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Label-free visual biosensor based on cascade amplification for the detection of *Salmonella*

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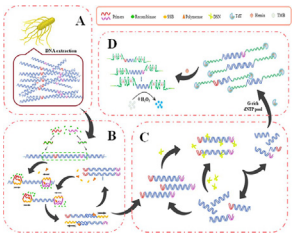
HIGHLIGHTS

- Label-free visual biosensor developed based on triple cascade amplification.
- TdT extension achieves a third signal increase and forms a G-quadruplex for the visualized signal output.
- RPA product used as a template for DSN to generate more 3′-OH to achieve the second signal amplification.
- TdT extension using the 3′-OH product achieves a third signal increase and forms a G-quadruplex for the visualized signal output.
- There is an ultra-low detection limit of 6 cfu/mL.

GRAPHICAL ABSTRACT



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ABSTRACT

Salmonella is a widely distributed, extremely harmful bacteria, the presence of which requires confirmation via an on-site visual biosensor. In this study, we constructed a label-free, cascade amplification visualization biosensor for the sensitive and rapid detection of *Salmonella enterica* subsp. *enterica serovar typhimurium* based on the RDTG principle (recombinase polymerase amplification (RPA), duplex-specific enzyme (DSN) cleavage, terminal deoxynucleotidyl transferase (TdT) extension and G-quadruplexes output). Following DNA extraction of *Salmonella* spp., the first step in the construction involved the recognition and amplification of nucleic acids, carried out by RPA, to achieve the first signal amplification within 10 min. This RPA product was then specifically cleaved by DSN to produce a large number of small double-stranded DNA (dsDNA) products with 3′-OH within 15 min to achieve the second signal amplification. Thereafter, TdT was employed to empower these small 3′-OH dsDNA products to extend and produce a large number of long G-rich single-stranded DNAs (ssDNAs) within 20 min, thus realizing the third signal increase. These long G-rich ssDNA products displayed a color change that could be directly observed through the naked eye by adding H_2O_2/3,3′,5,5′-tetramethylbenzidine (TMB). The RDTG biosensor for the detection of *Salmonella* spp. has several advantages, including a low limit of 6 cfu/mL. It is an isothermal-free instrument, simple to operate, with a rapid detection time of less than 1.5 h.

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Furthermore, it can be visually characterized and quantified by a microplate reader to detect *Salmonella* spp., in food and environmental samples, and it has broad application prospects.

1. Introduction

Salmonella, one of the most common and widely distributed foodborne pathogenic microorganisms, is a major cause of foodborne salmonellosis [1]. The World Health Organization (WHO) reports that *Salmonella* is one of the four key global reasons of diarrhoeal diseases, causing 550 million people, including 220 million children, falling ill [2]. To ensure food safety for human health, many methods for detecting *Salmonella* have been developed. However, the standard culture-based methods for *Salmonella* detection requires a series of complicated steps, such as pre-incubation, and cannot meet the optimum detection speed required before rapid treatment can be initiated in the disease prevention process [3]. As a result, numerous methods have been developed and proposed to replace traditional detection, such as the enzyme-linked immunosorbent assay (ELISA) [4], PCR technology [5], isothermal amplification technology [6,7], and biosensor technology [8,9]. Among these alternatives, biosensor technology is most favorably considered to be more cost-effective, faster and reliable than other *in situ* detection methods [10].

Studies have also shown that, with biosensor technology, the pre-amplification step can be used to obtain more products than with other methods. In order to further increase the sensitivity and specificity of the biosensor, a pre-amplification step is usually performed using classical polymerase chain reaction (PCR) or isothermal amplification, and the biosensor is then used to detect the amplification product [11]. While PCR is, indeed, the most commonly used amplification technique, it requires specialized equipment and, due to the high complexity of the reaction setup and the precise temperature control required for amplification, it is limited in field detection. Isothermal nucleic acid amplification technology can overcome the above shortcomings and can be used to replace PCR technology. Isothermal nucleic acid amplification technology includes loop-mediated amplification (LAMP) [12], helicase-dependent amplification (HDA) [13] strand replacement amplification (SDA) [14], rolling circle amplification (RCA) [15] and recombinase polymerase amplification (RPA) [16]. Among these technologies, RPA offers the advantages of a relatively simple primer design, short reaction time and low reaction temperature compared with other constant temperature amplification techniques. It is a preferred technique for simple and rapid nucleic acid-based diagnosis. The method is based on two specific oligonucleotide primers and a mixture of recombinase, polymerase and single-stranded DNA binding protein (SSB), which can bind to the template and generate double stranded products. Amplification of less than 10 copies of genomic DNA can be done in less than 30 min [17,18]. Based on the more rapid and more sensitive characteristic of RPA than those of PCR [19,20], many RPA-based biosensor has been developed to detect pathogens [21,22], virus [23], plant genome [24] and single-nucleotide polymorphisms (SNPs) [25] with end-point detection and real time detection. Among these biosensors, several lateral flow platforms have been established [10,26–28] for the detection of *Salmonella* based on gold nanoparticles (AuNPs). However, these immunochromatographic methods only provide qualitative analysis and are not able to afford accurate quantitative results. On the other hand, magnetism-based concentration [29] and microfluid-based enrichment [30] have been explored to improve the sensors' detection performance in complicated matrix while the detection limit is still high with an order of magnitudes of 10^2-10^3 cfu/mL. Therefore, it is necessary to build a more accurate quantitative RPA-based method to detect *Salmonella*.

As an important biocatalyst with a distinctive structure and functions, enzymes have been used to detect various foodborne pathogens in numerous biosensors. In this assay, we constructed a novel enzyme-mediated visualization sensor for the rapid and sensitive detection of *Salmonella* spp.. Duplex-specific nuclease (DSN) is a kind of protein enzyme that can effectively identify and cleave double-stranded DNA (dsDNA) and strands of hybridized DNA/RNA complex, but which have no effect on single-stranded DNA (ssDNA) or single/double-stranded RNA [31,32]. Based on the different cutting characteristics of DSN for single-double-stranded nucleic acids, researchers have installed different signal output and amplification methods and established a series of biosensing technologies, such as optical biosensors [33,34], electrochemical biosensors [35,36] and magneto-optical biosensors [37].

Terminal deoxynucleotidyl transferase (TdT) is a template-free polymerase catalyzing the addition of deoxynucleotides to DNA at 3′-hydroxyl (3′-OH). The addition of deoxynucleotides to the nucleic acid strand is possible as long as the acting nucleic acid is longer than three bases [38,39]. The DNA sequences produced by TdT are non-specific and random, with their composition highly dependent upon that of the substrate deoxyribonucleotide triphosphate (dNTP) pool. The unique ability of TdT to originate genomic materials makes it one of nature's most fascinating DNA polymerases. The signal output products formed by the extension are diverse and various modes can be realized, thus satisfying diverse biosensor requirements, such as fluorescent biosensor [40,41], colorimetric biosensor [42,43] and surface ion resonance biosensor [44], in addition, TdT also mediates many biosensors that use electrochemical signals for signal output [10,45].

Based on the abovementioned properties, we designed and constructed a label-free and cascading-amplified visualization biosensor based on RDTG for the sensitive and fast detection of *Salmonella* spp., as shown in Scheme 1. The first step in the construction process, following DNA extraction of *Salmonella* spp., involved the recognition and amplification of nucleic acids by RPA to achieve the first signal amplification. Since DSN can specifically cleave dsDNA, the products of RPA as a cleavage template for DSN produced a large number of dsDNA products with 3′-OH, which could be further extended as tails by TdT as a template, thereby achieving the second signal amplification. In a G-rich dNTP pool, randomly arranged G-rich tails are elongated at the 3′-OH end of DSN/RPA products via TdT polymerization. Then, the combination of hemin induces such G-rich sequences to form G-quadruplex DNAzymes with a mimic horseradish peroxidase activity. Next, the mimetic enzyme can catalyze chromogenic substrate (H_2O_2/TMB) to produce a visible blue color with a characteristic optical absorbance at 450 nm. In this enzymatic reaction, G-quadruplex DNAzymes catalyze H_2O_2 to produce single-state oxygen to oxide TMB for signal output.

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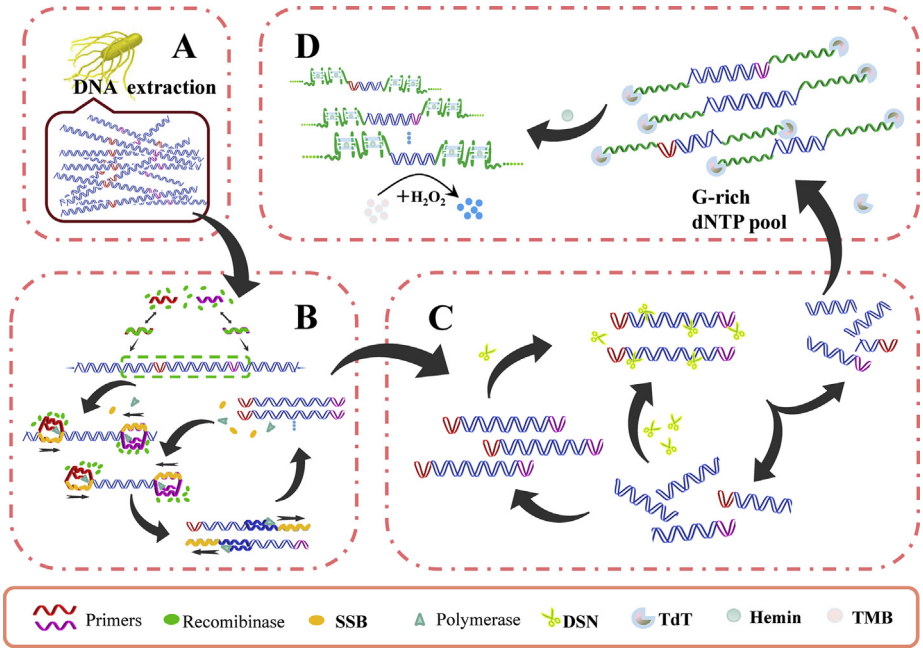

Scheme 1. Schematic diagram of a visualized biosensor based on RPA amplification, duplex-specific nuclease (DSN) cleavage and terminal deoxynucleotidyl transferase (TdT) extension for detection of *Salmonella* spp.. (A) DNA extraction of *Salmonella* spp.. (B) RPA amplification for the first signal amplification. (C) RPA products as a cleavage template are digested by DSN for the second signal amplification. (D) TdT-catalyzed polymerization for the third signal amplification and output.

2. Materials and methods

2.1. Reagents and apparatus

All synthetic DNA primers were synthesized at Invitrogen (Beijing, China). *Salmonella* spp., *E. sakazakii, L. monocytogenes, S. aureus, V. Parahemolyticus* and *E. coli* were supplied by the Laboratory of Food Safety at the College of Food Science and Nutritional Engineering at China Agricultural University. A TwistDx TwistAmp Basic kit was purchased from TwistDx Ltd. (TwistAmp Basic, TwistDx, Cambridge, UK). TdT and DSN were bought from New England Biolabs Ltd., (Beijing) and Evrogen Joint Stock Company (Russia), respectively. TMB was purchased from Beijing Biyuntian Co., Ltd., (Beijing, China).

2.2. DNA extraction from samples

The deposited *Salmonella* spp. was added to the LB medium for cultivation for 16 h. The concentration of the strain was determined by the dilution plate method. Take 1 mL of the bacterial solution in a 1.5 mL EP tube, centrifuge at 12000 r/min for 5 min at 4 °C, and discard the supernatant. Then add 50 μL of ddH_2O in an electric thermostatic water bath at 100 °C for 10 min, then immediately set on ice for 20 min. Then, centrifuge at 12000 r/min, 4 °C for 5 min, and take the supernatant as real sample DNA. The concentration of the extracted real sample DNA was measured on NanoDrop, the initial concentration of *Salmonella* spp. DNA was 80 ng/μL.

2.3. RPA reactions

In order to obtain the optimal RPA amplification products, we first designed eight pairs of primers for screening the preferred primers, and selected a relatively good pair of primers for the next experiment. RPA amplification was performed using the TwistAmp Basic kit with a system of 50 μL per reaction. The system contained 29.5 μL of rehydration buffer, 2.4 μL of forward and reverse primers and 1 μL of DNA template. This mixture was added to a freeze-dried tube to completely dissolve the powder in the tube, with a further addition of 2.5 μL of 280 mM magnesium acetate (MgAc). It should be noted that the RPA reaction was started immediately after the addition of MgAc. Under normal conditions, the tube was immediately incubated at 37 °C for 30 min in a thermocycler and, after the reaction, the product was analyzed by 2% agarose gel electrophoresis.

2.4. Production of large quantities of 3′-OH through DSN

The 10 μL reaction system included: 1 μL of RPA reaction product, 1 μL of TdT reaction buffer (50 mM KAc, 20 mM Tris-Ac, 10 mM $Mg(Ac)_2$, pH 7.9) and 0.25 U/mL DSN. After incubating for 15 min at 42 °C in a thermocycler, the reaction was terminated by heat inactivation at 85 °C for 10 min.

2.5. Formulation of G-quadruplex by TdT

10 μL of the DSN digestion product, prepared above, was mixed with 1.5 μL of dGTP (10 mM), 1.0 μL of dATP (10 mM), TdT (20 U/μL) and $CoCl_2$ (0.25 mM) in 2 μL of TdT reaction buffer. This mixture was incubated at 37 °C for 20 min and finally, the reaction was terminated by heat inactivation at 85 °C for 10 min. The reaction product was analyzed by 12% polyacrylamide gel electrophoresis (PAGE).

3. Results and discussion

3.1. Primer design and screening

RPA primers are usually 30 to 35 nucleotides long, and the amplified product is less than 500 bp in length and preferably about 200 bp in length. For specific analysis of *Salmonella* spp., the *inv A* gene(Table S2) was selected for detection and identification. Since the group of *Inv* genes, composing *inv A*, *inv B*, *inv C*, *inv D* and *inv E*, influence the bacteria to enter host epithelial cells and is closely

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related to the pathogenicity of *Salmonella*. Of all, *inv A*, the major virulence factor of *Salmonella*, is of high homology in different strains of *Salmonella* and is highly distinctive compared with other pathogens [46]. Eight pairs of primers were designed in this study. All primer sequences can be found in Table S1 and the RPA experiments with different primer-pairs are shown in Fig. 1A. All of eight pairs of primers show obvious positive bands, especially primer 4 with the longest amplification, thus establishing that RPA-F4/R4 is well suited for the amplification of *Salmonella* spp. with RPA for the subsequent experiment.

3.2. Optimization of RPA reaction conditions

The reaction rate of *Salmonella* spp. detection is very important. In order to achieve detection quickly, the reaction time should be shortened as much as possible. Compared with the traditional PCR nucleic acid amplification method, the rate of the RPA reaction was found to be significantly improved. However, in order to achieve the goal of even more rapid detection, we shortened the reaction times of the RPA to 10 min, 20 min and 30 min, respectively. Therefore, we compared the effects of different concentrations of the forth primer set on the RPA amplification under different reaction time conditions. The results of the reactions are shown in Fig. 1B, C and 1D, corresponding to the reaction times of 10 min, 20 min and 30 min, respectively. It is evident in Fig. 1B–D that, with the increase in the concentration of primer, the brightness of the strip also gradually increased, thus indicating that the amount of RPA product was gradually increasing. Some differences were noted in the product of RPA at different reaction times. After 10-min RPA (Figs. 1B), 249-bp amplification bands darkened with the increase of concentrations of the forth primer set with negligible non-specific bands at 750 bp. After 20-min RPA (Fig. 1C), target bands darkened with the increase of primers but the products began to diffuse. After 30-min RPA (Fig. 1D), target amplicons turned to weak while the non-specific bands started to be dark. Therefore, 5 μM of the forth primer set and 10-min RPA time were selected for the subsequent analysis.

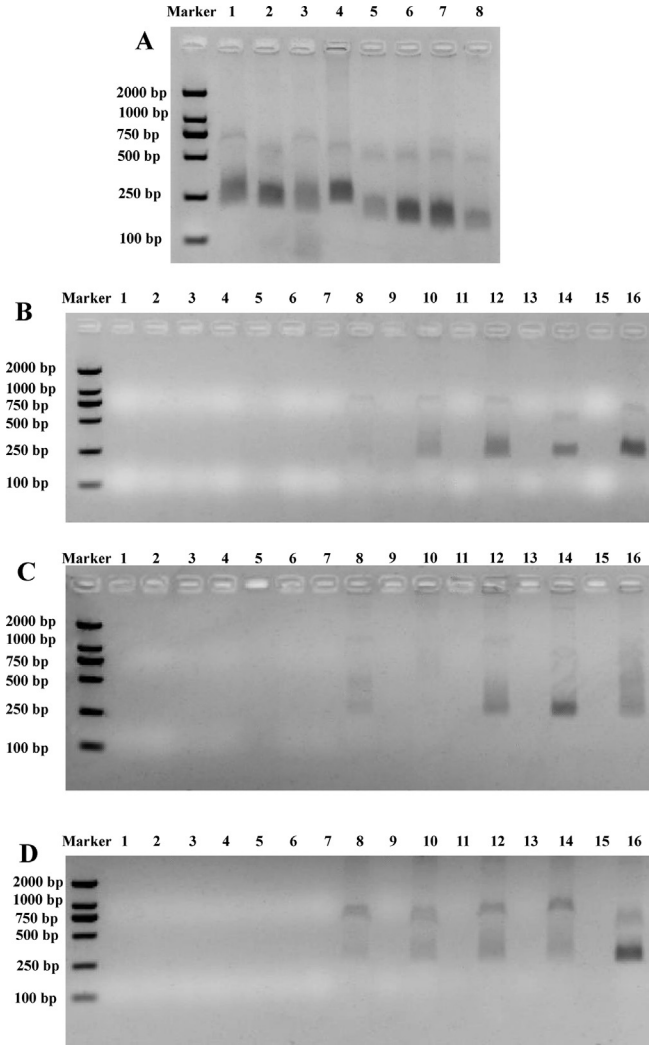

Fig. 1. Primer design and optimization of RPA reaction conditions. 2% agarose gel electrophoresis of RPA-amplified products with (A) eight different pairs of primers; (B) eight concentrations of the primers of negative control (no-target amplification) and positive sample (target-contained amplification) with the forth pair of primers in 10 min; (C) eight concentrations of the primers of negative control (no-target amplification) and positive sample (target-contained amplification) with the forth pair of primers in 20 min; (D) eight concentrations of the primers of negative control (no-target amplification) and positive sample (target-contained amplification) with the forth pair of primers in 30 min. In lanes 1–16 of B, C, D, the concentrations of the primers are 0.1 μM (Lane 1–2), 0.25 μM (Lane 3–4), 0.5 μM (Lane 5–6), 1 μM (Lane 7–8), 2 μM (Lane 9–10), 3 μM (Lane 11–12), 4 μM (Lane 13–14) and 5 μM (Lane 15–16), respectively.

3.3. Reduction of the non-specificity of the RPA reaction

When the RPA reaction conditions were investigated, a non-specific band was always evident at around 750 bp. Various attempts were made to improve the specificity of the reaction, including RPA amplification experiments under different reaction temperatures. Fig. 2A shows the electrophoresis patterns of RPA amplification products under different concentrations of primers. Under reaction conditions for amplification of 42 °C for 10 min, the non-specific band of approximately 750 bp in length was barely visible in the electropherogram, and further experimental results show that increasing the temperature can effectively reduce the generation of non-specific bands in the reaction. We speculate that the reason for this phenomenon is that increasing the reaction temperature reduces non-specific binds between the primers and also reduces non-specific binding between the primers and the template.

3.4. Optimization of formation reaction conditions of TdT-induced G-quadruplex

As the DNA fragment of *Salmonella* spp. amplified by RPA was longer than three bases, it could be used as a template strand for the TdT extension. In order to shorten the overall reaction time of the sensor, different extension times (10 min, 20 min, 30 min, 40 min) under 37 °C were optimized for TdT catalysis. As shown in Fig. 2B, when the RPA amplification product was directly extended, it was found that none of the color changes of the DNAzyme-catalyzed TMB/H_2O_2 were obvious, with a low absorbance at 450 nm under different reaction times. This suggested that the enzyme used in the RPA amplification process inhibits the extension of TdT and, therefore, the product of RPA amplification was diluted and extended by the TdT at different reaction times. The RPA products diluted four- and eight-fold are shown in Fig. 2C and D, respectively, with reaction times of 10 min, 20 min, 30 min and 40 min, respectively. As shown in Fig. 2B, C and 2D, the TdT extensions were carried out after dilution. At the same reaction time, the color of the final products was significantly altered with the stronger absorbance at 450 nm, compared to the non-diluted samples, thus indicating that the enzyme used in the RPA reaction may have an inhibitory effect on the elongation of TdT. In view of the inconspicuous color changes of TdT-catalyzed samples over the different extension times of 20 min, 30 min and 40 min, 20 min

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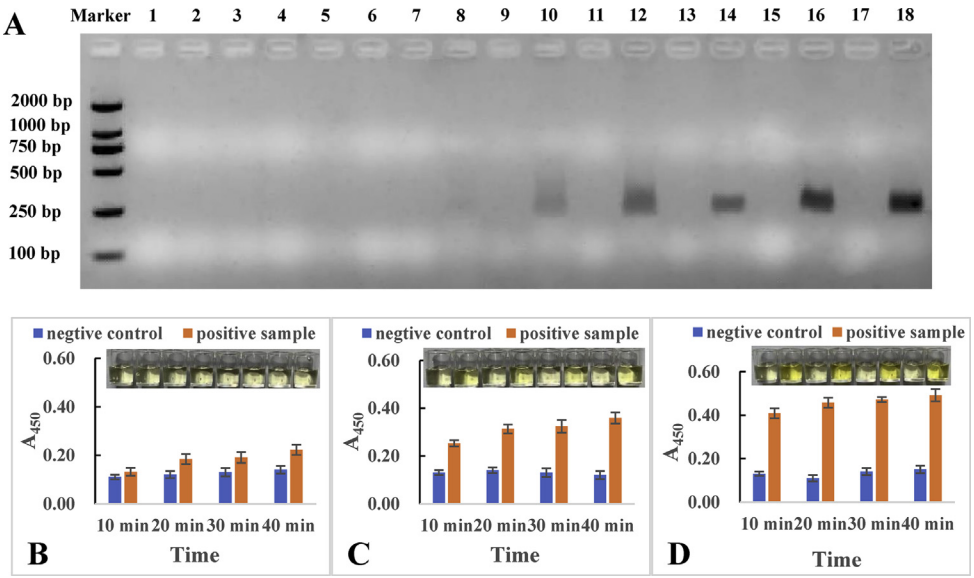

Fig. 2. The elimination of non-specific amplification of RPA and optimization of TdT extension conditions. (A) 2% agarose gel electrophoresis for RPA reaction at different concentrations of negative control (no-target amplification) and positive sample (target-contained amplification) with the forth primer set at 42 °C in 10 min. The concentrations of the primers are 0 μM (Lane 1–2), 0.1 μM (Lane 3–4), 0.25 μM (Lane 5–6), 0.5 μM (Lane 7–8), 1 μM (Lane 9–10), 2 μM (Lane 11–12), 3 μM (Lane 13–14), 4 μM (Lane 15–16) and 5 μM (Lane 17–18), respectively. The absorbance measured at 450 nm after the sample was extended by TdT. The forth primer set-mediated RPA amplification products were diluted at different folds and measured at different reaction times; (B) samples without diluting; (C) samples diluted four-fold; and (D) samples diluted eight times.

was selected as the extension time of TdT of the constructed biosensor.

3.5. Enzyme-mediated optimization of overall conditions for biosensors

The novel biosensor comprises a combination of two enzymes for the rapid detection of *Salmonella* spp.. In order to reduce the effect of the 1 × DSN buffer (50 mM Tris-HCl, 5 mM $MgCl_2$,1 mM DTT, pH 8.0) on the TdT extension, an experimental attempt was made to use the 1 × TdT buffer instead of the DSN buffer for the DSN cleavage reaction. The experimental results, in Fig. 3A, show that the buffer of TdT can be used effectively in place of the DSN buffer in different reaction times, and that the reaction of the TdT buffer was, in fact, even better (Fig. S5). The target fragment was well cleaved by the DSN within 15 min. Therefore, the TdT buffer was used henceforth during the entire enzyme reaction and the reaction time of DSN was 15 min.

Following the selection of the reaction time and buffer, in order to ascertain the optimal reaction conditions, we optimized the DSN's reaction concentration and explored the optimal reaction conditions for eliminating the effect of the enzyme used in the RPA reaction on the TdT extension. The template (the cleaved product of the DSN) was diluted before performing the TdT extension. As shown in Fig. 3B, the TdT extension was performed without dilution after treatment with different concentrations of DSN, and bright diffusion bands were produced in all four lanes. The product strip was brighter than the product strip that was extended after dilution (Fig. S1). This phenomenon may have been caused by the DSN cleavage, which is equivalent to dilution of the RPA product, thereby reducing the inhibition of the TdT extension. Moreover, the results of the polyacrylamide gel electrophoresis (Fig. 3A) indicate that the enzyme in the RPA reaction did not affect the cleavage of the DSN. The experimental results described above indicate that when the concentration of DSN is 0.25 U/μL, the amount of product is optimized and the corresponding band is the brightest. At this concentration, the color after the action with TMB was also brighter than the other concentrations and, after the addition of the stop solution, as shown in Fig. 3C, the color of the solution changed from blue to yellow. At this time, the corresponding absorbance value was also the largest. The experimental results are shown in Fig. 3D. In order to exclude the false positive results of our proposed biosensor, detailed controlled trials were conducted by both the gel analysis and the absorbance determination in Fig. S2. Firstly, negative control and positive trial of RPA were performed, as shown in lane 1, lane 2 and their corresponding optical absorbance, respectively. No amplified band appeared in lane 1 whereas a clear and distinct amplified band indicated the large amount of RPA amplification. Similarly, both of them had no optical absorbance at 450 nm. Secondly, negative control and positive trial of DSN-cleaved RPA were conducted. As shown in lane 3, the negative amplification of RPA had no DSN-cleaved products but a dispersed band existed due to DSN-cleaved RPA positive amplification in lane 4. Likewise, they had no absorbance value at 450 nm. Thirdly, negative control and positive trial of DSN/TdT-catalyzed products were conducted. Compared with a weak diffuse band in lane 5, the dual enzyme catalyzed products presented a clear diffuse band formation in lane 6, indicating that there is a large amount of G-quadruplex. Correspondingly, the optical absorbance of the positive result is about 3.5 times that of the negative result. Because TdT does not have a specific recognition site, the DNA can be extended by the action of TdT when 3′-OH is present, and the resulting randomly arranged G-rich DNA strand can form a G-quadruplex structure. The G-quadruplex DNAzyme structure formed by TdT was further confirmed by circular dichroism spectra. In Fig. S3, a positive peak at 215 nm and a negative peak at 252 nm both existed, indicating a parallel structure of G-quadruplex DNAzyme in accord with

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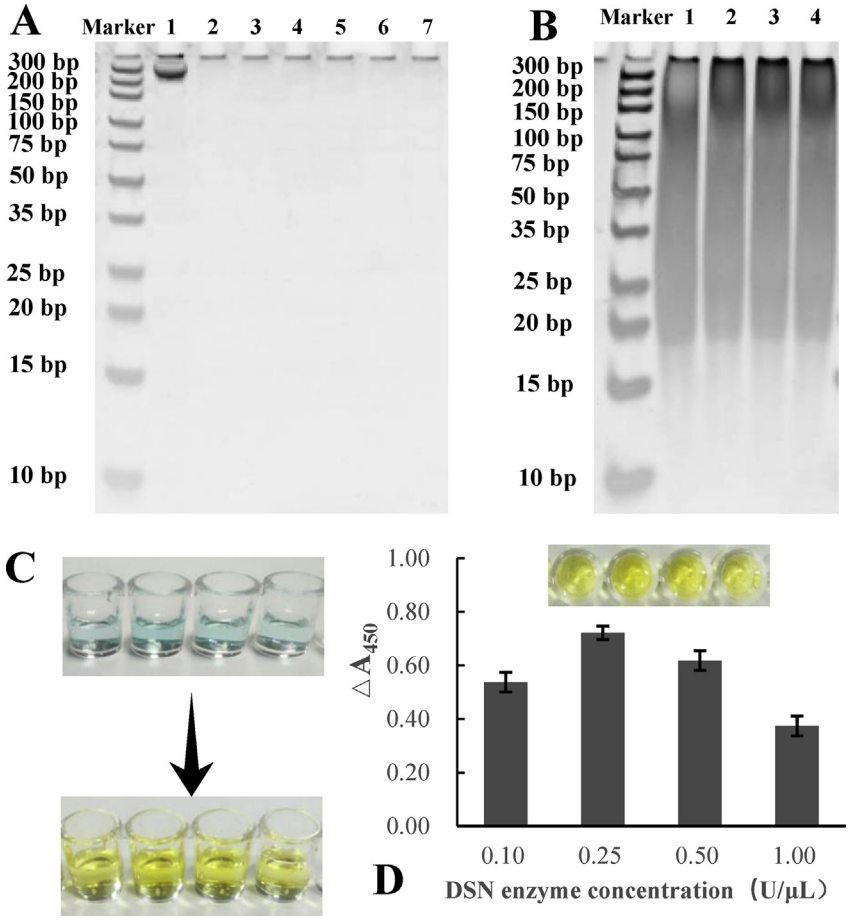

Fig. 3. (A) 12% polyacrylamide gel electrophoresis pattern of DSN cleavage product. Lane 1: Product of RPA; 2: The reaction time in 1 × TdT buffer solution was 15 min; 3: The reaction time in 1 × DSN buffer solution was 15 min; 4: Reaction time was 25 min, reaction in 1 × TdT buffer; 5: Reaction time was 25 min, reaction in 1 × DSN buffer; 6: Reaction time was 35 min, reaction in 1 × TdT buffer; 7: Reaction time was 35 min, reaction in 1 × DSN buffer. (B) After DSN cleavage, the 12% polyacrylamide gel electrophoresis pattern of the product after TdT extension for 20 min Lanes1-4: The concentrations of DSN were 0.1 U/μL, 0.25 U/μL, 0.5 U/μL and 1 U/μL. (C) The final product of the reaction was observable to the naked eye under different concentrations (0.1 U/μL to 1 U/μL) of DSN. Above panel: Reaction image of G-quadruplex and TMB solution based on cascade amplification. Bottom panel: The image of the RDTG-TMB reaction was terminated by H_2SO_4 solution. (D) The absorbance of the final product of the reaction of different concentrations of DSN at 450 nm.

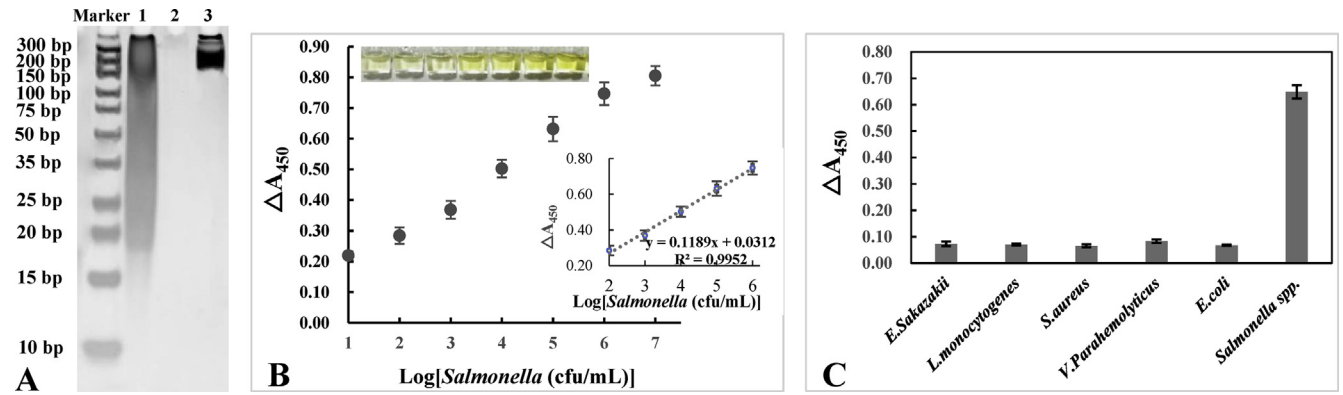

Fig. 4. (A) 12% polyacrylamide gel electrophoresis with 5 μM primer for RPA amplification. Lane 1: Product of RPA + DSN + TdT; 2: Product of RPA + DSN; 3: Product of RPA. (B) Absorbance values of *Salmonella* spp. at different concentrations (cfu/mL) at 450 nm. Inset: The color change of *Salmonella* spp. concentration vs. absorbance signal, and the calibration curve of *Salmonella* spp. at a concentration of 10^2 cfu/mL to 10^6 cfu/mL at $\triangle A_{450}$. (C) Specificity test. $\triangle A_{450}$ in response to *Enterobacter sakazakii, Listeria monocytogenes, Staphylococcus aureus, Vibrio parahemolyticus, Escherichia coli* and *Salmonella* spp. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

previous studies [47,48]. It suggested that both the single-stranded primer I and the DSN-cleaved RPA product can be used as templates for TdT extension to form G-quadruplex DNAzyme. As shown in Fig. 4A, lane 1 was a disperse band of RPA + DSN + TdT products. There were no bands in Lane 2 due to DSN-mediated cleavage of RPA products. There was a single bright band of RPA products in Lane 3, thus indicating that the detection of *Salmonella* spp. by the constructed biosensor was accurate.

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3.6. Sensitivity and specificity of the biosensor

It is well established that sensitivity and specificity are particularly important parameters for assessing the success of an assay system. In order to evaluate the sensitivity of the detection platform, the relationship between the final measured optical density value and a series of different concentrations of *Salmonella* spp. detection was evaluated. As is evident in Fig. 4B, color changes from single cell to 10^7 cfu/mL can be observed with the naked eye. As the concentration of *Salmonella* spp. increased, so, did the absorbance of the biosensor. Especially, a strong linear relationship was evident between $\triangle A_{450}$ and the logarithm of the *Salmonella* spp. concentrations, with a linear range of 10^2 cfu/ml-10^7 cfu/ml, linear equation: $y = 0.1189x + 0.0312$, and the regression coefficient $R^2 = 0.9952$. The lowest concentration of 6 cfu/mL being detected according to the 3σ rule [49]. In comparison with the reported *Salmonella* detection methods summarized in Table S3, the RDTG strategy is not only sensitive but also simple and convenient. In order to ensure the reproducibility of the biosensor, the linear detection range of the sensor was performed once again in Fig. S4, which is in good agreement with Fig. 4B. To assess the specificity of the enzyme-mediated *Salmonella* detection platform, six common pathogens, namely *Salmonella* spp., *E.sakazakii, L. monocytogenes, S. aureus, V. Parahemolyticus* and *E. coli*, were selected for experimentation under the same conditions. As shown in Fig. 4C, under the same experimental conditions, the absorbance of *Salmonella* spp. was significantly higher than that of other strains, thus displaying the detection platform's excellent specificity. Based on the above results, the feasibility of the detection platform we constructed is proved, with excellent selectivity and high sensitivity.

3.7. Detection of Salmonella spp. In yoghurt starter samples

In order to test the feasibility of the biosensing platform we built in practical applications, *Salmonella* spp. was test detected in a yoghurt starter. The experimental results are shown in Table S4. Different concentrations of *Salmonella* spp. were added to the samples and the biosensor test results proposed by our method were consistent with the traditional colony count results. It shows the good applicability of the RDTG biosensing platform in actual sample detection.

4. Conclusion

In summary, we have developed a label-free and visual cascade-amplified biosensor for the sensitive and selective detection of *Salmonella* spp. It enables the specific detection of *Salmonella* spp. in a relatively short period of time. The cascaded biosensor has the following advantages: (1) The RDTG design. The *Salmonella* spp. DNA amplified by RPA is cleaved by DSN to form a large number of dsDNA with 3′-OH, which can then be used as a TdT extension template. The content of *Salmonella* spp. is converted to a visual signal output by the action of a double enzyme, accompanied by cascade amplification of the detection signal. (2) Visualization. TdT forms a G-rich sequence after the extension of the nucleic acid tail, which then induces the formation of a G-quadruplex structure and TMB binding to generate a visible blue color for qualitative analysis. After the addition of the stop solution, the absorbance is measured at 450 nm. Quantitative analysis can, therefore, be achieved. (3) Rapidity and high sensitivity. *Salmonella* spp. can be detected by the detection platform we built in approximately 1.5h. Furthermore, the *Salmonella* spp. concentration responds linearly in the range of 10^2-10^6 cfu/mL, thus enabling detection at a single cell level. (4) Versatility. Other pathogenic bacteria can be detected by the biosensor constructed with the corresponding sequence of the primers. The biosensor, therefore, has the advantages of simple operation, short detection time and qualitative judgment of the naked eye, and it offers good prospects for food site safety assessments.

Declaration of interests

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

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Appendix A. Supplementary data

The tables of the primers of RPA used in this work, the polyacrylamide gel electrophoresis pattern of TdT extensions after cleavage with different concentrations of DSN and *Salmonella* spp. analysis in real samples.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2019.05.020.

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