RESEARCH ARTICLE

Rapid Molecular Detection of Multidrug-Resistant Tuberculosis by PCR-Nucleic Acid Lateral Flow Immunoassay

Hatairat Kamphee1, Angkana Chaiprasert2, Therdsk Prammananan3, Natpapas Wiriyachaiporn4, Airin Kanchanatavee1, Tararaj Dharakul1,4*

1 Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, 2 Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, 3 National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand Science Park, Pathumthani, Thailand, 4 National Nanotechnology Center, National Science and Technology Development Agency, Thailand Science Park, Pathumthani, Thailand

* tararaj.dha@mahidol.ac.th

Abstract

Several existing molecular tests for multidrug-resistant tuberculosis (MDR-TB) are limited by complexity and cost, hindering their widespread application. The objective of this proof of concept study was to develop a simple Nucleic Acid Lateral Flow (NALF) immunoassay as a potential diagnostic alternative, to complement conventional PCR, for the rapid molecular detection of MDR-TB. The NALF device was designed using antibodies for the indirect detection of labeled PCR amplification products. Multiplex PCR was optimized to permit the simultaneous detection of the drug resistant determining mutations in the 81-bp hot spot region of the \( rpoB \) gene (rifampicin resistance), while semi-nested PCR was optimized for the S315T mutation detection in the \( katG \) gene (isoniazid resistance). The amplification process additionally targeted a conserved region of the genes as \( Mycobacterium tuberculosis \) (Mtb) DNA control. The optimized conditions were validated with the H37Rv wild-type (WT) Mtb isolate and Mtb isolates with known mutations (MT) within the \( rpoB \) and \( katG \) genes. Results indicate the correct identification of WT (drug susceptible) and MT (drug resistant) Mtb isolates, with the least limit of detection (LOD) being \( 10^4 \) genomic copies per PCR reaction. NALF is a simple, rapid and low-cost device suitable for low resource settings where conventional PCR is already employed on a regular basis. Moreover, the use of antibody-based NALF to target primer-labels, without the requirement for DNA hybridization, renders the device generic, which could easily be adapted for the molecular diagnosis of other infectious and non-infectious diseases requiring nucleic acid detection.

Introduction

Multidrug-resistant tuberculosis (MDR-TB) is defined by the resistance of \( Mycobacterium tuberculosis \) (Mtb) to at least the two most potent antimicrobials against TB infection,
rifampicin (RIF) and isoniazid (INH) [1]. According to the WHO drug resistant TB surveillance report of 2014, MDR-TB occurred in 3.5% of new TB cases and 20.5% in previously diagnosed TB cases, with the incidence of MDR-TB estimated to be 5% of the overall TB cases on a global scale [2]. Every year, at least half a million new cases continue to emerge, adding to the existing MDR-TB burden [2]. The traditional culture based drug susceptibility testing (DST) remains the primary diagnostic platform in most developing countries. The consequent diagnostic time-delay is a major cause of escalating incidence. The key to preventing further spread is early detection and treatment.

A range of molecular diagnostic methods have been introduced into developing countries through the endorsement of the WHO [3,4]; however, several limitations hamper their popularity. The foremost drawback to molecular tests such as real-time PCR is the associated expense. Even though technologies like Xpert MTB/RIF (Cepheid, USA), a real-time PCR based detection system first endorsed by the WHO in 2010 [3], is sensitive enough to detect MDR-TB in HIV infected patients [5–7], the widespread use is unaffordable. Other molecular tests are largely PCR based endpoint detection systems such as INNO-LiPA Rif (Innogenetics, Belgium) and GenotypeMTBDRplus (Hains Lifesciences, Germany) that are DNA based strip tests. The test strips are lined with a wide array of mutation specific detection probes [8], which complicates result presentation. This strip design feature may be suitable for epidemiological surveys, but potentially impedes their practical use in routine diagnostics.

The primary objective of this proof of concept study was to develop a molecular diagnostic alternative for MDR-TB, targeting low-resource and peripheral healthcare settings that already routinely perform nucleic acid amplification. The aim was to create a highly simple, rapid, and easy-to-use detection tool, and to optimize its compatibility with conventional thermocycling technology. This limits the requirement for additional expenditure on instruments. The detection device developed is a one-step antibody-based Nucleic Acid Lateral Flow (NALF) immunoassay designed for the selective detection of specifically labeled nucleic acid within a PCR amplicon mixture.

The target Mtb genes for the PCR-NALF test in this study are \( rpoB \), with mutations conferring RIF resistance [9,10], and \( katG \), with mutations conferring INH resistance [11]. \( rpoB \) and \( katG \) mutation detection has been divided into two separate assays. Multiplex PCR was optimized for the \( rpoB \) assay, allowing for a simultaneous detection of multiple RIF resistance determining codons (531, 526 and 516) within the \( rpoB \) gene. Site- and mutation-specific primers for \( rpoB \) were designed and combined into one single assay. At any one time, only the primer specific to the mutation type binds to the target, from the multitude of primers, to register RIF resistance. This design strategy is practical because a simultaneous occurrence of more than one drug resistance conferring mutation in a single gene is uncommon. For the \( katG \) assay, semi-nested PCR was optimized for the detection of a single mutation (S315T), to register INH resistance. Primers were designed and evaluated in this study for their performance in identifying mutant (MT) templates, corresponding to drug resistant Mtb isolates, and the H37Rv wild-type (WT) template, corresponding drug susceptible Mtb isolate. All synthesized primers were labeled with specific tags for a rapid and easy detection by NALF antibodies. Both \( rpoB \) and \( katG \) assays follow the same test protocols, including the same thermocycling conditions. All NALF results were compared with the results of agarose gel electrophoresis for laboratory evaluation.

**Materials and Methods**

**Template Preparation**

Genomic DNA extracts from Mtb isolates (Table 1) were obtained from the Drug Resistant Tuberculosis Fund Laboratory, Department of Microbiology, Faculty of Medicine Siriraj
Hospital, Mahidol University, Thailand. Outer primers were designed, using NCBI Primer-BLAST [12], to obtain specific regions of the target genes; RpoB-OF (5'-CGCTGTGGGAAAACCTGTTC-3'), RpoB-OR (5'-CTCCAGGAAGGAATCATCG-3'), and KatG-OF (5'-GGCGGACCTGATTGTTTCG-3'), KatG-OR (5'-GAGACAGTCAATCCCGATGC-3'). The amplified products of the target genes were ligated with pGEM T-Easy Vector (Promega, USA) (Table 1) and incubated overnight at 4°C, as per the protocol provided by the manufacturer. Competent *E. coli* DH5-α cells were transformed with the ligated plasmids and then grown on LB agar culture plate containing ampicillin, IPTG, and X-gal at 37°C overnight (16–18 hrs) for blue/white selection. The white colonies were picked and inoculated in LB broth with ampicillin and incubated overnight (12–16 hrs) at 37°C, with vigorous shaking (250 rpm). The overnight grown bacterial culture was then used for extracting the plasmid DNA (pDNA), using a plasmid extraction mini-kit (Favorgen, Taiwan). The presence of inserted genes was confirmed using restriction enzyme EcoR1-HF and by DNA sequencing (First Base, Singapore). Stocks of plasmids in DH5-α *E. coli* cells were made in 50% glycerol and stored at -80°C. The stored *E. coli* cells were regrown in LB broth (ampicillin) and pDNA extracted for use (mini-kit, Favorgen). The concentrations of the extracted pDNA were measured using a spectrophotometer (NanoDrop 8000, Thermo Scientific, USA).

**PCR Amplification**

Amplification of the target genes, *rpoB* (locus Rv0667) and *katG* (locus Rv1908c) [13,14], were performed in two separate assays using labeled primers (Table 2). For the *rpoB* assay, RpoB-IF' and RpoB-IR' (Table 2) were designed to amplify a conserved region within the *rpoB* gene as Mtb DNA control. A set of *rpoB* mutant primers (forward) (Table 2) [15], together with RpoB-IR', were also designed for specific mutation detection to determine Mtb RIF resistance in the *rpoB* assay. For the *katG* assay, KatG-IF' and KatG-IR' (Table 2) were designed for the amplification of a conserved region within the *katG* gene as Mtb DNA control, together with 315T-F' (*katG* mutant forward) and KatG-IR' (Table 2), designed for the S315T mutation detection to determine Mtb INH resistance in the *katG* assay (Table 2). RpoB-IF' and RpoB-IR', as well as KatG-IF' and KatG-IR' have also been designed to recognize a conserved region within the *rpoB* and *katG* genes, within the Mycobacterium tuberculosis complex.

| Table 1. Bacteria and plasmid; source and function in template preparation. |
|-----------------------------|--------------|---------------|----------------|-------------------|--------------------|
| Material                    | Type         | Gene          | Codon          | Mutation type     | Mtb Strain          | Source/Strain      | Function          |
| Mtb genomic DNA extracts with known mutations | bacteria | *rpoB* | 516 | (D) Asp to Val (V) | DS 10216, DS 3315, DS 6279, DS 4230 | clinical isolates | for target gene amplification |
| Mtb genomic DNA extracts with known mutations | bacteria | *rpoB* | 526 | (H) His to Tyr (Y), (H) His to Arg (R), (H) His to Asp (D), (H) His to Leu (L) | DS 6308, DS 8417, DS 5904, DS 0502, DS 4224, DS 9442, DS 6646 | clinical isolates | for target gene amplification |
| Mtb genomic DNA extracts with known mutations | bacteria | *rpoB* | 531 | (S) Ser to Leu (L) | DS 9469, DS 6354, DS 6088, DS 6000 | clinical isolates | for target gene amplification |
| Mtb genomic DNA extracts with known mutations | bacteria | *katG* | 315 | (S) Ser to Thr (T) | DS 12791, DS 11964, DS 10477, DS 12168 | clinical isolates | for target gene amplification |
| Mtb reference strain | bacteria | - | - | - | - | - | H37Rv | experimental control |
| pGEM T-Easy Vector | plasmid DNA | - | - | - | - | - | commercial target gene cloning |
| *E. coli* | bacteria | genome | - | - | - | - | DH5-α | host cell |

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All primers were designed using NCBI Primer-BLAST [12]. For the rpoB assay, individual forward primers targeting rpoB codons 531, 526 and 516 were first experimented separately to evaluate their efficacy and stringency. The individual rpoB primers were then combined into one single assay for multiplex PCR, for the simultaneous detection of all three codons. For the katG assay, a single forward primer (315T-F') targeting the S315T mutation was combined with KatG-IF' and KatG-IR' for semi-nested PCR. All tests were initially performed using pDNA, with a concentration of 10^8 copies (1 ng) added per PCR reaction. The experimental conditions were optimized for primer concentrations and amplification parameters. The optimized conditions were validated with Mtb isolates with known mutations; for the rpoB gene, 4 isolates for codon 516, 6 for codon 526, and 4 for codon 531 were used for the validation of the rpoB assay, and for the katG gene, 4 isolates with S315T mutation were used for the validation of the katG assay (Table 1). The rpoB and the katG assays were also validated with the H37Rv WT Mtb isolate. For each PCR reaction, 10^5 DNA copies (1 ng) of genomic DNA extracts from Mtb isolates were added. PCR was performed in 25 μl reaction mixtures containing commercial 10x PCR buffer (composition of 1x buffer; 7.5 mM Tris-HCl (pH 8.75), 25 mM KCl, 1 mM MgCl2), 100 μM dNTP mix, 1 U Taq DNA Polymerase (Gen- eaid, Taiwan), and 1 ng purified pDNA/ 1 ng Mtb genomic DNA as amplification template. RpoB and katG assays were subjected to the same thermocycling conditions; initial denaturation at 95°C for 5 min, followed by 5 cycles of 94°C for 1 min and 72°C for 1 min, then 30 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min (T-Professional Thermocycler, Biometra, Germany). For each experiment, the negative

| Primer                  | Label-Sequence     | Conc. (μM) | Product Length (bp) |
|-------------------------|--------------------|------------|---------------------|
| **katG primers**        |                    |            |                     |
| KatG-IF'                | DIG-AGCGGTAAGC GGAATCTGGAGAA | 0.25       | 630                 |
| KatG-IR'                | Biotin-CATGCTCTGGATGGATGCTTGA | 0.25       | 335                 |
| 315T-F'                 | FITC-GTAAGGACGCGATCACCAc | 0.1        |                     |
| **rpoB primers**        |                    |            |                     |
| RpoB-IF'                | DIG-GGAGGCCGATCACACCGAGACGT | 0.1        | 314                 |
| RpoB-IR'                | Biotin-TTTCGATGAACCCGAACG | 1          |                     |
| **Single mutation (SM) primers** |                |            |                     |
| SM-516V-F'              | FITC-CTGA GCAA TCCATGgt | 1          | 235                 |
| SM-526Y-F'              | FITC-GTCGGGGTTGACct | 1          | 203                 |
| SM-526D-F'              | FITC-GTCGGGGTTGACcg | 1          | 203                 |
| SM-526R-F'              | FITC-GTCGGGGTTGACcc | 1          | 203                 |
| SM-526L-F'              | FITC-GTCGGGGTTGACct | 1          | 203                 |
| SM-531L-F'              | FITC-ACAAGGCCGACTGgt | 1          | 189                 |
| **Double mutation (DM) primers** |            |            |                     |
| DM-516V-F'              | FITC-CTGA GCAA TCCATGgt | 1          | 235                 |
| DM-526Y-F'              | FITC-GTCGGGGTTGACatA | 1          | 203                 |
| DM-526D-F'              | FITC-GTCGGGGTTGACagA | 1          | 203                 |
| DM-526R-F'              | FITC-GTCGGGGTTGACacg | 1          | 203                 |
| DM-526L-F'              | FITC-GTCGGGGTTGACct | 1          | 203                 |
| DM-531L-F'              | FITC-ACAAGGCCGACTaTt | 1          | 189                 |

Reference

\[15\].

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(MTBC). All primers were designed using NCBI Primer-BLAST [12]. For the rpoB assay, individual forward primers targeting rpoB codons 531, 526 and 516 were first experimented separately to evaluate their efficacy and stringency. The individual rpoB primers were then combined into one single assay for multiplex PCR, for the simultaneous detection of all three codons. For the katG assay, a single forward primer (315T-F') targeting the S315T mutation was combined with KatG-IF' and KatG-IR' for semi-nested PCR. All tests were initially performed using pDNA, with a concentration of 10^8 copies (1 ng) added per PCR reaction. The experimental conditions were optimized for primer concentrations and amplification parameters. The optimized conditions were validated with Mtb isolates with known mutations; for the rpoB gene, 4 isolates for codon 516, 6 for codon 526, and 4 for codon 531 were used for the validation of the rpoB assay, and for the katG gene, 4 isolates with S315T mutation were used for the validation of the katG assay (Table 1). The rpoB and the katG assays were also validated with the H37Rv WT Mtb isolate. For each PCR reaction, 10^5 DNA copies (1 ng) of genomic DNA extracts from Mtb isolates were added. PCR was performed in 25 μl reaction mixtures containing commercial 10x PCR buffer (composition of 1x buffer; 7.5 mM Tris-HCl (pH 8.75), 25 mM KCl, 1 mM MgCl2), 100 μM dNTP mix, 1 U Taq DNA Polymerase (Gen- eaid, Taiwan), and 1 ng purified pDNA/ 1 ng Mtb genomic DNA as amplification template. RpoB and katG assays were subjected to the same thermocycling conditions; initial denaturation at 95°C for 5 min, followed by 5 cycles of 94°C for 1 min and 72°C for 1 min, then 30 cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min (T-Professional Thermocycler, Biometra, Germany). For each experiment, the negative
control (-C), which consisted of PCR reagents without the addition of templates (NO TEM-
PLATE), were included to test for possible background appearance or false positives.

For the \textit{rpoB} assay, the FITC-labeled \textit{rpoB} forward primers, together with the biotin-labeled RpoB-IR' (Table 2), generate PCR products of 189 bp (codon 531), 203 bp (codon 526) and 235 bp (codon 516) in size (Fig 1). The DIG-labeled RpoB-IF' (Table 2) and the biotin-labeled RpoB-IR', generate PCR product of 314 bp in size (Fig 1). For the \textit{katG} assay, the FITC-labeled 315T-F' and the biotin-labeled KatG-IR' (Table 2), generate PCR product of 335 bp (codon 315) in size (Fig 1). The DIG-labeled KatG-IF' (Table 2), together with the biotin-labeled Kat-
G-IR', generate PCR product of 630 bp in size (Fig 1). With the use of the labeled primers, the PCR amplification products are rendered as dual labeled amplicons (either biotin-FITC, or bio-
tin-DIG, on either end of the amplicon). After PCR amplification, 10 μl of PCR product were used for NALF detection. For analytical comparison of the results, 5 μl of PCR product were also used to run gel electrophoresis, and were visualized using 3% agarose gel (SeaKem, Lonza Inc., USA) infused with Gel-Red dye (Biotium, USA).

\textbf{Nucleic Acid Lateral Flow (NALF) Development}

The NALF device has been designed for the indirect detection of amplified PCR products through the use of antibodies against primer-tags; FITC, DIG, and biotin (Fig 2). Monoclonal antibodies (mAbs) against each tag molecule were produced in-house using the mouse hybrid-
oma technology, with specific recognition to FITC, DIG, and biotin (data not shown).

The NALF device consists of sample application pad, conjugate pad, nitrocellulose mem-
brane and adsorption pad, which are assembled together in a plastic housing. NALF detection was performed individually for the \textit{rpoB} and \textit{katG} assays, using separate NALF devices for each assay. Following PCR, 10 μl of PCR product, either from the \textit{rpoB} or the \textit{katG} amplifica-
tion assay, were mixed with 90 μl of NALF buffer solution and added to the NALF device at the sample pad. The mixture was then dispensed onto the conjugate pad, which hosts gold-nano-
particles (AuNPs) conjugated with anti-biotin mAbs (AuNP-anti-biotin) to detect dual labeled amplicons by binding with biotin (Fig 2). The complex flows along the nitrocellulose mem-
brane towards the test-lines, T1 and T2.
The first test-line (T1) is composed of anti-FITC mAb for capturing biotin-FITC-labeled amplicons to indicate RIF resistance in the \textit{rpoB} assay or INH resistance in the \textit{katG} assay (Fig 3); for \textit{rpoB}, the appearance of T1 corresponds to PCR product sizes 189 bp, 203 bp or 235 bp on agarose gel, and for \textit{katG}, to 335 bp on agarose gel (Fig 1). The second test-line (T2) hosts anti-DIG mAb to capture biotin-DIG-labeled amplicons for Mtb DNA control, which must appear in all cases for the results to be valid (Fig 3); for \textit{rpoB}, the appearance of T2 correlates with 314 bp on agarose gel, and for \textit{katG}, to 630 bp on agarose gel (Fig 1). The excess AuNP-anti-biotin is captured at the control line (C) by anti-mouse IgG. NALF results were read after 10 minutes of PCR product addition.

**Least Limit of Detection (LOD) Determination**

The least limit of detection (LOD) is defined by the minimum number of copies of target genes required in each PCR reaction for a successful detection of the PCR products. The least limit of detection was determined using purified genomic DNA extracts from the H37Rv Mtb isolate, as well as Mtb isolates with known mutations (Table 1) as PCR amplification templates. To determine the LOD of the \textit{rpoB} and \textit{katG} assays, serial dilution of the templates was performed as follows; 10 ng (10^6 copies of DNA), 1 ng (10^5 copies of DNA), 0.1 ng (10^4 copies of DNA), 0.01 ng (10^3 copies of DNA), 0.001 ng (10^2 copies of DNA). All NALF results were compared with the results of agarose gel electrophoresis for confirmation.

**Results**

The Selection of Genes and Mutation Sites for MDR-TB Determination

In order to determine MDR-TB, this study focuses on the positive detection of RIF and INH resistance. Based on a broad compilation of MDR-TB global epidemiological statistics (Fig 4),
85–95% of RIF resistance has been found to naturally accompany INH resistance [17–23], making RIF resistance a widely used surrogate marker for MDR-TB; however, there is a 5–15% chance of RIF monoresistance development. 90–95% of RIF resistance confers mutation(s) in the 81-bp hot-spot region (codons 507–533) of the \( rpoB \) gene, called the rifampicin resistance-determining region (RRDR), with the highest global prevalence being at codons 531, 526 and 516. Multiple mutation types have been recorded per codon, with different statistical occurrence [24–28]. On the other hand, 60–80% of INH resistance is due to a single mutation (S315T) in the \( katG \) gene at codon 315 [29–34], with the rest occurring in other genes, such as...
in the promoter region of inhA and in ahpC genes [35–37]. INH resistance, however, is not commonly used as an independent marker for MDR-TB [38–42]. From the compiled data, the target mutation sites selected for MDR-TB determination in this study are rpoB codons 531, 526 and 516 for the rpoB assay in the determination of RIF resistance, with one mutation type of interest for codon 531 (L), four for codon 526 (Y, R, D and L), and one for codon 516 (V) (Table 1), with the highest statistical prevalence worldwide [17,18,23–28,43–47]. As for the katG assay, katG codon 315, with the S315T mutation, has been selected as the single mutation site in the determination of INH resistance [17–21,29–36,48–52].

**KatG PCR Amplification and Detection by NALF**

The determination of INH resistance in this study targets a single mutation (S315T) in the katG gene. Because the target mutation is singular, the use of just one mutation determining primer, 315T-F (Table 2), in the katG assay should be adequate. The functionality of katG primers and the ability to identify WT and MT templates were evaluated with the H37Rv Mtb isolate, along with four Mtb isolates containing the S315T mutation, via semi-nested PCR. The amplicons were detected using the NALF device and confirmed with agarose gel electrophoresis. The NALF results and the corresponding agarose gel electrophoresis results for all four Mtb isolates indicate the correct identification of MT and WT templates. Fig 5 is a representation of the results.

**Primer Design for the rpoB Assay**

RpoB, unlike katG, carries multiple mutation sites. To simultaneously detect all the target sites in one PCR reaction, the incorporation of several site- and mutation-specific primers into a single assay is required. The standard primer design of a single base mismatch between primer and non-target template, especially for codon 526 with four different target mutation types (Table 1), may not be enough to ensure correct template distinction. Therefore, the Yaku-Bonczyk primer design method, entailing an intentionally incorporated additional mismatch, has been explored in the study [15,53]. The added mismatch is expected to enhance specificity, leading to a better discrimination against non-complementary DNA. For the eventual selection of rpoB primers, for mutation detection within the rpoB assay, two primer sets were designed.
and assessed against each other; SM (single mutation) primers with the standard design of a single base mismatch at the 3'-terminus to complement MT templates, and DM (double mutation) primers with an additional strategically located mismatch at the third position from the 3'-terminus in accordance with the Yaku-Bonczyk method (Fig 6, Table 2).

**Mutant Primer Selection for the rpoB Assay**

The SM and DM primers were tested with MT and WT templates in semi-nested PCR. Each primer was tested in a separate assay composed of RpoB-IF', RpoB-IR', and the target primer of interest for mutation detection (Table 2). The test results (Fig 7) show that SM primers were able to anneal to their specific targets, and were able to correctly identify the MT and WT templates with high efficacy. The appearance of an unaccountable non-specific band of approximately 270 bp on agarose gel in Fig 7 did not lead to a false positive/negative or background on NALF, and therefore, did not interfere with the test. DM primers, on the other hand, did not perform as expected. They failed to anneal to their targets, providing negative results (data not shown). DM primers were, therefore, exempted from further experimentation.

**Multiplex PCR for the Simultaneous Detection of RIF Resistance Determining Codons in the rpoB Gene**

SM primers, which have been selected for the rpoB assay, were combined into a single assay for multiplex PCR, which would allow all RIF resistance determining mutations at target codons 531, 526 and 516 to be detected simultaneously. Initially, the combined assay was tested using pDNA for condition optimization (data not shown). The optimized conditions were then validated and tested for reproducibility using the H37Rv (WT) Mtb isolate and Mtb isolates with known mutations (4 isolates for codon 516, 6 for codon 526, and 4 for codon 531) (Table 1). Fig 8 is a representation of the results, which reaffirms the efficacy, stringency and the specificity of the SM primers in the rpoB assay.

**Fig 6. RpoB primer design strategy.** RpoB primer designs for codon 526 (mutations Y and R) have been demonstrated above as examples. SM (single mutation) primers are designed with a single base mismatch at the 3'-terminus to complement MT templates. DM (double mutation) primers, which have also been designed to complement MT templates, carry an added mismatch at the third position from the 3'-terminus. The red colored bases indicate an intentional mismatch and the blue colored bases indicate a complementary base to the SM and DM primers.

![Diagram of RpoB primer design strategy](https://example.com/rpoB_primer_design.png)

**Table 1.** Summary of Mtb isolates.

| Codon | WT | Known Mutations |
|-------|----|-----------------|
| 516   |     | Y               |
| 526   |     | R               |
| 531   |     | Y, R            |

**Fig 7.** Detection of MDR-TB by PCR-NALF.

![Detection of MDR-TB by PCR-NALF](https://example.com/pcr_nalf.jpg)

**Fig 8.** Representation of multiplex PCR results for the simultaneous detection of RIF resistance determining codons in the rpoB gene.

![Representation of multiplex PCR results](https://example.com/multiplex_pcr.jpg)

**Table 2.** Primer and template sequences.

| Primer/Template | Sequence |
|-----------------|----------|
| SM-526Y-F'      | 5'-FITC- GTC GGG GTT GAC C T -3' |
| SM-526R-F'      | 5'-FITC- GTC GGG GTT GAC A G A -A-3' |
| DNA template (MT)| 3'-CAG CCC CAA CTG G A T -5' |
| DNA template (WT)| 3'-CAG CCC CAA CTG G G T -5' |

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Least Limit of Detection (LOD) Determination

The least limit of detection (LOD), which has been described in the study as the minimum number of copies of target genes required in each PCR reaction for a successful detection of the PCR products, was determined for both the \textit{rpoB} (using SM primers) and the \textit{katG} assays. The results indicate the LOD to be 10^4 copies of DNA (0.1 ng) per PCR reaction via NALF detection, and 10^5 copies of DNA (1 ng) per PCR reaction via detection by gel electrophoresis (Fig 9). The NALF test-lines illustrate a clear loss of intensity as the concentration of template DNA in the reactions diminished. Below the concentration of 10^4 copies of DNA, the NALF test-lines were no longer visible. A lower LOD determined for NALF, compared to agarose gel electrophoresis, suggests a higher sensitivity of the device.

Discussion

NALF technology is an interesting tool for enabling simplification of molecular diagnosis. Recent studies have successfully field-tested NALF for point-of-care molecular detection of malaria with promising results [54–56]. Preliminary tests have also been conducted for a lateral
flow based quantitative detection of amplified HIV-RNA with clinical relevancy [57]. This proof of concept study, using the above principle, attempted to develop a molecular diagnostic alternative for MDR-TB to complement the use of conventional PCR, targeting low-resource and peripheral healthcare settings that already routinely employ PCR machines. The development of NALF, to meet the objective, was fixated upon simplicity and rapidity. These qualities were achieved through the strategic design to minimize operator involvement, and to present the results in the most non-complex manner. The approach uses gold nanoparticle (AuNP) conjugated with anti-biotin antibodies to detect dual labeled amplicons, and anti-FITC/DIG antibodies to capture and immobilize the amplicons on the test-lines for visual indication (Fig 2). This technique eliminates the requirement for operator intervention. Moreover, the simplicity is also projected through the concise display of NALF results, using only two test lines; T1 for RIF resistance indication in the rpoB assay or INH resistance indication in the katG assay, and T2 for Mtb DNA control (Fig 2).

This NALF design contrasts other commercially existing DNA strip tests for MDR-TB such as GenotypeMTBDR plus which has 27 reaction zones on a single test strip, with 21 DNA probes for mutation detection and 6 probes as control [8]. All 27 lines representing rpoB, katG and inhA are displayed on the same test strip and have to be individually interpreted by the operator. The detailed and complex nature of the results renders it more suitable for epidemiological rather than diagnostic work. In comparison, the NALF device has no technical specification or interference requirements, making it more appropriate for diagnostics.

Another important advantage of using anti-tag antibodies on NALF rather than DNA probes is that it permits the indirect capture of target amplicons without necessitating DNA hybridization. This advantage makes the device generic, allowing broader application. The device can, thus, be adapted for the diagnosis of any disease requiring nucleic acid detection, based on the strict use of primers conjugated with specific tags; biotin for detection and FITC/DIG for capture (Fig 2). This generic nature allows the device to be mass produced at low cost, serving the objective of targeting low-resource settings. This design differs from the lateral flow designs adopted in previous studies in the determination of HIV and malaria infection where sequence specific oligonucleotide probes were used to capture HIV-RNA and P. falciparum-DNA [54–57].
As for primer design, we initially reasoned that the standard design of incorporating a single 3’-terminal mismatch to avoid primer hybridization with non-complementary DNA may be inadequate for the determination of single-nucleotide polymorphisms (SNPs). For this study, incorrect hybridization could lead to the misidentification of Mtb drug resistance, and therefore, MDR-TB. To minimize the possibility, the study explored the Yaku-Bonczyk primer design strategy entailing an additional intended mismatch to enhance specificity [15,53]. The study compared the functionality of the SM (single mutation) and DM (double mutation) primers (Table 2, Fig 7) to evaluate their performance in the selection for the rpoB assay. The criterion was based on specificity, efficacy, stringency and also their technical influence on the design of the NALF device. DM primers, with an additional strategic mismatch (Fig 6), were predicted to perform with a higher discrimination effect based on their success in other studies [15,53,58]. The results, however, defeated the expectations wherein the DM primers failed to anneal to their targets, providing false negative results. In this study, the DM primers were 15–17 bases in length. Comparatively, the primers employed in a previous study, used as guidance in the design of DM primers, is about 38 bases in length [15]. This distinction could have been the root cause of the second mismatch considerably weakening the annealing bond between...

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the DM primers and their complementary MT template, resulting in complete non-binding [59]. SM primers, on the other hand, with the standard design of a 3’-terminal base complementing the MT templates (Fig 6), selectively annealed to their targets, resulting in a successful identification of the drug resistant (MT) and the drug susceptible (WT) Mtb isolates (Fig 7). The rpoB SM primers were, therefore, selected for use in multiplex PCR.

From the broad compilation of the global MDR-TB epidemiological statistics (Fig 4), 85–95% of RIF resistance was found to be accompanied by INH resistance; therefore, RIF resistance is popularly used by several existing commercial tests, such as XpertMTB/RIF and INNO-LiPA, as the surrogate marker for MDR-TB [43,46,60]. However, many studies point towards the benefit of testing for both RIF and INH resistance to improve the comprehensiveness of the results [38,61–63]. Retrospective studies have claimed that RIF monoresistance is on the rise in South-Africa, and testing for RIF resistance alone has led to the misidentification of MDR-TB [61,64]. Additionally, the incorporation of an INH assay would also support the determination of INH monoresistance. A recent study in China showed that early diagnosis of INH monoresistance allowed for the tailoring and implementation of specific therapies to prevent the development of MDR-TB [52]. The results of the study showed positive treatment outcomes for both patients diagnosed early with INH monoresistance as well as the drug-susceptible TB patients. A similar study conducted in the United States in 2009 also presented with comparable results [49]. INH resistance, however, is characterized by mutations in several genes (Fig 4) and aiming to detect all these genes would undesirably increase the complexity of the PCR-NALF test, based on the current design. This study, therefore, selected to focus only on the S315T mutation detection of the katG gene, an INH resistance conferring mutation with the highest global prevalence [29–34].

The current PCR-NALF test also presents an additional benefit of choice by dividing rpoB and katG into two separate assays. The option offers convenience. This design contrasts the test feature of GenotypeMTBDRplus where multiple probes for rpoB, katG and inhA are all pre-lined on one single test strip [8]. RpoB and katG assays in this study also share the same PCR thermocycling conditions, permitting the PCR amplification of both assays at the same time, if the user chooses to. The same test design could also be adapted for the determination of resistance to second-line drugs for the indication of extensively drug-resistant tuberculosis (XDR-TB), such as for the detection of fluoroquinolone(s) resistance, which is caused by single base mutations in the gyrA and gyrB genes [65,66]. The addition would add to the choice, as well as the coverage of the test.

Another important factor defining the validity of a molecular test is the least limit of detection (LOD), which signifies the sensitivity of the test. The LOD of the current test, for both the rpoB and katG assays, was found to be $10^4$ genomic copies per PCR reaction (0.1 ng of DNA) (Fig 9) using NALF. Even though the least LOD is relatively high, suggesting the need for further optimization, the current result of $10^4$ genomic copies could potentially be considered within the clinically relevant range. Sputum samples from TB patients with positive AFB staining of 1+ and 2+, based on the standard manual for laboratory technicians, is estimated to contain approximately $1-3 \times 10^4$ and $3-5 \times 10^5$ Mtb bacilli per ml of sputum, respectively [67]. Moreover, further studies using sputum samples are underway to help define the sensitivity and specificity of the test.

In spite of the promising results of the study, several aspects could still be improved. The LOD, which reflects the analytical sensitivity of the PCR-NALF test, could further be reduced through additional optimization, to enable a feasible detection of lower DNA concentrations. The extent of rpoB mutation coverage in the study could also be widened by incorporating the detection of more codons such as codons 533, 522, 513 and 511 within the rifampicin-resistance determining region [27,28,44,47]. In addition, further incorporation of inhA and ahpC...
genes, which account for 20–40% of INH resistance (Fig 4), could also improve the overall comprehensiveness of the test results.

In conclusion, this proof of concept study demonstrates the potential use of PCR-NALF as a molecular diagnostic alternative for the detection of MDR-TB. The simplicity, rapidity and ease-of-use could prove beneficial for low-resource settings that already employ PCR machines. Furthermore, the generic nature of the NALF device provides great diagnostic potential in adapting the test for the detection of other infectious and non-infectious diseases that require nucleic acid identification.

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Author Contributions
Conceived and designed the experiments: HK TD AC TP. Performed the experiments: HK TD AK. Analyzed the data: HK TD AC TP NW AK. Contributed reagents/materials/analysis tools: TD AC TP. Wrote the paper: HK TD AC TP.

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