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Computational Design and Validation of a Loop-Mediated Isothermal Amplification (LAMP) Assay for the S450W *rpoB* Mutation in *Mycobacterium tuberculosis*

Dian R. Setyorini[1], Irvan Faizal[2,4,*], Kiki Kurniawan[2], Jessie S. Liusarany[4], Selin F. Satiya[5] , Tri Wibawa[1,3]

[1]*Biotechnology Study Program, The Graduate School of Universitas Gadjah Mada, Yogyakarta 55281;* [2]*Centre for Vaccine and Drug Research, National Research and Innovation Agency Republic of Indonesia (BRIN), Kawasan Puspiptek Serpong, Serpong 15314;*[3]*Department of Microbiology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta 55281;* [4]*School of Bioscience, Technology, and Innovation, Universitas Katolik Indonesia Atma Jaya, Jl. Raya Cisauk Lapan, Sempora, Kec. Cisauk, Kabupaten Tangerang, Banten 15345;* [5]*Biomedical Science Study Program, Universitas Andalas, Limau Manis, Pauh, Padang City, West Sumatra 25175*

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Abstract

Tuberculosis (TB) in Indonesia is exacerbated by rising resistance to first-line drugs like rifampicin, often linked to point mutations in the *rpoB* gene, such as S450W. While Loop-Mediated Isothermal Amplification (LAMP) is used for resistance detection, its application for specific point mutations requires further optimization. This study aimed to design and validated a LAMP assay specifically for the S450W *rpoB* mutation using computational and in vitro methods. Computational analysis first confirmed the S450W mutation as a viable biomarker. Subsequently, in vitro validation demonstrated high analytical sensitivity and specificity. The real-time LAMP assay achieved a lower limit of detection (LOD) of 581.4 copies per reaction, demonstrating a tenfold increase in sensitivity than the colorimetric assay, which had an LOD of 58,140 copies per reaction. The designed primer set also showed high specificity, successfully differentiating the S450W mutant sequence from the wild-type and non-template controls. These results establish our LAMP assay as a sensitive and specific tool for the rapid detection of the S450W rifampicin resistance mutation, offering significant potential for clinical diagnostics in high-burden settings.

Keywords: Loop Mediated Isothermal Amplification, *Mycobacterium tuberculosis*, point mutation, rifampicin resistant, S450W.

1. Introduction

Indonesia is the second country with the highest rate of tuberculosis cases in the world after India (Kaaffah et al., 2023). According to Indonesia's tuberculosis control report (Indonesia Ministry of Health, 2023), tuberculosis cases in Indonesia reached at least 638.646 in 2023, with a total estimated number of known cases of MDR-TB/RR-TB is 24.637. Yogyakarta Province has a high achievement of confirming rifampicin resistance tests (222 cases), along with 3 other provinces, specifically South Sumatra (442 cases), Bali (422 cases) and Papua (222).

Multidrug-resistant tuberculosis is a growing public health concern in many parts of the world, especially in low-income countries where most cases occur. However, current conventional drug susceptibility testing methods are either time-consuming, such as the proportion method on solid media, or costly, such as the BACTEC 960 System (Abdul-Imam Almazini, 2013). MDR-TB potentially detected using rifampicin resistant marker, as 90% of rifampicin-resistant isolates are also found to be resistant to isoniazid (Raoot & Dev, 2015).

The *rpoB* gene encodes the RNA polymerase β Sub-unit (RNAP) protein which plays a role in the synthesis and transcription of bacterial oligo-ribonnucleotides (Campbell et al., 2001). Rifampicin works by blocking and inhibiting the transcription process (Wulandari et al., 2024). Mutations in *rpoB* are known to inhibit the interaction between rifampicin (RIF) and RNAP (Li et al., 2021).

A recent study from Filipenko et al. (2019) showed S450L *rpoB* mutation can be used as Allele-Specific LAMP biomarker. Although the resistance profile of S450L *rpoB* gene is already known, another promising mutant variant, especially S450W as LAMP biomarker, needs to be further investigated.

Loop Mediated Isothermal Amplification (LAMP) is one of the pathogen detection methods based on nucleic acid amplification (Chaouch, 2021; Kashir & Yaqinuddin, 2020). LAMP is known to be able to accurately detect pathogens so that it can be used as a Point-of- Care (POC) diagnostic test (Augustine et al., 2020; Notomi et al., 2000). The results of colorimetric LAMP detection can be visually observed (Wong et al., 2018). The LAMP amplification process takes less than 65 minutes, has a high level of accuracy, low cost (about $2.5 or

* Corresponding author. e-mail:irvan.faizal@brin.go.id.

$40,139.75/test), and requires simple instruments (Jaroenram et al., 2020). LAMP has potential to be used in limited sources area due to the minimum instrument needed (Jekloh et al., 2022; Sreedeep et al., 2020).

Identification of resistance profiles is usually carried out using in vitro study at the laboratory, specifically with the drug susceptibility test (DST) or minimum inhibitory concentration (MIC) method (Seid et al., 2024). However, characterization using conventional method tends to take a long time to analyze (Geethalakshmi et al., 2024). In addition, areas of mutation and changes in protein stability cannot be analyzed directly. Sanger sequencing and whole-genome sequencing followed by machine learning-based computational analysis have great potential for predicting tuberculosis drug resistance (Ayaş et al., 2024; Paredes-Gutierrez et al., 2025). The use of sanger sequencing is still considered quite promising in determining the location of mutations in clinical DNA samples.

This study focused on identified S450W in *rpoB* gene of MTB as rifampicin resistant biomarker. Computational analysis using molecular docking is expected to provide more in-depth information about the mutation effects occurring at the molecular level. Mutation sequence is used as template in primer set design for colorimetric and real-time LAMP reaction.

2. Materials and Methods

2.1. DNA Sample Collection

Total of three DNA clinical samples were collected from sputum specimen of a tuberculosis patient during 2024 until 2025 and one H37Rv as reference sample was collected from culture media (Table 1). Sample collection was carried out at M. Djamil Hospital, Padang Pariaman, West Sumatra, and Microbiology Laboratory (Biosafety level 2+), Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada, Yogyakarta, Indonesia. Study have been approved by Medical and Health Research Ethics Committee (MHREC) of Universitas Gadjah Mada (Ref: KE/FK/1288/EC/2024). DNA isolation process of the sample using Sputum DNA Isolation Kit (Slurry)™ (Norgen Biotek Corp, Canada). Samples that confirmed positive on GeneXpert-RIF test and drug susceptibility test were labelled as presumptive antibiotic resistant.

Samples were included in this study if they met the following criteria simultaneously: (1) The sample was identified as presumptively Rifampicin-resistant based on initial testing using either GeneXpert MTB/RIF or the MGIT Drug Susceptibility Testing (DST) system; and (2) The isolated DNA from the sample exhibited adequate concentration and purity, defined as an A260/280 ratio within the range of 1.8–2.0, to ensure robust LAMP amplification. Samples were excluded from the study if: (1) The extracted DNA showed poor quality, specifically defined as having an A260/280 ratio outside the acceptable range of 1.8–2.0, which suggests potential contamination or the presence of PCR/LAMP inhibitors

Table 1. Sample identity

Code	Presumptive Profile Assay	Resistance profile	Concentration (ng/µL)	Purity (260/280)	Geo Location
Y6	MGIT DST+	RIF	28.0	1.98	Indonesia: Yogyakarta
S1h	GeneXpert+	RIF	28.4	2.00	Indonesia: Padang
R1	GeneXpert+	RIF	30.5	1.94	Indonesia: Padang
H37Rv	wild type	-	29.3	1.83	Indonesia: Bandung

2.2. PCR Primer Design

The *rpoB* gene was amplified using the Polymerase Chain Reaction (PCR) primer design based on previous reference with target modification (Vo et al., 2024). PCR primer design was made based on the *Mycobacterium tuberculosis* H37Rv reference sequence template (NC_000962.3). Primer designed to amplify RRDR (rifampicin resistant determining region) in *rpoB* gene NC_000962.3 (759807...763325). PCR primer created using SnapGene, was examined using BLAST (*blast.ncbi.nlm.nih.gov*) and quality was checked using OligonucleotideAnalysis (*geneinfinity.org*).

The quality control criteria were strictly maintained to ensure optimal amplification efficiency, specifically by confirming that: (1) Self-annealing was negligible; (2) Hairpin formation was avoided; and (3) The melting temperatures (Tm) of the Forward and Reverse primers were closely matched (ideally within ±3 °C) to guarantee simultaneous and efficient binding during the isothermal reaction. Self-customized primer pair used in this study can be seen in Table 2.

Table 2. PCR primer set

Antibiotic	Gene	Primer Design (5' to 3')	Amplicon (bp)	Tm (°C)	Ta (°C)
Rifampicin (RIF)	*rpoB*	F:CAATTCATGGACCAGAACAA R: GCATCACAGTGATGTAGTCG	673	59.2 61.3	59,2

2.3. PCR Reaction

The PCR reaction using high-fidelity mastermix (Colman et al., 2015). PCR mixture was prepared using Nuclease-Free Water (Himedia, India), Phusion Plus 2X High-Fidelity Mastermix (Thermo Scientific, USA), a working primer solution (10 µM), and DNA samples. The final reaction volume was 10 µL, composed of 3 µL of Nuclease-Free Water, 5 µL of Mastermix, 1 µL of primer solution, and 1 µL of DNA sample. PCR program includes pre-denaturation in 98°C (30 seconds), denaturation in 98°C (10 seconds), annealing temperature 59,2°C (30 seconds), extension of 72°C (15 seconds) with a cycle repeat 30 times, and the extension step in 72°C (10 minutes). The entire PCR process was carried out with a Biometra Thermal Cycler (Jena Analytics, Germany). This was followed by electrophoresis, which was conducted for 30 minutes at 100 V using the Mupid-eXu® Submarine (Advance, Japan). For gel preparation, 0.4 g of agarose was dissolved in 40 mL of TAE buffer (Himedia, India), heated, and then supplemented with 2.5 µL of RedSafe™

DNA staining (South Korea) to achieve a final concentration of 1% agarose. The electrophoresis results were visualized using the AlphaImager™ Mini Gel Documentation System (ProteinSimple, USA).

2.4. DNA Purification

Purification was necessary to increase the concentration and purity of DNA from clinical samples collected in Yogyakarta. This process was performed using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). DNA purification process was based on previous protocol (Arunachalam & Sreeja, 2025).

2.5. DNA Sequencing

Positive samples from the PCR step were used for DNA sequencing. Each sample consisted of 35 µL of unpurified PCR product with a minimum concentration of 100 ng/µL. Single-pass DNA sequencing was performed by Apical Scientific (Malaysia) using an Applied Biosystems Genetic Analyzer (Thermo Fisher Scientific, USA) and a BigDye® Terminator V3.1 sequencing kit (Thermo Fisher Scientific, USA). For quality control, the resulting electropherograms were visualized using FinchTV and Geneious Prime. Sequences with a low-quality electropherogram (confidence score lower than 50%) were excluded. High-quality forward and reverse sequences were then used to generate a consensus sequence after a pairwise alignment process in BioEdit. Multiple alignment of the consensus sequences was performed with the MTB H37Rv reference sequence using SnapGene. Sequence identity was confirmed with BLAST, and the multiple sequence alignment was visualized using MSA viewer.

2.6. In Silico Analiysis: Protein and Ligand Preparation

This preparation method was adopted from Maladan et al., (2023); Singh et al., (2021) with some modifications. In silico mutagenesis was carried out with substitution of amino acid residues in mutant amino acid sequence manually using notepad and visualized using SnapGene translation tool. The 3D structure of native proteins was obtained from homology modelling using SWISS MODEL (*swissmodel.expasy.org*) based on protein template database. The protein conformations with identity value close to 100%, GMQE closes to 1, and the best ramachandran plot are selected as the receptor. Meanwhile, the crystal structure of rifampicin as ligand was collected from protein-ligand complex separation with PDB ID: 5UHB in RSCB PDB (*rcsb.org*). Protein and ligand preparation were performed by eliminating water molecules and irrelevant protein chain structures using Biovia Discovery Studio (*3ds.com/products/biovia/discovery-studio*). Hydrogen atoms were added using PyMol (*pymol.org*) and energy minimization using Swiss PDB Viewer software (*spdbv.unil.ch/disclaim.html)*.

2.7. Molecular Docking

Molecular docking process using CB-Dock2 program (*cadd.labshare.cn/cb-dock2/index.php*) by entering protein receptor (PDB format) and ligand (SDF format) on the website. The best protein-ligand conformations are selected based on the suitability of amino acid residue bonds and docking scores. The structure of protein-ligand complexe in wild type and mutant isolate were then compared. Docking results are visualized in 2D form using Biovia Discovery Studio.

2.8. LAMP Primer Design

Primer set was designed using web-based program PrimerExplorer V5 (*primerexplorer.eiken.co.jp/e/*). LAMP primer design method used in this study is based on some previous references (Aulia et al., 2023; Varona & Anderson, 2021) with several modifications. Mutant sequence being input as template in PrimerExplorer. This primer design strategy specifically placed the mutation S450W (C1349G) in 3' end of F2 primer (Table 2), without any probe and nucleotide base mismatch included. Primer set with low GC rate, similar Tm, minimum dimer, and low self-annealing based on oligonucleotide analysis is preferable as final primer design. This design set does not include LF primer in LAMP reaction to minimize missamplification issue (Khumwan et al., 2022).

2.9. Colorimetric LAMP Reaction

LAMP reaction was carried out using WarmStart Colorimetric 2X Mastermix (#M1800, Biolabs, UK) using previous research design method with several modification (Liu et al., 2012). LAMP mixing using single 02 mL PCR tube containing 6,5 µL mastermix, 1,25 µL LAMP primer 10X stock (Table 2) (concentration each primer: 8 µM FIP, 8 µM BIP, 2 µM F3, 2 µM B3, 4 µM LB), 4,5 µL nuclease free water, and 0,5 µL purified DNA template (total 12,5 µL mixing solution). The primer concentration was decided based on optimization result to minimize missamplification. LAMP temperature and time optimization was carried out using Biometra® (Analytik Jena, Germany) that suitable for temperature gradient purposes. Temperature used for LAMP reaction is 68,5°C for 35 minutes. Limit of detection depends on last detectable color changes by visual and electrophoresis using five serial dilutions of DNA template control.

Table 3. LAMP primer set

Mutant	Primer code	Sequence (5' to 3')	Tm (°C)	GC rate
rpoB S450W	RM2_F3	CGCTGTCGGGGTTGACC	61.87	0.71
	RM2_B3	TGACCCGCGCGTACAC	61.01	0.69
	RM2_FIP	GGTGCACGTCGCGGACCT-ACAAGCGCCGACTGTG	66.84	0.63
			58.73	0.72
	RM2_BIP	GTCGCACTACGGCCGGATGT-GACAGCGAGCCGATCAGA	66.55	0.61
			60.66	0.65
	RM2_LB	CCGATCGAAACCCCTGAGG	61.09	0.61

2.10. Real-Time LAMP Reaction

Procedure used for this step is adopted from related references (Alhamid et al., 2023; Khumwan et al., 2022) with simple modification. Real-time reaction mix using 6,25 µL WarmStart Colorimetric 2X Mastermix (#M1800, Biolabs, UK), 2,5 µL nuclease free water (Himedia, India), 2 µL Sensifast™SYBR-No Rox (Meridian bioscience, USA), 1,25 µL LAMP primer stock 10X, and 0,5 µL DNA template. Real-time LAMP was performed using Quant Studio™ 5-Real Time PCR System (ThermoFisher Scientific, USA). Real-time program setting used 12,5 µL reaction mix, temperature 68,5°C, quantification time every 1 minute for 50 cycles, in duplicate. Limit of detection (LOD) was based on last positive detectable

amplification curve with cut off Cq value ≤ 35. LOD calculation using formula from Ma et al., (2019), number of copies/test = $(M\times6.02\times10^{23}\times10^{-9})/(n\times660)$ with M is amount of DNA in nanogram, n is length of *M. tuberculosis* genome (4.411.529 bp), 660 is average weight of single basepair (Da), and $6{,}02\times10^{23}$ is Avogadro's constant.

2.11. Statistical analysis

Statistical analysis of the qLAMP data was focused on analytical validation (Bates et al., 2025). The Limit of Detection (LOD) determination was performed in duplicate (n=2) for each concentration in the serial dilution. All quantitative data, including the Real-Time LAMP Amplification Bar (Figure 8B), are presented as the Mean ± Range Standard Deviation (SD) to demonstrate the precision and reproducibility of the assay at different target concentrations. All graphical and descriptive statistical analyses were performed using GraphPad Prism Software, Inc. (USA).

3. Results and Discussions

3.1. The rpoB Gene Amplification

PCR primer set are designed to amplify 673 bp of *rpoB* gene (1294…1966) containing common mutation at RRDR (Figure 1). RRDR site located in 81 bp long sequence from 1276 to 1356 bp of *rpoB* gene based on MTB numbering system. PCR primer located in 50-57% GC content region to increase amplification efficiency.

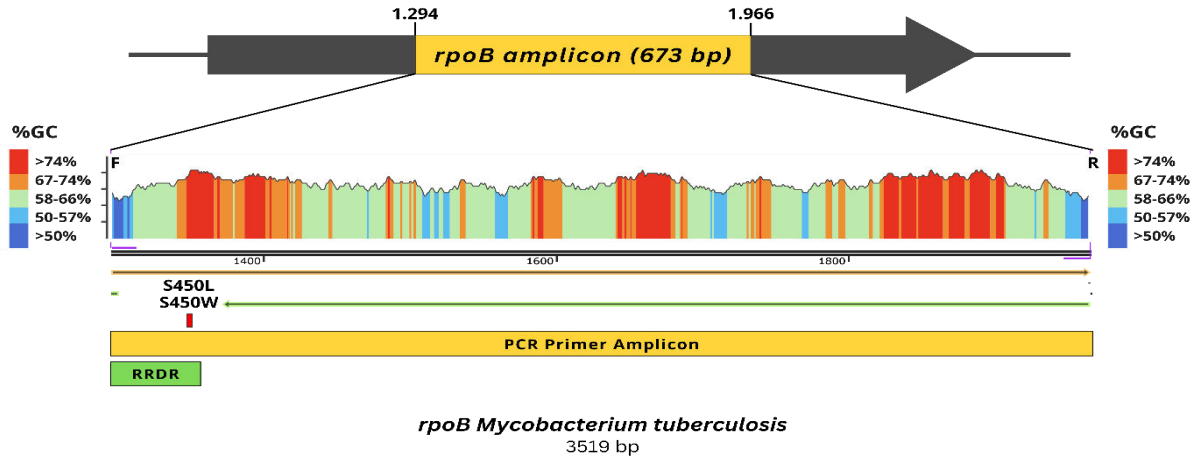


Figure 1. *rpoB* PCR primer map shows amplification area in Rifampicin Resistant Determining Region (RRDR). S450L and S450W found as point mutation in this study, annotated with red-color line. GC content represented in color gradient. Open reading frame (ORF) of coding strand visualized as orange arrow, meanwhile the template strand visualized as green dashed arrow (illustration edited using SnapGene software and *canva.com*).

The three of sputum DNA samples, when amplified using the PCR primer set, produced a single band at 673 bp on agarose gel electrophoresis (Figure 2). Meanwhile, the reference sequence H37Rv was considered as a negative result. This single, confident band indicates that the primers successfully and specifically amplified the RRDR, which includes the S450 codon, a known high-prevalence mutation site. The single band also suggests that the primers did not form dimers.

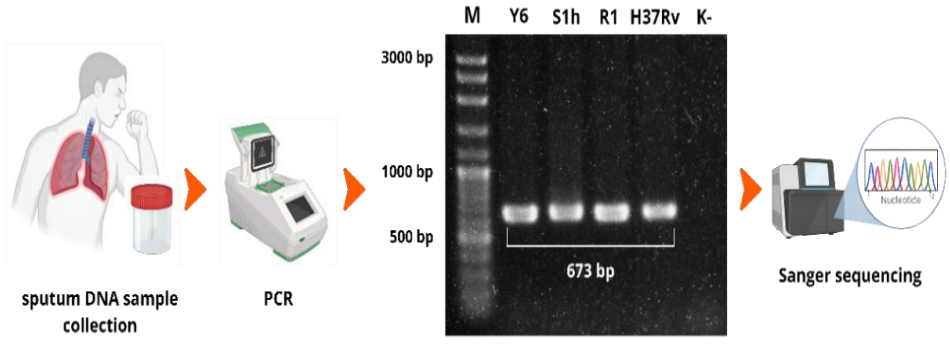


Figure 2. Unpurified PCR product used for DNA sequencing. Visualization of control samples electrophoresis result (icon assets and illustration editing using *biorender.com* and *canva.com,* PCR visualization using research documentation).

3.2. Mutation Profiling

DNA sequencing is used to determine sample mutation profile, along with multiple alignments for each sample sequence with reference sequence (H37Rv) to locate the mutation position. Samples used for DNA sequencing included one Yogyakarta sample (Y6), and two Padang samples (S1h and R1). All samples have single point mutation at codon 450 in RRDR (Figure 1,3). Conkle-Gutierrez et al. (2022) stated that rifampicin resistance is generally caused by mutations in the RRDR of the *rpoB*

gene. Based on multiple alignments, Y6 showed single point mutation in S450W, changing the nucleotide base from TCG (Serine) into TGG (Tryptophan) (Figure 3). Meanwhile, the S1e, S1h, S1m and R1 showed different mutations, specifically S450L that changed nucleotide base from TCG (Serine) into TTG (Leucine) (Figure 3). Electropherogram quality control using GeneiousPrime showed confident curve peak appear with phred score (Q20, Q30, Q40) at 100% and HQ% score per sequence higher than 90% indicate low error reading rate in mutation identification.

The S450W mutation in the *rpoB* gene is an uncommon mutation but is known to cause significant drug resistance. Although its frequency is low compared to the more common S450L mutation, it is considered a highly resistant mutant. The frequency and type of mutations in MTB can vary by geographical location. The effect of this mutation on drug resistance was investigated using molecular docking to analyze changes in protein conformation at the drug binding site.

Figure 3. Multiple sequence alignment result. Location of mutation in *rpoB* gene based on alignment with H37Rv as reference sequence. Electropherogram comparison between mutant and non-mutant variant showed high phred score, indicates good quality of *rpoB* sequences.

3.3. Resistance Prediction Based on Molecular Docking

Molecular docking analysis revealed significant conformational changes in mutant proteins compared to the wild type. The binding affinity between the protein and the rifampicin ligand changed drastically. In the wild-type protein, the RNA polymerase β subunit binds directly to rifampicin through several amino acid residues, including S450 (serine). This finding is consistent with research by Li et al. (2021), which confirmed that S450 directly binds to rifampicin via a hydrogen bond in its native state.

In this study, the docking scores directly correlate with binding affinity, with more negative values indicating a stronger bond. The wild-type protein showed the strongest binding affinity with rifampicin at -8.8 kJ/mol (Figure 4A), supported by five conventional hydrogen bonds. The S450L mutant variant had a reduced binding affinity of -7.4 kJ/mol (Figure 4C), forming only two hydrogen bonds. In contrast, the S450W mutant showed the lowest binding affinity at -4.7 kJ/mol (Figure 4B), with only a single hydrogen bond. These results indicate that the S450W mutation most effectively disrupts the binding of rifampicin.

The analysis revealed a significant difference in binding affinity. The S450W mutation resulted in a much larger change in binding affinity (4.1 kJ/mol difference) compared to the S450L mutation (1.4 kJ/mol difference), indicating that S450W more effectively disrupts the protein-drug interaction. In the S450L mutant, the substituted leucine (L) residue still forms a direct alkyl bond with rifampicin, allowing for a degree of binding. Conversely, in the S450W mutant, the new threonine (W) residue does not form a direct chemical bond with rifampicin. Although another residue, arginine (R), maintains a hydrogen bond, this is not enough to preserve the original binding strength. This disruption in binding and changes in protein conformation collectively suggest that S450W is more resistant to rifampicin. The molecular docking results, binding affinity, and resistance analysis prediction are summarized in Table 4.

Table 4. Samples mutation profile

Code	Mutation	Annotation	Binding Affinity (kJ/mol)	Resistance associated	Reference
Y6	TCG > TGG	S450W	-4.7 (low)	+	(World Health Organization, 2023)
S1h	TCG > TTG	S450L	-7.4 (low)	+	
R1	TCG > TTG	S450L	-7.4 (low)	+	
H37Rv	No mutation	S450	-8.8 (high)	-	

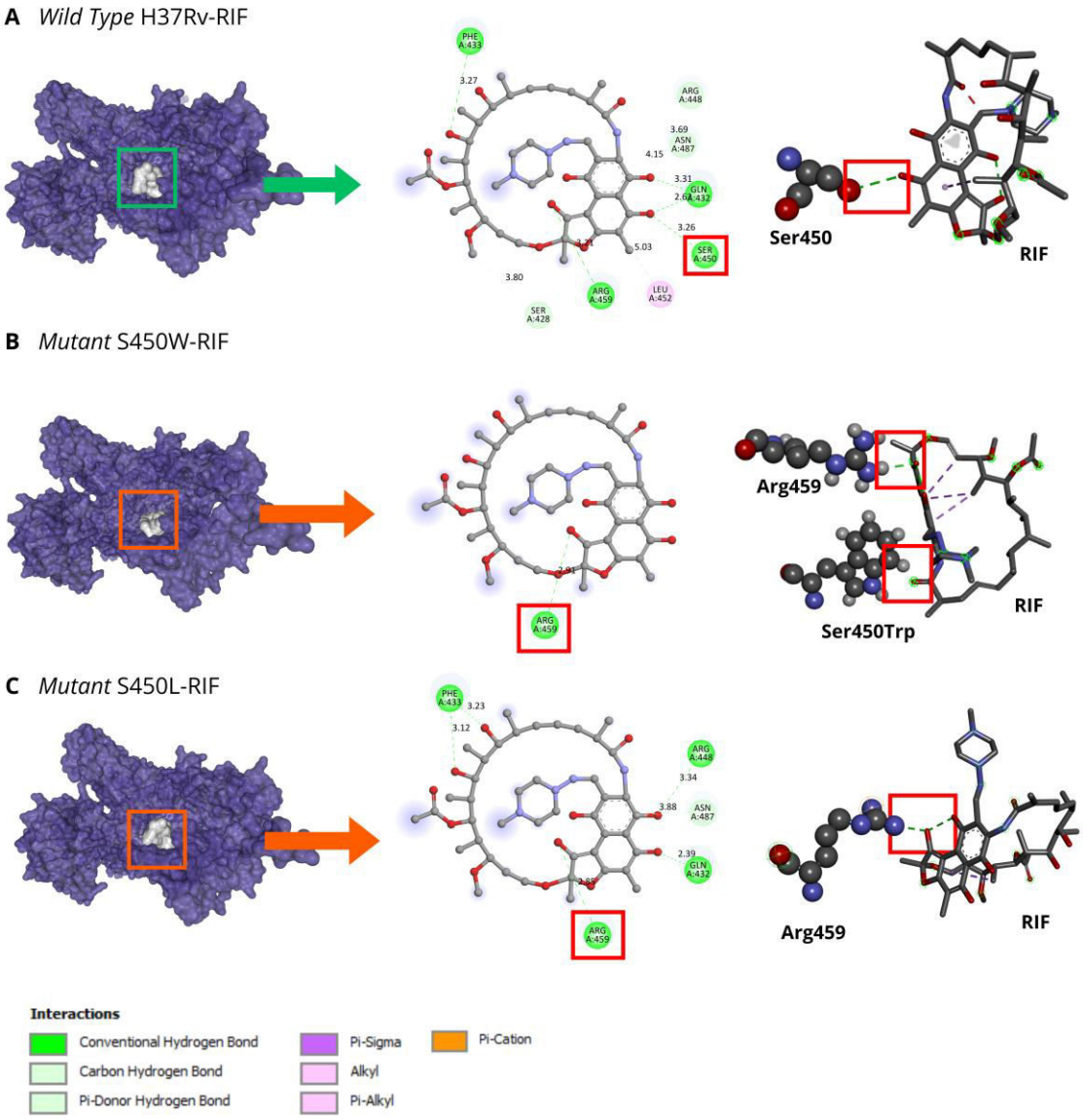


Figure 4. Molecular docking result. (A) Wild type-RIF showed Serine (S450) as residue that binding directly with rifampicin, indicates its location in RNA Polymerase β Sub-unit binding pocket. (B) S450W-RIF showed that the mutant variant no longer binding directly with rifampicin. (C) S450L-RIF still has ability to binding with rifampicin but with different type of molecular interaction.

3.4. LAMP Primer Optimization

The RM2 LAMP primer set was designed to amplify the S450W mutation in the *rpoB* gene (Figure 5). The F2 primer was specifically engineered to target the single point mutation site (TGG) in the S450W mutant. This mutation is strategically located at the 3' end of the F2 primer, a critical position for ensuring specificity. As noted in a previous study, a mutation in this position, particularly in the F2 region of the FIP primer, can differentiate between mutant and wild-type isolates (Khumwan et al., 2022). This is because the mutant nucleotide base in the F2 primer prevents it from annealing to the wild-type sequence, leading to a failure in the amplification initiation. While computational analysis confirmed the high quality of this primer set, further optimization is still necessary to screen for potential amplification issues that can arise from the long structure of the FIP and BIP primers.

The colorimetric LAMP reaction with phenol red dye will change color from red to yellow as pH changes (Peltzer et al., 2021; Selva Sharma & Lee, 2024). The buildup of protons at the end of the reaction will cause the pH to drop so that the positive sample will turn yellow, while the negative sample will remain pink (Tanner et al., 2015). Visual observation result showed that the optimum temperature for colorimetric LAMP reaction is 65°C and 70°C (Figure 6A). LAMP reaction in 65°C and 70°C also showed highly distinctive ladder-like band as unique pattern of LAMP reaction product in electrophoresis visualization (Figure 6D). The significance of color change in positive control samples compared with NTC was calculated using G/R ratio-temperature curve analysis in Microsoft Excel (Figure 6B).

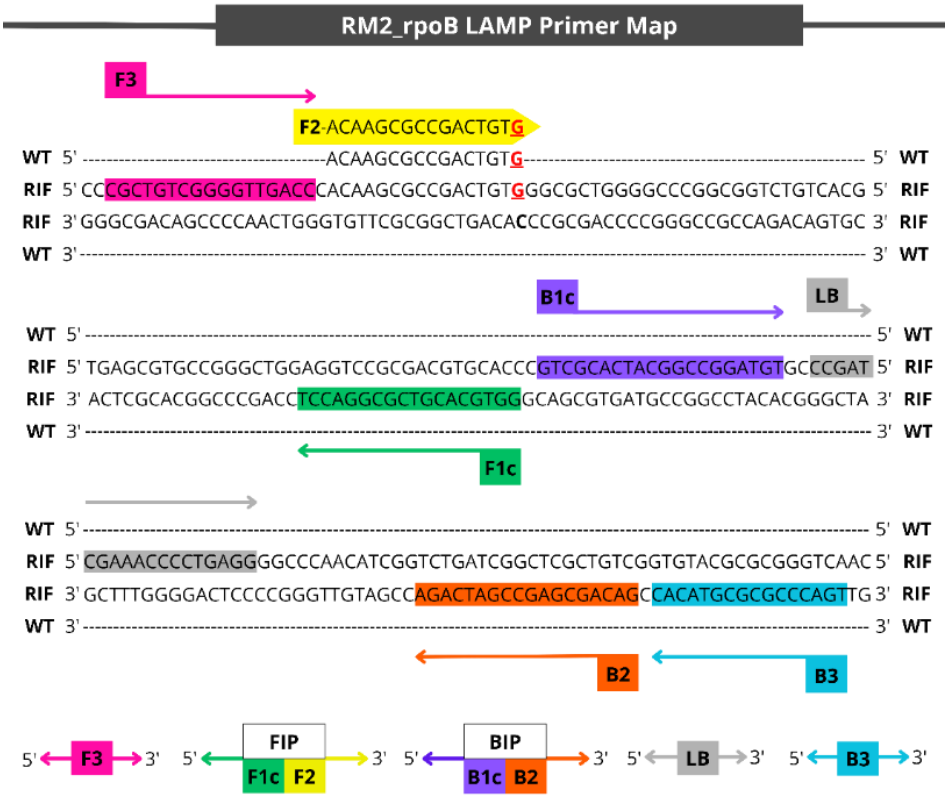


Figure 5. RM2 LAMP primer sets with color coded sequence. Mutation site located in 3' end of F2 primer to enhance the annealing specificity. F2 and F1c primer formation known as FIP, while the BIP primer contains B1c and B2.

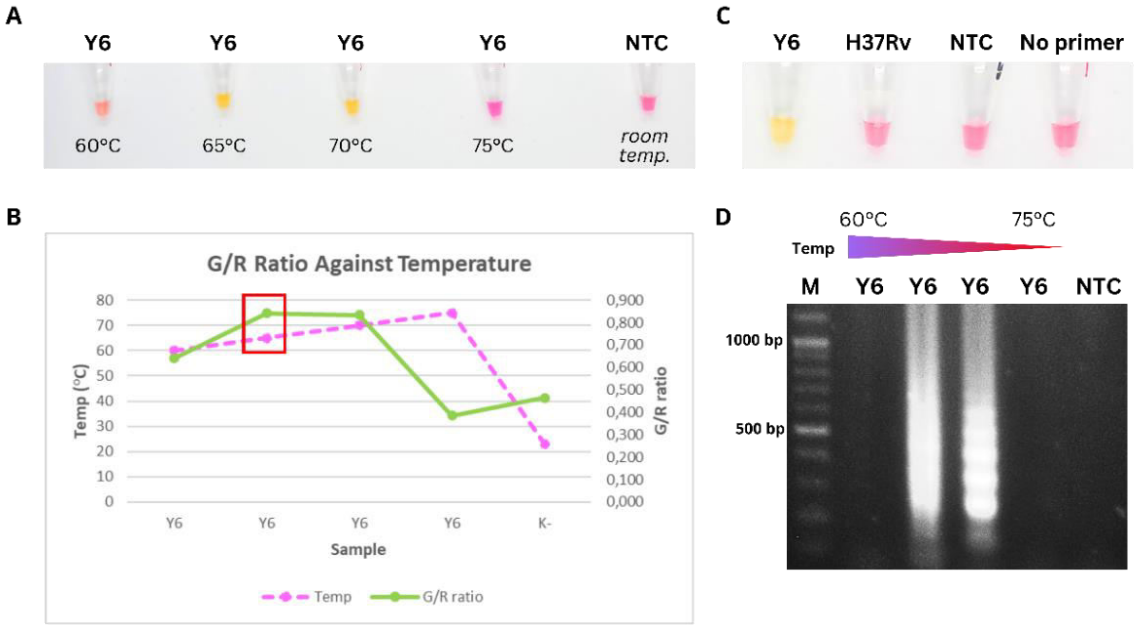


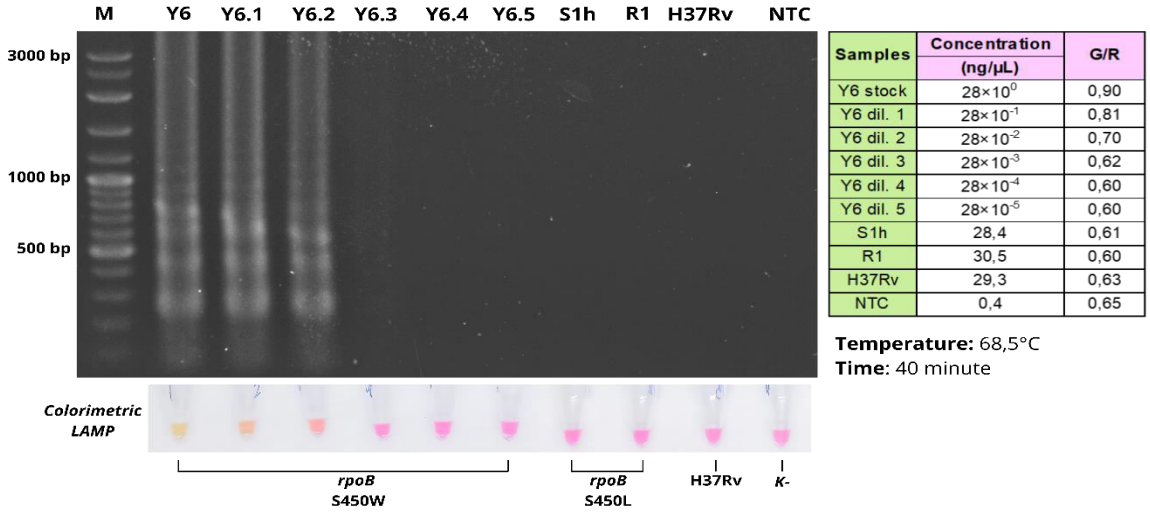
Figure 6. LAMP primer optimization. (A) Temperature optimization based on colorimetric LAMP visualization. (B) colorimetric LAMP G/R ratio curve optimization using 5°C gradient temperature increase. (C) colorimetric LAMP comparison with optimum temperature based on G/R Ratio-Temperature curve. (D) Colorimetric LAMP electrophoresis showed ladder-like band in temperature range at 65°C to 70°C. All the optimization running time is 35 minutes.

The analysis of the LAMP reaction at different temperatures revealed that the optimal amplification for the S450W mutant occurred at 68.5°C. The highest difference in the Green/Red (G/R) ratio curve between the mutant sample and the non-template control (NTC) was observed at 65°C. This finding was consistent with both the colorimetric observation and the electrophoresis results. Similarly, the LAMP reaction at 70°C also showed a high G/R ratio difference, though it was slightly lower than at 65°C. Based on the curve analysis, a G/R ratio threshold of ≥ 0.7 was effective in distinguishing between negative and positive samples. However, further optimization determined that 68.5°C was the ideal temperature for the amplification process. This temperature was found to consistently maintain clear

colorimetric differentiation between the S450W mutant (Y6) and the wild-type (H37Rv) samples.

3.5. Limit of Detection (LOD) Determination

Real-time LAMP amplification settings were based on the optimal conditions determined from the colorimetric LAMP reaction. The assay was performed at 68.5°C for 50 minutes using a five-step serial dilution. The Y6 (S450W) sample was used as a positive control to determine the limit of detection (LOD). The S1h and R1 (S450L) samples served as mutant (MT) controls, while H37Rv was the wild-type (WT) control. The colorimetric LAMP assay had a detection limit at the second dilution, with a concentration of 2.8×10^{-2} ng/µL, equivalent to 58,140 copies/test, as confirmed by electrophoresis (Figure 7). However, a clear visual color change from pink to yellow was only observed at a much higher concentration of 28 ng/µL (5,814,000 copies/test). At the next two dilutions (Y6.1 and Y6.2), the color remained orange, indicating that the amplification had not reached its endpoint. Further optimization showed that extending the incubation time to 40 minutes was feasible to achieve maximum color change. However, extending the reaction beyond 50 minutes is known to increase the risk of false positives due to mispriming and the accumulation of non-target products. Therefore, a 35-minute cut-off value was chosen for this test, a duration adopted from a previous study by Ma et al., (2019).



Samples	Concentration (ng/µL)	G/R
Y6 stock	28×10^{0}	0,90
Y6 dil. 1	28×10^{-1}	0,81
Y6 dil. 2	28×10^{-2}	0,70
Y6 dil. 3	28×10^{-3}	0,62
Y6 dil. 4	28×10^{-4}	0,60
Y6 dil. 5	28×10^{-5}	0,60
S1h	28,4	0,61
R1	30,5	0,60
H37Rv	29,3	0,63
NTC	0,4	0,65

Temperature: 68,5°C
Time: 40 minute

Figure 7. Colorimetric LAMP confirmation using electrophoresis. Limit of detection (LOD) determination using serial dilution compared with wild type H37Rv and S450L mutant sample as reaction control showed detectable band in second dilution.

The real-time LAMP assay in this study achieved a detection limit of 2.8×10^{-4} ng/µL, equivalent to 581.4 copies/test (Figure 8A). The amplification curve for the fifth dilution showed inconsistency, which is attributed to its low DNA concentration and high error rate, as indicated by the standard deviation bars (Figure 8B). The negative control (NTC) showed no amplification, even when the reaction was extended to 50 minutes. The detection limit achieved in this study is comparable to other real-time methods. For instance, Takarada et al., (2020) reported a detection limit of 200 fg/µL (41.53 copies/test) using a LAMP-specific allele system to detect mutations in the RRDR of *Mycobacterium tuberculosis*. In another study, J. Kim et al., (2021) used AS-RPA/SYBR to detect mutations in the *rpoB* and *katG* genes, reporting a detection limit of 5 ng/test (1,038,270 copies/test). While the mutation control sample (R1) showed late amplification after 45 minutes, this late detection suggests that the assay can distinguish between the S450W and S450L mutations. This is because a ΔCt value ≥ 20 cycles and a cut-off time of 35 minutes can effectively differentiate the two mutants. This specificity is crucial for identifying different resistance types.

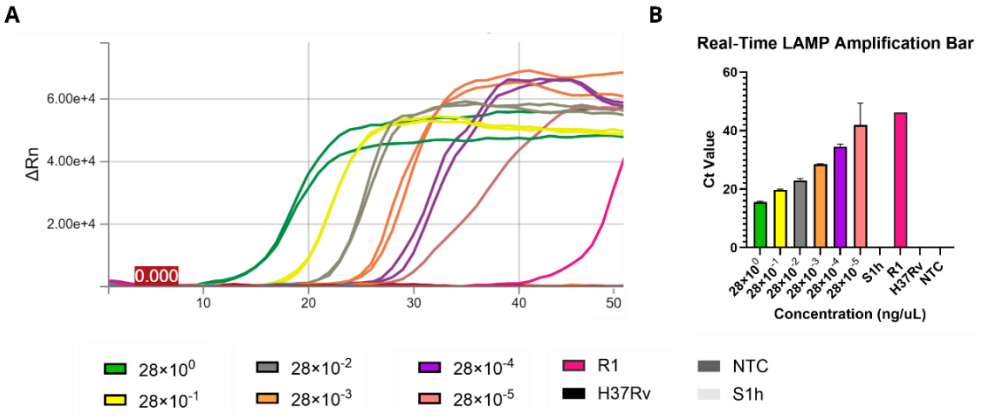


Figure 8. Real-time LAMP reaction results comparison. (A) LOD analysis based on real-time LAMP result. (B) Real-time LAMP ampplification deviation standard with error bar. Curve and bar using the same color coded showed below the data.

The colorimetric LAMP assay in this study had a higher limit of detection (LOD) than the one reported by Takarada et al. (2020), suggesting that the primer set's sensitivity still needs improvement. In contrast, the real-time LAMP method in this study showed a lower LOD than the AS-RPA/SYBR method (Kim et al., 2023). The higher sensitivity of the Takarada et al. (2020) study is likely due to its use of multiple mutation sites, whereas this study focused on a single site. Further research using multiple mutations or mismatches, as suggested by (Khumwan et al., 2022), could increase the sensitivity of the LAMP primer sets.

This study introduces a new LAMP assay to detect the S450W mutation in MTB, a method not previously reported for clinical isolates from Indonesia. While the S450W mutation is less common than S450L, our computational analysis using molecular docking suggests it confers a higher resistance profile (Rando-Segura et al., 2021). This finding highlights the value of computational tools for rapidly predicting resistance mechanisms and identifying new biomarkers for rifampicin-resistant TB.

This study provides insights into specific LAMP primer set design strategies for amplifying the S450W *rpoB* mutation. This was achieved by strategically placing mutated bases at the 3' end of the FIP primer and reducing the length of the LF primer to minimize non-specific amplification. The colorimetric LAMP assay successfully differentiated the S450W mutation from the wild type at 68.5°C within 35 minutes. The developed primer set also demonstrated the ability to distinguish S450W from S450L mutations at the same codon using a specific cut-off time, which highlights its high discriminatory power. For future development, it is recommended that additional triple mismatches be incorporated into the F2 primer to further enhance the assay's specificity, as demonstrated in another research.

Colorimetric LAMP is a promising tool for use in resource-limited areas due to its effective turnaround time and low cost. The primer set in this study was able to detect the S450W *rpoB* mutation in less than 40 minutes. The estimated time from sputum sample preparation to colorimetric LAMP visualization is less than 2 hours. Based on cost estimates for MTB tests in Indonesia, the LAMP colorimetric method costs less than Rp. 200,000 (US$12.32) per test. This is significantly more affordable than other screening methods, such as GeneXpert, which costs around Rp. 850,000 (US$52.41), or the gold-standard DST-MGIT culture test, which costs around Rp. 900,000 (US$55.50) per reaction. A study by Sohn et al. (2019) in Vietnam also confirmed the lower cost of TB-LAMP (US$14.17) compared to GeneXpert (US$40.62) in a medium-volume laboratory.

The affordability of the LAMP method makes it a promising screening tool, especially in countries like Indonesia with a high incidence of tuberculosis. Because it is more cost-effective than other methods, such as GeneXpert and DST-MGIT culture, LAMP can be used to screen a higher volume of samples in a single run. The real-time LAMP assay developed in this study demonstrated a superior limit of detection (LOD) of approximately 2.8×10^{-4} ng/µL (581.4 copies/test). This is significantly more sensitive than our colorimetric LAMP assay (2.8×10^{-2} ng/µL, or 58,140 copies/test) and other reported methods like the one from Kim et al. (2023) 1,038,270 copies/test. Although real-time platforms require more sophisticated instrumentation, their enhanced sensitivity provides a more robust detection capability, which is crucial for rapid point-of-care screening to guide initial treatment, early diagnosis and for detecting low bacterial loads.

The limitations of this study are twofold: first, the sample size was restricted to four double-validated clinical isolates (n=4), serving only for technical proof-of-concept; and second, the assay's specificity is strictly limited to the *rpoB* S450W mutation, meaning it cannot detect resistance conferred by mutations located outside the Rifampicin Resistance Determining Region (RRDR).

As this is an early development of the S450W biomarker, further in-depth exploration through a clinical trial is warranted. A deeper analysis should specifically focus on validating the primer set's sensitivity and specificity using a larger sample size. Additionally, cross-testing against non-tuberculosis bacteria is crucial to definitively confirm the primer set's ability to differentiate between *Mycobacterium tuberculosis* and other bacterial species.

4. Conclusion

This study successfully developed a specific, fast, and sensitive real-time LAMP assay for detecting S450W point mutations in the *Mycobacterium tuberculosis rpoB* gene. We have incorporated a colorimetric assay that exhibits high sensitivity (a low Limit of Detection - LOD). This method is advantageous for practical applications due to its rapidity (results in <40 minutes), cost-effectiveness, and independence from complex laboratory equipment. The assay also showed a strong ability to distinguish S450W mutations from both the wild-type and S450L mutations on the same codon using a cut-off time of 35 minutes. Overall, the LAMP assay developed in this study has great potential as an affordable and efficient molecular diagnostic tool for the rapid detection of rifampicin resistance, which can significantly support TB control efforts in Indonesia.

Conflict of Interest

The authors declare no conflict of interests.

Author Contribution

Dian R. Setyorini contributed to the data collection, analysis, and manuscript writing. Selin F. Satiya and Jessie S. Liusarany contributed to sample collection and sample preparation. Irvan Faizal, Kiki Kurniawan, and Tri Wibawa conducted major supervision, material preparation, and study design. Review and corrections for the previous version of the manuscript conducted by Irvan Faizal and Tri Wibawa. All authors read and approved the final manuscript.

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