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Universal linker Polymerase Chain Reaction-triggered Strand Displacement Amplification visual biosensor for ultra-sensitive detection of Salmonella

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ABSTRACT

Salmonella is a principal causal agent of pathogenic outbreaks via food. A universal, highly sensitive and visual *Salmonella* detection method was proposed in this paper, based on a universal linker PCR (UL-PCR)-triggered Strand Displacement Amplification (SDA). In this research, the UL-PCR achieved the primary amplification. The universal linker primer was ingeniously designed and composed of two parts, one of which was the binding sequence of the target, and the other was the universal linker. Complementary sequences of the G-quadruplex and the nicking endonuclease recognition sequence were included in the universal linker. Therefore, the G-quadruplexes and nicking sites were successfully introduced into the UL-PCR products, providing a basis for further SDA triggering. SDA achieved the secondary signal amplification and generated a large amount of label-free DNAzymes. Following SDA, DNAzymes catalyzed 3,3',5,5'-tetramethylaniline (TMB) into colored compounds visible to the naked eye. We obtained the best experimental conditions by univariate analysis. Under optimal conditions, this proposed universal label-free method could detect *Salmonella* genome at level as low as 22 copies mL^{-1}, with an excellent linear range between 10^2 copies mL^{-1} and 10^7 copies mL^{-1}. And the limit of quantification (LOQ) was 10^2 copies mL^{-1}. This strategy shows promise for broad applications.

1. Introduction

Bacteria are ubiquitous in nature, and pathogens are a potential threat to human health and safety. It is estimated that an average of 2.2 million people died each year due to foodborne infections [1]. According to the CDC, *Salmonella* is the primary pathogenic contributor to foodborne disease outbreak in the United States [2]. In China, about 60% of food poisoning events can be attributed to *Salmonella*, which is also the leading cause of bacterial food poisoning every year [3].

Pathogen detection technologies are critical in preventing foodborne disease outbreak and ensuring food safety. However, low efficiency, low accuracy, high equipment requirements, and high cost inhibit conventional biochemical analytical methods from meeting current detection requirements [4]. In contrast, with the development of functional nucleic acids (FNA) [5,6], FNA-based detection methods are capable of sensitively and rapidly detecting pathogens in a much shorter time at a low cost.

According to whether the temperature changes, nucleic acid amplification technologies are divided into non-isothermal nucleic acid amplification technologies such as Polymerase Chain Reaction (PCR) and Ligase Chain Reaction (LCR) [7], and isothermal amplification technologies. Developed by Kary Mullis in 1983, PCR has become one of the most widely used amplification strategies in molecular diagnosis, including molecular cloning, gene expression analysis, DNA or RNA sequencing, DNA fingerprinting, disease diagnosis, and target detection [8,9]. Due to its high efficiency and stable performance, PCR is one of the commonly used techniques in the detection of *Salmonella* [10,11]. Isothermal amplification methods include Loop-mediated Isothermal Amplification (LAMP) [12,13], Hybridization Chain Reaction (HCR) [14,15], Rolling Circle Amplification (RCA) [16], Strand Displacement Amplification (SDA) [17]. These technologies don't rely on temperature changes during the reaction process and don't require large temperature changing equipment; only a metal bath or constant temperature water bath pot is sufficient. Among them, SDA has the advantages of high efficiency, adaptability and simple operation [18], and provides exponential amplification of trace DNA or RNA sequences from complex

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mixtures [19]. It relies upon the coordinated activity of two enzymes, a strand-displacing DNA polymerase and a restriction endonuclease. The restriction endonuclease nicks one strand of double-stranded DNA, the polymerase extends the 3' end at the nick and displaces the downstream DNA strand. And this cycle of nicking, extension and displacement is repeated continuously, resulting in increasing in the number of displaced target strands [20].

PCR is more convenient and effective than technologies based on pathogens isolation and immunoassays. However, most methods employed for monitoring PCR require gels [21] or other devices, such as high-resolution fluorescence detectors. Therefore, it is extraordinarily costly and impossible to resolve directly with the naked eyes. G-rich nucleic acid sequence is prone to fold into G-tetrads and stack to a G-quadruplex structure [22]. When G-quadruplex binds with hemin, the complex formed has higher peroxidase-like activity than hemin only, catalyzing the redox reaction between H_2O_2 and 3,3',5,5'-tetramethylaniline (TMB) to produce colored compounds. In detection, this can be used for signal amplification and visual signal output.

Consequently, a universal linker PCR (UL-PCR)-triggered SDA biosensor is first constructed for the ultra-sensitive and visual detection of *Salmonella*. The complementary sequences of the G-quadruplex and Nt.BstNBI nicking endonuclease recognition sequence are embedded into the 5' end of the UL-PCR primer. When the targets present, the G-quadruplexes and nicking sites of Nt.BstNBI are successfully introduced into the UL-PCR products, which further trigger SDA to produce a large number of free G-quadruplex sequences. Thereby, achieving double signal amplification. Then, the G-quadruplexes interact with hemin to catalyze the chromogenic reaction of TMB. Therefore, realizing the visual detection of *Salmonella*. The best experimental conditions of this method were obtained by univariate analysis, which has a low detection limit and a wide linear range. There is excellent potential in developing this universal method to detect other food pathogens by replacing the target-specific binding sequence in the primer since it can provide useful information regarding risk supervision and the assessment of food safety.

2. Materials and methods

2.1. Materials

All DNA sequences in Table S1 were synthesized at Invitrogen Co., Ltd. (Beijing, China). Nt.BstNBI nicking endonuclease, *Bst* 2.0 DNA polymerase, rTaq DNA polymerase, 10 × PCR Buffer (20 mM Mg^{2+} Plus) and dNTPs Mixture (2.5 mM each) were purchased from Takara (Beijing, China). TMB coloring solution was purchased from Biyuntian Co., Ltd. (Beijing, China). The PCR thermal cycler was supplied by Bio-Rad (California, the USA), and the multimode microplate reader was supplied by Thermo Fisher Scientific (Beijing, China).

2.2. Sample preparation

The *Salmonella* spp. and other bacterial strains were preserved in the laboratory (Laboratory of Food Safety, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, China). All strains were stored in 20% (v/v) glycerol solution at −80°C until they were used. Strains were grown in Luria-Bertani broth medium (NaCl 5 g, yeast extract 5 g, tryptone 10 g and H_2O to 1000 mL) at 37°C for 16 h. This process was followed by the extraction of genomic DNA templates with a Bacteria DNA Kit (TianGen, Beijing, China).

2.3. Primer design

The specific genomic sequence of *Salmonella* (invA gene, NC_003197.2) was selected via the GenBank website (https://www.ncbi.nlm.nih.gov/). Primer design was performed using Primer 5.0 design software (Premier Biosoft, Palo Alto, USA). Universal linker primer has two sections: the first is the target binding sequence at the 3' end, the second is the universal linker at the 5' end. A universal linker consists of two parts: the complementary sequence of G-quadruplex and the complementary sequence of the Nt.BstNBI nicking endonuclease recognition sequence.

2.4. UL-PCR assay

The reaction was conducted in an S1000 Thermal Cycler (BioRad, California, USA) with a volume of 25 μL. The reaction system consisted of 1 × PCR buffer, 400 μM dNTPs mixture, 5 μL DNA, 1 μM forward primer, 1 μM reverse primer, and 0.05 U μL^{-1} rTaq DNA polymerase. The reaction was performed at 95°C for 5 min; 20 s at 95°C, 20 s at 57°C, and 30 s at 72°C for 40 cycles; then 72°C for 5 min.

2.5. SDA assay

During this process, 1.6 mM dNTPs mixture, 0.4 U μL^{-1} *Bst* 2.0 DNA polymerase, 0.6 U μL^{-1} Nt.BstNBI nicking endonuclease, 1 × isothermal amplifications buffer, 5 μL PCR product and 8 mM $MgSO_4$ were mixed evenly and incubated at 62°C for 15 min. Then, the mixture was kept at 95°C for 20 min to inactivate the enzyme and terminate the reaction.

2.6. Visual detection

For this reaction, 80 μL reaction buffer (pH 8.4), 10 μL of 40 μM hemin, and 10 μL SDA product were mixed evenly and incubated at 37°C for 30 min. This process was followed by the addition of 30 μL TMB coloring solution, and incubation for a further 30 min, after which 30 μL of 2 M H_2SO_4 solution was added to terminate the reaction. The color change was observed, and the absorption spectrum was obtained with multimode microplate reader.

2.7. Sensitivity analysis and calculation method of the LOD

The genomic mass concentration was measured using a micro-ultraviolet analyzer, and converted according to the following equation:

$$C(copies/mL) = \frac{NA(copies/moL) \times \rho(g/mL)}{Mr(g/moL)}$$

wherein NA represents the Avogadro constant (6.02×10^{23}), ρ represents the mass concentration, and Mr represents the average molecular weight. Mr (dsDNA) = number of haploid genomic bases × 660. Concentrations of 10 copies mL^{-1}, 10^2 copies mL^{-1}, 10^3 copies mL^{-1}, 10^4 copies mL^{-1}, 10^5 copies mL^{-1}, 10^6 copies mL^{-1}, 10^7 copies mL^{-1}, 10^8 copies mL^{-1}, and 10^9 copies mL^{-1} of *Salmonella* genomic samples were prepared. The amplification and color reaction were carried out under the optimal experimental conditions. After the color rendering experiment was completed, a microplate reader was used to detect the absorbance at 450 nm, and the results were analyzed.

The LOD in this study was calculated according to the calculation formula: the minimum detection limit = the average blank absorbance + 3σ (the standard deviation of the blank signals (3 replicates)), also known as the 3σ principle. Then, the minimum concentration of the quantitative detection system for *Salmonella* was derived from the standard curve.

2.8. Specificity analysis

The genomes of *Salmonella*, *Shigella*, *V. parahaemolyticus*, *E. coli*, *S. aureus* and *B. cereus* were used at a concentration of 10^7 copies mL^{-1}. The amplification and color reaction were carried out under the optimal experimental conditions. After the color rendering experiment was completed, a microplate reader was used to detect the absorbance at 450 nm, and the results were analyzed.

3. Results and discussion

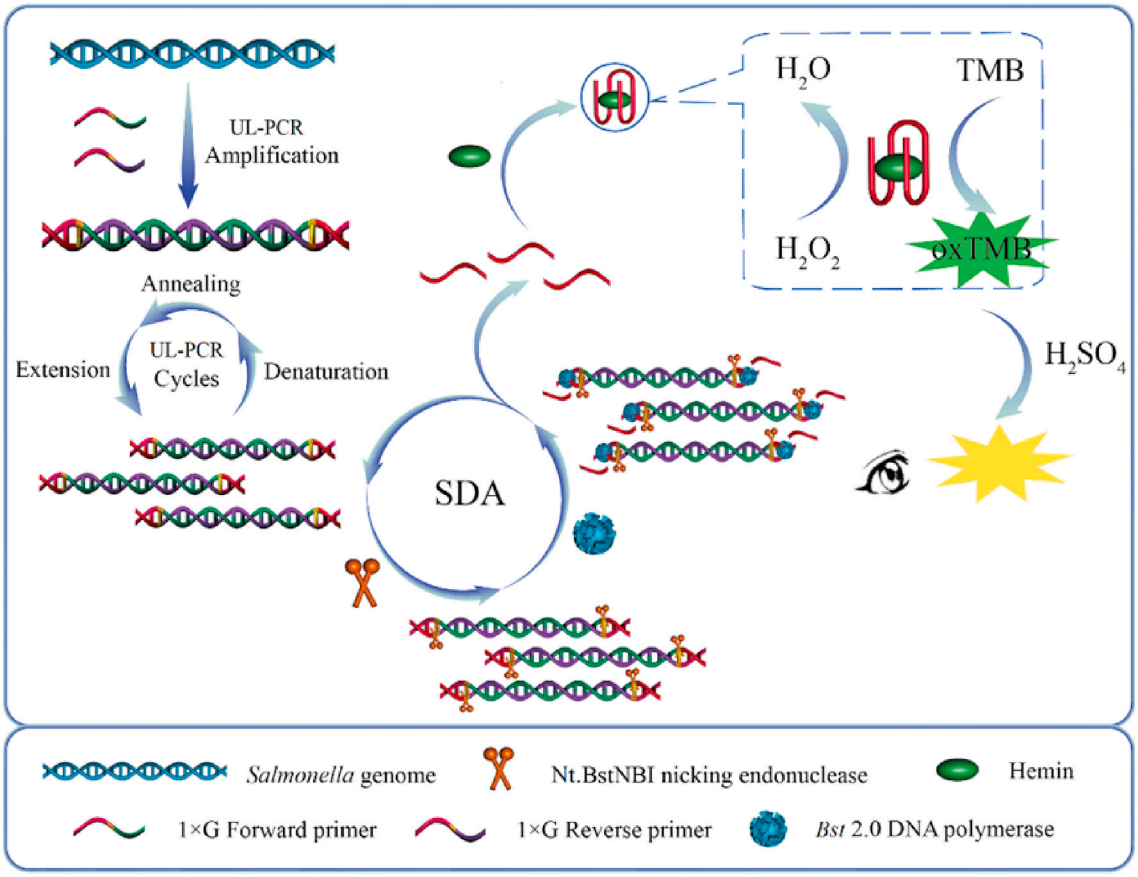
3.1. The principle of the assay

The principle of the UL-PCR triggered SDA visual biosensor for ultrasensitive detection of *Salmonella* is shown in Scheme 1. First, the UL-PCR achieved the primary signal amplification to produce a large number of double-stranded products. The UL-PCR primer was specially designed and was composed of two parts, one of which was the binding sequence of the target, while the other was the universal linker. Complementary sequences of the G-quadruplex and nicking endonuclease recognition sequence were included in the universal linker. When the targets appeared, the binding sequences in the primers recognized and hybridized with the targets. The complementary sequences of the universal linkers in the primers, namely the G-quadruplexes and nicking sites of endonuclease, were successfully introduced into the products by UL-PCR amplification. Then, during SDA, the recognition sites on the UL-PCR products triggered the nicking of the Nt.BstNBI nicking endonuclease to generate nicks, and *Bst* 2.0 DNA polymerase extended the 3' ends of the nicks and displaced the downstream G-quadruplex sequences. Plenty of single-stranded free G-quadruplex sequences were produced in continuous cycles of nicking, extension and displacement. Consequently, double signal amplification was realized. Following the SDA, the G-quadruplexes were co-incubated with hemin to form DNAzymes to exert peroxidase-like activity and catalyzed a TMB color reaction to produce visible color change.

3.2. Feasibility verification and universal linker length optimization

The universal linker was the key to this research. It allowed for a flexible design to use the PCR product to trigger SDA and perform double signal amplification. Furthermore, the conversion of the PCR double-stranded products into a large number of single strands and visual signal output were realized. In order to achieve the best level of biosensor performance, the length of the universal linker needed to be verified and optimized. The results are shown in Fig. 1. Fig. 1(a) represents the agarose gel electrophoresis of the UL-PCR products, indicating that different lengths of linkers lead to different positions of the bands. The longer the linker, the closer it is to the sample hole. The results are consistent with the theory, which indicates that the universal linkers have been successfully introduced into the UL-PCR products. Furthermore, the melting peaks of the real-time quantitative PCR (RT-qPCR) products in Fig. 1(b) show that the Tm of the products with the universal linkers are slightly higher than that without a universal linker. This is because the lengths of the double-stranded products increased with the introduction of the linkers in the primers, and the products were more stable, which in turn leaded to an increase in Tm. Moreover, All the melting curves have only one peak and no other hetero-peaks, indicating that the primers did not form dimers and there was no non-specific amplification. Fig. 1(c) is the amplification result of RT-qPCR. The results show that the quantitative curves are basically the same regardless of whether there is a universal linker or not in the primer and the length of the universal linker. The Ct values are all very low, the addition of the universal linker did not affect the amplification. These results illustrate the rationality and feasibility of primer design, which is beneficial to the subsequent reactions, reduce the interference of the background signal, and improve sensitivity.

In addition, a color rendering reaction was also conducted. The coloration results in Fig. 1(d) show that the absorbance at 450 nm ($OD_{450\ nm}$) is the maximum level, and the yellow is the deepest when the universal linker contains only one intact G-quadruplex complementary sequence. Since the introduction of the linkers did not affect the



Scheme 1. The principle of the *Salmonella* detection system based on Universal linker PCR-triggered Strand Displacement Amplification.

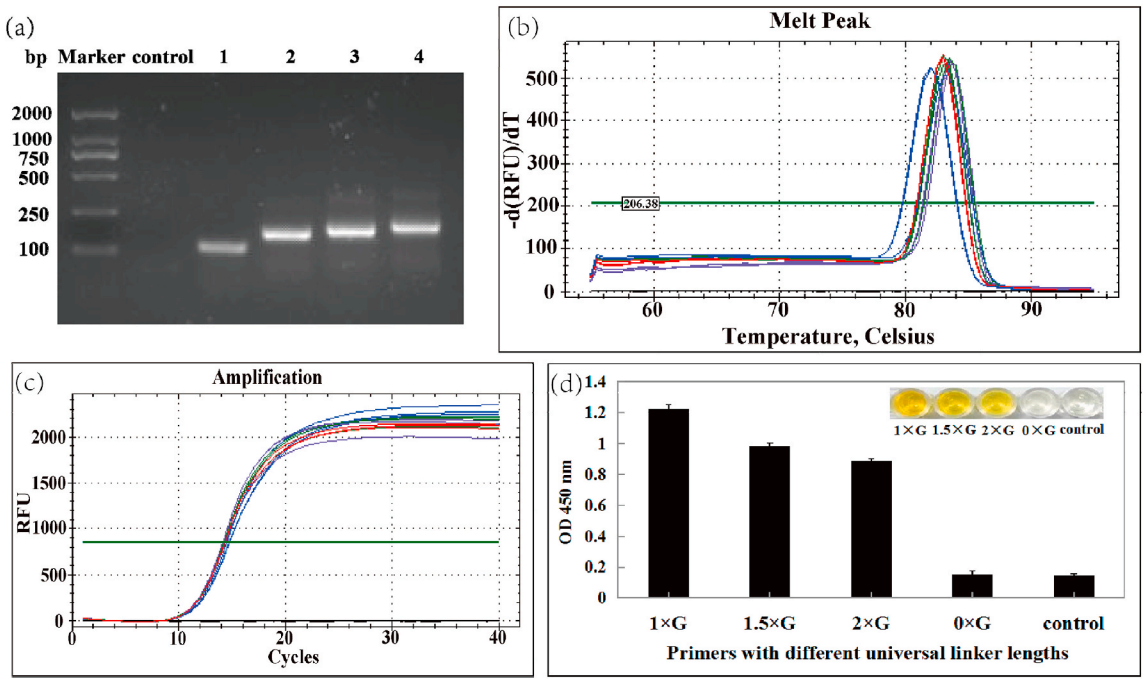


Fig. 1. The results of the feasibility verification and universal linker length optimization: (a) results of the agarose gel electrophoresis of the UL-PCR products with different universal linker lengths (the universal linker lengths from lane 1 to lane 4 were 0 × G (conventional primers), 1 × G, 1.5 × G, and 2 × G, respectively. The control group had no target and was amplified using 1 × G primers.); (b) the melt peaks of RT-qPCR products (the blue curve represents 0 × G primer, the red curve represents 1 × G primer, the green curve represents 1.5 × G primer, and the purple curve represents 2 × G primer); (c) amplification results of RT-qPCR; (d) the change in absorbance at 450 nm. The inset presents a photograph of the corresponding system. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

amplification efficiency, it was speculated that the difference in color was due to the higher SDA efficiency in short strand amplification, resulting in a higher concentration of the G-quadruplex sequences. Based on these results, the feasibility of this strategy was verified, and the optimal universal linker sequence was obtained. 1 × G primer was selected for subsequent experiments.

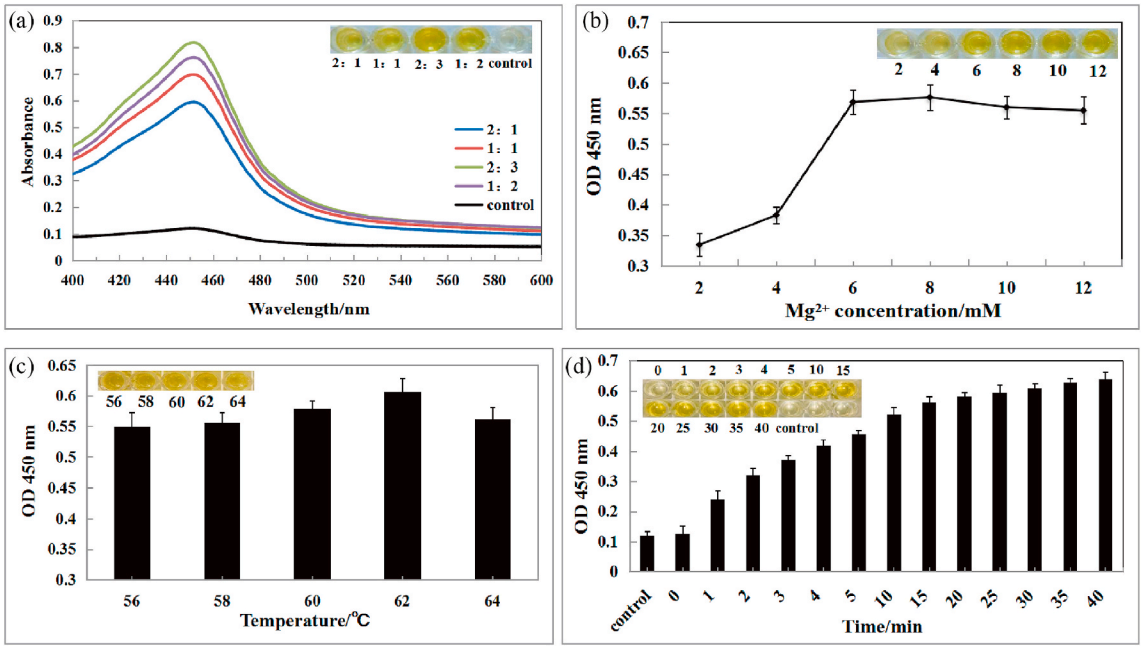


Fig. 2. The results of experimental conditions optimization: (a) optimization of the *Bst* 2.0 DNA polymerase and Nt.BstNBI nicking endonuclease ratio; (b) optimization of Mg^{2+} concentration; (c) optimization of SDA incubation temperature; (d) optimization of SDA incubation time. The insets present photographs of the corresponding systems.

3.3. Optimization of experimental conditions

PCR is a notably mature technology. Therefore, the capital conditions of SDA were optimized to improve the performance of detection. *Bst* 2.0 DNA polymerase is a DNA polymerase exhibiting strand replacement activity. Nt.BstNBI nicking endonuclease can recognize specific sequence and nick at specific site, producing gap that create condition for the amplification of the *Bst* 2.0 DNA polymerase. These two enzymes achieve amplification by cooperating with each other. If the proportion of polymerase is too large, its effect will not be fully exerted. Furthermore, if the ratio of endonuclease is too high, the amplified fragments will be too short to form G-quadruplex structures. Therefore, to fully exploit the effects of the two enzymes, the ratio was optimized. According to Fig. 2(a), when the ratio is 2:3, the absorbance value at 450 nm is the highest, and the color development is the best. Therefore, 2:3 was selected as the optimal ratio of *Bst* 2.0 DNA polymerase and Nt.BstNBI endonuclease.

Enzyme play a crucial role in the reaction system. In addition to the concentration, the enzyme activity has a great impact on amplification efficiency and detection sensitivity. Mg^{2+} in the reaction system plays a vital role in enzyme activity, and the optimum concentrations of different enzymes varied. Fig. 2(b) manifests that the absorbance enhance with the increase in Mg^{2+} concentration at low concentration range. However, when it reaches 8 mM, the absorbance is the largest, the further increase in concentration does not increase the absorbance but instead has a weak inhibitory effect. Consequently, the enzyme activity was strongest when the Mg^{2+} concentration was at 8 mM, which was, therefore, used in subsequent experiments.

Moreover, the temperature also significantly influences the enzyme activity. Too low a temperature might inhibit the activity, while too high a temperature might lead to the denaturation and inactivation of enzymes. The optimum temperature of *Bst* 2.0 DNA polymerase is 65°C, while that of Nt.BstNBI endonuclease is 55°C. The optimum temperatures for the two are not uniform, and it is, therefore, essential to optimize the reaction temperature of SDA to improve detection performance. The results in Fig. 2(c) show that the best color rendering is obtained at 62°C.

Prolonging the reaction time allows the SDA product to accumulate, improving the coloration effect. However, during the actual detection process, the reaction time should be shortened as much as possible. Consequently, the reaction time was optimized. The results shown in Fig. 2(d) manifest that an extended reaction time improves the coloration effect. Between 0 min and 15 min, the absorbances rise rapidly. While after 15 min, the SDA products accumulate to a certain extent, the rise trend slows down, the prolonged time has no significant effect on the absorbance. In summary, to ensure better color development and shorter time, 15 min was carefully selected.

3.4. Sensitivity analysis

If a biosensor exhibits high sensitivity and a wide linear range, it has an absolute advantage over similar detection strategies. To assess the sensitivity of this method for detecting *Salmonella*, samples containing different concentrations of the *Salmonella* genome were tested under the optimal conditions, and the relationship between the absorbance and concentration was analyzed. Fig. 3 suggests that, with an increase in concentrations, the absorbances at 450 nm continue to rise, while the systems continue to darken. The experimental results display a good linear relationship between $OD_{450\ nm}$ and the *Salmonella* concentration in a range of 10^2–10^7 copies mL^{-1}, while the linear equation is $y = 0.082x+0.0494$. R^2 is 0.9915. The LOD level is as low as 22 copies mL^{-1}. Furthermore, the limit of quantification (LOQ) refers to the lowest concentration of the analyte in the sample that can be quantified. Therefore, the LOQ of this method is 100 copies mL^{-1}, the lowest concentration in the linear range.

Furthermore, the detection limits of different *Salmonella* biosensors are compared and the results are shown in Table S2. The biosensor established in this paper displays clear advantages relating to both high sensitivity and visualization.

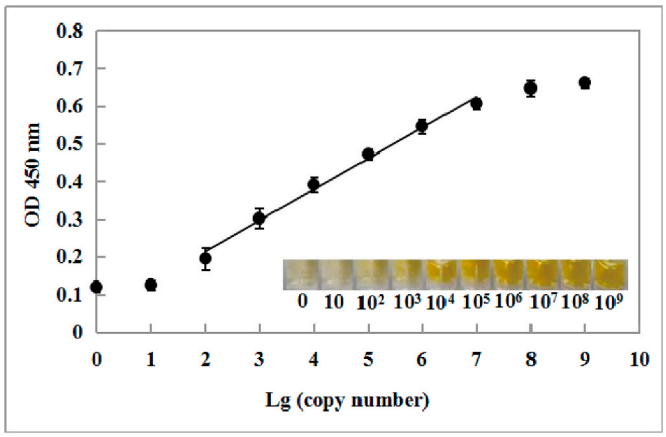


Fig. 3. The changes in absorbances at 450 nm with the concentrations of the *Salmonella* genome. The inset presents a photograph of the corresponding system.

3.5. Specificity analysis

The specificity of a method is essential for its application in actual detection. Different representative pathogens, including gram-negative bacteria such as *Shigella*, *V. parahaemolyticus*, *E. coli*, and gram-positive bacteria such as *S. aureus*, *B. cereus*, were tested under optimal conditions to assess the specificity of this method. The genomic concentrations of all pathogens were 10^7 copies mL^{-1}. As shown in Fig. 4, under the same concentration and other conditions, the absorbance of *Salmonella* at 450 nm is significantly higher than the others, and the visible yellow is also deeper. These results make clear that this method displays excellent specificity for *Salmonella* detection and can be directly discriminated with the naked eyes.

3.6. Analytical performance evaluation

In order to further illustrate the excellent performance of this biosensor, the repeatability and uncertainty of this method were also verified. The relevant data are shown in Table S3. The uncertainty varies from 0.009 to 0.018, and repeatability varies from 1.87% to 5.93%. This method newly established has low uncertainty and great repeatability.

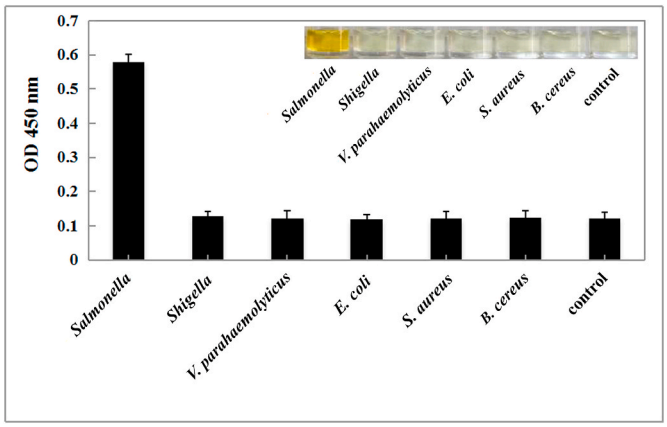


Fig. 4. Results of specificity verification, with an inset photograph of the corresponding system. The control group had no target.

4. Conclusion

In summary, this study provides a universal, highly sensitive, label-free visual method for the detection of *Salmonella* based on UL-PCR triggered SDA. The universal linker primer was designed to contain complementary sequences of the G-quadruplex and nicking endonuclease recognition sequence, which could further trigger SDA in the UL-PCR products under enzymes action. The double-stranded UL-PCR products were converted into single strands, and a large number of G-quadruplex sequences were generated to realize double signal amplification and visual signal output. Under the optimal experimental conditions obtained by univariate analysis, the linear range of this biosensor is between 10^2–10^7 copies mL^{-1}, the LOQ is 10^2 copies mL^{-1}, and the LOD level can drop as low as 22 copies mL^{-1}. In addition, this detection method has favourable specificity and repeatability, and has the potential to be used to detect other targets by replacing the binding sequence in the universal linker primer. This strategy displays exceptional potential for extensive applications in food safety.

Author statement

Yuan Zhang: Methodology, Investigation and Writing-original draft. **Shuting Li:** Investigation, Validation and Manuscript revision. **Jingjing Tian:** Investigation and Manuscript revision. **Kai Li:** Validation and Software. **Zaihui Du:** Investigation and Manuscript revision. **Wentao Xu:** Resources, Supervision, Conceptualization, Writing-review & editing and Funding acquisition.

Declaration of competing interest

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2020.121575.

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