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A One-Pot Toolbox Based on Cas12a/crRNA Enables Rapid Foodborne Pathogen Detection at Attomolar Level

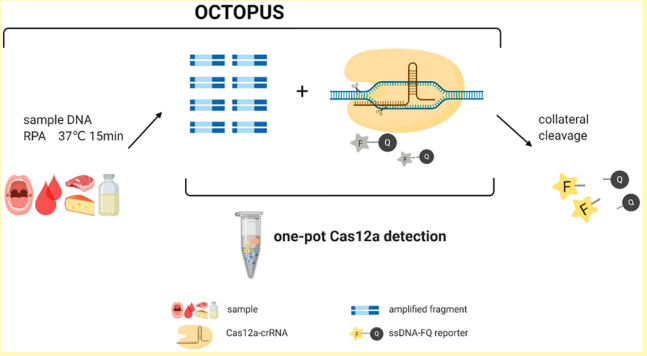
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ABSTRACT: Bacterial contamination accounts for more than half of food poisoning cases. Conventional methods such as colony-counting and general polymerase chain reaction are time-consuming, instrument-dependent, and sometimes not accurate. Herein, we developed a novel one-pot toolbox with precision and ultra sensitivity (OCTOPUS) platform for foodborne pathogen detection based on the mechanism in which Cas12a nontarget binding unleashes its collateral DNase activity. We demonstrated its application on two widespread foodborne bacteria, namely, *E. coli* O157:H7 and *Streptococcus aureus*, using specific crRNA targeting rfbE and nuc gene, respectively. For better sensitivity, recombinase polymerase amplification (RPA) was integrated without product purification. This one-pot detection, that is, RPA reagent, crRNA, and ssDNA-FQ reporter are all in one tube with the subsequent addition of Cas12a enzyme, was able to detect genomic DNA at the attomolar level. It omits an extra cap-opening process to avoid practical inconvenience and possible cross-sample contamination. Moreover, we demonstrated this platform for a real food matrix. A simple water boiling method for genome extraction together with one-pot assay achieved a limit of detection value of 1 CFU/mL in less than 50 min. This OCTOPUS technique integrates bacterial genome extraction, preamplification based on RPA, and Cas12a/crRNA cleavage assay.



KEYWORDS: *Cas12a, CRISPR, recombinase polymerase amplification, foodborne pathogen, real matrix*

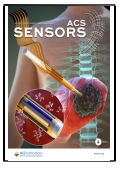
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Food safety is of great concern in human health, where bacterial contamination is a major risk factor.[1] Common foodborne pathogens are Shiga toxin-producing *Escherichia coli* (STEC), *Staphylococcus aureus*, *Salmonella enterica*, *Listeria monocytogenes*, *Vibrio* spp., and *Clostridium perfringens*. The clinical manifestations of patients with mild illness are mainly vomiting and diarrhea, and patients with severe illness may even die. Minimizing economic loss is also an incentive for effective pathogen detection.[2] Bacterial contamination may occur during any step in the farm-to-table continuum from industrial, environmental, animal, or human sources.[3] To prevent foodborne illness, it is necessary to accurately and rapidly detect the causative bacteria. Despite the fact that the traditional culture methods followed by colony counting are time-consuming and labor-intensive, they remain the standard for identifying bacterial pathogens and are generally adopted by the quality inspection departments.[4] Therefore, alternative techniques for rapid, sensitive, and practical detection of foodborne pathogenic bacteria are needed.

Current methods mainly include nucleic acid-based, immunologically based, and biosensor-based techniques.[5] Nucleic acid-based methods target specific genes in bacteria. Simple and multiple polymerase chain reaction (PCR) operates by cycles of three-step amplification. The amplified products are visualized by electrophoresis.[6] Real-time PCR or quantitative PCR adopts a TaqMan probe, SYBR green fluorescent dye, and molecular beacon, so that the PCR instrument can instantly show the detection results.[7,8] Loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) are novel methods that work under isothermal conditions, which is of great importance to on-site detection.[9−11] Nonetheless, detection methods that are more sensitive, time-efficient, and labor-saving and less dependent on instrumentation are required to meet all objectives for pathogen detection.[12]

The advent of CRISPR-Cas has provided brand new technologies that led to the emergence of rapid diagnostic methods. Cas12 and Cas13 among the Cas family are reported to generate collateral cleavage on DNA and RNA, respectively.[13−15] By designing a specific crRNA probe, Cas12a will activate its DNase activity upon binding to the target substrate; thus, a dual-labeled reporter will be cleaved and the separated

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fluorophore can emit fluorescence. This detection method could be used to distinguish target sequences of concentrations in the picomolar range. To expand the limited sensitivity, novel nucleic acid detection techniques for different purposes based on this mechanism have been developed in combination with other gene amplification methods. DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter), which combines RPA with Cas12a/crRNA based ssDNA-FQ reporter cleavage, can rapidly and accurately distinguish two genotypes of human papillomavirus in patient swab samples.[16] HOLMES (1 h low-cost multipurpose highly efficient system) employed the DNA collateral effect of Cas12a and PCR. HOLMES can be used to detect human single nucleotide polymorphism (SNP) genotypes and DNA viruses, and the sensitivity reached the attomole level.[17] Similarly, Gootenberg et al. integrated isothermal amplification of nucleic acid sequences and a Cas13a-based detection platform to develop SHERLOCK (Specific High-sensitivity Enzymatic Reporter UnLOCKing).[13] In a follow-up study, they characterized a four-channel platform, namely, SHERLOCKv2. Using four separate reporter sequences, each exploiting the orthogonal sequence or nucleic acid specificities of four Cas13 and Cas12a variants, SHERLOCKv2 demonstrated multiple RNA and DNA detection within a single sample.[18] Their applications have expanded to microbe detection, SNP genotyping and point of bed diagnostics, yet new techniques with more prominent features are still needed.[19−23]

Though Cas12a alone is less sensitive than Cas13, it omits the request of a transcription process when used in DNA detection and has the potential to push the detection sensitivity down to the attomolar level when combined with RPA.[16,17,24] However, in current platforms, isothermal nucleic acid amplification and Cas12a based target detection are two separate steps. One needs to transfer the amplification product to the Cas cleavage system for target detection, which is practically inconvenient and potentially leads to cross-contamination among samples.[25] Herein, we developed a novel one-pot toolbox with precision and ultra sensitivity (OCTOPUS) platform, based on Cas12a/crRNA, in which all reagents are added simultaneously in order to avoid an uncapping operation which increases the risk of aerosol contamination. This one-pot detection is challenging for Cas12a, because Cas12a and enzymes included in RPA share the same DNA substrate. Chen et al. intended to address this challenge by taking out some sample and then adding Cas12a, crRNA, and ssDNA-FQ. In this study, we introduced another strategy by storing Cas12a at the tube lid and spinning it 15 min post RPA preamplification.

Moreover, for one-pot detection's field application, the genomic DNA (gDNA) extraction process needs to be simple and practical. Also, interference from chemical components in the food itself shall be avoided. To address these two challenges, we adopted a water boiling method for bacterial genome extraction and isolated supernatant after centrifugation as the template for RPA preamplification and Cas12a/crRNA cleavage. Our data collected from milk samples were consistent with that of water, suggesting the stability of the one-pot toolbox.

With the presented OCTOPUS integrating bacterial genome extraction, RPA preamplification, and Cas12a/crRNA cleavage assay, we demonstrated its application on the two most common Gram-negative and positive bacteria in food matrix, namely, *Escherichia coli* O157:H7 and *Staphylococcus aureus*. *Escherichia coli* O157:H7 is a highly pathogenic serotype of Shiga-toxin-producing *E. coli* (STEC) with a foodborne etiology that poses a severe public health threat as well as related tremendous societal costs.[26,27] Patients show symptoms like diarrhea and hemorrhagic enteritis, while multiple complications may lead to death.[28] *Staphylococcus aureus* has strong viability and can cause infections worldwide, and thus there are many opportunities for food contamination. Both bacteria produce enterotoxin that resists high temperature, which can cause local suppurative infection, pseudomembranous enteritis, pneumonia, pericarditis, and, even worse, sepsis.[29] Thus, a technology that overcomes the need for laboratory facilities and is relatively low cost, fast, and sensitive would be highly valuable. Herein, we evaluated the practical applicability of OCTOPUS (Figure 1) for extremely rapid and sensitive detection of pathogenic bacterial strains in food samples.

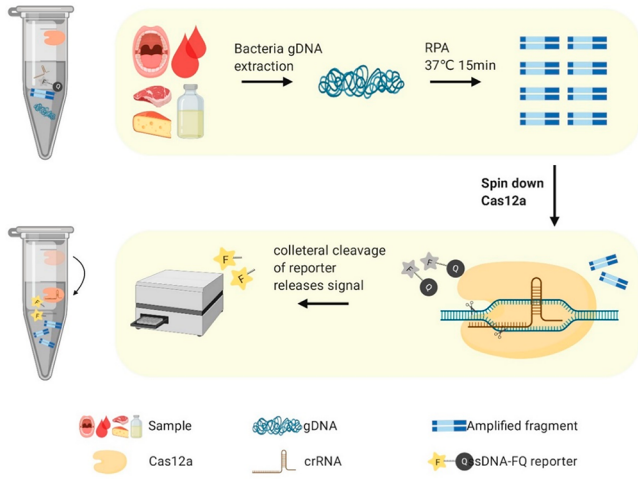


Figure 1. Schematic illustration of OCTOPUS showing the process of genomic DNA extraction, RPA preamplification, Cas12a/crRNA cleavage assay, and fluorescence readout. Sample extraction is to yield the bacterial genome, which can be finely acquired by 10 min boiling and 2 min centrifugation. The supernatant is then used for RPA preamplification and Cas12a/crRNA cleavage detection. RPA reagent, crRNA, and ssDNA-FQ reporter are all in one tube, with subsequent addition of Cas12a enzyme. Target binding of Cas12a will unleash its ability to digest ssDNA. The fluorophore HEX (F) is quenched by a quencher (Q) if intact, and emits fluorescence when cleaved, which is measured under microplate reader.

EXPERIMENTAL SECTION

In Vitro Transcription and Purification of Cas12a crRNAs. DNA template for crRNA was synthesized (Sangon Biotech), with a T7 promotor sequence added at its 3′ end. The DNA template was annealed with T7−3G oligonucleotide and 10× anneal buffer (50 μM each), by performing a 5 min denaturation, followed by slowly cooling the reaction to room temperature. Then, crRNAs were synthesized by incubating at 37 °C overnight with the High Yield RNA Synthesis Kit (NEB). Translation products were treated with DNase and purified using RNAXP clean beads following the manufacturer's protocol. crRNA was assessed via 200−300 nm absorbance spectrum on a Nanodrop spectrophotometer.

Bacteria Strains and Genome Extraction. We used the following bacterial strains: *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Salmonella enterica*, and *Lactobacillus casei*. Genomic DNA of the bacteria was extracted using Bacterial Genomic (Solarbio). As for one-pot detection, genomic DNA was extracted by using the water boiling method. One milliliter of bacteria solution, ranging from 10^8 to 10^0 CFU/mL, was centrifuged at 8000 × g for 2 min, and the supernatant

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was removed. The bacteria were resuspended in sterile deionized water and then centrifuged at 8000 × g for 2 min to remove the supernatant. Then, the bacteria were resuspended in 100 μL sterile deionized water and heated for 10 min at 100 °C. After that, the bacterial solution was centrifuged at 8000 × g for 2 min and the supernatant was used as the substrate.

Milk Sample Preparation. Milk sample was used for the simulated food matrix detection. *E. coli* or *S. aureus* was added in a milk sample (sterile 10% milk powder) to achieve different concentrations from 10^8 to 10^0 CFU/mL using 10-fold serial dilutions. This food matrix was incubated in a table concentrator at 37 °C for 0 or 4 h. Then, the DNA extracted at each time point was used as the template for one-pot detection.

Cas12a/crRNA Nucleic Acid Detection. The Cas12a-mediated cleavage assay contained 50 nM LbCas12a, 50 nM crRNA, 100 nM ssDNA-FQ reporter, and 2 μL of NEBuffer 2.1 with 2 μL of substrate dsDNA in a 20 μL reaction volume. LbCas12a was purchased from New England Biolabs, and ssDNA-FQ reporter was synthesized with poly-A nucleotides labeled with HEX at the 5′ end and a quencher at the 3′ end. The reaction was performed in a 384 well microplate and incubated in Infinite 200 Pro (Tecan) for 90 min at 37 °C with fluorescence measurements taken every 5 min (λ_{ex}: 533 nm; λ_{em}: 559 nm).

Recombinase Polymerase Amplification. The primer sequences for rfbE and nuc have been published previously.[30,31] Standard RPA reactions were performed according to the instructions of the TwistAmp Basic Kit (TwistDx). Each reaction contained one pellet, 14.75 μL of rehydration buffer, 0.48 μM forward and reverse primers, an appropriate amount of genomic DNA, 1.25 μL of magnesium acetate (MgAc), and sterile water up to 25 μL. The mixture was incubated at 37 °C for 30 min.

One-Pot Detection. One-pot detection combines RPA preamplification and Cas12a/crRNA cleavage detection in the same reaction. Briefly, RPA preamplification was allowed to proceed with all the reagents (listed in Table 1) except Cas12a for 15 min at 37 °C, in an Eppendorf tube. Then, Cas12a was spun down and 25 μL one-pot reaction assay was transferred to a 384-well plate. Experimental groups used solutions containing different concentrations of genomic DNA, while a nontarget control group used sterile water without genomic DNA and went thorough the same procedure.

Table 1. One-Pot Detection System

component	volume to add (μL)
Forward primer, 50 μM	0.24
Reverse primer, 50 μM	0.24
TwistAmp Rehydration Buffer	14.75
MgAc, 280 mM	0.53
NEB 2.1	2
LbCas12a, 1 μM	1.25
crRNA, 1 μM	1.25
Detection substrate	2.5
ssDNA-FQ reporter, 2 μM	1.25
nuclease free water	0.99

qPCR and Standard Curves. Standard qPCR reactions were performed according to the instructions of the Master qPCR Mix (SYBR Green I) (Tsingke). Each reaction contained one 10 μL aliquot of 2× Mix, 0.4 μM forward and reverse primers, an appropriate amount of genomic DNA, and sterile water up to 20 μL. qPCR analysis for different concentrations of bacteria in water or milk sample was run and analyzed by Roche Light Cycler following manufacturer's instruction.

RESULTS AND DISCUSSION

Cas12a/crRNA Complex Can Directly Detect Bacterial DNA. We first verified the feasibility of using Cas12a/crRNA on bacterial target sequence detection. rfbE for *Escherichia coli* and nuc for *Staphylococcus aureus* were chosen as specific genes (Figure 2a and Figure S1). To acquire high-quality crRNA, we translated and purified them as described in the Experimental Section and Figure S2. A final 20 μL detection system was composed of LbCas12a, crRNA, ssDNA-FQ reporter, NEB 2.1, different substrate, and nuclease-free water. We experimentally optimized concentrations of LbCas12a, crRNA, and ssDNA-FQ reporter as 50 nM, 50 nM, and 100 nM, respectively (Figure 2b and Figure S3).

Using the optimized detection system, we determined whether the designed crRNA probes were able to detect bacterial genome specifically. In a cross panel of 1 nM gDNA isolated from *E. coli* O157:H7, *Staphylococcus aureus*, *Salmonella enterica*, and *Lactobacillus casei*, crRNA correctly genotyped bacterial strains and cross-reactivity is not statistically significant (Figure 2c). Next, we explored the detection sensitivity of Cas12a/crRNA. A final concentration of gDNA ranging from 10^9 to 10^5 aM was tested. Reactions were allowed to proceed for 90 min at 37 °C, with fluorescence measured every 5 min. The end-point fluorescence and kinetics results showed that Cas12a has a detection sensitivity around 1 pM (Figure 2d and Figure 2e). Our results implied that Cas12a/crRNA can indiscriminately cleave ssDNA-FQ reporter when the target sequence is present. It alone works as a rapid and portable test for directly reading bacterial gDNA.

One-Pot Detection without Extra Sample Purification. To improve the robustness of the detection and reduce the likelihood of a false-positive result, we combined recombinase polymerase amplification (RPA) preamplification with Cas12a/crRNA cleavage to develop OCTOPUS. Cas12a with its crRNA and ssDNA-FQ reporter was directly added in the RPA reactions without any purification procedure. The buffer used in one-pot detection was a mixture of MgAc and NEB 2.1.

Fluorescent signals can be observed at 10^7 aM gDNA input, however, disappeared at 10^5 aM gDNA (Figure S4). Thus, we hypothesized that Cas12a cuts the trace gDNA before its successful amplification, and failed to cleave the ssDNA-FQ reporter to generate fluorescence. To verify such a hypothesis, Cas12a was added on the tube lit and spun down after 15 min post-amplification with 10^5 aM gDNA input. A robust fluorescent signal could now be observed. Also, successful RPA preamplification was validated by SYBR Green I (Figure 3a). These results were in accordance with previous literature.[32] The step-by-step procedure is described in the Experimental Section.

We further analyzed the selectivity of the presented one-pot detection platform in two aspects (Figure 3b,c). After preamplification at 37 °C for 15 min, 1 μL of each reaction was taken out and electrophoresed. Then, Cas12a was added to products left for further cleavage. Only reaction with corresponding gDNA showed the correct amplification band and robust fluorescent signal. Next, to test the dynamic range of the one-pot detection method, *E. coli* and *S. aureus* gDNA were tested on a series dilution from 10^6 to 10^0 aM. Every reaction was repeated three times. As depicted in Figure 3d,e and Figure S5, the one-pot assay is robust for aM nucleic acid detection. We performed linear regression for the logarithm of gDNA concentration and relative fluorescence units. The R^2 value for *E. coli* and *S. aureus* was 0.9371 and 0.9747, respectively. The definite limit of detection (LOD) for *E. coli* and *S. aureus* was 1.291 aM and 1.287 aM, respectively, which was calculated as three times the residual standard deviation of a regression line divided by the slope of linear regression. The results indicated

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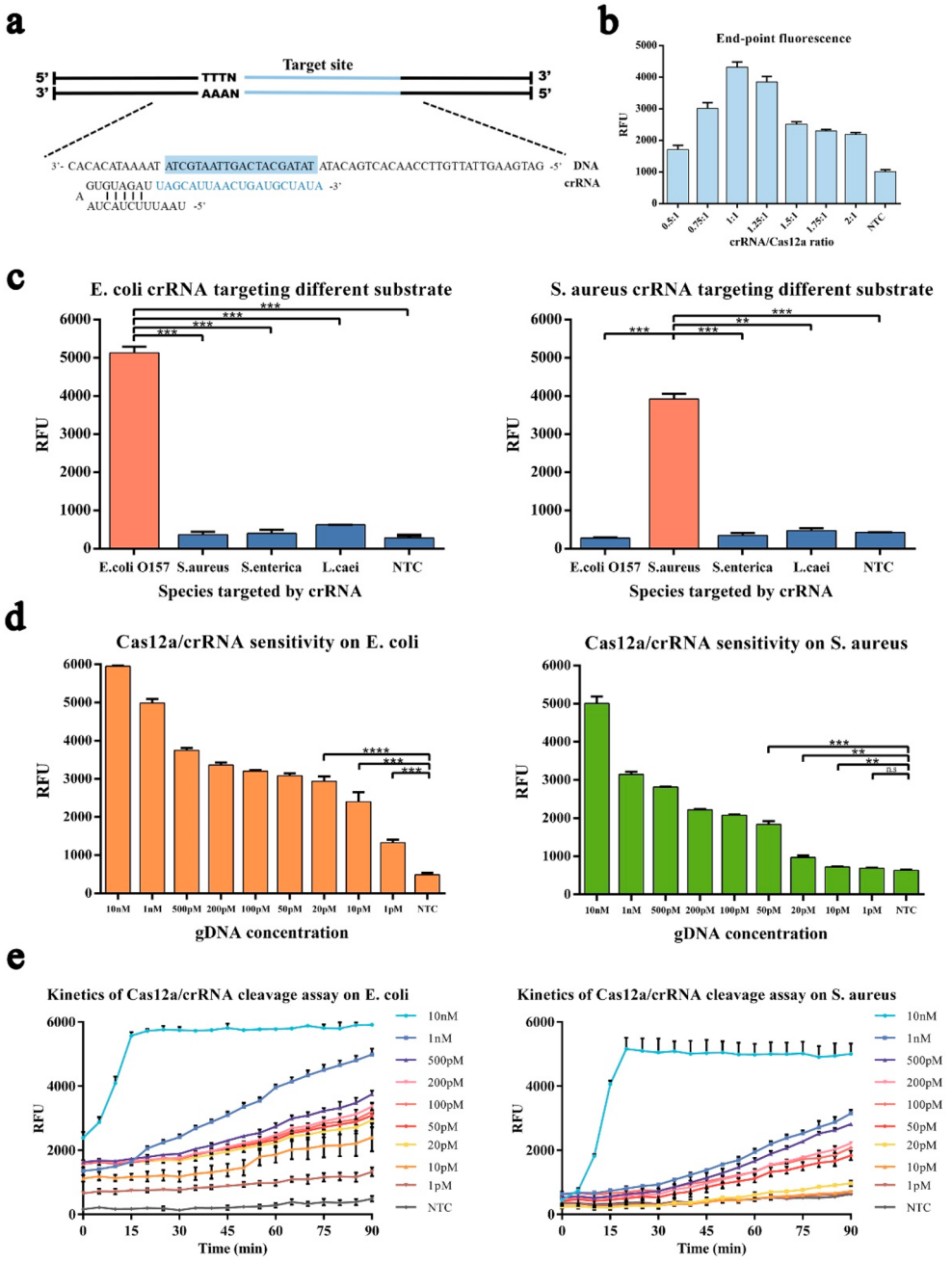


Figure 2. Cas12a/crRNA direct cleavage assay. (a) Schematic of dsDNA target detected with the Cas12a/crRNA. crRNA is provided in detail. The dsDNA target site is highlighted in blue. crRNA targeting *E. coli* is shown here and crRNA targeting *S. aureus* is available in Figure S1. (b) Fluorescence generated from different crRNA/Cas12a ratios. The kinetics curve is available in Figure S3. (c) Cas12a/crRNA assay specificity analysis among four different bacteria, namely, *E. coli* O157:H7, *S. aureus*, *S. enterica*, and *L. caei*. (d) Cas12a/crRNA assay sensitivity analysis on *E. coli* and S. aureus. End-point fluorescence for two targets is shown. (e) Kinetics of Cas12a/crRNA assay sensitivity analysis on *E. coli* and *S. aureus*. $n = 3$ biological replicates, two-tailed Student's t test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$; NTC nontarget control; n.s. not significant; bars represent mean ± S.D.

that OCTOPUS detection quantitation was sustainable across a large range of nucleic acid concentrations (Figure 3d and e).

Feasibility of One-Pot Detection Applied to Real Food Matrix. Since the presented one-pot assay has demonstrated high specificity, sensitivity, and stability, we further investigated the potential application to actual food samples. The primary challenge for real-world application is that the genomic template extraction process should not be sophisticated or require too many instruments, in order to be practical in the field. Therefore, we chose a water boiling method, as described in the Experimental Section. Bacteria solutions in sterile deionized water, with their concentration ranging from 10^8 to 10^0 CFU/mL, were used as substrate. End-point fluorescence and kinetic curves depicted in Figure 4a,b showed a robust signal and reached saturation quickly. The LOD was down to 10^0 CFU/mL. The whole procedure took less than 50 min.

Moreover, complicated components of food matrices may interfere with RPA preamplification or Cas12a/crRNA cleavage. To address this concern, one-pot detection was assessed with milk inoculated with *E. coli* or *S. aureus* at concentrations ranging

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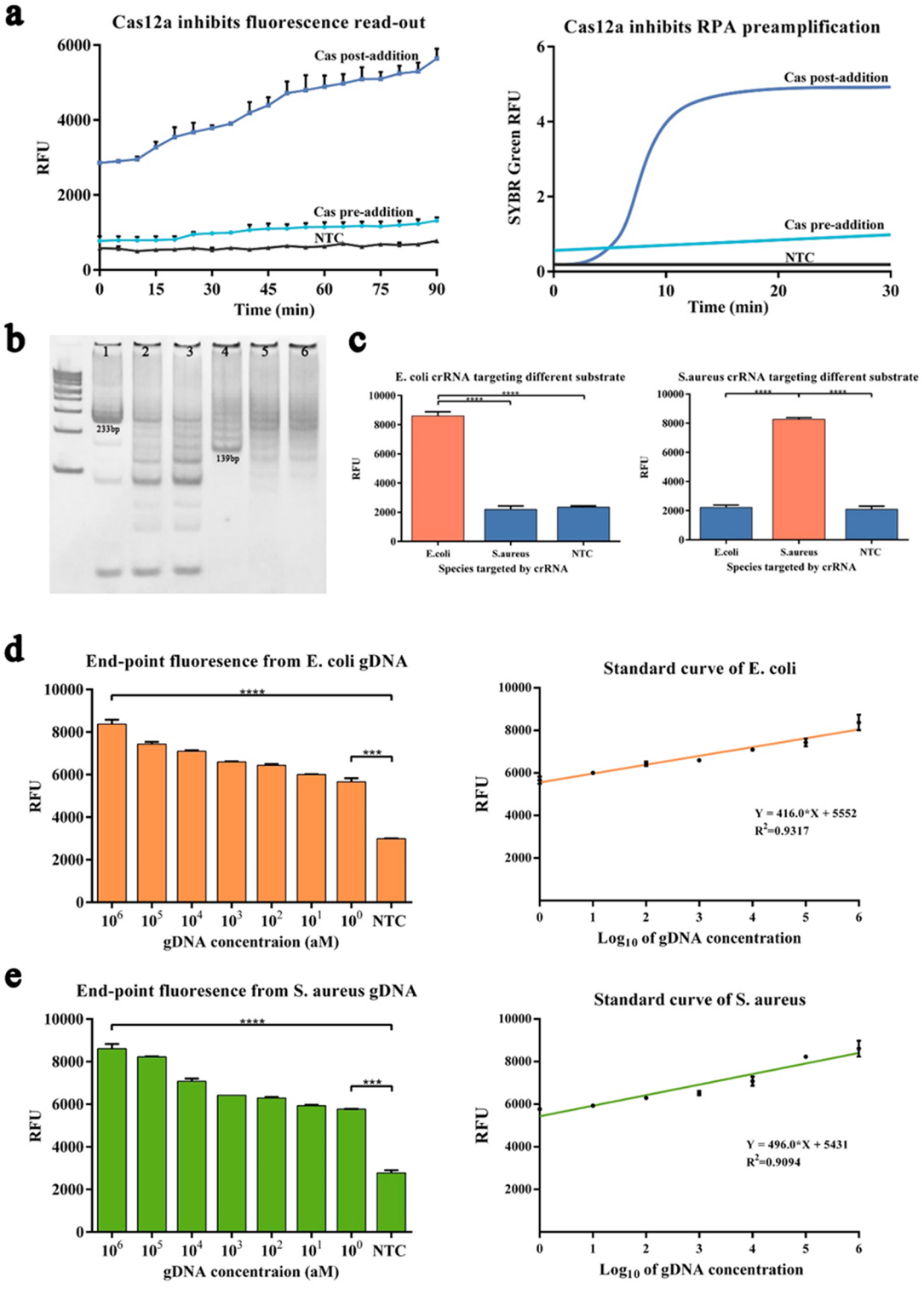


Figure 3. OCTOPUS combines RPA preamplification and Cas12a/crRNA cleavage. (a) Kinetics of samples with different Cas12a addition order. Only the test with Cas12a addition 15 min after RPA reaction exhibits fluorescence and successful preamplification indicated by SYBR GreenI. (b) Specificity analysis of RPA preamplification. Lanes 1, 2, 3: *E. coli* RPA primers and crRNA, with the addition of *E. coli* gDNA, *S. aureus* gDNA, and water, respectively. Lanes 4, 5, 6: *S. aureus* RPA primers and crRNA, with the addition of *S. aureus* gDNA, *E. coli* gDNA, and water, respectively. (c) Specificity analysis of one-pot detection. (d) Sensitivity analysis of one-pot detection on *E. coli*. (e) Sensitivity analysis of one-pot detection on *S. aureus*. End-point fluorescence and correlation of gDNA concentration with detected fluorescence are shown here. Kinetic curves are available in Figure S5. $n = 3$ biological replicates, two-tailed Student's t test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$; NTC nontarget control; n.s. not significant; bars represent mean ± S.D.

from 10^8 to 10^0 CFU/mL. Then, the food matrix was incubated at 37 °C for 0 or 4 h. Figure 4c and Figure S6 demonstrated a similar LOD, compared with those in water. We also evaluated the end-pot fluorescence with or without enrichment, suggesting

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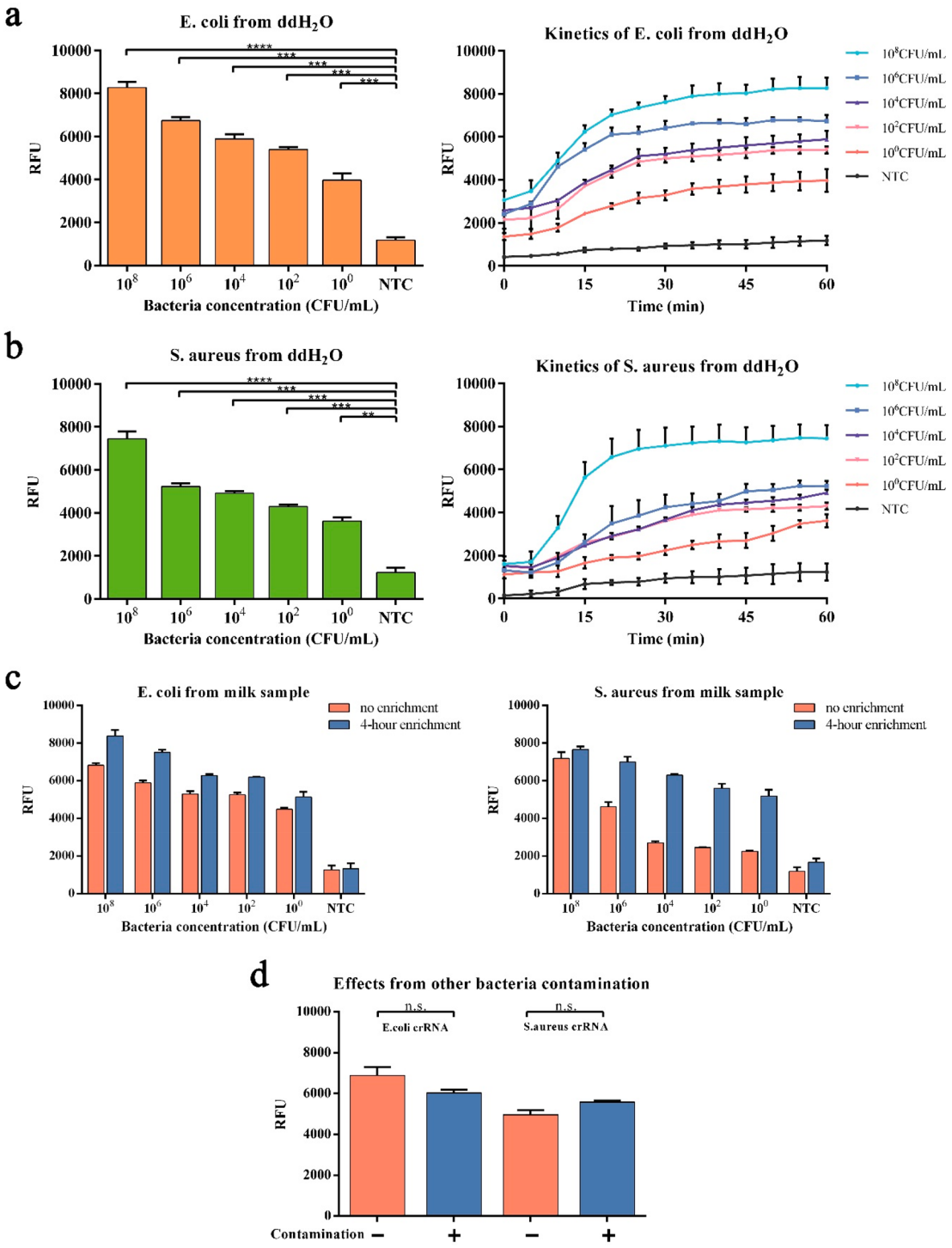


Figure 4. OCTOPUS integrating genome extraction and its application to real food matrix. (a) Toolbox sensitivity analysis on *E. coli*. (b) Toolbox sensitivity analysis on *S. aureus*. End-point fluorescence and kinetics for two bacteria are shown. (c) Toolbox practicability and sensitivity analysis on contaminated milk sample. The end-point fluorescence in the food matrix before and after 4 h enrichment is provided. Kinetic curves are available in Figure S6. (d) Assessment of the effects from contaminating bacteria. Contamination indicates the milk sample inoculated with *E. coli*, *S. aureus*, *S. enterica*, and *L. casei* at 10^6 CFU/mL each. $n = 3$ biological replicates, two-tailed Student's t test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$; NTC nontarget control; n.s. not significant; bars represent mean ± S.D.

that a 4 h enrichment can provide a more significant readout especially for samples with lower contamination level (Figure 4c).

Another fact is that food samples often risk multiple bacterial contamination. To investigate the effects of other contaminating bacteria, we also inoculated milk samples with *E. coli*, *S. aureus*, *S. enterica*, and *L. casei* at 10^6 CFU/mL each and performed one-pot detection. There was no statistical difference in fluorescence readout, indicating that OCTOPUS's performance is stable on a real food matrix (Figure 4d).

Better Sensitivity Compared with qPCR. We further compared OCTOPUS with quantitative PCR (qPCR), which is

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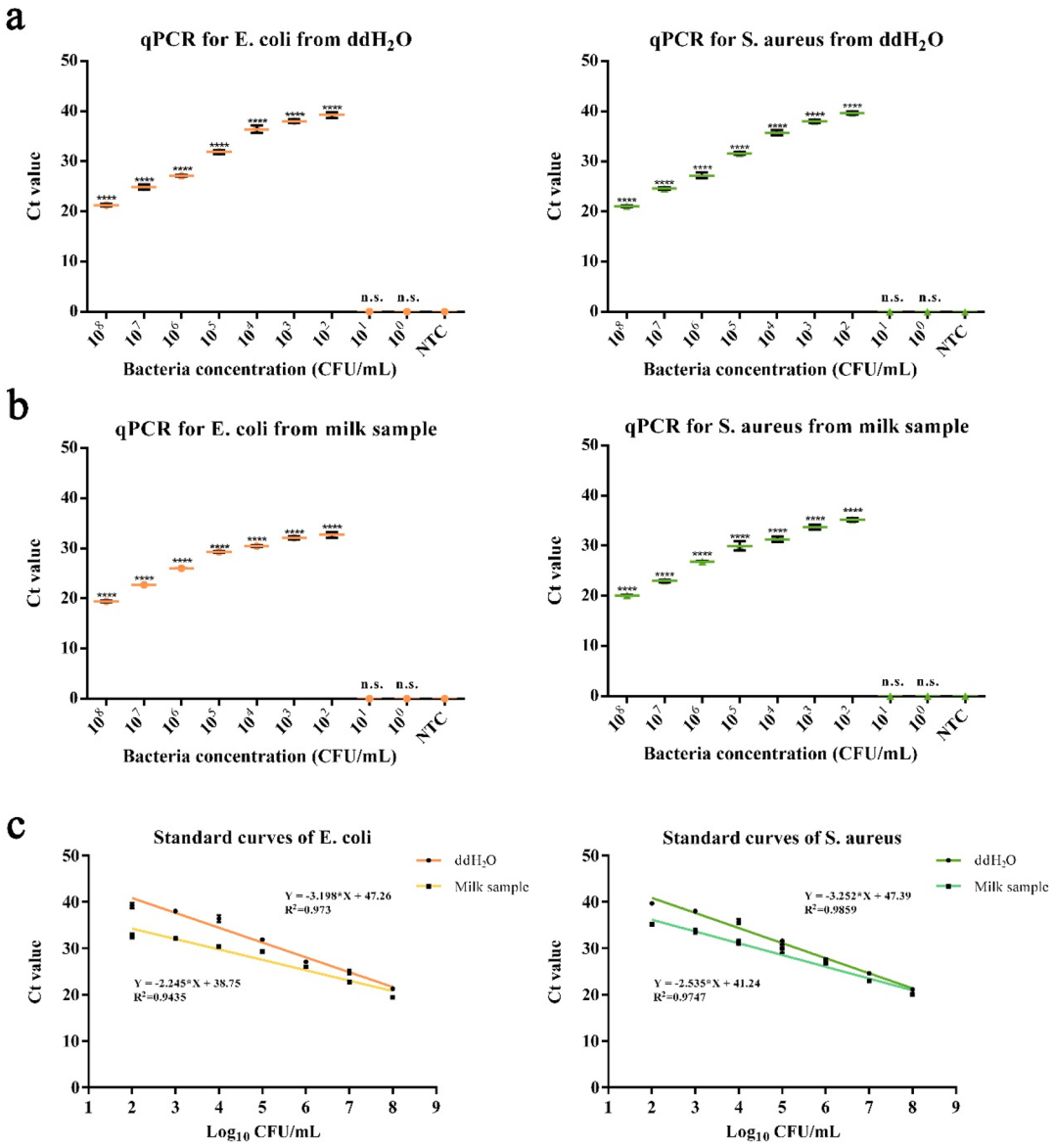


Figure 5. General qPCR analysis. (a) C_t value of *E. coli* and *S. aureus* from water sample in standard qPCR using a serial dilution ranging from 10^8 to 10^0 CFU/mL. (b) C_t value of *E. coli* and *S. aureus* from milk sample. (c) Standard curves of *E. coli* and *S. aureus* from water and milk sample. $n = 3$ biological replicates, two-tailed Student's *t* test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$; NTC nontarget control; n.s. not significant; bars represent mean ± S.D.

the most commonly used detection method as the gold standard. Primers targeting the same genes are generated using an online Web site (http://bioinfo.ut.ee/multimprimer3). For determination of the analytical sensitivity of the rfbE and nuc qPCRs, serial dilutions were prepared from 10^8 to 10^0 CFU/mL, both in water and in raw milk. We used the water boiling method for bacterial genome extraction, and 1 μL supernatant was added into each qPCR reaction, with each performed in quadruplicate.

The C_t values for *E. coli* and *S. aureus* from water or milk samples are shown in Figure 5a and b. Corresponding linear aggression analysis is shown in Figure 5c. The LOD of qPCR is 10^2 CFU/mL. For better understanding and references, we compared these two methods, in terms of sensitivity, time, cost, equipment, and procedure simplicity (Table 2).

Table 2. Comparison between OCTOPUS and Standard qPCR

	OCTOPUS	qPCR
Sensitivity	High (10^0 CFU/mL)	Low (10^2 CFU/mL)
Time	50 min	60 min
Cost	$5.2	$0.7
Equipment	Fluorescence reader	Light cycler
Procedure	Easy	Moderate

CONCLUSION

In this study, a rapid and sensitive Cas12a/crRNA based toolbox was developed for foodborne pathogen detection. The obtained results indicate that this toolbox has achieved high specificity among different bacteria strains and extremely high sensitivity. The LOD is down to 10^0 CFU/mL, which is superior to general qPCR (10^2 CFU/mL) and other commonly used methods.[12] RPA as an isothermal amplification technology is much more favorable than PCR for onsite detection. Many published studies have demonstrated contaminated food detection using basic or exo RPA kit,[9,33,34] indicating that OCTOPUS should work as

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well with different food matrices. By changing crRNA and RPA primers, this toolbox can be used on other intended bacteria. Of note, this rapid and sensitive method only requires an incubator and a portable fluorescence reader. In this way, OCTOPUS has promising potential for translation into real daily life. In the past few years, human beings have suffered from several vicious global diseases, including super bacteria, SARS, Ebola, and the ongoing tragedy caused by coronavirus in China. The presented sensitive and rapid one-pot platform can be applied for broad disease and epidemic prevention and control with slight modification.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.0c00320.

Figure S1: Schematic of dsDNA target and crRNA of *S. aureus*. Figure S2: Electrophoresis of crRNAs from in vitro transcription. Figure S3: Fluorescence curves of different crRNA/Cas12a concentrations. Figure S4: Fluorescence curves of different concentrations of *E. coli* genome. Figure S5: Fluorescence curves of one-pot detection of genomic DNA. Figure S6: Fluorescence curves of our toolbox detection on milk sample with or without enrichment. Table S1: Sequences of oligonucleotides used in this study. (PDF)

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Notes

The authors declare no competing financial interest.

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