



# Improvement of Loop-Mediated Isothermal Amplification Combined with Chromatographic Flow Dipstick Assay for *Salmonella* in Food Samples

Jia Yu<sup>1</sup> · Jiahua Xing<sup>2</sup> · Xijing Zhan<sup>3</sup> · Zhen Yang<sup>2</sup> · Jun Qi<sup>3</sup> · Yuxi Wei<sup>1</sup> · Yin Liu<sup>2</sup>

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## Abstract

Loop-mediated isothermal amplification (LAMP) had been employed as a powerful tool to facilitate genetic tests for various food pathogens, as it is easy to perform. Recently, various methods of detecting the LAMP amplicon were developed. In this study, we improved two LAMP assays by combining LAMP with chromatographic flow dipstick (LFD) assays for *Salmonella* (targeting *phoP* and *invA*, respectively). We evaluated different labeled primer sets, then selected the optimal sets to perform the LFD assays. We compared the optimal LFD and LAMP assays with the ISO standard method. The results showed that LFD was more sensitive and quicker than LAMP. Furthermore, enrichment broths of 225 food samples were tested. The sensitivity of two LFD assays was 100%. The specificity of LFD assay targeting *phoP* was 99.1%, and LFD assay targeting *invA* was 99.5%. For the LFD assay targeting *phoP*, the estimate of limit of detection (LOD) 50% was 0.061 CFU/g and the estimate of LOD 95% was 0.265 CFU/g. For the LFD assay targeting *invA*, the estimate of LOD 50% was 0.040 CFU/g and the estimate of LOD 95% was 0.172 CFU/g. We validated this method in a primary laboratory, where we accomplished the assay only using an incubator and a heating block. It suggested that the LFD assay had the potential to become a suitable diagnostic method in field test and primary labs.

**Keywords** Loop-mediated isothermal amplification · Chromatographic flow dipstick · *Salmonella* · *phoP* · *invA*

## Introduction

*Salmonella* is an important foodborne pathogen for the public health (Gomez et al. 1997; Sanchez-Vargas et al. 2011). It was estimated that 21 million people were infected by *Salmonella* every year worldwide (Crump and Mintz 2010). The World Health Organization mentions *Salmonella* as one of the four crucial global causes of diarrheal diseases. Undercooked food, including various meats, fruits, and vegetables, can be

contaminated with the pathogen during the lengthy farm-to-fork process and transmit it to humans (Park et al. 2012; Pires et al. 2010). Therefore, multifaceted detection methods of *Salmonella* are needed to better ensure food safety, especially during the production, processing, and distribution of agricultural food.

These assays should be rapid, simple, and accurate. Traditional culture methods of detecting *Salmonella* in foods are reliable but labor-intensive and time-consuming. The ISO standard method (ISO 2017) consists of many steps that include non-selective and selective pre-enrichment, differential plating, and biochemical identification. This method requires at least 5 days to confirm the results. Thus, it is important to develop and validate faster screening methods for *Salmonella*. Immunoassays, such as chromatographic flow dipstick and enzyme-linked immunosorbent, are simple and rapid, but the low specificity limited their application widely (Eriksson and Aspan 2007). Recently, molecular methods such as PCR, real-time PCR, and various isothermal amplification assays have been developed and used widely to detect *Salmonella* DNA markers (*invA*, *phoP*, and *fimC*) in food samples (Kasturi and Drgon 2017; Levy et al. 2008; Li et al. 2009; Pikhova et al.

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✉ Yin Liu  
liuyin@nankai.edu.cn

<sup>1</sup> College of Life Sciences, Qingdao University, Qingdao 266071, People's Republic of China

<sup>2</sup> School of Medicine, Nankai University, Tianjin 300071, People's Republic of China

<sup>3</sup> Tianjin International Travel Health Care Center, Tianjin 300456, People's Republic of China

2005). Most of these methods reduce the detection time to 2–3 days. Moreover, loop-mediated isothermal amplification (LAMP) has emerged as a promising molecular method and been used broadly to detect *Salmonella* (Domesle et al. 2018; Fan et al. 2015; Yang et al. 2015; Zhuang et al. 2014), since it can be performed in a simple isothermal instrument such as a water bath or heating block.

The LAMP was performed by *Bst* DNA polymerase and a set of four specially designed primers, termed inner and outer primers (Mori et al. 2001; Notomi et al. 2000). The LAMP can also be accelerated by a further pair of loop primers (Nagamine et al. 2001). LAMP product analysis is usually performed by agarose gel electrophoresis, dsDNA-specific fluorescent dye, or turbidity of magnesium pyrophosphate. However, electrophoresis analysis usually brings aerosol pollution. The aerosol leads to the false positive results in later assays (Chen et al. 2016). The operator may make misjudgment by the observation of fluorescent signal and turbidity. LAMP combined with chromatographic flow dipstick (LFD) assay can avoid these disadvantages (Choi et al. 2016; Jaroenram et al. 2009). Generally, a generic LFD dipstick can detect a dual-labeled LAMP amplicon (Kaewphinit et al. 2013; Khunthong et al. 2013; Lalle et al. 2018). Consequently, the LFD techniques meet the need of point-of-care testing (POCT) and field-test better, and the results from the dipstick are more reliable.

In the last decade, many LAMP assays were developed to detect the foodborne pathogens (Domesle et al. 2018; Niessen et al. 2013). However, only a few of LAMP assays were improved to the corresponding LFD assays. One of the reasons is the primer set composition for LFD assay is still ambiguous. Different primers were labeled and composed to perform the LFD assays, such as labeled LF + LB to detect avian-origin influenza A (H7N9) virus (Ge et al. 2013); labeled FIP + BIP to detect foot-and-mouth disease virus (Waters et al. 2014), *Taenia solium* (Nkouawa et al. 2016), and *Xanthomonas citri* (Rigano et al. 2010); labeled BIP + LF to detect *Mycobacterium tuberculosis* (Roskos et al. 2013); labeled FIP + LF to detect *Leptospira* (Nurul Najian et al. 2016); an additional FITC-labeled probe combined a labeled primer to detect *Mycobacterium tuberculosis* (Kaewphinit et al. 2013), *Candidatus Liberibacter* species (Rigano et al. 2014), *Vibrio parahaemolyticus* (Prompamorn et al. 2011), *Pseudomonas aeruginosa* (Manajit et al. 2018), and canine parvovirus (Sun et al. 2014). In our previous studies, we analyzed dual-labeled amplicon using different primer set LFD assays and evaluated the specificity and sensitivity of these assays (Yu et al. 2019). The results showed that an optimal primer sets can avoid the false positive results and shorten the amplification time. Consequently, the procedure of selecting primer sets and optimizing the LFD protocol plays a significant role in expanding application of LFD assay. Theoretically, any LAMP assay can be upgraded to the corresponding LFD assay

following a proper procedure. The objective of this study was to develop a method for improving LAMP to LFD assay for *Salmonella* and verify using inoculated food matrices.

## Materials and Methods

### Bacterial Strains and DNA Extraction

A total of 38 known bacterial type strains, including 16 type strains of *Salmonella* and 22 non-*Salmonella* type strains of *Enterobacteriaceae*, were collected from culture centers (listed in Table 1, and more details of *Salmonella* listed in Supplementary Table S1) and employed to evaluate the LFD assay. Additionally, we collected 12 *Salmonella* isolates and 5 *Citrobacter freundii* isolates from food samples and also utilized them to analyze the assays. All of the bacterial strains and isolates are listed in Table 1. All the strains and isolates were cultured in Luria-Bertani broth or Luria-Bertani agar. The *Salmonella* isolates were identified by the procedure based on the ISO 6579:2017 (ISO 2017). The *C. freundii* isolates were identified by the VITEK 2 Compact system (BioMérieux, Inc., Maray l'Etoile, France). Bacterial strains were stored in 30% (w/v) glycerol broth at  $-80\text{ }^{\circ}\text{C}$ . For DNA extraction, a single colony of the bacterium was picked from a Luria-Bertani agar plate and inoculated in 10-ml LB broth. The broth was incubated at  $37\text{ }^{\circ}\text{C}$  for 12 h to obtain about  $10^7$  CFU/ml. One milliliter of the enriched broth was extracted DNA by a Bacterial Genomic DNA Extract Kit (Tiangen Co., Beijing, China). The extracted DNA was diluted by TE buffer and stored at  $-20\text{ }^{\circ}\text{C}$ .

### LAMP and LFD Assays

The LAMP assays were both performed as the previous studies with some optimization (Li et al. 2009; Techathuvanan et al. 2010). In brief, the LAMP reaction was performed in a 25- $\mu\text{l}$  volume containing 0.2  $\mu\text{M}$  each of F3 and B3, 1.6  $\mu\text{M}$  each of FIP and BIP, 0.8  $\mu\text{M}$  each of LF and LB, 2.5 mM each deoxynucleoside triphosphate, 0.8 M betaine (Sigma, B0300, St. Louis, USA), 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM  $(\text{NH}_4)_2\text{SO}_4$ , 8 mM  $\text{MgSO}_4$ , 0.2% Tween 20, 5 U of the *Bst* DNA polymerase large fragment (New England Biolabs, M0275S, Beverly, USA), and 5  $\mu\text{l}$  of DNA template. The mixture was incubated at  $63\text{ }^{\circ}\text{C}$  using a heating block for 60 min. The LAMP products were analyzed by observing the white turbidity and the mixture of 1.0  $\mu\text{l}$  of 1/10-diluted original SYBR Green I (ThermoFisher Scientific Co., S-7567, Waltham, USA) detection described in a previous study (Li et al. 2009). The primers targeting *phoP* gene in *Salmonella* were designed according to genomic DNA of the *S. enterica* subsp. *enterica* serovar Paratyphi A str. ATCC 9150 (GenBank accession no. CP000026.1). The *phoP* gene

**Table 1** Bacterial strains used in this study

Species	Strains	Number of isolates
<i>Salmonella enterica</i>	ATCC 10719, ATCC 13312, ATCC 19585, ATCC 43976, CMCC 47014, CMCC 50166, CMCC 50167, CMCC 50213, CMCC 50215, CMCC 50338, CMCC 50341, CMCC 50730, CMCC 50822, CMCC 50879, CMCC 50919, ATCC 9150	9
<i>Salmonella bongori</i>	–	3
<i>Citrobacter freundii</i>	CGMCC 1.1732, CMCC 48001, CMCC 48002, CMCC 48005, CMCC 48006, CMCC 48010, CMCC 48016, CMCC 48019	5
<i>Enterobacter aerogenes</i> <sup>a</sup>	ATCC 13048, CGMCC 1.876	0
<i>Enterobacter cloacae</i>	ATCC 13047, CGMCC 1.57	0
<i>Pantoea agglomerans</i>	ATCC 27155	0
<i>Escherichia coli</i>	ATCC 8739, ATCC 11229	0
<i>Hafnia alvei</i>	ATCC 9760	0
<i>Klebsiella pneumoniae</i>	ATCC 9345, ATCC 10031	0
<i>Proteus vulgaris</i>	ATCC 13315	0
<i>Serratia marcescens</i>	ATCC 13880	0
<i>Shigella dysenteriae</i>	CMCC 51630	0
<i>Yersinia enterocolitica</i>	CMCC 52301 2	0

ATCC American type culture collection, CMCC China medical culture collection, CGMCC China general microbiological culture collection

<sup>a</sup> *Enterobacter aerogenes* is also known as *Klebsiella mobilis*

location is between 1680130 and 1680804. The LAMP products are a mixture of multiples of various sizes, and the primary product is about 240 bp (Li et al. 2009). Meanwhile, the primers targeting *invA* gene in *Salmonella* were designed according to genomic DNA of the *S. enterica* subsp. *enterica* serovar Braenderup str. ATCC BAA-664 (GenBank accession no. CP034773.1). The *invA* gene location is between 3690856 and 3692913. The LAMP products are a mixture of multiples of various sizes, and the primary product is about 180 bp (Techathuvanan et al. 2010). The primer sequences used in this study are listed in Supplementary Table S2.

For LFD assays, we labeled the FITC at the 5'-end of FIP, BIP, LF, and labeled BIO at the 5'-end of BIP, LF, LB, respectively. All unlabeled and labeled primers were synthesized by Sangon Biotech Co., Shanghai, China. Then, we organized these labeled primers and unlabeled primers to six primer sets, each set containing two labeled primers and four unlabeled primers (listed in Table 2). The LFD assays were performed by these primer sets with the same concentration as the LAMP assays. For evaluation of the shortest amplification time of LFD assays, we incubated the LFD mixtures at 63 °C from 10 to 60 min. The dual-labeled amplicons of LFD assays were detected by a Type II BEST Cassette (Ustar, Hangzhou, China) at room temperature. This device detected the amplicons with the test (T) line for capturing dual-labeled amplicons and the control (C) line for capturing streptavidin-conjugated color particle-labeled antibody. In brief, the LFD reaction tube was placed in an amplicon cartridge of a Type II BEST Cassette immediately after amplification. The cartridge was then closed and inserted into the detection chamber. The

handle of the detection chamber was closed to seal and start amplicon detection with the chromatographic flow dipstick. The assay result was read by eye to score from the detection window of the chamber after 5 min. A positive result was scored when both the T line and the C line were visible. A negative read result was scored when only the C line was displayed. The assay was regarded as invalid when C line was invisible.

### Comparison of the Sensitivity of Different Labeling Methods for LFD Assays

The sensitivity of the assays was evaluated with the type strains of *S. enterica* subsp. *enterica* serovar Paratyphi A

**Table 2** Composition of LFD primer sets

Primer set name		Labeled primer <sup>a</sup>	Unlabeled primer	
<i>phoP</i> <sup>b</sup>	<i>invA</i> <sup>b</sup>			
PP1	IP1	FIP-FITC	BIP-BIO	LF, LB, F3, B3
PP2	IP2	FIP-FITC	LB-BIO	LF, BIP, F3, B3
PP3	IP3	LF-FITC	LB-BIO	FIP, BIP, F3, B3
PP4	IP4	LF-FITC	BIP-BIO	FIP, LP, F3, B3
PP5	IP5	FIP-FITC	LF-BIO	BIP, LB, F3, B3
PP6	IP6	BIP-FITC	LB-BIO	FIP, LF, F3, B3

<sup>a</sup> FITC, the primer was labeled fluorescein isothiocyanate at 5'-end; BIO, the primer was labeled biotin at 5'-end

<sup>b</sup> Detection target

str. ATCC 9150. We determined the sensitivity by the method described previously (Li et al. 2009). Briefly, we picked a single colony from a freshly prepared Luria-Bertani agar plate and suspended it in 200- $\mu$ l phosphate-buffered saline (pH 7.0). Fifty microliters of the suspension was transferred into two tubes labeled as samples A and B. Ten-fold serial dilutions, to  $10^{-9}$ , were made from samples A and B. We spread 50  $\mu$ l of each dilution of sample A series on Luria-Bertani agar plates in triplicate and counted colonies on these plates after incubation for 48 h at 37 °C. Meanwhile, sample B was utilized to make the artificial inoculation samples. We homogenized 25-g minced food samples using a conical flask with 225 ml of buffered peptone water (BPW). We inoculated 50  $\mu$ l of sample B serial dilutions ( $10^{-6}$  to  $10^{-9}$ ) into the prepared food samples. These artificially inoculated food samples were subjected to pre-enrichment and DNA extraction for LAMP and LFD analysis (see part 2.5). Genomic DNA was also extracted from 50- $\mu$ l suspension and utilized as positive control. The concentration of extracted genomic DNA solution was detected at 260 nm using a NanoDrop 2000 (ThermoFisher Scientific Co., Waltham, USA). Then, the concentration of mass was converted to the concentration of genomic DNA copies. The formula was referred a previous study (Bowe et al. 2009). The amount of genomic DNA copies equals [the mass (in grams)  $\times$  Avogadro's number] / (average molecular weight of a base  $\times$  genomic DNA length). We prepared four 10-fold dilutions containing about  $10^3$  to  $10^0$  genomic copies per microliter as template DNA solution.

### Optimization of the LFD Assay Protocol

For optimization of the LFD assay protocol, we evaluated the specificity, sensitivity, and the shortest amplification time of the LFD assays performed by different primer sets.

The genomic DNA templates from 38 bacterial type strains and 17 isolates were chosen to investigate the LFD assay specificity. These LFD mixtures were incubated at 63 °C using a heating block for 60 min. For simplification, we evaluated the specificity of LFD assays' primer sets by four steps. In each step, we excluded the primer sets that cause a false positive or false negative result in triplicate assays. (1) Ultra-pure water (Amplification grade water, Promega, WI, USA) was used as the negative reference to evaluate the all LFD primer sets. (2) The rest primer sets were adopted to detect the *C. freundii* strains, which is the closest to *Salmonella* in phylogenetic analysis by *phoP* gene (Li et al. 2009). (3) The specificity of the rest primer sets was evaluated by the other non-*Salmonella* reference strains. (4) Then, we confirmed the specificity of the rest primer sets using all of the *Salmonella* strains and isolates. Finally, we selected the primer sets which performed the

true positive and negative results as the candidate primer sets.

We compared the sensitivity of LFD assays by DNA serial dilutions of *S. enterica* (see part 2.3). All the sensitivity tests were identified in triplicate.

The primer sets with the highest sensitivity were selected to evaluate the shortest amplification time. Firstly, we amplify the template DNA with the selected primer set at its lowest concentration for 50 min. The reaction mixture was detected by a chromatographic flow dipstick. If it is positive, we shortened the amplification time for another 5 min, until at least one negative result displayed in triplicate assays. We recorded the shortest amplification time of three positive results. Therefore, the optimized LFD assay was performed by the primer set with its shortest amplification time. In addition, agarose gel electrophoresis (Li et al. 2009) was performed to analyze the amplicons of these LFD reactions.

### Detection of Salmonella in the Food Samples

We used minced pork for artificially contaminated samples. Firstly, we confirmed these samples being free of *Salmonella* contamination by a procedure based on the ISO 6579:2017 method. We inoculated 50- $\mu$ l serial cell dilutions ( $6.2 \times 10^0$  CFU to  $3.75 \times 10^{-1}$  CFU) of *S. enterica* to 25-g minced pork samples. We incubated these food samples and extracted the DNA sample from them using the method described by a previous study (Malorny et al. 2007). In brief, we homogenized 25-g minced food samples using a conical flask with 225 ml of buffered peptone water (BPW), then enriched the mixtures for 20 h at 37 °C without shaking. Then, 1-ml culture was centrifuged for 5 min at 6000 $\times$ g. The pellet was resuspended in 1 ml ddH<sub>2</sub>O. Cells in this mixture were lysed by boiling at 100 °C for 10 min, followed by centrifugation at 3000 $\times$ g for 30 s. Finally, 5  $\mu$ l of each supernatant was removed and subjected to the LFD assays.

We collected and tested 225 food samples, including 85 minced pork, 50 raw milk, 90 chicken breast as natural food samples. We detected these samples using the ISO method and LFD assays individually. For the LFD assays, the food samples were pre-enriched and extracted DNA following the method described by Malorny et al. (Malorny et al. 2007). The LFD assays were performed as the optimized protocol (see part 2.4). The limit of detection (LOD) of the assays was analyzed with the probability of detection (POD) function (PODLOD\_ver9.xls), described by Wilrich and Wilrich (2009). The total LFD assay time of food sample was shorter than 24 h.



## Results and Discussion

### Specificity of the LFD Assays

Firstly, we evaluated the specificity of the LAMP assays. Then, we evaluated the specificity of the LFD assays, which performed by all the 12 primer sets as listed in Table 2. The specificity was evaluated by four steps, and the results are shown in Table 3.

- Step 1. All of the primer sets were employed to assess the specificity using ultra-pure water as the negative control. Among the six primer sets targeting *phoP*, PP1 presented false positive results in the LFD assays. We got the similar results in primer sets targeting *invA*, IP1 set presented false positive results, and other five sets presented negative results.
- Step 2. The rest five primer sets targeting *phoP* evaluated the specificity using eight strains and five isolates of *C. freundii*. For *phoP* detection, we got three false positive results (*C. freundii* CMCC 48001, CMCC 48002, and CMCC 48005), as the LFD assays were performed by PP4 primer set. The LFD assays by other primer sets, PP2, PP3,

PP5, and PP6, showed the true negative results. Similarly, for *invA* detection, all of the five primer sets, IP2-IP6, showed true negative results of the LFD assays.

- Step 3. Then, we evaluated the selected primer sets, PP2, PP3, PP5, PP6, and IP2-IP6, by all the rest non-*Salmonella* strains. All the LFD assays showed true negative results.
- Step 4. These selected primer sets were utilized to amplify DNA template, which were extracted from 16 type strains and 12 isolates of *Salmonella*. All the primer sets showed specificity for *Salmonella* and were chosen for sensitivity and efficiency evaluation.

In present study, we chose *C. freundii* to analyze the primer set specificity prior to other bacteria. The reason is that *C. freundii* is the closest to *Salmonella* in phylogenetic analysis by *phoP* gene (Li et al. 2009). Furthermore, we collected the target sequence between primers F3 and B3 and aligned the sequence in GenBank by BLAST. The BLAST results showed that *C. freundii* contains the most similar sequence to *Salmonella* both in *phoP* and *invA*. This step can simplify the evaluation process.

**Table 3** Specificity of various primer sets

Step <sup>a</sup>	Templates	LAMP		LFD primer sets											
		<i>phoP</i> <sup>c</sup>	<i>invA</i> <sup>c</sup>	PP1 <sup>b</sup> <i>phoP</i>	IP1 <i>invA</i>	PP2 <i>phoP</i>	IP2 <i>invA</i>	PP3 <i>phoP</i>	IP3 <i>invA</i>	PP4 <i>phoP</i>	IP4 <i>invA</i>	PP5 <i>phoP</i>	IP5 <i>invA</i>	PP6 <i>phoP</i>	IP6 <i>invA</i>
1	Ultra-pure water	–	–	+	+	–	–	–	–	–	–	–	–	–	–
2	<i>Citrobacter freundii</i>	0/13	0/13	NA <sup>d</sup>	NA	0/13 <sup>e</sup>	0/13	0/13	0/13	3/13	0/13	0/13	0/13	0/13	0/13
3	<i>Enterobacter aerogenes</i>	0/2	0/2	NA	NA	0/2	0/2	0/2	0/2	NA	0/2	0/2	0/2	0/2	0/2
3	<i>Enterobacter cloacae</i>	0/2	0/2	NA	NA	0/2	0/2	0/2	0/2	NA	0/2	0/2	0/2	0/2	0/2
3	<i>Pantoea agglomerans</i>	0/1	0/1	NA	NA	0/1	0/1	0/1	0/1	NA	0/1	0/1	0/1	0/1	0/1
3	<i>Escherichia coli</i>	0/2	0/2	NA	NA	0/2	0/2	0/2	0/2	NA	0/2	0/2	0/2	0/2	0/2
3	<i>Hafnia alvei</i>	0/1	0/1	NA	NA	0/1	0/1	0/1	0/1	NA	0/1	0/1	0/1	0/1	0/1
3	<i>Klebsiella pneumoniae</i>	0/2	0/2	NA	NA	0/2	0/2	0/2	0/2	NA	0/2	0/2	0/2	0/2	0/2
3	<i>Proteus vulgaris</i>	0/1	0/1	NA	NA	0/1	0/1	0/1	0/1	NA	0/1	0/1	0/1	0/1	0/1
3	<i>Serratia marcescens</i>	0/1	0/1	NA	NA	0/1	0/1	0/1	0/1	NA	0/1	0/1	0/1	0/1	0/1
3	<i>Shigella dysenteriae</i>	0/1	0/1	NA	NA	0/1	0/1	0/1	0/1	NA	0/1	0/1	0/1	0/1	0/1
3	<i>Yersinia enterocolitica</i>	0/1	0/1	NA	NA	0/1	0/1	0/1	0/1	NA	0/1	0/1	0/1	0/1	0/1
4	<i>Salmonella enterica</i>	25/25	25/25	NA	NA	25/25	25/25	25/25	25/25	NA	25/25	25/25	25/25	25/25	25/25
4	<i>Salmonella bongori</i>	3/3	3/3	NA	NA	3/3	3/3	3/3	3/3	NA	3/3	3/3	3/3	3/3	3/3

<sup>a</sup> The step number is corresponding to that of in part 3.1

<sup>b</sup> Name of primer set, see Table 2

<sup>c</sup> Detection target

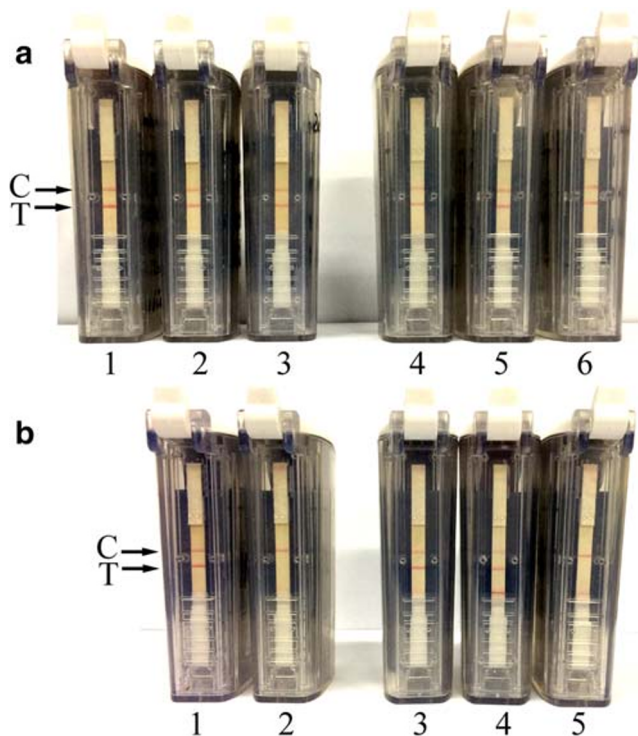
<sup>d</sup> NA, not assay

<sup>e</sup> 0/8, number of positive results/number of total assay

## Comparison the Sensitivity of Different Labeling Methods for LFD Assays

We prepared four 10-fold genomic DNA dilutions to evaluate the sensitivity (see part 2.3). The concentrations of dilutions were from  $9.2 \times 10^{-3}$  to  $9.2 \times 10^{-6}$  ng/ $\mu$ l, which contained about  $1.75 \times 10^3$  to  $1.75 \times 10^0$  genomic DNA copies/ $\mu$ l. We detected 5  $\mu$ l of each prepared dilution, with the LFD assays. After incubating at 63 °C for 60 min, four primer sets, PP5, PP6, IP5, and IP6, showed the highest sensitivity. The LFD assays performed by PP5, PP6, IP5, and IP6 can detect the  $9.2 \times 10^{-5}$  ng/ $\mu$ l DNA dilution (containing about  $4.6 \times 10^{-4}$  ng DNA). Partial of these results is shown in Fig. 1. The sensitivity results of all the assays are listed in Table 4.

In the present study, we found that the most efficient LFD assays were carried out with PP5, PP6, IP5, and IP6 primer sets. All of them contain a labeled inner primer, and the labeled loop primer next to the inner primer, such as BIP-LB, FIP-LF. The results were consistent with our previous study (Yu et al. 2019). These selected primer sets can produce various dual-labeled intermediates (some short dsDNA molecules) in the early several LFD steps. We verified this mechanism in the present study.



**Fig. 1** The sensitivity of LFD assay performed by PP5 and PP6. **a** The sensitivity of LFD supported by primer sets on  $9.2 \times 10^{-5}$  ng/ $\mu$ l. The results of A1–3 were replicate assays of  $9.2 \times 10^{-5}$  ng/ $\mu$ l using PP5. The results of A4–6 were replicate assays of  $9.2 \times 10^{-5}$  ng/ $\mu$ l using PP6. C, control line; T, test line. **b** The sensitivity of LFD supported by primer sets on  $9.2 \times 10^{-6}$  ng/ $\mu$ l. The results of B1–2 were replicate assays of  $9.2 \times 10^{-6}$  ng/ $\mu$ l using PP5. The results of B3–5 were replicate assays of  $9.2 \times 10^{-6}$  ng/ $\mu$ l dilution using PP6. C, control line; T, test line

**Table 4** Sensitivity of LFD assays in pure culture

Primer set	Sensitivity ( $9.2 \times 10^0$ ng/ $\mu$ l)			
	$n = -6$	$n = -5$	$n = -4$	$n = -3$
PP2	0/1 <sup>a</sup>	0/1	3/3	NA <sup>b</sup>
PP3	0/1	0/1	2/3	3/3
PP5	1/2	3/3	NA	NA
PP6	2/3	3/3	NA	NA
IP2	0/1	0/1	3/3	NA
IP3	0/1	0/1	0/1	3/3
IP4	0/1	0/1	3/3	NA
IP5	1/2	3/3	NA	NA
IP6	0/1	3/3	NA	NA

Pure culture was from *S. enterica* subsp. *enterica* serovar Paratyphi A str. ATCC 9150

<sup>a</sup> 0/1, number of positive results/number of total assay

<sup>b</sup> NA, not assay

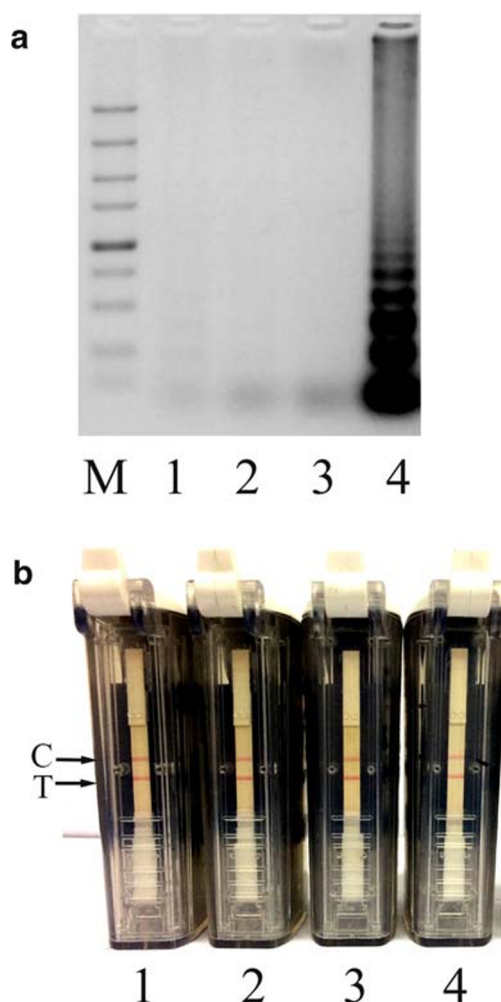
## The Shortest Amplification Time of the LFD Assays

The  $9.2 \times 10^{-5}$  ng/ $\mu$ l DNA dilution was used as the template for evaluation of the shortest amplification time of the LFD assays. In *phoP* detection, PP5 showed the shortest amplification time of 15 min, which is 5 min lesser than PP6. In *invA* detection, IP5 and IP6 showed the same shortest amplification time of 20 min. All the shortest amplification times are shorter than the amplification time of their corresponding LAMP assays. We also utilized agarose gel electrophoresis to analyze the amplicons of these LFD reactions at the shortest amplification time. The electrophoresis showed negative results, while LFD assays were positive (see Fig. 2).

Many *Salmonella* LAMP assays have been developed since 2005 (Hara-Kudo et al. 2005). LAMP amplicons were detected with multiple methods, including turbidity, colorimetry, gel electrophoresis, fluorescence, electrochemical sensors, enzyme-linked immunosorbent assay (ELISA), and lateral flow dipstick (Yang et al. 2018; Zhang et al. 2014). The amplification time for *Salmonella* LAMP assays ranges from 25 min (real-time fluorescence-based LAMP) to 2 h (traditional LAMP) (Mashooq et al. 2016; Yang et al. 2018; Ye et al. 2011). In the present study, the optimized LFD assays showed the shorter amplification time than those above methods.

## LFD Assays in Artificial Contaminated Samples

We optimized a LFD protocol for detecting *Salmonella* in food samples (shown in Fig. 3) and verified it in artificial contaminated samples. The LFD assays can be performed with an incubator, a centrifugal machine, and a heating block in 24 h. We utilized two primer sets, PP6 and IP6, to perform



**Fig. 2** LFD amplicons detected by electrophoresis and chromatographic flow dipstick. **a** Detection by electrophoresis. Lanes: M, DNA marker; 1,  $9.2 \times 10^{-5}$  ng/ $\mu$ l amplified by primer set PP5 for 15 min; 2, 20 min; 3, 25 min; 4, 1 h. **b** Same amplicon detection by chromatographic flow dipstick. Samples 1–4 are corresponding to the samples 1–4 in Fig. 2a. 1,  $9.2 \times 10^{-5}$  ng/ $\mu$ l amplified by primer set PP5 for 15 min; 2, 20 min; 3, 25 min; 4, 1 h. C, control line; T, test line

the LFD assays. We used a gradient solution of *Salmonella* cells to artificially contaminate a 25-g food sample (details are listed in Supplementary Table S3). The extracted DNA was specifically detected by PP6 and IP6 sets, respectively. The LOD of the assays were analyzed with the probability of detection (POD) function. For the LFD assay by PP6 sets, the estimate of LOD 50% was 0.061 CFU/g and the estimate of LOD 95% was 0.265 CFU/g. For the LFD assay by IP6 sets, the estimate of LOD 50% was 0.040 CFU/g and the estimate of LOD 95% was 0.172 CFU/g. The statistical analysis results are listed in Supplementary Table S4 and Fig. S1.

In the present study, we utilized the POD function and the LOD of a binary microbiological measurement method to describe the sensitivity of our LFD assays (Wilrich and Wilrich 2009). The sensitivity is described by its probability of detection as a function of the contamination of the test food

samples or by the LOD with a specified probability. It supported more information than a traditional representation method. It is also easy-to-perform with an excel program.

### LFD Assays in Food Samples

A total of 225 food samples, including 85 minced pork, 50 raw milk, and 90 chicken breast, were collected as naturally food samples. All the samples detected *Salmonella* using ISO 6579:2017 method as the Golden Standard. We also detected 25 g of each food sample using the LFD assays (see Fig. 3). The results are listed in Table 5. A total of 15 positive samples were detected using ISO 6579:2017 method, including 3 samples of minced pork, 1 sample of raw milk, and 11 samples of chicken breast. The LFD assays performed by PP6 detected all the 15 true positive samples (tp), but also two false positive samples (fp) of minced pork. The number of false negative samples (fn) was 0, and the number of true negative samples (tn) was 208. The LFD assay performed by IP6 showed the similar results. The numbers of tp, fp, tn, and fn were 15, 1, 209, and 0, respectively. Consequently, the sensitivity of LFD assay targeting *phoP* was 100% [ $tp/(tp + fn) = 15/(15 + 0)$ ], and the specificity of the assay was 99.1% [ $tn/(tn + fp) = 208/(208 + 2)$ ]. The sensitivity and specificity of LFD assay targeting *invA* were 100% [ $tp/(tp + fn) = 15/(15 + 0)$ ] and 99.5% [ $tn/(tn + fp) = 209/(209 + 1)$ ], respectively.

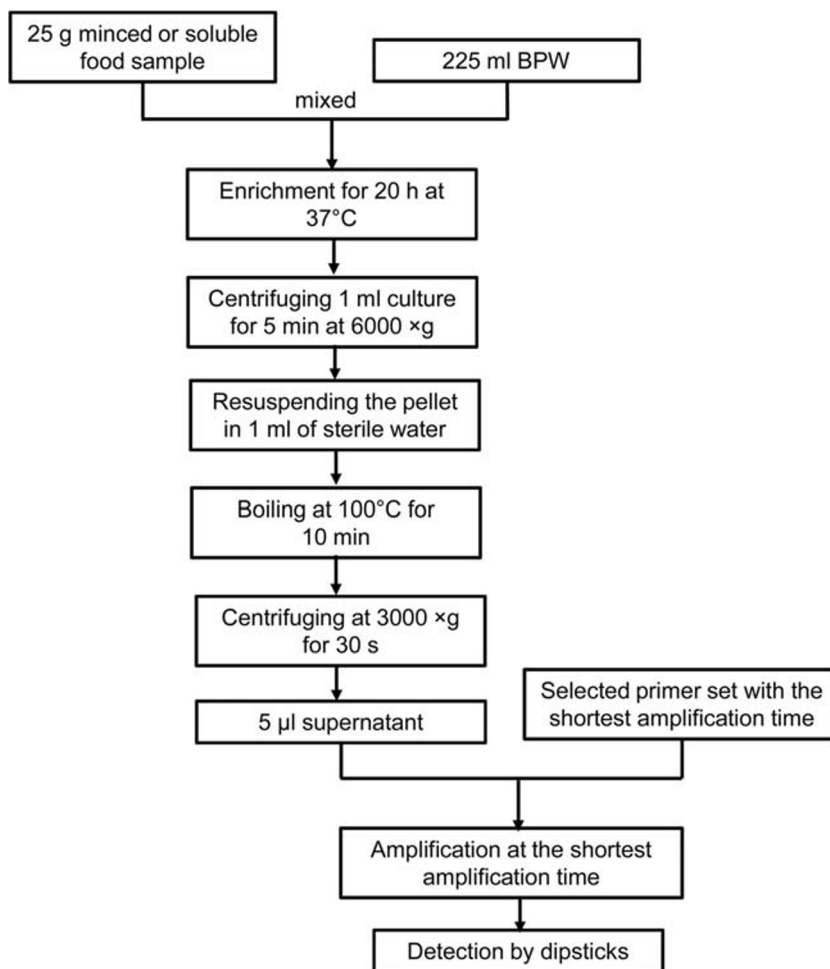
### The Evaluation Procedure of Salmonella LFD Assays

The evaluation procedure is illustrated in Fig. 4. This process can efficiently improve a LAMP assay to its corresponding LFD assay.

Firstly, the primer sets should be evaluated by negative control, ultra-pure water. Several previous studies proved that LFD is more sensitive than LAMP (Chen et al. 2016; Choi et al. 2016; Jaroenram et al. 2009). It was suggested that the LFD assay may get more false positive results than LAMP. Therefore, we evaluated the specificity by negative control at first. Both of the FIP + BIP labeled primer sets showed the false positive results. This may be due to the length of FIP and BIP. It is generally recognized that primers can bind each other and generate dimer or cross-dimer. The cross-dimer generated by FIP + BIP may be the most stable one, because both FIP and BIP are much longer than LP and LB. If the cross-dimer or its amplification products is dual-labeled dsDNA, it can be detected as a false-positive result by the chromatographic flow dipstick.

Secondly, we evaluated the specificity by similar strains. We chose *C. freundii* to analyze the primer set specificity

**Fig. 3** LFD protocol for detecting *Salmonella* in food samples



before other bacteria, because the BLAST results showed that *C. freundii* contains the most similar sequence to *Salmonella* both in *phoP* and *invA*. This step can simplify the evaluation process.

Thirdly, we evaluated the specificity by the other reference strains. Various non-specific dual-labeled dsDNAs can be generated in an amplification reaction (Yu et al. 2019). The quantity of these non-specific amplicons may be less than that of specific amplicons. It is possible, therefore, that they cannot be identified by traditional detection methods of LAMP, such as observation of turbidity and color of fluorescent dye.

However, the dual-labeled non-specific amplicons can be detected as a false positive result by an LFD assay. As a result, the specificity of a LAMP assay should be better than a corresponding LFD assays.

Fourthly, we analyzed the LFD assay results of the target bacterial strains, *Salmonella* in this study, by the selected primer sets. This step can confirm the specificity of the LFD assays. In addition, whether LFD assay is more sensitive than ISO 6579:2017 method needs further study.

Fifthly, we evaluated the sensitivity of the LFD assays performed by the rest primer sets. We selected the most

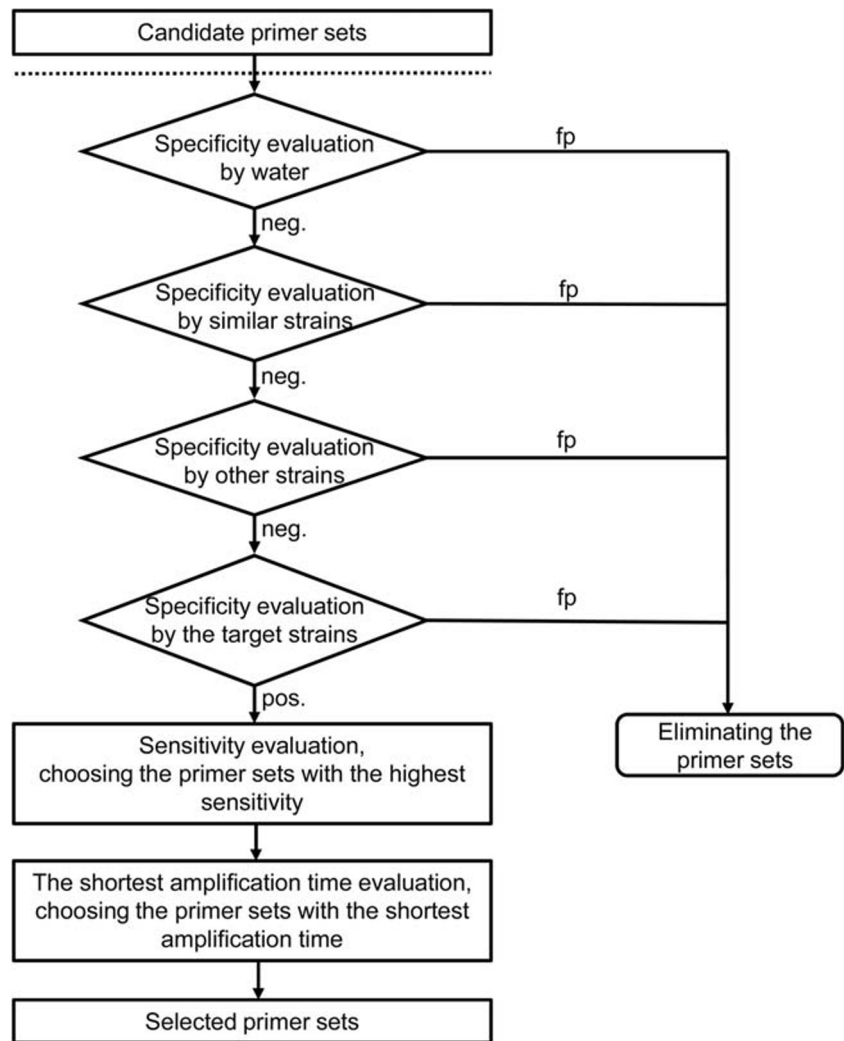
**Table 5** LFD assays in food samples

Number of samples	total	ISO positive	ISO negative	LFD targeting <i>phoP</i> by PP6				LFD targeting <i>invA</i> by IP6			
				tp	fp	tn	fn	tp	fp	tn	fn
Minced pork	85	3	82	3	2	80	0	3	1	81	0
Raw milk	50	1	49	1	0	49	0	1	0	49	0
Chicken breast	90	11	79	11	0	79	0	11	0	79	0
total	225	15	210	15	2	208	0	15	1	209	0

tp true positive, fp false positive, tn true negative, fn false negative



**Fig. 4** Optimal protocol for LFD evaluation approach. fp, false positive result; neg., negative result; pos., positive result



sensitive primer sets as the candidates. After identifying the sensitivity, we found that the sensitivity of LFD performed by these selected primer sets is better than the corresponding LAMP assay. It suggested that the LFD assay suits for detection of the low pathogen concentration sample, such as food.

Finally, we shortened the amplification time and got the shortest amplification time of each primer set. Each shortest amplification time of the LFD assays is shorter than 1 h, and the shortest amplification time value is only 15 min. The result suggested that the specific dual-labeled dsDNAs had been synthesized in the early amplification.

Theoretically, we can improve a LAMP assay to a more sensitive, time-saving, and easy-to-perform LFD assay with this procedure. The optimized LFD can improve the sensitivity limit and shorten the amplification time. However, it has only verified in several studies (Yu et al. 2019; Xing et al. 2020). Further study would be necessary to evaluate and optimize this procedure.

## Conclusion

This study reported a process of improving a LAMP assay to an optimized LFD assay and applied it to two improving LFD assays for *Salmonella* in food samples. We also established the evaluation protocol to improve the LFD assay. It is a rapid, easy to conduct, and inexpensive analytical approach for foodborne pathogen detection, and expected to apply in other foodborne pathogen detection. Therefore, the optimized LFD assay is poised to play a significant role in food safety monitoring and surveillance due to its capacity for field test and simple laboratory test, especially in developing countries.

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**Authors' Contributions** All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Jia Yu, Jiahua Xing, Xijing Zhan, and Zhen Yang. The first draft of the manuscript was written by Jia Yu, Yin Liu, and all authors

commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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## Compliance with Ethical Standards

**Conflict of Interest** Jia Yu declares that she has no conflict of interest. Jiahua Xing declares that he has no conflict of interest. Xijing Zhan declares that she has no conflict of interest. Zhen Yang declares that he has no conflict of interest. Jun Qi declares that he has no conflict of interest. Yuxi Wei declares that he has no conflict of interest. Yin Liu declares that he has no conflict of interest.

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

**Informed Consent** Not applicable.

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