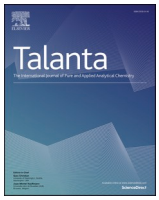
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Rapid and sensitive detection of nucleic acids using an RAA-CRISPR/Cas12b one-pot detection assay (Rcod)

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ABSTRACT

Rapid, sensitive and specific methods are crucial for nucleic acid detection. CRISPR/Cas12b has recently been widely used in nucleic acid detection. However, due to its thermophagic property, DNA isothermal recombinase-aided amplification (RAA) and subsequent CRISPR/Cas12b detection require two separate reactions, which is cumbersome and inconvenient and may cause aerosol pollution. In this study, we propose an RAA-CRISPR/Cas12b one-pot detection assay (Rcod) for *Bordetella pertussis* detection without additional amplification product transfer steps. The time from sample processing to response time was less than 30 min using nucleic acid extraction-free method, and the sensitivity reached 0.2 copies/μL. In this system, *Alicyclobacillus acidoterrestris* Cas12b protein (AacCas12b) exhibited strong and specific *trans*-cleavage activity at a constant temperature of 37 °C, while the *cis*-cleavage activity was weak. This characteristic reduces the interference of AacCas12b with nucleic acids in the system. Compared with real-time PCR, our Rcod system detected *B. pertussis* in 221 clinical samples with a sensitivity and specificity of 97.96 % and 99.19 %, respectively, with nucleic acid extraction-free method. The rapid, sensitive and specific Rcod system provides ideas for the establishment of CRISPR-based one-step nucleic acid detection and may aid the development of reliable point-of-care nucleic acid tests.

Importance: Pertussis is an acute respiratory infection caused by *B. pertussis* that is highly contagious and potentially fatal, and early diagnosis is essential for the treatment of whooping cough. In this study, we found that AacCas12b has high and strongly specific *trans*-cleavage activity at lower temperatures. A RAA-CRISPR/Cas12b one-step detection platform (Rcod) without interference with amplification was developed. In addition, the combination of Rcod and nucleic acid extraction-free method can quickly and accurately detect the qualitative detection of *B. pertussis*, and the detection results are visualized, which makes the pathogen nucleic acid detection and analysis process simpler, and provides a new method for the rapid clinical diagnosis of *B. pertussis*.

1. Introduction

Respiratory infections caused by viruses or bacteria are among the most common diseases in humans worldwide, and identifying the causative viral pathogens of respiratory viral infections is of great importance for selecting appropriate treatments and preventing

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epidemics [1]. Pertussis, or "whooping cough", is an acute respiratory infection caused by *Bordetella pertussis*, and early diagnosis is crucial for the treatment of pertussis [2–4]. At present, the commonly used methods for the diagnosis of pertussis in the laboratory include culture-based methods, direct fluorescent antibody detection, polymerase chain reaction (PCR), pairing and single serological techniques [5]. Isolation of *B. pertussis* by culture is the traditional "gold standard" for the diagnosis of pertussis and exhibits approximately 100 % specificity [6]. However, the sensitivity of culture-based methods is very poor, ranging from 12 % to 60 %, and these methods are very time-consuming, taking on average 5 days [7–9].

The traditional PCR or reverse transcription PCR (RT−PCR) method is the gold standard for nucleic acid detection with high sensitivity [10–14]. Since the 1990s, PCR has been used as a diagnostic tool for *B. pertussis* in clinical and nonclinical research environments [3,15,16]. However, PCR technology requires skilled personnel and equipment infrastructure, and the sampling-to-result time is also very long, which is not suitable for simple and rapid point-of-care (POC) testing [8]. Isothermal nucleic acid detection technologies, such as recombinase polymerase amplification (RPA) [17], recombinase-aided amplification (RAA) [18] and loop-mediated isothermal amplification (LAMP) [19,20] have great potential in POC detection with the advantages of simple operation, constant temperature, and rapidity. Frustratingly, their application is limited because their lower sensitivity compared with qPCR and their possible false positive results [21,22].

In recent years, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas)-based nucleic acid detection methods have emerged as attractive options for molecular POC detection [23–25]. The RNA-guided class 2 CRISPR/Cas system proteins, when recognizing the target nucleic acid, not only *cis*-cleave the target nucleic acid but also indiscriminately *trans*-cleave nearby single-stranded nontarget nucleic acids [26–30]. Taking advantage of this property, CRISPR/Cas nuclease proteins have been combined with isothermal amplification technologies, to develop a series of simple, fast, sensitive, and visualized nucleic acid detection methods that do not require special precision instruments [31–34]. For example, Cas13a- or Cas13 b-based specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) [31], Cas12a-based DNA endonuclease-targeted CRISPR trans reporter (DETECTR) [32], and Cas12b-based Cas12b-mediated DNA detection (CDetection) [34] enable rapid detection of nucleic acids. However, these methods require a two-step process, with amplification of the target sequence followed by CRISPR detection. Such multiple processing steps increase the operating time and the risk of cross-contamination. The STOPCovid (SHERLOCK Testing in One Pot) and STOPCovid. v2 based on the development of thermostable Alicyclobacillus acidiphilus Cas12b (AapCas12b) and RT-LAMP are designed to solve the problem of two-step method [35]. However, these one-pot detection based on LAMP require a long reaction time and the reaction temperature is 60 °C, which limits the primer design of LAMP, and the high temperature is easy to cause burns, which is not suitable for detection in homes and other places.

In this study, we propose an RAA-CRISPR/Cas12b one-pot detection assay (Rcod) with the advantages of simplicity, rapidity and high sensitivity. We found that AacCas12b still had high *trans*-cleavage activity in the temperature range of 30 °C–39 °C, although its *cis*-cleavage activity was extremely weak. In the Rcod system, AacCas12b cleavage and isothermal amplification occurred simultaneously in one tube, and AacCas12b with high and specific *trans*-cleavage activity and low *cis*-cleavage activity, exhibited weaker interference with nucleic acids in the system than AacCas12b with high *cis*-cleavage activity, showing higher sensitivity and reliability. The Rcod system detected 221 *B. pertussis* clinical samples with the nucleic acid-free extraction method within 30 min. Compared with the PCR detection results, the sensitivity and specificity were 97.96 % and 99.19 %, respectively. Rcod is a hyperthermic CRISPR/Cas12b-mediated detection method that has the advantages of simplicity, rapidity, high sensitivity, and good specificity. We also provide an understanding of the CRISPR one-pot method, provide ideas for establishing a rapid one-step method based on Cas12b or other CRISPR systems, and apply CRISPR detection to clinical POC diagnosis.

2. Experimental section

Materials. All DNA targets, primers and ssDNA reporters used in this study were synthesized by Tsingke Biotechnology Co., Ltd. (Beijing, China), and the sequence information is listed in Table S1 in the Supporting Information. All otherwise unspecified chemical reagents were obtained from Sangon Biotechnology Co., Ltd. (China). The ZC-RAA® Basic kits were purchased from ZC Bio-Sci & Tech Co., Ltd. (China). Gloria Nova HS 2× Master Mix used for PCR amplification was from ABclonal Technology Co., Ltd. (Wuhan, China). Purification of plasmid DNA was performed using the TIANprep Midi Plasmid Kit from Qiagen (Shanghai, China). PCR amplification products were recovered, and RAA products were extracted using the FastPure Gel DNA Extraction Mini Kit from Vazyme (Nanjing, China). A *B. pertussis* nucleic acid detection kit (PCR-Fluorescence Probing) was purchased from Shenzhen YILIFANG Biological Co., Ltd. (Shenzhen, China). A viral nucleic acid extraction-free release agent kit was provided by BioDetect (Xiamen, China). The HiScribe T7 Quick High Yield RNA Synthesis Kit and DNase I (RNase-free) used to prepare sgRNA were purchased from New England Biolabs (Beijing, China). Glycerol stocks of AacCas12b-harboring bacteria were presented by Professor Liang-Liu of School of Life Sciences, Xiamen University. Ni Sepharose was purchased from GE Healthcare. Clinical samples of *B. pertussis* were provided by Beijing Children's Hospital affiliated with Capital Medical University.

Target DNA preparation. The sequence of *B. pertussis* IS1663 (GenBank accession CP046995.1) was synthesized by Tsingke Biotechnology Co., Ltd. (Beijing, China) and inserted into the pUC-57 vector. To verify *cis*-cleavage, a 632-bp IS1663 dsDNA fragment was PCR amplified from pUC57-IS1663 with Gloria Nova HS 2× Master Mix from ABclonal Technology Co., Ltd. (Wuhan, China). In addition, 40-nt dsDNA templates with consecutive single-point or double-point mutations were formed by the annealing of two complementary pairs of single strands at a 1:1 ratio. The annealing process was executed in 1 × annealing buffer containing 50 mM HEPES (pH 7.4) and 100 mM NaCl at 94 °C for 4 min followed by gradual cooling to 25 °C at a rate of 0.1 °C/s.

Preparation of sgRNA. To prepare the sgRNA targeting *B. pertussis* IS1663, we inserted the T7 transcript and the DNA sequence corresponding to the sgRNA into the pUC-57 vector, which was synthesized by Tsingke Biotechnology Co., Ltd. (Beijing, China) to obtain the pUC57-sgRNA plasmids. Next, to prepare DNA templates for in vitro transcription, sgRNA-F and sgRNA-R were used as primers for the plasmid pUC57-sgRNA to amplify linear dsDNA fragments containing T7 transcripts. Next, the purified PCR products were used as templates for the synthesis of the sgRNA with the HiScribe T7 Quick High Yield RNA Synthesis Kit overnight at 37 °C. Then, the transcription products were treated with DNase I (RNase-free) to degrade the template DNA, and the sgRNA was extracted and purified using a phenol-chloroform extraction method and quantified with a NanoDrop 2000C spectrophotometer (Thermo Fisher).

AacCas12b expression and purification. Glycerol stocks of bacteria containing the AacCas12b plasmid (gifted by Liang-Liu Lab) were activated overnight at 37 °C, and then the activated cells were expanded to OD600 = 0.6 before expression was induced with 1 mM IPTG treatment at 25 °C for 6 h. Cell culture precipitates were collected by centrifugation and resuspended with lysis buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM DTT, 5 % glycerol, and 0.1 % Triton X-100; pH 8.0) and disrupted by sonication. The sonicated solution was then centrifuged at 12,000 rpm for 30 min at 4 °C, and the supernatant was run through Ni Sepharose (GE Healthcare) preequilibrated with lysis buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM DTT, 5 % glycerol, and 0.1 % Triton X-100; pH 8.0). Then, the column was washed with washing buffer (50 mM

Tris-HCl, 0.2 M NaCl, 5 mM DTT, 5 % glycerol, 0.1 % Triton X-100, and 50 mM imidazole). After the column was equilibrated, AacCas12b was eluted with elution buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM DTT, 5 % glycerol, 0.1 % Triton X-100, and 250 mM imidazole), and the eluted components were dialyzed in storage buffer overnight (20 mM Tris-HCl, 0.5 M NaCl, 5 mM DTT, and 1 mM EDTA). Next, dialysis fractions containing the AacCas12b protein were collected, adjusted to a final concentration of 4 mg/mL and stored at −20 °C in 50 % glycerol.

***Cis*-cleavage assay.** AacCas12b and sgRNA were mixed at a molar ratio of 1:1 (0.6 μM AacCas12b and 0.6 μM sgRNA) in cleavage buffer containing 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM MgCl2 and 1 mM DTT. Then, 200 ng of PCR amplicons was added to the system and incubated at 30 °C, 32 °C, 35 °C, 37 °C, 39 °C, 42 °C, 45 °C, 48 °C, 52 °C, 56 °C, 60 °C, 64 °C, 68 °C and 72 °C for 30 min. Finally, the products were analyzed by 1 % agarose gel electrophoresis, and the results were analyzed by ImageJ software.

***Trans*-cleavage assay.** The *trans*-cleavage activity of AacCas12b was determined by cleavage of ssDNA reporters modified with fluorophores and quenchers, including FAM fluorophore-modified 6C, 6 T, 6 A, and 6G, as well as HEX fluorophore- and ROX fluorophore-modified forward and reverse primers. AacCas12b, sgRNA, ssDNA reporter molecules and 200 ng of the target were incubated in a SLAN-96 P real-time PCR instrument (Shanghai Hongshi Medical Technology Co., Ltd.), and the fluorescence intensity was measured every minute at 20 °C, 25 °C, 30 °C, 32 °C, 35 °C, 37 °C, 39 °C, 42 °C, 45 °C, 48 °C, 52 °C, 56 °C, 60 °C, 64 °C, 68 °C and 72 °C for 30 min each. Next, the samples were scanned and imaged using a THBC-470 LED blue light (TUOHE) and a Clinx gel documentation system (Clinx).

RAA assay. The RAA and RPA are recombinase-based isothermal amplification assays, and it has been reported that RPA/RAA had consistent clinical performance [18]. The lyophilized RAA system was resuspended in 37.5 μL of reaction buffer containing 12.5 μL of buffer A, 2.4 μL of 10 μM forward primer and 2.4 μL of 10 μM reverse primer and mixed. Then, the resuspended RAA reaction solution was divided into two parts, and the targets and 1.25 μL of buffer B were added to a volume of 25 μL and incubated at 37 °C immediately.

Rcod assay. The Rcod assay was performed in one tube in a reaction volume of 25 μL, including the above 18.75 μL RAA components, 0.23 μM AacCas12b, 0.23 μM sgRNA, 1.2 μM 6C-FQ reporters and target DNA. AacCas12b, sgRNA, 6C-FQ reporters and RAA components were first added to each reaction tube, and subsequently, the target and 1.25 μL of buffer B were added and immediately incubated in a SLAN-96 P real-time PCR instrument (Shanghai Hongshi Medical Technology Co., Ltd.), and the fluorescence intensity was measured every minute for 30 min. After incubation, the end-point fluorescence signals were observed by the naked eye under LED blue light and UV irradiation.

Clinical sample collection. The *B. pertussis* clinical samples were provided by Beijing Children's Hospital affiliated with Capital Medical University. A viral nucleic acid extraction-free release agent kit (Bio-Detect (Xiamen) Biotechnology Co., Ltd.) was used to extract clinical samples for Rcod. Specifically, 20 μL of liquid sample was added to the same volume of nucleic acid extraction buffer. After shaking for 5 s, the lysed sample was used as the template for amplification.

Real-time PCR assay. The *B. pertussis* nucleic acid detection kit (PCR-Fluorescence Probing) (Shenzhen YILIFANG Biological Co., Ltd.) was used as a comparison reagent for the Rcod method to test *B. pertussis*. One milliliter of sample was concentrated to 10 μL by centrifugation at 12,000×*g* for 5 min, and then 50 μL of DNA extraction solution from the kit was added to the concentrated samples and treated at 100 °C for 10 min. After cooling, the samples were centrifuged at 12,000×*g* for 5 min. Then, 4 μL of the treated samples was added to a volume of 16 μL containing 15.2 μL of PCR solution A and 0.8 μL of PCR solution B. Finally, the real-time PCR assay was carried out on an ABI7500 or Roche instrument.

Statistical analysis. Statistical analysis was performed by two-tailed Student's *t*-test using GraphPad Prism 8.0. Each experiment was repeated three times. The results are presented as the means ± S.D.

3. Results and discussion

Cas12b exhibits strong *trans*-cleavage activity in a wider temperature range. We purified the AacCas12b and verified that the Cas12b protein had both *cis*-cleavage activity for double-stranded DNA (dsDNA) and collateral *trans*-cleavage activity for single-stranded DNA (ssDNA) at 48 °C (Fig. S1). Previous studies have shown that AacCas12b was a thermophagic endonuclease [36]. To further expand the application of AacCas12b, we have explored the active temperature range of AacCas12b. We first designed single-guide RNA (sgRNA) for the conserved sequence of *B. pertussis* IS1663 (Fig. S2). In addition, we investigated the *cis*-cleavage activity of AacCas12b at different reaction temperatures using the designed sgRNA and IS1663 target. After incubation at 30 °C, 32 °C, 35 °C, 37 °C, 39 °C, 42 °C, 45 °C, 48 °C, 52 °C, 56 °C, 60 °C, 64 °C, 68 °C, 72 °C for 30 min, the results showed that AacCas12b could cleave target dsDNA with greater than 60 % *cis*-cleavage activity in the temperature range of 45 °C–60 °C. However, between 30 °C and 39 °C, the *cis*-cleavage activity of AacCas12b was very weak, and it could hardly cleave the target dsDNA (Fig. 1b–c).

We further tested the *trans*-cleavage activity of AacCas12b under different reaction temperature conditions. Previous studies indicated that Cas12b exhibited a preference for different homopolymerization reporters [34]. Therefore, to enhance the *trans*-cleavage of AacCas12b, we first investigated the cleavage preference of AacCas12b for ssDNA-fluorophore quencher (FQ) reporters. The results showed that AacCas12b preferred the cytosine polymer (6C), followed by the thymine polymer (6 T) and adenine polymer (6 A), while the guanine polymer (6G) could hardly be cleaved (Fig. S3). In addition, we also optimized the structure and length of sgRNA, but the results showed that the *trans*-cleavage activity of Cas12b decreased or inactivated when the structure or length of sgRNA changed (Fig. S4). Next, the polymer 6C was used as the ssDNA- FQ reporter for testing *trans*-cleavage activity. After incubation at 20 °C, 25 °C, 30 °C, 32 °C, 35 °C, 37 °C, 39 °C, 42 °C, 45 °C, 48 °C, 52 °C, 56 °C, 60 °C, 64 °C, 68 °C, 72 °C for 30 min, the real-time fluorescence results showed that AacCas12b had extremely high *trans*-cleavage activity between 30 °C and 68 °C, and the fluorescence reached saturation within 15 min (Fig. 1d–e and Fig. S4). More specifically, there was no significant difference in *trans*-cleavage activity between 37 °C and 48 °C (Fig. S5). In addition, after 30 min of incubation, the intense endpoint fluorescence signal could be observed by the naked eye under a blue LED or ultraviolet (UV) light illuminator (Fig. S6).

As shown in Fig. 1a, these results indicated that AacCas12b exhibited potent *cis*-cleavage and *trans*-cleavage activities at higher temperatures (45 °C–60 °C). However, the *trans*-cleavage activity of AacCas12b was still very strong at lower temperatures (30 °C–39 °C), while the *cis*-cleavage activity was extremely weak. Previous study has shown that Cas12b prefers to cleave non-target strand at lower temperatures [36]. The flexible 6C sequence may, be more accessible to the RuvC active site of AacCas12b for cleavage, just like the non-target strand, but further structural studies are required.

Lower temperatures enhance the specificity of AacCas12b *trans*-cleavage activity. We next investigated the effect of temperature on the specificity of the AacCas12b *trans*-cleavage activity targeting dsDNA. First, we designed a series of consecutive single-point mutated and double-point mutated dsDNA sequences as templates for detection at 37 °C and 48 °C, respectively (Fig. 2a and c and Table S1). Then, 21 single-point mutation targets and 22 double-point mutation targets, as well as the wild-type control and negative control, were added in turn in the system containing AacCas12b, sgRNA and ssDNA- FQ, and then incubated at 37 °C and 48 °C respectively to detect their *trans*-cleavage activity. Real-time fluorescence and LED blue light analysis results showed that AacCas12b tolerated the mutation at 48 °C and produced a stronger fluorescence signal than at 37 °C (Figs. S7–S10). In addition,

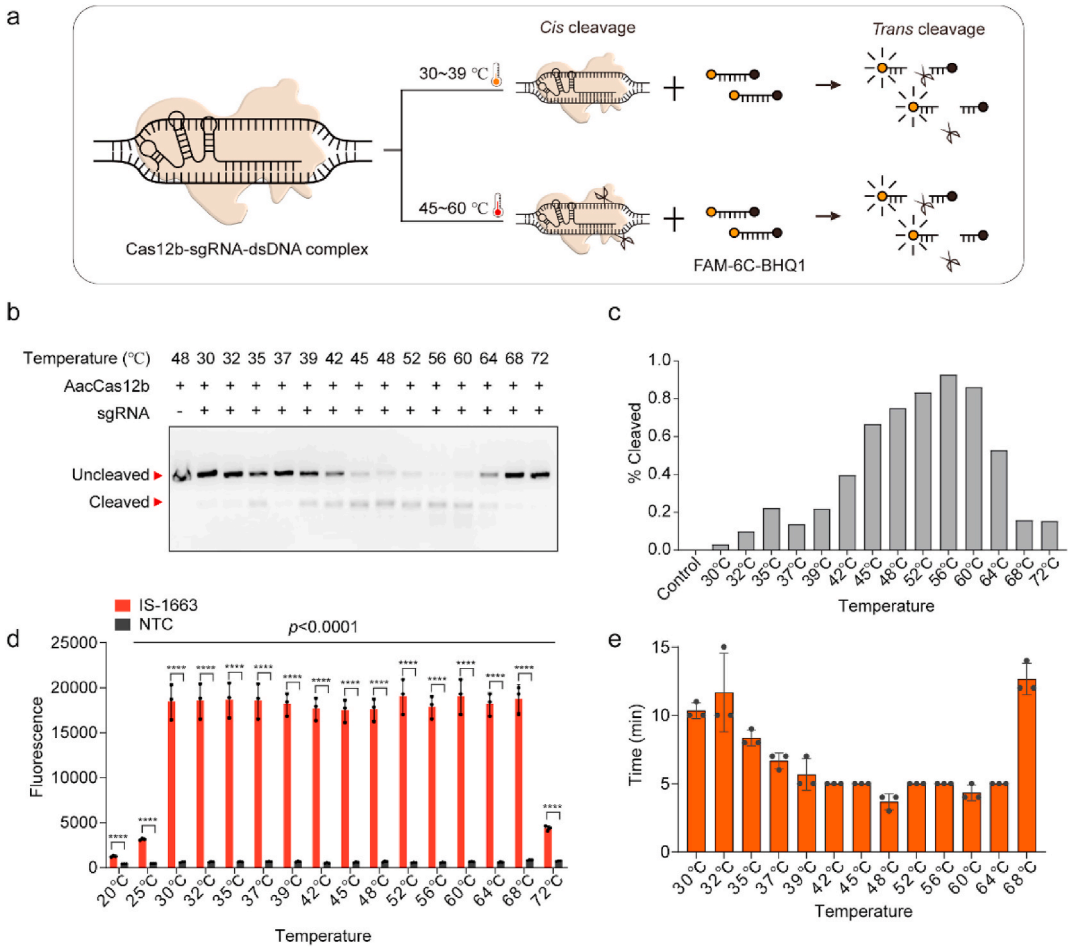


Fig. 1. *Cis*- and *trans*-cleavage activities of AacCas12b at different temperatures. a. Display of *cis*- and *trans*-cleavage of AacCas12b at different temperatures. b, c. *Cis*-cleavage activity of AacCas12b at different temperatures. d. *Trans*-cleavage activity at different temperatures after 30 min of incubation. e. Comparison of the time to reach fluorescence saturation at different temperatures. NTC, nontemplate control reaction. Error bars represent the means ± S.D. of n = 3 replicates, ns = not significant with p > 0.05, and asterisks (*, **, ***, and ****) denote significant differences based on the p values listed above.

the single-point mutants were more easily tolerated than the double-point mutants by AacCas12b/sgRNA3 at 37 °C and 48 °C (Fig. 2b and d). Similarly, the *trans*-cleavage efficiency relative to the wild type (WT) also showed that the *trans*-cleavage efficiency of the detection of single-point mutants and double-point mutants at 37 °C was lower than that at 48 °C (Fig. 2b and d). These results indicated that the specificity of the AacCas12b/sgRNA *trans*-cleavage activity could be significantly improved at 37 °C.

Establishment of the isothermal one-step Rcod system. To apply AacCas12b for nucleic acid detection, we next determined the sensitivity of the AacCas12b/sgRNA system. Without preamplification, a significant difference in the fluorescence signal of Cas12b and Cas12a was observed only when the target concentration was greater than 0.4 nM, and the fluorescence of Cas12b at 37 °C and 48 °C was stronger than that of Cas12a (Fig. S11). These results meant that the difference in *cis*-cleavage activity of AacCas12b at 37 °C and 48 °C would not affect the detection sensitivity of AacCas12b protein, both are 0.4 nM, and its detection fluorescence discrimination is slightly better than that of LbCas12a.Since AacCas12b exhibited strong *trans*-cleavage activity high specificity, and sensitivity consistent with 48 °C at 37 °C, and to avoid contamination caused by cap opening and further improve the test sensitivity of Cas12b, we integrated and RAA into a single reaction system to establish an RAA-CRISPR/Cas12b one-pot detection assay (Rcod). The Rcod workflow is shown in Fig. 3a. Briefly, all reagents, including the RAA mix, primers, sgRNA/AacCas12b complex and 6C reporter, were premixed in one tube, and the amplification and detection steps were performed simultaneously in one tube. After 20–30 min of incubation, the results were observed by real-time fluorescence or directly visualized under a blue LED or UV light illuminator at the end point.

Previous findings indicated that the cleavage activity of CRISPR/Cas would inhibit the target amplification in one-tube reactions [25,37]. Therefore, we further verified the compatibility of the AacCas12b detection system and amplification system. We first tested whether the *cis*-cleavage activity of AacCas12b would cleave the target IS1663 fragment within 30 min. The results showed that the *cis*-cleavage activity of AacCas12b for the dsDNA target was weak at 37 °C, with a value of approximately 20 %, but at 48 °C, this activity increased over time, with the highest activity of more than 80 % observed (Fig. 3b–c).

In addition, we also tested whether the *trans*-cleavage activity of AacCas12b would randomly cleave the primers of the amplification system. First, we designed a pair of labeled primers. The upstream primer was labeled with a 5′-HEX fluorophore and 3′-black hole quencher (BHQ1); correspondingly, the downstream primer was labeled with a 5′-ROX fluorophore and 3′-BHQ1 (Fig. S12a and Table S1). Then, the 5′-HEX fluorophore- and 5′-ROX fluorophore-labeled primers, 5′-FAM fluorophore-labeled 6C and AacCas12b/sgRNA were incubated at 37 °C and 48 °C for 30 min. The real-time fluorescence results showed that at 37 °C, only the FAM channel exhibited strong fluorescence signals, while at 48 °C, the fluorescence of the FAM, HEX and ROX channels could be clearly observed (Fig. 3d–e and Figs. S12b–c). We additionally tested the *trans*-cleavage of AacCas12b for six random ssDNA Taqman

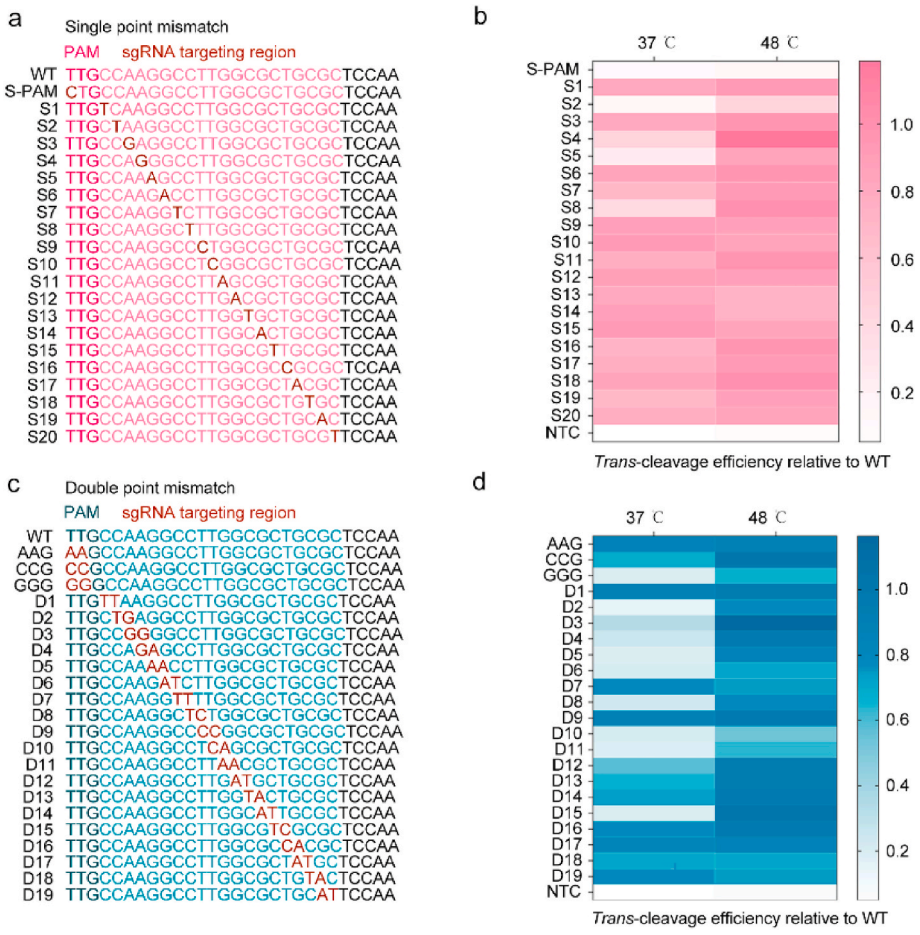


Fig. 2. Lower temperatures enhance the specificity of the AacCas12b *trans*-cleavage activity. a. Single-point mismatch on the IS1663 dsDNA fragment. b. Comparison of the specificity of the *trans*-cleavage activity of AacCas12b for single-point mutations at 37 °C and 48 °C. c. Double-point mismatch on the IS1663 dsDNA fragment. d. Comparison of the specificity of the *trans*-cleavage activity of AacCas12b for double-point mutations at 37 °C and 48 °C.

probes at 37 °C and 48 °C, and observed only a significant difference in fluorescence at 48 °C (Fig. S13). These results indicated that the *cis*-cleavage of AacCas12b had little interference with the target and the *trans*-cleavage activity only specifically cleaved the ssDNA 5′-FAM-6C reporter, implying that AacCas12b had no obvious interference effect on the amplification system at 37 °C.

Since AacCas12b exhibited weak *cis*-cleavage activity at lower temperatures (Fig. 1a–b), the Rcod system was next tested at temperatures from 30 °C to 45 °C to determine the best reaction temperature for the one-step Rcod method. For comparison, the protein was first incubated at 37 °C for 10 min, and then the temperature was increased to 48 °C for detection. In addition, 200 copies/μL plasmid containing the 1017-bp IS-1663 sequence of *B. pertussis* was used as the target sequence (Table S1). As shown in Fig. 3f–g and Fig. S14, the Rcod system showed significantly enhanced fluorescence signals at temperatures from 32 °C to 39 °C. In addition, the fluorescence signal at 37 °C was almost identical to that of the control at 37 °C and then at 48 °C. Furthermore, after 30 min of incubation, the end-point fluorescence signals showed consistent results by LED blue light illumination or ultraviolet irradiation (Fig. 3h). In addition, the Rcod system was also tested at room temperature, but no signal was observed (Fig. S15). Therefore, we chose 37 °C as the reaction temperature for the one-step Rcod system.

Limit of detection (LoD) of the Rcod system. Next, to enhance the sensitivity of Rcod detection, we optimized the components of the system. We first optimized the AacCas12b/sgRNA ratio because the activity of AacCas12b is critical to the system. The results showed that when the optimal AacCas12b/sgRNA ratio (1:1, 0.23 μM each) was exceeded, the higher the AacCas12b/sgRNA ratio was, the lower the fluorescence signal generated by cleavage of the 6C reporter molecule (Fig. S16). It may be that the high AacCas12b/sgRNA ratio led to high *cis*-cleavage activity, which inhibited the RAA reaction. By then choosing a 1:1 ratio of AacCas12b/sgRNA, we investigated the effect of primer concentration and 6C ssDNA probe concentration on the Rcod system. As shown in Figs. S17–18, the optimal primer concentration and 6C ssDNA probe concentration were 0.48 μM and 1.2 μM, respectively.

Further, we tested the LoD of our one-step Rcod system under the conditions of the optimal system. A series of 10-fold dilutions of the IS-1663 plasmid were used as templates, with 4 assays per gradient. As shown in Fig. 4a–c, Figs. S19 and S21a, our Rcod system at 37 °C could stably detect as low as 0.2 copies/μL input plasmid DNA. In addition, we compared the isothermal method with the variable-temperature one-step Rcod method, in which the amplification reaction was performed at 37 °C first and then the temperature was raised to 48 °C for detection. Real-time fluorescence and LED blue light results showed that the sensitivity of the variable-temperature one-step Rcod was also 0.2 copies/μL (Fig. 4d–f and Figs. S20–S21b). To further determine whether the relative standard deviation between parallel samples was acceptable, plasmid DNA with a concentration of 0.2 copies/μL was tested 20 times by the Rcod system at 37 °C, and the results showed a detection rate of 100 % (20/20), indicating that these standard deviations were within reasonable limits (Fig. S22). In addition, our Rcod platform also exhibited high specificity, and could only specifically detect *B. pertussis* without cross interference from other respiratory pathogens (Fig. S23). These results indicate that our Rcod platform shows high sensitivity and specificity in nucleic acid detection, and is convenient, rapid, isothermal and practical in one-step reaction.

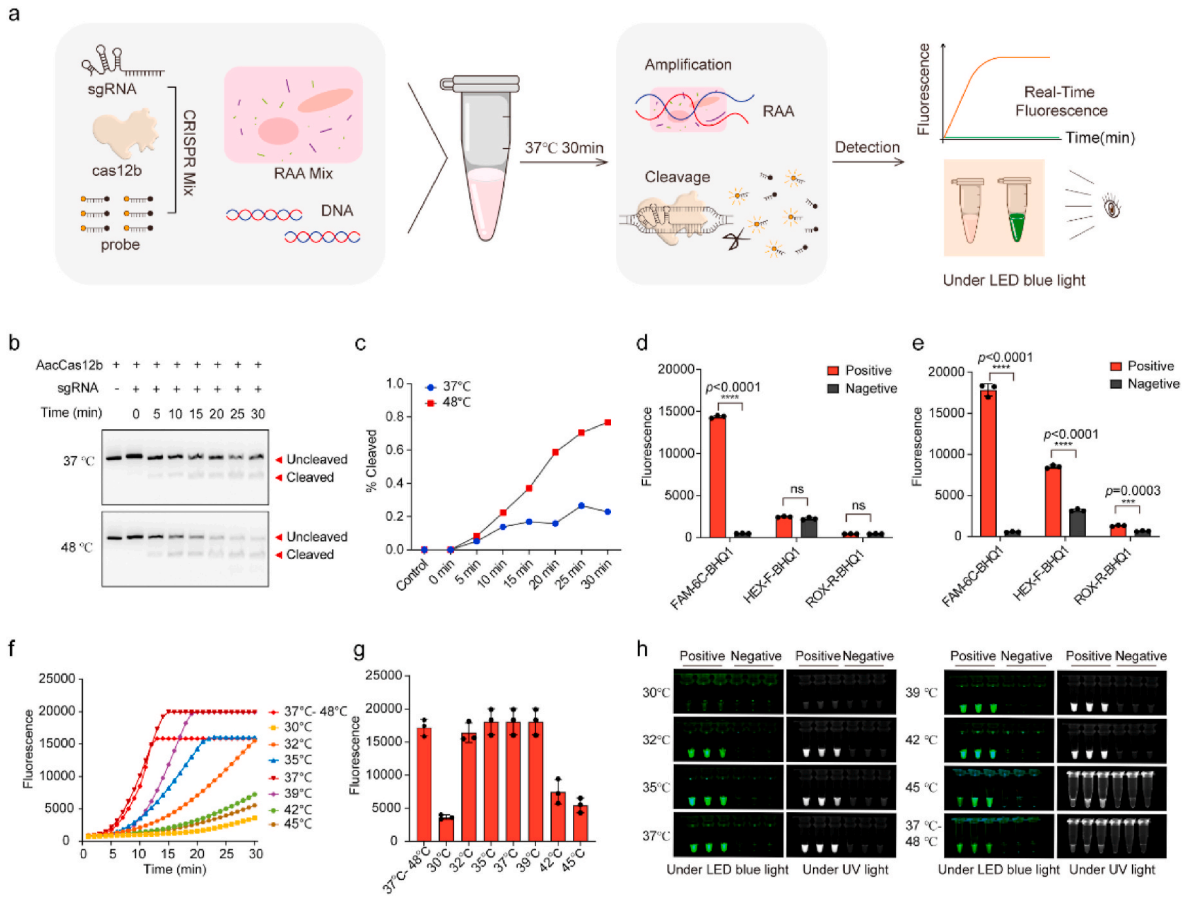


Fig. 3. Establishment of the isothermal one-step Rcod system. a. Schematic showing the Rcod assay system for rapid and visual nucleic acid detection. b,c. *Cis*-cleavage activity of AacCas12b at 37 °C and 48 °C at different times. d, e. *Trans*-cleavage activity of AacCas12b for primers at 37 °C (d) and 48 °C (e). f, g, h. Evaluation of the Rcod system at different temperatures by real-time fluorescence and endpoint imaging. Error bars represent the mean ± S.D. with n = 3 replicates. ns = not significant with p > 0.05, and the asterisks (*, **, ***, and ****) denote significant differences with the p values listed above.

Clinical validation of the Rcod platform. To shorten the sample pretreatment time and increase the speed of testing clinical samples, the Rcod platform was combined with the nucleic acid extraction-free release agent method, which could extract samples and test within 30 min. The workflow from sample extraction to detection was shown in Fig. 5a. Specifically, 20 μL of clinical samples were mixed with an equal volume of nucleic acid release agent 1:1. After shaking for 5 s, 5 μL of lysate was added to the premixed Rcod system, and the results were monitored by fluorescence signals in real time, or the fluorescence signals were observed with the naked eye through LED blue light and ultraviolet light, so as to perform qualitative analysis on the tested samples.

To validate the clinical applicability of the Rcod platform with a nucleic acid extraction-free release agent, we tested 98 whooping cough culture-positive samples and 123 whooping cough culture-negative samples from Beijing Children's Hospital Affiliated to Capital Medical University with the isothermal and variable-temperature Rcod system. Infection status was independently confirmed by Beijing Children's Hospital Affiliated to Capital Medical University. The 221 samples were divided into two parts, one for the Rcod system and the other for real-time PCR.

As shown in Fig. 5b–c, we compared the original fluorescence intensity of Rcod with the cycle threshold value (Ct) of real-time PCR, which is proportional to the concentration of the target gene. The two detection methods showed good consistency. 96 whooping cough-positive culture samples were identified as *B. pertussis*-positive by our isothermal temperature Rcod system, and the original fluorescence intensity was greater than 2699 a. u. (3 × the average of all samples' initial fluorescence values) (Fig. 5b, d-e and Fig. S24). In contrast, 122 whooping cough culture-negative samples were identified as *B. pertussis*-positive with our method, and the endpoint fluorescence value was less than 2000 a. u. (Fig. 5c–e and Fig. S25). In addition, the end-point visual detection results were consistent, and there was a significant difference in the end-point fluorescence values of *B. pertussis* positive and negative samples, which could be effectively distinguished by end-point fluorescence (Fig. 5d and Fig. S25). Furthermore, we calculated the sensitivity and specificity of the isothermal Rcod system for testing *B. pertussis* clinical samples. Our Rcod system showed 97.96 % sensitivity, 99.19 % specificity, and 98.64 % accuracy compared to PCR, while the detection time was 20–30 min, compared to 2 h for PCR (Fig. 5f and Table S2).

To further improve the objectivity and accuracy of visual observation, we have introduced a mobile isothermal amplification analysis system, which fluorescence signal results and positive or negative result interpretation were displayed on the mobile software Fullgene01 (Fig. S26a). As shown in Figs. S26b–c, positive and negative samples showed consistent results when tested on a mobile isothermal amplification analysis system compared with real-time fluorescence and LED blue light. Furthermore, the mobile isothermal amplification analysis system was more convenient to use than real-time fluorescence, and the result interpretation was more objective and accurate than LED blue light instruments.

These results indicate that Rcod combined with the nucleic acid extraction-free release agent extraction method shows high sensitivity, specificity and accuracy in the clinical application of *B. pertussis*, and is highly consistent with the qPCR method. Moreover, Rcod detection is very rapid and simple, with a time of 30 min and a reaction temperature

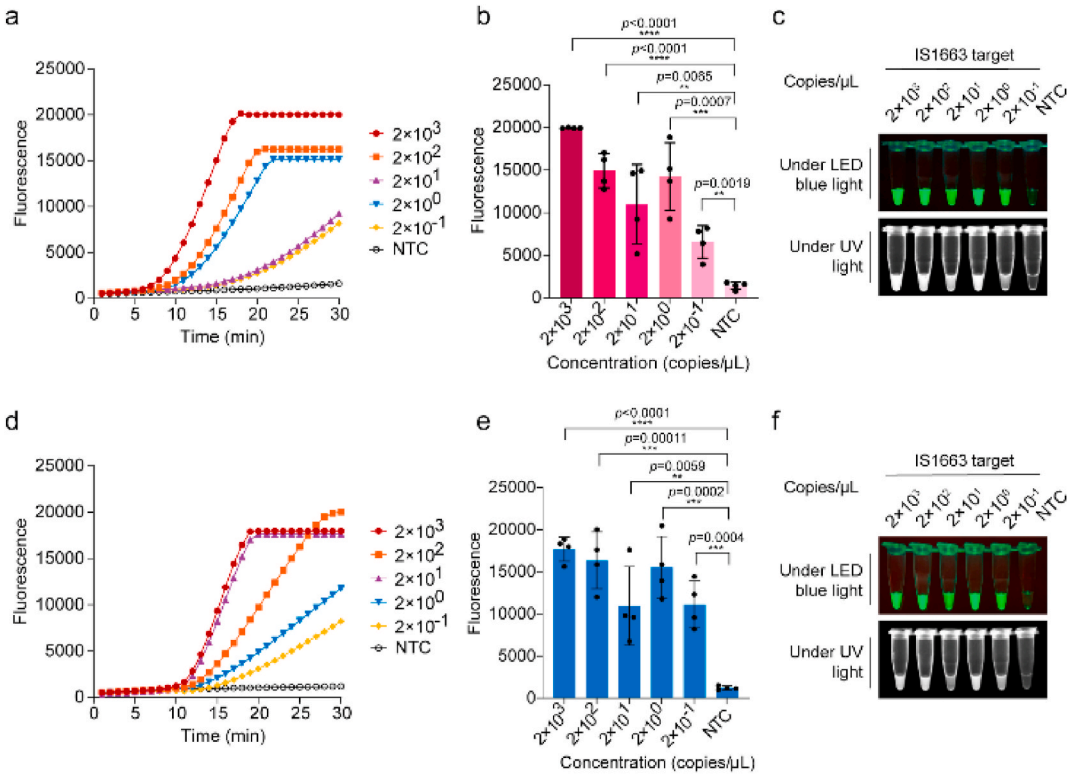


Fig. 4. Limit of detection (LoD) of the Rcod system a, b, c. Real-time fluorescence and endpoint imaging results showing the LoD of the Rcod system at a constant temperature of 37 °C. d, e, f. Real-time fluorescence and endpoint imaging showing the LoD of the ORCD system at variable temperatures. Error bars represent the mean ± S.D. with n = 4 replicates. the asterisks (*, **, ***, and ****) denote significant differences with the p values listed above.

of 37 °C. After the reaction, the fluorescence signal can be observed by naked eyes through LED blue light, ultraviolet irradiation or mobile handheld isothermal PCR analysis system, while the detection time required by qPCR is 2 h, and rely on repeated temperature changes, indicating that our Rcod system has greater application value in clinical diagnosis and detection.

4. Conclusion

In this study, we established an RAA-CRISPR/Cas12b one-pot detection assay (Rcod) system for rapid pathogen detection. Compared with traditional CRISPR-based detection methods [31,32,34], the system in which RAA amplification and AacCas12b cleavage are directly performed in one tube is more convenient to implement, avoids the tedious operation of the two-step method, and reduces the possibility of contamination caused by opening the cap. Compared with other Cas12b one-step methods, such as STOPCovid which combines LAMP and Cas12b detection in one step, Our system has the advantages of higher sensitivity (0.2 copies/μL) and shorter time consumption (within 30 min), while STOPCovid has a sensitivity of 2 copies/μL within 60 min [35]. Moreover, the combination of Rcod with a nucleic acid-free extraction method could detect *B. pertussis* in 221 clinical samples within 30 min with a sensitivity of 97.96 %, specificity of 99.19 % and accuracy of 98.64 % compared to those of real-time PCR.

In addition, our Rcod system exhibited excellent *trans*-cleaving specificity for point mutation targets and random ssDNA at low temperatures. This high *trans*-cleaving specificity reduced the cleavage interference of Cas12b to the primers in the Rcod system. In contrast to the flexible 6C sequence, we reasoned that complex and macromolecular ssDNA might be poorly accessible the RuvC active site of AacCas12b at low temperature. We are doing further structural research and verification.

Moreover, we investigated the *cis*-cleavage and *trans*-cleavage activities of AacCas12b at different temperatures. At lower temperatures, AacCas12b still maintained high *trans*-cleavage activity, while the *cis*-cleavage activity was extremely weak. This low *cis*-cleavage activity and high *trans*-cleavage activity of CRISPR−Cas proteins is the key to one-step detection combined with nucleic acid amplification. Low *cis*-cleavage activity can reduce the possibility of CRISPR−Cas proteins cleaving nucleic acids in one pot. In this study, the *cis*-cleavage activity of AacCas12b was reduced by reducing the reaction temperature. Furthermore, the strong *cis*-cleavage activity of CRISPR−Cas could be reduced by reducing the concentration of Cas proteins or designing an sgRNA targeting a nonclassical protospacer-adjacent motif (PAM), which is more convenient to combine with nucleic acid amplification for one-step detection.

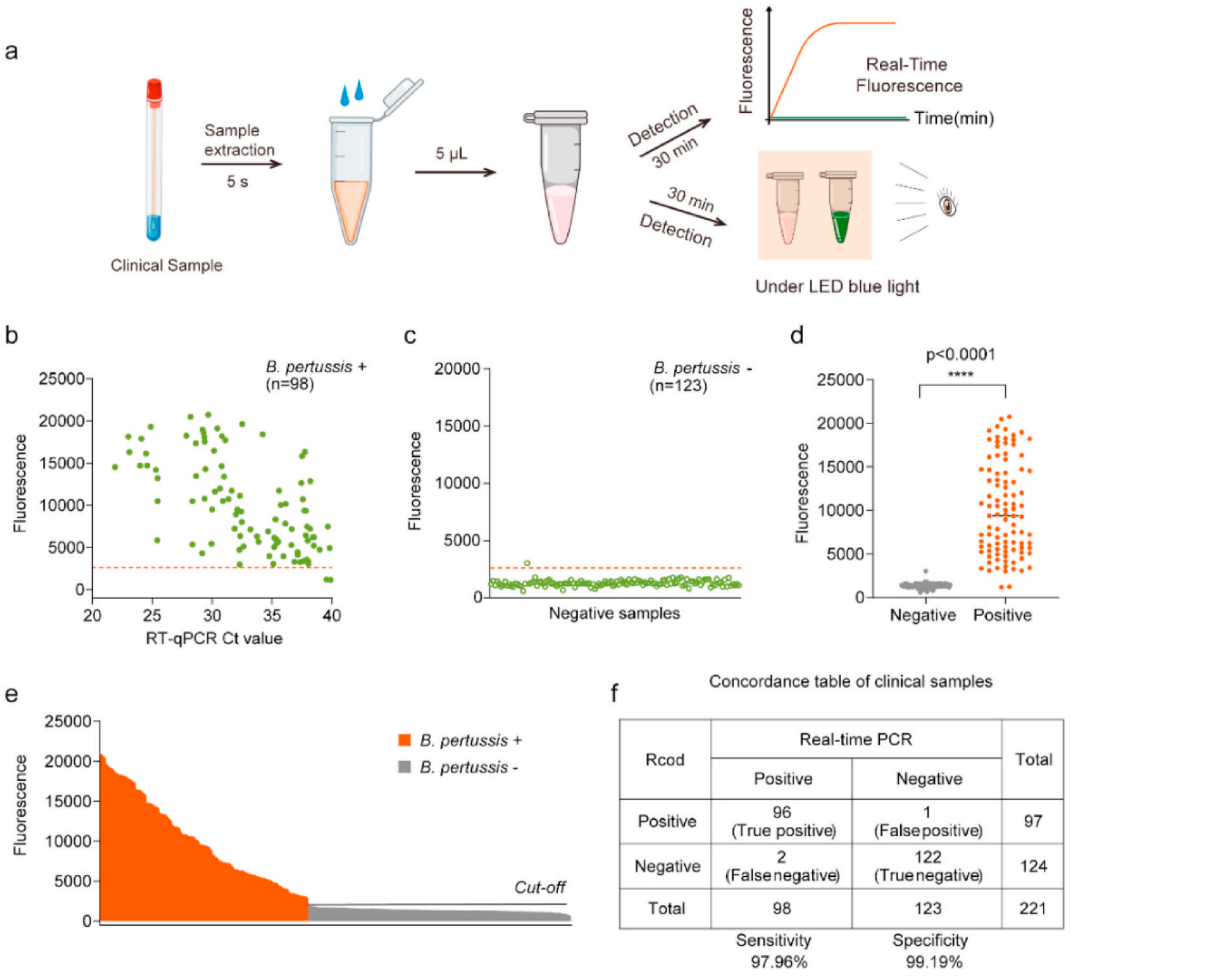
In summary, the Rcod system we established is a one-step, simple and fast nucleic acid detection method with high sensitivity and specificity and provides a rapid diagnostic methodology for POC diagnosis.

Author Contributions

Weixin You: Resources. Xiangju Guo: Data curation, Resources. Kaihu Yao: Data curation, Resources. Qinghan Li: Data curation, Resources. Boan Li: Conceptualization, Supervision, Validation, Writing – review & editing. Xiao Li: Data curation, Resources. Zhiyong Li: Conceptualization, Validation, Writing – review & editing. Kangfeng Lin: Data curation, Methodology, Validation, Writing – original draft. Xiaoqing Yang: Conceptualization, Validation, Writing – review & editing. Rui Zhang: Formal analysis. Zhen Sun: Formal analysis. Jianguang Guo: Formal analysis. Ya Bian: Formal analysis. Wenjing Ren: Formal analysis

Declaration of competing interest

The authors declare that they have no known competing financial



Rcod	Real-time PCR		Total
	Positive	Negative	
Positive	96 (True positive)	1 (False positive)	97
Negative	2 (False negative)	122 (True negative)	124
Total	98	123	221
	Sensitivity 97.96%	Specificity 99.19%	

Fig. 5. Detection of *Bordetella pertussis* clinical samples by Rcod. a. Workflow of Rcod combined with the nucleic acid extraction-free release agent extraction method. b,c. Rcod results for 221 *Bordetella pertussis* clinical samples (a, 98 positive samples; b, 123 negative samples). The threshold (2699 a. u) was determined as 3 × the average of all samples' initial fluorescence values. d. Comparison of fluorescence values in patients with *Bordetella pertussis* (+) and controls (−). e. Waterfall distribution of all *Bordetella pertussis* test samples (n = 221). f. Concordance table between Rcod and real-time PCR for 221 samples.

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2023.125616.

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