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PAPER IN FOREFRONT

Single-tube analysis for ultra-fast and visual detection of *Salmonella*

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Abstract

Herein, we developed an ultra-fast and visual single-tube nucleic acid detection approach, which combined the advantages of self-settling characteristics of chitosan-functionalized diatomaceous earth (CDE) and accelerated PCR (AC-PCR). DNA was rapidly extracted by CDE within 3 min for the next nucleic acid amplification based on the nucleic acid attached on the chitosan in pH = 5.0. Under the action of gravity, the DNA-enriched CDE self-sediments to the bottom of the tube could be directly used for AC-PCR to achieve single-tube extraction and amplification. Our method detected *Salmonella* culture fluids with a detection limit of 1 CFU/mL, which was 100-fold more sensitive than conventional method that have not undergone nucleic acid enrichment. Furthermore, it also displayed high specificity and sensitivity for a variety of spiked samples. The entire process could be completed within 17 min in a single tube, and in particular, the result was visualized by the naked eyes. Overall, it is an all-in-one detection strategy without the requirement of redundant procedure, which greatly improved the detection efficiency, and saved the time and the cost. With these advantages, the approach will supply a promising tool in the field of point-of-care testing for *Salmonella* and other foodborne pathogens.

Keywords Single-tube · Diatomaceous earth · Nucleic acid amplification · Visual detection · *Salmonella* · Nucleic acid extraction

Introduction

Infectious diseases caused by foodborne pathogens pose a major threat to public health and have caused countless diseases and huge economic losses [1, 2]. *Salmonella* is one of the most common pathogens, causing more symptomatic food poisoning infections than any other pathogens [3], and it is very easy to contaminate foods such as meat, eggs, and dairy products and the surface of vegetables and fruits [4]. People may experience diarrhea, abdominal pain, vomiting, fever, and occasionally death within 12–72 h after ingesting contaminated food [5, 6]. Conventional culture-based method for *Salmonella* detection is time-consuming, and it usually takes 2–5 days [7, 8]. Real-time fluorescence amplification detection of nucleic acid like polymerase chain reaction (PCR) [9] has shown excellent performance in the diagnosis of pathogens. However, PCR takes 1.5–2 h to realize temperature rise and cooling, which is difficult to be used in field detection scenarios that require quick result readout. Therefore, some accelerated PCR (AC-PCR) methods have been developed to provide faster detection and a powerful alternative method for rapid on-site detection [10].

Current nucleic acid amplification methods face the problem of being time-consuming or expensive sample preparation. Traditional nucleic acid extraction methods, such as CTAB method [11] and SDS method [12], require organic solvent extraction and precipitation, which are time-consuming and have high requirements on the laboratory environment. The spin column method and magnetic

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bead method used in commercial kits shorten the nucleic acid extraction time to less than 30 min, but the high cost limits their application in rapid on-site testing, especially in some developing countries. For low-concentration samples, these methods generally take 16–18 h of pre-enrichment growth to enrich to reach the detection limit of the amplification method [8, 13]. Therefore, making the extraction step simpler and achieving effective enrichment, it is of great significance for point-of-care detection.

In recent years, a series of studies have reported diagnostic platforms that integrate nucleic acid extraction, amplification, and result reading into an integrated device [14–16]. For example, Dong et al. proposed a closed-type cassette system that integrates magnetic bead extraction and amplification detection [17]. Song et al. proposed a microfluidic cassette for sample filtration, extraction, and detection [18]. However, these platforms normally require external components (pumps, microvalves, mixers, etc.) for liquid operations, and the conventional extraction schemes used are still difficult to enrich low-concentration targets. Diatomaceous earth (DE) is a low-cost natural silica with good biocompatibility and strong adsorption capacity [19–21]. It has been reported to use modified DE for nucleic acid and pathogen enrichment [22, 23]. Utilizing the natural self-sedimentation ability of DE, pathogenic bacteria could be efficiently enriched without ultracentrifugation device [24, 25]. However, this program still requires washing and elution [26]. The micro-upgrading targets that finally are added to the amplification system are still a certain amount of sample loss.

In situ analysis has been reported in recent years [27]. The nucleic acid extraction matrix was analyzed directly to maximize the enrichment of all targets in the sample [28, 29]. Here, a single-tube analysis for *Salmonella* diagnosis was proposed. The entire detection process including sample enrichment, nucleic acid amplification, and result readout was completed within 17 min. We reported for the first time that a chitosan-modified diatomaceous earth (CDE) material is used for low-cost nucleic acid enrichment of *Salmonella*, which can achieve DNA isolation and enrichment in large-volume samples within 3 min. Chitosan enriches nucleic acids via electrostatic absorption at pH < 6.3 with an amino group pKa of 6.3 [30]. All enriched nucleic acid could be concentrated in AC-PCR reaction (pH = 8.8) and trigger the amplification process. The test results could be read directly with the naked eye through ultraviolet light. This protocol improved the performance of AC-PCR by 100 times, and at the same time, the rapid nucleic acid amplification of *Salmonella* did not have any sample loss. Therefore, the combined protocol provided a fully integrated system for on-site detection of *Salmonella* within 17 min.

Materials and methods

Materials and reagents

DE for nucleic acid extraction with a density of 0.47 g/cm^3 was bought from Sangon Biotech (Shanghai, China). Chitooligosaccharide was purchased from Yunzhou Biotechnology Co., Ltd. (Qingdao, China). Chemicals for electrophoresis gels, 20-bp DNA ladders, and EvaGreen dye were bought from Takara Biomedical Technology Co., Ltd. (Beijing, China). Genomic DNA was extracted with TIANamp bacteria DNA kit, which was purchased from Tiangen Biotech Beijing Co., Ltd. (Beijing, China). Fast PCR reagents (Item No. KD3101) were obtained from Qingdao Navid Biotechnology Co., Ltd. (China). All other chemicals are analytical grade unless otherwise stated.

Reference strains of *Salmonella typhimurium*, *Bacillus cereus*, *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Vibrio parahaemolyticus* were preserved in our laboratory. The primers were designed based on *fimY* sequences of *Salmonella* using the NUPACK web tool (http://www.nupack.org/) and the DNAMelt Web Server (http://unafold.rna.albany.edu/?q¼DINAMelt). The synthesis of primers was performed in Sangon Biotech Co., Ltd. (Shanghai, China).

Preparation of GPTMS-chitosan

The GPTMS-chitosan solution contains 1% (3-glycidoxypropyl) methyldiethoxysilane (GPTMS), 0.01 g/mL chitosan, and 50 mM acetic acid. The molecular weight of chitosan (Fig. S1) and the concentration of chitosan (Fig. S2) were optimized. After applying 50-Hz ultrasound for 20 min, the solution was incubated at 37 °C for 2 h and ready for use.

Functionalization of DE

DE was washed with piranha solution (2:1, H_2SO_4/H_2O_2) at 70 °C for 10 min. After being washed with distilled water, clean DE was collected by centrifugation. Then, the DE was dried thoroughly in the oven. To modify chitosan to DE, 0.02 g DE was added into 0.1 mL of GPTMS-chitosan solution and incubated for 8 h at room temperature. Then, the DE was rinsed with 50 mM acetic acid solution to remove unbound chitosan, followed by rinsing to neutrality with water, and dried thoroughly in the oven.

AC-PCR reaction

The AC-PCR was performed in 20 μL reaction volume, containing fast DNA polymerase, specific primers, and fluorescence probe in the reaction solution. The thermal cycling program included initial denaturation at 95 °C for 2 min and 35 rapid cycles of 95 °C for 3 s and 60 °C for 10 s. Fluorescence signals of amplification process were monitored by fluorescence quantitative PCR instrument (ND260, Qingdao Navid Biotechnology Co., Ltd., China) at 1-cycle intervals. The sequence information used in the experiment is shown in Table S1.

Single-tube analysis

For nucleic acid extraction from *Salmonella*, 150 µL of sample solution was added into a tube containing 50 µL binding buffer (50 mM MES, pH = 5.0) and 2.25 mg of CDE, mixed gently with a pipette, and incubated at 95 °C for 3 min. The pH of the binding buffer (Fig. S3) and the quantity of CDE in the reaction mixture (Fig. S4) have been optimized. During this period, the CDE settled to the bottom of the tube due to its self-sedimentation ability. After removing the supernatant, 20 µL AC-PCR reaction mixture (pH = 8.8) was sucked into the tube with the nucleic acid-bound CDE precipitate. Then, after placing the tube in a CFX96™ Real-Time detection system (Bio-Rad, CA, USA), AC-PCR amplification was performed within 14 min. Finally, irradiating with an ultraviolet lamp, the result was directly measured under ultraviolet light.

Detection of Salmonella in artificially contaminated samples

Samples of fresh oysters, fish, and lamb were purchased from the local market. According to the Chinese National Standard (GB 4789.4–2016); three samples were detected to be negative for *Salmonella*. Three artificially contaminated samples were obtained as follows: first, 25 g sample was added to 225 mL buffered peptone water (10.0 g/L peptone, 5.0 g/L sodium chloride, 9.0 g/L disodium hydrogen phosphate dodecahydrate, 1.5 g/L potassium dihydrogen phosphate) and homogenized for 2 min. After being contaminated with 1.0×10^0 to 1.0×10^6 CFU/mL of *Salmonella*, each homogenate was centrifuged at 1000 × *g* for 1 min to remove larger debris, and then the supernatant was transferred to a new tube and centrifuged at 12,000 × *g* for 5 min. Subsequently, the precipitate was resuspended in 100 µL of MES buffer (pH = 5.0), and

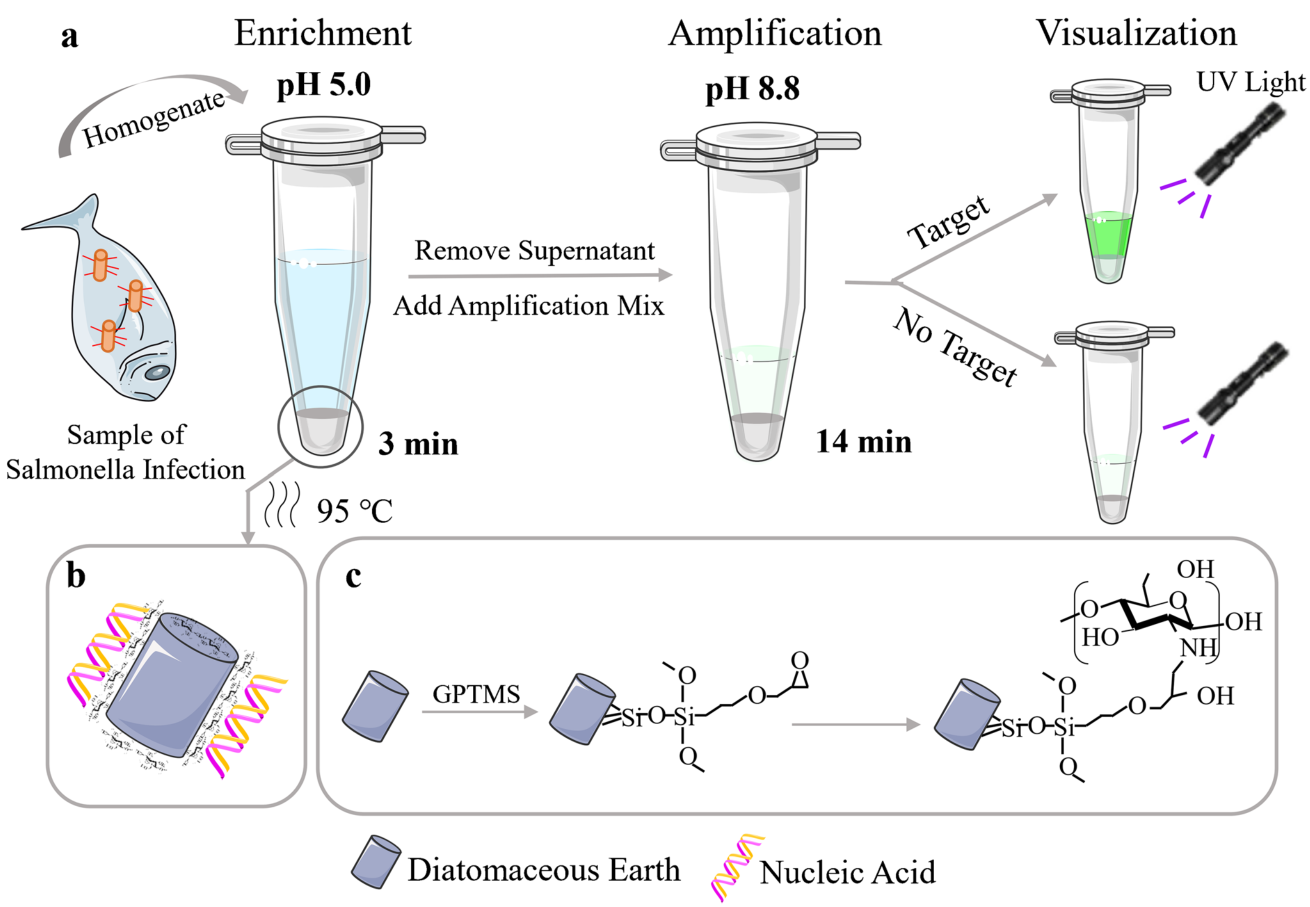

Fig. 1 A schematic illustration of the suggested protocol of single-tube platform. The single-tube platform substantially simplifies the steps and time of genetic analysis, completing within 17 min without centrifugation. **a** showed the operation process of the single-tube protocol, **b** represented the isolation of nucleic acids by CDE, and **c** showed the functionalization steps of CDE.

directly tested by the CDE-based single-tube protocol. Each assay was carried out in triplicates.

Results and discussion

Working scheme of the CDE-based single-tube protocol

Figure 1 shows the working scheme of the CDE-based single-tube protocol. The whole detection process only includes three steps of enrichment, amplification, and visualization (Fig. 1a). DE was used for nucleic acid enrichment after functionalization with chitosan (Fig. 1c). Homogenization of the sample was first mixed with lysis and binding buffer (50 mM MES, pH = 5.0) and loaded on CDE. DNA was released, and absorbed on the chitosan layer by electrostatic action after heat incubation (Fig. 1b), and precipitated to the bottom of the tube along with the self-sedimentation of the DE during this period. Then, AC-PCR reaction mixture (pH = 8.8) was directly added to the CDE enriched with nucleic acids. The chitosan molecules then became negatively charged due to pH changes, and the captured nucleic acids were eluted as a result. Next, the AC-PCR amplification was triggered; nucleic acids were amplified exponentially under the action of DNA polymerase. The result can be read out directly by the naked eyes through UV light according to the color change.

Characterization of nucleic acid enrichment performance using CDE

FT-IR was used to characterize the modification of DE by chitosan (Fig. 2A). Compared with the blank DE, chitosan-modified DE showed an obvious absorption band at 1635 cm^{-1}, which is the characteristic absorption peak of chitosan [31], suggesting that chitosan is successfully modified on the DE. Next, the CDE was directly added to the AC-PCR reaction system to verify its effect on amplification. Figure 2B shows the AC-PCR reaction with CDE which is only 1 Ct smaller compared with the DNA added to AC-PCR reaction system directly, and there is no effect on the amplified products (Fig. 2C). Therefore, CDE can be used in AC-PCR reaction system. In order

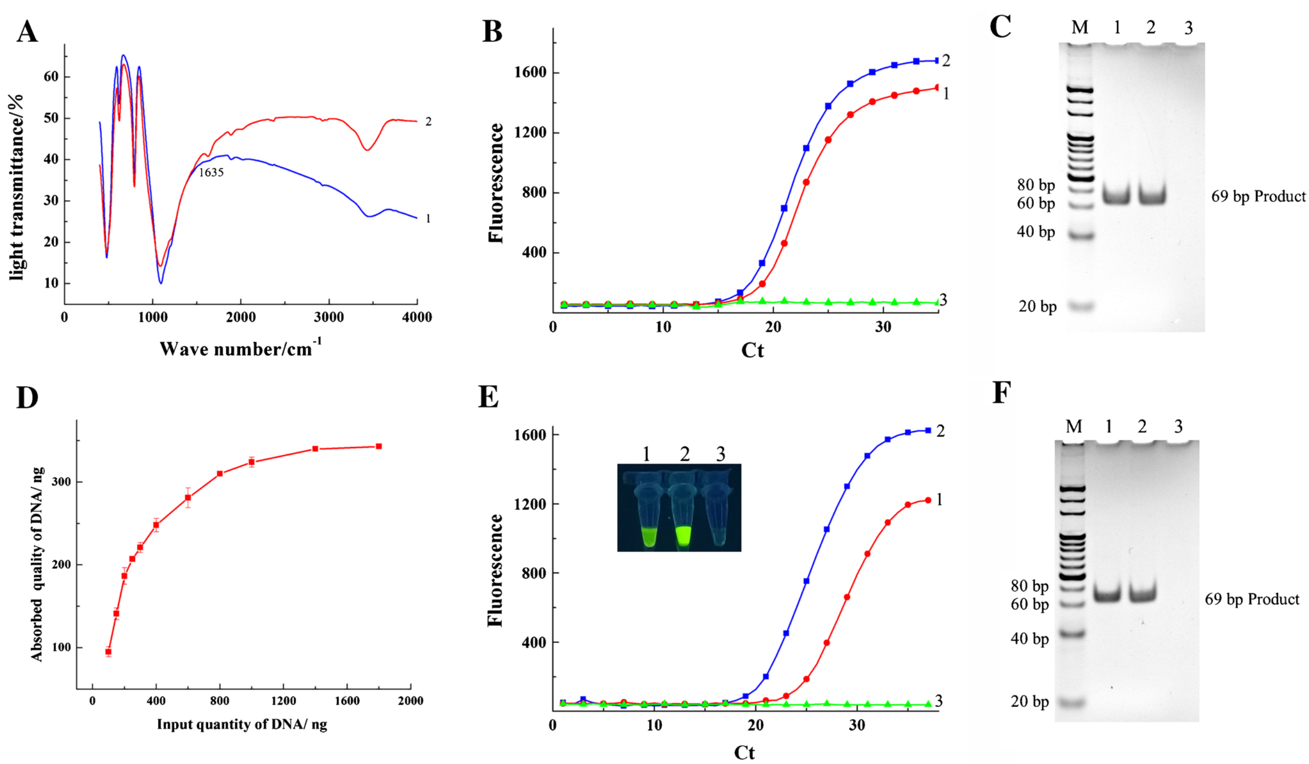

Fig. 2 Chitosan functionalization of diatomaceous earth and its detection application. A FT-IR spectrum of chitosan-specific functional groups on the DE. Line 1 represented DE, and line 2 represented CDE. B Real-time fluorescence curves of AC-PCR with (curve 1) and without (curve 2) DE; curve 3 represented NTC. C PAGE image for AC-PCR products after amplification from B, M was 20-bp DNA ladder. D DNA recovery efficiency and binding capacity of CDE. E Real-time fluorescence curves of AC-PCR before (curve 1) and after (curve 2) CDE enrichment, curve 3 represented NTC. Inset represented the corresponding colorimetric result for the amplification. F PAGE image for AC-PCR products after amplification from E, M was 20-bp DNA ladder

to prove that, the CDE could adsorb nucleic acid; 2.25 g CDE was loaded into different quantities of DNA. Quickdrop™ Micro-Volume Spectrophotometer (Molecular Devices, LLC, USA) was used to determine the amount of the remaining nucleic acid after the adsorption process. The absorbed mass of DNA was the input mass minus the remaining mass. Figure 2D shows that CDE could absorb up to 343 ng of nucleic acid. The amount of CDE in the amplification system had been optimized (Fig. S1). In addition, CDE was used to enrich nucleic acids, and AC-PCR was used for detection. Figure 2E shows 4 Ct advanced of the enriched sample, and it could be clearly seen that the amplified products showed green fluorescence by ultraviolet light irradiation, even brighter than before enrichment. There was the same length of the amplified product before and after the enrichment (Fig. 2F), which proved that the CDE enrichment step has no effect on the AC-PCR amplification process. Therefore, DNA of *Salmonella* can be directly enriched and detected by CDE-based single-tube AC-PCR protocol within 17 min.

Specificity of single-tube AC-PCR for detection of Salmonella

In order to determine the specificity of the single-tube AC-PCR for the detection of *Salmonella*, 5 species of bacteria including *Bacillus cereus*, *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Listeria monocytogenes,* and *Vibrio parahaemolyticus* were tested. The genomes of *Salmonella* and five other bacteria were extracted using TIANamp bacteria DNA kit according to the manufacturer's instructions. As shown in Fig. 3A, no fluorescence signal was observed for the other strains and no template control (NTC) except for *Salmonella*, indicating that the single-tube AC-PCR could specifically identify *Salmonella* rather than the other stains. The specificity could be further demonstrated by the UV light irradiation result (Fig. 3B). Only the amplification product of *Salmonella* has obvious green fluorescence, while the others have no fluorescence.

Sensitivity of single-tube AC-PCR for detection of Salmonella

To evaluate the sensitivity of the single-tube protocol for *Salmonella* detection, different concentrations of *Salmonella* culture fluids were detected. As shown in Fig. 4A, the fluorescence signals gradually increased with the increasing concentrations of culture fluids ranging from 1.0×10^5 to 1.0×10^0 CFU/mL. This result was consistent with the target 39-bp amplification products in gel electrophoresis (Fig. 4B). In addition, as shown in Fig. 4C, the Ct value increased linearly with the increasing negative logarithm (lg) value of *Salmonella* culture fluid concentrations, ranging

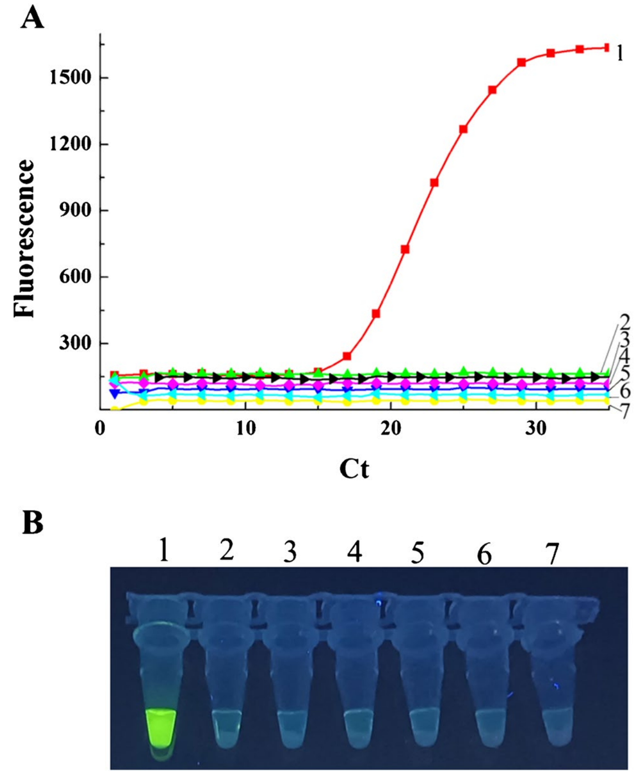

Fig. 3 Specificity of the CDE-based single-tube platform. A Real-time fluorescence curves of AC-PCR. Curves 1–6, respectively, represented that the targets were genomic DNA of *S. typhimurium, S. aureus, L. monocytogenes, E. coli* O157: H7, *B. subtilis*, and *V. parahaemolyticus*. Curve 7 represented the NTC. B Corresponding colorimetric result for AC-PCR products after amplification from A

from 1.0×10^5 to 1.0×10^1 CFU/mL. The regression equation was $Ct = 13.299\ (-\lg C) - 134.35$ (*C* is the concentration of *Salmonella* culture fluids, $R^2 = 0.9796$). It means the single-tube AC-PCR showed good linearity and sensitivity in detecting *Salmonella*. Moreover, tenfold serial dilutions of culture fluids before being enriched were detected using AC-PCR. The UV light irradiation result showed that the single-tube AC-PCR could be used to detect *Salmonella* as low as 1.0×10^0 CFU/mL (Fig. 4D(a)), while conventional AC-PCR without single-tube enrichment could only detect 1.0×10^2 CFU/mL (Fig. 4D(b)). This result is consistent with the Ct value obtained by amplification (Fig. 4E). As such, it is successful for the proposed single-tube analysis of *Salmonella* detecting based on CDE. Besides, the lowest detectable concentration was 100 times lower than the conventional method without nucleic acid enrichment.

Validation of Salmonella detection in artificially spiked samples

Additionally, a major bottleneck for PCR-based detection of foodborne pathogen is the necessity to purify the biospecimen

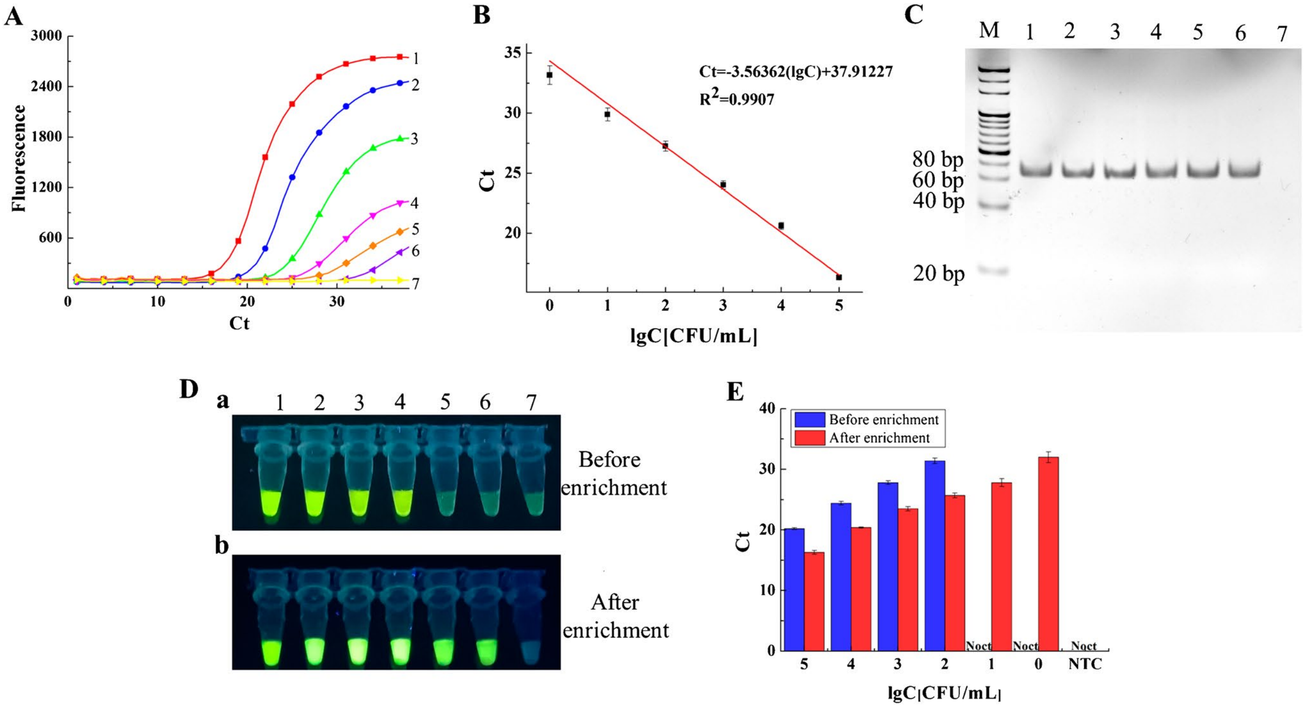

Fig. 4 Sensitivity of the CDE-based single-tube platform. A Real-time fluorescence curves of AC-PCR. Curves 1–6, respectively, represented that the concentration of Salmonella bacteria solution was 10^5–10^0 CFU/mL. Curve 7 represented the NTC. B Relationship between the Ct values and the logarithmic values of concentration of Salmonella bacteria solution. Error bars showed mean standard deviations of three determinations. C PAGE image for AC-PCR products after amplification from B, M was 20-bp DNA ladder. D Corresponding colorimetric result for AC-PCR products before (a) and after (b) CDE enrichment. 1–6, respectively, represented that the concentration of *Salmonella* bacteria solution was 10^5–10^0 CFU/ mL. 7 represented the NTC. E Corresponding Ct value of D

prior to reaction. Therefore, we assess the detection performance of the single-tube AC-PCR on artificially spiked samples. Oysters, fish, and lamb artificially contaminated by *Salmonella* were prepared as the test samples. As the content of *Salmonella* in the spiked samples decreased, the Ct value of each sample commonly increased at regular intervals. The dynamic detection range of *Salmonella* in oyster (Fig. 5A) and fish (Fig. 5B) samples was 1.0×10^1 to 1.0×10^5 CFU/mL. The regression curve respectively showed correlation coefficients (R^2) which were 0.9839 and 0.9929, which confirmed the highly linear relationship between the Ct value and the logarithm of *Salmonella*. The detection sensitivity of the single-tube AC-PCR for oyster and fish samples was 1.0×10^1 CFU/mL, which is sufficient to detect the human infective dose of foodborne *Salmonella* which causes clinical gastrointestinal symptoms ($\approx 1.0\times10^5$ cells) [32]. The dynamic detection range of *Salmonella* in lamb (Fig. 5C) was 1.0×10^2 to 1.0×10^5 CFU/mL. The regression curve showed that the correlation coefficients (R^2) was 0.9910. The detection limit in lamb was 1.0×10^2 CFU/mL, which is also enough to detect the human infective dose of foodborne *Salmonella*. Figure 5D–F show that the detection limit of the single-tube protocol using UV light irradiation for *Salmonella* is as low as 1.0×10^1 CFU/mL in oysters and fish, and 1.0×10^2 CFU/mL in lamb. It was consistent with the amplification results which was interpreted by the real-time fluorescence curve. In particular, the colorimetric results could be interpreted visually. Additionally, we sought to compare the extraction efficiency from infected fish samples using our CDE-based single-tube protocol with a commercial DNA extraction kit. As shown in Table 1, 32 samples were detected with *Salmonella* infection by conventional culture method. Of these specimens, 32 were known to be *Salmonella*-positive based on the commercial kit extraction, whereas only 31 of these specimens were identified as *Salmonella*-positive with the single-tube analysis. This result demonstrated that the single-tube analysis yielded a sensitivity of 96.88%. In contrast, all 74 specimens with negative culture method results were identified as *Salmonella*-negative both with the commercial kit extraction and single-tube analysis, which corresponded to a specificity of 100.0%, which demonstrated the reliability of detection results. Especially the single-tube protocol extracted DNA within 3 min, which is faster than the commercial DNA extraction kit > 30 min (Table 2). Therefore, DNA of *Salmonella* could be directly extracted and PCR-quantified from complex-infected sample with our single-tube protocol, at efficiencies comparable to DNA extraction with the commercial DNA extraction kits.

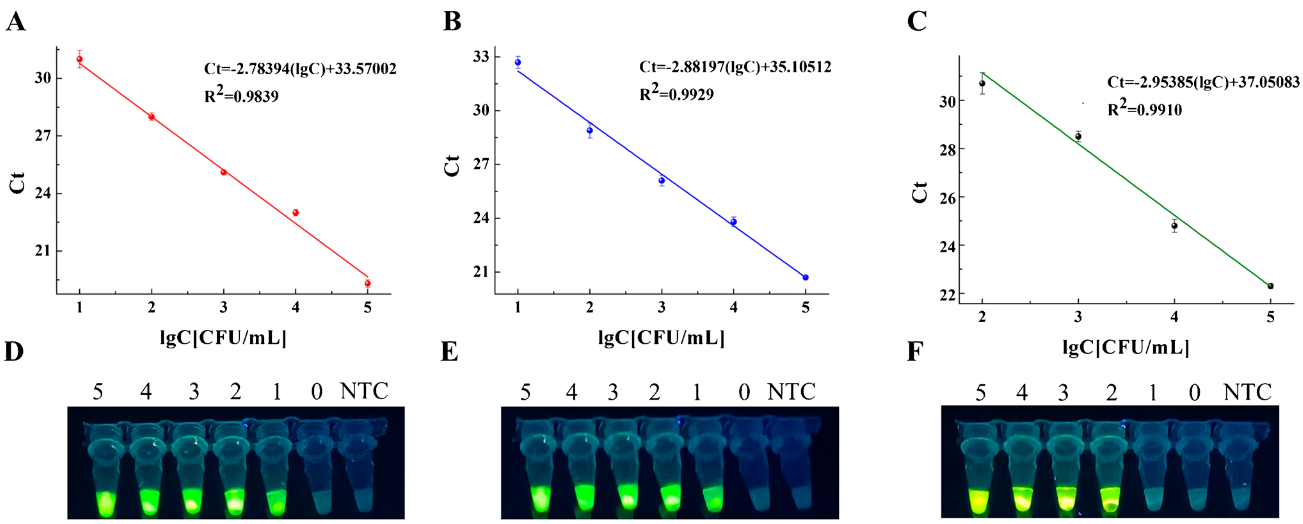

Fig. 5 Sample-to-answer detection in artificially spiked with *Salmonella* using the CDE-based single-tube platform. Standard curves of PCR and colorimetric result of the artificially spiked samples of oysters (A, D), fish (B, E), and lamb (C, F). 5–0, respectively, represented that the concentration of *Salmonella* in artificially spiked was 10^5–10^0 CFU/ mL

Table 1 Comparison of *Salmonella* detection by single-tube analysis and commercial kit directly from fish samples

	Positive ($n=32$)	Negative ($n=74$)	Total ($n=106$)
Single-tube analysis	31	75	106
Commercial kit	32	74	106

Table 2 Overall comparison of the various extraction procedures

Method	Total extraction time	Cost
Single-tube analysis	3 min	$0.001
Centrifugal column kit	30 min	$1.318
Magnetic bead-based assay	30 min	$2.384

Conclusion

The ability to extract and quantify nucleic acids forms the foundation of testing and tracking infections, such as that for the current SARS-CoV-2 pandemic and some foodborne pathogenic bacteria infection to trace the source. In this study, a CDE-based single-tube AC-PCR protocol was developed. The protocol integrated in situ DNA extraction, amplification, and visualization for ultra-fast detection of *Salmonella*. Chitosan, which is positively charged at pH = 5.0, enabled the efficient extraction of the *Salmonella* DNA through electrostatic interaction. To simplify the extraction steps, our single-tube protocol made use of self-settling characteristics of chitosan-functionalized DE, achieving centrifugal-free extraction within 3 min. Therefore, our protocol could extract DNA directly from biological samples, avoiding the time-consuming operation and reagent wasting such as numerous binding, washing, and eluting by silica-based columns. The CDE-based single-tube protocol not only overcame many inherent problems of traditional nucleic acid extraction methods, but also reduced the detection limit of subsequent amplification methods by 100 times. The CDE-based single-tube AC-PCR protocol met all the major requirements for *Salmonella* detection, including no amplification interference, nucleic acid enrichment ability, and high sensitivity. This system also showed excellent performance in the detection of artificial infection samples, and the significant distinction between the positive and negative samples could be easily and visually obtained. We are sure the system would provide new clues for foodborne pathogen detection and benefit point-of-care testing (POCT).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00216-022-03904-z.

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Declarations

Conflict of interest The authors declare no competing interests.

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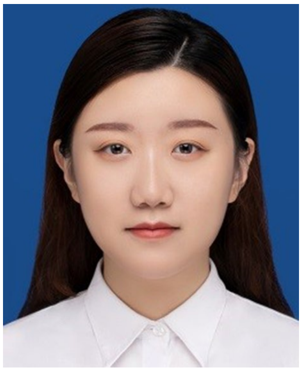

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