1 **Title:** An isothermal method for sensitive detection of *Mycobacterium tuberculosis*
 2 *complexes* using CRISPR/Cas12a *cis*- and *trans*-cleavage

3 **Short title:** Ultrasensitive MTB diagnosis method

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32 **Keywords:**

33 *Mycobacterium tuberculosis*; RPA; CRISPR/Cas12a; diagnostic

34 **Abbreviations:**

35 TB: Tuberculosis

36 MTB: *mycobacterium tuberculosis*

37 CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

38 CRISPR-Cas: Clustered Regularly Interspaced Short Palindromic Repeats associated

39 protein

40 gRNA: guide RNA

41 RPA: recombinase polymerase amplification

42 HIV: human immunodeficiency virus

43 AIDS: acquired immune deficiency syndrome

44 NTM: *non-tuberculosis mycobacteria*

- 45 PCR: polymerase chain reaction
- 46 WHO: World Health Organization
- 47 LAMP: loop mediated amplification
- 48 HDA: helicase dependent amplification
- 49 SNP: single nucleotide polymorphism
- 50 PAM: protospacer adjacent motif
- 51 LOD: limit of detection
- 52 ssDNA: single-stranded DNA
- 53 **Genes:**
- 54 IS6110: Mycobacterium tuberculosis IS6110 IS-like element
- 55 IS1081: Mycobacterium tuberculosis IS1081 IS-like element
- 56

57 ***Abstract***

58 Tuberculosis is still one of the most serious infectious diseases resulting in lethal
 59 death worldwide. The traditional method is still not enough to meet the clinical
 60 requirements of rapid diagnosis, high specificity and sensitivity. Fast, sensitive and
 61 accurate detection of *mycobacterium tuberculosis* (MTB) is an urgent need for the
 62 treatment and control of tuberculosis disease. Clustered Regularly Interspaced Short
 63 Palindromic Repeats (CRISPR)-associated proteins (Cas12a) exhibits strongly
 64 nonspecific degradation ability of exogenous single-strand nucleic acid
 65 (trans-cleavage) after specific recognition of target sequence. We purified Cas12a
 66 protein and selected a proper guide RNA (gRNA) based on conserved sequences of
 67 MTB from gRNA library we designed. Then, we proposed a novel method based on
 68 recombinase polymerase amplification (RPA) and CRISPR/Cas12a nuclease system
 69 for specific and sensitive detection of MTB DNA. The assay based on fluorescence
 70 detection pattern showed 4.48 fM of limit of detection (LOD) and good linear
 71 correlation of concentration and fluorescence value ($R^2=0.9775$). Also, it showed
 72 good performance in distinguishing other bacteria. Furthermore, its clinical
 73 performance was evaluated by 193 samples and showed sensitivity of 99.29%
 74 (139/140) and specificity of 100% (53/53) at 99% confidence interval, respectively,
 75 compared with culture method. The CRISPR/Cas12a system showed good specificity,
 76 excellent sensitivity and accuracy for MTB detection, and it meets requirements of
 77 MTB detection in clinical samples and has great potential for clinical translation.

78

79 Tuberculosis (TB), which is caused by *Mycobacterium tuberculosis* (MTB) and could
80 spread through aerosol in the air, for example by coughing, remains one of the top 10
81 causes of death and the leading cause of death from a single infectious agent (ranking
82 above HIV/AIDS) worldwide ¹. Therefore, the fast, sensitive and accurate diagnosis
83 of active TB and drug resistance (for example, rifampicin, isoniazid, pyrazinamide) is
84 crucial both for the individual and population level to reduce morbidity, mortality and
85 transmission among patients. Sputum smear microscopy is the primary test for TB
86 diagnosis, but it is significantly limited by its laborious detection process and
87 relatively low detection sensitivity of only 56% to 68%^{2, 3}. In addition, the microscopy
88 performs poorly on HIV co-infected specimens because the amount of MTB is low in
89 sputum specimen. Meanwhile, it cannot distinguish MTB and *non-tuberculosis*
90 *mycobacteria* (NTM) which of patients are treated totally differently due to different
91 pathogenicity and drug susceptibility profiles ⁴⁻⁶, although they show similar clinical
92 manifestation. So far, culture-based mycobacterial detection method is still the gold
93 standard for the laboratory diagnosis of tuberculosis due to its high sensitivity,
94 specificity and accuracy⁷. But, it needs extensive laboratory experiences and usually
95 takes up to 4-6 weeks to achieve satisfied results, which does not meet the rapid test
96 requirement, and it significantly hindered the prompt treatment and isolation patients
97 which is crucial for disease control. Moreover, it exists the cases that some bacilli
98 cannot be successfully cultured, which might arise error results ⁸.

99 Genetic sequencing of MTB DNA was reported to be a more accurate strategy
100 for TB diagnosis. The Xpert MTB/RIF system (Cepheid, USA), a polymerase chain

101 reaction (PCR) based test, is the only one accepted by WHO from 2010 for detecting
102 TB infection and rifampicin resistance. Though it has many advantages including full
103 automation, integration and better accuracy than microscopy or culture methods, its
104 sensitivity for some bacilli clinical specimen is still inadequate. Furthermore, the cost
105 of cartridges, instrumentation and maintenance of Xpert MTB/RIF system make it
106 impossible to set-up in many high TB burden countries, which further limited its
107 usefulness^{9, 10}.

108 In recent years, isothermal amplification assays using loop mediated
109 amplification (LAMP)¹¹, helicase dependent amplification (HDA)¹² and recombinase
110 polymerase amplification (RPA)¹³⁻¹⁵ have been extensively applied in the field of
111 molecular diagnosis including pulmonary TB diagnosis¹⁶⁻¹⁸. Especially, the RPA
112 reaction which replaces the thermal cycles needed for PCR reaction and works at
113 temperatures between 25°C and 42°C with three core enzymes, permits rapid DNA
114 amplification in minutes. Recently, the RPA based methods have been used for
115 detection of MTB and other pathogen, while it always had high false positive results
116 in clinical samples¹⁹. To further improve specificity, long oligonucleotide probe with
117 fluorophores and quenchers or other modifications of probe, such as biotin,
118 tetrahydrofuran, etc, have been designed to recognize RPA amplicon for specific
119 cleavage by nucleases, and these newly developed methods have been successfully
120 applied for pathogen diagnosis²⁰⁻²², liquid biopsy²³, and SNP/genotyping²⁴. However,
121 the synthesis of long oligonucleotide probes with multiple modifications is cost, and
122 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^{19, 25}.

Microbial Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated (CRISPR-Cas) adaptive immune system, consists of guide RNA(s) for target recognition and a Cas enzyme for target cleavage²⁶⁻²⁹. The sequence-specific recognition capabilities of the CRISPR-Cas system make it as a promising genome engineering tool for genome editing^{29, 30}, cancer target therapy³¹, and gene therapy³². Recently, the CRISPR-Cas nuclease systems (Cas12, Cas13 and Cas14)^{26, 33-35} 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 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 nucleases systems have been used for highly sensitive and specific detection of nucleic acids and became as powerful tools for developing molecular diagnostic techniques^{26, 33, 34}.

To achieve prompt, sensitive and specific confirmed diagnosis of MTB, herein we combined the RPA reaction with the CRISPR-Cas nuclease (Cas12a) system to develop a novel MTB DNA detection method (Fig. 1). Firstly, the sequence of MTB with protospacer adjacent motif structure (5'-TTTN-3') for Cas12/guide RNA (gRNA) recognition was selected for gRNA design. The gRNA consists of two parts, universal

145 spacer sequence (5'-UAAUUUCUACUAAGUGUAGAU-3') at the 5' end (also near
146 the PAM structure end) and specific sequence at the 3' end. And gRNAs were selected
147 via evaluating by multiple softwares, such as CRISPR-DT, NUPACK, Nucleotide
148 BLAST etc. Then the feasibility of Cas12a/gRNA trans-cleavage reaction was
149 validated by the purified Cas12a. Subsequently, the suitable amplicon screening was
150 performed and tested to effectively avoid false amplification of RPA. In the
151 Cas12a/gRNA trans-cleavage fluorescence for MTB assay, in the presence of MTB
152 DNA, they could be amplified by RPA reaction. Later, through Cas12a/gRNA
153 complexes binding to amplified MTB DNA, Cas12a mediated nonspecific
154 degradation of nonspecific fluorescent probes, which were polythymidine
155 oligonucleotides with fluorophore at the 5' end and quencher at the 3' end as MTB
156 DNA reporters, could release the fluorophores from the quenchers to increase
157 fluorescence intensities for detection. Here proposed a novel isothermal method based
158 on RPA and CRISPR-Cas system which has very low limitation on MTB detection in
159 our model testing system, such as 4.48 fM of LOD, range over 6 orders of magnitude
160 ($R^2=0.9775$), and could be applied to practical applications on clinical samples with
161 excellent specificity, sensitivity and accurate, which are comparable with the result to
162 the gold standard of culture method.

163

164 ***Materials and Methods***

165 ***Target Selection and gRNA Design for MTB-Cas12a System***

166 MTB-specific fragment of the insertion sequence IS1081, which exists in all

167 *mycobacterium tuberculosis complexes*³⁶, was selected as the target sequence for
 168 design of gRNA to target MTB and RPA reaction primers. To obtain the suitable guide
 169 RNA for MTB, gRNAs targeting IS1081 dsDNA were designed and scored by
 170 CRISPR-DT online software for Cpf1 (Cas12a)
 171 (http://bioinfolab.miamioh.edu/CRISPR-DT/interface/Cpf1_main.php), and target
 172 efficiency less than 0.6 was excluded. Meanwhile, the second structure and homology
 173 of remaining gRNAs were further evaluated by NUPACK (<http://www.nupack.org/>)
 174 and Nucleotide BLAST
 175 (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), respectively. Afterwards, RPA primers were designed
 176 through Primer Premier 5.0 software and selected using agarose gel electrophoresis
 177 according to the design manual of TwistAmp Assay (TwistDx Ltd., England)
 178 [https://www.twistdx.co.uk/docs/default-source/RPA-assay-design/twistamp-assay-design-](https://www.twistdx.co.uk/docs/default-source/RPA-assay-design/twistamp-assay-design-manual-v2-5.pdf?sfvrsn=29)
 179 [gn-manual-v2-5.pdf?sfvrsn=29](https://www.twistdx.co.uk/docs/default-source/RPA-assay-design/twistamp-assay-design-manual-v2-5.pdf?sfvrsn=29).

181 All RNA, IS1081 or IS6110 and modified DNA oligo were synthesized by
 182 General Biosystems (Anhui, China). Plasmids were obtained from the construct that
 183 the insert sequence IS1081 (Genbank ID: CP003248.2) and IS6110 (Genbank ID:
 184 X17348.1) were put into vector PUC57 respectively. Sequence information can be
 185 seen in the Supplement Data. The fluorescent polyoligonucleotides probe (ssDNA-FQ)
 186 was labeled with a fluorescent dye at the 5' end and a quenching group at the 3' end
 187 then purified with HPLC. The fluorescence could be detected only when the
 188 fluorescent dye and quenching group were separated. Forward and Reverse primers

189 for RPA and non-specific single strand DNA (ssDNA, non-target) for Cas12a/gRNA
190 trans cleavage reaction were obtained from Sangon Biotech (Shanghai, China) with
191 PAGE purification. All DNA/RNA oligonucleotides and plasmids were diluted with
192 RNase-free water and the concentrations were determined by Nanodrop 200 (Thermo
193 Fisher, USA). All of these were stored at -20°C until use. The sequences of
194 DNA/RNA oligonucleotides used in the assay were shown in Table 1.

195

196 ***Feasibility of Purified Cas12a for MTB Detection***

197 The DNA plasmid encoding Cas12a (also named Cpf1) with 6×His-tag,
198 maltose-binding protein (MBP) and TEV protease cleavage site was selected for
199 LbCas12a expression. The plasmid 6His-MBP-TEV-huLbCpf1 (plasmid # 90096)
200 (see Figure.1 and Supplementary M3 in the Supplement Data) was purchased from
201 Addgene. After extracted from amplified strains, the plasmids were transformed into
202 BL21(DE3) pLysS Chemically Competent Cells (TransGen Biotech). Then
203 transformed strains were recovered from Luria-Bertani solid growth media and turned
204 to 5 mL Luria-Bertani liquid growth media. After that, the LbaCas12a protein was
205 expressed and purified according to Chen's method ²⁶. Briefly, a 5 mL starter culture
206 was grown in Luria-Bertani liquid growth media (LB, containing of 10 g/L tryptone, 5
207 g/L yeast extract, 10 g/L NaCl, pH 7.0, Sigma) at 25°C overnight, and then further
208 transferred into 1 L of LB and inoculated for growth at 37°C under 300 rpm shaking
209 until the OD₆₀₀ approximately reach to 0.6. Afterwards, all bacterium was cooled
210 down and supplemented with 500 µM isopropyl-1-thio-β-D-galactopyranoside (IPTG,

TransGen Biotech) at 16°C for 16 h to allow protein expression. After centrifugation at 5200 rpm for 30 min at 4°C, the bacterial precipitation was harvested and stored at -80°C until use.

The protein purification was performed at 4°C. Firstly, the bacterial precipitation was crushed and lysed by sonication in Lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 5% (v/v) glycerol, 1 mM TCEP, 0.5 mM PMSF and 0.25 mg/mL lysozyme, pH 7.5). After centrifugation, the supernatant was filtered via a Stericup 0.22 µm filter (EMD Millipore). The filtered supernatant was pipetted to Ni-NTA resin (GE) for 30 min, and washed with lysis buffer. Then, the resin was resuspended in 1×TEV digestion buffer (50 mM Tris-HCl, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, pH 8.0) with 250 units of TEV protease (Beyotime Biotechnology) overnight at 4°C with rotation. To obtain purer proteins, the steps on Ni-NTA resin was repeated. The protease treated LbCas12a was dialyzed in Buffer A (20 mM Tris-HCl, 125 mM KCl, 5% glycerol, 1 mM TCEP, pH 7.5) for three times at 4°C, and further purified on a 5 mL HiTrap SP HP sepharose column (GE Healthcare). After washing with three column volumes of Buffer A, LbCas12a was eluted using a linear gradient from 0-100% Buffer B (20 mM Tris-HCl, 1 M KCl, 5% glycerol, 1 mM TCEP, pH 7.5) over 20 column volumes. The eluted proteins were further purified on Superdex 200 column (GE Healthcare) with elution Buffer (20 mM Tris-HCl, 200 mM KCl, 5% glycerol, 1 mM 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 usage. To test the *cis*- and *trans*-cleavage activity of

233 purified LbCas12a protein and the feasibility of Cas12/gRNA trans cleavage for MTB
234 detection, we were observing the alteration of amount and integrity of substrates and
235 nonspecific single strand DNA by 4% agarose gel electrophoresis and the
236 fluorescence intensity was collected by detector with/out target plasmid, gRNA or
237 non-target ssDNA. The Cas12a/gRNA trans-cleavage for MTB detection assay was as
238 follows: 50 nM LbCas12a incubating with 36 nM IS1081-gRNA, 80 ng IS1081
239 plasmid and 50 nM non-target ssDNA (replaced with ssDNA-FQ in Cas12a/gRNA
240 fluorescence assay) in 1× reaction buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM
241 MgCl₂, 100 µg/mL BSA, pH 7.9), incubated at 37°C for 2 h.

242

243 ***Mycobacterium tuberculosis (MTB) Detection by Cas12a Fluorescence Assay***

244 To improve signal amplification efficiency and simplify operation, 1 µL of DNA
245 extracted from amplified clone strains as template was added into RPA reaction
246 mixture of 12.5 µL volume. RPA reaction mixture including 0.24µM~0.72µM
247 forward and reverse primers, 1.8 mM dNTP, 1×Reaction Buffer, 1×Basic E-mix,
248 0.625 µL 20×Core Reaction Mix, 1 µL genome, and 14 mM MgOAc and DEPC water
249 up to 12.5 µL and incubation at 37°C~42°C for 1 h. According to the design manual
250 of TwistAmp (TwistDx Ltd., England) assay, multiplex sets of RPA primers were
251 selected as candidates, including primers from published literature^{18,37} (see Table.1 in
252 the Supplemental Data) and tried to get best conditions from RPA reaction *via*
253 optimization for concentration of dNTP, Mg²⁺, primers and reaction temperature with
254 TwistAmp® Liquid Basic/Basic RT (TwistDx, England).

255 After RPA reaction finished at one hour, 7.5 μ L Cas12a/gRNA mixtures of 36
256 nM gRNA, 50 nM ssDNA-FQ, 50 nM Cas12a in 1 $\times$ reaction buffer was added into
257 the cap of RPA reaction tube to the final volume of 20 μ L. Then transient high-speed
258 centrifugation was performed to mix RPA productions with Cas12a/gRNA system.
259 The Cas12a/gRNA trans-cleavage fluorescence reaction was incubated at 37°C for 2
260 hours and its fluorescence was collected every 1.25 minutes by fluorescence detector
261 (Applied Biosystems 7500 Real-Time PCR System in this study) during reaction.

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

266

267 ***Performance of MTB-Cas12a Fluorescence Assay***

268 For evaluation the sensitivity of Cas12a/gRNA for MTB fluorescence assay, a series
269 of gradient dilution concentration of IS1081 plasmid from 8960 pM to 44.8 fM were
270 prepared with 1 μ L volume as RPA template. Meanwhile, no-MTB DNA was used as
271 negative control and all of them were performed in three replicates. Total DNA mass
272 for the assay was determined following formula: $M/5-7 \times (4.38 \times 10^6 \times 660) = \mu\text{g}/\mu\text{L}$. M
273 is concentration of IS1081 plasmid, 5-7 is number of copies of IS1081 per MTB
274 genome and 4.38×10^6 is approximately number of DNA length of M. bovis BCG. The
275 specificity of the assay was obtained by analyzing at least 448 fM genomic DNA of
276 target MTB, with/ or not closely related species from clinical isolates and standard

277 strains.

278

279 ***Clinical Performance Evaluation of MTB-Cas12a Fluorescence assay***

280 The practical performance evaluation of the MTB detection assay was carried out via
 281 Cas12a trans-cleavage fluorescence system using a total of 194 clinical sputum
 282 samples, which were randomly collected from Fuzhou Center for Disease Control and
 283 Prevention in 2013~2016 with one sample came from one person, including patients
 284 with similar signs and symptoms consistent with pulmonary TB. All sample culture
 285 results, including 140 culture-positive results and 54 culture-negative results, were
 286 unknown for operator firstly. Genome of all samples were extracted using QIAGEN
 287 nucleic acid extraction Kit and the concentrations were measured via Nanodrop 2.0
 288 and then stored at -20°C before test. The 194 clinical samples were detected by
 289 Cas12a/gRNA trans-cleavage fluorescence assay and the data analysis was carried out
 290 according to the above criteria.

291

292 ***Data Analysis***

293 All the data analysis was performed with SPSS version 2.2 and Origin Lab version 8.0,
 294 including ROC area, R square etc. Each experiment was repeated at 3 times for each
 295 sample.

296

297 ***Results***

298 ***Design of Guide RNA of MTB***

299 To ensure the coverage on all MTB species, the target insertion sequence IS1081 was
 300 selected for the assay and gRNAs targeting *mycobacterium tuberculosis* were
 301 designed. Principally, there are numbers of gRNAs corresponding to amounts of PAM
 302 structure existing in the insert sequence IS1081. Actually, different gRNAs had
 303 different target efficiency of Cas12a/gRNA system and not all predicted gRNAs were
 304 suitable for targeting MTB. Scores of target efficiency for gRNAs were evaluated by
 305 Cpf1-CRISPR-DT online software and range from 0.7061 to 0.97 was selected after
 306 excluding that of less than 0.6, and nine gRNAs were remained. In the end, we
 307 selected one that the score of target efficiency was 0.7061 based on un conspicuous
 308 secondary structure under reaction temperature and specificity of gRNA spacer for
 309 MTB. Especially, the spacer of MTB-specific sequence of gRNA was conserved
 310 region of IS1081 according to previous study and our sequence blasting. The
 311 sequences of designed IS1081 gRNAs and corresponding score of target efficiency for
 312 MTB genome were shown Table.2 in Supplemental Data.

313

314 ***Feasibility Analysis of Purified Cas12a for MTB Detection***

315 The gRNA-directed DNA binding and following activation of single-stranded DNA
 316 (ssDNA) trans-cleavage activity of Cas12a protein are vital properties for accurate
 317 recognition of target DNA for detection. The activity level of Cas12a protein directly
 318 determines the limit of detection of our Cas12a/gRNA trans-cleavage detection assay.
 319 As seen in Fig 2, when it exists IS1081 plasmid as template, the Cas12a/gRNA system,
 320 mixture of IS1081 gRNA and Cas12a protein, could degrade the target IS1081

321 plasmid *in cis* and single strand DNA *in trans*, resulting in reduction of the number of
 322 targets and non-target ssDNA (as reporter) in Cas12a/gRNA system or increase
 323 fluorescence intensity in Cas12a/gRNA fluorescence detection system. Excitingly, we
 324 observed IS1081 plasmid and ssDNA reporters were cleaved almost at the same time.
 325 These results suggested that the cis- and trans-cleavage activity ability of purified
 326 Cas12a protein is quite high and Cas12a/gRNA system for MTB detection is feasible.

327

328 ***Optimization of Cas12a Fluorescence Assay for MTB***

329 In order to simplify operation to achieve “one-pot” detection pattern and improve
 330 reaction efficiency, the volume of RPA reaction was controlled under 12.5 μ L and then
 331 added compositions of Cas12a/gRNA system to the final volume of 20 μ L for
 332 Cas12a/gRNA trans-cleavage fluorescence assay. Prominently, the RPA reaction
 333 conditions were optimized to maximize amplification efficiency, including reaction
 334 temperature, concentration of dNTP and primers and Mg^{2+} . As shown in Fig. 3
 335 (details can be seen in Figure.2 in the Supplemental Data), we found 0.48 μ M forward
 336 and reverse primers and 28 mM MgOAc of RPA reaction were mainly influence
 337 factors for Cas12a/gRNA trans-cleavage fluorescence assay, and the assay obtained
 338 higher fluorescence intensity under optimized conditions using the same concentration
 339 of target. Therefore, these conditions were used in the following experiments for MTB
 340 detection.

341

342 ***Performance of Cas12a Fluorescence Assay for MTB***

343 To evaluate the ability of MTB-Cas12a trans-cleavage fluorescence assay, a series of
344 gradient concentration of diluted IS1081 plasmids and genome of pathogens that may
345 infect human respiratory tract were tested. For the sensitivity of MTB-Cas12a
346 fluorescence assay, the fluorescence accumulation value of the assay decreased
347 gradually with the decrease of concentration of IS1081 plasmid, and the limit of
348 detection (LOD) for IS1081 was 4.48 fM (Fig. 4A). According to the formula
349 depicted above, it was equivalent to 2.59-1.85 µg/L of genome of *M. bovis* BCG
350 strain based on formula above from clinical samples. Comparing to RPA method¹⁸ for
351 IS1081 of MTB detection with LOD of 20 fg for 50µL volume, it achieved four
352 orders of magnitude higher and got attogram level which would dramatically promote
353 positive detection rate. Meanwhile, the fluorescence value was proportional to the
354 logarithm value of target concentrations over 6 orders of magnitude. The regression
355 equation was $Y=87983.47X+1.29307E6$ with R square of 0.9775 (Fig. 4B). It was
356 helpful to quantify MTB DNA accurately, especially regarding to low concentration
357 of MTB DNA as substrate.

358 To assess the specificity of the MTB-Cas12a fluorescence assay, genome of
359 samples from a panel of NTM and pathogens which may infect human respiratory
360 tract or their clinical symptoms similar to TB were tested. Three replicates were
361 performed and 500 pg of DNA from either *M. bovis* BCG or *M. tuberculosis* H37Rv
362 was used as positive template controls for each batch. Consequently, as shown in Fig.
363 4C, according to the judgment of criteria above, no positive results were obtained
364 from the tested DNA except for the genome of MTB strains, indicating the high

365 specificity of MTB-Cas12a fluorescence assay for MTB detection.

366 To evaluate the clinical diagnostic performance of the MTB-Cas12a assay, 193
 367 patient samples (140 positive and 53 negative for MTB) which had been confirmed by
 368 the gold standard culture method were tested. Consequently, the fluorescence assay
 369 successfully detected 139 positive samples and 53 negative samples among 140
 370 positive samples and 53 negative samples for MTB culture method. The detection
 371 accuracy of the assay based on 193 samples showed sensitivity of 99.29% (139/140)
 372 and specificity of 100% (53/53) (Table 2). Its performance was nearly similar to
 373 culture method except that one of culture-positive sample was failed to be detected
 374 whose fluorescence intensity was lower 1.5 times than that of negative result. Maybe
 375 this one failed to be detected by the assay due to the low concentration of genome
 376 and/or it was degraded in storage or during deliver process. Especially, the major
 377 reason could be that the MTB target for guide RNA recognition exist mutation
 378 resulting in none-complete hybridization, namely “turn-off” effect. As far as we can
 379 infer, it may be resolved by the modification of gRNA covering mutation site, such as
 380 LNA or leap over, similar to the processed methods of PCR and RPA for detection of
 381 mutation. But it needs to be proved in the future. In addition, Cas12a/gRNA trans
 382 cleavage fluorescence assay for MTB detection was required an average time of 4h,
 383 which including 1h of MTB DNA extraction, 1h for MTB DNA amplification by RPA
 384 and 2h for detection of Cas12a/gRNA system. It showed a significant advantage over
 385 culture method which needs few days, and a little slow compared to RPA method for
 386 MTB DNA detection which needs 1~2 h totally.

387 ***Discussion***

388 Rapid and sensitive detection method remains a challenge for clinical MTB diagnosis.
 389 Positive results of sputum smear microscopy were required at least five thousand of
 390 bacteria every milliliter and skilled technician. And culture method for MTB detection
 391 takes up to a few weeks and relatively low detection rate. They are not feasible for
 392 rapid molecular diagnostic of MTB in clinical applications. Rapid and sensitive MTB
 393 DNA detection method was attempted to be improved previously, such as automatic
 394 integration plate based PCR method—GeneXpert, RPA, etc. Unfortunately, they also
 395 brought some side effects which were similar to the original disadvantages and were
 396 not benefit for clinical MTB diagnosis. For example, continuous power input for
 397 temperature rising and falling and sample volume of 500 μ L were required for
 398 GeneXpert. In contrast, Cas12a trans cleavage fluorescence assay for MTB can occur
 399 at 37°C which not needs a thermos-cycler and it could obtain positive results under
 400 low volume of MTB sample (100~200 μ L). Meanwhile, it estimated to be 0.6138
 401 dollars every sample³³ comparing to 35~50 dollars for one sample of one cartridge
 402 using GeneXpert.

403 In this study, by optimizing the conditions of RPA reaction and Cas12a/gRNA system,
 404 we established one ultrasensitive method for Cas12a trans-cleavage fluorescence
 405 assay that could obtain 2.59-1.85 μ g/L of limit of MTB genome (5-7 copies of IS1081
 406 per MTB genome) detection from clinical samples. Although IS6110 is in multiple
 407 copies of up to 25 per genome of MTB and was used to be target in several
 408 commercial kits, such as Xpert MTB/RIF, the assay integrated the advantages of

409 polymerase mediated DNA amplification and Cas12a mediated enzymatic signal
 410 amplification to compensate relatively low copies of IS1081 in 5-7 repeats per
 411 genome, and we proved that our Cas12a/gRNA system could detect MTB DNA with
 412 ultralow LOD. Also, Cas12a/gRNA system for IS1081 can strictly recognize MTB
 413 genome with PAM structure, which showed high specificity for target. Moreover, our
 414 study on 193 samples highlights the potential of Cas12a/gRNA as an ultra-sensitive,
 415 promising assay for diagnosis of clinical tuberculosis, in spite of miss detection of one
 416 sample of culture-positive for MTB which might due to the existence of mutation in
 417 the recognition sequence of Cas12-gRNA or low concentration of MTB DNA. So, in
 418 order to reduce the false negative possibility, spacer sequence of gRNA should be
 419 avoided from region with mutation sequences or SNP, especially mutation closes to
 420 PAM sequence.

421 In addition, there are also some limitations in our established Cas12a
 422 fluorescence assay. Firstly, it takes nearly twice time to get result compared with
 423 Xpert MTB\RIF. This may be reason the assay got ultra-sensitivity. But the time
 424 consumed totally could be controlled under the acceptable range for clinical diagnosis.
 425 Secondly, the Cas12a trans-cleavage fluorescence is detected by fluorescence detector
 426 but not visual readout that requires no additional devices. Finally, apart from
 427 diagnosis, drug resistance is also increasingly severe in the world for treating MTB
 428 patients. It is an urgent need to analyze mutations of drug susceptibility for one- or
 429 even second-line drugs via sensitive detection methods. With the natural
 430 characteristics of sensitive signal amplification of Cas/gRNA system, it has the

431 potential to detect multiple mutations related to drug response. Ultra-sensitive and
432 specific Cas12a/gRNA system combining polymerase mediated DNA amplification
433 for both pathogen and drug resistance detection would permit the precise approach to
434 control tuberculosis infection.

435 In a word, we have developed a CRISPR-Cas12a based fluorescence assay by
436 combining isothermal recombinase polymerase amplification with Cas12a
437 trans-cleavage activity, which could be activated by target specific DNA with high
438 sensitivity and selectivity for rapid detection of pathogen *Mycobacterium tuberculosis*
439 from clinical samples comparing to gold standard culture method which needs few
440 days and extensive labor work. It is helpful to use this assay to prompt the confirmed
441 diagnosis of mycobacterium tuberculosis complexes from none mycobacterium
442 including NTM in clinical settings. But, in future, multi-center prospective study is
443 needed to provide deeper understanding on its potential usage for clinical diagnosis.

444

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550

Table 1. The oligonucleotide sequences of RPA primers, gRNA and non-specific single strand DNA for MTB Cas12a/gRNA trans-cleavage assay

Name	Sequence (5' → 3')
RPA-F	CCAAGCTGCGCCAGGGCAGCTATTTCCCGGAC
RPA-R	TTGGCCATGATCGACACTTGCGACTTGGA
IS1081-gRNA*	UAAUUUCUACUAAGUGUAGAUG <u>GACCAGGCGCUCCAUCCGGC</u>
ssDNA	GCTTGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAGGAT
(Non-target)	GGGCACCACCCCGGCTTTTTTTTTTTTTTTTTTTTTTTTTTTT
ssDNA-FQ	FAM-TTTTT-BHQ1

* The underlined sequence is the specific sequence targeting insert sequence IS1081 of MTB; RPA-F and RPA-R are the forward and reverse primers of RPA, respectively. And ssDNA-FQ was used in the Cas12a/gRNA trans-cleavage fluorescence assay

Table 2. Clinical diagnostic performance of MTB-Cas12a assay on clinical samples

		MTB-Cas12a assay		
		Positive	Negative	Total
Culture Method	Positive	139	1	140
	Negative	0	53	53
Total		139	54	193

Figure 1. Schematic diagram of the Cas12a/gRNA trans cleavage fluorescence signal amplification system for MTB detection.

Genome of mycobacterium tuberculosis complex (MTB) was extracted into RPA reaction for amplification. Positive fluorescent signals were captured when probes were cleaved by activated Cas12a under target with PAM sequence at 5' end that recognized by gRNA.

Figure 2. Feasibility analysis of Cas12a/gRNA system for MTB detection.

4% agarose gel electrophoretic analysis of the feasibility of Cas12a/gRNA system for MTB detection. 79 nt ssDNA (Non-Target) as substrate of Cas12a/gRNA trans cleavage (Figure 2A); Real-time fluorescence of Cas12a/gRNA fluorescence assay. ssDNA-FQ was served as the reporter probe. The concentration of the reaction components was as follows: 50nM purified LbCas12a, 36 nM IS1081-gRNA, 50 nM ssDNA (Non-Target) or ssDNA-FQ, 80 ng IS1081 or IS6110 plasmid (Figure 2B).

577

578 **Figure 3. Optimization of MTBC-Cas12a trans-cleavage fluorescence assay.**

579 Figure 3A, Figure 3B, Figure 3C and Figure 3D are optimized results of assay on

580 reaction temperature, dNTP concentration, primers concentration, and Mg²⁺

581 concentration, respectively.

Figure 4. Detection performance of MTB-Cas12a fluorescence assay.

Real-time fluorescence intensity alteration of MTB-Cas12a assay of different concentration of target(Figure 4A); The calibration plots of fluorescence intensity versus the logarithm of target concentration(Figure 4B); The specificity of the MTB-Cas12a fluorescence assay, including NTM and other pathogens of respiratory tract(Figure 4C).