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METHODS AND PROTOCOLS

# An effective established biosensor of bifunctional probes-labeled AuNPs combined with LAMP for detection of fish pathogen *Streptococcus iniae*

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## Abstract

In purpose of valid *Streptococcus iniae* detection, we established a colorimetric biosensor using gold nanoparticles (AuNPs) labeled with dual functional probes and along with loop-mediated isothermal amplification (LAMP) assay (LAMP-AuNPs). Based on the characteristics of self-aggregation and bio-conjugation with ligands, AuNPs were chosen for observable color change in tandem with LAMP amplification method to reach high sensitivity and easy operation. Meanwhile, the improvement of dual probes that could fully utilize the LAMP product gave the biosensor a stable result exhibition. LAMP-AuNPs targeting gene *ftsB*, one of the ATP transporter-related genes, turned out favorable specificity in cross reaction among other fish pathogens. The detect limit of $10^2$ CFU revealed a better sensitivity compared with polymerase chain reaction (PCR) method and AuNPs lateral flow test strip (LFTS). It was also proved to be effective by zebrafish infection model trials with less than 2-h time consumption and nearly no devices which make it a convenient biosensor for point-to-care *S. iniae* detection.

**Keywords** LAMP · Gold nanoparticles · Bifunctional probes · *Streptococcus iniae*

## Introduction

*Streptococcus iniae* is a beta-hemolytic, Gram-positive pathogen which is one of the contributing factors to global epidemic fish streptococcosis disease both in fresh and marine water (Klesius et al. 2006) over the past decades. On the east and south coasts of China, *S. iniae* has already caused large damage in the fishing industry in aspects of no matter aquatic ecosystem and commercial earnings (Deng et al. 2017). As one of the main fish pathogens, *S. iniae* could cause two typical *S. iniae* decease symptoms including multiple subcutaneous abscesses and skin lesions (Agnew and Barnes 2007). Looking back on the development of *S. iniae* detection methods, molecule-based and immunity-related assays have been continuously evolved such as polymerase chain reaction (PCR) (Mata et al. 2004), real-time PCR (RT-PCR), and enzyme-linked immunosorbent assay (ELISA) (Shelby et al. 2002). Unlike original PCR assays that needs laboratory instruments to fulfill various temperature conditions requirements, loop-mediated isothermal amplification (LAMP) can accomplish rapid and specific amplification of target DNA sequences without complex temperature variation (Liang et al. 2009), that the whole process can be conducted with a water bath. The target gene *ftsB* is one of the iron transporter-related genes partly transcribed adenosine triphosphate (ATP)-binding-cassette (ABC) transporter system of *S. iniae* (Zou et al. 2010). Two sets of LAMP primers were designed according to the target gene *ftsB*, which has a quite favorable specificity.

Colloidal gold synthesized from reduction of hydrogen tetrachloroaurate and trisodium citrate is a potential nano material in medicine and biotechnology due to its visible wine red color and good affinity between gold surface and various ligands (David et al. 2010). It was widely applied as the label of electron microscopy imaging for gene or cellular locating

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(Rosi et al. 2006) and carrier of amino acid drug delivery in past decades. With more research of its optical and electronic features, focus was tended to explore its properties of bioconjugation with ligands including oligonucleotide probes, antibodies, and others both in biological and therapeutic detections. The application of gold nanoparticles in detection approaches used to be associated with principle of cross-linking (CL) aggregation based on their physical and chemical properties alteration such as chemiluminescent detection during the past decades (Sato et al. 2003). However, this kind of detection system is exposed to be a disadvantage when it comes to field diagnosis that it requires massive and precise equipment. The self-aggregation is found to be a great property of ssDNA-labeled gold nanoparticles (AuNPs) that can visualize the result of target DNA test by naked eyes. This is based on the weak electrostatic/steric repulsion between ssDNA probes when surface density of oligonucleotides is less than 26 pmol/cm$^2$ (Song et al. 2010) while the formation of duplexes intensifies this force that solution remains wine red. With the screening effect of the salt, AuNPs-ssDNA will assemble which leads to a visible color change from wine red to blue gray (Saha et al. 2012) while AuNPs-dsDNA stay homogeneous and dispersed. Therefore, it is evidenced of the possibility for AuNP's utilization in establishing an efficient colorimetric biosensor without extra devices like agarose gel electrophoresis and gel imaging system.

The detection of target gene is based on hybridizing between LAMP products and oligonucleotide probes that labeled on the Au surface through thiol functional groups (Gao et al. 2012). However, single probe corresponds half of target oligonucleotide sequence which might have obstacles to fully combine with both strands of target products when the LAMP template is relatively little. To address this demerit, bifunctional ssDNA probes (Dharanivasan et al. 2016) was brought into the system to hybridize with both strands of partial LAMP amplicon of *ftsB*, which led to more stable result exhibition and more distinct color comparison between positive and negative ones. In this study, we attempted to develop a valid LAMP-AuNPs biosensor that was able to distinctively detect *ftsB* gene of *S. iniae* and distinguished it from other pathogens in an observable way with less time consumption and higher sensitivity.

## Materials and methods

### Strains and DNA extraction

Two strains of *S. iniae* were used in this study including an isolated *S. iniae* strain HN given by Prof. Anxing Li from Sun Yat-sen University and standard strain ATCC 29178 purchased from ATCC which was the strain our LAMP-AuNPs assay was based on. In addition to *S. iniae* strains, six strains including *Streptococcus agalactiae* CMCC 32116, *Streptococcus dysgalactiae* ATCC 27957, *Lactococcus garvieae* CTCC 20392 (Itsaro et al. 2012), *Aerononas hydrophila*, *Vibrio alginolyticus* (Zhenyu et al. 2013), and *Vibrio harveyi* (Austin and Zhang 2006) were used in this study for cross detection experiments (Table 1). Bacteria strains were stored with glycerin at − 80 °C. *Streptococci* and other gram-positive bacteria were activated on brain heart infusion (BHI) (OXOID, UK) solid agar medium and gram-negative bacteria were recovered on Luria-Bertani solid agar medium (LB) (Sigma, USA), following with overnight amplified cultivation in BHI or LB medium at 37 °C for subsequent DNA extraction. The resources of those strains were listed on Table 1. The whole genomic DNA extraction was operated with Bacteria DNA kit purchased from Tiangen. The corresponding extracted *S. iniae* DNA genome of every 10-fold diluted bacteria solution was measured by nanodrop (ThermoFisher, USA) and stored at − 20 °C for later LAMP and PCR sensitive test usage.

### Design of LAMP primers and bifunctional probes

*ftsB* gene in a length of 927 bp was chosen to be target sequence for LAMP with a detection region of 217 bp. Two sets of LAMP primers were designed online via primer Explorer ver. 5 website. The sequences of *Probe 1* and *Probe 2* ranged from F1c to B1c of two separate strands of dsDNA that were non-complementary to one and another. The 5′ end of both probes were conjugated with thiol groups as well as hexyl groups for stability (Liu and Lu 2006). All the primers and probes were synthesized in Shanghai Generay Biotech Company and their particular sequences information were presented in Table 2.

### Establishment of optimal LAMP reaction conditions

The LAMP assay was comprised of 0.2 μM each outer primers F3 and B3, 1.2 μM each inner primers FIP and BIP,

**Table 1** Strains used in this study

| Bacteria | References or sources |
|---|---|
| *Streptococcus iniae* standard strain | ATCC 29178 |
| *Streptococcus iniae* HN | Isolated by Sun Yat-sen University |
| *Streptococcus agalactiae* | CMCC 32116 |
| *Streptococcus dysgalactiae* | ATCC 27957 |
| *Lactococcus garvieae* | CTCC 20392 |
| *Aerononas hydrophila* | Stored in laboratory |
| *Vibrio alginolyticus* | Stored in laboratory |
| *Vibrio harveyi* | Stored in laboratory |

**Table 2** Information of LAMP primers and probes involved in this study

| Name | Sequence (5′ to 3′) | Length (bp) |
|---|---|---|
| *ftsB*-F3 | GGTAAAATTCCAGAAAAC | 18 |
| *ftsB*-B3 | CATTTGACCCTACCACAATG | 20 |
| *ftsB*-FIP | TGGCAACTATAGGCAAACCTAATTTTTTTGTGTGGTCAGCTTATCATCA | 49 |
| *ftsB*-BIP | ACTAAAGGTGCAAAAGTTGTTTCAGTTTTAGATCGGGTTTTAAGGCAG | 48 |
| Probe 1 | SH-$(CH_2)_6$-$A_{10}$TGACCTCTTATGATGCGAA | 29 |
| Probe 2 | SH-$(CH_2)_6$-$A_{10}$TAGTCTTTTAAAATAGGAT | 29 |

1.2 mM dNTP (New England Biolabs, USA), 6 mM $MgSO_4$ (New England Biolabs, USA), 1 U Bst DNA polymerase (New England Biolabs, USA), 1 × reaction buffer (New England Biolabs, USA), 2 μl DNA complete, and $ddH_2O$ to build a reaction system of 25 μl altogether (Han et al. 2011). For effective LAMP amplification, the optimum reaction temperature and time were investigated initially. The reaction temperature was investigated from 61 to 65 °C for 1 h, while the reaction was conducted in best temperature for 30, 35, 40, 45, 50, and 55 min with 50 ng *S. iniae* genome as DNA template. The results were observed by UV imaging system after running 2% agarose gel electrophoresis (AGE).

## Preparation of AuNPs and labelling with bifunctional probes

As 13~15-nm diameter of gold nanoparticles is appropriate for labelling oligonucleotide probes (Storhoff et al. 2003), AuNPs were prepared by trisodium citrate reduction of $HAuCl_4$ in water with 2 ml 1% (*w*/*v*) trisodium citrate and 1 ml 1% (*w*/*v*) hydrogen tetrachloroaurate ($HAuCl_4$; Sigma-Aldrich, USA) were added into 98-ml boiling ultrapure water with continuously stirring and heating for 15 min. The whole process was accorded to Mao et al. (2009) with some modifications. The size of nanoparticles was determined by UV-Vis spectrometry with microplate reader for maximum absorbance wavelength. The bifunctional probes were labeled on the AuNPs surface through thiol linkages of ssDNA probes. The process started with mixing 3 nmol probe 1, 3 nmol probe 2, and 1 ml AuNP solution into one microcentrifuge tube which was incubated at 50 °C with shaking at 200 rpm for 24 h, Add 100-μl labelling buffer (100 mM sodium phosphate buffer, 1 M NaCl) with 100 μl 1% sodium-dodecyl sulfate (SDS; Sigma-Aldrich, USA) for another 4 h shaking. The labeled AuNP solution was centrifuged at 13,000 *g* at 4 °C for 30 min and washed using 500 μl phosphate buffer (100 mM sodium phosphate buffer, 100 mM NaCl, 0.1% SDS). The AuNP probe solution was concentrated 10 times and stored at 4 °C for later use. Meanwhile, the UV-visible absorbance peak of probe-labeled AuNPs was measured as well.

## Establishment and optimization of LAMP-AuNPs colorimetric biosensor

After LAMP reaction, the products were transformed into two single strands through heat denaturation at 95 °C for next complementary hybridization process between single-stranded LAMP amplificon and ssDNA-AuNPs. The 10-μl mixture system was placed in a PCR tube and was incubated in thermostatic water bath at 65 °C for 5 min to facilitate AuNPs-dsDNA formation. Add 5 μl 0.5 M $MgCl_2$ solution (Watthanapanpituck et al. 2014) of each system to exhibit the visible results based on the salt-induced AuNP self-aggregation. The positive and negative samples were also conducted with both UV-visible absorbance spectrum and HR-TEM scanning as references of characteristics changing. The dual probes and two single-probe labeled system of LAMP-AuNPs were both tested of the same positive sample. The best hybridization volume ratio of AuNPs-ssDNA and LAMP product was tested varying from 1:9 to 8:2 (Arunrut et al. 2016).

## Sensitivity and specificity of LAMP-AuNPs

The total DNA genome of tenfold serial diluted *S. iniae* from $10^6$ to 10 CFU was used as template in both sensitivity and specificity experiments. The strain used in sensitive test was *S. iniae* ATCC 29178, which was cultured in BHI culture medium. The templates of cross detection were the whole genome of *S. iniae* ATCC 29178, *S. iniae* HN, *S. agalactiae*, *S. dysgalactiae*, *L. garvieae*, *A. hydrophila*, *V. alginolyticus*, and *V. harveyi*. The genomic DNA of those strains was used in the procedure of LAMP reaction, followed with hybridization and salt-induced aggregation. Both AGE and color observation were photographed as result exhibitions.

## Sensitivity of PCR and AuNP-MAb lateral flow test strip detection methods

Two detection approaches including PCR and lateral flow test strip detection (LFTS) were introduced for intention of sensitivity comparison with LAMP-AuNPs system. PCR was performed using Taq polymerase with LAMP outside primers which amplified a target gene of 217 bp in a total reaction volume of 20 μl. PCR result was obtained by running 2%

agarose gel electrophoresis and UV-Vis imaging. The AuNPs-MAb LFTS detection, another common colorimetric assay, was based on two *S. iniae* monoclonal antibodies (MAb) of 6C11 and E1H8 which were prepared previously according to the protocol described by Omidfar et al. (2011). AuNPs solution (0.5–0.6 OD) of 25 nm was adopted to label with MAb 6C11 that 1 ml AuNP mixed with 3 μl 1 mg/ml 6C11. The five parts purchased for strip assembling were backing card, sample pad, conjugate pad, nitrocellulose membrane (NC), and absorbent pad (Sartorius, Germany). The test line was conjugated with MAb E1H8 while the goat anti mouse IgG was coated onto control line using the dispenser (HM 3030, Shanghai Kinbio Tech). Then, 0.4 cm widths assembled cards (Wang et al. 2017) were packaged into plastic boxes and were tested by $10^7$, $10^6$, $10^5$, $10^4$, $10^3$, and $10^2$ CFU of *S. iniae*. Alien from LFTS assay, PCR was carried out by testing the genomic DNA of $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, and 10 CFU of *S. iniae* with an extra step of DNA extraction. The corresponding amount of genomic DNA extracted from bacteria was measured as well.

### LAMP-AuNPs detection in fish samples

The zebrafish was chosen to be infected with $10^8$ CFU/ml *S. iniae* via soaking approach to establish a field fish infection model. After 2-h soaking produce, the fish were cultivated in clear reverse osmosis water for 12 h. Then, the kidneys, livers, intestine, and spleen were taken out and treated with 100 μl 1% triton each as well as grinding. The supernatant was taken out carefully for DNA extraction, 2 μl of each sample was added into LAMP reaction system to conduct LAMP-AuNPs test.

## Results

### React conditions optimization of LAMP-combined detection

In order to establish LAMP-combined colorimetric biosensor using AuNPs labeled with probes, the optimal temperature and reaction time for LAMP were tested firstly. The temperature and reaction time for LAMP targeting gene *ftsB* were tested in varying conditions with 50 ng *S. iniae* genome as DNA complete. The LAMP product detected by agarose gel electrophoresis showed that the band pattern in 63 °C was better than those temperatures below 63 °C in intensity. The reaction time was confirmed to have a clear and bright pattern until 45 min in 63 °C (Fig. 1). Thus, 63 °C and 45 min were chosen to be the optimal conditions for LAMP process.

### Optimization and characteristic of bifunctional probes conjunction and hybridization process

In the case of little LAMP products that hybridized with single probe contributing to an indistinct color observation, the detection system is suggested to be improved in aspects of probes labelling and hybridization ratio. Figure 2b gives a color comparison between dual probes system and single probe systems (probes 1 and 2). The trials with single probe had weaker color difference between positive and negative results than dual probes did. Nevertheless, the positive sample of bifunctional probes remained wine red, while single-probe solution turned out to be purple red. The formation of AuNP-ssDNA and AuNP-dsDNA would alter the optical properties and features of gold nanoparticles. Thus, samples of AuNP-based solution including untreated one, samples right after probes conjunction, positive and negative samples after hybridizing along with salt treatment showed different states through UV spectra and HR-TEM. The UV spectra posed a gradual increase in maximum absorption wavelength that the untreated AuNPs gave an absorption peak at 518 nm while AuNPs labeled with dual probes was at 520 nm. The samples containing dsDNA after hybridizing had a maximum UV-Visible absorption of 532 nm which was considered as positive result, while negative sample ssDNA-AuNP solution was gradually self-assembled and showed a nearly flat absorbance spectra (Fig. 2c). In the other hand, it can be clearly observed by electron microscopy that presence and absent of dsDNA-AuNPs turned to particle dispersed and clustered, respectively (Fig. 2d). Furthermore, the volume ratio of AuNPs and LAMP product was investigated varying from 1:9 to 8:2 and it turned out that 5:5 had the strongest color contrast between positive and negative results (Fig. 2e).

### Sensitivity of LAMP-AuNPs assay and comparison with other assays

Six 10-fold serial dilution of *S. iniae* were tested by LAMP-AuNPs system. As DNA template of LAMP and PCR was genomic DNA extracted from *S. iniae*, the corresponding DNA amount was known by previous trials that genome amount of $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, and 10 CFU *S. iniae* were 13.3 and 1.9 ng; 246, 61, and 1.8 pg; and 500 fg, respectively. Agarose gel electrophoresis image and color comparison came out with a detection limit of $10^2$ CFU (1.8 pg) simultaneously (Fig. 3). Subsequently, PCR method using Taq polymerase had patterns when the amount of bacterial was above $10^3$ CFU (61 pg) by AGE. Meanwhile, AuNP-MAb lateral flow test strip had lower sensitivity than LAMP-AuNPs that gave a detection limit of $10^4$ CFU (Fig. 4), which is 100 times higher than LAMP-AuNPs colorimetric biosensor.

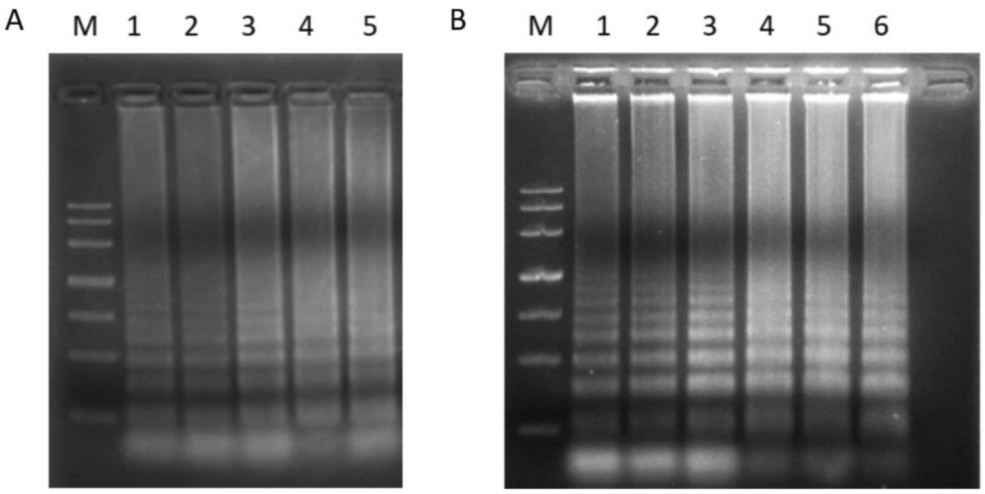


**Fig. 1** React conditions optimization of LAMP-based *S. iniae* detection. **a** The reaction temperature and **b** time are tested. **a** Lines 1–5: 61, 62, 63, 64, and 65 °C. **b** Lines 1–6: 30, 35, 40, 45, 50, and 50 min. M = Maker III

### Specificity of LAMP-AuNPs assay for detection of *S. iniae*

Two pairs of LAMP primers of *ftsB* gene of *S. iniae* and dual ssDNA probes were utilized for specificity test of *S. iniae* isolate and other six common fish pathogens including *S. agalactiae*, *S. dysgalactiae*, *L. garvieae*, *A. hydrophila*, *V. alginolyticus* and *V. harveyi.* The LAMP AGE image showed there were only two clear ladder patterns in line of *S. iniae* ATCC 29178 and *S. iniae* HN. At the same time, LAMP-AuNPs results of two *S. iniae* strains presented positive with red color while other six pathogens were all gray (Fig. 5).

### Field detection in zebrafish samples by LAMP-AuNPs

For exhibiting a good applicability of LAMP-AuNPs assay in field conditions, we performed an infectious simulation of *S. iniae* on zebrafish. After soak infected with *S. iniae*, kidneys, livers, spleens, and intestines of zebrafish were taken and the bacterial DNA of these organ suspension was extracted as LAMP template. The result showed that the organ samples exhibited positive phenomena except the kidney sample from both color change observation and AGE (Fig. 6). It is suggested that LAMP-AuNPs assay could be applied to fish tissue sample detection in accordance with pure cultured bacteria with no concern of background interference.

## Discussion

The damage to aquatic ecosystem and economic losses of fish farming caused by *S. iniae* make it urgent to deal with this destructive microbe pathogens pollution issue starting with identifying. Conventional detecting methods such as culture techniques, PCR, and immunoassays like ELISA can no more satisfied the requirements of point-of-care and readily operation for practical usage. The culture method (Vandenberghe et al. 2003) would normally take 3~4 days for selective culture and biochemical tests (Chen et al. 2016). ELISA assay would last 1~2 days to get final results while PCR method need additional expensive laboratory devices to conduct the reaction with extra step of running gel electrophoresis observed through UV imaging apparatus. Alien from traditional methods, LAMP-AuNPs can be a favorable colorimetric detection assay with much less time spending, higher sensitivity, and specificity attributing to LAMP amplification and hybridization between complementary oligonucleotides. Another superiority of this biosensor stands on its observable characteristic by naked eyes and quick presentation of color change in seconds. It is attributed to the introduction of gold nanoparticles with its unique thiol group bio-conjugation affinity and self-aggregation property. Recently, the related colorimetric methods constantly occur and have been developed all along. Fu et al. (2013) demonstrated a method associated with PCR gene amplification and gold nanoparticles for *Listeria monocytogenes* and *Salmonella enterica* detection in food samples and Kaewphinit et al. (2013) combined LAMP assay with FITC-labeled DNA probe for *Mycobacterium tuberculosis* detection, which both give a satisfactory detection limit but lack of convenience especially in usage in field environment where it is short of instruments for operation and result presentation that whole process might be performed by untrained staff. In this article, LAMP-AuNPs is suggested to largely reduce the time consumption to less than 2 h with 40 min of DNA extraction, 45 min of LAMP process, 15 min of hybridization, and few seconds of color presentation after salt adding. Besides, there is no need for possessing sophisticated equipment, just a water bath for LAMP reaction and DNA product denaturation is feasible which is another merit of this biosensor for its practicality and convenience. Unlike PCR-related approaches, requiring expensive PCR thermocycle instrument, just a water bath for LAMP reaction and DNA product

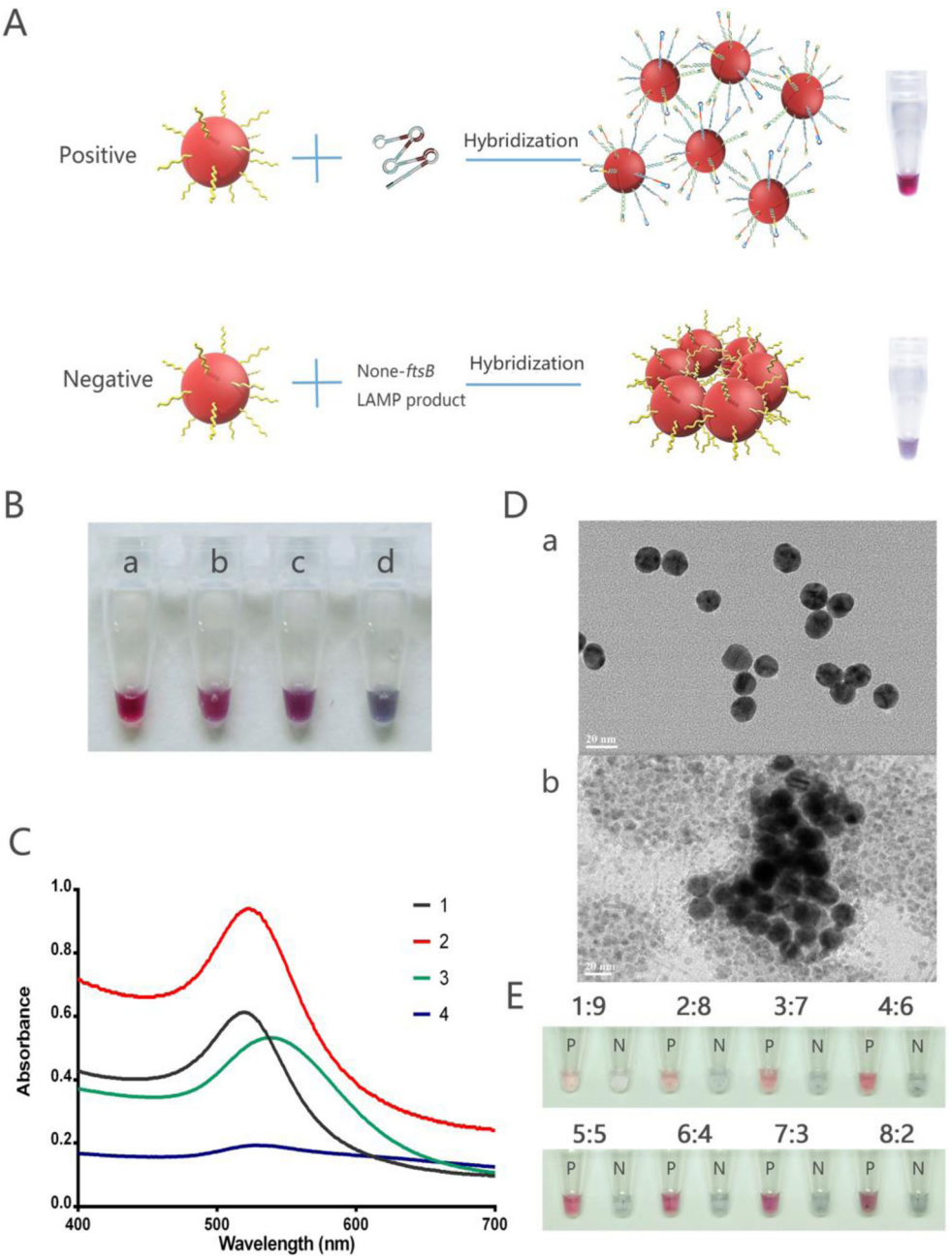


**Fig. 2** The optimization and characteristics of dual probes labelling and hybridization. **a** Schematic illustration of LAMP-AuNPs colorimetric detection of *ftsB* gene through hybridization and self-assembly processes. **b** Color comparison between dual probes (a) and two single probes which are probe 1 (b) and probe 2 (c) in the LAMP-AuNPs biosensor. **c** UV absorption spectra peak alteration after probes labelling and hybridization, samples including 1 = untreated AuNPs, 2 = AuNPs labeled with dual probes, 3 = AuNP-oligonucleotides hybridized with target LAMP products, and 4 = AuNP-oligonucleotides in absent of target LAMP products. **d** TEM images of positive (a) and negative result (b) after hybridization and salt treatment. **e** The test for best hybridization ratio of AuNPs probe solution volume and LAMP products volume

denaturation is feasible to carry out LAMP-AuNPs detection which largely relives the financial burden of the fish farming industry. On the other hand, AuNP as a reproducible and readily obtained material is relatively cheaper than those conjugation materials like quantum dots (QDs), latex, and anti-hapten antibodies in detection biosensors. Meanwhile, its optical property excludes the need for additional result presentation devices. Due to the lack of expensive laboratory instruments and cheap label material and reagents, the cost of LAMP-AuNPs detection is quite reasonable.

In circumstances of practice use, little pathogen infecting in aquatics environment is usually a critical period for infection resource identifying that can make corresponding efforts to prevent diseases outbreak. Therefore, LAMP-AuNPs could provide an accurate color contrast between positive and negative samples when LAMP product is insufficient. Dharanivasan et al. (2016) has applied dual probes in AuNPs biosensor for detection of tomato leaf curl New Delhi virus (ToLCNDV) in which dual probes is superior to single probe in color observation. In order to fully utilize two strands of dsDNA and make the LAMP-AuNPs detection result distinctive especially when the amount of amplificon is few, we have designed dual functional probes of which sequences are in the region of F1C-B1C in separate strands of *ftsB* gene which are non-complementary with each another to avoid mispairing. The result showed that bifunctional probes

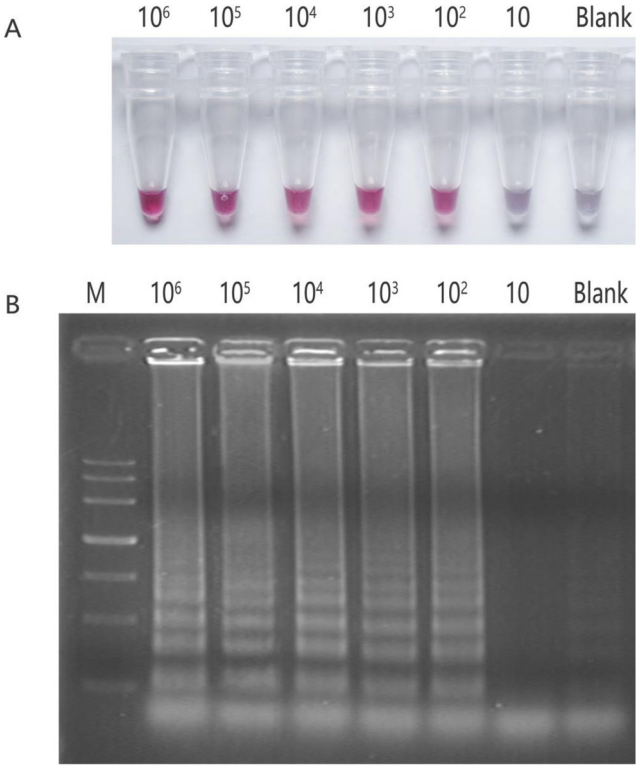


**Fig. 3** Sensitivity of LAMP-AuNPs colorimetric detection of *S. iniae* ATCC 29178. The visual observation (**a**) and AGE image (**b**) results of LAMP-AuNPs conducted with varying DNA genome template of 10-fold serial diluted *S. iniae* ATCC 29178 which are $10^6$ (13.3 ng), $10^5$ (1.9 ng), $10^4$ (246 pg), $10^3$ (61 pg), $10^2$ (1.8 pg), and 10 (500 fg) CFU and a blank control ($ddH_2O$) give a detection limit of $10^2$ (1.8 pg)

system has a redder color than single probe system which tends to be purple red with the same *S. iniae* positive sample. In field detection, it is vital to detect potential pathogen diseases in advance when the number of bacteria is not enough to give an obvious pathogenic symptom to get rid of the possibility of massive loss. Thus, the bifunctional probes help the LAMP-AuNPs system completed and lead to a stable result exhibition.

In this biosensor, we choose *ftsB* gene as a target gene which is partly transcribed adenosine triphosphate (ATP)-binding-cassette transporter system (*ftsABCD*). The region of LAMP amplification ranges 217 bp length with little sequence similarity from nucleotide blasting. Meanwhile, the six regions of target gene corresponding to two sets primers makes LAMP reaction more specific (Wong et al. 2017). The specific test turns out *S. iniae* ATCC 29178 and isolated strain HN have positive results no matter through agarose gel electrophoresis and color change observation while other pathogens are negative confirmed by multiple retrials. This indicates that this *S. iniae* detection biosensor has superior specificity. The result is confirmed by AGE image to support LAMP-AuNPs color observation. The least quantity of bacteria that can be tested by LAMP-AuNPs is $10^2$ CFU which contains approximately 1.8 pg DNA genome that there will be tiny up and down variation of DNA genome value because of variable conditions of actual extraction operation such as temperature, absorbing, and elution effect. To evaluate the LAMP-AuNPs sensitivity of *S. iniae*, we have simultaneously conducted limit

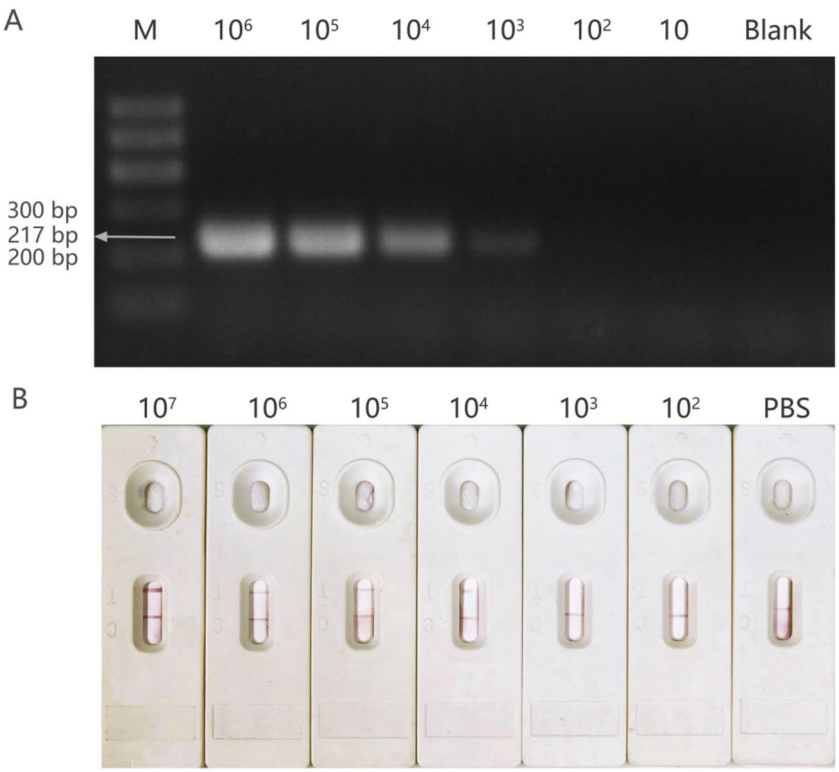


**Fig. 4** Sensitivity comparison with other assays. There are two assays that have been eluvated of *S. iniae* detection sensitivity involving **a** PCR with a detection limit of $10^3$ CFU (61 pg) M = Maker III. and **b** AuNP-MAbs lateral flow test strip with a detection limit of $10^4$ CFU. **a** The template is extracted DNA genome of 10-fold serial diluted *S. iniae.* Lines 1–7: $10^6$ (13.3 ng), $10^5$ (1.9 ng), $10^4$ (246 pg), $10^3$ (61 pg), $10^2$ (1.8 pg), and 10 (500 fg) CFU and a blank control ($ddH_2O$). **b** The 10-fold diluted cultured *S. iniae* was used for LFTS detection. Lines 1–7 = $10^7$, $10^6$, $10^5$, $10^4$, $10^3$, and $10^2$ CFU and PBS control

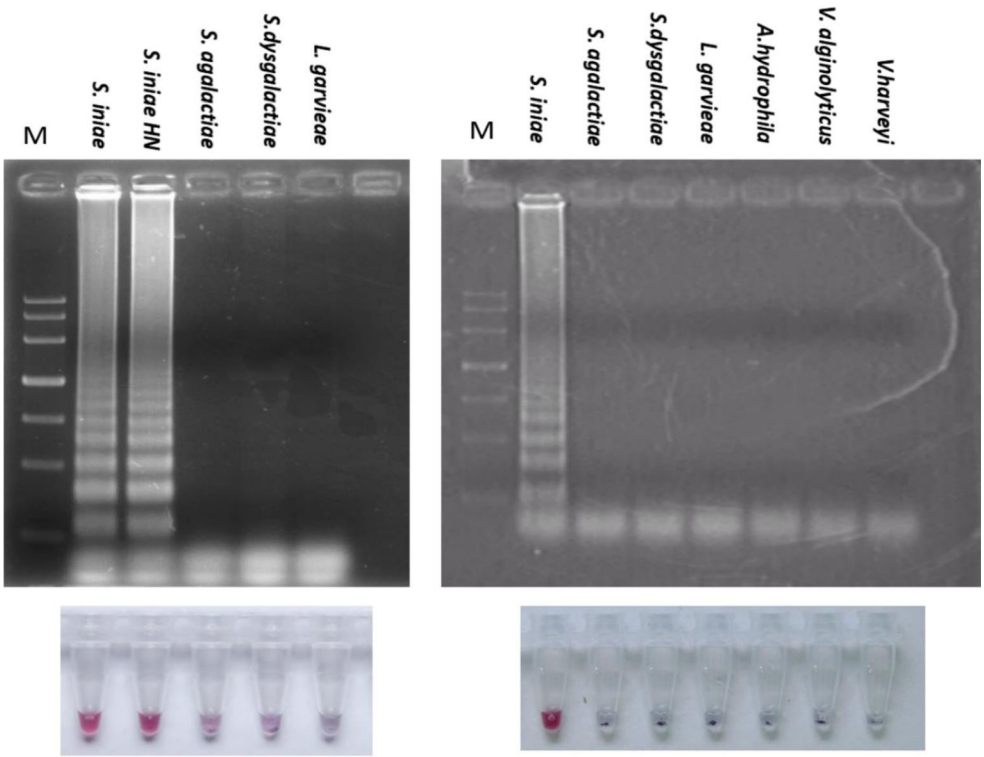


**Fig. 5** Specificity of LAMP-AuNPs assay for detection of *S. iniae* ATCC 29178. The cross reaction contains standard *S. iniae* strain ATCC 29178, *S. iniae* isolate HN and six other fish pathogens including *S. agalactiae*, *S. dysgalactiae*, *L. garvieae*, *A. hydrophila*, *V. alginolyticus*, and *V. harveyi*. AGE and color observation exhibit consistent results that both *S. iniae* strains have LAMP patterns and remain red color with no cross reaction of other pathogens

tests on other two typical detection methods, PCR and AuNPs lateral flow biosensor. The result revealed the detecting limit of PCR of $10^3$ CFU (61 pg) and LFTS of $10^4$ CFU. Although AuNP-MAbs LFTS is widely recognized to be commercial advantageous, LAMP-AuNPs has 100-fold sensitivity than LFTS in identifying of *S. iniae* as well as relatively less costing than other non-AuNP-related methods. All features mentioned above completes LAMP-AuNPs biosensor to be an efficient method not only sensitive and specific, but also convenient and stable.

In addition to the laboratory trials of LAMP-AuNPs efficiency, it is necessary to evaluate this biosensor in fish infection model in order to simulate a more complex detection situation. This model established through soak infection in zebrafish gives a verification for valid field testing in fish farming environment. Compared with laboratory cultured bacteria testing results, the DNA template extracted from organs of infected zebrafish exhibit positive result in tissues including liver, spleen, and intestine except kidney. This phenomenon can be explained that accumulation of *S. iniae* differentiates in zebra fish organs (Zhou and Li 2016). Besides, the fish genome might be extracted along with bacterial genome that could interfere the reaction system in certain ways. Considering the contamination possibility of LAMP reaction (Kundapur and Nema 2016) in places without biosafety cabinet and air circulation system that results in false positive, the introduce of lyophilized LAMP which mentioned by Hayashida et al. (2015) and Carter et al. (2017) that contains dried form of LAMP reagents might be a good choice to address this potential problem in commercial application aspects. The positive results of liver, spleen, and intestine in fish samples confirms LAMP-AuNPs assay can be effectively used in field detection. The *S. iniae* LAMP-AuNPs detection biosensor we established is a novel accurate and

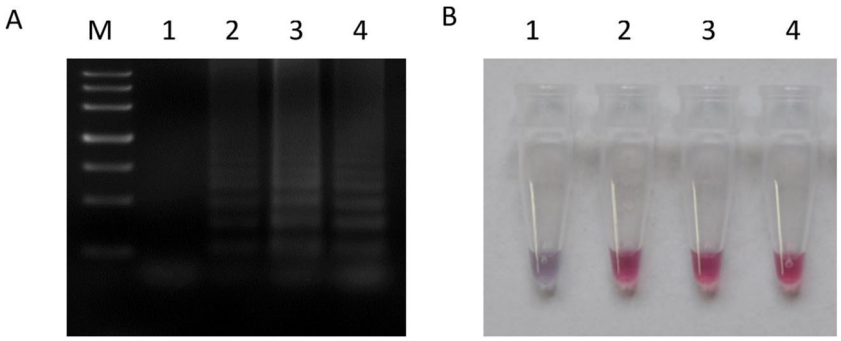


**Fig. 6** Field detection of zebrafish organs by LAMP-AuNPs assay. There are four organs including 1 = kidney, 2 = liver, 3 = intestine, and 4 = spleen of adult zebrafish chosen for field detection. Lines 2–4 exhibit positive phenomena except for Line 1 from both visual observation and AGE image

effective method. As a molecular-associated method, it gets rid of the traditional PCR assay demerit of laboratory operation only and can be readily used in fish farming environment with just a heating device with 2-h time consumption and nearly no cost on experimental instruments. Thus, it has much potential to be the next applicable detection tool in field detection after gold nanoparticle LFTS but much more sensitive and specific.

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## Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

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