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An Isothermal Method for Sensitive Detection of *Mycobacterium tuberculosis* Complex Using Clustered Regularly Interspaced Short Palindromic Repeats/Cas12a *Cis* and *Trans* Cleavage

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Tuberculosis is one of the most serious infectious diseases, resulting in death worldwide. Traditional detection methods are not enough to meet the clinical requirements of rapid diagnosis, high specificity, and high sensitivity. Fast, sensitive, and accurate detection of *Mycobacterium tuberculosis* (MTB) is urgently needed to treat and control tuberculosis disease. Clustered regularly interspaced short palindromic repeats (CRISPR)—associated proteins (Cas12a) exhibit strong nonspecific degradation ability of exogenous single-strand nucleic acids (*trans* cleavage) after specific recognition of target sequence. We purified Cas12a protein and selected a proper guide RNA based on conserved sequences of MTB from designed guide RNA library. Then, we proposed a novel detection method based on recombinase polymerase amplification and CRISPR/Cas12a nuclease system for specific and sensitive detection of MTB DNA. The assay, based on fluorescence detection, showed 4.48 fmol/L of limit of detection and good linear correlation of concentration with fluorescence value ($R^2 = 0.9775$). It also showed good performance in distinguishing other bacteria. Furthermore, its clinical performance was evaluated by 193 samples and showed sensitivity of 99.29% (139/140) and specificity of 100% (53/53) at 99% CI, compared with culture method. Taken together, the CRISPR/Cas12a system showed good specificity, excellent sensitivity, and excellent accuracy for MTB detection, and it meets requirements of MTB detection in clinical samples and has great potential for clinical translation. *(J Mol Diagn 2020, 22: 1020—1029; https://doi.org/10.1016/j.jmoldx.2020.04.212)*

Tuberculosis (TB),[1] which is caused by *Mycobacterium tuberculosis* (MTB) and could spread through aerosol in the air (eg, by coughing), remains one of the top 10 causes of death and the leading cause of death from a single infectious agent (ranking above HIV/AIDS) worldwide (global tuberculosis report 2018, *https://www.who.int/tb/publications/2018/en/*, last accessed April 24, 2020). Therefore, the fast, sensitive, and accurate diagnosis of active TB and drug resistance (eg, rifampicin, isoniazid, and pyrazinamide) is crucial both for the individual and population level to reduce morbidity, mortality, and transmission among patients. Sputum smear microscopy is the primary test for TB diagnosis, but it is significantly limited by its laborious detection process and relatively low detection sensitivity of only 56% to 68%[2] (global tuberculosis control: a short update to the 2009

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report, https://www.who.int/tb/publications/2009/en/, last accessed April 24, 2020). In addition, the microscopy performs poorly on HIV co-infected specimens because the amount of MTB is low in sputum specimen. Meanwhile, it cannot distinguish MTB and nontuberculosis mycobacteria, which are treated totally differently because of different pathogenicity and drug susceptibility profiles,[3–5] although they show similar clinical manifestation. So far, culture-based mycobacterial detection method is still the gold standard for the laboratory diagnosis of tuberculosis because of its high sensitivity, specificity, and accuracy.[6] But it needs extensive laboratory experience and usually takes up to 4 to 6 weeks to achieve satisfied results, which does not meet the rapid test requirement. This has significantly hindered the prompt treatment and isolation of patients, which is crucial for disease control. Moreover, some bacilli cannot be successfully cultured, which might cause error results.[7]

Genetic sequencing of MTB DNA was reported to be a more accurate strategy for TB diagnosis. The Xpert MTB/RIF system (Cepheid, Sunnyvale, CA), a PCR-based test, is the only one accepted by the World Health Organization from 2010 for detecting TB infection and rifampicin resistance. Although it has many advantages, including full automation, integration, and better accuracy than microscopy or culture methods, its sensitivity for some bacilli clinical specimens is still inadequate. Furthermore, the cost of cartridges, instrumentation, and maintenance of Xpert MTB/RIF system make it impossible to set up in many high TB burden countries, which further limited its usefulness.[8,9]

In recent years, isothermal amplification assays, using loop-mediated amplification,[10] helicase-dependent amplification,[11] and recombinase polymerase amplification (RPA),[12–14] have been extensively applied in the field of molecular diagnosis, including pulmonary TB diagnosis.[9,15,16] Especially, the RPA reaction, which replaces the thermal cycles needed for PCR and works at temperatures between 25°C and 42°C with three core enzymes, permits rapid DNA amplification in minutes. Recently, the RPA-based methods have been used for detection of MTB and other pathogens, whereas it always had high false-positive rates in clinical samples.[17] To further improve specificity, long oligonucleotide probe with fluorophores and quenchers or other modifications of probe, such as biotin and tetrahydrofuran, has been designed to recognize RPA amplicon for specific cleavage by nucleases, and these newly developed methods have been successfully applied for pathogen diagnosis,[18–20] liquid biopsy,[21] and single-nucleotide polymorphism/genotyping.[22] However, the synthesis of long oligonucleotide probes with multiple modifications is costly, and different probes with the same amplified region may also affect the efficiency of RPA. Especially, the base mismatches near the middle of probes or the 3′-OH end of primers might significantly affect the efficiency of RPA reaction, which could lead to reduced amplification efficiency and false-positive results.[17,23]

Microbial clustered regularly interspaced short palindromic repeats (CRISPR)—associated protein (Cas) adaptive immune system consists of guide RNA(s) [gRNA(s)] for target recognition and a Cas enzyme for target cleavage.[24–27] The sequence-specific recognition capabilities of the CRISPR-Cas system make it a promising genome engineering tool for genome editing,[27,28] cancer target therapy,[29] and gene therapy.[30] Recently, the CRISPR-Cas nuclease systems (Cas12, Cas13, and Cas14)[24,31–33] also have been developed for nucleic acid detection with high sensitivity and specificity. These CRISPR-Cas nuclease systems use the gRNA to recognize the RNA (Cas13a and Cas13b) or DNA (Cas12a and Cas14) targets, then are followed by activating cleavage capacity of Cas nuclease to degrade multiple foreign nucleic acid reporters for target detection. Combining with nucleic acid amplification strategy, the Cas nuclease systems have been used for highly sensitive and specific detection of nucleic acids and became powerful tools for developing molecular diagnostic techniques.[24,31,32]

To achieve prompt, sensitive, and specific confirmed diagnosis of MTB, herein the RPA reaction is combined with the CRISPR-Cas nuclease (Cas12a) system to develop a novel MTB DNA detection method (Figure 1). First, the sequence of MTB with protospacer adjacent motif structure (5′-TTTN-3′) for Cas12/gRNA recognition was selected for gRNA design. The gRNA consists of two parts, universal spacer sequence (5′-UAAUUUCUACUAAGUGUAGAU-3′) at the 5′ end (also near the protospacer adjacent motif structure end) and specific sequence at the 3′ end. And gRNAs were selected via evaluating by multiple software programs, such as CRISPR-DT, NUPACK, and Nucleotide BLAST. Then, the feasibility of Cas12a/gRNA *trans*-cleavage reaction was validated by the purified Cas12a. Subsequently, the suitable amplicon screening was performed and tested to effectively avoid false amplification of RPA. In the Cas12a/gRNA *trans*-cleavage fluorescence assay for MTB, in the presence of MTB DNA, they could be amplified by RPA reaction. Later, through Cas12a/gRNA complexes binding to amplified MTB DNA, Cas12a mediated non-specific degradation of non-specific fluorescent probes, which were polythymidine oligonucleotides with fluorophore at the 5′ end and quencher at the 3′ end, as MTB DNA reporters, that could release the fluorophores from the quenchers to increase fluorescence intensities for detection. The current article proposes a novel isothermal method based on RPA and CRISPR-Cas system, which has low limitation on MTB detection in the model testing system, with 4.48 fmol/L of limit of detection (LOD), ranging over six orders of magnitude ($R^2 = 0.9775$), and could be applied to practical applications on clinical samples with excellent specificity, sensitivity, and accuracy, which are comparable with the result of the gold standard of culture method.

Materials and Methods

Target Selection and gRNA Design for MTB-Cas12a System

MTB-specific fragment of the insertion sequence IS*1081*, which exists in all MTB complexes,[34] was selected as the target

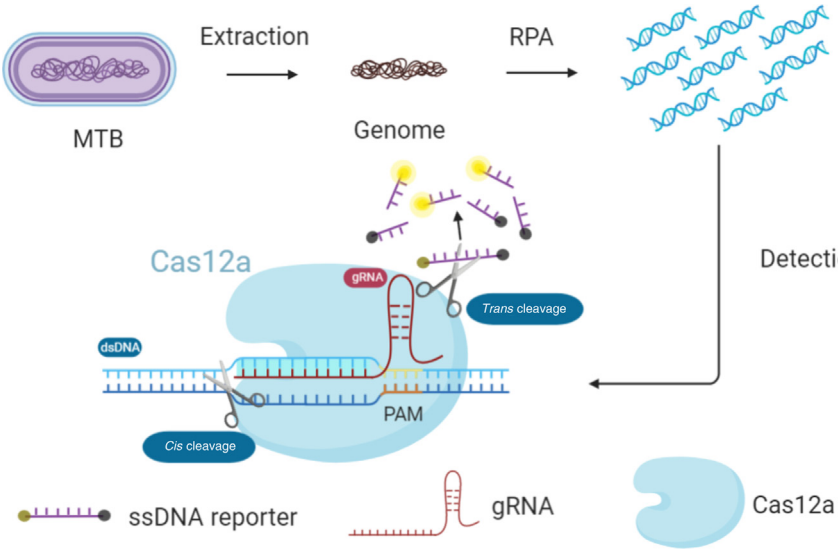


Figure 1 Schematic diagram of the Cas12a/guide RNA (gRNA) *trans*-cleavage fluorescence signal amplification system for *Mycobacterium tuberculosis* (MTB) detection. Genome of MTB complex was put into recombinase polymerase amplification (RPA) reaction for amplification. Positive fluorescent signals were captured when probes were cleaved by activated Cas12a under the targeting with protospacer adjacent motif (PAM) sequence at 5′ end that was recognized by gRNA. dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

sequence for design of gRNA to target MTB and RPA reaction primers. To obtain the suitable guide RNA for MTB, gRNAs targeting IS*1081* double-stranded DNA were designed and scored by CRISPR-DT online software for Cpf1 (Cas12a; *http://bioinfolab.miamioh.edu/CRISPR-DT/interface/Cpf1_main.php*, last accessed April 24, 2020), and target efficiency <0.6 was excluded. Meanwhile, the second structure and homology of remaining gRNAs were further evaluated by NUPACK (*http://www.nupack.org/*, last accessed April 24, 2020) and Nucleotide BLAST (*https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome*), respectively. Afterward, RPA primers were designed through Primer Premier software version 5.0 (Premier Biosoft International, San Francisco, CA) and selected using agarose gel electrophoresis, according to the TwistAmp Assay Design Manual (TwistDx Ltd, Cambridge, MA).

All RNA, IS*1081* or IS*6110* segments and modified DNA oligonucleotides were synthesized by General Biosystems (Anhui, China). Plasmids obtained from the constructs of the segment of *Mycobacterium bovis* transposase insertion sequence element, IS*1081* (gene ID: 32286187, *https://www.ncbi.nlm.nih.gov/gene/*) and IS*6110* (GenBank: X17348.1, *https://www.ncbi.nlm.nih.gov/nuccore/*), were put into vector PUC57. Sequence information can be seen in Supplemental Table S1. The fluorescent polyoligonucleotides probe [single-stranded DNA (ssDNA)—fluorophore quencher (FQ)] was labeled with a fluorescent dye at the 5′ end and a quenching group at the 3′ end and then purified with high-performance liquid chromatography. The fluorescence could be detected only when the fluorescent dye and quenching group were separated. Forward and reverse primers for RPA and non-specific ssDNA (nontarget) for Cas12a/gRNA *trans*-cleavage reaction were obtained from Sangon Biotech (Shanghai, China) with PAGE purification. All DNA/RNA oligonucleotides and plasmids were diluted with RNase-free water, and the concentrations were determined by Nanodrop 200 (Thermo Fisher Scientific, Waltham, MA). All of these were stored at −20°C until use. The sequences of DNA/RNA oligonucleotides used in the assay are shown in Table 1 and Table 2.

Feasibility of Purified Cas12a for MTB Detection

The DNA plasmid encoding Cas12a (alias Cpf1) with 6× His-tag, maltose-binding protein, and tobacco etch virus (TEV) protease cleavage site was selected for LbCas12a expression. The plasmid 6His−maltose-binding protein−TEV-huLbCpf1 (plasmid 90096) (Supplemental Figure S1 and Supplemental Table S1) was purchased from Addgene (Cambridge, MA). After extraction from amplified strains, the plasmids were transformed into BL21 (DE3) pLysS Chemically Competent Cells (TransGen Biotech Co, Ltd, Beijing, China). Then, transformed strains were recovered from Luria-Bertani solid growth media and turned to 5 mL Luria-Bertani liquid growth media. The LbaCas12a protein was then expressed and purified, according to the method described by Chen et al.[24] Briefly, a 5-mL starter culture was grown in Luria-Bertani liquid growth media (containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, pH 7.0; Sigma, St. Louis, MO) at 25°C overnight, and then further transferred into 1 L of Luria-Bertani liquid growth media and inoculated for growth at 37°C under 300 rpm shaking until the OD 600 reached approximately 0.6. Afterward, all bacterium was cooled down and supplemented with 500 μmol/L isopropyl-1-thio-b-D-galactopyranoside (TransGen Biotech Co, Ltd) at 16°C for 16 hours to allow protein expression. After centrifugation at 2600 × *g* for 30 minutes at 4°C, the bacterial precipitation was harvested and stored at −80°C until use.

The protein purification was performed at 4°C. First, the bacterial precipitation was crushed and lysed by sonication in lysis buffer [50 mmol/L Tris-HCl, 500 mmol/L NaCl, 5% (v/v) glycerol, 1 mmol/L Tris(2-carboxyethyl)phosphine

Table 1 The Oligonucleotide Sequences of RPA Primers and Non-Specific ssDNA for Setup of MTB Cas12a/gRNA *Trans*-Cleavage Assay

Name	Sequence
RPA-F1*	5′-CCAAGCTGCGCCAGGGCAGCTATTTCCCGGAC-3′
RPA-F2	5′-CAAGCGAGCTGAACGCGCACTGACCAGCGT-3′
RPA-F3	5′-GGTGGCGACCTGCTACCTGCTGGGAGTAT-3′
RPA-R1*	5′-TTGGCCATGATCGACACTTGCGACTTGGA-3′
RPA-R2	5′-CGAAACGCCTCTACGGCTTCGTCGAGCTC-3′
RPA-R3	5′-GGCGTCGGCGGCGAGGAAGGTATACGG-3′
ssDNA (nontarget)	5′-GCTTGTGGCCGTTTACGTCGCCGTCCAGCTCGACC-AGGATGGGCACCACCCCGGCTTTTTTTTTTTTTTTTTTTTTTT-3′
ssDNA-FQ	5′-FAM-TTTTT-BHQ1-3′

ssDNA (nontarget) was used in the Cas12a/gRNA assay, and ssDNA-FQ was used in the Cas12a/gRNA *trans*-cleavage fluorescence assay.
*RPA-F and RPA-R are the forward and reverse primers of RPA, respectively. The final sequences of RPA primers are marked.
FQ, fluorophore quencher; gRNA, guide RNA; MTB, *Mycobacterium tuberculosis*; RPA, recombinase polymerase amplification; ssDNA, single-stranded DNA.

(TCEP), 0.5 mmol/L phenylmethylsulfonyl fluoride, and 0.25 mg/mL lysozyme, pH 7.5]. After centrifugation, the supernatant was filtered via a Stericup 0.22-μm filter (EMD Millipore, Hong Kong, China). The filtered supernatant was pipetted to Ni-NTA resin (GE Healthcare, Chicago, IL) for 30 minutes, and washed with lysis buffer. Then, the resin was resuspended in 1× TEV digestion buffer (50 mmol/L Tris-HCl, 50 mmol/L NaCl, 0.5 mmol/L EDTA, and 1 mmol/L dithiothreitol, pH 8.0) with 250 units of TEV protease (Beyotime Biotechnology, Shanghai, China) overnight at 4°C with rotation. To obtain purer proteins, the steps on Ni-NTA resin were repeated. The protease-treated LbCas12a was dialyzed in buffer A (20 mmol/L Tris-HCl, 125 mmol/L KCl, 5% glycerol, and 1 mmol/L TCEP, pH 7.5) for three times at 4°C, and further purified on a 5-mL HiTrap SP HP sepharose column (GE Healthcare, Boston, MA). After washing with three column volumes of buffer A, LbCas12a was eluted using a linear gradient from 0% to 100% buffer B (20 mmol/L Tris-HCl, 1 mol/L KCl, 5% glycerol, and 1 mmol/L TCEP, pH 7.5) over 20 column volumes. The eluted proteins were further purified on a Superdex 200 column (GE Healthcare, Boston, MA) with elution buffer (20 mmol/L Tris-HCl, 200 mmol/L KCl, 5% glycerol, and 1 mmol/L TCEP, pH 7.5). The existence of LbCas12a protein was confirmed by SDS-PAGE and Coomassie Blue staining, and then mixed with equal volume of glycerol after quantification and stored at −20°C for further use. To test the *cis*- and *trans*-cleavage activity of purified LbCas12a protein and the feasibility of Cas12/gRNA *trans* cleavage for MTB detection, the alteration of amount and integrity of substrates and non-specific single-stranded DNA by 4% agarose gel electrophoresis was observed, and the fluorescence intensity was collected by detector with/out target plasmid, gRNA, or nontarget ssDNA. The Cas12a/gRNA *trans* cleavage for MTB detection assay was as follows: 50 nmol/L LbCas12a incubating with 36 nmol/L IS*1081*-gRNA, 80 ng IS*1081* plasmid, and 50 nmol/L nontarget ssDNA (replaced with ssDNA-FQ in Cas12a/

Table 2 The Sequences of Designed gRNAs Targeting IS*1081* and the Corresponding Target Efficiency Score for MTB Genome

Name	Sequence	Target strand	Target efficiency score
gRNA 1	5′-UAAUUUCUACUAAGUGUAGAUGUCAACCCAGCACCUGCCAG-3′	Antisense	0.9725
gRNA 2	5′-UAAUUUCUACUAAGUGUAGAUGACACCCGTGCCGCAACCAT-3′	Antisense	0.9639
gRNA 3	5′-UAAUUUCUACUAAGUGUAGAUGAAGGAUCACGCGAUGACCG-3′	Sense	0.8954
gRNA 4	5′-UAAUUUCUACUAAGUGUAGAUACCACACCUUGGGGCACCUU-3′	Antisense	0.8461
gRNA 5	5′-UAAUUUCUACUAAGUGUAGAUGCCAUGAUCGACACUUGCGA-3′	Sense	0.838
gRNA 6	5′-UAAUUUCUACUAAGUGUAGAUCAAGUCGCAAGUGUCGAUCA-3′	Antisense	0.7736
gRNA 7	5′-UAAUUUCUACUAAGUGUAGAUUCGGUCAGAGCGUCGAGUAC-3′	Sense	0.7692
gRNA 8	5′-UAAUUUCUACUAAGUGUAGAUGGACCCGCCCGCUCGAUGCC-3′	Antisense	0.7227
gRNA 9*	5′-UAAUUUCUACUAAGUGUAGAUGACCAGGCGCUCCAUCCGGC-3′	Sense	0.7061
gRNA 10	5′-UAAUUUCUACUAAGUGUAGAUGCGCCAGAUCUGCUUGGGGA-3′	Sense	0.5644
gRNA 11	5′-UAAUUUCUACUAAGUGUAGAUUCACACCAAGUGUUUCGACC-3′	Sense	0.5353
gRNA 12	5′-UAAUUUCUACUAAGUGUAGAUCUGGUCGUUUCGAAGGAUCA-3′	Sense	0.4128
gRNA 13	5′-UAAUUUCUACUAAGUGUAGAUCCGGACUGGCUGCUGCAGCG-3′	Antisense	0.2013
gRNA 14	5′-UAAUUUCUACUAAGUGUAGAUCUGCUUGGCGGGUUCUUCGG-3′	Sense	0.0606
gRNA 15	5′-UAAUUUCUACUAAGUGUAGAUUUUCCUUCGAUUGACUCUGG-3′	Sense	0.0107

*The final sequence of gRNA used in the MTB Cas12a *trans*-cleavage fluorescence assay.
gRNA, guide RNA; MTB, *Mycobacterium tuberculosis*.

gRNA fluorescence assay) in 1× reaction buffer (50 mmol/L NaCl, 10 mmol/L Tris-HCl, 10 mmol/L $MgCl_2$, and 100 μg/mL bovine serum albumin, pH 7.9), incubated at 37°C for 2 hours.

MTB Detection by Cas12a Fluorescence Assay

To improve signal amplification efficiency and simplify operation, 1 μL of DNA extracted from amplified clone strains as template was added into RPA reaction mixture of 12.5 μL volume. RPA reaction mixture, including 0.24 to 0.72 μmol/L forward and reverse primers, 1.8 mmol/L dNTP, 1× reaction buffer, 1× basic E-mix, 0.625 μL 20× core reaction mix, 1 μL genome, and 14 mmol/L magnesium acetate and diethyl pyrocarbonate water up to 12.5 μL, was incubated at 37°C to 42°C for 1 hour. According to the TwistAmp Assay Design Manual, multiplex sets of RPA primers were selected as candidates, including primers from published literature[9,35] (Table 1), and the best conditions were sought from RPA reaction via optimization for concentration of dNTP, Mg^{2+}, primers, and reaction temperature with TwistAmp Liquid Basic/Basic RT (TwistDx Ltd).

After RPA reaction finished at 1 hour, 7.5 μL Cas12a/gRNA mixtures of 36 nmol/L gRNA, 50 nmol/L ssDNA-FQ, and 50 nmol/L Cas12a in 1× reaction buffer were added into the cap of RPA reaction tube to the final volume of 20 μL. Then, transient high-speed centrifugation was performed to mix RPA productions with Cas12a/gRNA system. The Cas12a/gRNA *trans*-cleavage fluorescence reaction was incubated at 37°C for 2 hours, and its fluorescence was collected every 1.25 minutes by fluorescence detector (Applied Biosystems 7500 Real-Time PCR System in this study; Thermo Fisher Scientific) during reaction.

As defined herein, positive result of sample was refined as the fluorescence intensity collected by detector and was 1.5 times more than that of negative control. Others were negative for samples. Negative control was non-MTB DNA as template for Cas12a/gRNA *trans*-cleavage fluorescence assay.

Performance of MTB-Cas12a Fluorescence Assay

For evaluation of the sensitivity of Cas12a/gRNA for MTB fluorescence assay, a series of gradient dilution concentrations of IS*1081* plasmids from 8960 pmol/L to 44.8 fmol/L were prepared with 1 μL volume as RPA template. Meanwhile, no-MTB DNA was used as negative control, and all of them were performed in three replicates. Total DNA mass for the assay was determined with following formula: M/5-7×(4.38 × 10^6 × 660) = μg/μL. M is concentration of IS*1081* plasmid, 5-7 is the number of copies of IS*1081* per MTB genome, and 4.38 × 10^6 is the approximate number of DNA length of *M. bovis* bacillus Calmette-Guérin. The specificity of the assay was obtained by analyzing at least 448 fmol/L genomic DNA of target MTB, with/or not closely related species from clinical isolates and standard strains.

Clinical Performance Evaluation of MTB-Cas12a Fluorescence Assay

The practical performance evaluation of the MTB detection assay was performed via Cas12a *trans*-cleavage fluorescence system using a total of 194 clinical sputum samples, which were randomly collected from Fuzhou Center for Disease Control and Prevention in 2013 to 2016, with one sample from one individual, including patients with similar signs and symptoms consistent with pulmonary TB. All sample culture results, including 140 culture-positive results and 54 culture-negative results, were unknown for operator first. Genome of all samples was extracted using QIAamp One-For-All Nucleic Acid Kit (Qiagen, Germantown, MD), and the concentrations were measured via Nanodrop 2.0 (Thermo Fisher Scientific) and then stored at −20°C before test. The 194 clinical samples were detected by Cas12a/gRNA *trans*-cleavage fluorescence assay, and the data were analyzed according to the above stated criteria. To further compare the performance of this Cas12a-based assay with other reported strategies, a real-time PCR method was built based on the TaqMan probe for clinical sample detection, according to a previous report.[36] The self-built real-time PCR for MTB detection was with total volume of 20 μL, including 1× Premix Ex Taq (probe real-time quantitative PCR; 2×; Takara Bio, Dalian, China), 0.2 μmol/L PCR forward primer (5′-TCGCGTGATCCTTCGAAACG-3′; Ultrapage) and PCR reverse primer (5′-GCCGTTGCGCTGATTGGACC-3′; Ultrapage), 0.4 μmol/L probe (5′-FAM-CCGCGAACGCAGCGATGAGC-BHQ1-3′; high-performance liquid chromatography), 0.5× ROX Reference Dye II (50×; Takara Bio), and 2 μL template. Then, it runs at following condition: 30 seconds for 95°C and 50 cycles of 5 seconds for 95°C and 34 seconds for 60°C in Applied Biosystems 7500 Real-Time PCR System.

Data Analysis

All data were analyzed with SPSS version 2.2 (IBM, Armonk, NY) and OriginLab version 8.0 (OriginLab Corp, Northampton, MA), including receiver operating characteristic curve area and R^2. Each experiment was repeated at least three times for each sample.

Results

Design of Guide RNAs for MTB

To ensure the coverage on all MTB species, the target insertion sequence IS*1081* was selected for the assay, and gRNAs targeting MTB were designed. Principally, there are numbers of gRNAs corresponding to the amounts of protospacer adjacent motif structure existing in the insertion sequence of IS*1081*. Actually, different gRNAs have different target efficiency of Cas12a/gRNA system, and not all predicted gRNAs were suitable for targeting MTB. Therefore, scores of target efficiency for gRNAs were

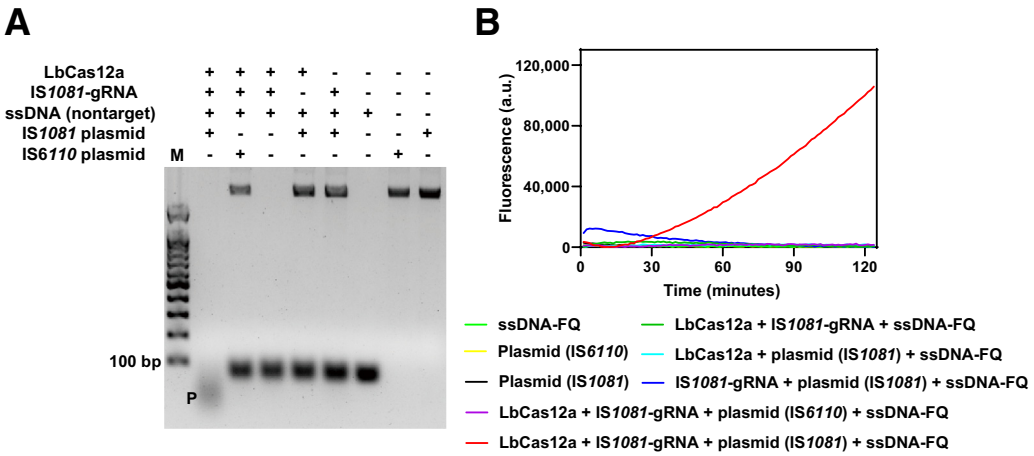


Figure 2 Feasibility analysis of Cas12a/guide RNA (gRNA) system for *Mycobacterium tuberculosis* (MTB) detection. A 4% agarose gel electrophoretic analysis of the feasibility of Cas12a/gRNA system for MTB detection. **A:** A 79-nucleotide single-stranded DNA (ssDNA; nontarget) as substrate of Cas12a/gRNA *trans* cleavage; real-time fluorescence of Cas12a/gRNA fluorescence assay. ssDNA—fluorophore quencher (FQ) served as the reporter probe. **B:** The concentration of the reaction components was as follows: 50 nmol/L purified LbCas12a, 36 nmol/L IS*1081*-gRNA, 50 nmol/L ssDNA (nontarget), or ssDNA-FQ, 80 ng IS*1081* or IS*6110* plasmid. a.u., arbitrary unit; M, marker; P, product.

evaluated by Cpf1-CRISPR-DT online software, and range from 0.7061 to 0.97 was selected after excluding that of <0.6, and nine gRNAs remained. In the end, one was selected in which the score of target efficiency was 0.7061 based on unconspicuous secondary structure under reaction temperature and specificity of gRNA spacer for MTB. Especially, the spacer of MTB-specific sequence of gRNA was the conserved region of IS*1081*, according to previous study and to the current sequence blasting.[35] The sequences of designed IS*1081* gRNAs and corresponding score of target efficiency for MTB genome are shown in Table 2.

Feasibility Analysis of Purified Cas12a for MTB Detection

The gRNA-directed DNA binding and following activation of ssDNA *trans*-cleavage activity of Cas12a protein are vital properties for accurate recognition of target DNA for detection. The activity level of Cas12a protein directly determines the limit of detection of the Cas12a/gRNA *trans*-cleavage detection assay. When IS*1081* plasmid exists as template, the Cas12a/gRNA system, mixture of IS*1081* gRNA and Cas12a protein, could degrade the target IS*1081*

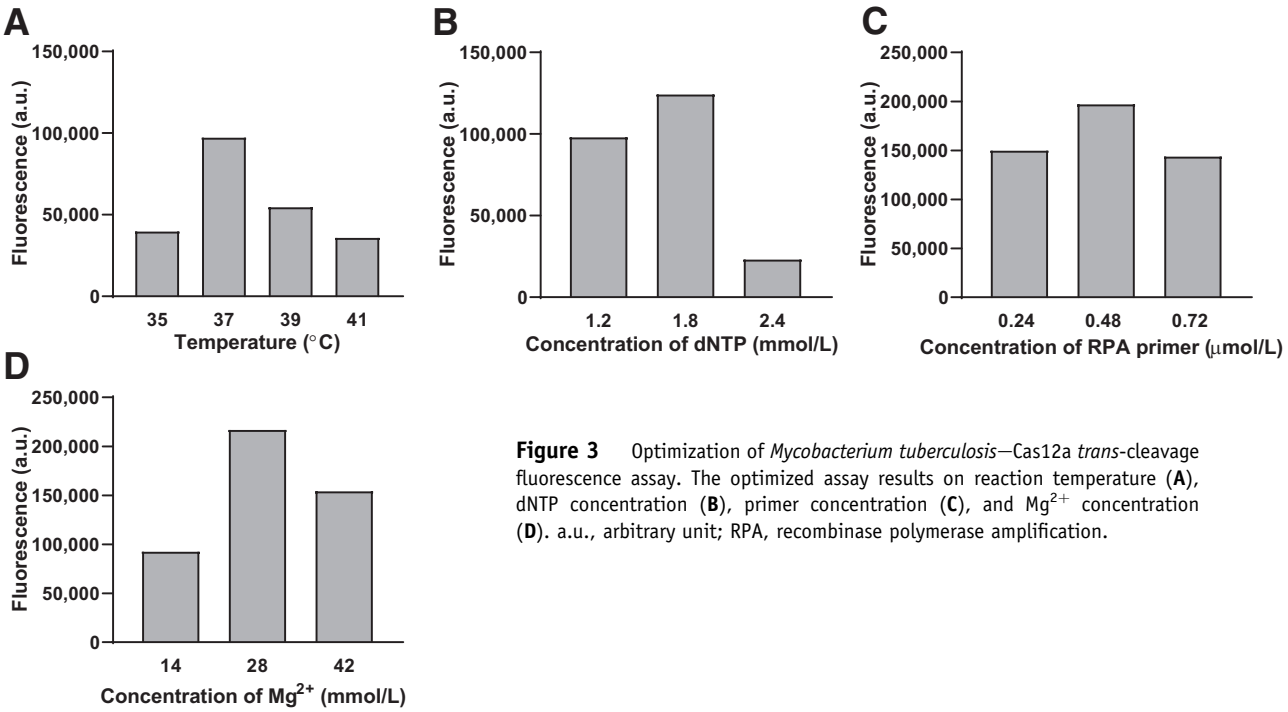


Figure 3 Optimization of *Mycobacterium tuberculosis*—Cas12a *trans*-cleavage fluorescence assay. The optimized assay results on reaction temperature (**A**), dNTP concentration (**B**), primer concentration (**C**), and Mg^{2+} concentration (**D**). a.u., arbitrary unit; RPA, recombinase polymerase amplification.

plasmid in *cis* and single-stranded DNA in *trans*, resulting in reduction of the number of targets and nontarget ssDNA (as reporter) in Cas12a/gRNA system or increased fluorescence intensity in Cas12a/gRNA fluorescence detection system (Figure 2). Excitingly, IS*1081* plasmid and ssDNA reporters were observed to be cleaved almost at the same time. These results suggested that the *cis*- and *trans*-cleavage activity ability of purified Cas12a protein is high, and Cas12a/gRNA system for MTB detection is feasible.

Optimization of Cas12a Fluorescence Assay for MTB

To simplify operation to achieve one-pot detection pattern and improve reaction efficiency, the volume of RPA reaction was controlled under 12.5 μL and then added compositions of Cas12a/gRNA system to the final volume of 20 μL for Cas12a/gRNA *trans*-cleavage fluorescence assay. Prominently, the RPA reaction conditions were optimized to maximize amplification efficiency, including reaction temperature and concentration of dNTP, primers, and Mg^{2+}. Amounts of 0.48 μmol/L forward and reverse primers and 28 mmol/L magnesium acetate of RPA reaction were found to be the main influencing factors for Cas12a/gRNA *trans*-cleavage fluorescence assay, and the assay obtained higher fluorescence intensity under optimized conditions using the same concentration of target (Figure 3 and Supplemental Figure S2). Therefore, these conditions were used in the following experiments for MTB detection. To further verify that the predicted target efficiency scores correlate with laboratory testing results in the Cas12a-based assay, five MTB-IS*1081* gRNAs with different target efficiency scores were synthesized, including two high (gRNA4 and gRNA9), one middle (gRNA10), and two low (gRNA13 and gRNA15), and their fluorescence signals were compared using the Cas12a/gRNA *trans*-cleavage fluorescence system. No signal was detected in gRNA13 and gRNA15 with low target efficiency score, and median fluorescence signal was detected in gRNA10 with middle target efficiency score, whereas high fluorescence signal was detected in gRNA4 and gRNA9 with high target efficiency score (the signal for both high score gRNAs is similar) (Supplemental Figure S3). From these results, it can be concluded that the target efficiency score correlates well linearly with laboratory testing results. Therefore, the gRNA9 was selected for the following analysis of MTB.

Performance of Cas12a Fluorescence Assay for MTB

To evaluate the ability of MTB-Cas12a *trans*-cleavage fluorescence assay, a series of gradient concentrations of diluted IS*1081* plasmids and genome of pathogens that may infect human respiratory tract were tested. For the sensitivity

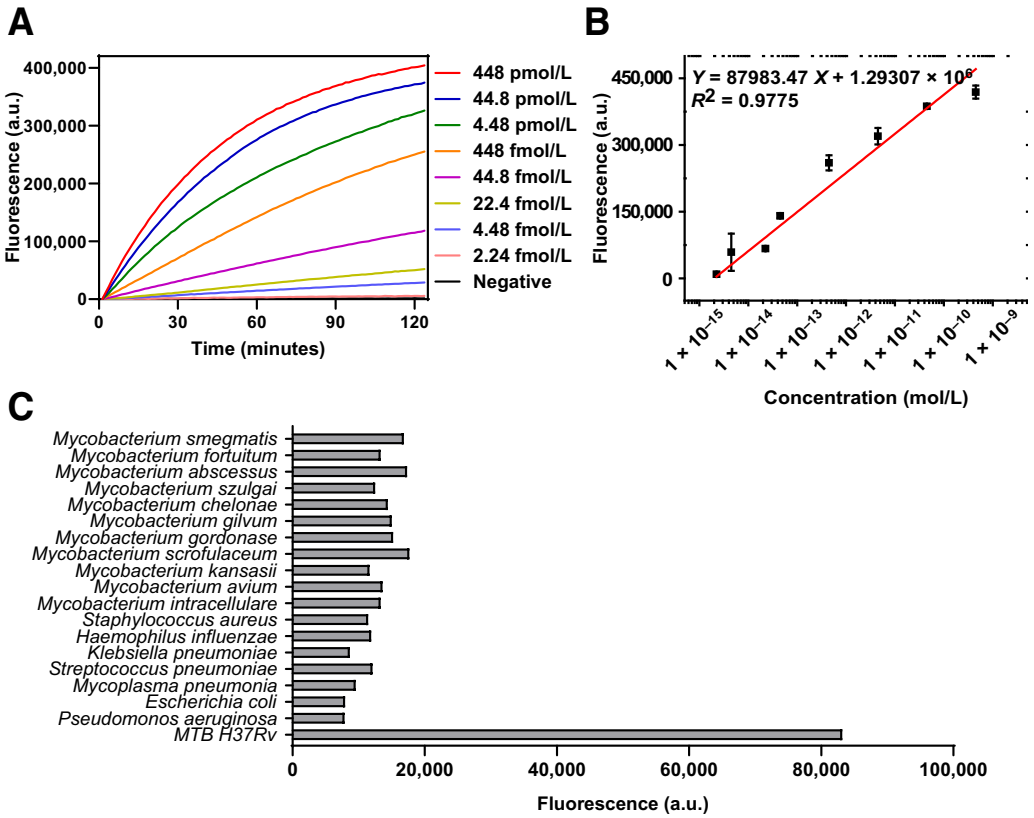


Figure 4 Detection performance of *Mycobacterium tuberculosis* (MTB)—Cas12a fluorescence assay. Real-time fluorescence intensity alteration of MTB-Cas12a assay for different concentration of targets (**A**); the calibration plots of fluorescence intensity versus the logarithm of target concentration (**B**); and the specificity of the MTB-Cas12a fluorescence assay, including nontuberculosis mycobacteria and other pathogens of respiratory tract (**C**). a.u., arbitrary unit.

Table 3 Clinical Diagnostic Performance of MTB-Cas12a Assay on Clinical Samples

Reference method	Results of reference method	MTB-Cas12a assay		
		Positive	Negative	Total
Culture method	Positive	139	1	140
	Negative	0	53	53
Total		139	54	193

MTB, *Mycobacterium tuberculosis*.

of MTB-Cas12a fluorescence assay, the fluorescence accumulation value of the assay decreased gradually with the decrease of concentration of IS*1081* plasmid, and the LOD for IS*1081* was 4.48 fmol/L (Figure 4A). According to the formula depicted above, it was equivalent to 2.59 to 1.85 μg/L of genome of *M. bovis* bacillus Calmette-Guérin strain from clinical samples. Comparing with RPA method[9] for MTB detection of IS*1081* with LOD of 20 fg for 50 μL volume, it achieved four orders of magnitude higher and got attogram level, which would dramatically promote positive detection rate. Meanwhile, the fluorescence value was proportional to the logarithm value of target concentrations over six orders of magnitude. The regression equation was $Y = 87983.47X + 1.29307 \times 10^6$, with R^2 of 0.9775 (Figure 4B). It was helpful to quantify MTB DNA accurately, especially regarding low concentration of MTB DNA as substrate.

To assess the specificity of the MTB-Cas12a fluorescence assay, genome of samples from a panel of nontuberculosis mycobacteria and pathogens that may infect human respiratory tract or their clinical symptoms similar to TB were tested. Three replicates were performed, and 500 pg of DNA from either *M. bovis* bacillus Calmette-Guérin or MTB (H37Rv) was used as positive template control for each batch. Consequently, according to the judgment of criteria above, no positive results were obtained from the tested DNA, except for the genome of MTB strains, indicating the high specificity of MTB-Cas12a fluorescence assay for MTB detection (Figure 4C).

To evaluate the clinical diagnostic performance of the MTB-Cas12a assay, 193 patient samples (140 positive and 53 negative for MTB), which had been confirmed by the gold standard culture method, were tested. Consequently, the fluorescence assay successfully detected 139 positive samples and 53 negative samples among 140 positive samples and 53 negative samples for MTB culture method. The detection accuracy of the assay based on 193 samples showed sensitivity of 99.29% (139/140) and specificity of 100% (53/53) (Table 3). Its performance was nearly similar to culture method, except that one of the culture-positive samples could not be detected (whose fluorescence intensity was <1.5 times that of negative result), likely due to the low concentration of genome and/or because it was degraded during storage or delivery process. It is possible that MTB target for guide RNA recognition harbors mutation, resulting in incomplete hybridization (namely, turn-off effect). To further demonstrate the performance of the Cas12a-based assay, a real-time PCR method was built based on TaqMan probe to validate detection results, according to a previous report,[36] with the LOD of one copy per microliter (Figure 5A). Some different concentration samples detected by the Cas12a assay were randomly selected, including two of high (sample 1 and sample 2), two of middle (sample 3 and sample 4), and two of low concentration (sample 5 and sample 6), and the clinical sample (sample 7), which was negative by the Cas12a assay but positive by MTB culture. The results of real-time quantitative PCR–based assay are consistent with the Cas12a-based assay: samples 1 and 2 were high concentration, samples 3 and 4 were middle concentration, samples 5 and 6 were low concentration, and sample 7 was negative (Figure 5B). As discussed above, this may result from the genome of one culture-positive sample being degraded during transportation and/or storage.

In addition, Cas12a/gRNA *trans*-cleavage fluorescence assay for MTB detection required an average time of 4 hours, including 1 hour of MTB DNA extraction, 1 hour for MTB DNA amplification by RPA, and 2 hours for detection of Cas12a/gRNA system. It showed a significant advantage over culture method, which needs a few days, and was a

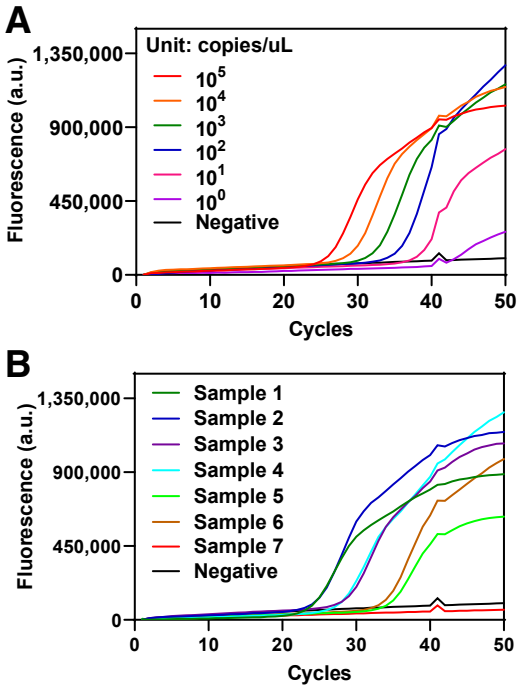


Figure 5 Validating the detection performance of different concentration samples by self-built real-time PCR method. **A:** The sensitivity of self-built real-time PCR detection method. **B:** The results of different concentration samples detected by the real-time PCR method. a.u., arbitrary unit.

little slow compared with RPA method for MTB DNA detection, which needs 1 to 2 hours totally.

Discussion

A rapid and sensitive detection method remains a challenge for clinical MTB diagnosis. Positive results of sputum smear microscopy required at least 5000 bacteria every milliliter and a skilled technician. And the culture method for MTB detection takes up to a few weeks and has a relatively low detection rate. They are not feasible for rapid molecular diagnosis of MTB in clinical applications. A rapid and sensitive MTB DNA detection method attempted to be improved previously, such as automatic integration plate-based PCR method—GeneXpert and RPA. Unfortunately, some adverse effects that were similar to the original disadvantages still remained, limiting its application in clinical MTB diagnosis. For example, continuous power input for temperature rising and falling and sample volume of 500 μL were required for GeneXpert. In contrast, Cas12a *trans*-cleavage fluorescence assay for MTB can occur at 37°C, which does not need a thermocycler, and it could obtain positive results under low volume of MTB sample (100 to 200 μL). Meanwhile, it was estimated to be $0.6138 for every sample,[31] compared with $35 to $50 for one sample of one cartridge using GeneXpert.

In this study, by optimizing the conditions of RPA reaction and Cas12a/gRNA system, one ultrasensitive method for Cas12a *trans*-cleavage fluorescence assay was established that could obtain 2.59 to 1.85 μg/L of limit of MTB genome (five to seven copies of IS*1081* per MTB genome) detection from clinical samples. Although IS*6110* is in multiple copies of up to 25 per genome of MTB and was used to be target in several commercial kits, such as Xpert MTB/RIF, the assay integrated the advantages of polymerase-mediated DNA amplification and Cas12a-mediated enzymatic signal amplification to compensate relatively low copies of IS*1081* in five to seven repeats per genome, and the ability of the Cas12a/gRNA system to detect MTB DNA with ultralow LOD was proved. Also, Cas12a/gRNA system for IS*1081* can strictly recognize MTB genome with protospacer adjacent motif structure, which showed high specificity for target. Moreover, this study on 193 samples highlights the potential of Cas12a/gRNA as an ultrasensitive, promising assay for diagnosis of clinical tuberculosis, despite missed detection of one sample of culture positive for MTB, which might due to the existence of mutation in the recognition sequence of Cas12-gRNA or low concentration of MTB DNA. So, to reduce the false-negative possibility, spacer sequence of gRNA should be avoided from region with mutation sequences or single-nucleotide polymorphism, especially mutation close to protospacer adjacent motif sequence.

In addition, there are also some limitations to the established Cas12a fluorescence assay. First, it takes nearly twice the amount of time to get the result compared with Xpert MTB/RIF. This may be the reason the assay got ultrasensitivity. But the time consumed totally could be controlled under the acceptable range for clinical diagnosis. Second, the Cas12a *trans*-cleavage fluorescence is detected by fluorescence detector but not visual readout, which requires no additional devices. Finally, apart from diagnosis, drug resistance is also increasingly severe in the world for treating MTB patients. It is an urgent need to analyze mutations of drug susceptibility for one or even second-line drugs via sensitive detection methods. With the natural characteristics of sensitive signal amplification of Cas/gRNA system, it has the potential to detect multiple mutations related to drug response. Ultrasensitive and specific Cas12a/gRNA system, combining polymerase-mediated DNA amplification for both pathogen and drug resistance detection, would permit the precise approach to control tuberculosis infection.

In a word, a CRISPR-Cas12a–based fluorescence assay was developed by combining isothermal recombinase polymerase amplification with Cas12a *trans*-cleavage activity, which could be activated by target-specific DNA with high sensitivity and selectivity for rapid detection of pathogen MTB from clinical samples, compared with the gold standard culture method, which needs a few days and extensive labor work. It is helpful to use this assay to prompt the confirmed diagnosis of MTB complex from no *Mycobacterium*, including nontuberculosis mycobacteria in clinical settings. But, in the future, a multicenter prospective study is needed to provide deeper understanding on its potential use for clinical diagnosis.

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Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2020.04.212*.

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