16S rRNA-functionalized multi-HCR concatemers in a signal amplification nanostructure for visual detection of *Salmonella*

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Abstract

To prevent foodborne diseases and minimize their impacts, it is extremely important to develop a cost-effective and efficient bacterial detection assay for diagnostics, particularly in resource-poor settings. In this study, 16S rRNA from foodborne *Salmonella* was coupled with multiple HCR (hybridization chain reaction) concatemers and functionalized in a signal structure for lateral flow nucleic acid biosensor (LFNAB) detection. The 16S rRNA was incubated with two specific capture probes and multiple helper probes carrying the same initiator, to unwind its secondary structure and form an “initiators-on-a-string” complex. Through use of the initiators, each target 16S rRNA yielded multiple HCR concatemers tethered to numerous biotins, and numerous streptavidin-labeled gold nanoparticles were introduced on the LFNAB. The limit of detection was 53.65 CFU/mL for *Salmonella*. Notably, this method has high specificity and applicability for the detection of *Salmonella* in food and water samples.

Key words: hybridization chain reaction; lateral flow nucleic acid biosensor; visual detection

Introduction

Foodborne diseases caused by pathogenic bacteria have become a serious threaten to human health and to the global economy [1]. The World Health Organization [2] has estimated that the consumption of food and water contaminated by pathogenic microorganisms causes 1.8 million deaths per year worldwide [3]. According to surveillance data on foodborne disease from the U.S. Centers for Disease Control and Prevention (Atlanta), *Salmonella* is the most commonly reported infection, and its incidence is not weaken for over a decade [4, 5]. Worldwide, non-typhoidal *Salmonella*e have been estimated to arouse 94 million cases of enterogastritis and 155,000 deaths per year [6], most of which result from the consumption of contaminated food and water. *Salmonella* infection is a common bacterial disease throughout the developed and developing world, causing abundant morbidity and mortality [7]. In order to satisfy the requirements of mass screening and cost efficiency, sensitive, low cost, and convenient method for *Salmonella* point-of-care (POC) detection is of utmost importance for food safety and clinical diagnosis.

Traditional methods for the detection of pathogenic bacteria rely on either the gold standard of culture growth or on real-time quantitative PCR (qPCR) [8]. These methods are not suitable for rapid screening and POC testing for foodborne bacteria, because they require highly professional personnel and labor-intensive manipulation, thus hindering their

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wide application. To overcome the drawbacks of conventional methods, detection of nucleic acids by hybridization is a promising alternative [9]. The method through Watson-Crick base pairing, uses a short oligonucleotide probe to bind a specific DNA or RNA analyte and detects the probe-analyte complex [10]. Direct detection of nucleic acids of pathogenic bacteria is of great importance in POC diagnostic formats. Nucleic acid hybridization-based techniques would enable straightforward and simple tests that do not require expensive instruments and skilled staff. Analysis of 16S rRNA sequences is used in the classification of bacterial species as well as in molecular diagnostics of infectious diseases [11-13]. The 16S rRNA is often selected as a target molecule in the identification of bacteria, and it shows great promise for application in POC diagnostics [14]. Immunoassay or PCR amplification detection methods are unable to distinguish between live and dead bacteria. Each living bacteria cell contains up to 18,000 copies of rRNA, providing an inherent amplification effect for the direct detection of 16S rRNA, which could distinguish between living and dead bacteria [15]. Furthermore, multiple copies of 16S rRNA exist in a single bacterial cell, making it an attractive target for the detection of low abundance bacteria without PCR amplification.

However, 16S rRNA is often inaccessible for hybridization with complementary probes, because regions of interest are often found in stable RNA secondary structures. This drawback creates a major limitation in the analysis of 16S rRNA of pathogenic bacteria [16]. In order to expedite direct detection of bacteria, dissociation of the secondary structure of 16S rRNA is vital for efficient hybridization [17]. Hybridization of multiple functional strands

to 16S rRNA unwinds its secondary structure to form complexes suitable for direct detection, and the entire length of the 16S rRNA molecule is used for signal production [18]. As an efficient amplification platform, the hybridization chain reaction (HCR) [19], developed by Dirks and Pierce [20], has attracted substantial attention in the fields of detection of various analytes, such as nucleic acids, proteins [21], metal ions [22], and cells [23]. HCR, as a probe-amplification technique, can effectively reduce false-positive results and cross contamination from amplicons, which often occur in target amplification PCR.

Inspired by these previous studies, to design a highly sensitive biosensor for bacteria detection, we combined a multiple HCR concatemers (mHCR) approach with a probe set for assisted unfolding of a long nucleic acid 16S rRNA. In our assay, 16S rRNA was incubated with a probe set containing two specific capture probes and multiple helper probes carrying the same initiator to unwind its secondary structure and then form an "initiators-on-a-string" complex. This process yielded multiple tethered amplification polymers per target 16S rRNA, thus functionalizing the signal structure, and then a mass of gold nanoparticles (AuNPs) modified streptavidin (SA) were introduced on LFNAB for visual detection.

Materials and Methods

Reagents

Salmonella. enteritidis (*S. enteritidis*, CICC 24119), *Escherichia coli* O157:H7 (*E. coli* O157:H7, CICC 21530), *Proteus mirabilis* (*P. mirabilis*, CICC 22928), *Listeria monocytogenes*

(*L. monocytogenes*, CICC 21529), *Staphylococcus aureus* (*S. aureus*, CICC 21600), and *Citrobacter freundii* (*C. freundii*, CICC 10404) were obtained from the China Type Culture Collection. The 16S rRNA sequences were searched from GenBank (https://www.ncbi.nlm.nih.gov/) and aligned with Clustal W, as shown in the supporting information. The target-specific sequence site for *S. enteritidis* (positions 422–439 and 440–457) and the corresponding probe sequences are listed in Table 2.

Oligonucleotide probes were synthesized by Takara Biotechnology Co. Ltd. (Dalian, China). All oligonucleotide sequences are listed in Table 1. DNA molecular weight markers were from Takara Biotechnology Co. (Dalian, China). Anti-Fam antibodies and bovine serum albumin (BSA)-biotin conjugate were from Ruiqi Biotech Co., Ltd. (Shanghai, China). SA was obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Colloidal AuNPs were obtained from Hualan Chemical Co., Ltd. (Shanghai, China). All chemicals were from Sigma-Aldrich, Inc. (St. Louis, MO, USA) and were used without further purification

Polyacrylamide gel electrophoresis (PAGE)

Prior to use, H1-biotin and H2-biotin, diluted with sodium phosphate sodium chloride buffer (150 mM Na_2HPO_4 and 0.75 M NaCl, pH 7.4), were heated at 95°C for 5 min and incubated at room temperature for 1 h. Then, 10 μL of various concentrations of helper probes were incubated with the same volume of H1-biotin/H2-biotin in reaction buffer for 1 h. During this process, the HCR was triggered and progressed to generate long DNA

products with numerous biotins. The sample solutions were subjected to electrophoretic analysis on 9% nondenaturing polyacrylamide gels. Before loading of samples, 10 μL of oligonucleotides was mixed with 2 μL of 6× loading buffer. Gel electrophoresis was run at 25°C for 1 h under a voltage of 140 V/cm at pH 6.0. The gel was stained with 0.5 μg of ethidium bromide per mL for 30 min and photographed with a digital camera under UV illumination.

Preparation of the lateral flow nucleic acid biosensor

The LFNAB was prepared through a previously described procedure with some modifications [21]. The LFNAB consisted of four parts: an absorbent pad, a nitrocellulose (NC) membrane, a sample pad, and a conjugate pad. SA (18 μg/mL) was added to 20 nm AuNPs (0.01%, m/v) to form AuNPs-SA conjugates, which were then impregnated in the absorbent pad of the LFNAB. The BSA-biotin conjugate (1.2 mg/mL) was incubated on the surface of the NC membrane to form the control line, and anti-Fam antibody (4 mg/mL) was incubated on the surface of the NC membrane to form the test line. The LFNAB was cut into pieces 5 mm wide and 50 mm long. The sealed LFNABs were kept at room temperature before use.

Bacterial culture and total RNA extraction

Exponential growth phase culturing and other strains were prepared by incubating them overnight at 37°C and 180 rpm in LB medium (10 g/L NaCl, 5 g/L yeast extract and 10 g/L tryptone, pH 7.0). *S. enteritidis* counts were determined by a series of decimal dilution

on LB agar plates and incubating overnight at 37°C in thermostatic incubator. Colony forming units (CFU) was determined at optical density 600 nm (OD_{600}). Total RNA was extracted with a Bacteria Total RNA Purification Kit from bacterial samples, the kit was from Sangon Biotech Co., Ltd. (Shanghai, China). The RNA quality and quantity were evaluated by UV-Vis spectrophotometer (Thermo Fisher NanoDrop). The extracted RNA was kept at -80°C in RNase/DNase-free water till use.

Procedure for 16S rRNA-based *S. enteritidis* detection

For the assay, the helper probes (0.25 μM) and capture probes (CPs, 0.1 μM) were annealed with isolated 16S rRNA (0.16–1.3 ng/mL) by incubation in sodium phosphate sodium chloride buffer at 95°C for 5 min. The mixtures were incubated at room temperature, and mixed with H1-biotin/H2-biotin probes by incubation at room temperature for 30 min to allow HCR to generate long nicked DNA polymers. The 16S rRNA–mHCRs complexes formed between 16S rRNA and the helper probes/capture probes/mHCRs were analyzed by LFNAB. Then ideal amount of buffer (25% saline sodium citrate buffer including 4% BSA) with 10 μL of 16S rRNA-mHCRs/CPs signal structure was added to the sample pad. The mixture solution flowed upward by capillary force. The LFNAB results were read after 10 min. The positive test result of *S. enteritidis* was indicated the presence of red lines at control zone and test zone. The presence of red line only at the control zone was considered a negative detection of *Salmonella*. For the quantitative analysis, the signal intensity of peak areas of test line/control line(T/C) of LFNAB was measured using the software of Image J [19].

Detection of the target bacteria in spiked milk

Pasteurized milk was purchased from a local supermarket and used as a real sample model. Prior to use, the milk was diluted 10 times with sterile PBS. *S. enteritidis* cells with concentrations ranging from 50 to 10^7 CFU/mL were mixed with the diluted milk to prepare the *Salmonella*-spiked milk samples. The *S. enteritidis* cells in the spiked milk samples were first separated, and then total RNA was obtained from *S. enteritidis* with Bacterial Total RNA Purification Kit. Finally, the 16S rRNA was collected and detected, and the amount of *S. enteritidis* cells in the spiked milk was calculated by using a calibration curve for the biosensor.

Results and discussion

Operating principle of the assay

The sandwich system of two capture probes/target 16S rRNA/multiple HCR concatemers was fabricated as the sensing platform. The scheme of this assay is described in Fig.1. The 16S rRNA was incubated with a series of probes, containing two Fam-modified capture probes and multiple helper probes carrying the same initiator, to unwind its secondary structure and then form an “initiators-on-a-string” complex in the signal structure. Addition of the helper probe triggered a chain reaction between H1-biotin and H2-biotin that resulted in assembly of a long-nicked double-stranded amplification polymer with numerous biotins [19]. Target 16S rRNA was functionalized in a signal structure through tethering of mHCR concatemers with numerous biotins. The 16S rRNA in the signal

structure carried numerous AuNPs-SA on the LFNAB for visual detection. The presence of target 16S rRNA was determined according to the test line's color in the LFNAB, and semi-quantitative detection of the target was based on the color depth of the test line.

Optimal assay conditions

Optimization of the ratio of helper probes (initiators)/H1-biotin and H2-biotin for HCR

Under this process, it was imperative that the initiator domain on each helper probe-activated a cascade of hybridization between two designed hairpin probes to generate double chain DNA polymers. In view of these requirements, we used gel electrophoresis to demonstrate the results of introducing the ratio of each helper probe and H1-biotin /H2-biotin. As shown in Fig. 2, low-molecular-weight bands in lane 8 (H1-biotin/H2-biotin) indicated that HCR self-assembly of H1-biotin and H2-biotin could not be triggered in the absence of helper probes. However, consecutive bands with high-molecular weight could be observed once a series of concentrations of helper probe was separately added, indicating the occurrence of HCR (in lanes 1–7). A series of ratios of helper probe mixture (initiators)/H1-biotin/H2-biotin produced diverse long double-stranded HCR products. As shown in Fig.2, the best ratio of helper probe mixture (initiators) and H1-biotin /H2-biotin was 1:8.

Optimization of capture probe concentration

In this experiment, two specific capture probes were used to improve the sensitivity of the detection. The capture probe is one of the most important factors for sensitivity in this

experiment. The result showed in Fig.3, as the amount of two capture probes mixture (capture probe 1 and capture probe 2 in the same proportion) increased, the signal reached a maximum at 0.1 μM of capture probes mixture, and then dropped down. Deficient capture probes affected the binding efficiency of 16S rRNA/mHCRs on LFNAB test line. Competitive inhibition from excess free capture probes reduced the amount of 16S rRNA/mHCRs on the test line. In consequence, 0.1 μM concentration of capture probes was used in subsequent experiments.

Optimization of helper probes

As shown in Fig.4, the signal intensity increased with the number of helper probes. Each target 16S rRNA using a probe set containing five helper probes, each carrying the same initiator, yielded five tethered amplification polymers. In the detection stage, all helper probes and DNA hairpins were introduced in parallels, and unused helper probes and hairpins did not reduce the complex on the test line. More helper probes may be used to increase the signal gain, decrease cost, and increase the signal to background ratio by decreasing nonspecific probe binding.

Specificity

To determinate the specificity of this assay, the specific part of 16S rRNAs of several bacteria in Table 2 were purified. The amount of each bacterium was approximately 10^9 cells. The band for *S. enteritidis* was clear, and there was no signal for *P. mirabilis*, *S. aureus*, *C. Freundii*, *L. monocytogenes*, or *E. coli* O157:H7 with this assay in Fig.5. This result

indicated that the two capture probes used in this study were specific to *S. enteritidis*. Two specific capture probes were used to improve the specificity of this assay.

Sensitivity

Under the optimized conditions, different concentrations of the *S. enteritidis* cells from 50 CFU/mL to 1.0×10^7 CFU/mL were determined through this method. As shown in Fig. 6, the calibration plots showed a good linear relationship between the red color intensity and the concentration of *S. enteritidis* in the range from 10^2 to 10^4 CFU/mL. The regression equation was y = 0.1602 LgX - 0.2288, R^2 = 0.9760, in which y was the peak area ratio of T/C, and X was *S. enteritidis* concentration (CFU/mL). The limit of detection of this biosensor was determined to be 53.65 CFU/mL(at three times the signal-to-noise ratio), which was lower than those of several recently reported biosensors for *S. enteritidis* detection [5, 18]. The high sensitivity of this proposed biosensor may be attributed to two aspects: (1) Direct 16S rRNA detection provided an inherent amplification effect, because there were as many as 18,000 copies of 16S rRNA per cell in growing bacteria. (2) Each target 16S rRNA yielded mHCRs concatemers with biotins, tethered to numerous SA-labeled AuNPs on the LFNAB. We have fabricated sandwich system of capture probe/target 16S rRNA/HCR complexes as the sensing platform for detecting *S. enteritidis*, and the detection limit of *S. enteritidis* was 3×10^3 CFU/ mL. Each target 16S rRNA only yielded one capture probe and one HCR concatemer with biotins, tethered to numerous SA-labeled AuNPs on the LFNAB. At the same time, the secondary structure target 16S rRNA was not easy to unwind to bind probe at room temperature. All these factors affected the sensitivity of the detection method.

Compared with our published paper [18] , the sensitivity of this method was improved about 60 times, mainly because multiple auxiliary probes in this method could specifically capture more signal probes, thus effectively improving the sensitivity of this method.

Detection of *S. enteritidis* in spiked milk

To further evaluate the applicability of our method to a real food matrix, its performance was tested with whole milk contaminated with *S. enteritidis*. Milk samples from a local supermarket were spiked with *S. enteritidis* at concentrations ranging from 10^2 to 10^4 CFU/mL. A milk sample without spiked *S. enteritidis* was used as the control. The concentration of *S. enteritidis* in the milk was determined on the basis of the obtained standard curve. The recoveries of *S. enteritidis* ranged from 90.36%–99.94% (Table 3). These results successfully demonstrated the feasibility and reliability of our method, which could be applicable for food sample detection.

Conclusions

In this study, a novel assay combining signal amplification based on mHCRs concatemers and LFNAB-based visual signal detection was successfully developed for sensitive and rapid detection of *S. enteritidis* 16S rRNA. The 16S rRNA from *S. enteritidis* was coupled with mHCRs concatemers and functionalized in a signal structure for LFNAB detection. This assay had a wide linear detection range from 10^2 to 10^4 CFU/mL and was able to detect *S. enteritidis* levels as low as 53.65 CFU/mL. This technique provided a novel

assay for diagnostics for foodborne pathogenic bacteria. In addition, it will be provided a new possibility in biological analysis field.

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Table 1 Probes designed from alignments of 16S rRNA sequences from *S. enteritidis*

Name	Sequences (5'-3')
H1-biotin	biotin-TTTTTTTTAACCCACGCCGAATCCTAGACTCAAAGTAGTCTAGGATTCGGCGTG
H2-biotin	AGTCTAGGATTCGGCGTGGGTTAACACGCCGAATCCTAGACTACTTTGTTTTTT-biotin
Capture probe 1	FAM-GTCAATTGCTGCGGTTAT
Capture probe 2	TAACCACAACACCTTCCT-FAM
Helper probe 1	CTGAAAGTACTTTACAACCCGAAGGCCTTTTTCATAGTCTAGGATTCGGCGTGGGTTAA
Helper probe 2	CTGCTGGCACGGAGTTAGCCGGTGCTTCTTTTGCGAGTCTAGGATTCGGCGTGGGTTAA
Helper probe 3	AATTCCGATTAACGCTTGCACCCTCCGTATTACCGAGTCTAGGATTCGGCGTGGGTTAA
Helper probe 4	CGCGGCATGGGTGCATCAGGCTTGCGCCCACTGTGAGTCTAGGATTCGGCGTGGGTTAA
Helper probe 5	TGACTTGACAGACCGCCTGCGTGCGCTTTACGCCCAGTCTAGGATTCGGCGTGGGTTAA

Table 2 Specific probes from alignments of 16S rRNA sequences from *S. enteritidis* and other foodborne bacteria

Name	Specific probe 1	Specific probe 2
S. enteritis	AGGAAGGTGTTGTGGTTA	ATAACCGCAGCAATTGAC
E. coli O157：H7	AGGAAGGGAGTAAAGTTA	ATACCTTTGCTCATTGAC
P. mirabilis	AGGAAGGTGAGAAGGTTA	ATACCCTTGTCAATTGAC
S. aureus	AAGAACATATGTGTAAGT	AACTGTGCACATCTTGAC
L. monocytogenes	AAGAACAAGGATAAGAGT	AACTGCTTGTCCCTTGAC
C. freundii	AGGAAGGCGTTGTGGTTA	ATAACCGCAGGATTGAC

Table 3 Recoveries of *S. enteritidis*-spiked samples

Original value (CFU/ mL)	*S. enteritidis* added (CFU/mL)	*S. enteritidis* found (mean ± RSD, CFU/ mL)	Recovery (100%)
0	1×10^2	$(0.9036 \pm 0.028) \times 10^2$	90.36 ± 2.8
0	2×10^2	$(0.9993 \pm 0.013) \times 10^2$	99.93 ± 1.3
0	5×10^2	$(0.9724 \pm 0.022) \times 10^2$	97.24 ± 2.2
0	1×10^3	$(0.9726 \pm 0.013) \times 10^3$	97.26 ± 1.3
0	1×10^4	$(0.9994 \pm 0.041) \times 10^4$	99.94 ± 4.1

Fig.1. Schematic of the LFNAB assay for 16S rRNA-functionalized multi-HCR concatemers.

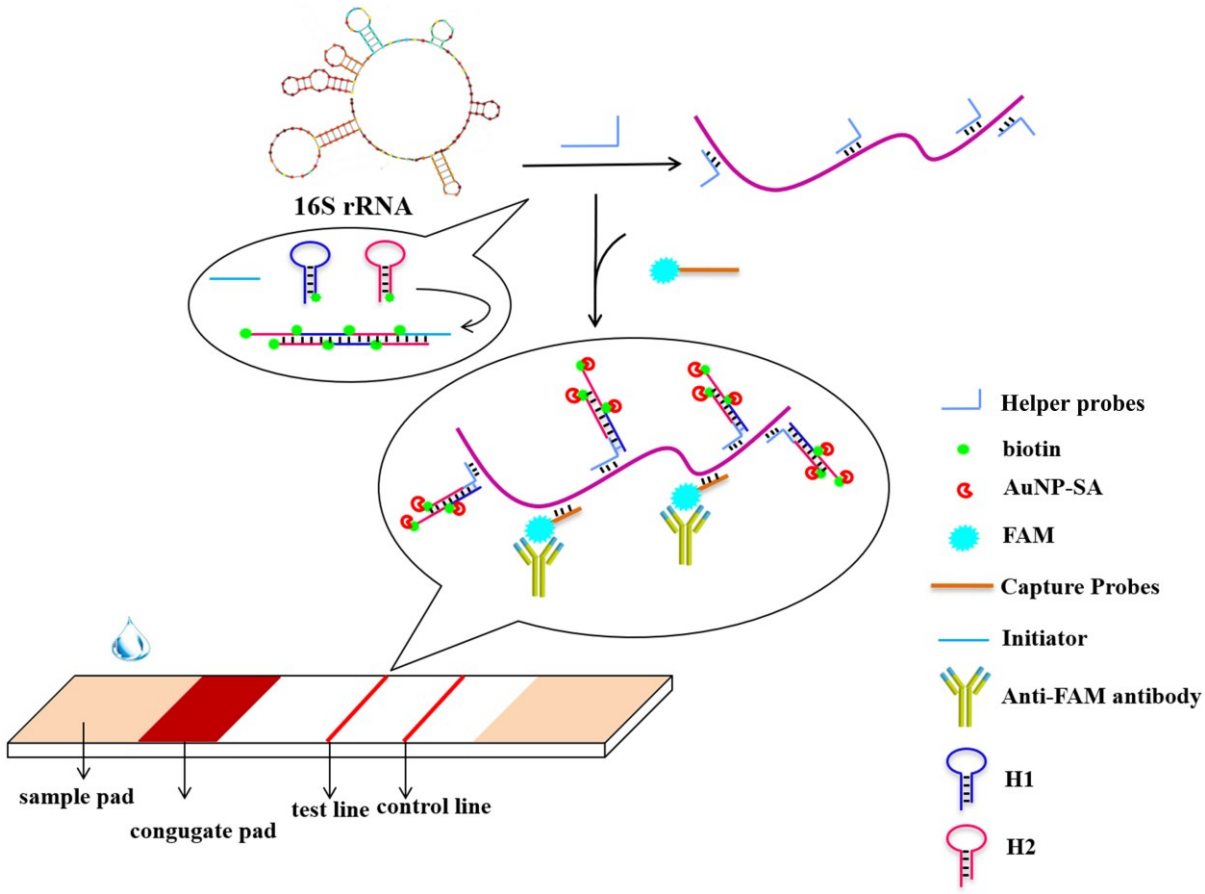

Fig.2. Analysis of HCR reaction conditions by polyacrylamide gel electrophoresis. The lanes show ratios of

helper probes and H1-biotin/H2-biotin at 1:1 (line 1), 1:2 (line 2), 1:4 (line 3), 1:5 (line 4), 1:6 (line 5), 1:8

(line 6), 1:10 (line 7) applied successively, and the concentration of H1-biotin/H2-biotin was 2 ìM. Line 8

shows the mixture of H1-biotin/H2-biotin without helper probes.

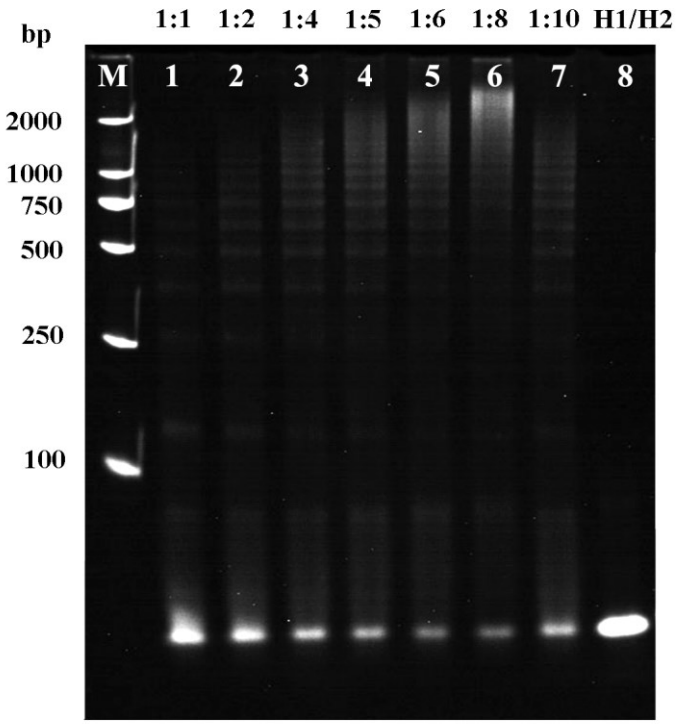

Fig.3. Analysis of concentrations of CPs by using LFNAB. The tested concentrations of CPs were 0.05 μM, 0.1

μM, 0.3 μM, 0.5 μM, 0.7 μM, 0.9 μM, 1.2 μM, and 1.5 μM. The concentration of five helper probes was 0.25

μM. The error bars are standard deviations from three repeated measurements.

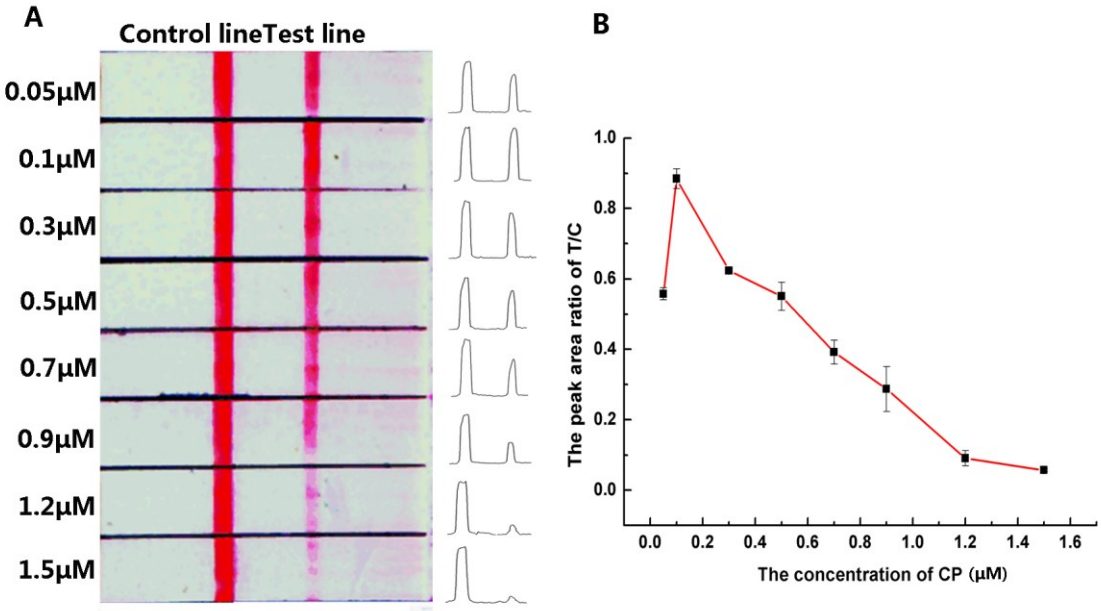

Fig.4. Influence of different numbers of helper probes on Salmonella detection. A. LFNAB detection with 1–5
helper probes. The error bars are standard deviations from three repeated measurements.

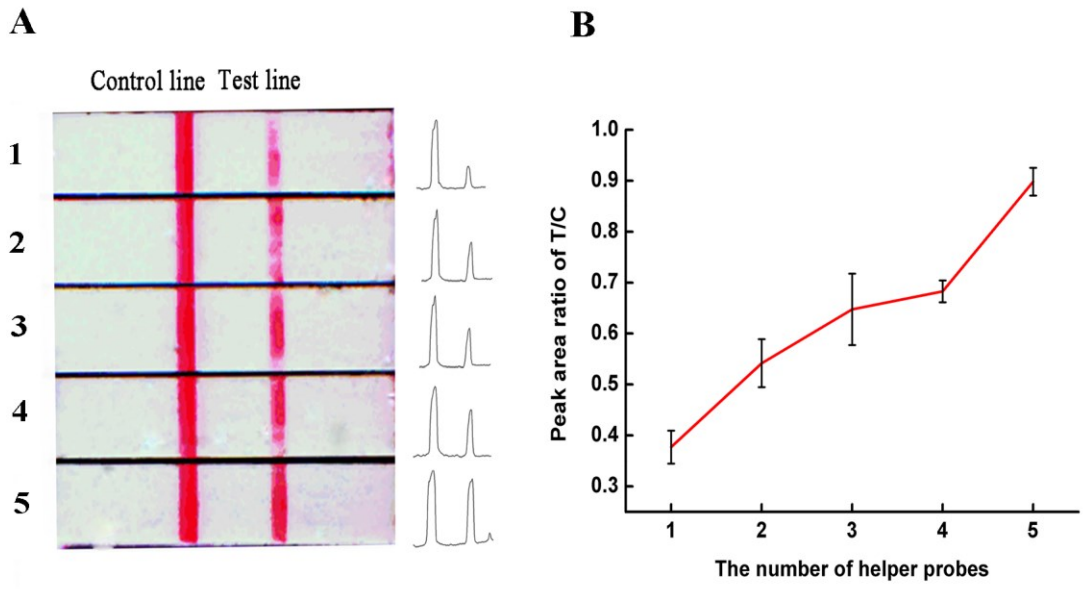

Fig.5. Specific detection of different bacteria by LFNAB. A. These bacteria are enteritis, Escherichia coli O157:H7, Proteus mirabilis, Staphylococcus aureus, Listeria monocytogenes, and Citrobacter freundii.

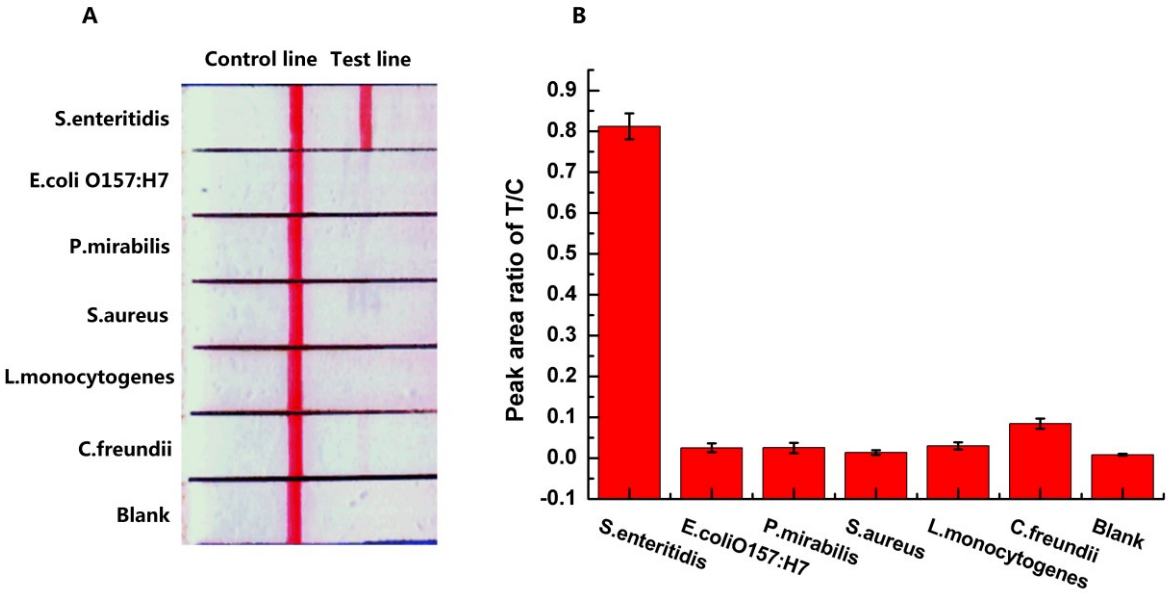

Fig.6. Sensitive detection of Salmonella in standard solution. A. Salmonella detection with LFNAB. B. Calibration plot of the peak area ratio of T/C versus Lg (Salmonella). The error bars are standard deviations from three repeated measurements.