

Development and application of loop-mediated isothermal amplification label-based nanoparticles lateral flow biosensor for detection of *Mycobacterium tuberculosis*

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Abstract

Tuberculosis is a serious disease with high morbidity and mortality, thus rapid and cost-effective diagnostic test for *Mycobacterium tuberculosis* (MTB) is urgently needed. Here, a novel detection diagnostic technique, termed as loop-mediated isothermal amplification label-based nanoparticles with lateral flow biosensor (LAMP-LFB), was developed and evaluated for rapid, reliable and objective detection of MTB. Two sets of primers, which targeted IS 6110 and IS 1081 sequences of MTB, were simultaneously designed for establishment of LAMP-LFB assay. The optimal reaction conditions of MTB-LAMP-LFB assay confirmed were 66°C for only 50min. The analytical sensitivity of MTB-LAMP-LFB is 10fg of genomic templates in pure culture, and the detection results obtained from LFB was in conformity with agarose gel electrophoresis. No cross-reactivity with other common bacteria and non-tuberculous mycobacteria strains (NTM) was obtained. A total of 158 clinical samples were collected from presumptive 158 TB patients, were used for evaluating the feasibility of MTB-LAMP-LFB assay. Among 98 TB patients diagnosed with composite reference standard, the positive rate for MTB detection using liquid culture, Xpert MTB/RIF and LAMP-LFB were 40.0% (39/98), 50.0% (48/98), and 86.7% (85/98), respectively. Among 39 culture confirmed samples, 84.6% (33/39) cases were Xpert MTB/RIF-positive and 92.3% (36/39) were LAMP-LFB-positive. For the 59 clinically diagnosed TB cases 25.4% (15/59) and 83.0% (49/59) were Xpert MTB/RIF-positive and LAMP-LFB positive, respectively. Therefore, MTB-LAMP-LFB assay is a simple, reliable, and sensitive method for MTB detection and maybe prospective in early diagnosis of MTB.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), is now the leading cause of death from an infectious disease, which kills the people even more than malaria and HIV worldwide. A major reason for TB causing for so many deaths is the difficulty of rapid and reliable diagnosis, with 40% of estimated incident cases failing to be diagnosed and reported (World-Health-Organization 2018). Rapid and reliable identification of TB is a prerequisite for successful treatment, thus alternative accurate screening and diagnostic techniques are urgently needed for sensitive, effective and specific detection of MTB.

Traditionally, smear microscopy is the routine tool applied for identification of TB in resource-limited endemic regions because the test is simplicity and low cost. However, smear microscopy lacks sensitivity and often requires more than a single visit of a patient to be completed (Steingart et al. 2006). Mycobacterial culture is still the gold standard assay for diagnostic of MTC due to its high sensitivity and specificity, while this method is time-consuming and technically demanding assay, limiting its wider application at reference centers. Particularly, PCR-based techniques (such as conventional PCR, real-time PCR, multiplex PCR et, al) are the attractive and promising methods for detection of MTB (Kabir et al. 2018; Molaudzi and Molepo 2019; Lekhak et al. 2016; Nyaruaba et al. 2019), while expensive laboratory instruments and complex technical skill needed to conduct these tests make PCR-based assays inaccessible in resource-poor areas where the majority of TB cases occur.

Isothermal amplification techniques, such as loop-mediated isothermal amplification (LAMP), eliminated the shortcomings posed by traditional smear microscopy, culture- and PCR-based assays, and has been endorsed by the WHO (World Health Organization) in 2016 (Notomi et al. 2000; World-Health-Organization 2016). Although many LAMP-based assays have been reported for reliable diagnostic of MTB (Yadav et al. 2017; Rakotosamimanana et al. 2019; Shete et al. 2019; Bojang et al. 2016), the TB-LAMP results were determined by agarose gel electrophoresis, colorimetric indicator (SYBR green I or calcein dyes) and real-time turbidity. Particularly, analysis of LAMP result using agarose gel electrophoresis requires an additional process, and easily leads to carryover contamination; recognition of LAMP amplification using colorimetric indicator is potentially subjective, and easily produces ambiguous judgment; detection of the LAMP products using real-time turbidity needs an expensive optical instrument (Zhang et al. 2014). Furthermore, these monitoring techniques did not differentiate specific amplification and non-specific amplification. Most importantly, most of TB-LAMP assays reported only targeted IS6110 gene to design the specific LAMP primer set. Unfortunately, the copy number of the target sequence is absent or lower (even only one copy) in certain proportion of MTB isolates (Kolia-Diafouka et al. 2019; McEvoy et al. 2007; Kim et al. 2010), thus IS6110-LAMP-based assays can lead to false-negative results when they are applied for diagnosing these strains.

In order to overcome these posed by reported LAMP-based methods, we firstly designed a LAMP combined with nanoparticles-based lateral flow biosensor (LFB; LAMP-LFB) for rapid, visible, sensitive and reliable diagnostic of MTB using two target genes (IS6110 and IS1081). In this report, we optimized the novel LAMP assay and evaluated its sensitivity and specificity using pure culture, and also demonstrated its potential clinical application using patients' samples.

Materials And Methods

Reagents and Apparatus

The Isothermal amplification kit, and lateral flow biosensor were purchased from BeiJing-HaiTaiZhengYuan Technology Co., Ltd (Beijing, China). QIAamp DNA Mini Kit was purchased from Qiagen Co., Ltd (Beijing, China). Glass beads based kits (CapitalBio Co.) were bought from BoaoJingxin Biotechnology Co., Ltd (Chengdu, China). The NanoDrop ND-1000 was bought from Thermo Fisher Scientific (China) Co., Ltd (USA). The Loopamp LA-320C Realtime Turbidimeter was purchased from Eiken Chemical Co., Ltd (Tokyo, Japan). Gel Doc TM XR+ Image System was bought from Bio-Rad Co., Ltd (USA). The PCR apparatus was bought from Dongsheng Innovation Biotechnology Co., Ltd (Beijing, China). Bectec MGIT 960 system was purchased from BD Co., Ltd (USA), and GeneXpert Dx System bought from Cepheid Co., Ltd (USA).

Primer design

Two sets of LAMP primers based on the insertion sequence *IS6110* and *IS1081* were designed by Primer Premier 5.0. The specificity of primers was confirmed by sequence alignment analysis using the basic

local alignment search tool (BLAST). More details of primers were shown in **Figure 1** and **Table 1**. Primers used in this study were synthesized by TianyiHuiyuan Biotechnology Co., Ltd (Beijing, China).

Bacterial strains and clinical samples

A total of 45 bacterial strains, including 1 MTB reference strain H37Rv, 1 *Mycobacterium bovis* BCG strain, 12 clinical isolates strains of MTB, 25 non-mycobacteria and 8 NTM strains were used in this study (**Table 2**).

Our study was approved by Ethical committee of Beijing Chest Hospital and Beijing Children's Hospital. Informed consent has been obtained from all adult patients and guardians of all enrolled children. A total of 158 clinical samples were collected from 98 TB patients and 60 non-TB patients. TB patients were enrolled from Beijing Chest Hospital during January 2, 2019 to January 15, 2019, the other 60 were from Beijing Children's Hospital during January 1, 2019 to February 22, 2019. The types of clinical samples of TB patients included sputum, bronchial lavage fluid, and pleural fluid, while in non-TB patients was sputum only. 3ml of clinical samples were collected, 1 ml each for culture, Xpert MTB/RIF assay and DNA extraction.

Patients in this study were divided into 3 groups according to the Diagnosis for Pulmonary Tuberculosis WS288-2017 (National-Health-and-Family-Planning-commission-of-the-People's-Republic-of-China 2017). (1) Definite TB patients: confirmed by bacteriological examinations, such as smear microscopy, culture, molecular biology methods, and pathological examinations. (2) Clinically diagnosed TB patients: diagnosed by radiographic evidence that consistent with TB and at least one of the following conditions: suspected symptoms of TB, positive tuberculin skin test, interferon- γ release test or MTB antibodies, positive histopathology or bronchoscopy. (3) Non-TB patients: symptomatic or laboratory testing not fitting the above definitions and confirmed as infection with virus, mycoplasma, or bacteria.

DNA extraction

Bacteria DNA was extracted with QIAGEN DNA Mini Kit according to the manufacture instructions. For clinical samples, 1 ml of volume were prepared with adding equal volume of 4% NaOH to digest, and the genomic DNA was extracted with glass bead-based kits. Finally, 50 μ l of DNA was obtained and quantified in a NanoDrop ND-1000.

The Standard LAMP Assay

The singlex LAMP reaction for IS6110 or IS1081 was conducted in the 25 μ l amplification mixtures comprised 0.8 μ M each FIP* and FIP primers, 1.6 μ M BIP primers, 0.8 μ M each LF and LB primer, 0.4 μ M each F3 and B3 primers, 12.5 μ l 2 \times reaction mix, 1 μ l (8 U) of Bst DNA polymerase, 1 μ l Biotin-14-dATP (0.4 mM), 1 μ l Biotin-14-dCTP (0.4 mM) and 1 μ l DNA template.

The multiplex LAMP was conducted in a 25 μ l mixture comprised 0.4 μ M each of 6110-FIP*, 6110-FIP, 6110-LF, 1081-FIP*, 1081-FIP, and 1081-LF, 0.8 μ M each of 6110-BIP and 1081-BIP, 0.2 μ M each 6110-F3,

6110-B3, 1081-F3 and 1081-B3 primers, 12.5 µl 2 × reaction mix, 1 µl (8 U) of Bst DNA polymerase, 1 µl Biotin-14-dATP (0.4 mM), 1 µl Biotin-14-dCTP (0.4 mM) and DNA template (1 µl for isolated strains, 5 µl for clinical specimens). The mixtures were heated at 66°C for 1h, then the amplification products were detected by LFB and agarose gel electrophoresis. The LFB was prepared and performed as previous study (Wang et al. 2017). In brief, 0.5 µl LAMP products and 70 µl running buffer were added into the immersion pad, then two red lines could be seen at TL (test line) and CL (control line), respectively, otherwise only one red line at CL. The colorimetric lines were readily visible within 2 min.

Then the optimum reaction temperatures of two sets of LAMP primers were performed. The amplifications were carried out at the temperature from 61°C to 68°C at 1°C intervals for 1h, the results were monitored by real-time Turbidimeter. Genomic DNA of H37Rv (100fg) was used as positive control, *Streptococcus pneumoniae* (1ng) and *Escherichia coli* (1ng) were used as negative control, and double distilled water (DW) as blank control.

Analytical Sensitivity and Specificity of the LAMP-LFB Assay

To evaluate the analytical sensitivity of singlex (IS6110-LAMP-LFB and IS1081-LAMP-LFB) and multiplex LAMP-LFB assays for MTB identification, serially diluted (10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg) genomic DNA extracted from H37Rv were prepared. The detection of the limit (LoD) was confirmed as the last positive dilution. Amplification products were monitored by agarose gel electrophoresis and LFB.

To assess the analytical specificity of multiplex LAMP-LFB assay for MTB detection, reactions were performed under the conditions depicted above with DNA templates extracted from MTB reference strain H37Rv, 10 MTB clinical strains, 1 BCG strain, 25 non-mycobacteria and 8 NTM strains (**Table 2**).

Practical Application of LAMP-LFB assay in clinical specimens

To evaluate the applicability of LAMP-LFB assay in clinical samples, 5 µl of DNA templates extracted from each of the 158 clinical samples were added into the LAMP reaction mixtures. Among 98 TB patients, the results of LAMP-LFB assay for MTB detection were compared with MGIT liquid culture and Xpert MTB/RIF.

Results

Confirmation and Detection of amplification of LAMP assay

In order to validate the availability of two sets of LAMP primers for MTB detection, singlex LAMP and multiplex LAMP reactions were conducted in the absence or presence of H37Rv genomic templates (**Figure 2**). The LAMP results were indicated using agarose gel electrophoresis and LFB. Many different sizes of the bands were seen in positive control using agarose gel electrophoresis, but not in negative and blank controls. As shown in LFB, there were two red lines TL and CL in positive tubes and only one red line (CL) in negative and blank controls. The results demonstrated that the two LAMP primers were able to specifically identify MTB synchronously by targeting IS6110 and IS1081.

The optimal reaction temperature of LAMP assay

To assess the optimal reaction temperature of LAMP assays, IS6110-LAMP and IS1081-LAMP were carried out at temperatures from 61°C-68°C with an interval of 1°C. According to the real-time turbidimeter (**Figure 3**), the optimum amplification temperature ranged from 66°C-67°C for the IS6110-LAMP, and 65°C-68°C for IS1081-LAMP, respectively. Given this, amplification temperature of 66°C was chosen as the reaction temperature in subsequent LAMP-based experiments.

Sensitivity of singlex LAMP-LFB assay

Serial dilutions (1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg) of H37Rv genomic DNA were used for LAMP reactions, and amplification products were analyzed by LFB and agarose gel electrophoresis. As shown in **Figure 4A**, the LoD of IS6110-LAMP for MTB identification was 10fg analyzing by LFB, which was consistent with agarose gel electrophoresis analysis (**Figure 4B**).

The LoD of IS1081-LAMP for MTB detection was 100fg monitored by LFB (**Figure 4C**), as well as agarose gel electrophoresis (**Figure 4D**).

Sensitivity of multiplex LAMP-LFB assay

Analytical sensitivity of multiplex LAMP-LFB assay for MTB detection was determined under the optimal conditions. As shown in **Figure 5**, the LoD was 10fg per reaction for MTB detection monitored by LFB and agarose gel electrophoresis.

The Optimal reaction of Time for multiplex LAMP-LFB Assay

To optimized the reaction time of multiplex LAMP assay, the assays were amplified at 66°C for 30min, 40min, 50min, and 60min with template at the LoD level (10fg). There were two red lines becoming visible when the multiplex LAMP assay lasted 50min (**Figure 6**). Thus, 50min was used as the optimal duration time for MTB detection. Hence, the whole course, including DNA preparation (15min), LAMP reaction (50min), and result interpretation (2min), could be accomplished within 70min.

Specificity of multiplex LAMP-LFB assay

For specificity evaluation, DNA templates extracted from 33 common pathogens and 12 MTB clinical strains were analyzed by multiplex LAMP-LFB assay. Positive results were obtained only from MTB strains, two red lines (TL and CL) simultaneously appeared on the biosensor. However, only one band (CL) were shown in non-MTBC strains, which indicated that multiplex LAMP assay has a high specificity (100%) for MTBC detection (**Figure 7**).

Application of multiplex LAMP-LFB assay in clinical samples

To further test the availability of MTB-LAMP-LFB assay in clinical samples, 98 TB patients and 60 non-TB patients were recruited in our study. The sensitivity and specificity were calculated by using composite

reference standard including culture results and clinical evidences. The 98 samples were simultaneously tested by MGIT liquid culture, Xpert MTB/RIF and LAMP-LFB assay, the sensitivity was 40.0% (39/98), 50.0% (48/98), and 86.7% (85/98), respectively. In liquid culture positive patients, 84.6% (33/39), and 92.3% (36/39) patients were confirmed positive by Xpert MTB/RIF and LAMP-LFB, respectively. In liquid culture negative samples, the sensitivity of Xpert MTB/RIF and LAMP-LFB was 25.4% (15/59) and 83.0% (49/59), respectively. The specificity of Xpert MTB/RIF and LAMP-LFB were both 100% (60/60). These results demonstrated that MTB-LAMP-LFB could improve the detection rate of negative culture samples with high sensitivity and specificity (**Table 3**).

Discussion

TB is a severe infectious disease which can be easily transmitted by airborne droplets. Therefore, speedy diagnosis and treatment are essential to control TB. Here, we designed a novel LAMP-LFB for rapid diagnostic of MTB, which was proved to be a simple method with high sensitivity and specificity. The whole process, including template preparation (15 min), isothermal amplification (50 min) and LFB visual detection (2 min), can be completed within 70 minutes.

In this report, LFB (lateral flow biosensor) was used for rapid, objective and visual analysis of LAMP results. Comparing with the other analysis methods (ie, agarose gel electrophoresis, turbidity and colorimetric indicator) used in previous reports (Rodríguez-García et al. 2018; Pandey et al. 2008; Thapa et al. 2019), the biosensor exhibited its superiority on rapid results, simple operation, and ease of use in clinical, basic and field laboratories. Most importantly, indicating MTB-LAMP results by LFB was able to eliminate the use of additional procedure, expensive optical instrument instruments, and special reagent, thus, the novel LAMP-LFB method was more suitable than other MTB-LAMP assays established by previous researches for rapid, visual, reliable and objective detection of target pathogens.

For the purpose of improving the sensitivity of LAMP assay, multi-copy insertion sequences IS6110 and IS1081 which exclusively exist in MTBC were selected as target sequences for MTB detection. IS6110 was most widely used for MTB detections (PCR, Xpert MTB/RIF, TB-LAMP, et al) because of its highly conserved and multiple copies in MTB strains, however, the copy number of IS6110 element is ranging from 0 to 25 depending on the strain (McEvoy et al. 2007). Therefore, IS1081 was selected as a subsidiary marker since its multiple copy number in all MTBC strains (5 to 6) (van Soolingen et al.1992). As a result, the combination of IS1081 and IS6110 could improve the detection rate of those IS6110 low-copy strains and reduce false negative results. Further, seven primers were designed for each target to ensure the high specificity, including two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers (LF and LB), and a FITC labeled FIP primer, which can specifically bind to anti-FITC on the sensor.

The practicality of MTB-LAMP-LFB had been verified in clinical samples, which demonstrated excellent sensitivity and specificity. The sensitivities of LAMP and Xpert MTB/RIF were 86.7% and 50%, respectively, for the 98 TB patients; and 92.3% and 84.6%, respectively, for the culture-confirmed patients.

These results suggest that the MTB-LAMP assay in our study has a higher sensitivity than Xpert MTB/RIF, and also higher than previous meta-analysis which showed a 62% sensitivity in children younger than 16 years old (Detjen et al. 2015) and a multicenter study which showed a 46% sensitivity in adults (Dorman et al. 2018). Importantly, LAMP showed a superior detection rate in culture-negative patients than Xpert MTB/RIF (83% & 25.4%), which indicated that LAMP enhances of the detection rate in culture-negative samples. In addition, the specificity of LAMP reached 100% in non-TB patients. In summary, MTB-LAMP-LFB is of great significance for the early detection of MTB, so as to achieve early diagnosis and early treatment.

In conclusion, we firstly developed and validated MTB-LAMP-LFB assay simultaneously detected IS6110 and IS1081 for MTB detection in pure cultures and clinical samples. MTB-LAMP-LFB assay can be a new diagnostic technology with advantages of simple, rapid, efficient, and cost-effective for early detection of MTB in health care settings, especially in rural areas.

Abbreviations

MTB: *Mycobacterium tuberculosis* **TB:** tuberculosis **LAMP:** loop-mediated isothermal amplification **LFB:** nanoparticle-based lateral flow biosensor

Declarations

Acknowledgements

Not applicable.

Ethics approval and consent to participate

Our study was reviewed and approved by Ethical committee of Beijing Chest Hospital and Beijing Children's Hospital. Informed consent has been obtained from all adult patients and guardians of all enrolled children.

Consent for publication

No applicable.

Availability of data and materials

The data and materials supporting the conclusions of this manuscript are freely available for the scientific community.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Yi Wang, Xingyun Wang, Hairong Huang and Adong Shen conceived and designed the experiments; Yi Wang, Xingyun Wang, Weiwei Jiao, Yacui Wang, Shuting Quan performed the most experiments; Guirong Wang, Hui Qi, Lin Sun, Chen Shen, Jieqiong Li, Hairong Huang, Adong Shen contributed the reagents and materials; Xingyun Wang and Yi Wang performed the software; Xingyun Wang, Hairong Huang and A-Dong Sheng analyzed the data; Xingyun Wang, Yi Wang, Hairong Huang and A-Dong Shen wrote the manuscript. All authors contributed and approved the final manuscript.

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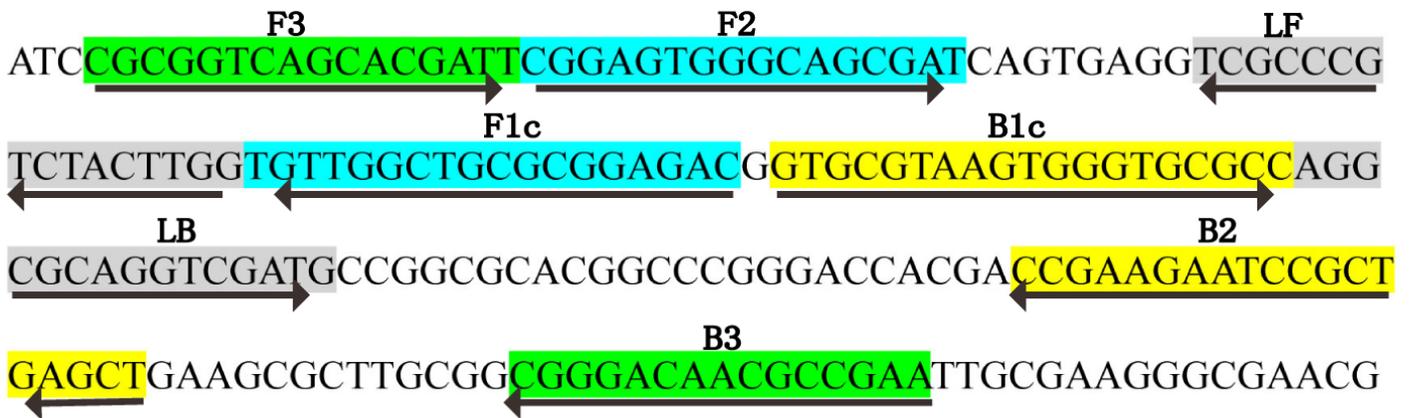
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Tables

Due to technical limitations, the tables could not be displayed here. Please see the supplementary files section to access the tables.

Figures

A primers for IS6110



B primers for IS1081

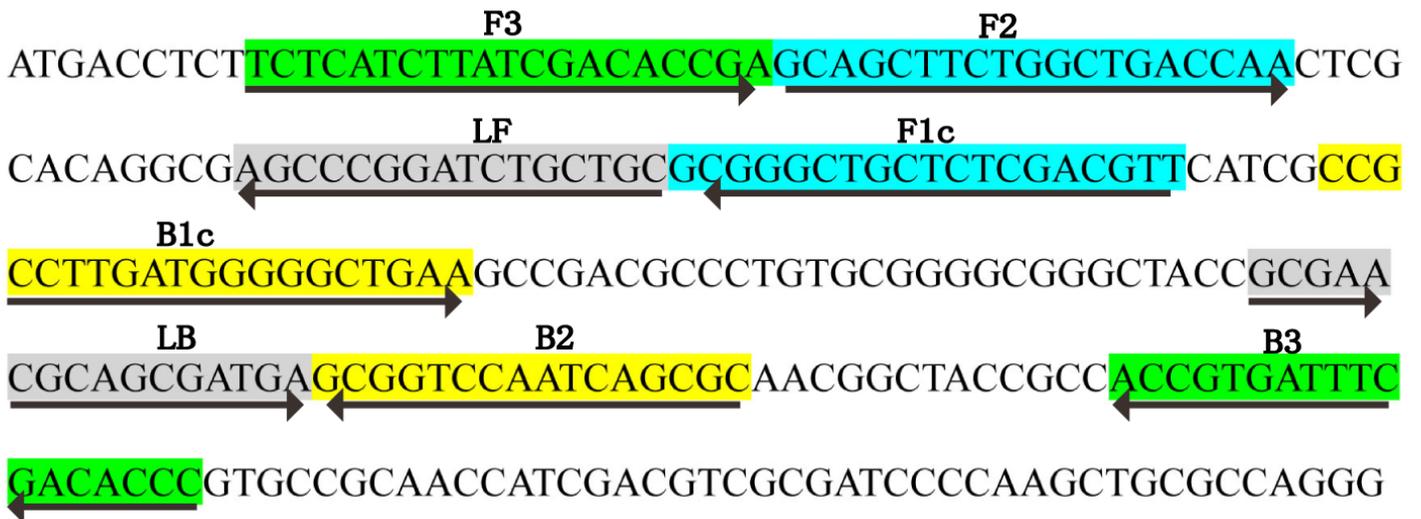


Figure 1

Primers specific to IS6110 and IS1081 of MTB used for LAMP-LFB assay. Sequence and locations of insertion sequences IS6110 and IS1081 used to design LAMP primers. The nucleotide sequences of IS6110 (A) and IS1081(B) are shown. Primer FIP included F2 and F1c; Primer BIP included B1c and B2. The direction of arrows indicated the primer from 5' to 3'.

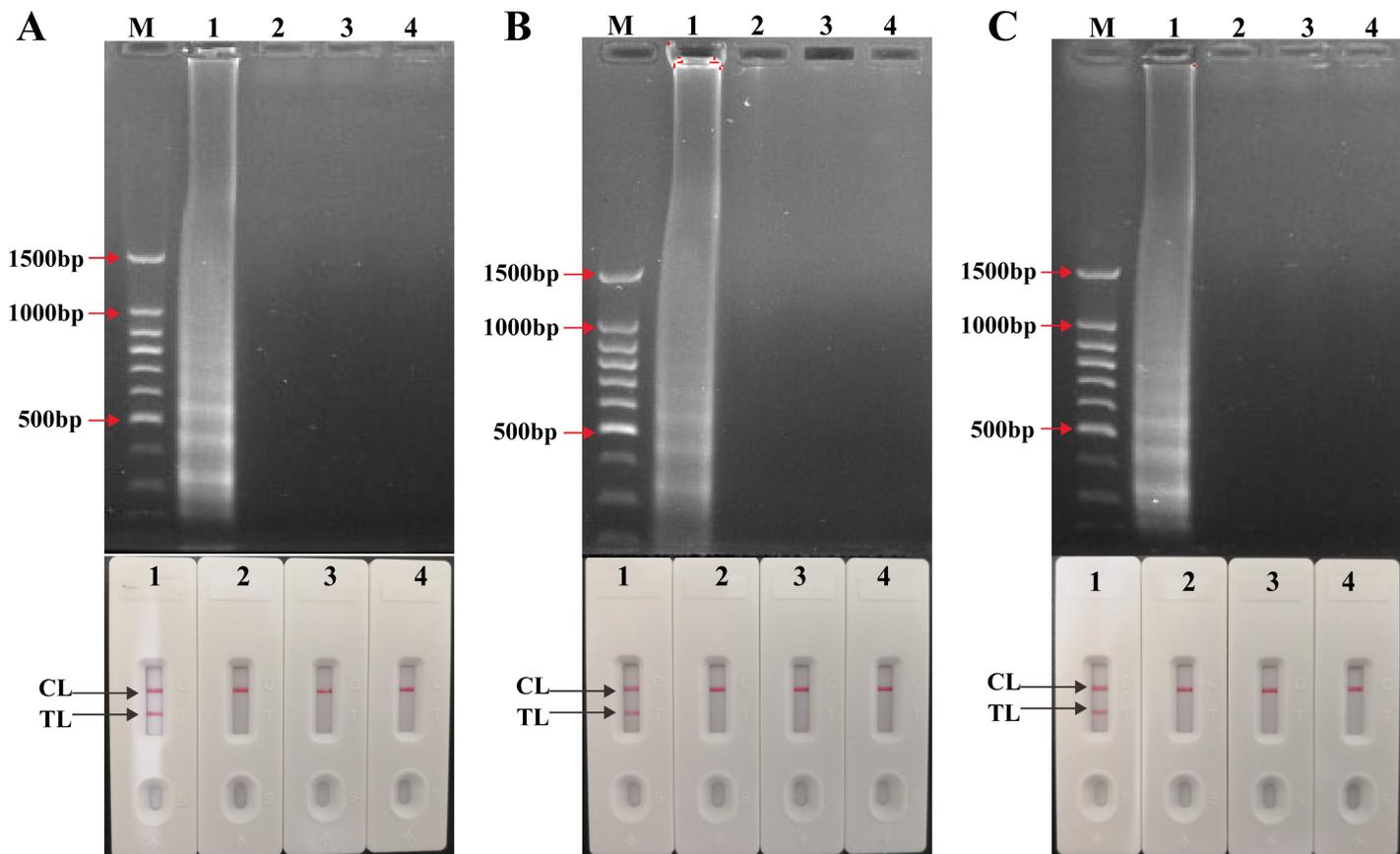


Figure 2

Confirmation and detection of singleplex LAMP and multiplex LAMP products. (A) Confirmation and detection the amplicons of IS6110-LAMP and (B) IS1081-LAMP and (C) multiplex LAMP. Top: 1.5% agarose gel electrophoresis using for amplification products analysis. Bottom: LFB applied for visual detection of amplicons analysis. Biosensors/lanes 1-4: positive products of *H37Rv*, *Streptococcus pneumoniae* and *Escherichia coli* as negative control, DW as blank control.

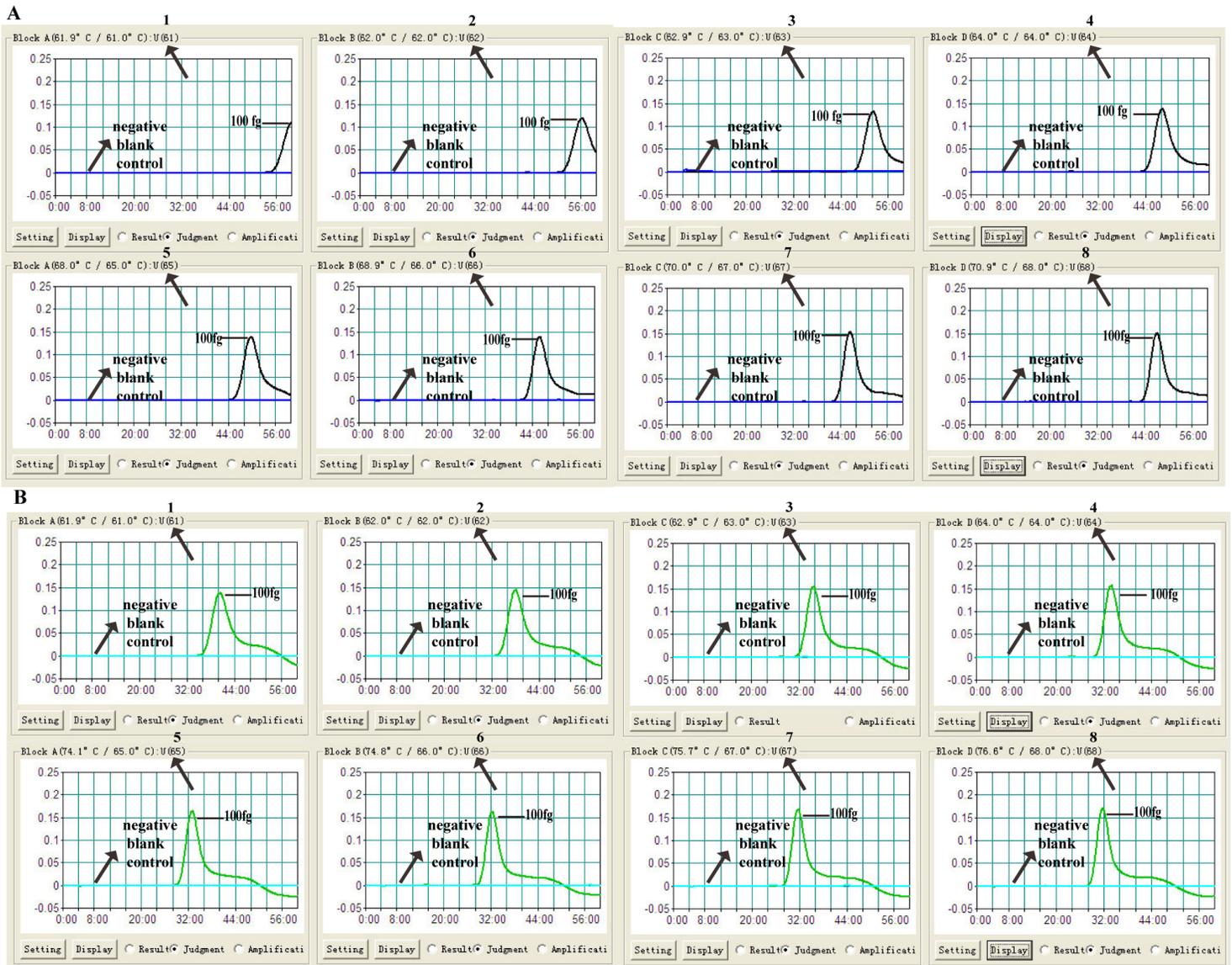


Figure 3

Optimization of reaction temperature for LAMP assay. The LAMP reactions were monitored by real-time turbidimeter. (A) IS6110-LAMP. (B) IS1081-LAMP. Turbidity of >0.1 was deemed to be positive. Eight kinetic graphs (1-8) were acquired at different temperatures (61°C to 68°C, 1°C intervals) with 100fg of H37Rv DNA template per reaction.

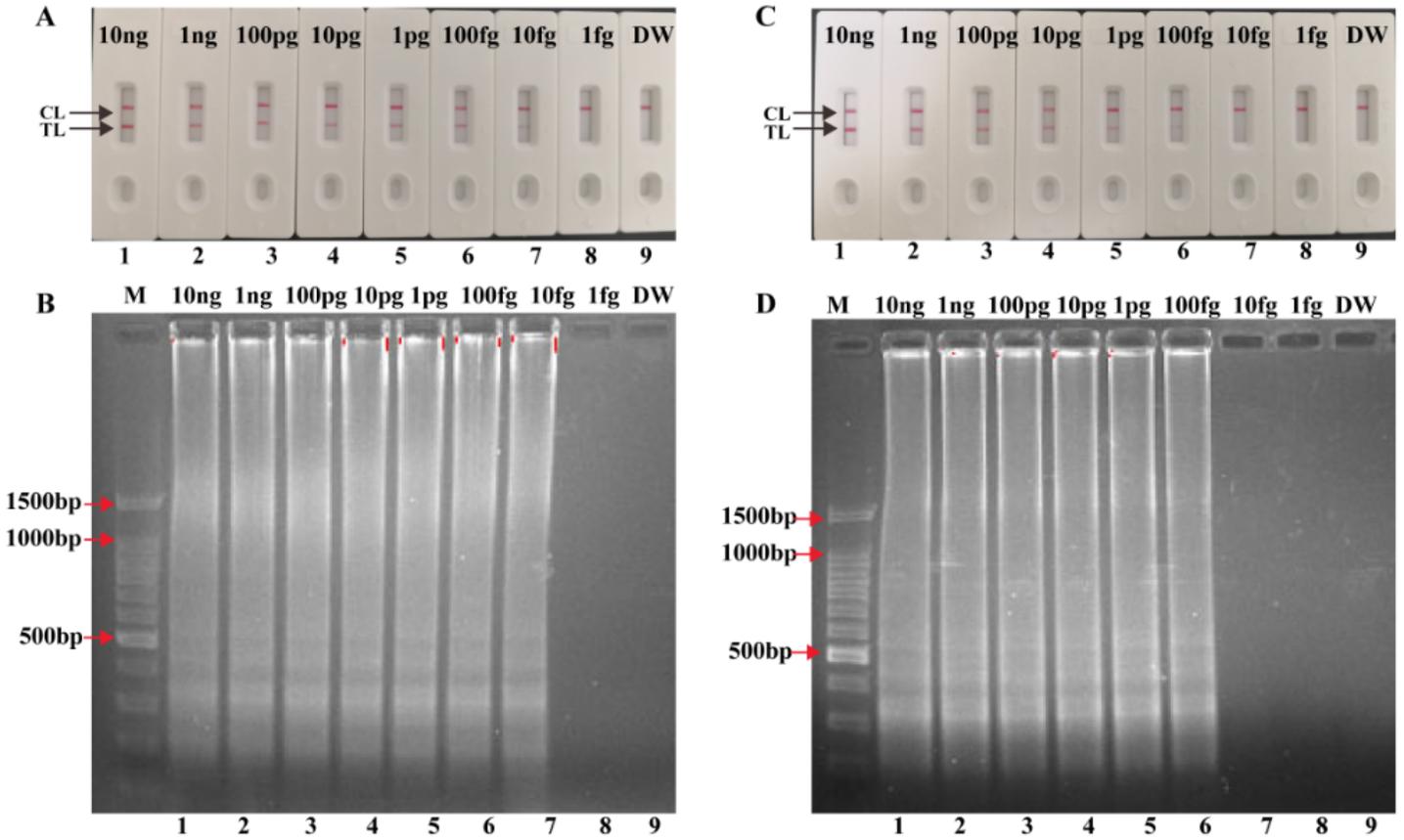


Figure 4

Analytical Sensitivity of singlex LAMP-LFB assay. Serial dilutions (10 ng, 1 ng, 100pg, 10pg, 1pg, 100fg, 10fg, 1fg) of genomic DNA of H37Rv were used for sensitivity determination of IS6110-LAMP (A,B) and IS1081-LAMP (C,D) assay. (A) and (C) Visual results of LFB for LAMP assay. (B) and (D) 1.5% agarose gel electrophoresis applied for LAMP products. Biosensors/lanes 1-8: H37Rv genomic DNA (10ng-1fg), Biosensor/lane 9: blank control (DW).

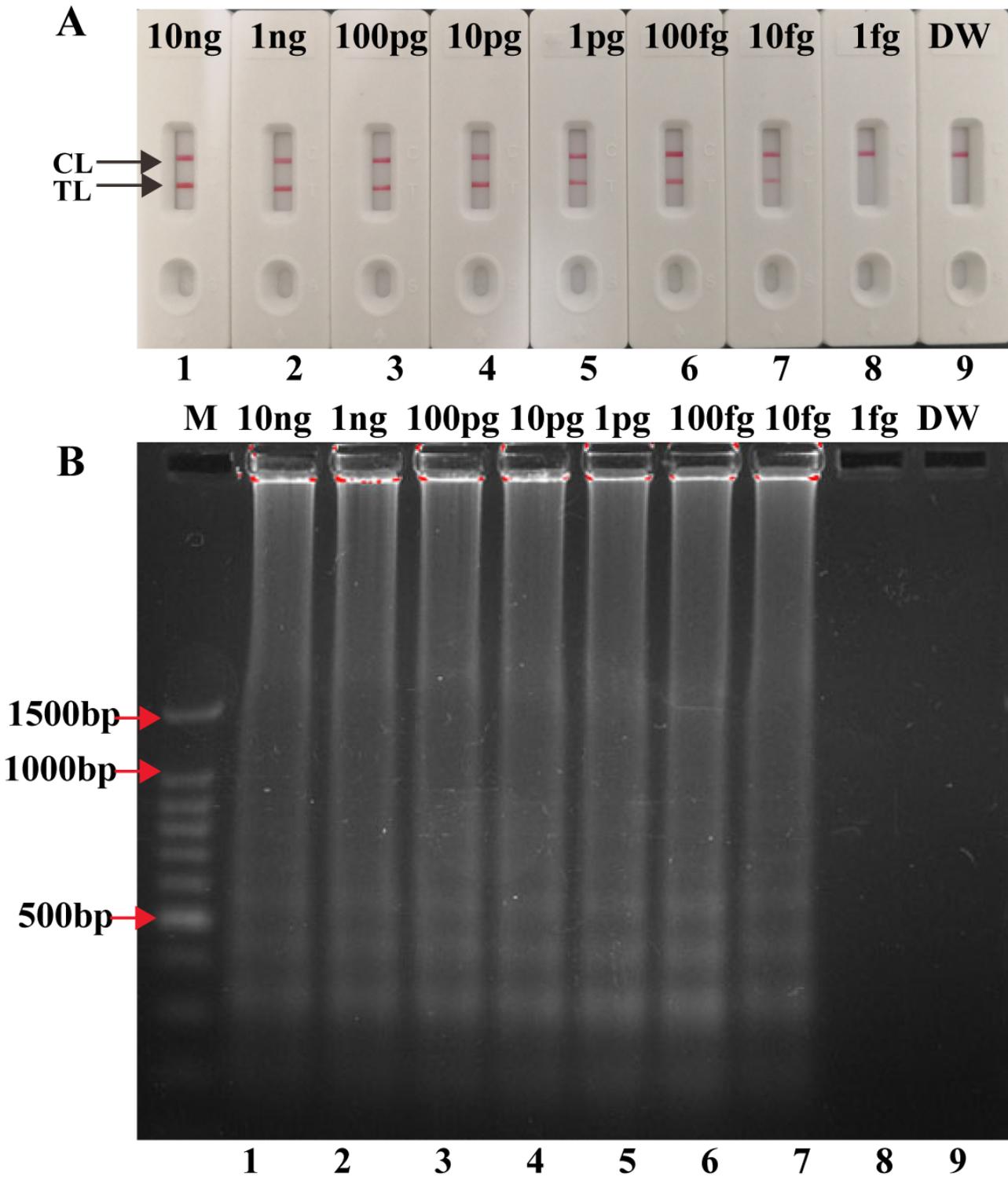


Figure 5

Analytical Sensitivity of multiplex LAMP-LFB assay. Two sets of primers targeting IS6110 and IS1081 were simultaneously added to one reaction tube to evaluate the sensitivity of LAMP assay. Two methods were used for amplification products analysis (A) LFB, (B) 1.5% agarose gel electrophoresis. Numbers 1-9: H37Rv genomic DNA 10ng, 1ng, 100pg, 10pg, 1pg, 100fg, 10fg, 1fg, DW.

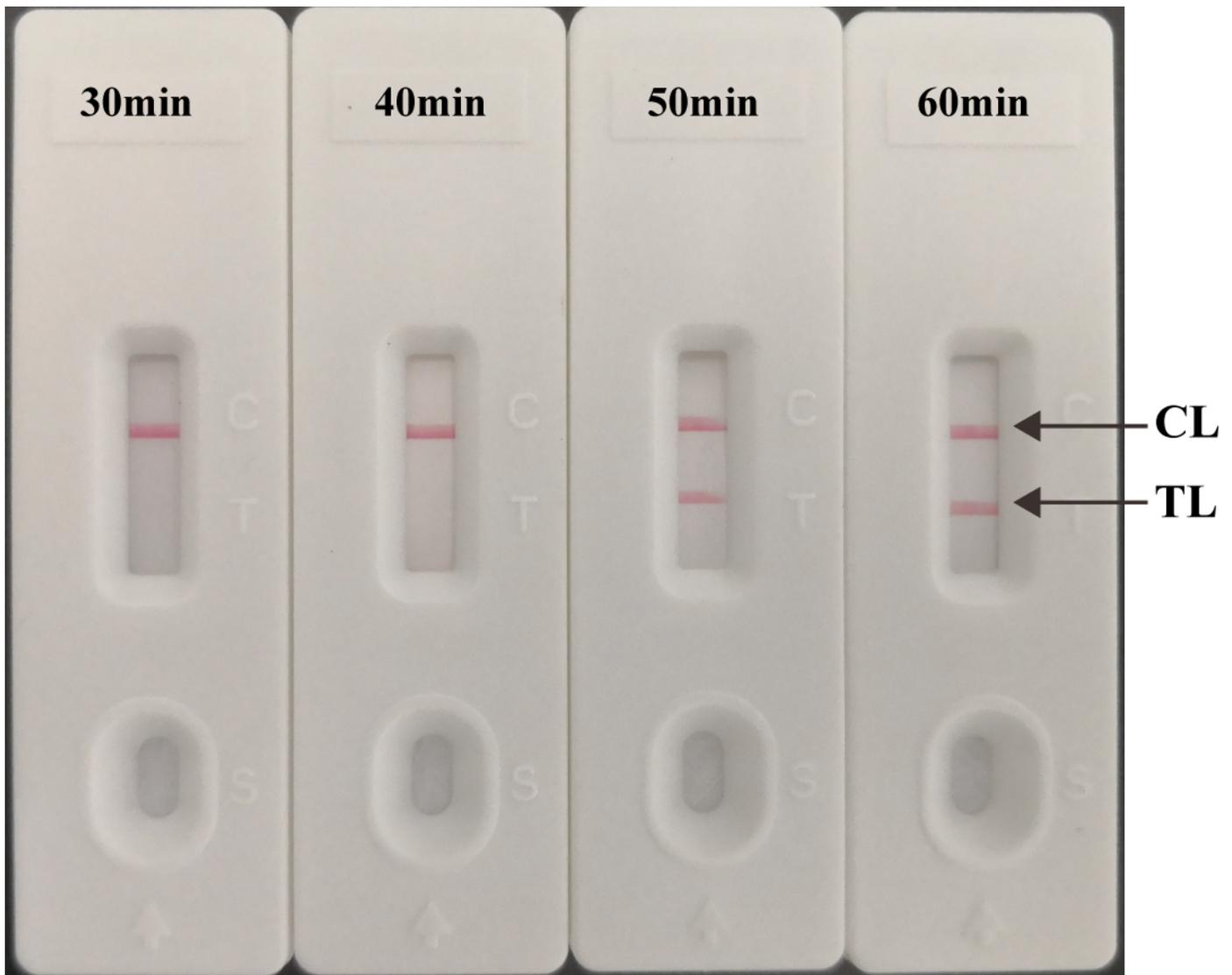


Figure 6

Optimized reaction Time for multiplex LAMP-LFB Assay The LAMP reactions with template at the LoD level (10fg) were amplified at 30min, 40min, 50min, and 60min at the optimal temperature (66 °C).

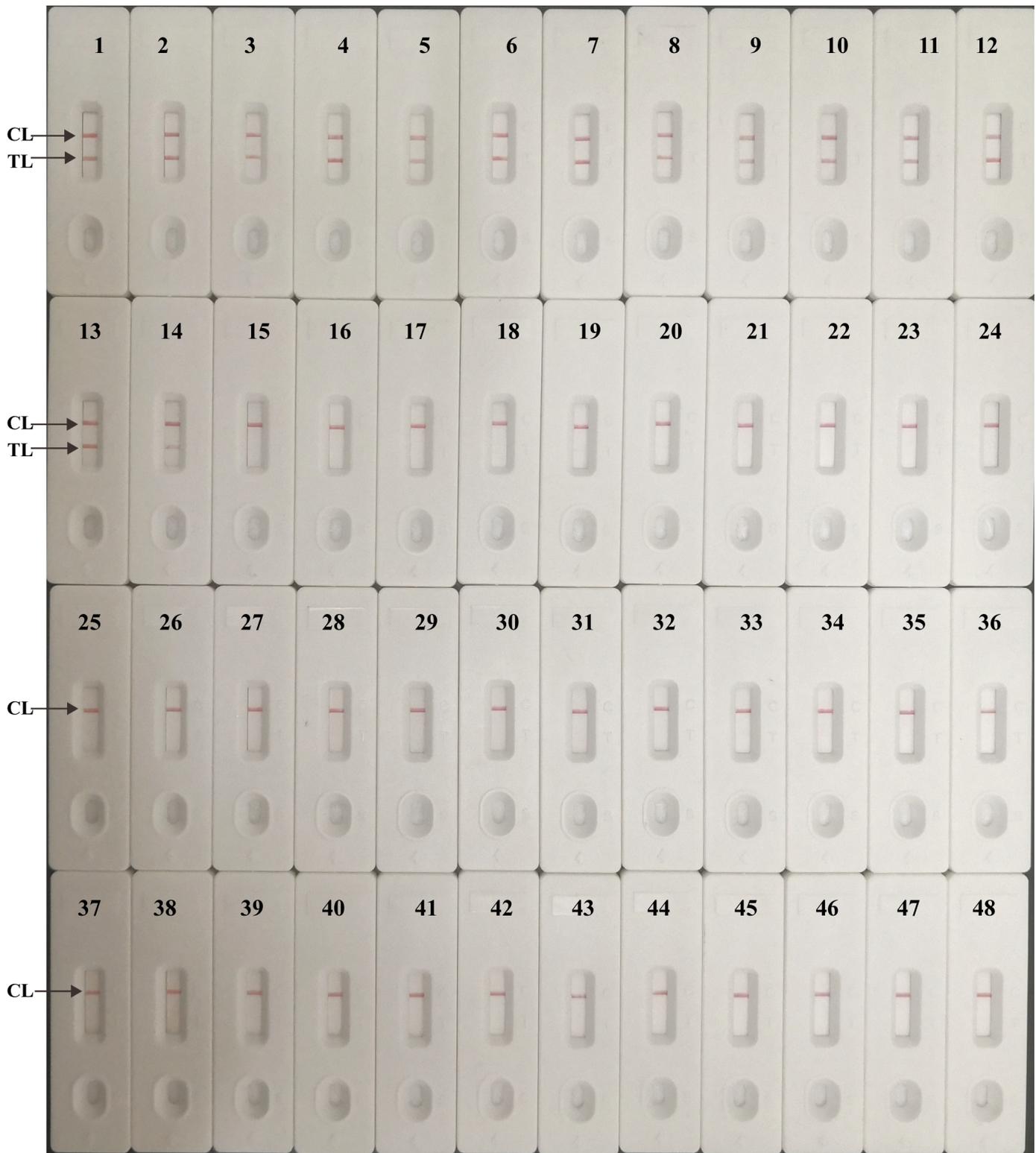


Figure 7

Analytical specificity of multiplex LAMP-LFB assay. The multiplex LAMP assays were carried out using various genomic DNA templates, and were monitored by LFB. Biosensor 1: H37Rv; Biosensor 2: BCG; Biosensors 3-14: 12 clinical isolated MTB strains; Biosensors 15-22: 8 different NTM strains (details shown in Table 2); Biosensor 23-47: 25 non-mycobacteria bacteria strains (details shown in Table 2); Biosensor 48: DW.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Tables.docx](#)