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Point-of-care and visual detection of *P. aeruginosa* and its toxin genes by multiple LAMP and lateral flow nucleic acid biosensor

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ABSTRACT

This study describes a simple and sensitive approach for visual and point-of-care detection of *P. aeruginosa* and its toxin genes based on multiple loop-mediated isothermal amplification (mLAMP) and lateral flow nucleic acid biosensor (LFNAB). Differentiation of the internal standard gene *ecfX* and toxin genes (*ExoS* and *ExoU)* in *P. aeruginosa* was determined using FITC-, hex-and digoxin-modified primers in the mLAMP process. In the presence of biotin-and FITC- (hex-, digoxin-) modified primers and Bst DNA polymerase large fragments, the mLAMP produced numerous biotin- and FITC- (hex-, digoxin-) attached duplex DNA products. The products were detected by LFNAB through dual immunoreactions (anti-biotin antibodies on the gold nanoparticle (Au-NP) and biotin on the duplex, anti-FITC (hex, digoxin) antibodies on the LFNAB test line and FITC (hex, digoxin) on the duplex). The accumulation of Au-NPs produced a characteristic red band, enabling visual detection of *P. aeruginosa* and its toxin genes without instrumentation. After systematic optimization of LFNAB preparation and detecting conditions, the current approach was capable of detecting concentrations as low as 20 CFU/mL *P. aeruginosa* or its toxin genes within 50 min without complicated instrument, which is more sensitive than PCR. Therefore, this approach provides a simple, pollution free, sensitive, and low-cost point-of-care test for the detection of *P. aeruginosa* and its toxin genes.

1. Introduction

P. aeruginosa is a ubiquitous environmental bacterium and one of the top three causes of opportunistic human infections, causing serious infections such as septicemia, pneumonia, endocarditis, otitis, and keratitis (Silby et al., 2011). One particular feature of *P. aeruginosa* is its tolerance in low-nutrient water. In addition, *P. aeruginosa* is often monitored as an indicator of other bacterial contamination of fecal origin. It is also one of the most frequently identified bacteria in tap water and bottled water (Warburton, 1992). Issues with *P. aeruginosa* in bottled water as well as in hospital infections have drawn worldwide attention. According to previous reports, the *ecfX* gene can be used as an internal standard gene for detecting *P. aeruginosa* (Lavenir et al., 2007; Cattoir et al., 2010). *P. aeruginosa* uses a type III secretion system (T3SS) to promote the development of severe disease, particularly in patients with impaired immune defenses. *P. aeruginosa* delivers toxins to host cells directly through its T3SS and changes the function of host cells, thus creating a favorable environment for bacteria to survive and spread. Although the genes encoding the *P. aeruginosa* type III secretion apparatus are similar to corresponding genes in other bacteria, such as *Yersinia*, *Salmonella*, and *Shigella spp*, the effector proteins transported by this apparatus are distinct (Frank, 1997). The *P. aeruginosa* T3SS system mainly produces four types of toxins, including *ExoU* (also called *PepA*), *ExoS*, *ExoT,* and *ExoY* (Rabin and Hauser, 2003; Sun et al., 2012). Characterization of *ExoU* and *ExoS* in particular has been the focus of much investigation. Measurements of mortality, bacterial persistence in the lung, and dissemination indicated that *ExoU* had the greatest impact on virulence, while secretion of *ExoS* had an intermediate effect, and *ExoY* and *ExoT* had little effect (Shaver and Hauser, 2006; Hu et al., 2013). *ExoU* is a potent cytotoxin with phospholipase A2 activity (Phillips et al., 2003; Toska et al., 2014). *ExoS* and *ExoT* are bifunctional enzymes that have 75% amino acid identity and encode both GTPase-activating protein (GAP) and ADP-ribosyltransferase (ADPRT) activities. *ExoY* is an adenylate cyclase (Liu et al., 1997). The relative virulence associated with each of these type III effector

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proteins may have important prognostic implications for patients infected with *P. aeruginosa*. Interestingly, very few strains encoded both *ExoS* and *ExoU* (Angus et al., 2010). Therefore, rapid and sensitive detection methods are needed to identify and better control potential *P. aeruginosa* infections through accurate and multiple detections of its standard and toxin genes.

It is time-consuming and labor-intensive to use conventional culture-based methods for detecting *P. aeruginosa*, which generally requires 3–4 days for selective culture and biochemical tests. PCRs, especially real-time PCR, have been widely established and employed for rapidly detecting *P. aeruginosa*. However, it is not easy to analyze the results. In addition, conventional PCR uses tedious and time-consuming gel electrophoresis. Real-time PCR assays require a real-time PCR instrument, which is expensive and not yet widely available. Recently, a novel isothermal nucleic acid amplification method, identified as loop-mediated isothermal amplification (LAMP), was developed for rapid and sensitive nucleic acid amplification. LAMP assays targeting *ecfX* genes (Zhang et al. 2013), *oprL* genes and *oprI* genes (Goto et al. 2010) (internal standard genes and outer membrane peptidoglycan-associated lipoproteins) were even more recently adopted to detect *P. aeruginosa*. These LAMP assays were reported to be very specific and sensitive for the detection of *P. aeruginosa* in environmental water and clinical specimens; however, there are issues with analyzing the results. Moreover, LAMP is advantageous because of its aerosol pollution. The analysis of LAMP products is mainly based on a dsDNA-specific fluorescent dye, the electrophoresis of amplicons, the turbidity of magnesium pyrophosphate, and the metal ion indicator. However,, these methods need to be improved. None of these analyses were used to multiply the identification of *P. aeruginosa*'s internal standard gene and its toxin genes even though this test has been shown to be selective and necessary. All of these detection methods are not specific to template sequences; therefore, the readout is identical for amplicons from any sequence. Unlike PCR, LAMP generates amplicons with various lengths, making detection very complicated. To enable multiple loop-mediated isothermal amplification (mLAMP), a conventional technique is to introduce an endonuclease recognition site into the LAMP primers and to make the length of the endonuclease-digested amplicons specific to the target species (Iseki et al., 2007; Shao et al., 2011). An alternative technique for mLAMP is to use a fluorophore-labeled primer combined with an intercalator dye (Aonuma et al., 2010; Kouguchi et al., 2010). Another technique to allow simultaneous detection of multiple LAMP products is with sequence-based barcodes coupled with nicking endonuclease-mediated pyrosequencing (Liang et al., 2012). This is a reliable technique for multiple target identification. However, this method cannot be used for point-of-care testing (POCT). For mLAMP, it is necessary to develop a method that can discriminate target-specific amplicons from the mixture of mLAMP products.

For POCT, it is necessary to use compact instruments with a small size that are lightweight. Because LAMP operates at a constant temperature, it is easy to construct a portable instrument. In recent years, the lateral flow nucleic acid biosensor (LFNAB) has attracted significant attention due to its characteristic affordability and rapid visual detection, therefore presenting great potential for point-of-care diagnostics in resource-limited settings (Hu et al., 2014; Ge et al., 2014). However, no previous reports have identified multiple detection methods for *P. aeruginosa* and its toxin genes using LFNAB. Moreover, to the best of our knowledge, no research efforts have found a method that combines these strategies into one system, specifically for the production of mLAMP, which is extremely suitable for LFNAB to amplify the signal and obtain accurately proportioned results.

In this study, we used a dual strategy that combined competitive mLAMP with LFNAB to develop a mLAMP-LFNAB cascade for the multiple detection of *P. aeruginosa* and its toxin genes with accuracy and usability superior to previously reported techniques. The accumulation of Au-NPs produced a characteristic red band, enabling visual detection of *P. aeruginosa* and its toxin genes without instrumentation. This method is also rapid, simple, pollution free and low-cost point-of-care.

2. Methods and materials

2.1. Reagents and instrumentation

Bst DNA polymerase large fragments were purchased from New England BioLabs (Beijing, China). DNA polymerase was purchased from TaKaRa (Dalian, China). Gold Chloride Trihydrate, Tri-Sodium Citrate Dihydrate, phosphate buffer saline (PBS, 0.01 M), Bovine Serum Albumin (BSA), Tween®20 (polyoxyethylene-20-sorbitan monolaurate), sucrose, 1 M Tris–HCl, 1.5% LB agar, 20% LB media, the anti-digoxin, anti-hex, anti-FITC, and anti-biotin were obtained from Sigma (St. Louis, MO). All the other reagents were analytical reagents or guaranteed reagents. All solutions were prepared with deionized and sterilized H_2O. The plastic backing, conjugate pad, Nitrocellulose membranes, sample pad and absorption pad were acquired from Millipore (Billerica, MA). The screw type of PCR-tube is the independent research of our laboratory (China Patent No. 201510293982.2) and produced by Beijing Food Safety Tech. Co., Ltd. (Beijing, China). A portable test strip reader (DT2050), which connected to a laptop, was purchased from Shanghai Goldbio Tech. Co., Ltd. (Shanghai, China) to collect assay signals from LNFAB.

2.2. Samples

A total of 60 *P. aeruginosa* strains and 15 other bacterial strains from various sources were used to determine the specificity of the mLAMP assay in this study (Table 1). These strains were obtained from the American Type Culture Collection (ATCC), China Microbiological Culture Collection (CMCC), China General Microbiological Culture Collection Center (CGMCC), and Chinese Academy of Science (CAS). All strains were stored in 20% (v/v) glycerol solution at −80 °C until they were used. We chose bacterial strains that naturally secreted the internal standard gene *ecfX* for our studies

Table 1
Bacterial strains used in this study.

Species	Sources
Pseudomonas aeruginosa (n=60)	PAO1 (ATCC 47085) PA103 (ATCC 47086) PAK (ATCC 53308) ACCC 10647 PA14 PA99 Environmental isolates
Other Pseudomonas (n=5)	
Pseudomonas fluorescens	2P24
Pseudomonas stanieri	PA1501
Pseudomonas putida	NB1
Pseudomonas mendocina	ATCC 25411
Pseudomonas cepacia	G63
None Pseudomonas (n=10)	
Salmonella spp.	CGMCC 1.0090
Yersinia	CO92
Shigella flexneri	CMCC51572
Escherichia coli	O157:H7 EDL933
Staphylococcus aureus	ATCC 25923
Lactobacillus brevis	AS1.12
Bifidobacterium longum	CGMCC 2265
Acetobacter aceti	AS1.48
Clostridium butyricum	CGMCC 1.209
Bacteroides fragilis	ATCC 25285

and constructed mutants that secreted *ExoS* alone or *ExoU* alone. PA99 is a *P. aeruginosa* clinical isolate that naturally carries the *ExoU* and *ExoS* genes. *P. aeruginosa* strains PA103 and *P. aeruginosa* strains PAO1 carry the *ExoU* and *ExoS* genes, respectively. All strains were grown at 37 °C overnight in Luria–Bertanibroth (LB). DNA templates were extracted with a Bacteria DNA Kit (TianGen, Beijing, China).

2.3. Multiplex LAMP reaction

The mLAMP reaction was carried out with a 25 μL reaction mixture containing 2.3 μL (each) FIP and BIP, 0.3 μL (each) F_3 and B_3, 2.3 μL deoxynucleoside triphosphates (dNTPs), 0.3 μL $MgSO_4$, 0.5 μL ddH_2O, 3 μL betaine, 1.5 μL Thermopol buffer, 0.6 μL Bst DNA polymerase, and 1.2 μL DNA template. Amplification was performed in a water bath at 65 °C for 40 min and then at 80 °C for 2 min to terminate the reaction.

2.4. Primer design

Three conservative segments from *ecfX*, *ExoS* and *ExoU* were selected as target sequences for this method. A set of four primers were designed using Primer Explorer version 4 (https://primerexplorer.jp/lamp4.0.0/index.html) to target six distinct regions of the *ecfX* gene (GenBank Accession No. Q996559), the *ExoS* toxin gene (GenBank L27629.1), and the *ExoU* toxin gene (GenBank U97065.1), as shown in Table 2A. The forward inner primer (FIP) consisted of F_1c (the complementary sequence of F_1) and F_2. The reverse inner primer (BIP) consisted of B_1c and B_2 (the complementary sequence of B_2c). The outer primers consisted of the forward outer primer F_3 and the reverse outer primer B_3 (the complementary sequence of B_3c). A series of target-specific labels were designed and tagged with the FIP primers and BIP primers. The primer sequences are indicated in Table 2B. Primers were synthesized commercially by Sangon Biotech (Shanghai, China). The detection of LAMP products was performed by agarose gel electrophoresis. The electrophoretogram of specificity experiment about these primers is shown in Fig. S1, indicating that the primers of the *ecfX* gene (GenBank Accession No. Q996559), the *ExoS* toxin gene (GenBank L27629.1), and the *ExoU* toxin gene (GenBank U97065.1) had good specificity.

2.5. Synthesis of antibody conjugated AuNPs

Au-NPs with an average diameter 15 ± 3.5 nm were prepared according to optimized reported methods (He et al., 2011). Conjugation reactions were conducted by adding 1 μg anti-biotin antibody into 1 mL of a tenfold-concentrated Au-NP solution (pH 8) followed by incubation at room temperature with periodic gentle mixing for 1 h. Then, a certain volume of 10% BSA and 10% NaCl was slowly added to the mixture solution. After gentle stirring for 120 min, the solution was centrifuged at 12,000*g* for 20 min. The pink supernatant of unbound antibodies was discarded, and the dark red soft sediment of anti-biotin–Au-NP conjugates was stored at 4 °C before being used.

2.6. Assembly of the paper based lateral flow

The test strip consisted of four components assembled together on a plastic backing: the conjugate pad, the NC membranes, the sample pad and the absorption pad. The conjugate pad was prepared by spraying anti biotin Au-NP conjugates on a glass fiber and dried at room temperature. Purified FITC, hex, digoxin antibodies (1.0 mg mL^{-1}) and anti-biotin antibodies (1.0 mg mL^{-1}) were dispensed on the NC membrane with 5–8 lm pore size to form test lines 1–3 and a control line, respectively. The distance between the two lines was approximately 5 mm. The membrane was then air dried at 37 °C for 12 h. For test strip assembly, the NC membrane was pasted onto the center of the plastic plate and covered with the conjugate pad and the absorption pad on each side. The sample pad receiving the liquid sample to be analyzed was attached to the other side of conjugate pad. Then, they were cut into 3 mm wide pieces and stored in a desiccated container at 4 °C.

Table 2
Primer sequences used in this study.

Primer name	Sequence (5′-3′)	
ecfX-F_3	GGATGAGCGCTTCCGTG	
ecfX-B_3	AAGTTGCGGGCGATCTG	
ecfX-FIP	CTTGCGCAGAAGCGCAGCGTTCCGTCTCGCATGCCTA	5′ modified biotin
ecfX-BIP	GCCGACCTCGCCCAGGATAGCTCGACCGATTGCCG	5′ modified FITC
ExoS-F_3	GGCATCCACAGTGACGC	
ExoS-B_3	GTGCCACGGAAAGTCTTCA	
ExoS-FIP	CAGAGCGCGATTCAGGTCCGGAAGTGATGGCGCTTGGTCT	5′ modified biotin
ExoS-BIP	CAGGGGCAGGAGCTGGATGCCGCTCTTCTCGAAGGCC	5′ modified hex
ExoU-F_3	TCGCTGAAAAAGGGCTTGG	
ExoU-B_3	CAATCTGCTGGCGATGGC	
ExoU-FIP	CCGCGAATCTATGCGTGGGAGCAACAAGATCGGCGGCTT	5′ modified biotin
ExoU-BIP	CCTAGAACGCCTATTGCGCGAGCGTGCAACCTCTGGATG	5′ modified digoxin

P. aeruginosa target DNA sequences

ecfX gene (GenBank Q996559)

1 atggatgagc gcttccgtgg ttccgtctcg catgcctatc aggcgttcca tggcgagttg 60
F_3 F_2
61 ctgcgcttcc tgcgcaagca cctgggcagt tcggcggacg ccgccgacct 120
cgcccaggat
F_1 B_1c
121 actttcgacc agtggctgaa atggccgggc cggcaatcgg tcgagcagcc 180
gcgcgcattt
181 cttttccaga tcgcccgcaa cttgttgcgc gaccattggc gccggcagca 240
gagccgcggc

ExoU toxin gene (GenBank L27629.1)

1 ggtggcatcc acagtgacgc cgaagtgatg gcgcttggtc tctacaccgg cattcactac 60
F_3 F_2
61 gcggacctga atcgcgctct gcgtcagggg caggagctgg atgcgggaca aaagctgatc 120
F_1 B_1c
121 gaccaaggta tgtccgcggc cttcgagaag agcggacagg ctgaacaggt agtgaagact 180
181 ttccgtggca cccgtggcgg ggatgccttc aacgcagtgg aagagggcaa ggttggccac 240

ExoU toxin gene (GenBank U97065.1)

1 catcgctgaa aaagggcttg ggcaacaaga tcggcggctt ctctgagttg ctgctcaatg 60
F_3 F_2
61 tactcccacg catagattcg cgggctgagc ccctagaacg cctattgcgc gacgagacac 120
F_1 B_1c
121 gcaaggccgt gctcggacag atcgctacgc atccagaggt tgcacgccag ccgaccgttg 180
181 ccgccatcgc cagcagattg cagtccggct ccggagtcac ctttggcgat ctagatcggt 240

2.7. Detecting the P. aeruginosa gene with the LFNAB-based mLAMP assay

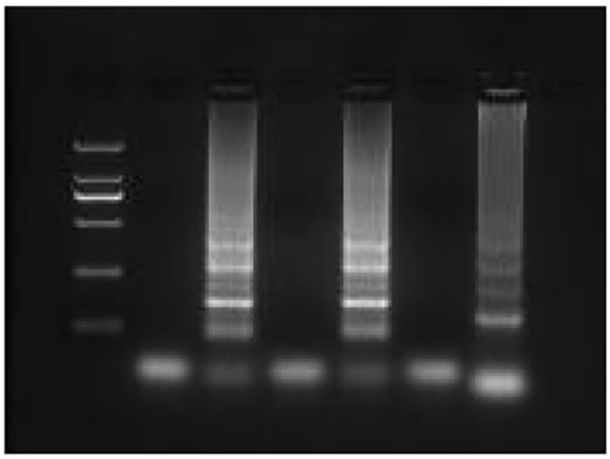
A 50 μL running buffer (4 × SSC, 2% BSA, and 0.05% Tween-20, pH 7.0) was mixed with 25 μL mLAMP product solution; then, a test strip was dipped into the mixture. The bands were visualized within 5 min. The intensities of the red bands on the test strip were quantified with a portable strip reader.

3. Results and discussion

3.1. Principle of multiplex LAMP coupled with lateral flow nucleic acid biosensor

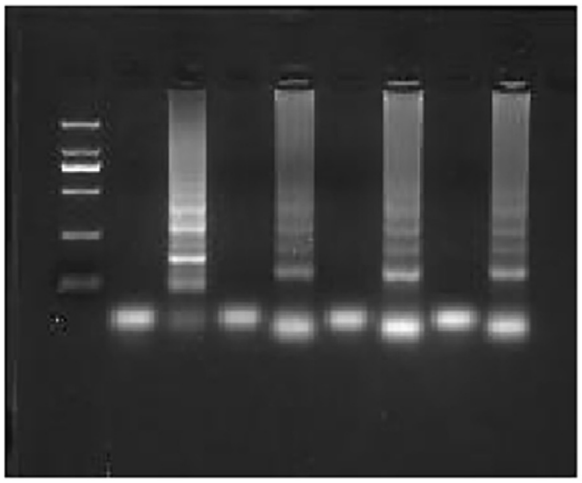
The protocol in this study combines the amplification features of mLAMP with the convenient visual detection of mLAMP

A



	2-plex	3-plex	1-plex	2-plex	1-plex	1-plex
ecfx primer	–	+	–	–	–	–
ExoS primer	+	+	–	+	+	+
ExoU primer	+	+	+	+	–	+
ecfx template	+	+	–	–	–	–
ExoS template	–	–	+	+	–	–
ExoU template	–	–	–	–	+	+

B

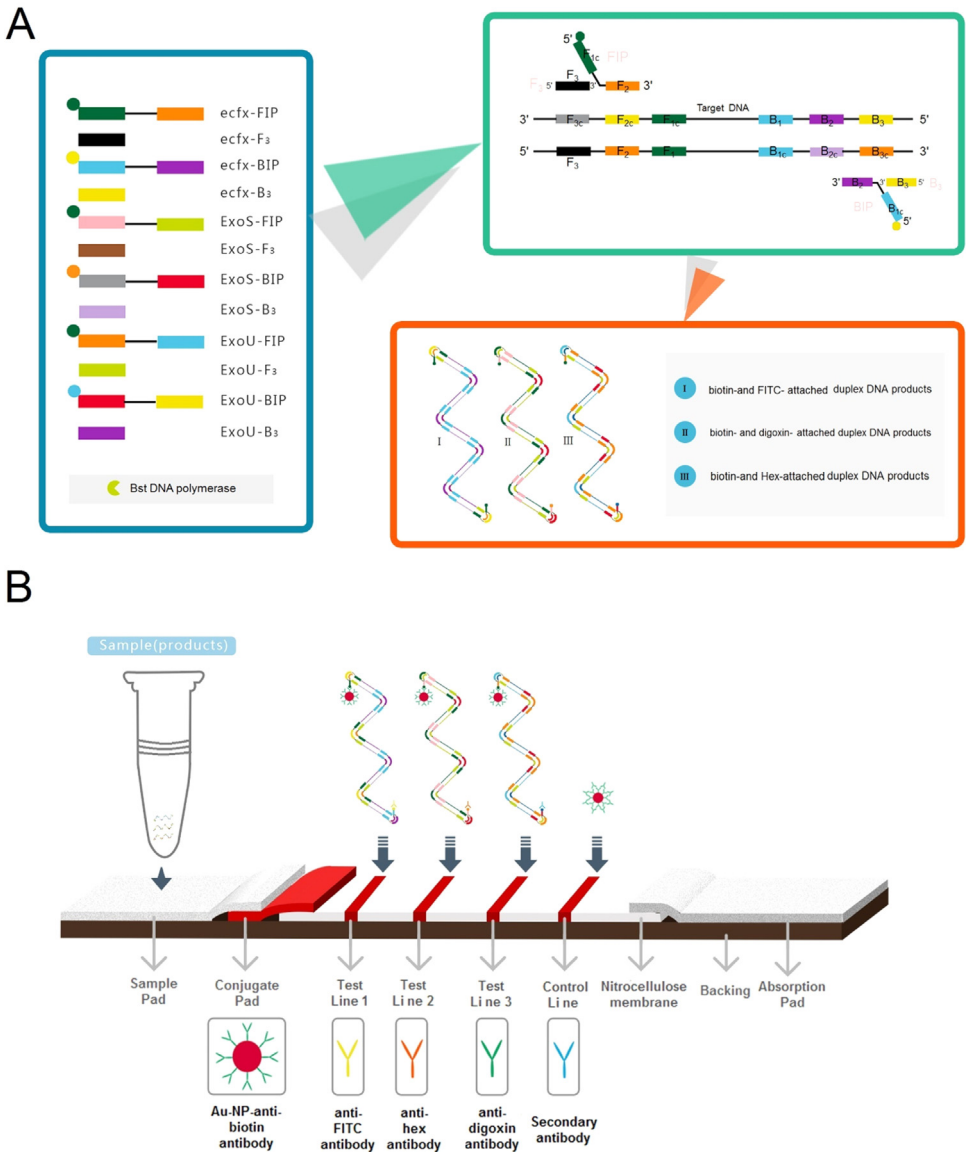


	1-plex	1-plex	2-plex	2-plex	2-plex	2-plex	3-plex	3-plex
ecfx primer	+	+	+	+	+	+	+	+
ExoS primer	–	–	+	+	–	–	+	+
ExoU primer	–	–	–	–	+	+	+	+
ecfx template	–	+	–	+	–	+	–	+

Fig. 1. (A) The electropherograms for investigating specificity of the mLAMP reaction. Template type and primers used for the LAMP reactions are listed under the electropherogram. No positive results were found when no target-specific primers were used in the LAMP reactions. This figure indicates that coexisting primers did not cause false-positive results in mLAMP detection. (B) Electropherograms for investigating the interference of multiple primers on the sensitivity of LAMP reactions for ATCC27853 templates. Template type and primers used for LAMP reactions are listed under the electropherograms. All mLAMP reactions had similar results compared with the single-plex LAMP. This figure indicates that the sensitivity of mLAMP was not affected by coexisting primers.

products on a LFNAB. The principle is shown in Fig. 1. mLAMP was used to generate numerous biotin- and FITC-attached duplex DNAs, biotin- and hex-attached duplex DNAs, biotin- and digoxin-attached duplex DNAs (Scheme 1A). This relies on an auto-cycling strand displacement of DNA synthesis performed by the Bst DNA polymerase large fragment. A set of four primers, including two inner primers and two outer primers, were used in the reaction to recognize six distinct regions on the target gene. LAMP was able to synthesize large amounts of DNA with primer-dimers as a by-product at 60–65 °C in less than an hour. The detection of LAMP products was performed by agarose gel electrophoresis. Because the LAMP assay was carried out under isothermal conditions, a simple heater that maintains a constant temperature was sufficient. Therefore, LAMP assays are faster, easier to perform, and more specific compared with conventional PCR assays. LAMP assays have been successfully applied to detect various food-borne pathogens. In an *n*-plex LAMP reaction, the number of primers is 4n; therefore, we used 12 types of primers in one tube when 3-plex LAMP was used to identify 3 different toxin genes (*ecfX, ExoS,* and *ExoU*). The main issue for the *n*-plex LAMP was the interaction among these primers. To evaluate the interaction, 3 different toxin genes (*ecfX, ExoS,* and *ExoU*) were individually analyzed by 3-plex LAMP with 3 sets of primers that identified all 3 targets and 3-plex LAMP without target-specific primers but with all other primers, respectively. Theoretically, no amplicon should be observed in any 3-plex LAMP with no target-specific primers in the LAMP reaction. As expected, in the presence of the *ecfX* template, the 3-plex LAMP gave positive results, but no positive result was observed in 2-plex LAMP, in the absent of the *ecfX* template, all 2-plex LAMPs gave positive results, but no positive result was observed in all 1-plex LAMPs (Fig. 1A). Therefore, the coexisting primers did not cause any false-positive results in mLAMP detection, indicating that it is possible to use LAMP for identifying multiple targets simultaneously. The other issue we should address is whether multiple primers in mLAMP interfere with the sensitivity of regular LAMP. A sample with 10 copies of *ecfX* templates (ATCC27853) was used as an example for the investigation. First, we performed a conventional single-plex LAMP with only the *ecfX*-primers on the *ecfX*-positive sample, and then, we performed two sets of 2-plex LAMPs with primers specific to *ExoS* and one of the other two toxins (*ExoS, ExoU*), and one set of 3-plex LAMP with primers specific to all three toxins (*ecfX, ExoS,* and *ExoU*), respectively. As shown in Fig. 1B, all the 2-plex LAMPs, and the 3-plex LAMP gave the same positive amplicons as the single-plex LAMP, which was only specific to *ecfX*. The detection limit of the regular LAMP was detected for 10 copies of templates per reaction; therefore, the sensitivity of mLAMP was not reduced when multiple primers coexisted.

The visual detection of the formed duplex DNA complexes was performed on a LFNAB, and the principle is shown in Scheme 1B. Anti-biotin-labeled Au-NPs and anti-FITC antibodies, anti-hex antibodies and anti-digoxin antibodies were pre-immobilized on the conjugate pad and test line (TL 3–1) of the LFNAB, respectively. The secondary antibody, which was against the anti-biotin antibody, was immobilized on the control line (CL). The LFNAB was dipped into a mixture of running buffer and mLAMP products. When the solution migrated by capillary action and passed the conjugate pad, it rehydrated the anti-biotin–Au-NP conjugates. The biotin on the duplex DNA reacted with the anti-biotin on the Au-NP surface to form a FITC-duplex DNA-biotin–anti-biotin–Au-NP complex (digoxin-duplex DNA-biotin–anti-biotin–Au-NP complex, hex-duplex DNA-biotin–anti-biotin–Au-NP complex) and continued to migrate along the strip. The complexes were captured on the test line by the specific reaction between anti-FITC (anti-digoxin, anti-hex) on the test line (TL 3–1) and the FITC (digoxin, hex) in the complexes. The accumulation of Au-NPs on the test line was



Scheme 1. Principle of Multiplex LAMP coupled with Lateral flow nucleic acid biosensor. (A) Schematic illustration of the formation of FITC (hex, digoxin) and biotin attached duplex DNA complexes based on mLAMP. (B) Schematic illustration for the visual detection of mLAMP products on a LFNAB.

visualized as a characteristic red band. Then, excess anti-biotin–Au-NP conjugates continued to move and were captured on the control line by immunoreactions between the secondary antibody and anti-biotin on the Au-NP surface, forming another red band. In the absence of template, there was no duplex DNA in the sample solution; therefore, no anti-biotin–Au-NP conjugate was captured on the test line of the LFNAB, and no red band was observed on the test line. For this method, a red band on the control line showed that the LFNAB was working properly.

Based on the proposed approach, we used DNA templates extracted from bacterial strains ATCC 27853, PA103, PAO1, and PA99 as target DNA and designed a series of biotin-and FITC-modified primers, biotin-and hex-modified primers and biotin-and digoxin-modified primers for mLAMP. The products were tested on the LFNABs. As expected, no distinct red band was observed on the test line of LFNABs in the absence of any DNA templates. A bright red band was observed on the test line 3 and test line 2 of the LFNAB with 1-nM ATCC27853 DNA. The appearance of a red band implied that the Au-NPs were captured on the test line of the LFNAB through the duplex DNA via the immunoreactions of biotin-anti-biotin and FITC–anti-FITC. The difference in red bands on test lines 1–3 in the presence of PA103 DNA, PAO1 DNA, and PA99 DNA showed excellent specificity of the assay.

3.2. Optimization of the lateral flow nucleic acid biosensor

To improve sensitivity for the detection of the *P. aeruginosa* gene, the conditions of LFNAB preparation and detection were optimized systematically. We studied the effects of the reaction time and the components of LFNAB (membrane materials, concentration of the anti-biotin antibodies for preparing anti-biotin-AuNP conjugates, volume of anti-biotin–AuNP conjugates dispensed on the conjugate pad, concentration of antibodies dispensed on the lines, and running buffers) on the peak area by the portable test strip reader (DT2050) of the assay (Fig. 2). The highest peak area was obtained with the LFNAB from Millipore 135s nitrocellulose membranes (Fig. 2A). The concentration of anti-biotin antibodies used to prepare the anti-biotin-Au-NP conjugates was one of the most important factors affecting the reproducibility and sensitivity of LFNABs. The concentrations of anti-biotin antibodies, ranging from 0.5 to 2.0 μg mL^{-1}, were used to prepare the anti-biotin–Au-NP conjugates, and the conjugate

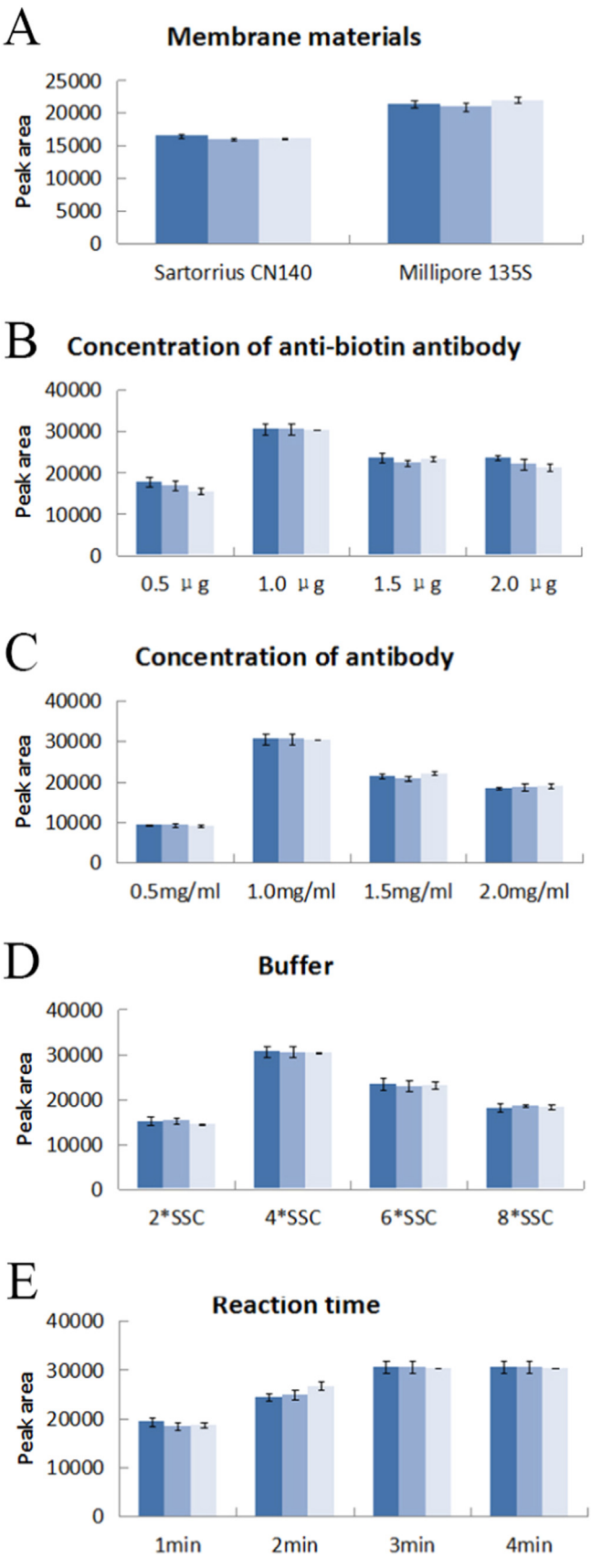


Fig. 2. Typical photo images from the optimization of LFNABs (A) Effect of membrane materials on the peak area of LFNABs; (B) Effect of anti-biotin antibody concentrations on the peak area of LFNABs; (C) Effect of antibody concentrations on the lines from the peak area of LFNABs; (D) Effect of running buffers on the peak area of LFNABs; and (E) Effect of reaction times on the peak area of LFNABs.

performances were evaluated by detecting PA99 DNA (100 CFU/mL). The best peak area was obtained for a concentration of 1 μg mL^{-1} anti-biotin antibody (Fig. 2B). The concentration of antibodies (anti-FITC antibody, anti-hex antibody, anti-digoxin antibody, and secondary antibody) dispensed on the lines affected the LFNAB response. The highest peak area was obtained with 1.0 mg mL^{-1} anti-FITC antibody, 1.0 mg mL^{-1} anti-hex antibody, 1.0 mg mL^{-1} anti-digoxin antibody, and 1.0 mg mL^{-1} secondary antibody, which were used to prepare the LFNAB in the following study (Fig. 2C). Another factor that affected the sensitivity and reproducibility of the assay was the use of various buffers. The highest peak area was given by 4 × SSC+2% BSA+0.05% Tween-20, which offered an appropriate environment to stabilize the LFNAB (Fig. 2D). Moreover, the reaction time for LFNAB detection was no less than 3 min (Fig. 2E).

3.3. Analytic performances of lateral flow nucleic acid biosensor detecting mLAMP products

3.3.1. Specificity

To implement the detection of the *P. aeruginosa* genes in real samples, it was essential to assess the analytical selectivity and specificity of LFNAB against possible interferences in biological samples. The sample solutions, including *P. aeruginosa* DNA and other bacterial strain DNA, *P. aeruginosa* DNA, other bacterial strain DNA, and ddH_2O, were tested after mLAMP with the LFNAB. The typical photo images of LFNABs are shown in Fig. 3A. These results suggested that there was intrinsic binding selectivity and specificity by the LFNABs in biological samples. Therefore, the fascinating LFNABs can be readily applied in the facile and rapid detection of *P. aeruginosa* genes in real biological samples.

3.3.2. Sensitivity

To investigate detection sensitivity of LFNAB, sample solutions containing different concentrations of PA99 samples were examined under optimized experimental conditions. The typical photo images of LFNABs are shown in Fig. 3B. For visual detection, the red band in the LFNAB test line was observed with concentrations as low as 20 CFU/mL of PA99 DNA.

3.3.3. Reproducibility

In addition to high sensitivity, LFNABs also exhibited high reproducibility. The reproducibility of the LFNABs were assessed by testing sample solutions in the presence of PA99 DNA (100 CFU/mL) 6 times. As shown in Fig. 3C, similar responses could be obtained each time. The corresponding RSD values of optical responses were 1.7%, indicating the excellent reproducibility of the measurements.

3.3.4. Stability

Stability was another important index in evaluating the biosensors. The LFNABs were stored at room temperature. Moreover, their responses did not change significantly after one month's storage at room temperature. The intensity of the test bands on the LFNABs for detecting PA99 DNA were almost the same to those obtained with the newly prepared LFNABs, indicating that the biosensors had good stability (Fig. 3D).

3.3.5. Application in real samples

To further confirm that mLAMP-LFNAB can be applied in biological samples, performances were challenged in the complex samples (see Table S1 in Supporting information). There are 113 (56%) positive samples of *P. aeruginosa* bacteria in the whole 201 environmental and drinking water samples, which include 36 river water samples, 52 drinking water samples and 25 soil samples. Then, 214 *P. aeruginosa* strains have been isolated from all the

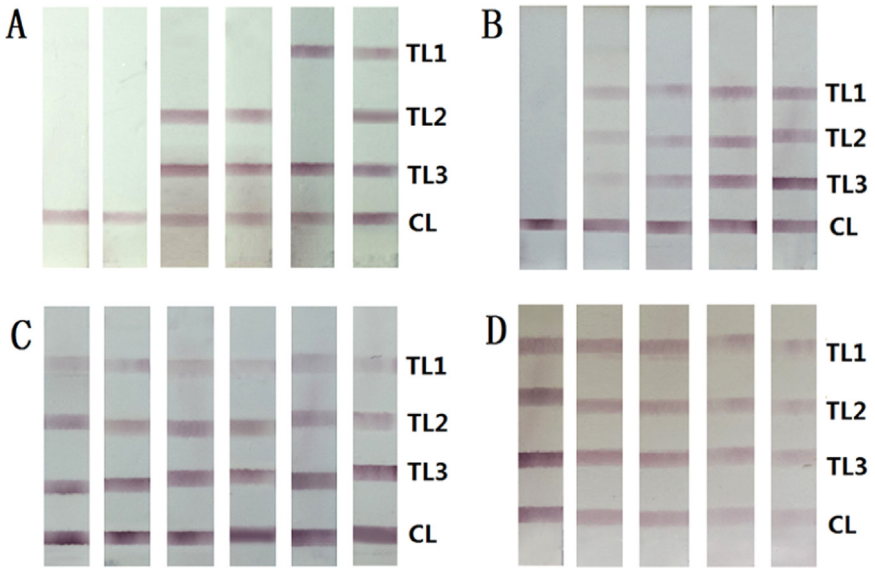


Fig. 3. Typical photo images from analytical performances of LFNABs. (A) Typical photo images from analytical specificity of LFNABs. From left to right, samples were produced by mLAMP for ddH_2O, other bacterial strain DNA, ATCC27853 DNA+ other bacterial strain DNA, PAO1 DNA+ other bacterial strain DNA, PA103 DNA, PA99 DNA; (B) Typical photo images from analytical sensitivity of LFNABs. From left to right, concentrations of PA99 in samples are 10 CFU/mL, 20 CFU/mL, 40 CFU/mL, 80 CFU/mL, 100 CFU/mL; (C) Typical photo images from analytical reproducibility of LFNABs. These tests occurred every other week; (D) Typical photo images from analytical stability of LFNABs. These tests occurred every other week.

above positive samples, the reference gene (*ecfX*) and the toxin genes (*ExoU* and *ExoS*) were detected for these isolated bacterial strains by our detection technique. There are 214 (100%) strains encoded ecfX, 180 (84%) strains encoded *ExoS* and 20 (9%) strains encoded *ExoU* (see Table S1 in Supporting information). The results indicated that the method can be successfully applied in the complex samples.

4. Conclusion

This study successfully combined the LFNAB and the mLAMP to design and test a multi-plex LFNAB for rapid detection of *P. aeruginosa* genes. The sensing platform used an inexpensive capillary action based on overlapping paper membrane systems with gold nanoparticles providing a colorimetric signal. The sensor displayed high specificity and detection for concentrations as low as 20 CFU/mL *P. aeruginosa* or its toxin genes within 50 min without instrumentation. Compared with conventional PCR and LAMP, the current approach is much simpler, pollution free, and avoids the use of specialized instruments and the requirement of highly qualified personnel. LFNAB can be used to rapidly test water and clinical specimens without any sample pretreatment or trained technicians. To make the system truly portable for POCT applications, we optimized the conditions of LFNAB preparation and detection systematically. The combination of LFNAB and mLAMP will provide a rapid, inexpensive, highly sensitive system for the diagnosis of *P. aeruginosa* genes in hospitals, war lines, developing countries, and inaccessible regions.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.03.006.

References

Angus, A.A., Evans, D.J., Barbieri, J.T., et al., 2010. Infect. Immun. 78 (11), 4500–4510.
Aonuma, H., Yoshimura, A., Kobayashi, T., et al., 2010. Exp. Parasitol. 125, 179–183.
Cattoir, V., Gilibert, A., Glaunec, J.L., et al., 2010. Ann. Clin. Microbiol. Antimicrob. 9, 21.
Frank, D., 1997. Mol. Microbiol. 26, 621–629.
Ge, X., Asiri, A.M., Du, D., et al., 2014. TrAC Trends Anal. Chem. 58, 31–39.
Goto, M., Shimada, K., Sato, A., et al., 2010. J. Microbiol. Methods 81 (3), 247–252.
He, Y., Zhang, S., Zhang, X., et al., 2011. Biosens. Bioelectron. 26 (5), 2018–2024.
Hu, H., Harmer, C., Anuj, S., et al., 2013. Clin. Microbiol. Infect. 19 (3), 266–272.
Hu, J., Wang, S.Q., Wang, L., et al., 2014. Biosens. Bioelectron. 54, 585–597.
Iseki, H., Alhassan, A., Ohta, N., et al., 2007. J. Microbiol. Methods 71, 281–287.
Kouguchi, Y., Fujiwara, T., Teramoto, M., et al., 2010. Mol. Cell Probe 24, 190–195.
Lavenir, R., Jocktane, D., Laurent, F., et al., 2007. J. Microbiol. Methods 70, 20–29.
Liang, C., Chu, Y., Cheng, S., et al., 2012. Anal. Chem. 84, 3758–3763.
Liu, S., Yahr, T.L., Frank, D.W., et al., 1997. J. Bacteriol. 179, 1609–1613.
Phillips, R.M., Six, D.A., Dennis, E.A., et al., 2003. J. Biol. Chem. 278, 41326–41332.
Rabin, S.D.P., Hauser, A.R., 2003. Infect. Immun. 71, 4144–4150.
Shao, Y., Zhu, S., Jin, C., et al., 2011. Int. J. Food Microbiol. 148, 75–79.
Shaver, C.M., Hauser, A.R., 2006. Infect. Immun. 72, 6969–6977.
Silby, M.W., Winstanley, C., Godfrey, S.A., et al., 2011. FEMS Microbiol. Rev. 35 (4), 652–680.
Sun, Y., Karmakar, M., Taylor, P.R., et al., 2012. J. Immunol. 188 (4), 1884–1895.
Toska, J., Sun, Y., Carbonell, D.A., et al., 2014. PLOS ONE 9 (1), e86829.
Warburton, D.W., 1992. Can. J. Microbiol. 38 (1), 12–19.
Zhang, S.H., Xu, X.K., Wu, Q.P., et al., 2013. Eur. Food Res. Technol. 236, 209–215.