nuclear DNA is limited or degraded (Yudianto et al., 2022).

Analysis of individuals using the mtDNA D-Loop involves integrated in silico and in vitro stages, including DNA extraction, specific primer design, PCR-based amplification, and sequencing. The resulting sequences are compared with the revised Cambridge Reference Sequence (rCRS) to identify nucleotide variation and haplogroup patterns, with amplification efficiency being highly dependent on primer specificity in the polymerase chain reaction (PCR) process (Li et al., 2024; McDonald et al., 2024). Current mtDNA testing involves amplification of HVS I and HVS II regions using specific primers that target more conserved regions (Sultana & Sultan, 2018). Thus, proper primer construction is crucial in determining the success of the PCR process (Kurniati et al., 2025).

Therefore, this study aims to construct and optimize primer pairs specific to the HVS II region of the mtDNA D-Loop to determine individual DNA profiles from human buccal swab samples. The resulting primers are intended to provide a molecular tool for supporting criminal investigations and tracing maternal lineage. The development of new primers is necessary to address limitations of existing primer sets when applied to genetically diverse populations and potentially degraded samples, as well as to improve amplification efficiency and reliability of mtDNA profiling in the Indonesian forensic context. Limited Indonesian research optimizes HVS II mtDNA primers for alternative markers amid diverse tribal genetics and in account for homoplasy.This study involved one segment, namely HVS II from a total of three segments of the mtDNA D-Loop control region. HVS II has a high level of genetic variation between individuals due to the presence of heteroplasmy, which is a condition when there is more than one type of mtDNA in one individual cell (Connell et al., 2023). Moreover, few studies have specifically explored the potential of the HVS II region as a genetic marker, with most previous research focusing predominantly on HVS I. This makes HVS II an important marker in genetic analysis, especially in maternal kinship studies (Bodner et al., 2022).

RESEARCH METHODS

This research involves both in silico and in vitro testing and includes elements of qualitative and quantitative research. The qualitative aspects include analysis of PCR amplification patterns through agarose gel electrophoresis, assessment of primer specificity, and evaluation of sequencing chromatograms. The quantitative aspects include DNA concentration and purity measurements, determination of amplicon sizes, and efficiency of primer amplification based on sequence identity percentages. Before conducting laboratory testing, ethical commission approval has been submitted to

the Health Research Ethics Commission (KEPK) unit of the Faculty of Medicine, Duta Wacana Christian University. The ethical clearance certificate was approved on March 21, 2025. Based on the research design that has been submitted, this research was declared to meet the ethical requirements and was reviewed in Ethical Clearance number 1740/C.I6/FK/2025. Written informed consent was obtained from all participants prior to sample collection, and all samples and associated data were anonymized to ensure participant confidentiality.

Primers were constructed using Primer3Plus program (https://www.primer3plus.com/index.html). Human mitochondrial DNA reference sequences were retrieved from the NCBI database with accession number: NC_012920.1 (https://www.ncbi.nlm.nih.gov/nucleotide/NC_012920.1). Nucleotide site numbers used to design HVS II primers, namely at site number 73-576 (Verma et al., 2018) for HVS II A primers, number 1-576 (Hong et al., 2015) for HVS II B primers, number 8-429 (Daud et al., 2014) for HVS II C primers, and number 73-340 (Anderson et al., 1981; Purkan et al., 2013) for HVS II D primers. Furthermore, the in silico characteristics of the constructed primer pairs were analyzed using the NetPrimer Biosoft (https://www.premierbiosoft.com/netprimer/) and UNAFold programs (https://www.unafold.org/). The parameters analyzed include primer length, GC content, melting temperature, annealing temperature, dimers, hairpin loop, and secondary structure stability (ΔG).

This study used biological samples of buccal swabs or epithelial cell swabs taken from seven different individuals with diverse ethnic and biological background. Sampling was performed using sterile cotton swabs (4N6 FLOQSwabs Genetics). For each participant, buccal sampling was conducted three times as independent biological replicates, with each collection consisting of 7-10 swabbing motions within the oral cavity. DNA extraction is performed using commercial kits that are available on the market and are widely applied in the field of forensic biology. The kit used was PrepFiler™ Forensic DNA Extraction Kit from Applied Biosystems™. The concentration and purity of the extracted DNA were subsequently measured using a NanoVue NanoDrop spectrophotometer. DNA samples showing A260/280 ratios of 1.8–2.0 and A260/230 ratios of 2.0–2.2 were deemed suitable for subsequent PCR amplification. Each NanoDrop measurement was performed in triplicate to ensure accuracy and reproducibility. Outlier values were identified based on significant deviation from replicate measurements and were excluded prior to calculating the mean concentration and purity values.

Furthermore, the *in silico*-designed primers were synthesized by Genewiz (Azenta Life Sciences, China) as 100% DNA oligonucleotides intended solely for research use.The forward and reverse primers synthesized from Genewiz are stock primers with an initial concentration of 100µM which was requoted to 5µM. Primer optimization was performed with gradient PCR technique using Thermocycler Gradient ProFlex PCR System. The PCR mixture used in this study consisted of Nuclease Free Water (3µL), PCR AmpliTaq Gold™ 360 Master Mix (10µL), 360 GC Enhancer (1µL), Forward Primer (1µL; 5µM), Reverse Primer (1µL; 5µM), and DNA template (5µL; 2.14ng/µL), so the total final volume of PCR mixture was 21µL. PCR running conditions include the following phases: stage 1, pre-denaturation at 95°C for 10 minutes (1 cycle); stage 2, denaturation at 95°C for 15 seconds, annealing with temperature variations of 54°C, 55.5°C, 57°C, 58.5°C, 60°C, and 61.5°C for 60 seconds, extension at 72°C for 60 seconds (30 cycles); and stage 3, post-extension at 72°C for 10 minutes (1 cycle). The expected PCR amplicon size ranged from approximately 400 to 600 bp. PCR products were analyzed by agarose gel electrophoresis using 2% agarose gels, run at a constant voltage of 70 V with a current ranging from 0.1 to 0.4 A for 45-70 minutes. Samples were prepared by mixing loading dye and DNA in a ratio of 1.5 µL to 3.5 µL, and a 100 bp DNA ladder was used as the molecular size marker to estimate the size of the amplified *HVS II*

mtDNA fragments ranging from approximately 400 to 600 bp. After the running process is complete, the results of the luminescence of DNA amplicons are observed under UV light using a gel documentation system.

The sequencing method used is Sanger sequencing which consists of 3 main stages, namely ExoSAP-IT, cycle sequencing, and purification cycle sequencing. Sanger sequencing was selected over next-generation sequencing due to its high accuracy and reliability for targeted mtDNA regions, cost-effectiveness for limited sample sizes, and suitability for routine forensic analysis requiring straightforward interpretation. ExoSAP-IT serves to clean DNA from PCR enzymatic components. The volume ratio used in making the PCR DNA mixture with ExoSAP-IT is PCR DNA: ExoSAP-IT = 8µL : 4µL (@ 12µL). After sample preparation for ExoSAP-IT is complete, the samples will be run using the Thermocycler ProFlex System. In the first stage, the samples were incubated at 37˚C for 15 minutes, followed by the second stage at 80˚C for 15 minutes (@ 30 minutes). Next, the quantification process is carried out again to see the concentration of amplified DNA and ensure that the DNA is clean from the PCR enzymatic component.

Cycle sequencing was performed using BigDye™ Terminator v3.1 under thermal cycling conditions. Sequencing reactions were prepared in a final volume of 20 µL, with M13 Forward Primer and pGEM™ plasmid included as positive controls to validate sequencing performance. The sequencing running cycle conditions consisted of stage 1 pre-denaturation at 96 °C for 1 minute (1 cycle); stage 2 denaturation at 96 °C for 10 seconds, annealing at 50 °C for 5 seconds, and extension at 60 °C for 4 minutes (25 cycles); and stage 3 post-extension at 72 °C for 10 minutes (1 cycle). Sequence quality was evaluated based on chromatogram clarity and base-calling confidence, and samples showing ambiguous peaks or low-quality reads were re-sequenced to ensure data accuracy and reliability. Purification of cycle sequencing products was performed using SAM™ Solution and XTerminator™ Solution according to the manufacturer's protocol. Purified samples were subsequently loaded into a 96-well format and analyzed using a 3500 Genetic Analyzer with a Fast Sequencing Assay for target sequences below 700 base pairs.

Sequencing results were stored in ABI file format and analyzed using BLAST via the NCBI database to identify sequence similarity with reference sequences in GenBank. Sequence alignment was performed using the ClustalW algorithm implemented in MEGA X-64, followed by manual inspection and editing in BioEdit. Low-quality regions at the 5' and 3' ends were trimmed based on chromatogram quality and base-calling confidence. Heteroplasmy was identified by the presence of double peaks at a single nucleotide position in both forward and reverse reads, with peak heights exceeding established background noise thresholds. The online program Mitomaster Mitomap (https://www.mitomap.org/MITOMAP) was used to predict the maternal lineage or haplogroup of the samples and compare the nucleotide variation of the samples with the rCRS (NC_012920.1). Meanwhile, a flowchart of haplogroup migration history constructed using the FamilyTreeDNA Discover program (https://discover.familytreedna.com/).

FINDING AND DISCUSSION

The primer location map of the HVS II region illustrates primer coverage within the mtDNA D-loop control region for PCR amplification. Mapping primer locations is important in forensic applications to ensure that primers target highly informative SNP sites, maximizing discriminatory power for individual identification and reliable haplotype determination. This visualization helps identify specific target areas of amplification and ensures primer precision in binding regions with high sequence variability. Such mapping supports the design and selection of primers that are effective for degraded DNA samples and for populations with diverse haplotypes, enhancing the reliability of forensic mtDNA profiling. The main

results of this study include the in silico construction of four primer pairs specific to the mitochondrial DNA control region (*D-Loop*), namely the *HVS II* region, of which two primer pairs were ultimately selected for further in vitro validation of their amplification performance. The primers pairs *HVS II* A, *HVS II* B, *HVS II* C, and *HVS II* D succesfully produced PCR products with DNA fragment sizes of 433 bp, 513 bp, 333 bp, and 166 bp, respectively (Figure 1). The four primer pairs correspond to standard DNA fragment sizes for amplification ranging from 100 to 1000 base pairs. Amplicons that are neither too short nor too long are preferred in forensic applications because they increase the likelihood of obtaining interpretable profiles from highly degraded DNA (Moeezullah et al., 2023). Compared to previous studies targeting the *HVS II* region or the mtDNA *D-Loop*, the amplicon sizes generated in this study fall within a similar optimal range, supporting their suitability for forensic mitochondrial DNA analysis (Daud et al., 2014; Verma et al., 2018).

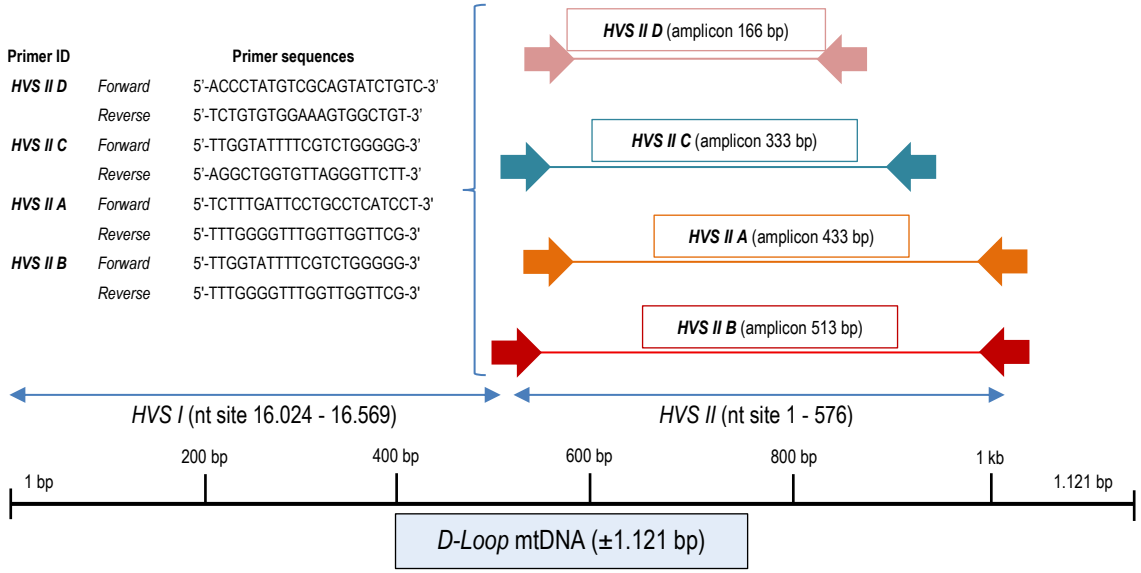

Figure 1. Primers mapping location of the *HVS II* region in the *D-Loop* region of mtDN

Analysis of in silico primer characteristics was conducted to determine primer conditions based on certain parameters (Putri et al., 2023) which will be used as a basis when optimizing (Abd-Elsalam, 2003). The parameters studied include primer sequence length, Guanine-Cytosine (GC) content, melting temperature (Tm), primer attachment temperature (Ta) (Rodríguez et al., 2015), and secondary structure parameters of DNA referred to as DNA fold (Sari et al., 2024; Yulita et al., 2025). Based on Table 1, primer pairs *HVS II* A and *HVS II* B have met the terms and conditions of ideal primers in all parameters studied. Primer *HVS II* C has a distance of Tm and Ta values close to the maximum limit of 5°C (Asif et al., 2021) between its forward and reverse primers. This makes the Ta value of the reverse primer too low referring to previous research, that the general Ta value is 52°C (Sharma, 2021). If the temperature distance is higher than 5˚C, it will cause a decrease in the amplification process (Nugraha et al., 2022). This near-limit difference may reduce amplification reliability in real forensic samples, particularly when DNA is degraded or present in low quantities, because large deviations in annealing temperature can lead to inefficient primer binding or non-specific amplification. While the *HVS II* D primer has too low PCR product coverage, which is 166 bp and its Ta value parameter is also low, both forward and reverse primers. Such a short amplicon limits the amount of informative sequence obtained, which can reduce the discriminatory

power in forensic analysis, and the low Ta values may further compromise amplification efficiency, especially in degraded or low-quantity DNA samples.

Table 1. In silico characterization of *HVS II* region primers

Parameter	*HVS II A*		*HVS II B*		*HVS II C*		*HVS II D*	
	F	R	F	R	F	R	F	R
Primer length [bp]	22	20	20	20	20	20	22	20
GC content [%]	45.45	50	50	50	50	50	50	50
Melting temperature (Tm) [°C]	60.43	62.88	59.85	62.88	59.85	55.58	56.1	55.79
Annealing temperature (Ta) [°C]	55.43	57.88	54.85	57.88	54.85	50.58	51.1	50.79
Self-dimer [kcal/mol]	-4.05	0	0	0	0	0	-4.55	0
Cross-dimer [kcal/mol]	0		0		0		0	
Hairpin loop [kcal/mol]	0	0	0	0	0	0	0	0

*Descriptions: red highlights indicate poorly characterized parts of the primer; F (Forward); R (Reverse)

In silico primers should adhere to universal standards to ensure reliable amplification and specificity in PCR-based applications, which is particularly critical in forensic contexts to avoid false positives and ensure accurate human identification. These standards include optimal ranges for melting temperature (Tm), GC content, primer length, and the absence of secondary structures such as dimers or hairpins. Detailed information on the in silico parameters used to evaluate primer quality is summarized in Table 2. However, not all designed primers met the desired criteria, as some showed suboptimal thermodynamic or structural characteristics. Therefore, based on the comprehensive in silico analysis, only the primer pairs *HVS II A* and *HVS II B* were selected for further in vitro testing. This careful selection helps minimize the risk of non-specific amplification, ensuring more reliable results in downstream forensic applications. Those primes were demonstrated the best compliance with universal primer design standards and are expected to yield specific and efficient amplification of the target HVS II region.

Table 2. Standard for in silico primers from previous studies

Parameter	Ideal standard	Over ideal standard	Under ideal standard
Primer length (bp)	14-28 (Asif et al., 2021)	Formation of secondary structures can inhibit DNA polymerization (Apte & Daniel, 2009; Garg, 2008; Pranata & Ahda, 2021)	Reduce primer specificity
GC content (%)	40-60 (Sasmito et al., 2014; Yulita et al., 2025)	Complicate the separation of DNA double-stranded chains (Hung & Weng, 2016; Maitriani & Wirajana, 2015; Sasmitha et al., 2018)	Not able to attach effectively to target DNA
Tm (˚C)	50-65 (Sasmitha et al., 2018)	Potential to form secondary structures that can inhibit the attachment process (Borah, 2011)	Primers attach to non-specific regions (Nuryady et al., 2024)
Ta (˚C)	Tm±5; ≥ 52; 55°C atau 60 °C (Daud et al., 2014; Erjavec, 2020)	The formation of unstable hydrogen bonds results in an inefficient hybridization process	Decrease in primer attachment intensity and power so that it becomes non-specific (Sasmito et al., 2014)
Self-dimer (kcal/mol)	Maximal -6 (Putri et al., 2023; Sasmito et al., 2014)	Primers complement with other primers of the same type; Low primer specificity and affects the reduction of primer concentration (Borah, 2011; Handoyo & Rudiretna, 2000)	
Cross-dimer (kcal/mol)	Maximal -5 (Putri et al., 2023; Sasmito et al., 2014)	Primers complement with their partner primers of different types; Low primer specificity and affects the reduction of primer concentration (Borah, 2011; Handoyo & Rudiretna, 2000)	
Hairpin loop (kcal/mol)	Maximal -3 (Putri et al., 2023; Sasmito et al., 2014)	Primer ends complement each other; Not allowed at all (Sasmitha et al., 2018)	

As complementary data, secondary structure prediction was carried out using the UNAFold program to assess the potential for DNA fold formation on primers *HVS II* A and *HVS II* B. This program visualizes the chemical structure of the primer single thread with one delta G (ΔG) value that indicates the stability of the secondary structure. The predicted delta G (ΔG) values were within commonly accepted thresholds for forensic primer design, indicating a low risk of primer–dimer formation or failed amplification, which supports their suitability for reliable PCR in forensic applications. The ideal value for PCR is above -9 kcal/mol (Integrated DNA Technologies, 2024). UNAFold prediction results show ΔG values ranging from +0.90 to +2.25, which reflects a less stable secondary structure and does not interfere with PCR amplification efficiency. In silico primer specificity was assessed using the NCBI BLAST database to evaluate potential binding to target sequences (Table 3). The analysis yielded exclusively *Homo sapiens*-specific HITS, with no detectable alignment to non-human sequences. This result is advantageous in forensic applications, as any alignment with non-*Homo sapiens* sequences would indicate insufficient primer specificity, potentially leading to cross-species amplification and unreliable identification. The absence of non-human hits therefore confirms that the primers are highly specific for human mtDNA, ensuring accurate intraspecies discrimination and enhancing the reliability of forensic DNA profiling.

Table 3. *HVS II* primers BLAST analyze

Primer *HVS II*	Description (Accession Number)	Max score	Total score	Query cover	E value	Percentage of identify
A Forward	*Homo sapiens* isolate 14 mitochondrion, complete genome (OP718239.1)	44.1	44.1	100%	0.091	100%
B Forward	*Homo sapiens* isolate AR-Arr027 *D-Loop*, complete seq; mt (MG832705.1)	40.1	40.1	100%	1.4	100%
A & B Reverse	*Homo sapiens* isolate S30 *D-Loop*, partial seq; mt (ON638353.1)	40.1	40.1	100%	1.4	100%

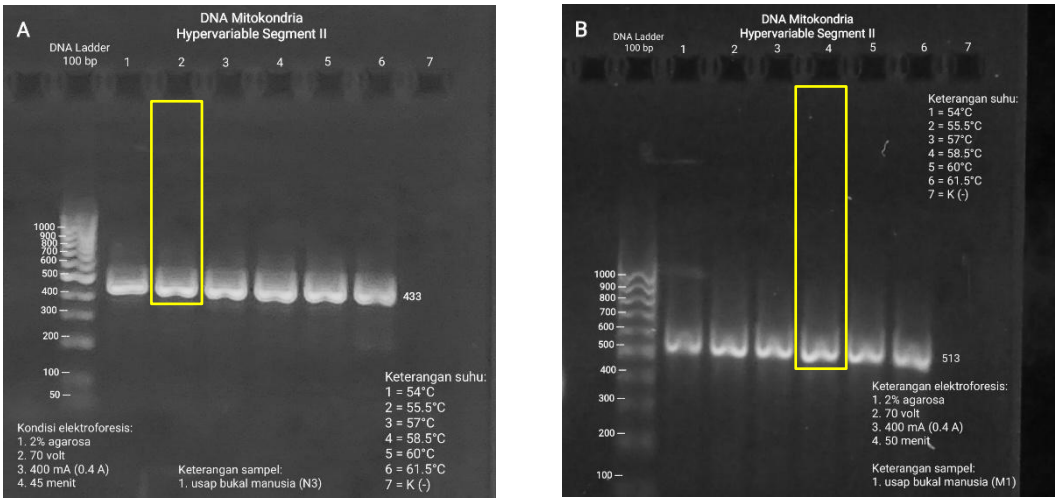

Figure 2. Optimization of *HVS II* primers using gradient PCR technique; (A) *HVS II* A 433 bp; (B) *HVS II* B 513 bp

BLAST results of *HVS II* primers showed that *HVS II* A and *HVS II* B primers have high specificity to human mitochondrial DNA, especially in the *D-Loop* region. BLAST results from NCBI identified about 100-103 HITS as *Homo sapiens* with consistent alignment distribution. Although these results are

promising, in vitro tests are still needed to confirm primer specificity in amplifying target DNA in forensic biology samples. In vitro testing was conducted to confirm the specificity of *HVS II* primers in amplifying target DNA using the gradient PCR technique. The determination of the variation of optimization temperature conditions was based on the results of silico analysis of *HVS II* A and *HVS II* B primers which showed that the optimal Ta value ranged from 54.85˚C - 57.88˚C (Table 1). Therefore, the optimization temperature starts from 54˚C to 61.5˚C with variations of 54˚C, 55.5℃, 57℃, 58.5℃, 60℃, and 61.5℃ with each temperature variation successively increased by 1.5℃.

Based on Figure 2, primer *HVS II* A is able to amplify target DNA at 433 bp, while primer *HVS II* B produces PCR products with a single amplicons size of 513 bp. Although the optimization results show that primers *HVS II A* and *HVS II B* produce visible DNA bands across a wide range of annealing temperatures, amplification occurs throughout the tested temperature range without a clearly defined optimum. Such broad, non-specific amplification may be a concern for stringent forensic protocols, as it can reduce reproducibility and compromise specificity, particularly when analyzing low-quality or degraded DNA samples. According to previous research, the most optimal temperature for amplifying the *HVS II* region is 56˚C (Rusu et al., 2018) which in this study was used as a positive primer (*HVS II* Control). This positive control was used as a comparison for the primer conditions that have been optimized and validated in previous studies. Therefore, to obtain more specific and optimal conditions without smear, further testing of both primer pairs was carried out at 56.5°C. This temperature was obtained based on visual observation of the electrophoresis and averaging some of the temperatures considered to show the most consistent and fluorescent amplification results, namely 55.5˚C (*HVS II* A) + 58.5˚C (*HVS II* B) + 56˚C (*HVS II* Control) = 56.67˚C = ~56.5˚C.

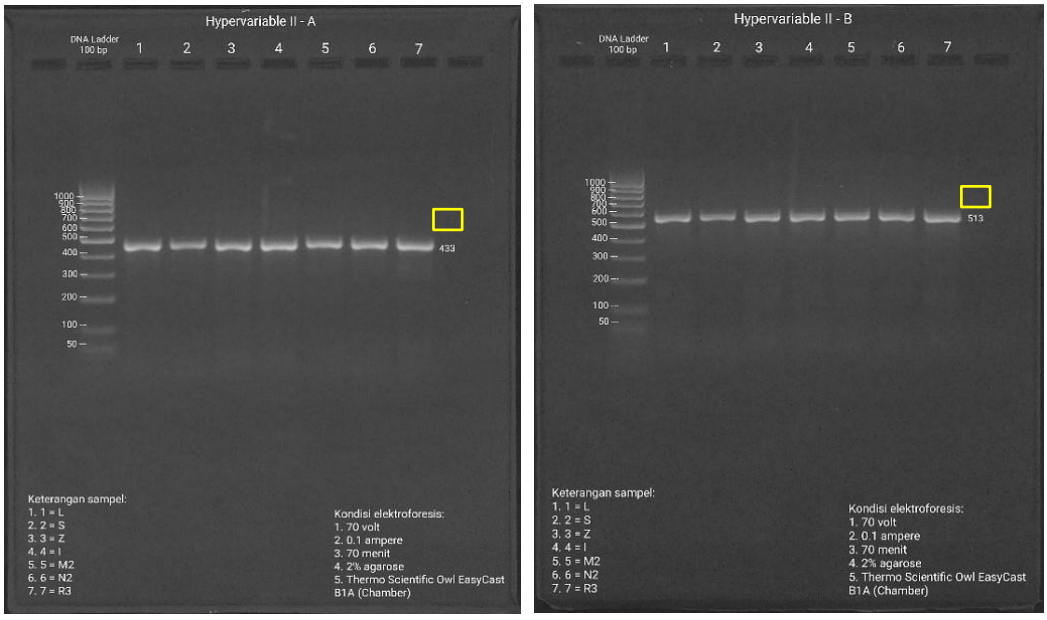

Figure 3. Sample assay using *HVS II* primers at an optimal temperature of 56.5˚C; (A) *HVS II* A 433 bp; (B) *HVS II* B 513 bp

The follow-up test was applied to 7 buccal swab samples belonging to seven individuals with different backgrounds. Although these results demonstrate initial validation of the primers, the limited sample size highlights the need for testing on larger and more genetically diverse populations to ensure

robust performance in forensic applications. Figure 3 shows the results of electrophoretic visualization testing the specificity of *HVS II* primers against each individual, namely seven *Homo sapiens* isolates, represented in wells 1 - 7 respectively. When compared with the results of electrophoretic visualization during primer optimization, the sample test results have a straight amplicon shape, fluorescent, showing no smear, and perfect DNA ladder separation. DNA bands have been formed consistently with the target DNA fragments using primers *HVS II* A and *HVS II* B, which are 433 bp and 513 bp respectively in well numbers 1 - 7. While these results demonstrate successful primer specificity and amplification, the limited sample size prevents generalization, and further testing on larger, genetically diverse populations is necessary to validate forensic applicability. The sample used for the subsequent sequencing test was a PCR DNA sample belonging to one individual "Z" (female) as a representative to confirm the specificity of the primers in amplifying human mitochondrial DNA. While this approach provides preliminary proof-of-concept, the use of a single sample constitutes a limitation, and further testing on multiple individuals is necessary to fully validate primer performance for forensic applications. Sequencing results were saved in AB1 format and analyzed using BioEdit software to assess the consistency of DNA reading peaks (Figure 4).

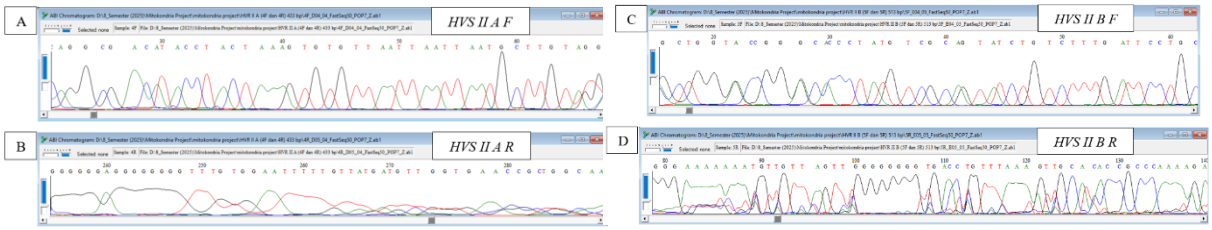

Figure 4. Chromatogram of sample Z sequencing results using *HVS* region II primers

The chromatogram results in Figure 4 show that not all primers work optimally in amplification, especially the reverse primers *HVS II* A and B which display distorted peaks. Reverse primer *HVS II* A produces peaks that are too sloping, while *HVS II* B shows a lot of stacking or background noise, which indicates non-specific primer binding or invalid readings. These distortions can be caused by contamination, column degradation, or instrument malfunction (Al-Shuhaib & Hashim, 2023). Only *HVS II* A and B forward primers that show equivalent or consistent peaks can be tolerated (Calloway et al., 2000). his indicates better performance in recognizing the target DNA. This imbalance may be influenced by the working direction of DNA polymerase which synthesizes from 5' to 3' (Anissa et al., 2023), so that the forward primer more efficiently initiates DNA synthesis. Due to the less than optimal performance of the reverse primer, specificity analysis was continued only on the forward primer, with sequence BLAST results shown in Table 4. Future work will be necessary to redesign or re-optimize the reverse primer to ensure reliable amplification for routine forensic applications.

Based on the sekuensing BLAST results (Table 4), forward primer *HVS II* A has a query cover of 99% and an identity percentage of 97.29%, while forward primer *HVS II* B has the same condition on the query cover with a lower identity percentage of 93.79%. The high percentage identity of forward primer HVS II A is 97.29% indicating a strong sequence match with the target DNA. The forward primer HVS II B shows a slightly lower identity of 93.79%. This value is still above the generally accepted threshold of 90% for forensic applications, suggesting that the minor reduction does not affect its effectiveness in human mtDNA profiling. This mismatch is thought to occur at the 3' end of primer *HVS II* B which has five

consecutive G bases and can interfere with attachment and elongation by DNA polymerase. The structure of the 3' end with more than three consecutive C or G bases can trigger the formation of secondary structures such as primer-dimer (Anissa et al., 2023).

Table 4. BLAST hasil sekuensing sampel Z menggunakan primer daerah *HVS II*

Primer ID	Sequences similarity	MtDNA region	Query cover	Persentase identitas
***HVS II* A Forward**	*Homo sapiens* isolate tzsw001, partial sequence; mitochondrial (Acc num: EF999599.1)	*D-Loop*	99%	97.29%
***HVS II* B Forward**	*Homo sapiens* isolate YXLPCT685, complete sequence; mitochondrial (Acc num: JQ037573.1)	*Control region*	99%	93.79%

This condition decreases the efficiency of PCR amplification and supports the importance of primer sequence matching. Despite the difference in identity percentage, BLAST results show that both primers still have above 90% similarity to the reference mitochondrial genome, so they remain valid for amplification of the *D-Loop* region. This is reasonable considering that no primer design program is perfect and biological factors such as heteroplasmy due to mutations can also affect results. The *D-Loop* control region of mtDNA is known as a region with a high level of genetic variation, both at the individual and population scale (Chaitanya et al., 2016). Therefore, mitochondrial DNA mutation variation analysis using *HVS II* forward primers and haplogroup prediction were conducted on individual Z test samples (Table 5).

Table 5. Mutational variation and haplogroup prediction of individual Z using *HVS II* region primers

Primer ID	Mutation sites and variations	Variations total	Haplogroup prediction
***HVS II* A Forward**	A189d(=A**188**d†), T195C, C231T, A237C, A244G, T245G, T246A, G247T, A248G, A263G, AA290-(=AA**286**d†), T310C, C311CTCC, A361d(=A**357**d†), C387d(=C**386**d†), T393C, CA394-, T408d(=T**405**d†), A426d(=A**425**d†), CA438-, TC455-, C476d	22	L4b (L4b2) Africa
***HVS II* B Forward**	C150A, A176d(=A**175**d†), A189d(=A**188**d†), T195C, A202d(=A**200**d†), A219d(=A**218**d†), T224C, A238d(=A**237**d†), AC241-, A243C, A249d(=A**248**d†), A263G, C299A, A302C, C315CC, A337d(=A**335**d†), CA356-	17	N21 (N21+195) Indonesia

*Descriptions: d† deletions (gray highlights), NT non-query deletions (blue highlights), transitions (green highlights), transversions (red highlights), insertions (purple highlights); similarity of site and mutation type between primers (underlines); d† = deletion substitution mutations that have been reported in previous studies

Analysis of nucleotide variation was carried out using the Mitomaster Mitomap program which compares sample sequences with rCRS (Gumilar et al., 2021). Individual Z showed 22 mutations with primer *HVS II* A and 17 mutations with *HVS II* B. Mutations found included deletion substitutions, transitions, transversions and insertions. Deletion substitution mutations were the most common and are marked with the symbol "d†" indicating the mutation sites. Transition mutations involve changes among purines or pyrimidines, such as A263G and T310C (Gumilar et al., 2021). Meanwhile, transversion mutations occur between purines and pyrimidines, such as C150A and A237C. Insertion mutations are the addition of one or more nucleotide bases into a DNA sequence and are found at sites C311CTCC (*HVS II* A) and C315CC (*HVS II* B). The results of this study showed the presence of common polymorphisms such as A263G and T310C. Despite originating from the same individual, only the same three mutations were found in both primers, reflecting the possibility of heteroplasmy in individual Z's

buccal cells. Heteroplasmy can appear as length variations in the homopolymer C pathway or other nucleotide sites in *HVS II* (Sturk-Andreaggi et al., 2020). Each haplotype has a unique geographic pattern and can be associated with disease risk Miura et al., 2022). Sample Z showed T195C, T310C, and C315CC mutations associated with melanoma and glaucoma diseases (Kumar et al., 2024; Young et al.2022),. N However, the involvement of T195C and C315CC in cancer is still debated due to limited data (Grandhi et al., 2017; Young et al., 2022). On the other hand, the m.310T>C variant actually serves as a protective variant against glaucoma (Kumar et al., 2024).

Mitochondrial haplogroups are distinguished by specific polymorphism characteristics and are often associated with specific geographic regions (Hahn & Zuryn, 2019). Based on Table 5, individual Z was predicted to belong to haplogroup L4b by primer *HVS II* A and N21+195 by primer *HVS II* B. Haplogroup N21 is known to originate from the Indonesian and Malaysian regions, and is part of macrohaplogroup N brought by early colonizers to Australia through Southeast Asia (Fregel et al., 2015; Nagle et al., 2017). This prediction is consistent with individual Z's Indonesian background. While the difference is scientifically interesting, it highlights the limitation of assigning haplogroups based on a single mitochondrial region and a single individual. Comprehensive haplogroup determination in forensic or population studies requires analysis of multiple mtDNA regions and larger sample sets to ensure accurate resolution. On the other hand, haplogroup L4b reflects lineages from East Africa and Saudi Arabia, and suggests genetic relatedness between Indonesian and Malagasy populations (Pierron et al., 2017; Uren et al., 2016).The Indonesian population has the largest shared identical fragment to Malagasy individuals, an island nation off the coast of Southeast Africa based on Identical by Descent (IBD). This means that most of the DNA fragments owned by individuals of African descent are owned by Indonesians (Pierron et al., 2017). Fragments of haplogroup L4b in individual Z were detected at nucleotide sites 195, 244, 263, and 310 according to the Mitomaster Mitomap program. Haplogroups N21 and L4b2 are still related through a common ancestor in human migration history (Figure 5).

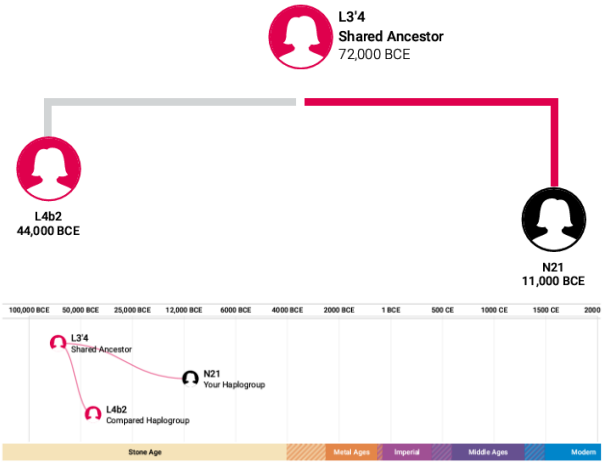

Figure 5. Migration history of haplogroup N21 and L4b2 (mtDNA Haplogroup N21, 2022)

CONCLUSION

This study concluded that the constructed primers showed good specificity in amplifying the *HVS II* region in the *D-Loop* region of mtDNA from human buccal swab samples. Primer *HVS II* region is able to produce single amplicons with sizes of 433 bp and 513 bp. These intermediate amplicon sizes are

advantageous for forensic applications because they are long enough to provide sufficient sequence information for haplotype analysis, yet short enough to allow successful amplification from degraded or low-quantity DNA samples. The optimum temperature in the mitochondrial DNA amplification process is 56.5˚C. Primer efficiency analysis through sequencing results showed that both forward *HVS II* primer threads were able to produce data that could be interpreted bioinformatically, although there were differences in analysis results. The reverse primers, however, exhibited suboptimal performance and were not fully validated, indicating that the complete primer sets are not yet ready for routine forensic use and require further optimization. The analysis showed that individual Z was classified into haplogroups L4b2 (*HVS II* A) and N21 (*HVS II* B), which are haplogroups from several populations in a region of Indonesia. This condition was accompanied by heteroplasmy occurring within one individual's buccal cell. Overall, the designed *HVS II* primers proved to be specific and efficient for use in genetic analysis of the *HVS II* region of human mtDNA, particularly on a population scale. These findings provide a valuable tool for forensic investigations and maternal lineage studies, while also contributing to broader population genetics research and the development of DNA-based applications that can benefit society.

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