Biosensors and Bioelectronics 159 (2020) 112143

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: http://www.elsevier.com/locate/bios

An ultrasensitive and specific point-of-care CRISPR/Cas12 based lateral flow biosensor for the rapid detection of nucleic acids

Omar Mukama [b,c,e], Jinghua Wu [a], Zhiyuan Li [b], Qiongxin Liang [a], Zhijian Yi [a], Xuewen Lu [b], Yujie Liu [b], Yumei Liu [a], Muzammal Hussain [d,e], Gaelle Guiewi Makafe [d,e], Jiaxin Liu [b], Ning Xu [f], Lingwen Zeng [a,b,*]

[a] School of Food Science and Engineering, Foshan University, Foshan, 528231, China
[b] Key Laboratory of Regenerative Biology, South China Institute for Stem Cell Biology and Regenerative Medicine, Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, 510530, China
[c] Department of Applied Biology, College of Science and Technology, University of Rwanda, Avenue de l'armée, P.O. Box: 3900, Kigali, Rwanda
[d] State Key Laboratory of Respiratory Disease, Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, 510530, China
[e] University of Chinese Academy of Sciences, 19 Yuquan Road, Shijingshan District, Beijing, 100049, PR China
[f] Department of Clinical Laboratory, Guangdong Provincial Hospital of Chinese Medicine (The Second Affiliated Hospital of Guangzhou University of Chinese Medicine), Guangzhou, China

ARTICLE INFO

Keywords:
CRISPR/Cas12
LAMP
Probe based lateral flow biosensor
Pseudomonas aeruginosa
Nucleic acid detection

ABSTRACT

CRISPR/Cas systems have displayed remarkable potential in developing novel biosensing applications for nucleic acid detection owing to the collateral cleavage activity of Cas effector proteins (Cas12, Cas13, etc.). Despite tremendous progress in recent years, the existing CRISPR/Cas based biosensing platforms have several limitations, including reliance on proper amplification methods, expensive fluorescence detection equipment, or lateral flow biosensor (LFB). Herein, we report a simple, inexpensive, and ultrasensitive DNA probe based LFB with CRISPR/Cas and loop-mediated Isothermal Amplification (namely CIA). The concept behind this approach is a non-detectable test line on the LFB when the Cas effector protein collaterally cleaves the cognate target and an ssDNA reporter sequence. The CIA based LFB can detect as low as a single copy cloned *Pseudomonas aeruginosa* acyltransferase gene, 1 cfu/ml plasmid containing *E. coli* DH5α pure cultures, as well as clinical samples without DNA extraction/purification or advanced apparatuses. No cross-reactivity with other non-target bacteria was observed. The naked eye result readout was obtained in 15 min of LAMP amplification, 30 min of Cas12 reaction, and 5 min of LFB readout. This platform is robust and of low cost for on-site testing.

1. Introduction

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (CRISPR/Cas) systems endow bacteria and archaea the adaptive immunity against invading nucleic acids (Barrangou et al., 2007; Jinek et al., 2012). These systems mainly work under the guidance of a guide-RNA (gRNA) that navigates Cas effector proteins to target and cleave an invader DNA sequence (Jinek et al., 2014). Over the last few years, CRISPR/Cas systems such as Type II CRISPR/Cas9 have become a prominent tool in transcription regulation, genome editing, and in situ DNA/RNA detection. Similar to CRISPR/Cas9, Type V CRISPR-Cas effectors such as Cas12 and Cas13 have also been used for genome editing (Tang and Fu, 2018).

Beyond genome editing ability, Cas12 and Cas13 effectors have recently demonstrated remarkable potentials in developing novel biosensing technologies for nucleic acid detection based on their unique property of collateral cleavage of target nucleic acids and non-specific single stranded nucleic acids (used as reporters) to provide fluorescence signal readout. For example, a Cas13-based RNA targeting method called SHERLOCK (Specific High Enzymatic Reporter UnLOCKing) has been used for the detection of pathogens and SNPs (Gootenberg et al., 2018; Myhrvold et al., 2018). Similarly, LbCas12a-based DETECTR (DNA Endonuclease Targeted CRISPR *Trans* Reporter) and HOLMES (One-Hour-Low cost Multipurpose highly Efficient System) have been reported for the diagnosis of human papillomavirus and SNPs, respectively (Chen et al., 2018; Li et al., 2018). In general, these biosensing

* Corresponding author. School of Food Science and Engineering, Foshan University, Foshan, 528231, China.
E-mail address: zeng6@yahoo.com (L. Zeng).

https://doi.org/10.1016/j.bios.2020.112143
Received 18 November 2019; Received in revised form 6 March 2020; Accepted 7 March 2020
Available online 14 March 2020
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platforms require several critical reaction components, including ssDNA activators, crRNAs, buffer, DNA amplification mixture, reverse transcription system (for SHERLOCK), and the single stranded nucleic acids. Besides, SHERLOCK approach requires dual nucleases (Csm6 and LwaCas13), reverse transcriptase RPA, lateral flow assay with an anti-FAM (6-carboxyfluorescein) antibody, streptavidin (SA), protein A, and fluorescence readout of bi-labeled reporters (Gootenberg et al., 2018). Although sensitive, all these CRISPR biosensing methods rely on target nucleic acid amplification through recombinase polymerase amplification (RPA) and PCR pre-amplification, and fluorescence readers to improve sensitivity (Gootenberg et al., 2018; Myhrvold et al., 2018). Therefore, they require sophisticated systems and tedious sample/reagent treatment steps, which further rely on expensive fluorescence reader instruments and well-trained technicians. This can cause practical inconvenience and make the system less robust.

LAMP (Huang et al., 2014; Notomi et al., 2000) has improved this drawback and allows the entire amplification at a single temperature (~65 °C), sensitive to amplify few copies to ~10^{10} copies in less than an hour. Given its 4 primers targeting 6 regions of the target sequence, high specificity can be achieved (Notomi et al., 2000). The LAMP amplicons can be detected using naked eye, dyes, lateral flow biosensors, obviating the need for expensive apparatuses but with susceptibility to false positives due to cross-contamination, non-specific amplification or primer dimerization. The use of various compounds such as DMSO (Watts et al., 2014), pullulan (Gao et al., 2019), betaine (Notomi et al., 2000) increased LAMP sensitivity and specificity. Recently, Li et al. employed LAMP with thermophilic Cas12b to develop a fluorescence based nucleic acid diagnostic termed HOLMESv2 (Li et al., 2019).

The hallmark of the CRISPR-Cas systems is that crRNA-mediated Cas effectors cleavage of reporter doesn't initiate in the absence of amplified target, overcoming previous limitations but with expensive fluorescence machinery. Herein, we integrated *Lachnospiraceae bacterium* Cas12a (LbaCas12a) and *Alicyclobacillus acidoterrestris* Cas12b (AaCas12b) effectors with LAMP to develop an ultrasensitive and specific lateral flow biosensor (LFB) for the detection of *Pseudomonas aeruginosa* (*P. aeruginosa*), characterized with CRISPR/Cas and loop-mediated Isothermal Amplification (termed CIA). *P. aeruginosa* is an opportunistic human pathogen with a multidrug-resistance, large and complex genome and critically infectious (Tacconelli et al., 2018). It is widely used as a model for CRISPR/Cas system studies (Gootenberg et al., 2018; Pawluk et al., 2016; van Belkum et al., 2015; Xu et al., 2018). Moreover, LFB is one of the foremost commonly exploited method for the detection of pathogens, small molecules, cancer and genotyping; owing to its rapidity, simplicity, stability, low cost, visual readout, and user-friendly characteristics (Bahadır and Sezgintürk, 2016; Gervais et al., 2011; Xu et al., 2014).

The principle of CIA method is based on the LFB detection of a biotinylated ssDNA reporter. By targeting the *P. aeruginosa* acyltransferase specific gene, we leveraged the CRISPR-Cas12 reaction conditions (buffer, crRNA, biotinylated ssDNA reporter, and activator) to enhance the collateral cleavage of the target gene and the reporter. The reporter cleavage was monitored on the DNA probe based LFB. A DNA probe sequence complementary to the reporter was fixed on the test line of the LFB to capture the reporter; while an inexpensive biotinylated rabbit polyclonal anti-IgG antibody (the antibody was used to fix biotin on the control line) was immobilized on the control line to capture the excessive gold nanoparticle-streptavidin (AuNP-SA) complex that was previously sprayed and dried on the conjugate pad. The AuNP-SA conjugate reacts with biotinylated ssDNA reporters and forms an AuNP-SA-biotin ssDNA reporter complex, which is captured by the capture probe at the test line. The excess complex subsequently reacts with the biotinylated rabbit polyclonal anti-IgG antibody on the control line to spot the biosensor performance. With LAMP amplification of the target at 65 °C for 15 min followed by Cas12 reaction, the LAMP products and the reporter were collaterally cleaved, resulting to trans-cleaved reporters that are undetectable at the LFB test line (Fig. 1). This provide a novel approach for visual detection of target gene on LFB with CRISPR/Cas mediated *trans*-cleavage. The CRISPR/Cas based fluorescence assay using a FAM fluorophore at one end and a fluorescence quencher TAMRA (tetramethylrhodamine) at the other end of the ssDNA reporter confirmed the single copy sensitivity. Moreover, our CIA based LFB discriminated *P. aeruginosa* from non-target bacteria (*Escherichia coli* O157:H7, *Staphylococcus aureus*, *Campylobacter jejuni*, *Salmonella typhimurium*, and *Shigella dysenteriae*), both in pure and complex samples. This technique is fast, accurate, robust, and inexpensive for clinical diagnosis of infectious diseases.

2. Material and methods

2.1. Reagents, chemicals and machines

All oligonucleotides, including *P. aeruginosa* specific target sequence, primers, crRNAs (designed using benchling), reporters, and lateral flow capture probes were synthesized by Sangon Biotech (Shanghai, China) (Table s1). Purified LbaCas12a, *Bst* 2.0 WarmStart polymerase was purchased from New England Biolabs (New England, USA). Deoxynucleoside triphosphates (dNTPs) and Taq DNA polymerase premix was purchased from Takara (Takara China). Betaine was purchased from Sigma–Aldrich (MO, USA). pET28a-AaCas12b plasmids gifted from Tolo Biotech (Shanghai, China) was expressed in BLD21 and purified accordingly using Ni column and gel filtration (Li et al., 2019).

$HAuCl_4.3H_2O$, trisodium citrate, and SA were purchased from Sigma–Aldrich (Steinheim, Germany). Nitrocellulose membranes CN140 (1UN14ER100025NT) were purchased from Sartorius (Göttingen, Germany). A dispenser HM3030, strip cutter ZQ2002, fiber glass for sample/conjugate pad SB-08 and absorbent pad CH37 (ABP-S370) were

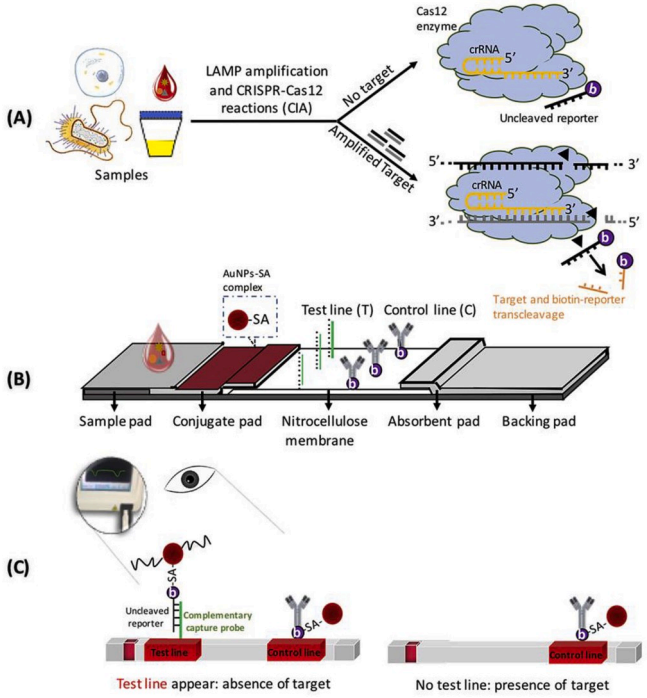


Fig. 1. A schematic illustration of the principle of CIA based LFB. (A) Samples are heat-treated, LAMP-amplified (65 °C, 15 min), and then subjected to Cas12a/b collateral cleavage reaction. (B) The reaction product mixture is directly loaded on the CIA based LFB. (C) In the absence of the target gene, AuNP-SA-biotin-ssDNA reporter complex is captured by a single strand capture probe that is complementary to the ssDNA reporter, and the test line becomes detectable. The excess of AuNP-SA is then captured at the control line by the biotinylated polyclonal antibody. The amount of uncleaved ssDNA reporter can be detected by naked eye or a portable strip reader.

purchased from Shanghai Kinbio (Shanghai, China). A portable strip reader is from Goldbio Technology Co. (Shanghai, China). Eppendorf centrifuge 5415R was from Eppendorf AG (Hamburg, Germany). Genome and plasmid extraction kits were purchased from Qiagen (Hilden, Germany) and Tiangen Biotech (Beijing, China), respectively. Other solutions and buffer used in this study were prepared in our laboratory.

2.2. Bacteria culture and crude genomic DNA extraction

P. aeruginosa acyltransferase DNA target was cloned into pUC57 vector and transformed into *E. coli* DH5α. Bacteria strains used in this study were *P. aeruginosa* and negative controls such as *S. aureus*, *E. coli*, *C. jejuni*, *S. typhimurium*, *S. dysenteriae* (ATCC, Manassas, VA). Bacteria were cultured in Luria-Bertani medium at 37 °C to >2 OD. Then, the culture was collected, washed and adjusted to 2, 1, 0.8, 0.6, 0.4, 0.2, 0.1 OD. Serial dilutions were made from the culture plate with 0.1 OD for the preparation of 10^8, 10^7, 10^6, 10^5, 10^2, 10, 1 colony forming units (cfu/ml).

To obtain a crude genomic DNA for subsequent amplification, bacterial cultures were heat-treated (95 °C, 5 min), centrifuged (6500 rpm, 10 min), followed by 10-fold serial dilutions, and measuring using nanodrop at $A_{260/280}$.

2.3. LAMP/PCR amplification

LAMP amplification was performed according to NEB instruction. Primers were designed using Premier Biosoft LAMP designer and NCBI-BLAST for template sequence specificity check. Briefly, 25 μl LAMP reaction mixture was composed of primers F3, B3 (0.2 μM) and FIB, FIP (1.6 μM), 1.4 mM dNTP, 1.0 M betaine, 6 mM $MgSO_4$, 1 × isothermal amplification buffer, various target DNA template concentrations, and 320 U/ml *Bst* 2.0 WarmStart DNA polymerase, which allows the reaction mixture set up at room temperature. The mixture was then LAMP amplified at 65 °C for 15–30 min and then terminated at 80 °C for 10 min.

For PCR amplification, 25 μl PCR reaction mixture was composed of 1 μl forward and reverse primers (0.3 μM), 12.5 μl 1 × rTaq premix, 1–2 μl template (200 ng) and ddH_2O to final reaction volume. The reaction mixture was incubated in a thermocycler using 3 steps PCR protocol: 95 °C, 10 min for denaturation; 95 °C, 10 s for denaturation; 62 °C, 10 s for annealing; 72 °C, 30 s for elongation; 35 cycles.

2.4. Target cleavage assays

For CIA based LFB analysis of trans-cleaved reporters, the cleavage assay (Chen et al., 2018) was carried out with slight modifications. A 25 μl set up of Cas12a/b cleavage assay consisted of 6 μl of cleavage buffer (150 mM KCl, 10 mM $MgCl_2$, 1% glycerol, 0.5 mM DTT, 20 mM HEPES (pH 7.5)), 3 μl crRNA (300 nM), 1 μl Cas12a/b (1 μM), 3 μl activator (400 nM) and 2 μl reporter (250 nM). Unless indicated otherwise, 1–2 μl target DNA and nuclease free water were added up to the final volume prior to incubation at 37 °C at different time intervals. For one-pot reaction, a 50 μl reaction was made of Cas12a/b premix as described above and double concentration of the LAMP amplification components. Then, the mixture was incubated at different temperature and time intervals prior to analyzing using LFB after adding ddH_2O up to 60 μl final volume.

For fluorescence analysis of trans-cleavage reaction, 25 μl set up was prepared as mentioned above with 5′FAM and 3′TAMRA labeled ssDNA reporter instead of biotinylated ssDNA reporter. The reaction was incubated at 37 °C in 96-well microplate and then read using the Mithras^2 LB 943 plate reader (Berthold Tech, Germany). The fluorescence from the cleavage reaction was measured at different time course with excitation at 495 nm and emission at 520 nm.

2.5. Preparation of AuNPs and conjugates

AuNPs of 15 nm average diameter were prepared by citrate reduction method (Zeng et al., 2013). To prepare AuNP–SA conjugate, 1 ml of prepared AuNP solution was collected by centrifugation (12,000 rpm, 25 min), concentrated 4 times, mixed with 100 μl of suspension buffer (20 mM Na_3PO_4, 5% BSA, 0.25% Tween-20, 10% sucrose, and 0.1% NaN_3) and subsequently added to 0.5 mg/ml of SA. The solution was gently shaken for 3 h at 4 °C, centrifuged (12,000 rpm, 25 min) and rinsed three times with suspension buffer to remove unbound SA. The red pellet was resuspended in 100 μl of suspension buffer before dispensing on the conjugate pad.

2.6. Construction of LFB

As shown in Fig. 1, the capture probe 3′-16A-T-AA-5’ (50 μM) and biotinylated rabbit polyclonal anti-IgG antibody (0.7 mg/ml) were dispensed on the LFB nitrocellulose membrane strip (25 mm in width) at 0.5 μl/cm flow rate to form a test and control zone, respectively. The membrane was dried at room temperature for 12 h and stored in low-humidity at 25 °C until use. To assemble the LFB, a fiberglass sample pad (16 mm in width) was immersed in sample pad buffer (1% Triton, 1% BSA, 2% glucose, 50 mM boric acid, pH 8.0), dried and stored in a desiccant container. The conjugate and absorbent pads (6 mm and 17 mm in width, respectively) were used without buffer treatment. Then, all pads were sequentially assembled along the adhesive part of the nitrocellulose membrane with an overlap of 2 mm and then cut into 0.4 cm wide strips.

2.7. LFB detection of reporter sequences

Prior to sample analysis, biotinylated reporter bio-8T-A-2T (5′–biotin-TTTTTTTTATT-3′) was used to hybridize with its complementary capture probe immobilized on the LFB test line. For the detection of cleaved reporter by LFB, 25 μl of the reaction was mixed with 35 μl of ddH_2O then loaded to the sample pad. After 5 min, the biosensor was read using a portable LFB reader and generated peak areas of the lines were analyzed using ImageJ software (Toley et al., 2015). Results were considered positive when the reporter sequence was entirely cleaved (no test line signal), and the control line captured the excess of AuNP-SA complex, indicating that the LFB works properly. Reproducibility of the approach was confirmed by testing each sample three times and the biosensor can be used for real sample determination.

2.8. Spiked and clinical isolates analysis

Clinical human serum and milk samples were confirmed *P. aeruginosa* negative using PCR. Twenty-five μl of each sample was 10 times diluted, spiked separately with different concentrations of *P. aeruginosa* crude genomic DNA or recombinant plasmid, and then subjected to CIA assay in a total volume of 50 μl.

For clinical sample test, 40 clinical sputa from *P. aeruginosa* suspected patients were collected. The samples were heated at 56 °C for 30 min for enzymatic inhibition. The heat-treated samples were either used for DNA extraction for PCR amplification or 10 times diluted and heated at 95 °C for 5 min and centrifuged (6500 rpm, 10 min) prior to LAMP amplification. Two μl of the heat extracted DNA was used in the CIA assay as mentioned above.

3. Results

3.1. Optimization of the length and concentration of the reporters and their complementary ssDNA capture probes on the LFB test line

Reporters with a certain nucleotide composition and length are crucial to provide optimal signal readout upon Cas effector mediated

collateral cleavage (Chen et al., 2018; Gootenberg et al., 2018; Li et al., 2018). Therefore, we adopted a 5′-TTATT-3′ ssDNA reporter (Chen et al., 2018) with slight modifications. The reporter was extended at the 5′-end with 1–9 thymines and biotinylated, and its complementary capture probes were immobilized on the LFB test line to achieve a detectable signal through hybridization (Table S1). The test lines were detected when the reporters and the capture probes were longer than 9 bases (Figs. S1A and B). As shown in Figs. S1C and D, strong hybridization was obtained with the combination of 11 bases reporter (5′-Bio--TTTTTTTTATT-3′) and 38 bases capture probe (3′-16ATAA-5′): 5′-AATAAAAAAAAAA AAAAAAAATAAAAAAAAAAAAAAA-3′, respectively. The highlighted sequence is repeated twice to increase the binding force with the reporter.

We subsequently tested different concentrations of the 11 bases reporter (5, 10, 15, 20, 25, 30, 50, 70, 100 nM) on the LFB (Fig. S1E). The test line was detected at a reporter concentration above 10 nM, which became prominent with a subsequent increase in concentration (Fig. S1E). However, 20 nM was found to be the optimum concentration to obtain a strong test line in the absence of the target, and non-detectable test line signal in the presence of the target (data not shown). The concentration of 20 nM and the reporter length 11 bases were then used for subsequent Cas12 cleavage experiments.

3.2. Sensitivity of CIA based LFB

Cas12a exhibits a PAM-dependent collateral cleavage property and target pre-amplification increases its sensitivity (Chen et al., 2018; Gootenberg et al., 2018; Li et al., 2018). The sensitivity of the CIA based LFB was evaluated using a cloned *P. aeruginosa* acetyltransferase gene with a conserved PAM (TTT) region cloned in pUC57 plasmid (Fig. 2A, Table S1) and verified with LAMP and PCR amplification (Fig. 2B). The cloned plasmid DNA was serially diluted to different concentrations (1×10^{-10}, 1×10^{-11}, 1×10^{-14}, 1×10^{-17}, 1×10^{-18}, 1×10^{-21} M). Two μl of each diluted sample was LAMP amplified and 1 μl of the amplified product was applied to Cas12a cleavage reaction. The product of the LAMP-Cas12a (CIA) reaction was directly loaded to the LFB. As shown in Fig. 2C and D, samples with concentrations of 1×10^{-18} M or higher target plasmid DNA showed no test line as a result of reporter trans-cleavage. However, samples with the concentration of 1×10^{-21} M target plasmid DNA showed a test line indicating no cleavage of the reporter sequence. This sensitivity was achieved with 15 min of LAMP amplification, 15 min of Cas12a cleavage (Fig. 2E and F) and 5 min of LFB readout. One aM (1×10^{-18} M) plasmid DNA in 2 μl contains 1.2 copies, depicting that the sensitivity of the CIA system reached single copy level. The sensitivity of the CIA based LFB was also tested using different amounts of pre-heated *E. coli* DH5α containing target recombinant plasmid DNA (1×10^{8}, 1×10^{7}, 1×10^{6}, 1×10^{5}, 1×10^{2}, 1×10, 1, 0 cfu/ml). None of the samples, except the control (0 cfu/ml), showed a test line on the LFB (data not shown). A sensitivity of 1 cfu/ml was achieved within 30 min of the CIA reaction and 5 min LFB readout (Fig. S4A). The same set of samples was also tested using FAM-TAMRA labeled reporters, and the same sensitivity was also achieved (Fig. S4B). The CIA based LFB reached single copy level sensitivity for plasmid DNA and 1 cfu/ml sensitivity for plasmid containing *E. coli*.

We further validated the CIA based LFB detection of target DNA from *P. aeruginosa* culture. *P. aeruginosa* culture was heat-treated at 95 °C for 5 min to release the genomic DNA for CIA reaction (Fig. 3). The genomic DNA was serially diluted to different concentrations (3.4×10^{-12}, 3.4×10^{-13}, 3.4×10^{-14}, 3.4×10^{-15}, 3.4×10^{-18}, 3.4×10^{-19}, 3.4×10^{-20}, 3.4×10^{-21}, 3.4×10^{-22} M) and then applied to CIA reaction. As shown in Fig. 3B and C, samples with crude genomic DNA of 3.4×10^{-18} M or higher didn't show any test line, the sample with 3.4×10^{-19} M showed faint test line and samples with lower input target DNA showed strong test lines indicating that attomolar crude genomic DNA from cultured *P. aeruginosa* was detected with the CIA based LFB. Two μl of crude genomic DNA sample with the concentration of 3.4×10^{-18} M contains 4 copies. This sensitivity was also obtained in plasmid DNA detection assay (1.2 copies in 2 μl) and Cas12a/b-based one-pot reaction (Figs. S5A–F). However, Cas12a could not withstand the LAMP reaction temperature higher than 55 °C and thus resulted in reduced reporter shearing (Fig. S6). This sensitivity is comparable to previously reported CRISPR/Cas based diagnostics (Chen et al., 2018; Gootenberg et al.,

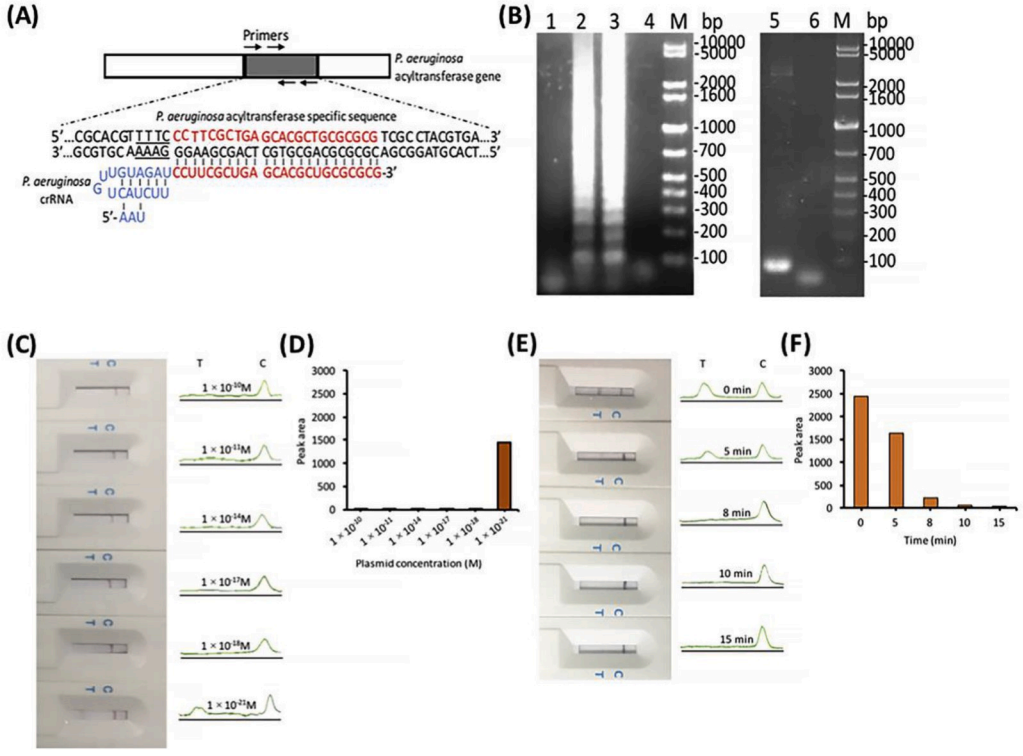


Fig. 2. Condition optimization and sensitivity of CIA assay using recombinant plasmid. (A) Design of *P. aeruginosa* cognate template and crRNA sequence. (B) Amplification of 2 μl *P. aeruginosa* acyltransferase gene (40 ng/μl). Lanes 1–3 correspond to LAMP amplification at 55, 60, and 65 °C; lanes 4 and M correspond to negative control and 1-kb marker, respectively; lanes 5 and 6 correspond to PCR amplification of the gene, its negative control, respectively; M is 1-kb marker. (C) CIA based LFB Sensitivity using different concentrations of recombinant plasmid DNA and (D) corresponding peak areas. (C) The CIA based LFB results for the detection of 1 cfu/ml plasmid containing *E. Coli* for different Cas12a trans-cleavage reaction time and corresponding peak areas (F).

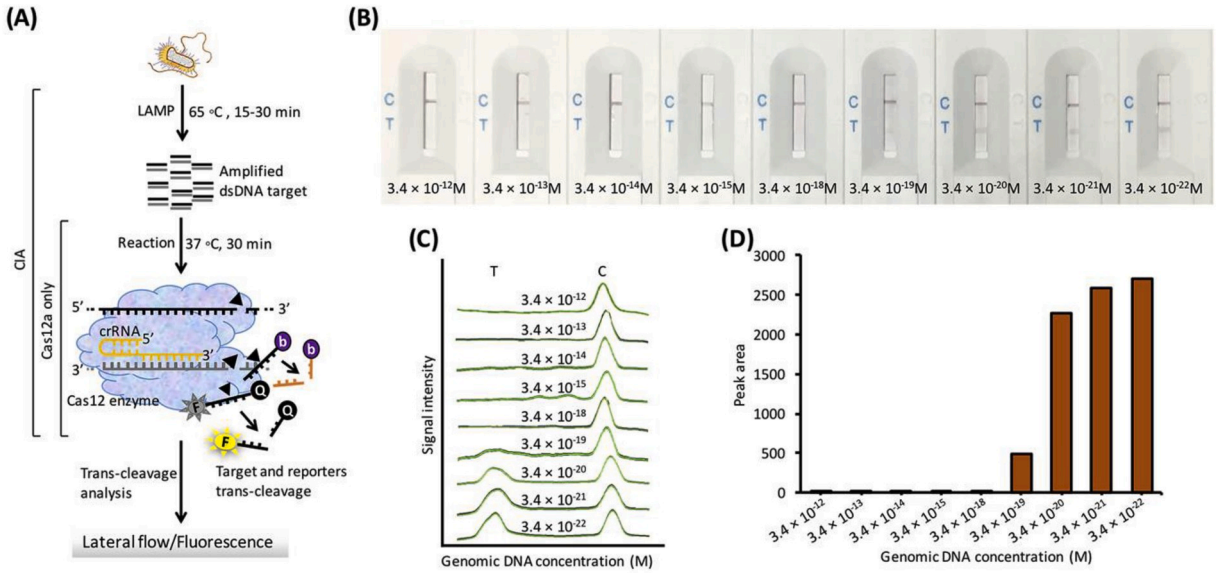


Fig. 3. Detection of *P. aeruginosa* crude genomic DNA. (A) Scheme illustration of LAMP coupled with Cas12 detection (CIA). (B) CIA based LFB detection of different concentrations of *P. aeruginosa* crude genomic DNA, corresponding signal intensities of the LFB (C) and calculated peak areas (D).

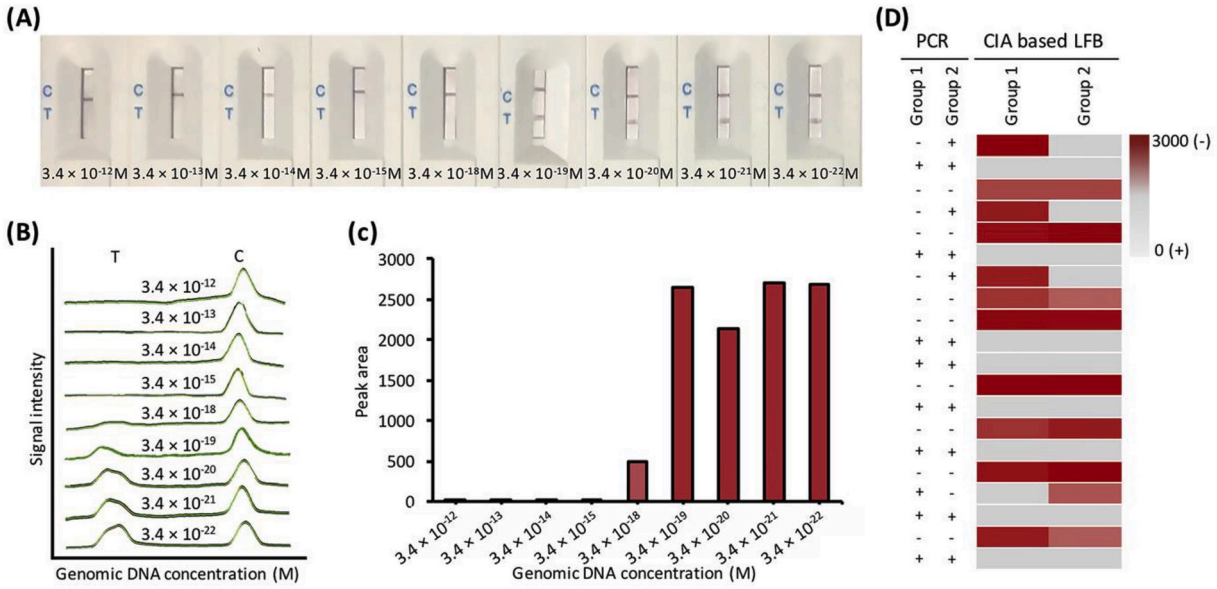


Fig. 4. Sensitivity of the CIA based LFB for the detection of *P. aeruginosa* from spiked and clinical samples. (A) CIA based LFB results for different concentrations of spiked genomic DNA and the corresponding signal intensities (B) and calculated peak areas (C). (D) Heatmap validating the detection of *P. aeruginosa* in 40 clinical sputa samples that were separated into two groups. The calculated peak areas of the LFB test line intensity is represented by red color (red cells: negative, gray cells: positive). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2018; Li et al. 2018, 2019).

3.3. Detection of P. aeruginosa in spiked and clinical samples with CIA based LFB

Milk and serum were spiked with serially diluted *P. aeruginosa* crude genomic DNA (3.4×10^{-12}, 3.4×10^{-13}, 3.4×10^{-14}, 3.4×10^{-15}, 3.4×10^{-18}, 3.4×10^{-19}, 3.4×10^{-20}, 3.4×10^{-21}, 3.4×10^{-22} M) and then subjected to CIA reaction. As shown in Fig. 4A–C, the LFB for the spiked samples with 3.4×10^{-18} M or higher crude genomic DNA showed invisible test lines as a result of cleavage of the reporter, samples with lower concentrations exhibited visible bands and high signal intensities on the test lines as a result of uncleaved reporters. Although the spiked sample with the concentration of 3.4×10^{-18} showed weak signal when a strip reader was used (Fig. 4C), the sensitivity results obtained here for complex samples (milk and serum) is similar as that for heat released crude genomic DNA from bacteria culture (Fig. 3). This sensitivity for target cleavage in complex samples (milk and serum) was also confirmed using the fluorescence assay (data not shown).

Moreover, we tested our CIA based LFB on 40 clinical samples that were previously confirmed *P. aeruginosa* sputa positive or negative by conventional culture and PCR (Fig. S4C). All samples were tested using CIA based LFB. The biosensor corroborated 100% with the conventional PCR method and conventional culture (Fig. 4D). The assay showed a fast response in 30 min and required no expensive materials such as electrophoretic, fluorescence apparatus, or monoclonal antibodies as in previous platforms (Chen et al., 2018; Gootenberg et al., 2018; Li et al. 2018, 2019; Myhrvold et al., 2018). Furthermore, apart from the

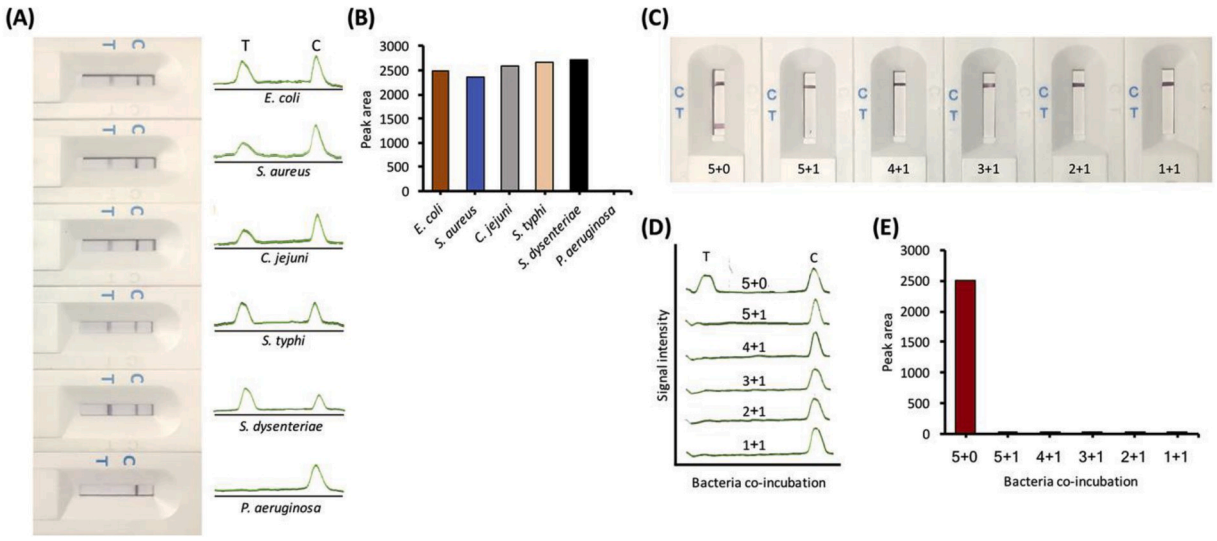


Fig. 5. Specificity assay. (A) CIA based LFB tests of the target *P. aeruginosa* (1 cfu/ml) and non-target bacteria (10^4 cfu/ml). (B) Test line signal peak areas of the CIA based LFB. (C) LFB detection of *P. aeruginosa* samples mixed with different non-target bacteria. The first number is the number of different types of non-target bacteria, and the second number indicates the presence (1) or absence (0) of the target bacteria. (D and E) Test line signal intensity plot (D) and bar chart (E) of the LFB detection results of mixed samples with and without *P. aeruginosa*.

fluorescent dyes, the usage of AuNPs based LFB enabled the visualization of results with naked eye. Thus, our method is applicable for on-site detection of nucleic acids in clinical samples.

3.4. Specificity of the CIA based LFB assay

The specificity of the CIA based LFB assay was tested with non-target bacteria species such as *E. coli* O157:H7, *S. aureus*, *C. jejuni*, *S. typhimurium*, *S. dysenteriae*. Two μl of heat-treated non-target bacteria (10^4 cfu/ml) and target bacteria (1 cfu/ml) were separately applied to CIA reaction. For the non-target bacteria samples, a strong signal was observed at the LFB test lines indicating no cleavage of the reporter, whereas in the presence of as low as 1 cfu/ml *P. aeruginosa*, the test line was not detected indicating complete cleavage of the reporter (Fig. 5A and B). The specificity of the assay was also tested using a mixture of different number of non-target bacteria species (10^4 cfu/ml) in the presence or absence of *P. aeruginosa* (1 cfu/ml). As shown in Fig. 5C, a detectable test line was observed only in the absence of the target bacteria, the test line was not observed as long as the target bacteria is present, even though the target bacteria were at a very low concentration (1 cfu/ml). These results were confirmed by signal intensities (Fig. 5D) and peak areas (Fig. 5E) of the test lines. The high specificity could also be attributed to the crRNA targeting since target sequences without PAM or 15 bases truncation near the PAM region exhibited no trans-cleavage or reduced trans-cleavage, respectively (Table S1, Fig. S7).

4. Conclusion

This work demonstrated a novel CRISPR/Cas12 based LFB for the detection of *P. aeruginosa*. The concept of this approach is a non-detectable test line on the LFB when Cas12 collaterally cleaves cognate target and ssDNA reporter sequences. By integrating LAMP amplification of target DNA to the CRISPR/Cas system, we developed a CRISPR/Cas and loop-mediated Isothermal Amplification (CIA) based LFB, which achieved a single copy sensitivity and high specificity both in pure and complex samples. Our system is robust because it doesn't require traditional DNA/RNA extraction, post product analysis like gel electrophoresis/instruments analysis, difficulty in multi-looped band analysis, use of toxic dyes, and longer amplification time. Moreover, our CIA based LFB is simple, portable, cheaper, and background-free. Furthermore, CIA based LFB is highly reproducible, rapid, and inexpensive. Notwithstanding that CRISPR-based techniques have shown some promiscuous or no trans-cleavage; thus, thorough buffer and activator optimization are of great importance. In the future, it could be programmable and multiplexed for point-of care detection of other pathogens, SNPs and genotyping by using specific crRNA(s) and necessary amplification reagents such as primers, and reverse transcriptase (for RNA detection).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Omar Mukama: Data curation, Writing - original draft. **Jinghua Wu:** Data curation. **Zhiyuan Li:** Project administration. **Qiongxin Liang:** Data curation. **Zhijian Yi:** Data curation. **Xuewen Lu:** Validation. **Yujie Liu:** Validation. **Yumei Liu:** Validation. **Muzammal Hussain:** Validation. **Gaelle Guiewi Makafe:** Validation. **Jiaxin Liu:** Validation. **Ning Xu:** Resources. **Lingwen Zeng:** Supervision, Conceptualization, Writing - review & editing.

Acknowledgment

We acknowledge the funding support the Basic and Applied Basic Research Programs of Science and Technology Department of Guangdong Province, China (911148427033). O.M, M.H, and G.G.M are sponsored by CAS-TWAS President's Fellowship for international Ph.D. students. We are also grateful to Fangcun-Guangdong Traditional Chinese Medicine Hospital for donating sputa samples, and Tolo Biotech (Shanghai, China) for providing pET28a-AaCas12b plasmid. We thank Rahma Hussein Adan and Dickson Adah for comments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2020.112143.

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