Direct Detection of Foodborne Pathogens via Proximal DNA Probe-based CRISPR-Cas12 Assay

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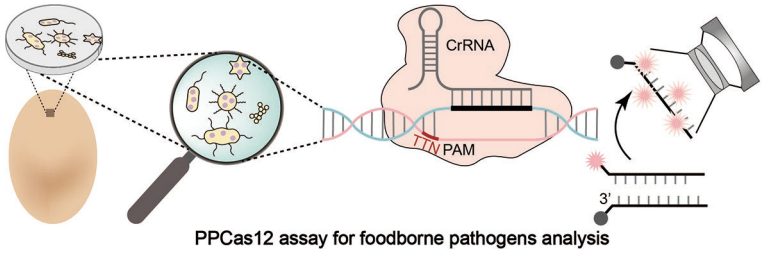
ABSTRACT

Foodborne pathogens can cause illness. Existing tools for detecting foodborne pathogens are typically lengthy and require complex protocols. Here, we report an assay to directly analyze pathogenic genes based on CRISPR-Cas12 system. This new test, termed Proximal DNA Probe-based CRISPR-Cas12 (PPCas12), makes possible the detection of foodborne pathogens without amplification steps. The elimination of the nucleic acid amplification reduced processing time, complexity and costs in the analysis of foodborne pathogens. The substitution of the frequently used dually labeled DNA reporter with proximal DNA probe in the PPCas12 assay led to a 4-fold sensitivity enhancement. PPCas12 offered a limit of detection of 619 Colony Forming Units in the detection of *Salmonella enterica* (*S. enterica*) without nucleic acid amplification process. The specific recognition of genes via PPCas12 allowed distinguishing *S. enterica* from other foodborne pathogens. The PPCas12 assay was applied in the screening of *S. enterica* contamination on fresh eggs with high precision. Hence, the new PPCas12 assay will be a valuable tool for on-site monitoring foodborne pathogens.

KEYWORDS

Salmonella enterica; CRISPR-Cas12; Genes; Nucleic acid analysis; Food safety



PPCas12 assay for foodborne pathogens analysis

INTRODUCTION

Foodborne diseases are of great concern globally due to their frequent occurrence and impact.[1-4] For instance, in the US, The Centers for Disease Control and Prevention (US CDC) estimated that there were 48 million food-related illness cases in the US each year, 128,000 of which requiring hospitalizition and resulting in 3,000 deaths.[3] *Salmonella enterica* (*S. enterica*) is one of the most common causes of foodborne infections worldwide.[3, 5] It causes typhoid fever，diarrhoea， vomiting and a life-threatening systemic infection due to *S. enterica* having several pathogenicity islands (SPI-1 and SPI-2) each including genes encoding for different virulence traits.[6-8] The SPIs encode secretion systems for the delivery of effector molecules into host cells, which leads to the systemic spread of disease.[7] Monitoring *S. enterica* contamination in food and water is therefore very important.

Currently, microbial culture methods, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) are the most powerful tools for *S. enterica* detection.[9-10] The microbial culture method is historically considered the golden standard to identify pathogens. This approach has the advantages of being inexpensive and simple to use, but one of its main impediments is the long sample-to-answer turnaround time: it requires at least 2-3 days to yield results.[11-13] Complementary to it, molecular tools mainly target proteins or genes of pathogens,

and this makes posible reducing the analysis time.[14] For example, ELISA, which uses antibodies to identify pathogens, can yield a color change as a response to the presence of a target pathogen.[15-16] ELISA is highly sensitive and rapid, which is promising for on-site detection of *S. enterica* contamination.[17] However, antibodies are costly, difficult to preserve, and environment-sensitive. Furthermore, antibodies can be very difficult to obtain for specific strains. In contrast, PCR allows detecting any bacterial gene, resulting in capability to potentially detecting specific foodborne pathogens. However, the need to carry out nucleic acid amplification at 3 different temperatures in PCR increases the cost and complexity of the analysis. Emerging isothermal nucleic acid amplification techniques, such as loop-mediated isothermal amplification (LAMP)[18], recombinase polymerase amplification (RPA)[19] and rolling circle amplification (RCA)[20], eliminate the use of costly temperature-control instruments and even allow detecting pathogens in a single test tube. Nevertheless, the amplification process still complicates the assay for foodborne pathogens and may cause amplicon pollution.[21]

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR associated proteins (Cas) modules are adaptive immunity systems that are present in archaea and bacteria.[22,23] CRISPR/cas12a complex shows target-induced single strands DNAse (ssDNAse) activation.[22, 24] Upon recognition of the DNA target, activated Cas nucleases indiscriminately cleaves single-stranded non-target nucleic acids. This feature has been exploited to detect the presence of specific DNA *in vitro* by non-specific degradation of reporter DNA.[25-26] Particularly, CRISPR/Cas assays have been developed for the detection of foodborne pathogens based on PCR, RPA and RCA technologies. [27-30]

Herein, we developed a nucleic acid amplification-free assay for detecting *S. enterica* by

using CRISPR-Cas12a to directly identify the signature regions of bacterial DNA. To improve the sensitivity of the assay, we designed a proximal DNA probe to serve as the substrate of Cas12a. We screened the gene sites in S. enterica that can effectively and specifically activate Cas12a via the hybridization of the CRISPR RNA (crRNA) and target sequences. The PPCas12 assay can specifically distinguish S. enterica from other foodborne pathogens. We further applied the PPCas12 assay to detect S. enterica in fresh eggs. The results indicate that the CRISPR-Cas system can enrich the toolbox for food safety analysis.

MATERIAL AND METHODS

Materials and reagents. DNA oligonucleotide sequences were chemically synthesized by Sangon Biotech (Shanghai, China) (Table S1, in the Supporting Information). Oligonucleotide sequences with less than 60 nucleotides (nt) were purified by PAGE. Chemically labeled *Rep 1*, *Rep 1A, Reporter* and oligonucleotide sequences over 60 nt were purified by HPLC.

LbCas12a (cat. no. M0653T), NEBuffer 2.1 (cat. no. B7202S), dNTP solution mix (cat. no. N0447S) rNTP solution mix (cat. no. N0466S), and DNase I (2000 U/mL) (cat. no. M0303S) were bought from New England Biolabs (Ipswich, MA, USA). T7 RNA Polymerase (20 U/μL) (cat. no. EP0111), phi 29 DNA Polymerase (10 U/μL) (cat. no. EP0094), and Platinum™ SYBR™ Green qPCR SuperMix-UDG (with ROX) (cat. no. 11744100) were acquired from Thermo Fisher Scientific (Waltham, USA). Agarose, 6 × Loading buffer, 50 × TAE buffer, and 10000 × Gelred dye were purchased from Beijing DingGuo Biotechnology Co., Ltd. (Beijing, China). Ezup Column Bacteria Genomic DNA Purification Kit (cat. no. B518255-0100) was

purchased from Sangon Biotech (Shanghai, China). Eggs were purchased from Chengdu Ito Yokado. All solutions were prepared using molecular-biology-grade H_2O (Corning, New York, USA).

Pathogenic bacteria preparation. *Salmonella enterica* (*S. enterica*) (ATCC 14028), *Vibrio parahaemolyticus* (*V. parahaemolyticus*) (ATCC 17802), *Bacillus cereus* (*B. cereus*) (ATCC 14579), *Staphylococcus aureus* (*S. aureus*) (ATCC 29213), *Listeria monocytogenes* (*L. monocytogenes*) (ATCC 19115) and *Escherichia coli* (*E. coli*) (ATCC 43889) were supplied by China Center of Industrial Culture Collection (CICC). *V. parahaemolyticus* strains were activated in peptone medium with 3% NaCl, and adjusting the medium to pH 8.5. Other strains were grown separately in nutrient broth medium for 6-20 h in an orbital shaker (37 ℃, 120 rpm).

DNA extraction. The suspension of bacteria (1 mL) was centrifuged (1 min, 8000 rpm). Bacteria was collected from the pellet and was sonicated at 70 Hz (1 min). Total DNA was extracted from the lysate using Ezup Column Bacteria Genomic DNA Purification Kit. Briefly, 180 μL of the digestion buffer and 20 μL proteinase K were firstly added to bacteria lysate and incubated at 56 ℃ for 1 h, followed by the addition of 200 μL buffer BD and incubation at 72 ℃ for 10 min. Then, 200 μL ethanol, 500 μL PW solution and 500 μL of washing solution were added and centrifuged to obtain DNA extract. Finally, DNA was dissolved in 20 μL H_2O, and kept under -20 ℃ until use.

***In vitro* transcription of crRNA.** CrRNAs were synthesized through *in vitro*-transcription. The synthesis reaction was carried in a volume of 40 μL with 4 μL 10×phi29 DNA Polymerase reaction buffer (33 mM Tris-acetate (pH 7.9 at 37 °C), 10 mM Mg-acetate, 66 mM K-acetate,

0.1% (v/v) Tween 20, 1 mM DTT), 4 μL promoter (10 μM), 4 μL L-crRNA (10 μM) and 8 μL H_2O. Then, the mixture was annealed at 90 °C for 5 min followed by an incubation at 20 °C for 30 min. Then, 4 μL phi29 Polymerase (1 U/μL) and 1 μL dNTP mix (25 mM each for dATP, dGTP, dCTP, and dTTP) were added into the mixture and incubated at 30 °C for 30 min to yield duplex DNA strands. 8 μL 5×transcription buffer, 1 μL T7 RNA polymerase (20 U/μL), 1 μL rNTP mix (25 mM each for rATP, rGTP, rCTP, and rUTP), and 5 μL H_2O were added and incubated at 37 °C overnight to obtain the transcription products of crRNA. To remove the DNA templates, 1 μL DNase I (2,000 U/mL) was added and incubated at 37 °C for 4 h, subsequently inactivated it at 75 ℃ for 5 min.

S. enterica **detection procedures.** PPCas12 assay analysis was carried 40 μL containing 4 μL 10 × NEB Buffer 2.1, 4 μL LbCas12a (10 μM), 4 μL crRNA (2 μM). The mixture remained at 37 °C for 5 min to allow the binding of crRNA with Cas 12a. Then, 2 μL Target RNA (5 μM)/ DNA extraction solution, and 4 μL *Rep 1* (3 μM) were incubated at 37 °C for 35 min for cleavage. Finally, 4 μL 10 × phi29 buffer, 4 μL *Rep 1*A (3 μM) and 14 μL H_2O were added and denatured at 90 °C for 5 min, followed by an incubation at 30 °C for 30 min. The sample was analysed in a microplate reader Synergy H1 (BioTek, USA). The excitation wavelength was 480 nm and the emission spectra was recorded from 510 nm to 600 nm.

Gel electrophoresis. Non-denaturing gel electrophoresis analysis was carried using 3% agarose and Gelred at a final reaction volume of 6 μL, containing 5 μL of oligonucleotides and 1 μL of gel loading buffer. Then, the separation was carried out at 150 V for 30 min. The gel wasimaged using a Gel Doc XR+ system (Bio-Rad, USA).

Detecting ***S. enterica*** **on fresh eggs.** *S. enterica (*100 μL 10^7 Colony Forming Units (CFU)

/mL, 10^6 CFU/mL, 10^5 CFU/mL, and 10^4 CFU/mL) was added on the surface eggshells to mimic relevant contamination. 500 μL of washing buffer (0.85% (w/v) NaCl) was used to collect *S. enterica* on the surface of the eggs. Total DNA was obtained from the *S. enterica*-contaminated eggshells using Ezup Column Bacteria Genomic DNA Purification Kit and dissolved in H_2O (20 μL). DNA solution (2 μL) was used for analysis based on the described protocols of the PPCas12 assay (reference).

Real-time PCR analysis. Real-time PCR was carried out using Platinum™ SYBR™ Green qPCR SuperMix-UDG (with ROX). The temperature program was: 50 °C for 5 min, 95 °C for 5 min, and then 45 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 45 s. The fluorescent signal emitted was captured at 72 °C. The system was carried inby missing 10 μL SYBR Green Supermix, 2 μL DNA extraction, 1 μL forward primer, 1 μL reverse primer and 6 μL H_2O. Primers were designed using Primer 5.0. The forward and reverse primers designed for amplifying 16S rDNA gene of *S. enterica* were 5'-GTGTAGCGGTGAAATGCGTAG-3' and 5'-CAAGGGCACAACCTCCAAG-3', respectively.

RESULTS AND DISCUSSION

Working principle of the PPCas12 assay. The PPCas12 assay allows amplification-free detection of pathogenic bacteria through direct recognition of pathogenic gene signatures using CRISPR-Cas12a (Scheme 1).

CrRNAs were screened to identify the gene sites with a protospacer adjacent motif (PAM) sequences (TTN) in pathogenic bacteria from food samples. CRISPR-Cas12a was recruited via specific hybridization between the crRNA and pathogenic genes. Instead of using doubly

158 labeled DNA reporter, a proximal DNA probe was developed by designing a 6-
159 carboxyfluorescein (FAM)-labeled single-stranded DNA (ssDNA) strand, *Rep 1*, and a Black
160 Hole Quencher-1 (BHQ1)-labeled ssDNA strand, *Rep 1A*. The proximity of the fluorogen, FAM,
161 and the quencher group, BHQ1 via the hybridization between *Rep 1* and *Rep 1A* lead to a highly
162 efficient quenching of the FAM fluorescence. The target-gene activated crRNA/Cas12a would
163 abundantly cleave the ssDNA *Rep 1*, thus released the fluorogen, FAM from the proximal DNA
164 probe. The presence of the target pathogenic bacteria can efficiently turn on the fluorescence of
165 the proximal DNA probe. Consequently, pathogenic bacteria can be quantified by measuring
166 the fluorescence of the PPCas12 assay. The substitution of the commonly used doubly labeled
167 DNA reporter by the proximal DNA probe increased the sensitivity of CRISPR-Cas12 system
168 for detecting pathogenic bacteria.

169 The structure of the proximal DNA probe and the process of target gene-activated
170 crRNA/Cas12a were firstly verified via fluorescence and electrophoretic analysis. The presence
171 of *Rep 1* emitted a strong fluorescence. The addition of *Rep 1A* shifted the fluorescence intensity
172 from 6115 to 364 (Figure 1A). The result manifested the proximity of *Rep 1* and *Rep 1A* probes
173 which led to a highly efficient quenching effect of FAM. The electrophoresis image also
174 indicated a successful hybridization of *Rep 1* and *Rep 1A* (lane 3) (Figure 1C).

175 We designed a crRNA targeting a fragment of 16S gene of *S. enterica* (ATCC 14028) and
176 confirmed its specificity using Blast database (Figure S1 and S2, in Supporting Information).
177 The presence of target gene sequences led to an important increase of the fluorescence intensity
178 to 5634 (Figure 1B). This indicates that Cas12a can be efficiently activated by target genes, and
179 the proximal DNA probe can offer a high turn-on ratio for sensing target genes. The

electrophoretic result further showed the activation process of Cas12a. In the absence of target DNA, the addition of Cas12a/crRNA did not reduce the amount of the proximal DNA probe (lane 2) (Figure 1C). In contrast, the presence of target DNA led to an efficient cleavage of the proximal DNA probe (lane 1).

crRNA Design. 16S rDNA segments of *S. enterica* were sequenced using the universal primer, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Figure S1, in the Supporting Information). Target DNA sites were chosen according to the report of Zhang group[22]. 13 DNA fragments were selected as candidates for *S. enterica* detection. CrRNA was composed of two parts: a 21 nt conserved scaffold that recognized and combined with Cas 12a protein, and a 20 nt spacer that was complementary to target DNA. Accordingly, 13 crRNAs were designed with target binding sites (highlighted in blue) and guide sites (highlighted in yellow) (Figure S2, in the Supporting Information). Target gene is composed with two single DNA strands, Target A (T-A) and Target B (T-B). T-A was the DNA strand of genes that hybridized with crRNA. T-B was the antisense strand that contained a PAM sequences (TTN).

To predict the binding capability of crRNAs, thermodynamic energy was calculated using NUPACK. The net thermodynamic energy for crRNA binding with target genes defined as $\Delta\Delta G=\Delta G_{crRNA\text{-}T\text{-}A} - \Delta G_{T\text{-}A/B}$, where $\Delta G_{crRNA\text{-}T\text{-}A}$ was the standard free energy of the hybridization of crRNA with T-A, and $\Delta G_{T\text{-}A/B}$ was the standard free energy of the hybridization of crRNA with T-A and T-B. The $\Delta\Delta G$ of 7 sites (03, 04, 05, 08, 10, 11, 12) was calculated to below 0 (Table S2, in Supporting Information). This suggests that the 7 crRNAs possess high affinities to target DNA. However, the complex structure of gene sequences and the sequence bias of

202 Cas12a/crRNA could also be affected by the activation process of Cas 12a protein. Thus, we
203 used the designed crRNAs to test the presence of 16S rDNA of *S. enterica* using a doubly
204 labeled DNA reporter (figure). The fluorescence intensity corresponding to the signal and
205 background from the DNA reporter changed by using different crRNAs. The ratios of the signal
206 to the background using crRNAs targeting 03, 04, 05, 08, 11 and 12 (varied from 28.6 to 34.7)
207 were significantly higher than crRNAs targeting other gene fragments (Figure S3, in the
208 Supporting Information). This indicates that these six DNA fragments presented higher
209 activation cleavage activity. The experiment result gave 85.8% concordance with the
210 calculation prediction. The structure and net thermodynamic energy of the six crRNA and target
211 DNA fragments are shown in Figure 2A. Furthermore, the six target DNA segments were
212 further tested using the doubly labeled DNA reporter and the proximal DNA probe in 100 nM
213 (Figure S4 in the Supporting Information). Lowering the concentration of the reporter reduced
214 the fluorescence background, but it resulted in weak ratio of signal to background (Figure S1,
215 Figure S2, in the Supporting Information). The concentration of the reporter was increased and
216 the presence of DNA (both synthesized *in vitro* and extracted from *S. enterica* at 10^6 CFU in
217 PPCas12 and traditional assay) was tested. The tests indicated that crRNAs target the site 04
218 yielded the highest ratio of signal to background (Figure 2B, C).

219 We further tested the use of crRNA targeting the site 04 to compare the performance of
220 assay using the doubly labeled DNA reporter and the proximal DNA probe under the optimal
221 conditions. Synthesized short DNA sequences and DNA extracted from *S. enterica* were both
222 used in the test. When using the double label DNA reporter, no significant differences were
223 found between the test of 100 CFU *S. enterica* and 0 CFU *S. enterica* (P=0.16, Figure 2E). In

224 contrast, when using of the proximal DNA probe, the presence of 100 CFU can be distinguished
225 from that of the background signal ($P < 0.01$, Figure 2D). These results indicate the potential
226 benefit of using the design of proximal probes would be an improvement of the sensitivity of
227 the CRISPR-Cas12-based assay.

228 **Detection of *S. enterica*.** Based on former experiments, the detection performance of the
229 PPCas12 assay was assessed with a range of concentrations of *S. enterica* (ranging from 1 CFU
230 to 10^8 CFU) (Figure 3A). Fluorescence intensity of the cleaved reporter gradually grew with
231 the concentration of *S. enterica* increasing from 1 CFU to 10^8 CFU (Figure 3B). The fluorescent
232 signal increased over the concentration range from 10^3 CFU to $10^{6.7}$ CFU ($y=791.1x+1758.6$,
233 $R^2=0.9809$). The detection limit (LOD),calculated from the concentration corresponding to the
234 fluorescence signal of three times the standard deviation of the blank without *S. enterica,* was
235 estimated to be 619 CFU. . The sensitivity of the PPCas12 assay, allowing to detect lower than
236 1000 CFU, was achieved without nucleic acid amplification process.

237 The CRISPR-Cas12-based gene test using the commonly used doubly labeled DNA reporter
238 was also examined as comparison. The LOD of the assay was 3110 CFU (Figure 3C). The
239 substitution of often used dually labelled DNA reporter with the proximal DNA probe, yielded
240 an enhancement of sensitivity by 4.02 times.

241 The specificity of the PPCas12 assay was tested for detecting *S. enterica*. Six predominant
242 foodborne bacteria *S. enterica*, *V. parahaemolyticus*, *B. cereus*, *S. aureus*, *L. monocytogenes*,
243 and *E. coli* were used as non-target pathogens. Taking into account the application of PPCas 12
244 assay in subsequent samples with different infection levels of pathogenic bacteria, three

concentrations (10^6 CFU, 10^4 CFU and 10^2 CFU) of bacteria were added in the PPCas12 assay under the same conditions done as for detecting *S. enterica* (Figure. 4A). For all concentrations, the addition of *S. enterica* could increase the fluorescence intensity of the PPCas12 assay. The addition of other bacteria, however, led to a fluorescence signal close to the background signals. (Figure. 4B). To assess the discrimination ability of the PPCas12 assay, the discrimination factor was defined as the ratio between the fluorescence intensity change generated by target bacteria and non-target bacteria (Figure. 4C). The result showed that the discrimination factor of the PPCas12 assay fell in 2.66 to 56.06. The result indicated that the PPCas12 assay offer a specificity to distinguish *S. enterica* from other bacteria.

Detection of *S. enterica* pollution in fresh eggs. The use of the PPCas12 assay was assessed for monitoring *S. enterica* pollution in fresh eggs. Four concentrations (10^5 CFU, 10^4 CFU, 10^3 CFU, 10^2 CFU) of *S. enterica* were spiked onto eggs' surface. Then, total DNA was extracted from the *S. enterica* -contaminated eggs using Ezup Column Bacteria Genomic DNA Purification Kit. The collected DNA was analyzed using the developed PPCas12 assay.

Figure 5B illustrates that target bacteria with a concentration falling in the detection dynamic range of the assay (10^3 CFU-10^5 CFU). The recovery ratios were 95.3% to 105.6%, and the relative standard deviation (RSD) < 6.22% (Table S3, in the Supporting information). qPCR was used to assess the results tested by the PPCas12 assay (Figure 5C). The result indicated that the PPCas12 assay can detect *S. enterica* pollution in complex food samples.

In summary, we constructed a nucleic acid amplification-free assay for detecting foodborne pathogens based on CRISPR-Cas12. The design of proximate DNA reporter allowed

to increase the sensitivity of the CRISPR-Cas12-based assay, making posible the detection of *S. enterica* below 1000 CFU. crRNA/Cas 12a can contribute to enhance the specificity to the PPCas12 assay for detecting *S. enterica* against other pathogenic bacteria. Due to the programmability of crRNA, the PPCas12 assay can be readily used to detect other foodborne pathogens via changing the binding sites of crRNA. Considering its wide applicability and simplicity, the PPCas12 assay is highly promising for monitoring pollution from foodborne pathogens, thus it is a powerful food safety tool.

Funding

This work was supported by the National Natural Science Foundation of China (No. 22074100), the Experimental Technique Funds of Sichuan University (No. SCU201130), the Green Manufacturing Project of Ministry of Industry and Information Technology of China, the Researchers Supporting Project Number (RSP-2021/138), King Saud University, Riyadh, Saudi Arabia.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. 16S rDNA sequence of *S. enterica*; sequences of 13 crRNAs and hybridized target DNA of *S. enterica*; fluorescence response using different crRNAs for detecting *S. enterica;* DNA oligonucleotide sequences, thermodynamic calculation using different crRNAs, and determination of *S. enterica* spiked in the eggshells.

Notes

The authors declare no conflict of interest.

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436

FIGURE CAPTIONS

Scheme 1. Scheme of the PPCas12 assay for detecting foodborne pathogens. Rep 1 was the substrate of Cas12a.

Figure 1. Validation of the PPCas12 assay. Fluorescence analysis of the proximal DNA probe (A) and (B) target DNA activation of Cas12a process. The concentrations of crRNA 04, Cas12a, target DNA 04, *Rep 1* and *Rep 1A* were 200 nM, 100 nM, 250 nM, 300 nM, and 300 nM, respectively. Fluorescent signal was detected with an excitation wavelength of 480 nm, and the emission ranged from 510 nm to 600 nm. (C) Electrophoresis analysis for the proximal DNA probe and the target DNA-activation of Cas12a process.

Figure 2. CrRNA design and significance analysis for *S. enterica* detection. (A) Sequences of 6 crRNAs and their target DNAs (numbering 03, 04, 05, 08, 11 and 12); Fluorescence analysis in the presence of target DNA synthesized *in vitro* and extracted from *S. enterica* using the proximal DNA probe (B) and the dually labeled DNA reporter (C). (D) The PPCas12 assay allowed detection of 100 CFU of *S. enterica*. (E) The assay using the dually labeled DNA reporter showed no significant difference in the presence 100 CFU of *S. enterica*. The concentrations of crRNA, Cas12a, and reporter were 200 nM, 100 nM, 250 nM, and 300 nM, respectively. Fluorescent signal was detected with an excitation wavelength was 480 nm, and the emission was ranged from 510 nm to 600 nm. Data in B, C, D and E was mean ± s.d. (n=3). Statistical significance data in D, E was obtained by two-tailed unpaired Student's t-test: $*P < 0.05$, $**P < 0.01$.

Figure 3. Quantification of *S. enterica* using crRNA/Cas12a. (A) Schematic illustration of pathogenic bacteria analysis via CRISPR-Cas12a. (B) Typical fluorescence spectra and

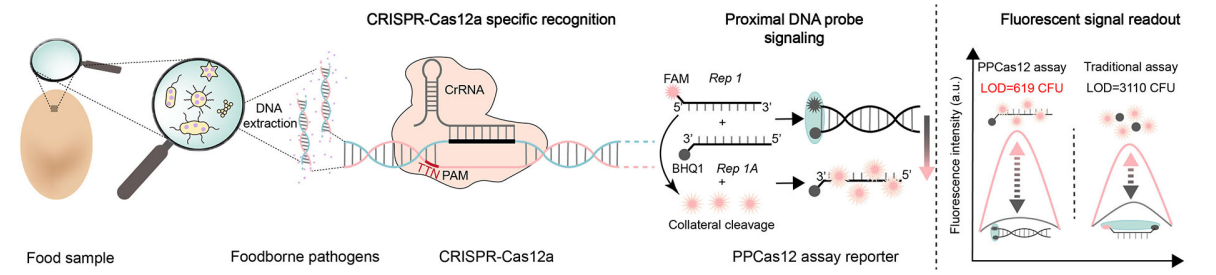
intensity of the PPCas12 assay upon addition of different concentrations of *S. enterica* (ranging from 10^0 CFU to 10^8 CFU); (C) Typical fluorescence spectra and intensity of the assay using doubly labeled DNA reporter assay upon addition of different concentrations of *S. enterica*. Inner: The linear relationship between *S. enterica* concentration and fluorescence response. The error bars indicate the standard deviation of three parallel measurements for each concentration of target *S. enterica*. The concentrations of crRNA, Cas12a, and reporter were 200 nM, 100 nM, 250 nM, and 300 nM, respectively. Fluorescent signal was detected with an excitation wavelength was 480 nm, and the emission was ranged from 510 nm to 600 nm. Data in B and C was mean ± s.d. (n=3).

Figure 4. Specificity of the PPCas12 assay for detecting *S. enterica*. (A) PPCas12 assay with the addition of target pathogens and non-target pathogens. (B) Fluorescence intensity of the PPCas12 assay upon addition different concentrations of foodborne bacteria. (C) Discrimination factor obtained using the PPCas12 assay for *S. enterica* detection. Discrimination factor was defined as the ratio between the fluorescence intensity change generated by target bacteria and that induced by non-target bacteria. The concentrations of crRNA, Cas12a, and reporter were 200 nM, 100 nM, 250 nM, and 300 nM, respectively.A fluorescent signal was screened from 510 nm to 600 nm with 480nm as excitation . Data in B and C was mean ± s.d. (n=3).

Figure 5. Detection of *S. enterica* contamination on fresh eggs. (A) Workflow for detecting *S. enterica* on fresh eggs. (B) Fluorescence response and recovery of *S. enterica* on fresh eggs using the PPCas12 assay. The concentrations of crRNA, Cas12a, and reporter were 200 nM, 100 nM, 250 nM, and 300 nM, respectively. Fluorescent signal was detected with an excitation

481 wavelength was 480 nm, and the emission was ranged from 510 nm to 600 nm. (C)

482 Determination of *S. enterica* on fresh eggs using qPCR. Data in B and C are mean ± s.d. (n=3).



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484 **Figure 1 1.** Illustration of the use of PPCas12 assay for detecting foodborne pathogens.

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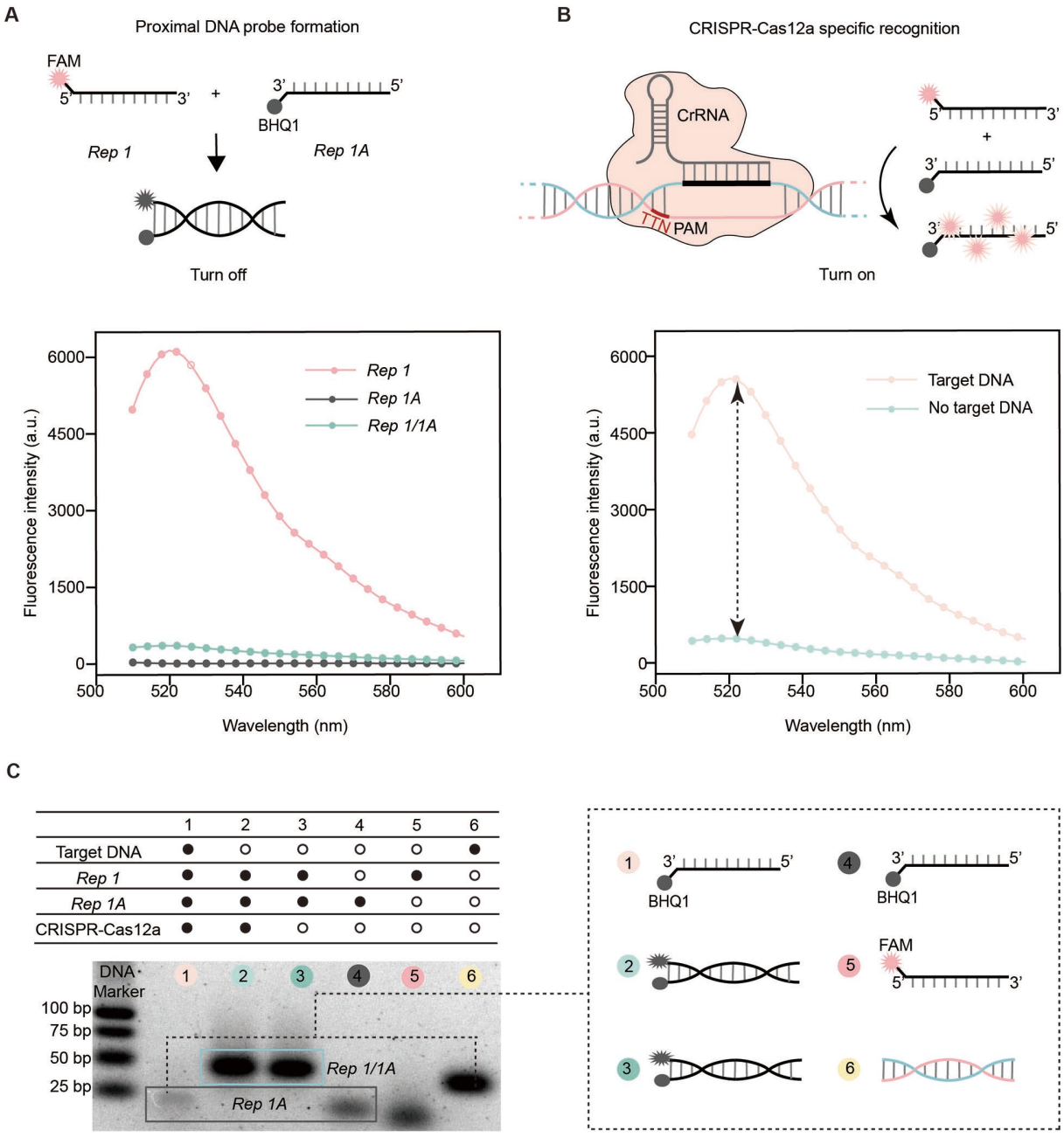

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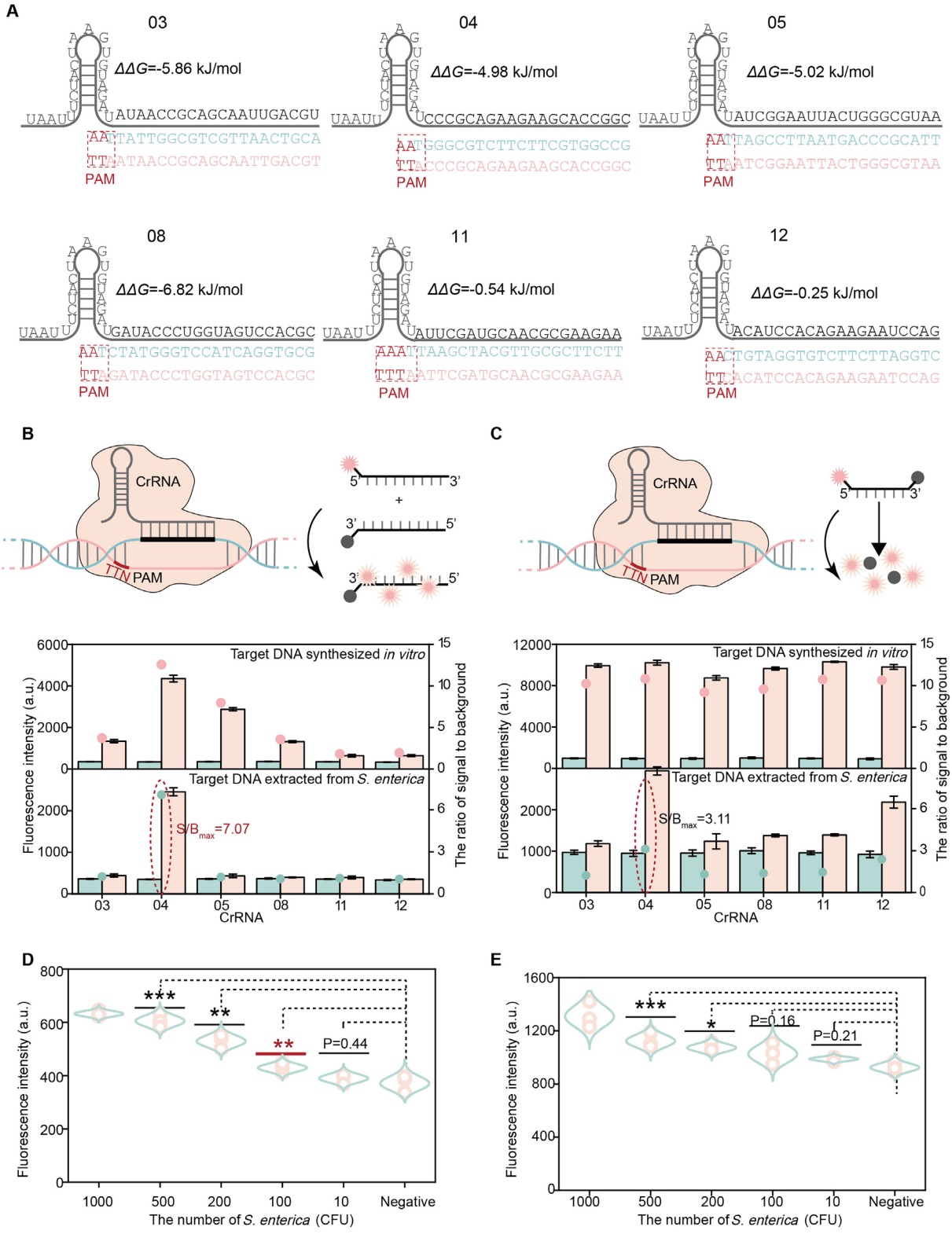

493

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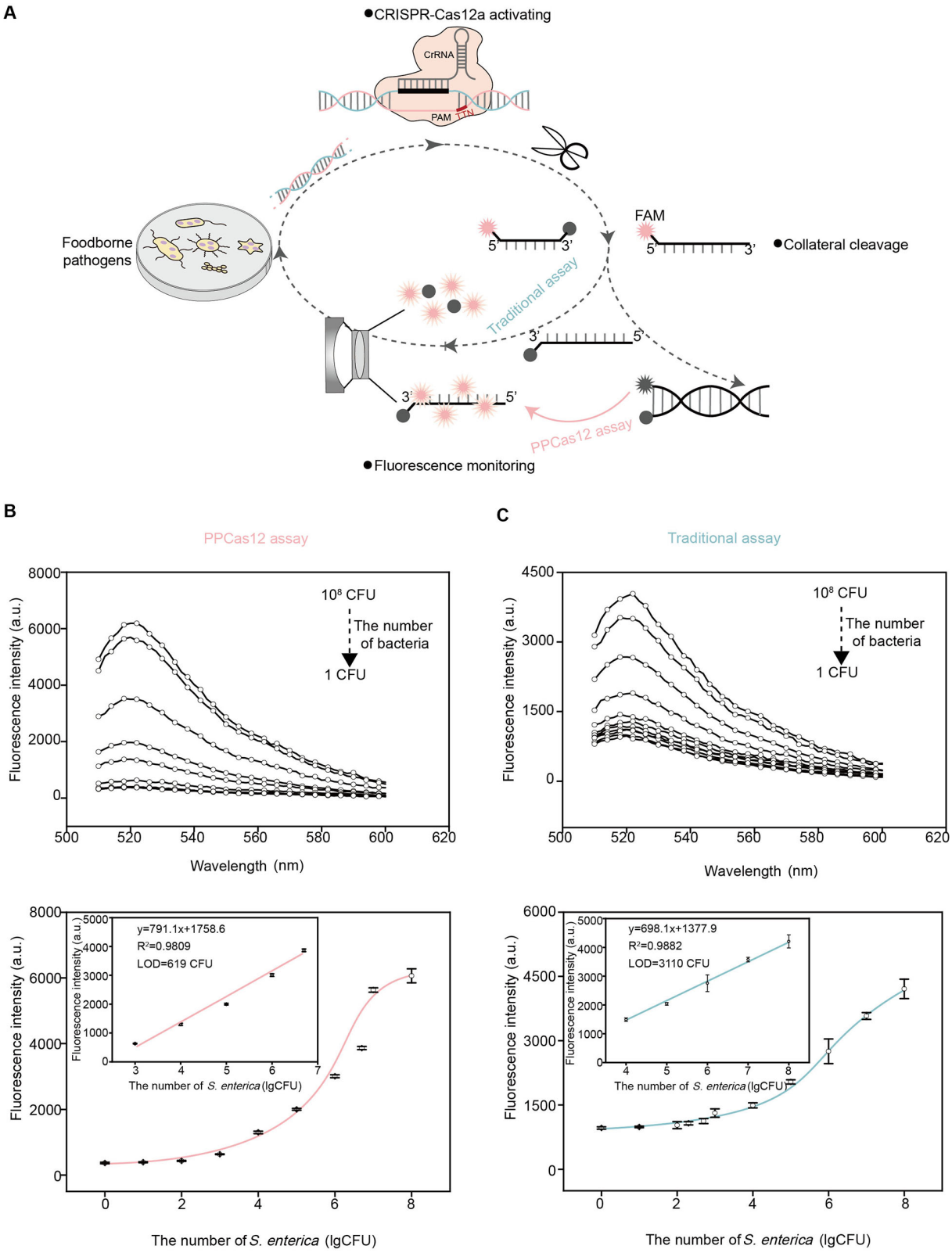
495 **Figure 2.** CrRNA design and significance analysis for *S. enterica* detection. (A) Sequences of

6 crRNAs and their target DNAs (numbering 03, 04, 05, 08, 11 and 12); Fluorescence analysis in the presence of target DNA synthesized *in vitro* and extracted from *S. enterica* using proximal DNA probe (B) and the dually labeled DNA reporter (C). (D) The PPCas12 assay allowed detecting 100 CFU of *S. enterica*. (E) The assay using the dually labeled DNA reporter showed no significant difference in the presence 100 CFU of *S. enterica*. The concentrations of crRNA, Cas12a, and reporter were 200 nM, 100 nM, 250 nM, and 300 nM, respectively. Fluorescent signal was detected with an excitation wavelength of 480 nm, and the emission ranged from 510 nm to 600 nm. Data in B, C, D and E was mean ± s.d. (n=3). Statistical significance data in D, E was obtained by two-tailed unpaired Student's t-test: *$P < 0.05$, **$P < 0.01$.



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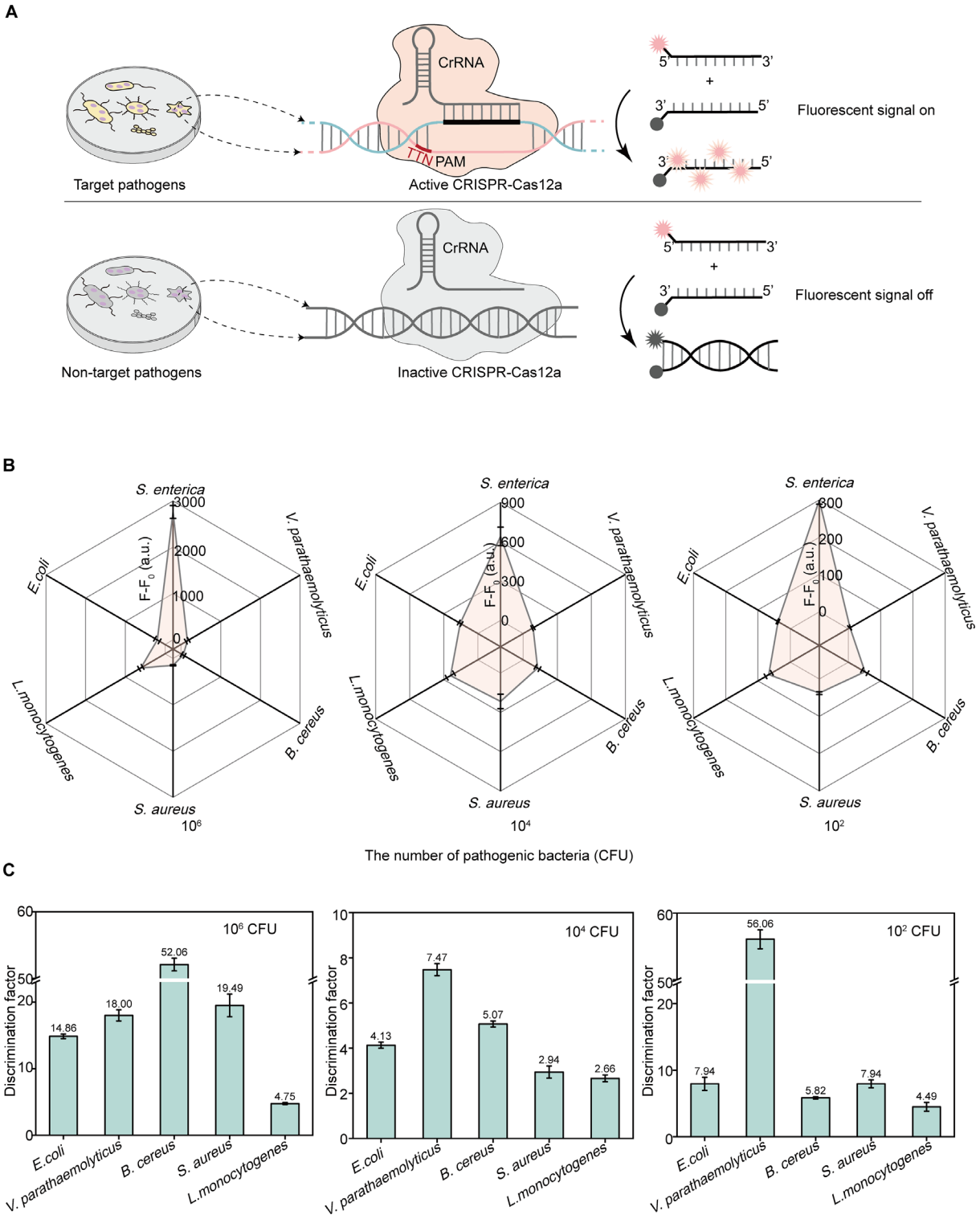

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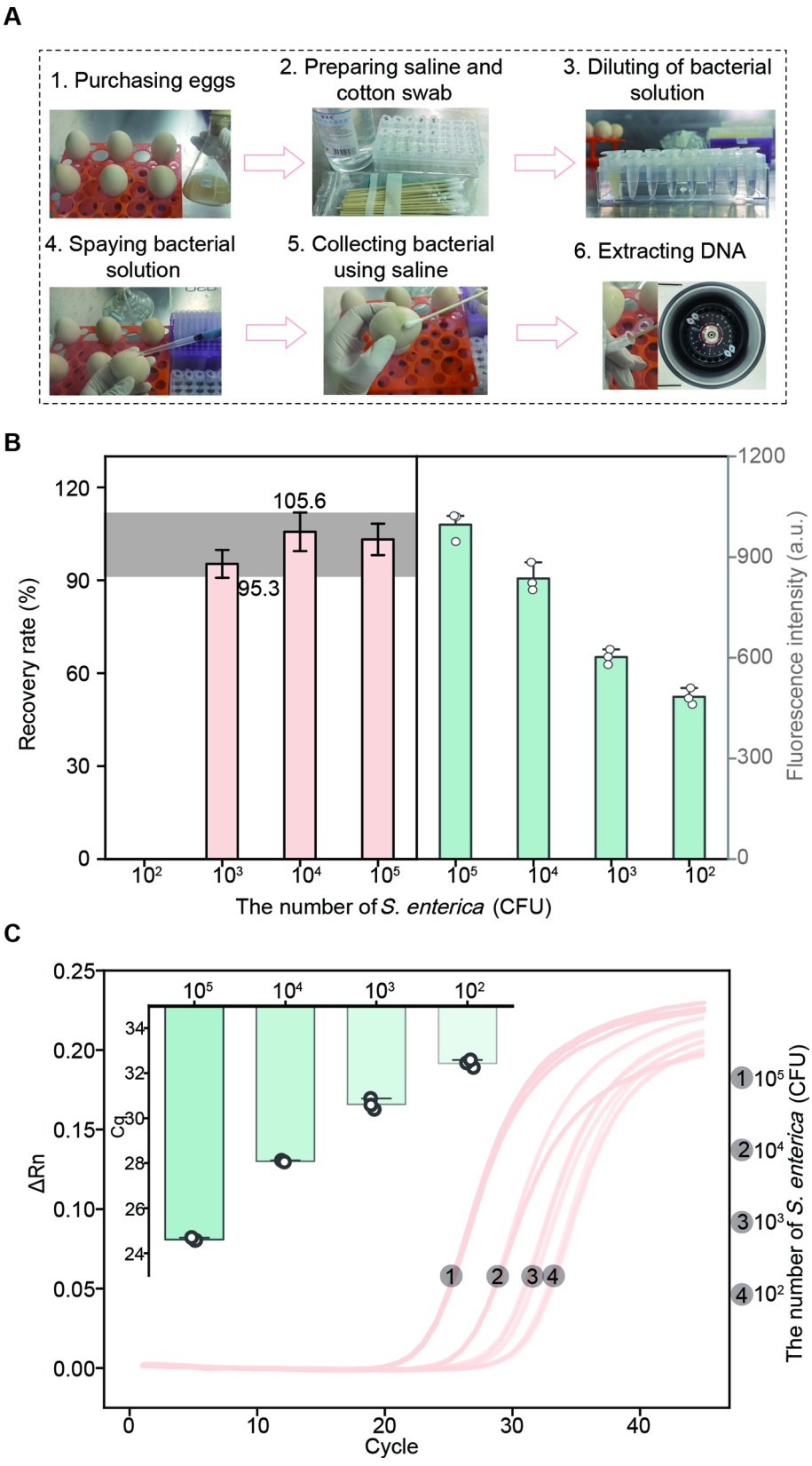

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using the PPCas12 assay. The concentrations of crRNA, Cas12a, and reporter were 200 nM, 100 nM, 250 nM and 300 nM, respectively. The excitation wavelength was 480 nm and the detection of the emitted radiation was between 510 nm and 600 nm. (C) Determination of *S. enterica* on fresh eggs using qPCR. Data in B and C are mean ± s.d. (n=3).

TABLE OF CONTENTS

Supporting Information

Direct Detection of Foodborne Pathogens via Proximal DNA Probe-based CRISPR-Cas12 Assay

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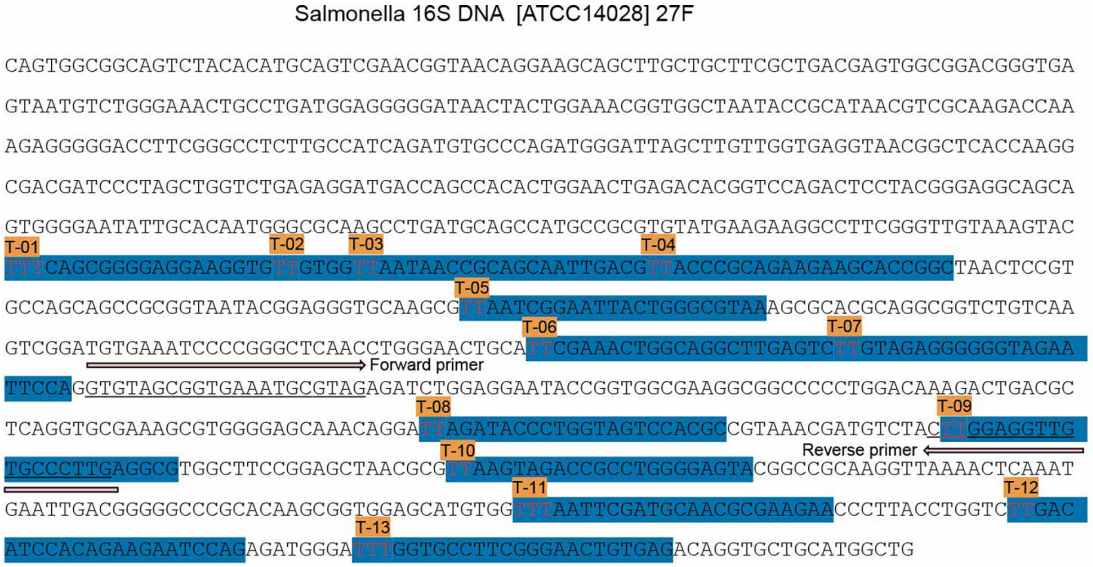

552

553 **Figure S1.** 16S rDNA sequence of *S. enterica*. 13 target DNAs were highlighted in blue and named

554 in yellow. The primers used for qPCR of *S. enterica*- contaminated eggshells were marked using a

555 pink arrow.

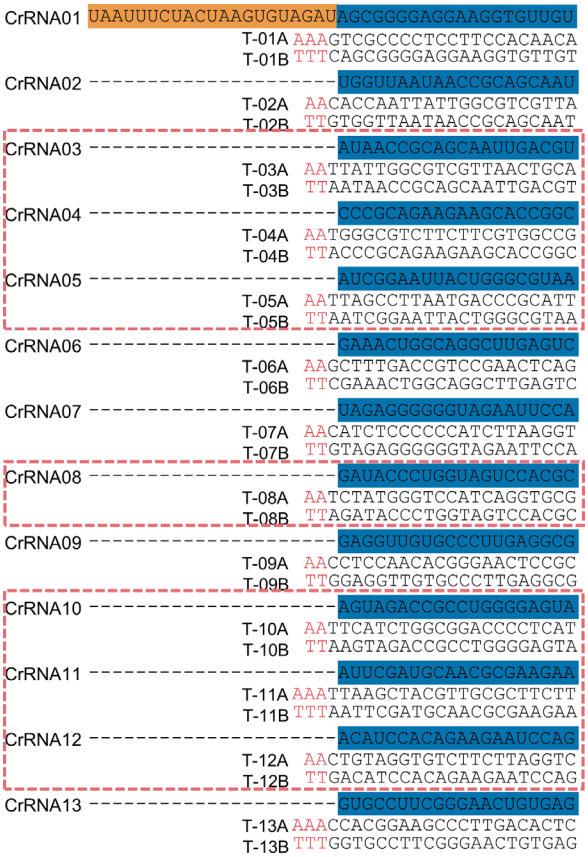

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Figure S2. The sequences of 13 crRNAs and hybridized target DNA of *S. enterica*. The binding sites and guide sites of crRNA were highlighted in blue and yellow, respectively. The PAMs of target DNA fragments were marked in red.

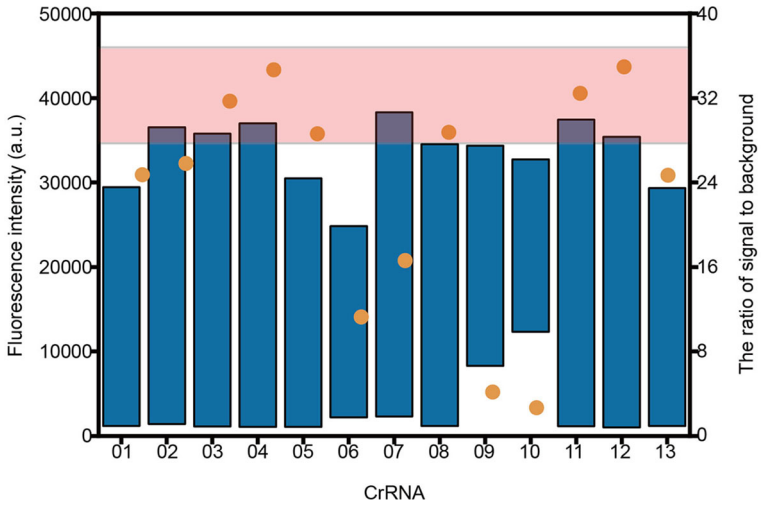

Figure S3. Fluorescence response and ratio of signal to background using different crRNAs for the detection of *S. enterica*. A dually labeled DNA reporter was used. The concentrations of crRNA, Cas12a, target DNA, and *Reporter* were 200 nM, 100 nM, 250 nM, and 500 nM, respectively. Fluorescent signal was detected with an excitation wavelength of 480 nm and emission ranging from 510 nm to 600 nm.

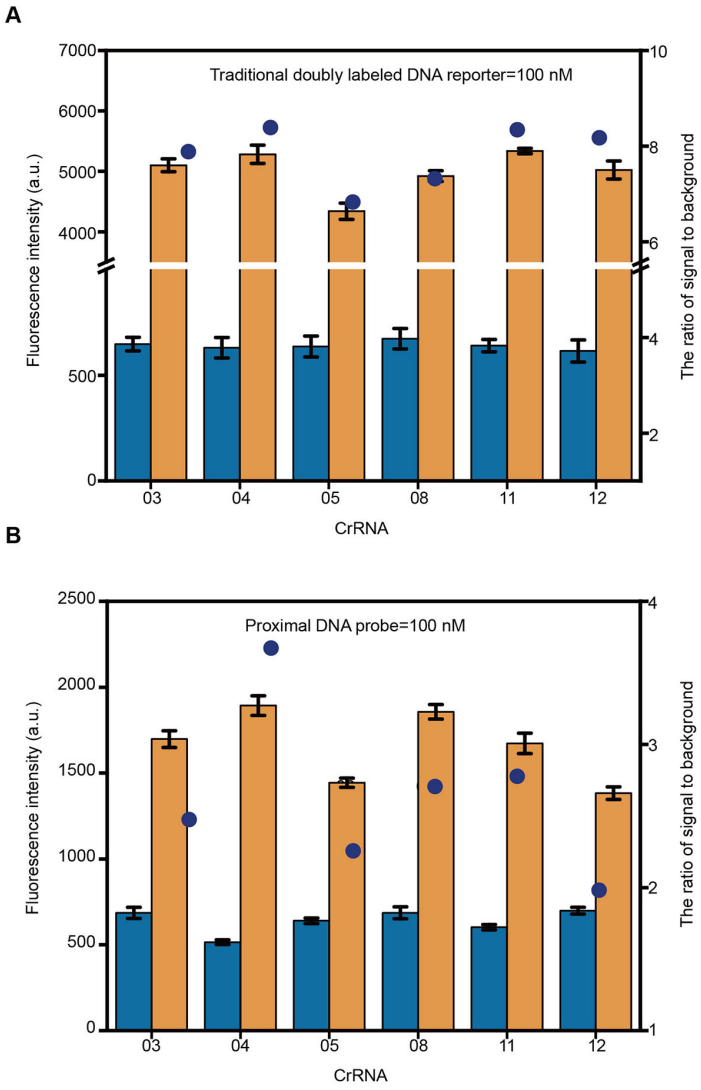

Figure S4. Fluorescence response and signal to background ratio using crRNAs targeting 03, 04, 05, 08, 11 and 12 sites using traditional doubly DNA reporter (**A**), and the proximal DNA probe (**B**). The concentrations of crRNA, Cas12a, target DNA, and reporter (or the proximal DNA probe) were 200 nM, 100 nM, 250 nM, and 100 nM, respectively. Fluorescent signal was detected with an excitation wavelength of 480 nm, and emission between 510 nm to 600 nm.

576 **Table S1. DNA oligonucleotide sequences**

	Oligonucleotide name	Sequence (5'-3')
01	L-crRNA-01	ACAACACCTTCCTCCCCGCTATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTA
02	L-crRNA-02	ATTGCTGCGGTTATTAACCAATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTA
03	L-crRNA-03	ACGTCAATTGCTGCGGTTATATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTA
04	L-crRNA-04	GCCGGTGCTTCTTCTGCGGGATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTA
05	L-crRNA-05	TTACGCCCAGTAATTCCGATATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTA
06	L-crRNA-06	GACTCAAGCCTGCCAGTTTCATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTA
07	L-crRNA-07	TGGAATTCTACCCCCCTCTAATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTA
08	L-crRNA-08	GCGTGGACTACCTGGGTATCATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTA
09	L-crRNA-9	CGCCTCAAGGGCACAACCTCATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTA
10	L-crRNA-10	TACTCCCCAGGCGGTCTACTATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTA
11	L-crRNA-11	TTCTTCGCGTTGCATCGAATATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTA
12	L-crRNA-12	CTGGATTCTTCTGTGGATGTATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTA
13	L-crRNA-13	CTCACAGTTCCCGAAGGCACATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTA
14	T-A-1	ACAACACCTTCCTCCCCGCTGAAA
15	T-B-1	TTTCAGCGGGGAGGAAGGTGTTGT
16	T-A-2	ATTGCTGCGGTTATTAACCACAA
17	T-B-2	TTGTGGTTAATAACCGCAGCAAT
18	T-A-3	ACGTCAATTGCTGCGGTTATTAA
19	T-B-3	TTAATAACCGCAGCAATTGACGT
20	T-A-4	GCCGGTGCTTCTTCTGCGGGTAA
21	T-B-4	TTACCCGCAGAAGAAGCACCGGC
22	T-A-5	TTACGCCCAGTAATTCCGATTAA

23	T-B-5	TTAATCGGAATTACTGGGCGTAA
24	T-A-6	GACTCAAGCCTGCCAGTTTCGAA
25	T-B-6	TTCGAAACTGGCAGGCTTGAGTC
26	T-A-7	TGGAATTCTACCCCCCTCTACAA
27	T-B-7	TTGTAGAGGGGGGTAGAATTCCA
28	T-A-8	GCGTGGACTACCAGGGTATCTAA
29	T-B-8	TTAGATACCCTGGTAGTCCACGC
30	T-A-9	CGCCTCAAGGGCACAACCTCCAA
31	T-B-9	TTGGAGGTTGTGCCCTTGAGGCG
32	T-A-10	TACTCCCCAGGCGGTCTACTTAA
33	T-B-10	TTAAGTAGACCGCCTGGGGAGTA
34	T-A-11	TTCTTCGCGTTGCATCGAATTAAA
35	T-B-11	TTTAATTCGATGCAACGCGAAGAA
36	T-A-12	CTGGATTCTTCTGTGGATGTCAA
37	T-B-12	TTGACATCCACAGAAGAATCCAG
38	T-A-13	CTCACAGTTCCCGAAGGCACCAAA
39	T-B-13	TTTGGTGCCTTCGGGAACTGTGAG
40	Promoter	TAATACGACTCACTATAGGG
41	*Rep 1*	5’ -FAM-GAGTCATTTGCCTTTAGGATTCTAGACAGACCTAAG -3’
42	*Rep 1A*	5’-GGTCTGTCTAGAATCCTAAAGGCAAATGACTC-BHQ1-3’
43	*Reporter*	5’-FAM-GGGTTTTTTGGG-BHQ1-3’
44	Forward primer	GTGTAGCGGTGAAATGCGTAG
45	Reverse primer	CAAGGGCACAACCTCCAAG

577

578 * L-crRNAs were synthesized to serve as templates in the *in vitro*-transcription of crRNAs. L-promoter

579 combined with promoter was marked in blue. The target gene was composed with two single DNA strand,

580 Target A (T-A) and Target B (T-B). T-A was the single DNA strand that would combine with crRNA. T-B

581 was the other single DNA strand that contained PAM sequences (TTN) highlighted in red.

582

583 **Table S2. Thermodynamic calculation using different crRNAs.**

	crRNA (5'-3')	T-A (5'-3')	T-B (5'-3')	$\Delta G_{T\text{-}A/B}$ (kJ/mol)	$\Delta G_{CrRNA\text{-}T\text{-}A}$ (kJ/mol)	$\Delta\Delta G$ (kJ/mol)
01	UAAUUUCUACUAAGUGUAGAUAGCGGGGAGGAAGGTGUUGU	ACAACACCTTCCTCCCCGCTGAAA	TTTCAGCGGGGAGGAAGGTGTTGT	-168.95	-166.27	2.68
02	UAAUUUCUACUAAGUGUAGAUUGGUUAAUAACCGCAGCAAU	ATTGCTGCGGTTATTAACCACAA	TTGTGGTTAATAACCGCAGCAAT	-149.61	-146.09	3.52
03	UAAUUUCUACUAAGUGUAGAUAUAACCGCAGCAAUUGACGU	ACGTCAATTGCTGCGGTTATTAA	TTAATAACCGCAGCAATTGACGT	-151.11	-156.98	-5.86
04	UAAUUUCUACUAAGUGUAGAUCCCGCAGAAGAAGCACCGGC	GCCGGTGCTTCTTCTGCGGGTAA	TTACCCGCAGAAGAAGCACCGGC	-169.49	-174.47	-4.98
05	UAAUUUCUACUAAGUGUAGAUAUCGGAAUUACUGGGCGUAA	TTACGCCCAGTAATTCCGATTAA	TTAATCGGAATTACTGGGCGTAA	-146.01	-151.87	-5.86
06	UAAUUUCUACUAAGUGUAGAUGAAACUGGCAGGCUUGAGUC	GACTCAAGCCTGCCAGTTTCGAA	TTCGAAACTGGCAGGCTTGAGTC	-159.57	-155.68	3.89
07	UAAUUUCUACUAAGUGUAGAUUAGAGGGGGGUAGAAUUCCA	TGGAATTCTACCCCCCTCTACAA	TTGTAGAGGGGGGTAGAATTCCA	-149.23	-147.39	1.84
08	UAAUUUCUACUAAGUGUAGAUGAUACCCUGGUAGUCCACGC	GCGTGGACTACCAGGGTATCTAA	TTAGATACCCTGGTAGTCCACGC	-153.25	-160.07	-6.82
09	UAAUUUCUACUAAGUGUAGAUGAGGUUGUGCCCUUGAGGCG	CGCCTCAAGGGCACAACCTCCAA	TTGGAGGTTGTGCCCTTGAGGCG	-168.07	-167.61	0.46
10	UAAUUUCUACUAAGUGUAGAUAGUAGACCGCCUGGGGAGUA	TACTCCCCAGGCGGTCTACTTAA	TTAAGTAGACCGCCTGGGGAGTA	-156.56	-162.42	-5.86
11	UAAUUUCUACUAAGUGUAGAUAUUCGAUGCAACGCGAAGAA	TTCTTCGCGTTGCATCGAATTAAA	TTTAATTCGATGCAACGCGAAGAA	-157.73	-158.27	-0.54
12	UAAUUUCUACUAAGUGUAGAUACAUCCACAGAAGAAUCCAG	CTGGATTCTTCTGTGGATGTCAA	TTGACATCCACAGAAGAATCCAG	-146.59	-146.84	-0.25
13	UAAUUUCUACUAAGUGUAGAUGUGCCUUCGGGAACUGUGAG	CTCACAGTTCCCGAAGGCACCAAA	TTTGGTGCCTTCGGGAACTGTGAG	-167.77	-134.66	33.11

*$\Delta G_{T\text{-}A/B}$ *and* $\Delta G_{crRNA\text{-}T\text{-}A}$ were defined as the free energy of hybridization of T-A and T-B and the hybridization of crRNA and T-A, respectively. Target gene is composed with Target A (T-A) and Target B (T-B). T-A was the DNA strand of genes that hybridized with crRNA. T-B was the antisense strand that contained a PAM sequence (TTN). The net thermodynamic energy for crRNA binding with target genes $\Delta\Delta G=\Delta G_{crRNA\text{-}T\text{-}A} - \Delta G_{T\text{-}A/B}$. The standard free energy of hybridization reaction was calculated using NUPACK. The calculation of the $\Delta G_{crRNA\text{-}T\text{-}A}$ and $\Delta G_{T\text{-}A/B}$ was based on the use of the hybridized part of sequences of genes in Figure S1. crRNAs with a $\Delta\Delta G<0$ were shadowed in pink.

Table S3. Determination of *S. enterica* pollution in fresh eggs.

Added CFU	Found CFU	RSD n=3	Recovery (%)	RSD (%) n=3
10^5	$10^{5.15}$	0.25	103.20	5.09
10^4	$10^{4.22}$	0.25	105.64	6.22
10^3	$10^{2.85}$	0.13	95.30	4.48
10^2	-	-	-	-

Table S4. Comparation of analytical performance of CRISPR/Cas-based Foodborne Pathogens assay.

Method	Type of amplification	Response time	LOD	Reference
Lateral flow strip-Cas9nAR	Lateral flow strip combined with Cas9 nickase-triggered isothermal DNA amplification	3 h	100 CFU/mL	1
CRISPR Cas12a based lateral flow platform	Integrating nucleic acid isothermal amplification	70 min	1×1^0 CFU/reaction	2
Cas12aFDet-based detection	Polymerase chain reaction (PCR), Recombinase-aided amplification (RAA)	15 min	3.37×10^1 CFU/mL, 1.35×10^2 CFU/mL	3
CRISPR/Cas12a based electrochemical biosensor	Recombinase-assisted amplification (RAA)	2 h	26 CFU/mL	4
CRISPR/Cas12a-PER	Primer exchange reaction (PER)	2.5 h	19 CFU/mL	5

CRISPR/Cas12a-PRA	Recombinase Polymerase Amplification (RPA)	1 h	6.5×10^4 CFU/mL	6
RPA-Cas12a-FS	Recombinase Polymerase Amplification (RPA)	45 min	10 copies	7
CRISPR-Cas13a based bacterial detection platform	Polymerase chain reaction (PCR)	4 h	1 CFU/mL	8
CRISPR-Cas13a-based assay	Polymerase chain reaction (PCR)	2 h	10^0 CFU/ml	9
PPCas12 assay	Amplification-free	70 min	619 CFU	This work

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