METHODS

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# High concentration of Cas12a effector tolerates more mismatches on ssDNA

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**Abstract**

Rapid pathogen detection is critical for prompt treatment, interrupting transmission routes, and decreasing morbidity and mortality. The V-type CRISPR system had been used for rapid pathogen detection. However, whether single-stranded DNA in CRISPR system can cause false positives remains undetermined. Herein, we show that high molar concentration of Cas12a effector tolerated more mismatches on ssDNA and activated its trans-cleavage activity at six base matches. Reducing Cas12a and crRNA molar concentration increased the minimal base-match number required for Cas12a ssDNA activation to 11, which reducing nonspecific activation. We then established a Cas12a-based *M tuberculosis* detection system with a primer having an 8 bp overlap with crRNA. This system did not exhibit primer-induced false positives, and minimum detection copy reached 1 copy/uL (inputting 1-μL sample) in standard strains. The Cas12a-based *M tuberculosis* detection system showed 80.0% sensitivity and 100.0% specificity in verification using clinical specimens, compared with Xpert MTB/RIF, which showed 72.0% sensitivity and 90.9% specificity. All these results prove that appropriate concentration of cas12a effector can effectively perform nucleic acid detection.

**KEYWORDS**

Cas12a, CRISPR/Cas, *Mycobacterium tuberculosis*, pathogen detection, trans-cleavage activity

## 1  |  INTRODUCTION

Rapid and sensitive pathogen detection cuts off routes of transmission and improves patient outcomes by enabling early treatment and appropriate antibiotic usage.[1] Several rapid methods having different adaptation, cost, sensitivity, and specificity, have been developed for pathogen detection.[2] Rapid pathogen detection, based on detection of nucleic acids via polymerase chain reaction (PCR), is often costly, requires specific equipment, and must be conducted in the laboratory environment.[3] Sequencing technology is still being perfected and cannot be directly used for clinical diagnosis.[4] This highlights the urgent need for a low-cost, simple, and highly

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**Abbreviations:** CMT, Catch *Mycobacteriumtuberculosis*; CRISPR/Cas, clustered regularly interspaced short palindromic repeats/CRISPR-associated; crRNA, CRISPR RNA; dsDNA, double-stranded DNA; MTB, *Mycobacterium tuberculosis*; NTM, non-tuberculous mycobacterium; PAM, protospacer-adjacent motif; PCR, polymerase chain reaction; RPA, recombinase polymerase amplification; ssDNA, single-stranded DNA.

sensitive and specific detection method requiring no additional equipment.

Over the past few years, the Clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) system has shown excellent performance in DNA editing[5-8] and regulation,[9] and RNA editing.[10] This system can also be potentially used for nucleic-acid detection.[11-14] The class 2V-type and VI-type CRISPR systems are currently used for gene detection. The V-type CRISPR system, including Cas12a,[15-17] Cas12b,[18,19] Cas14,[20,21] can be used directly for DNA detection after amplification of target gene by Recombinase Polymerase Amplification (RPA). In the VI-type CRISPR systems, especially those using Cas13a,[10,22] Cas13b,[23] and Cas13d,[24,25] DNA is detected via transcription step after amplification of the target gene. These two CRISPR system can also be used for RNA detection by adding a reverse-transcription step. Cas12a is more convenient and stable for DNA detection than Cas13a because Cas12a does not require an additional step involving reverse transcription or transcription.

Cas12a is guided by a single CRISPR RNA (crRNA) with a T-rich protospacer-adjacent motif (PAM) sequence to cleave double-stranded DNA (dsDNA), or without PAM to cleave ssDNA targets. Unlike Cas9, Cas12a cleaves both the target and nontarget strands of a targeted dsDNA using a single active site in the RuvC catalytic pocket. RNA-guided DNA binding generates indiscriminate cleavage of ssDNA by Cas12a, which completely degrades ssDNA molecules after target dsDNA or ssDNA is recognized; this is called trans-cleavage activity.[15,17] If trans-cleavage is used to degrade designed DNA to generate an identifiable signal, the target gene can be detected directly. The entire detection process can be completed within 1 hour and conducted at room temperature (25°C), with no additional temperature-control equipment required. This approach is also much more cost-effective than are other currently available methods for nucleic-acid detection.[15,16] CRISPR-based nucleic acid detection enables a straightforward, inexpensive, and rapid pathogen detection.[26] However, whether the ssDNA used in this system activates trans-cleavage activity remains undetermined. This may lead to systemic false-positive results, which impedes pathogen detection.

Herein, we investigated the minimum number of base matches needed to activate the trans-cleavage of ssDNA via Cas12a-mediated approach. We also established a Cas12a-based *Mycobacterium tuberculosis* (MTB) detection system to test the new mechanism.

## 2 | MATERIALS AND METHODS

### 2.1 | In vitro transcription and synthesis of crRNA

EuPaGDT was used to design crRNA. crRNA was also synthesized by Ruibiotech Co. (Beijing, China) or using in vitro transcription via HiScribe T7 Rapid and Efficient RNA Synthesis Kit (New England Biolab, Ipswich, MA, USA) according to manufacturer's instructions. Briefly, An oligonucleotide containing the T7 promoter and crRNA sequences was annealed with an overlapping reverse T7 promoter oligo used as a transcriptional starter. A 1 µg annealed product was added to the reaction mixture, and the mixture was incubated overnight. The DNA template was then removed by treatment with DNase, and in vitro transcribed crRNA was purified via Monarch RNA Cleanup Kit (New England Biolab). Then, 2-µL aliquots of crRNA were dispensed into PCR tubes at approximately 5-10 µg per tube.

### 2.2 | DNA cleavage assays

The Cas12a cleavage reaction was carried out at 37°C in a buffer containing 5 mM NaCl, 1 mM Tris-HCl, 1 mM MgCl2, and 10 µg/mL BSA (pH 7.9). Lb Cas12a was stored in a buffer containing 0.1 mM EDTA, 50% glycerol, 500 mM NaCl, 20 mM sodium acetate, and 0.1 Mm TCEP at a concentration of 20×. Lb Cas12a was also synthesized by New England Biolabs (EnGen Lba Cas12a). M13-targeting was performed using 35 nM-3.5 µM crRNA, 70 nM-7 µM Cas12a, 1-4 µM ssDNA or 40 nM dsDNA, and 30 nM M13mp18 Single Strand DNA (Takara, Dalian, China). The reaction was performed at 37°C for 30 minutes to 3 hours according to our experimental requirements. Detection via real-time fluorescence was performed using 35 nM crRNA, 70 nM Cas12a, 40 nM dsDNA (or 3 µL RPA product), and 50 nM FAM-ssDNA-BHQ1 (Ruibiotech Co). The reaction was performed at 37°C for 30 minutes to 1 hour according to our experimental requirements; fluorescence was detected using spark 100 (Tecan, Männedorf, Switzerland). Detection via lateral flow strips was performed using 35 nM crRNA, 70 nM Cas12a, 40 nM dsDNA (or 3 µL RPA product), and 50 nM FAM-ssDNA-BIO (Ruibiotech Co). The reaction was carried out at 37°C for 30 minutes to 1 hour according to the experimental requirements; then, the reaction mixture was added to 100 µL HybriDetect Assay Buffer. Dipsticks with sample application area were placed into the solution and incubated for 5-15 minutes in an upright position at 25°C.

### 2.3 | Recombinase polymerase amplification

Primers for recombinase polymerase amplification (RPA) were designed using NCBI PRIMER BLAST and synthesized by Ruibiotech Co. RPA was performed using the TwistAmp Basic (TwistDx, Cambridge, UK) kit according to the manufacturer's instructions; 1-3 µL genomic DNA extract was used as input.

## 2.4 | Extraction and preparation of template DNA

A DNeasy Blood & Tissue Kit (Qiagen, Dusseldorf, German) was used to extract genomic DNA from standard strains according manufacturer's instructions. Briefly, each boiled and inactivated strain was added to a grinding tube containing glass beads, and cell walls were broken by mechanical agitation. Then, DNA was extracted according to the manufacturer's instructions in Pretreatment for Gram-Positive Bacteria protocol. A Loopamp kit (Eiken Chemical Co., Tokyo, Japan) was used to extract MTB genomic DNA from clinical samples according to the manufacturer's instructions.

## 2.5 | Treatment and storage of clinical samples

Clinical samples usage was approved by the institutional review board of China-Japan friendship Hospital (2020-13-k10). Clinical samples including specimens, bronchoalveolar lavage fluid, hydrothorax, and homogenate of needle biopsy were divided into three parts. One part was combined with 4% NaOH for 15 minutes, centrifuged at $1500g$ for 5 minutes, washed once with PBS, resuspended in 1 mL PBS, dispensed into MGIT tubes at 0.5 mL per tube, and cultured using BACTEC MGIT960 for not longer than 40 days. Sanger DNA sequencing was used to detect Hsp65 of *M tuberculosis* in culture-positive specimens. The second part was combined with two-fold volume of Xpert specimen-treatment solution. This mixture was vortexed for 10 seconds and allowed to stand for 10 minutes at room temperature. The mixture was vortexed again for 10 seconds and allowed to stand for 5 minutes. Afterward, an appropriate amount of the mixture was added to the cartridge for detection. The third part was retained at −20° for CRISPR diagnose.

## 2.6 | Preparation of target-gene DNA fragment

Single-stranded target-gene DNA fragment was directly synthesized by Ruibiotech C o. Double-stranded target-gene DNA was obtained via PCR and gel electrophoresis. Primers were designed by NCBI PRIMER BLAST, and PCR was performed using PrimeSTAR HS DNA Polymerase with GC Buffer (Takara, R044B) according to the manufacturer's instructions. PCR reaction parameters were as follows: 98°C for 10 minutes, 35 cycles at 98°C with 10 seconds per cycle, 60°C for 5 seconds, 72°C for 1 minute, and an extension at 72°C for 5 minutes.

## 2.7 | RNA and DNA sequence used in this study

crRNA-6110:5′-UAAUUUCUACUAAGUGUAGAUucacc gacgccuacgcucgc-3′

crRNA-c0: 5′-UAAUUUCUACUAAGUGUAGAUucaca ccaaguguuucgacc-3′

crRNA-c1: 5′-UAAUUUCUACUAAGUGUAGAUucaau gacgccuacgauugc-3′

crRNA-c2: 5′-UAAUUUCUACUAAGUGUAGAUucaau gauaccuacgcucgc-3′

crRNA-c3:5′-UAAUUUCUACUAAGUGUAGAUucacac caagugucgcgacc-3′

crRNA-c4: 5′-UAAUUUCUACUAAGUGUAGAUucaau gacgccuacgcucgc-3′

RPA primer for amplification MTB is6110 gene, Forward primer: 5′-cgacccgccagcccaggatcctgcgagcgt-3′, Reverse primer:5′- tccagcgccgcttcggaccaccagcacctaac-3′

FAM-ssDNA-BIO:5′-FAM-TTAGGTTAGGATTGGTT-BIO-3′

FAM-ssDNA-BHQ1:5′-FAM-TTAGGTTAGGAT TGGTT-BHQ-3′

Target ssDNA used to match crRNA-c0:

15bp: 5′-GGTCGAAACACTTGG-3′
14bp: 5′-GGTCGAAACACTTG-3′
13bp: 5′-GGTCGAAACACTT-3′
12bp: 5′-GGTCGAAACACT-3′
11bp: 5′-GGTCGAAACAC-3′
10bp: 5′-GGTCGAAACA-3′
9bp: 5′-GGTCGAAAC-3′
8bp: 5′-GGTCGAAA-3′

## 2.8 | Statistical information

Exact McNemar test was used to compare sensitivity and specificity of clinical samples. To ensure reproducibility, experiments were performed using n = 3 independent samples. Statistical significance was defined as $P < 0.05$ for two-tailed. Statistical Analysis was performed using the SPSS software (version 19.0).

# 3 | RESULTS

## 3.1 | A minimum of 11 base matches was required to initiate trans-cleavage

Because the ssDNA and crRNA are not strictly matched, ssDNA primers may also activate the system. Therefore, we investigated the stringency of ssDNA matching by generating changes in ssDNA but not in matching crRNA
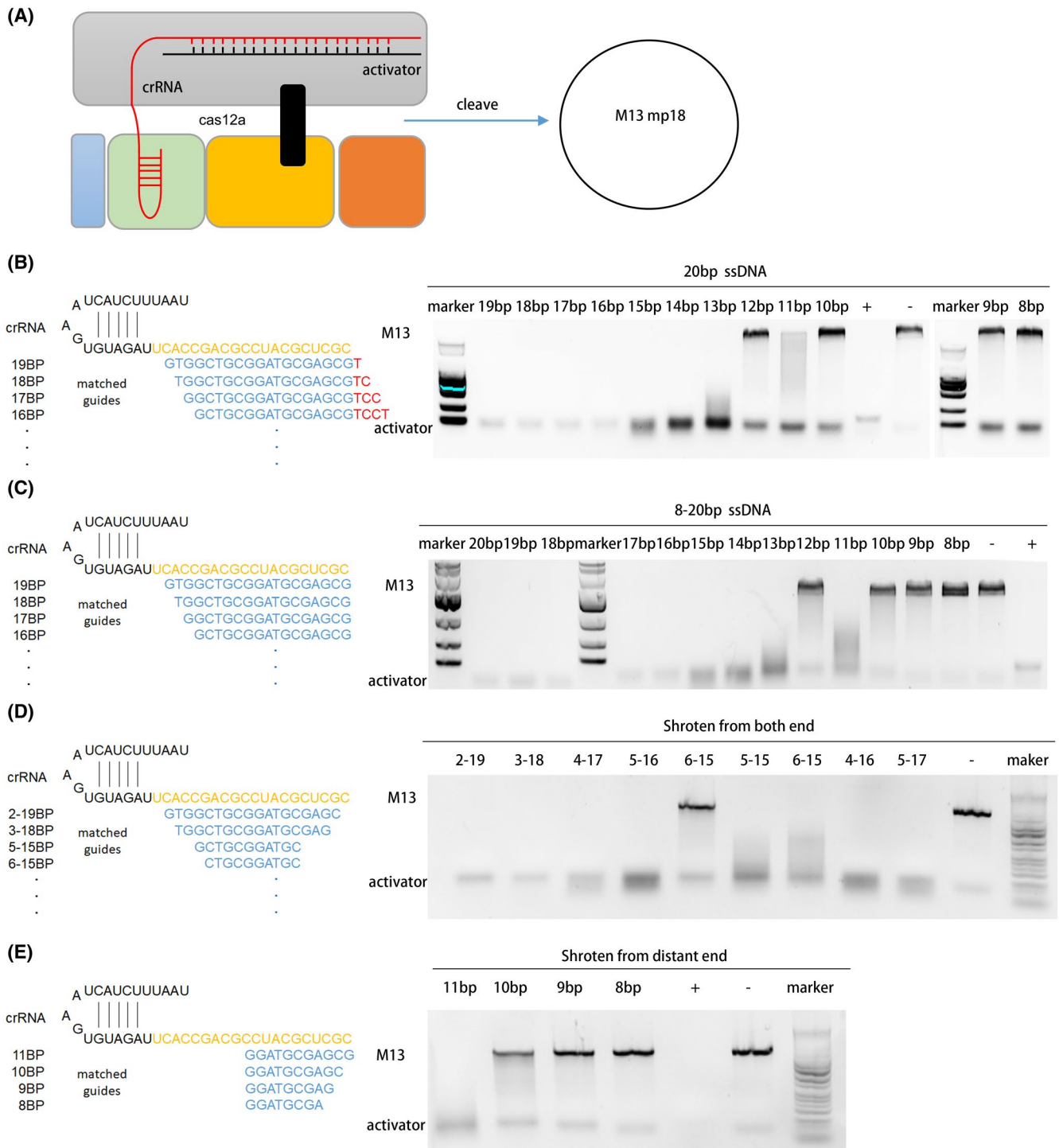
**(A)**



**(B)**



**(C)**



**(D)**



**(E)**



**FIGURE 1** Eleven minimum base matches are required for trans-cleavage. A, Schematic of Cas12a effector in the trans-cleavage of M13 ssDNA. B, A minimum of 11 base matches at the distal end (in the opposite direction of PAM) can activate trans-cleavage activity. C, Target gene activation with only matching sequences shows that 11 base matches at the distal end can activate trans-cleavage. D, Shortened target gene fragment from both ends shows that 11 base matches can activate trans-cleavage activity. E, Further shortened target gene fragment from the distal end shows that 11 bases matches are required to activate trans-cleavage activity. B-F, Left: Matching schematic of single-stranded DNA and crRNA. Right: Results of gel electrophoresis based on the schematic shown on the left. nt: indicates the matching number from the corresponding double-stranded PAM region. ssDNA: M13 single-stranded DNA. Activator: corresponding 20 bp target-gene fragment

(Figure 1A). A single base shift induced in ssDNA showed that a minimum of 11 base matches at the distal end (in the opposite direction of PAM) of ssDNA could activate

trans-cleavage activity (Figure 1B) when the molar concentration of crRNA and Cas12a was 70 and 35 nM. After redundant bases were deleted to reserve only the matching

bases, the minimum base-match requirement was still that of 11 bases (Figure 1C). By simultaneously reducing the matching bases from both ends of ssDNA, we were able to activate trans-cleavage using a minimum of 11 base matches (Figure 1D). Further reduction of matching bases from the distal end of ssDNA indicated that at least 11 base matches were required to completely degrade an M13 single-stranded DNA. However, partial degradation did occur at only 10 base matches (Figure 1E).
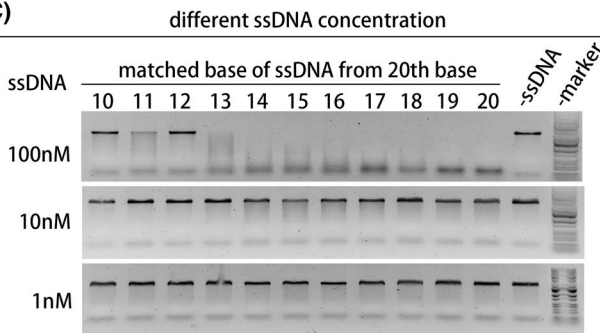
## 3.2 | Minimum activating base number is affected by the GC/ratio of crRNA or molar concentration of Cas12a and target ssDNA

We studied the effect of the GC/AT ratio of crRNA on the minimum activating base number. As shown in Figure 2A, we changed the GC/AT ratio of bases 6-20 of crRNA from 1/14 to 14/1. Interestingly, as the GC/AT ratio increased from 1/14 to 12/3, the minimum activating base number decreased
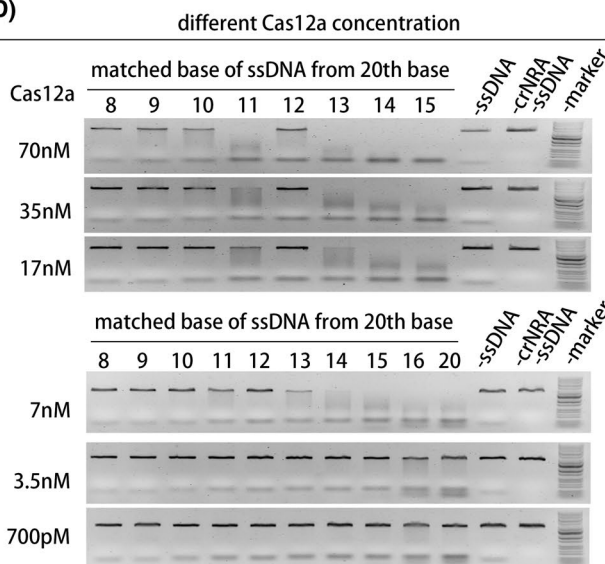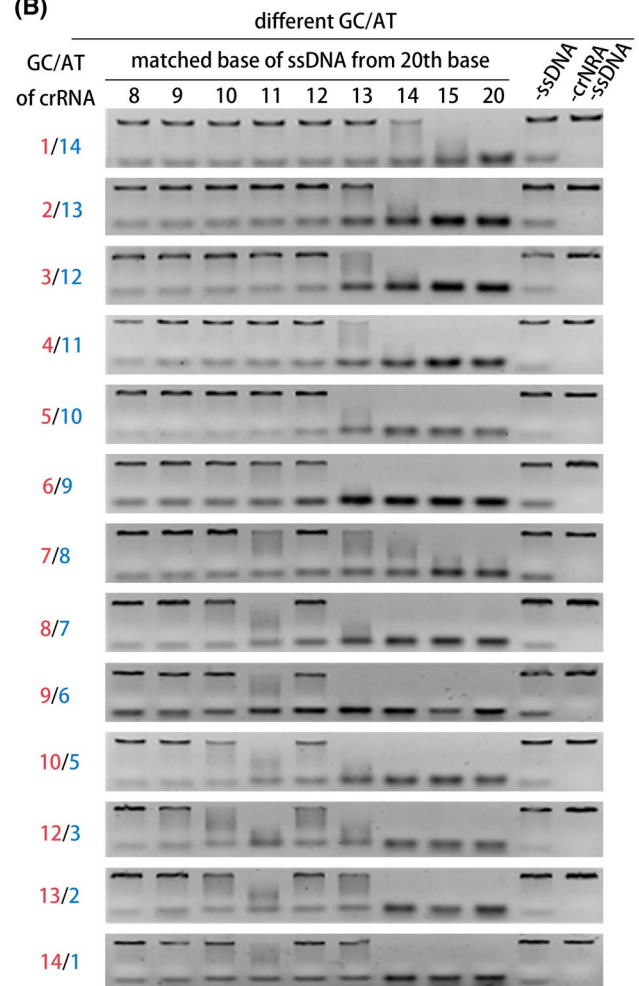


**FIGURE 2** Minimum activating base number is affected by the GC/ratio of crRNA or molar concentration of Cas12a and target ssDNA. A, Display of different GC/AT of crRNA. B, Corresponding gel electrophoresis image according to A. C, Effect of decreasing the concentration of ssDNA on the minimum activating base number. D, Effect of decreasing the concentration of Cas12a on the minimum activating base number

from 15 to 11 (Figure 2B). As the GC/AT ratio increased from 12/3 to 14/1, the 13-base match gradually failed to activate trans-cleavage activity (Figure 2B). Then, we studied the effect of decreasing the concentration of ssDNA on the minimum activating base number. The results showed that when concentration of ssDNA was reduced from 1 uM to

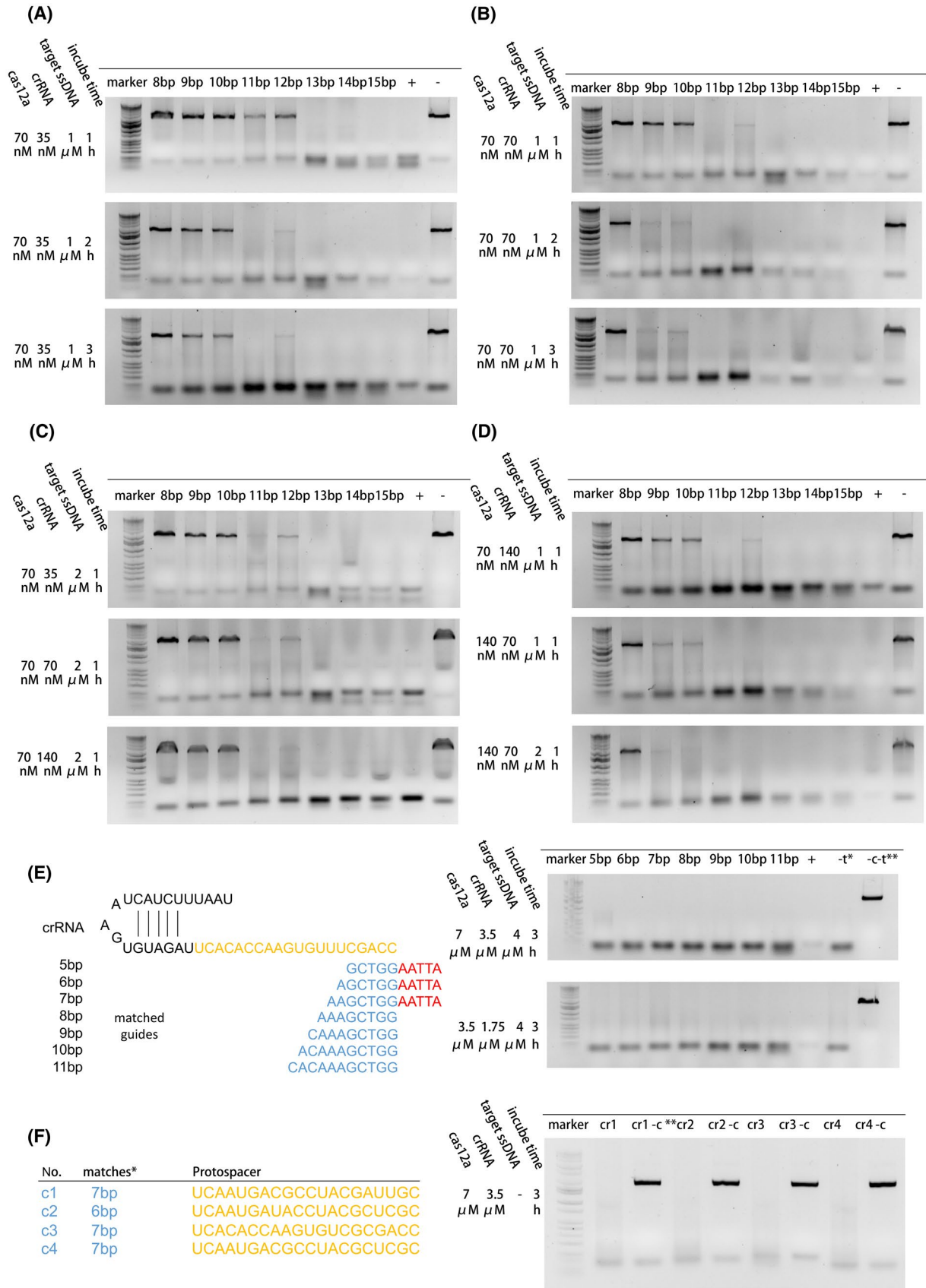

(A)

(B)

(C)

(D)

(E)

(F)

| No. | matches* | Protospacer |
|-----|----------|-------------|
| c1 | 7bp | UCAAUGACGCCUACGAUUGC |
| c2 | 6bp | UCAAUGAUACCUACGCUCGC |
| c3 | 7bp | UCACACCAAGUGUCGCGACC |
| c4 | 7bp | UCAAUGACGCCUACGCUCGC |

**FIGURE 3** Minimum base number required to activate trans-cleavage was decided by the molar concentration of the composition. A, Molar concentration of crRNA and Cas12a was 70 and 35 nM, respectively. Minimum number of bases required to activate trans-cleavage was 11 per 1 hour; 2-3 hours resulted in scant degradation of 9-10 bp or M13 ss DNA. B, Molar concentration of crRNA and Cas12a was 70 and 70 nM, respectively; minimum number of bases required to activate trans-cleavage was 11 in 1 hour, and 9-10 bp M13 ss DNA was degraded in 2-3 hours. C, Molar concentration was as indicated in 1 hour. The minimum number of bases required to activate trans-cleavage was 11. D, Molar concentration was as indicated in 1 hour. When crRNA was 70 nM, and Cas12a was 140 nM, and target ssDNA was 2 µm, the minimum number of bases required to activate trans-cleavage was 9 in 1 hour. E, Using 100 or 50 times the molar concentration of Cas12a and crRNA degraded all M13 ssDNA, while M13 ssDNA treated by a composition not containing Cas12a showed no degradation. *-t: without target DNA; **-c-t: without Cas12a or target DNA. F, M13 degradation using different crRNA. *: indicates that the base number of crRNA continuously matched with M13 ssDNA

100 nM, minimum activating base number was still 11, but when it was reduced to 10 nM, it was no longer able to activate trans-cleavage activity (Figure 2C). In addition, the decrease of Cas12a concentration also had a clear effect on the minimum number of activated bases. As its concentration decreased from 70 nM to 7 nM, the minimum activating base number increased to 13(Figure 2D).

## 3.3 | Minimum activating base number is determined by the molar concentration of the composition

Since we observed a partial degradation of 10 bp during the 1-hour incubation, we further observed the degradation of the delay time. Incubation for an additional 1 to 2 hours allowed for trans-cleavage with 9 or 10 base matches, but no difference for 8 base matches with the control group was observed in 3 hours (Figure 3A). We then adjusted the molar concentration of crRNA and Cas12a to 70 and 70 nM, respectively. Our results show that ssDNA was almost completely degraded in 3 hours with 9 and 10 base matches, while partial degradation was also observed at 1 and 2 hours using these numbers of base matches (Figure 3B). For a more rapid diagnosis, we increased the concentration of Cas12a and crRNA to 140 to 70 nM, respectively. Our results indicate that M13 single-stranded DNA was degraded entirely within 1 hour using 2 µM 9 bp ssDNA as activator (Figure 3C,D). It is possible that high concentration of Cas12a and crRNA may require shorter ssDNA to fully activate trans-cleavage. Therefore, we increased its concentration of Cas12a and crRNA by 100 times. As shown in Figure 3E, all ssDNA was degraded except for the line without Cas12a. This result demonstrates that M13 ssDNA could bind the crRNA to generate an effector at extremely high concentration of Cas12a effector. And we found the crRNA used in this step contained a 7 bp continuous match with M13. We then assessed another crRNA having a 6-7 bp continuous match with M13 ssDNA. Our results show that the entire M13 ssDNA was degraded in 3 hours (Figure 3F), indicating that high molar concentration of the Cas12a effector tolerated more mismatches in ssDNA.

## 3.4 | Characteristics of trans-cleavage of 70-nM crRNA and 35-nM Cas12a

Our results show that high molar concentration of the Cas12a effector tolerated more mismatches on ssDNA. Furthermore, 70-nM crRNA and 35-nM Cas12a used with 1-µM target ssDNA extended the minimal required number of activated bases to 11. We further examined the characteristics of trans-cleavage activity by extending or shortening the length of crRNA. When crRNA was shortened to 15 bp, full activation of trans-cleavage activity still required 11 base-pair matches (Figure 4A). When crRNA was extended to 25 bp, trans-cleavage activity required at least 16 bp matches (Figure 4B). These results indicate that a 20 bp match near the corresponding PAM region was critical for activating trans-cleavage. Next, we examined the continuity of ssDNA required to activate trans-cleavage. Our results indicate that activation of trans-cleavage activity required consecutively matched ssDNA and could not be activated with any base interruptions when using the minimal matched bases (Figure 4C). In terms of pathogen detection, our results show that dsDNA activate trans-cleavage activity and completely degraded the entire M13 single-stranded DNA within 15 minutes (Figure 4C). We then investigated the minimum dsDNA concentration needed to activate trans-cleavage activity. As shown in Figure 4E, even 10 pM target dsDNA could be used to degrade all of M13 single-stranded DNA within 30 minutes. Our results indicate that 70 nM Cas12a and 35 nM crRNA can be used for the detection of pathogens.

## 3.5 | Minimum detection capabilities of Cas12a *M tuberculosis* detection system as assessed using standard strains

To assess the utility of our Cas12a-mediated approach in pathogen detection, we designed an MTB identification system designated as Catch *M tuberculosis* (CMT). For this, nucleic acid was extracted and amplified for the target gene using RPA with reverse primers containing an 8-base overlap with crRNA (almost no trans-cleavage activity by 70 nM Cas12a and 35 nM crRNA). The entire process
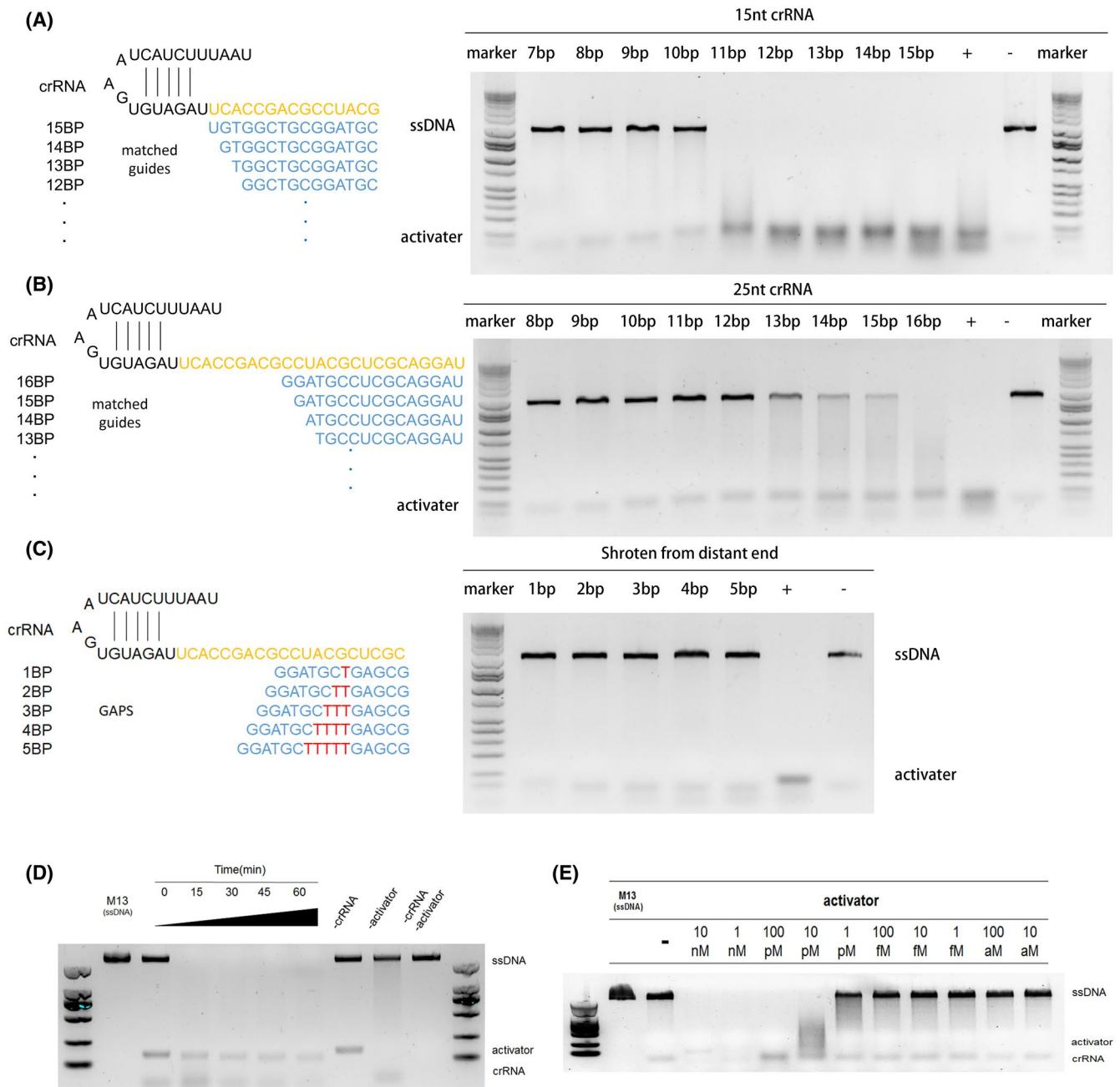
**FIGURE 4** Characteristics of trans-cleavage of 70-nM crRNA and 35-nM Cas12a. A, crRNA shortened to 15 nt still required 11 bp matches of crRNA and target gene to activate trans-cleavage activity. B, crRNA extended to 25 nt required 16 bp matches of crRNA and target gene to activate trans-cleavage activity. C, Consecutive matches without any base interruption were needed to activate trans-cleavage activity. D, crRNA activated trans-cleavage activity and completely degraded M13 single-stranded DNA within 15 minutes. Activator: IS6110 dsDNA. crRNA: crRNA-61010, ssDNA: M13 single-stranded DNA. F, At least 10 pM dsDNA was required to degrade all of M13 single-stranded DNA within 30 minutes. Activator: IS6110 dsDNA. crRNA: crRNA-6110, ssDNA: M13 single-stranded DNA

was completed within 40-60 minutes (Figure 5A). We then evaluated the minimum detection limit of MTB using genomic DNA of the H37Rv strain. As shown in Figure 5B, the minimum detection limit of our MTB system was 0.07 aM (0.7 aM≈1 copy) as determined using gel electrophoresis. Using fluorescent lateral flow test paper, we observed that 0.7 aM H37Rv genomic DNA was strongly positive, while 0.07 aM H37Rv genomic DNA showed a

weak positive band (Figure 5C). Real-time fluorescence showed a slight increase in the signal for 0.07 aM H37Rv genomic DNA and a significant increase in that for 0.7 aM H37Rv genomic DNA (Figure 5D,E). These results show that the minimum detection limit of our system for MTB was 1 copy/uL (using 1-μL sample as input). The cutoff values for negative and positive values were set to the lower limit of 95% confidence interval (95% CI) of the lowest
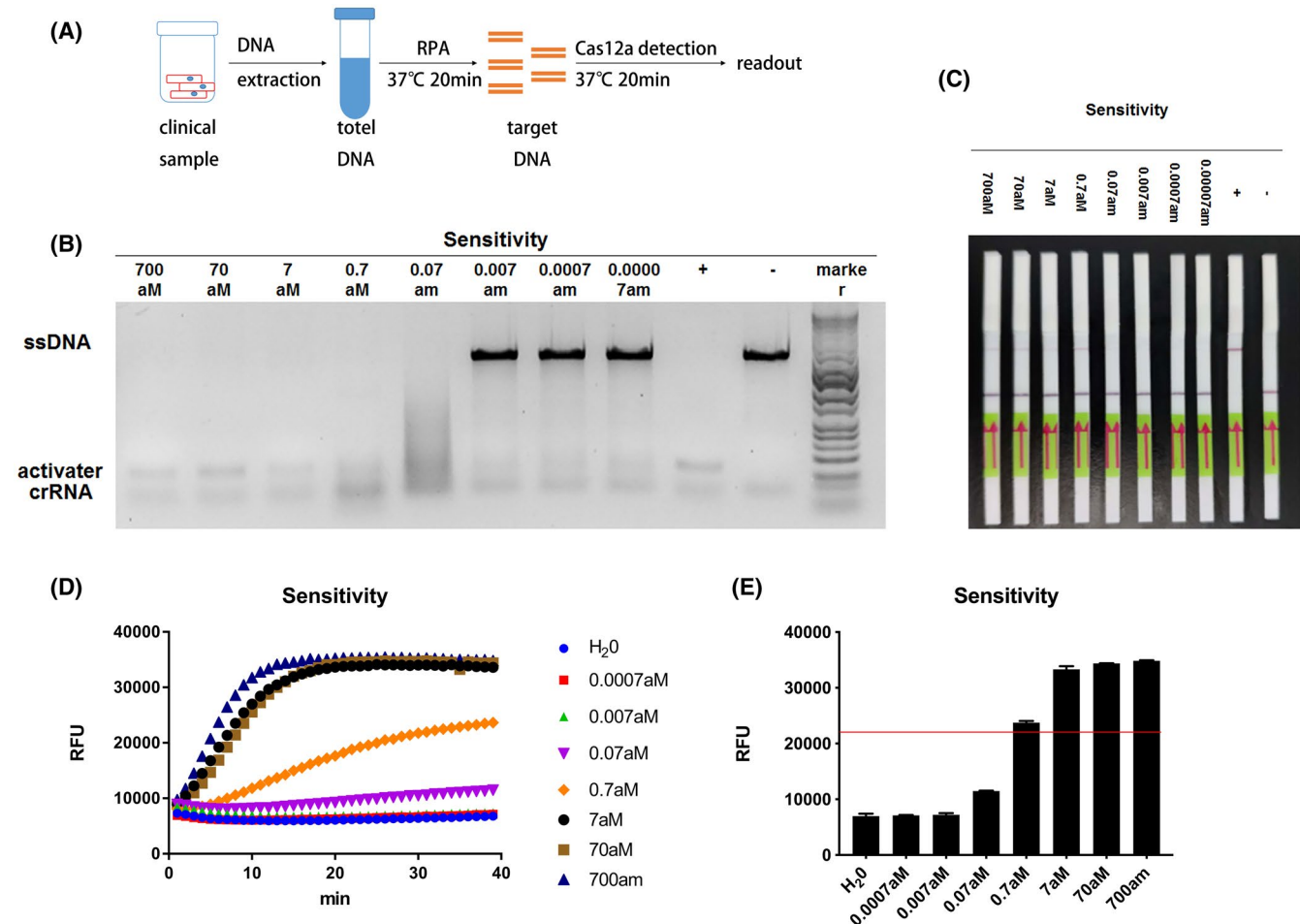
**FIGURE 5** Minimum detection limit of standard strains by Cas12a *M tuberculosis* detection system. A, Schematic diagram of the Cas12a *Mycobacterium tuberculosis* detection system. B, Minimum detection limit by *M tuberculosis* Cas12a detection system was 0.07 aM (0.7 aM≈1 copy) as assessed using gel electrophoresis with standard strains. ssDNA: M13 single-stranded DNA. Activator: H37RV RPA product. crRNA: crRNA-6110. C, Fluorescent lateral flow test strip test, used to assess the *M tuberculosis* Cas12a detection system using standard strains, showed a limit of 0.7 aM. D, Real-time fluorescence, used to assess the *M tuberculosis* Cas12a detection system using standard strains, showed slight increase in fluorescence for 0.07 aM H37Rv genomic DNA, and significant increase in fluorescence for 0.7 aM H37Rv genomic DNA. E, Final reading for real-time fluorescence. Red line: the cutoff values for negative and positive values are set to the lower limit of 95% CI of the lowest detection value

detection value (red line, Figure 5E). The specificity of our Cas12a-mediated MTB detection system was evaluated using non-tuberculous mycobacterium (NTM). As shown in Figure 6A, no degradation of M13 single-stranded DNA was observed in NTM samples, which agreed with the results obtained using real-time fluorescence and fluorescent lateral flow test strips (Figure 6B-D). No degradation occurred in additional 17 NTM strains, which were used to confirm the specificity of the system (Figure 6E).

## 3.6 | Verification of Cas12a *M tuberculosis* detection system using clinical samples

We next evaluated the performance of our Cas12a MTB detection system using 69 clinical samples by real-time

fluorescence. We additionally performed a tuberculosis culture test, which is the gold standard for the detection of MTB, and Xpert MTB/RIF test, which is the FDA-approved method for MTB based on multiplex PCR. In using culture and Xpert double-positive subset, only one sample showed a negative result as assessed via the Cas12a assay (Figure 7A). Four samples that were culture-negative and Xpert positive were all negative as assessed using our Cas12a MTB detection system Among the seven culture-positive samples that Xpert could not detect, three were shown positive using the Cas12a assay (Figure 7B-C). The 40 clinical samples, shown negative by both culture and Xpert, were also shown negative using the Cas12a assay (Figure 7D). Statistical analyses demonstrate that the Cas12a-based *M tuberculosis* detection system showed a high sensitivity of 80.0% and specificity of 100.0%, compared with the Xpert MTB/RIF system with its
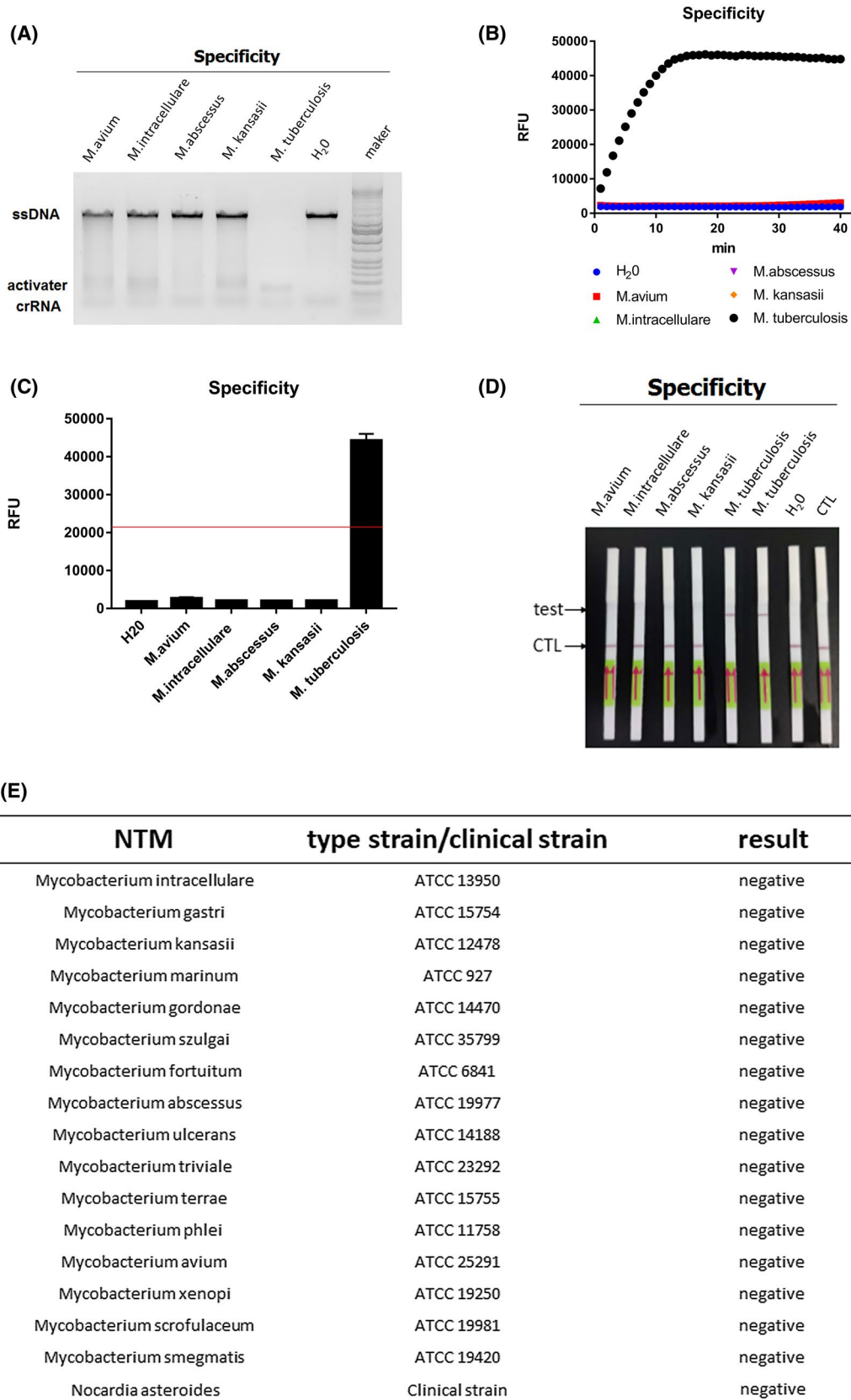
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**FIGURE 6** Specificity of the Cas12a *Mycobacterium tuberculosis* detection system. A, No degradation of M13 single-stranded DNA by the *M tuberculosis* Cas12a detection system was observed, as assessed via gel electrophoresis using four NTM strains. ssDNA: M13 single-stranded DNA. Activator: corresponding NTM RPA product. crRNA: crRNA-6110. B, Real-time fluorescence, used to assess the *M tuberculosis* Cas12a detection system using four NTM strains, showed no significant increase in fluorescence. C, Final reading of real-time fluorescence. D, No positive results were obtained via fluorescent lateral-flow test strips used to assess the *M tuberculosis* Cas12a identification system with four NTM strains. E, NTM type strain did not generate positive results with the Cas12a *M tuberculosis* detection system
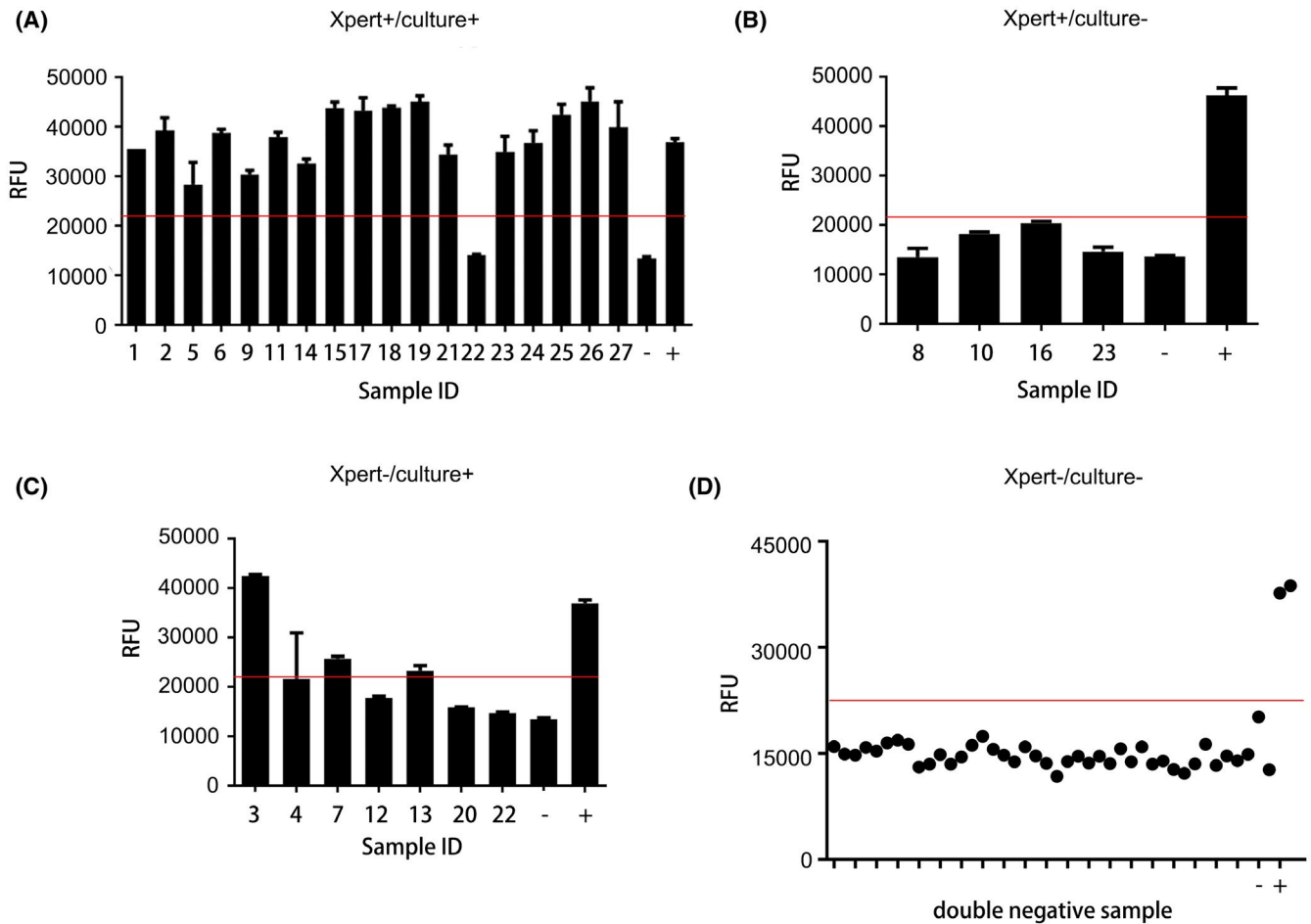
**FIGURE 7** Verification of Cas12a *Mycobacterium tuberculosis* detection system using clinical samples and real-time fluorescence. A, Only one sample showed negative results for Cas12a assay as assessed using Xpert[+]/Culture[+] clinical samples. Error bars represent the mean ± SD, n = 3 replicates. B, Cas12a assay negative results obtained using Xpert[+]/Culture[−] clinical samples. Error bars represent the mean ± SD, n = 3 replicates. C, Three samples showed positive results as assessed using the Cas12a system and Xpert[−]/Culture[+] clinical samples. Error bars represent the mean ± SD, n = 3 replicates. D, All samples showed negative results as assessed using Xpert[−]/Culture[−] clinical samples

72.0% sensitivity and 90.9% specificity but with no statistical difference ($P = .625$, exact McNemar test).

## 4 | DISCUSSION

The CRISPR/Cas system, one of the most important recent scientific breakthroughs, is a widely used genetic editing tool.[27] In addition to being used in gene therapy, the CRISPR/Cas system may also be useful for detection of pathogenic nuclei in translational medicine.[28] In this study, we aimed to eliminate the false positives caused by ssDNA by determining the minimum number of ssDNA base matches needed to activate Cas12a-mediated trans-cleavage. We then designed an MTB detection system based on this minimum number of ssDNA base matches required to activate trans-cleavage.

We hypothesized that shorter ssDNA sequences, combined with Cas12a and crRNA, would generate fewer effectors. Therefore, increasing the concentration of Cas12a and

crRNA would produce more effectors and accelerate the degradation of M13 single-stranded DNA. Sungchul et al have shown that the frequency of ssDNA binding to Cas1-Cas2 is related to the canonical pre-spacer DNA length.[29] Our study shows that Cas12a may use the same or similar mechanism for binding ssDNA.

The crRNA of Cas12a does not need to exceed 20 nt. Our results show that when crRNA length was less than 20 nt, the minimum number of bases required to activate trans-cleavage was 11 nt; however, when crRNA length was longer than 20 nt, the minimum number of bases needed to be increased by more than 20 nt on the basis of 11. This may be because Cas12a starts cleaving ssDNA at the 20nd-24nd base,[15] and bases after 20nd are considered invalid. Therefore, 20-nt crRNA maybe the optimal choice.[15]

Because Cas12a tolerates more ssDNA mismatches, we supposed that the gap would not affect the activation of its trans-cleavage activity. However, using the minimal activation sequence did not allow for a gap of even one base,

requiring continuous uninterrupted matching and demonstrating a need for stringency, in this case.

We observed a special result in the experiment. In some Figure, 11 base matches triggered trans-cleavage activity, but not 10 and 12 (Figures 1B,C, 3B-D). According to Takashi Yamano et al, bridge helix structure of AsCas12a, LbCas12a, and FnCas12a was located 9-10 bases from the PAM region[30] and our reverse 11th base was exactly on the position of the 9th base from the PAM region. In addition, according to the study of Majda Bratovič et al, arginines in the Cas9 bridge helix influenced guide RNA, and target DNA binding and cleavage.[31] There were two residues of arginine on the 9th base of Cas12a (951 and 955), so the residue of arginine might have an impact on the binding of single-stranded DNA and crRNA and the activation of trans-cleavage activity by this base. Of course, these required in-depth study of protein structure.

The CRISPR/Cas12a detection system has a wide range of applications. We established an MTB detection system which that could react at room temperature and complete the entire process in 40 minutes. Our system provided rapid analysis and was simpler to use than was the Xpert MTB/RIF with comparable sensitivity and specificity. Furthermore, our approach required no additional equipment, and even a lateral-flow test strip could be used to read the results. This renders our system useful in diagnosis and screening, which is not possible with PCR-based techniques.

In terms of results, gel electrophoresis offers the greatest sensitivity, followed by those of real-time fluorescence and lateral flow test strips. In our study, relatively stable results were obtained using gel electrophoresis and real-time fluorescence, while lateral flow test strips resulted in some false positives (data not shown). The reason may be that the test strips were adapted to the RPA reaction and not to CRISPR/Cas12a. Lateral flow test strips that are better suited for the CRISPR/Cas12a system are currently in development.

Because only a limited number of clinical samples were tested in this study, the sensitivity and specificity of our system need to be further validated in studies using larger sample numbers.

Overall, our study shows that Cas12a possessed a unique mechanism for activation of single-stranded trans-cleavage activity, which is useful in the design of pathogen detection systems and Cas12a knock-in system design using ssDNA. We developed a new pathogen-detection system that uses a Cas12a-mediated mechanism for the trans-cleavage of ssDNA and improves on the stability of existing Cas12a-based techniques. Validation using standard strains and clinical samples shows that our MTB detection system exhibited excellent sensitivity and specificity. Our MTB detection system can, therefore, be used clinically, as well as in various other fields, for the detection of pathogens.

## 5 | COMPETING INTERESTS

The authors declare no competing interests.

### AUTHOR CONTRIBUTIONS

H. Li, B. Cao, and B. Lu designed experiments and wrote the paper. H. Li performed most of the experiments and data analysis. X. Cui, B. Li, C. Wang, and Y. Wang assisted with clinical specimen collection. S. Liu, X. Deng, L. Sun, X. Zou, and Y. Liu assisted with some experiments.

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