Food Chemistry 334 (2021) 127608

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

RPA-Cas12a-FS: A frontline nucleic acid rapid detection system for food safety based on CRISPR-Cas12a combined with recombinase polymerase amplification

Hua Liu[a,b,1], Jinbin Wang[a,b,1,*], Haijuan Zeng[a,b], Xiaofeng Liu[c], Wei Jiang[a,b], Yu Wang[c], Wanbao Ouyang[c], Xueming Tang[a,b,*]

[a] Institute of Biotechnology Research, Shanghai Academy of Agricultural Sciences; Key Laboratory of Agricultural Genetics and Breeding, 2901 Beidi Road, Shanghai 201106, China
[b] Crops Ecological Environment Security Inspection and Supervision Center (Shanghai), Ministry of Agriculture and Rural Affairs, P.R.C., 2901 Beidi Road, Shanghai 201106, China
[c] School of Life Science and Engineering, Lanzhou University of Technology, Lanzhou 730050, China

ARTICLE INFO

Keywords:
CRISPR-Cas12a
Detection
Foodborne pathogenic bacteria
Genetically modified crops
Meat adulteration

ABSTRACT

Food analysis to ensure food safety and quality are relevant to all countries. This study aimed to develop a detection technique by combining recombinase polymerase amplification with CRISPR-Cas12a for food safety (termed RPA-Cas12a-FS). Our data showed that this novel method could be detected via fluorescence intensity for the molecular identification of foodborne pathogenic bacteria, genetically modified crops, and meat adulteration. After optimization, the sensitivity and stability of RPA-Cas12a-FS was further enhanced. The RPA-Cas12a-FS system could specifically detect target gene levels as low as 10 copies in 45 min at 37 °C. The RPA-Cas12a-FS system was sensitive both using standard samples in the lab and using samples from the field, which indicated that this detection method was practical. In conclusion, a simple, rapid, and highly sensitive detection method based on CRISPR-Cas12a was developed for molecular identification in the food safety field without requiring technical expertise or ancillary equipment.

1. Introduction

Food safety issues are closely related to human livelihood. For the effective prevention and control of food safety risks, the development of specific, sensitive, and rapid nucleic acid detection for foodborne pathogenic bacteria, genetically modified crops, and meat adulteration is imperative. Polymerase chain reaction (PCR) is a primary method used for detection systems that work at the DNA level, including qualitative PCR (Delong & Zhou, 2015), quantitative PCR (qPCR) (Cao et al., 2018), and droplet digital PCR (ddPCR) (Bogožalec Košir, Demšar, Štebih, Žel, & Milavec, 2019). However, these are costly, labor-intensive, and time-consuming. Furthermore, they are unsuitable for use in grass-root laboratories, due to their dependence on expensive instruments and complex detection steps to obtaine a final result. Rapid, simple, relatively low-cost, specific, and sensitive detection methods for nucleic acids that do not require special technical expertise and ancillary equipment are thus urgently needed (Chang et al., 2020).

The clustered regularly interspaced short palindrome repeats (CRISPR) system is widely used in cell imaging, expression regulation, gene editing, and DNA assembly (Lei et al., 2017; Li, Cheng, & Liu et al., 2018; Li, Cheng, & Wang et al., 2018; Li, Liu et al., 2018; Li, Liu, Zhao, & Wang, 2018; Rath, Amlinger, Rath, & Lundgren, 2015; Świat et al., 2017; Yao, Li, Cao, Liu, & Fan et al., 2018; Yao, Li, Cao Liu, & Qi et al., 2018; Zalatan et al., 2015). Recently, a CRISPR/Cas-based nucleic acid detection system has been described (Chen et al., 2018; Gootenberg et al., 2018; Gootenberg et al., 2017; Li, Liu, Zhao, & Wang, 2018). CRISPR Cas12a (LbCpf1) is a member of the Cas effector protein family and was first analyzed and reported in 2013 (Zetsche et al., 2015). CRISPR Cas12a can detect the presence of DNA target under the guidance of a CrRNA to form a 5′ sticky end (Fonfara et al., 2016) and has

* Corresponding authors at: Institute of Biotechnology Research, Shanghai Academy of Agricultural Sciences; Key Laboratory of Agricultural Genetics and Breeding, 2901 Beidi Road, Shanghai 201106, China.
E-mail addresses: wangjinbin@saas.sh.cn (J. Wang), xueming70@foxmail.com (X. Tang).
[1] Hua Liu and Jinbin Wang were considered as co-first author of this work. Hua Liu designed the study and interpreted the results. Jinbin Wang collected test data and drafted the manuscript.

https://doi.org/10.1016/j.foodchem.2020.127608
Received 29 February 2020; Received in revised form 12 July 2020; Accepted 16 July 2020
Available online 19 July 2020
0308-8146/ © 2020 Elsevier Ltd. All rights reserved.

non-specific ssDNA trans-cleavage activity of Cas12a (Li, Cheng, & Liu et al., 2018; Li, Cheng, & Wang et al., 2018; Li, Liu et al., 2018; Ma et al., 2015). The activity to cleave nearby non-targeted reporter ssDNA is activated by recognition of a suitable DNA target by the guiding CrRNA (Chen et al., 2018; Creutzburg, Swartjes, & van der Oost, 2020).

CRISPR-Cas12a-based detection has been successfully applied to detect high-risk HPV16 or HPV18 virus (ZIKV) (Tsou, Leng, & Jiang, 2019), *pseudorabies* virus (DENV) (Li et al., 2019), human *papillomavirus* (Gootenberg et al., 2018), cancer-related viruses (Huang, Lee, & Doudna, 2018), white spot syndrome virus (Chaijarasphong et al., 2019), African swine fever (Bai et al., 2019) and other molecules such as biallelic mutants (Xiao et al., 2020). In this study, we utilized the non-specific ssDNA cleavage characteristic of Cas12a, combined with RPA (Recombinase Polymerase Amplification), to design a detection technology platform called RPA-Cas12a-FS that did not require special technical expertise or ancillary equipment. RPA-Cas12a-FS detection, by combining RPA with guide CrRNA targeting and detection of FAM-BHQ1-labeled reporter ssDNA, allowed the development of a simple, relatively low-cost, rapid, specific, and highly sensitive detection system for the molecular identification of foodborne pathogenic bacteria, genetically modified crops, and meat adulteration.

2. Materials and methods

2.1. Materials

All primers, ssDNAs, and CrRNA were synthesized by Sangon Biotech (Shanghai, China) and RNase inhibitors were also purchased from Sangon Biotech. An RPA assay kit was purchased from TwistDx Ltd. (Cambridge, Massachusetts, USA). The various genetically modified organisms (GMOs) detection samples were provided by the Crop Ecological Environment Safety Monitoring and Test Center, Ministry of Agriculture, People's Republic of China. All food-borne microorganisms (*Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Vibrio parahaemolyticus*) were purchased from Sangon Biotech, and various meat products (pork, duck, and beef) purchased from Walmart supermarket.

2.2. Nucleic acid preparation

The DNA extraction used in this paper was extracted using the following kit. The DP350 extraction kit (Tiangen Biotech, Beijing, China) was used for rapid DNA extraction from commercialized transgenic crops (Table S1). The DP302 extraction kit (Tiangen Biotech, Beijing, China) was used for rapid DNA extraction from *E. coli*, *L. monocytogenes*, *S. aureus*, and *V. parahaemolyticus*. The DP323 extraction kit (Tiangen Biotech) was used for rapid DNA extraction from pork, duck meat, and beef. All DNA was stored at −20 °C until subsequent RPA experiments. When testing on-site samples, we use a super-fast release reagent (Ampfuture Co., Ltd, Shandong, China). First, if plant leaves or other parts were used, we shredded the tested sample, using 10 mg in 200 μL of lysis buffer. For meat and other animal tissues, we used 10 mg in 200 of μL lysis buffer, and for bacteria or other microorganisms, we used 20 μL of a cell culture solution and added it to 40 μL of lysis buffer. The samples were then mixed thoroughly or vortexed. After resting at 25–35 °C for 5 min, 2–3 μL of the lysate supernatant was used as a DNA template.

2.3. RPA primer design

Sequence alignment analysis was performed for different food safety indicators, and we designed 9 sets of highly specific amplified RPA primers using DNAMAN software (Version 6.0, Lynnon Co., USA). The fragment lengths and primer sequences are shown in Table 1.

2.4. CrRNA and ssDNAs preparation

The CrRNA sequences of various food safety indicators were designed based on the PAM site of Cas12a. The specific sequence information is shown in Table 2. In addition, based on the ancillary cleavage activity of Cas12a, ssDNAs of different lengths were designed (Table 2) for subsequent length optimization. All CrRNA and ssDNAs were stored at −80 °C until use.

2.5. Detection process

The detection process of RPA-Cas12a-FS is shown in Fig. 1. The DNA of the detected sample was quickly extracted, and the obtained DNA was used as a template for the RPA reaction. Using 2 μL of the DNA template, we made a 20 μL RPA-MIX reaction system, with 1 μL each of forward and reverse primers, and after constant temperature amplification at 37 °C for 4 min, the reaction tube was inverted 10 times and then centrifuged briefly. This was then placed at constant temperature, and after 12 min, the RPA reaction was complete. Subsequently, 1 μL of the above amplification product was added to a configured fluorescent reaction tube (20 μL volume, see Table S2). This was then incubated for 20 min, 180 μL ddH_2O was added, and it was placed in a microplate reader or handheld fluorometer for fluorescence intensity measurement.

2.6. Construction and purification of reference plasmid DNA

In sensitivity determination experiments, a reference plasmid was obtained by cloning RPA amplification product fragments into pUC57 (Sangon Biotech, Shanghai, China). A total of 9 plasmids with various indicators were created. The plasmid was extracted and identified by restriction digests and DNA sequencing. After purification with the SanPrep Column Plasmid Mini-Preps Kit (SanPrep, Sangon Biotech, China), the plasmid concentration was determined using the Nanodrop Spectrophotometer (Thermo scientific, Massachusetts, USA) and calculated in copy numbers. The solution was diluted in TE buffer to a concentration of 106–100 copies per 1 μL, from which a tenfold dilution series was made to serve as the detection sensitivity.

2.7. RPA reaction system optimization

The TwistAmp® Basic kit (TABAS03KIT, TwistDx Ltd, UK) was used for system optimization. This was carried out by adding 29.5 μL of buffer and 11.2 μL ddH_2O to strips of 8 tubes in the kit to completely dissolve the white solid in the tubes and prepare RPA-MIX for subsequent experiments. A 50 μL to 10 μL reaction system gradient was set up for system optimization. Table S2 shows the proportions of reagents added. For these three types of indicator factors, in Table 1, we list 9 pairs of RPA primers that were designed and optimized. The DNA template was fixed at 10 ng/μL of purified DNA.

The following procedure was carried out after the system was prepared. A constant temperature of 37 °C and duration of 4 min was used for incubations. Following incubation, tubes were removed from the thermostatic heater and inverted 10 times or for 15 s continuously. The strips were then centrifuged briefly before they were incubated in a thermostatic heater for 10 min until the RPA reaction had ended.

2.8. Fluorescent detection of Cas12a-mediated trans-cleavage

An LbCas12a cleavage experiment was used in all detection experiments. This Cas12a cleavage system was purchased from Tolo Biotech (Tolo Biotech, Anhui, China). The optional parameters for our 20 μL Cas12a enzyme digestion reaction system were as follows: 2.0 μL 10 × Cas12a reaction buffer, 0.5 μmol/L CrRNA, 0.05 μmol/L Cas12a nuclease, 0.5 mmol/L RNase inhibitor, 0.5 mmol/L reporter ssDNA, and 2 μL template DNA. The CrRNA used in this study is shown in the

Table 1
RPA Primers.

Primer name	Sequences (5′–3′)	Amplicon length (bp)	GenBank ID
RPA-*T*-NOS-F	5′-TGAATCCTGTTGCCGGTCTTGCGATGATTATC-3′	151	Vv00087.1
RPA-*T*-NOS-R	5′-TCTATCGCGTATTAAATGTATAATTGCGGGAC-3′		
RPA-P-35s-F	5′-CCTCTGCCGACAGTGGTCCCAAAGATGGACC-3′	123	V00141
RPA-P-35s-R	5′-CCCTTACGTCAGTGGAGATATCACATCAATCC-3′		
RPA-Beef-F	5′-TTAGAGATTGAGAGCCATATACTCTCCTTGGTGACA-3′	300	AF492351.1
RPA-Beef-R	5′-TTTGATGTTGGGAATAGTAGGCTTGGGAATAGTACGA-3′		
RPA-Duck-F	5′-ATCTATCCTGCTAGCCGCCGGCCTCTTATCAATGC-3′	227	MK770342.1
RPA-Duck-R	5′-TAGTTCGGGGGTTGGTACTAGGCTTGAGTGG-3′		
RPA-Pork-F	5′-ACTACCTATTGTCACCTTAATTATTATATTCCC-3′	383	NC_000845.1
RPA-Pork-R	5′-ATAGGGCGGGTGTTCCTTGTGGTAGAAAGTGGGC-3′		
RPA-Ec-F	5′-TCTTCATTTAGCTTTGTTAGCGTTAGGTATATCGG-3′	233	S83460.1
RPA-Ec-R	5′-CCATATCACATGGATGTCCGTATAAATGGACACAC-3′		
RPA-Vp-F	5′-ACTCGTATGAGAACGTGACATTGCGTATTT-3′	426	AB029908.1
RPA-Vp-R	5′-TTGAACTCAGAAGGAGAAACAAGCAGGTAG-3′		
RPA-Sa-F	5′-GCATCACAAACAGATAACGGCGTAAATAGAAG-3′	139	EF529607.1
RPA-Sa-R	5′-ACATTAATTTAACCGTATCACCATCAATCGCT-3′		
RPA-Lm-F	5′-TCGATCACTCTGGAGGATACGTTGCTCAATTC-3′	158	987,033
RPA-Lm-R	5′-GTTACCAGGCAAATAGATGGACGATGTGAAAT-3′		

Table 2
CrRNA sequences and reporter ssDNA sequences.

Oligo name	Sequences (5′–3′)
CrRNA-*T*-NOS-1	AAUUUCUACUGUUGUAGAUUGUUGAAUUACGUUAAGCAUGUAA
CrRNA-*T*-NOS-2	AAUUUCUACUGUUGUAGAUUGAGAUGGGUUUUUAUGAUUAGAG
CrRNA-*T*-NOS-3	AAUUUCUACUGUUGUAGAUUAUGAUUAGAGUCCCGCAAUUAUA
CrRNA-*T*-NOS-4	AAUUUCUACUGUUGUAGAUAUACGCGAUAGAAAACAAA
CrRNA-P-35s-1	AAUUUCUACUGUUGUAGAUAAGACGUGGUUGGAACGUCUUCUU
CrRNA-P-35s-2	AAUUUCUACUGUUGUAGAUUCCACGAUGCUCCUCGUGGGUGGG
CrRNA-P-35s-3	AAUUUCUACUGUUGUAGAUGGACCACUGUCGGCAGAGG
CrRNA-Ec-233-1	AAUUUCUACUGUUGUAGAUUUAGCGUUAGGUAUAUCGGAAGGA
CrRNA-Ec-233-2	AAUUUCUACUGUUGUAGAUCGUUGAUUCAGAUAAUGAAACUUG
CrRNA-Ec-2333	AAUUUCUACUGUUGUAGAUUACGGACAUCCAUGUGAUAU
CrRNA-Vp-426-1	AAUUUCUACUGUUGUAGAUCGCAGGUCAAAACGAUCCAACAGA
CrRNA-Vp-426-2	AAUUUCUACUGUUGUAGAUCAGUGGUUGGUUGUACUGGGGCAG
CrRNA-Vp-426-3	AAUUUCUACUGUUGUAGAUAGAGAAAGGUCGCUGGGAAGUGAG
CrRNA-Sa-139-ZX1	AAUUUCUACUGUUGUAGAUAUUAAAGCGAUUGAUGGUGAUACG
CrRNA-Sa-139-FX1	AAUUUCUACUGUUGUAGAUAUUAAAGUCGCAGGUUCUUUAUGU
CrRNA-Sa-139-FX2	AAUUUCUACUGUUGUAGAUAGUUGAAGUUGCACUAUAUACUGU
CrRNA-Lm-158-1	AAUUUCUACUGUUGUAGAUUUGGGAUGAAGUAAAUUAUGAUCC
CrRNA-Lm-158-2	AAUUUCUACUGUUGUAGAUACAUCGUCCAUCUAUUUGCCAGGU
CrRNA-Beef-1	AAUUUCUACUGUUGUAGAUGGUCUCCCUCUCGUAACCCUUAU
CrRNA-Beef-2	AAUUUCUACUGUUGUAGAUUCACAAUCCAGAACUGACACCAA
CrRNA-Beef-3	AAUUUCUACUGUUGUAGAUGUUGAAAGAUGAUAAAAA
CrRNA-Duck-1	AAUUUCUACUGUUGUAGAUGACUGUAGGUGUGUGGUGGCCUU
CrRNA-Duck-2	AAUUUCUACUGUUGUAGAUUUCUUCCTAGGAUUUUUCTGGGC
CrRNA-Duck-3	AAUUUCUACUGUUGUAGAUUGGGCAUUCUUCCACUCAAGCCU
CrRNA-pork-1	AAUUUCUACUGUUGUAGAUUACCACAAGGAACACCCGCCCUAUU
CrRNA-pork-2	AAUUUCUACUGUUGUAGAUUUGAGCCGAUGAAUAUAAUUAGAG
CrRNA-pork-3	AAUUUCUACUGUUGUAGAUGGUGUUGGGAAUAGUAAGCUUGG
HEX-12NT-BHQ1	HEX-TTTTTTTTTTTT-BHQ1
HEX-10NT-BHQ1	HEX-TTTTTTTTTT-BHQ1
HEX-8NT-BHQ1	HEX-TTTTTTTT-BHQ1
HEX-6NT-BHQ1	HEX-TTTTTT-BHQ1

sequences in Table 2. The DNA template was the RPA amplification product obtained above, and the ssDNA was a non-targeting fluorescent probe DNA that was composed of a random sequence of nucleotides. The 5′ end of this ssDNA was labeled with the fluorescent group 5′-Hexachloro-Fluorescein-CE Phosphoramidite (HEX), with an excitation wavelength of 535 nm and an emission wavelength of 553 nm, and the 3′ end was labeled with the quencher group BHQ1, with an absorption spectrum of 480 to 580 nm. The DNA fluorescent probe was HEX-NT-BHQ1, where N was 12, 10, 8, or 6 T base pairs.

After this Cas12a-mediated trans-cleavage fluorescence detection system was prepared, 1 μL of the RPA products above were added to it and ddH_2O was added to make the total volume 20 μL. The negative control amplification product from the RPA (NTC) was used as a blank control. The cleavage reaction temperature was 37 ˚C. After the aforementioned reaction was incubated at 37 ˚C for 20 min, 18 μL of ddH_2O was added to dilute the sample for fluorescence detection. An infinite M200 PRO (Tecan, Mannedorf, Switzerland) multi-purpose microplate reader was used with an excitation wavelength of 530 nm and fluorescence was detected at 565 nm. The difference in fluorescence between the multi-purpose microplate reader and handheld microplate reader was small and these results were also suitable for field tests. Unless specifically mentioned, the fluorescence intensity in these experiments was the results from a multi-purpose microplate reader.

2.9. RPA-Cas12a-FS system detection application to food safety

In food safety, the traceability of GMOs, food-borne microorganisms and meat products are three very important application goals. In global

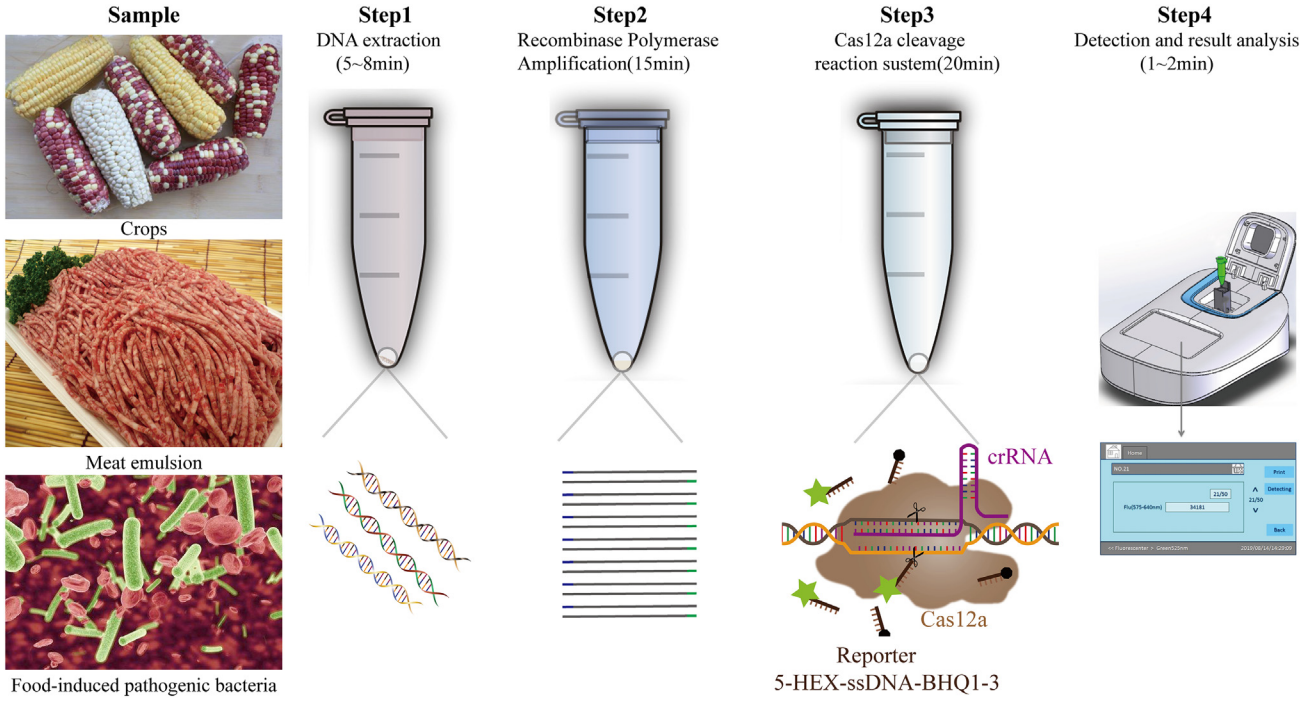


Fig. 1. Schematic diagram of the application of the RPA-Cas12a-FS detection system to genetically modified products, meat product traceability, and food-borne pathogens.

commercialized genetically modified products, more than 70% of the GMO species contain P-35S and *T*-nos, so these two screening elements are often used as indicating factors (Liu et al., 2020). The coverage of these two elements in GMO events reached 81.4%, of which P-35S accounted for 65.7% and *T*-nos accounts for 53.5% (Waiblinger, Ernst, Anderson, & Pietsch, 2008). In order to control food contamination by pathogenic bacteria and prevent the occurrence of microbial food-borne diseases, we selected the common pathogenic factors *E. coli*, *L. monocytogenes*, *S. aureus*, and *V. parahaemolyticus*. Focusing on the problem of meat adulteration, we selected pork, duck, and beef as our traceability verification meat products. After extraction of DNA from the above materials, the RPA reaction was completed in 15 min. 1 μL of the RPA amplification product was added to the detection system. Diluted fluorescence detection was carried out after 20 min. In our sensitivity experiments, we carried out the construction of standard molecular plasmids, and compared the results using real-time PCR and national standards. The detection results were then compared with the results of existing standards (General Administration of Quality Supervision and Inspection of the People's Republic of China, 2016; Ministry of Agriculture and Rural Areas of the People's Republic of China, 2012, 2018; National Standard of the People's Republic of China, 2018).

2.10. Statistical analysis

Each experiment was performed in triplicate. The fluorescence intensity measurement was performed using either an Infinite M200 PRO plate reader or a Fluo-380 handheld fluorimeter (Yuanpinghao, Beijing, China). Date analysis was performed using SPSS 23.0 (IBM Crop., Chicago, USA) multivariate analysis software. Data are expressed as the mean ± standard deviation (SD). For parametric data, comparison of different groups was performed by one-way analysis of variance, followed by the Tukey-Kramer HSD test ($\alpha = 0.05$) for multiple comparisons. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., Chicago, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. RPA system optimization

To reduce experiment costs, we attempted to reduce the volumes of our RPA reaction system. As the total system was continuously scaled down, the reaction stability gradually decreased (Fig. S1). ANOVA analysis classified all of these RPA reaction volumes into five groups (a, b, c, d and e in Fig. S1) with $p = 0.05$, meaning that there was a statistically significant difference. The fluorescence intensity of the positive sample and the negative sample showed a significant difference in each different RPA reaction volume. When the RPA reaction system was scaled down to 20 μL, fluorescence intensity was still stably produced. Thus, a 20 μL RPA reaction system was used for subsequent experiments, which decreased experiment costs to 40% of the original cost (The recommended system in the kit was 50 μL). There was no related literature for optimizing the RPA system. The cost advantage brought by downsizing this system was beneficial for rapid detection. After optimizing the RPA system to 20 μL in the laboratory, on the basis of ensuring the stability of RPA, the cost per sample was reduced, signal amplification at room temperature was realized, and a method for rapid detection was demonstrated.

3.2. Specificity and sensitivity of ssDNA and CrRNA length screening

With regards to reducing experiment costs, we also attempted to decrease the sequence length of the ssDNA used and designed HEX-12NT-BHQ1, HEX-10NT-BHQ1, HEX-8NT-BHQ1, and HEX-6NT-BHQ1 substrates. In fluorescence detection experiments, we used a qPCR fluorescence quantitation machine for recording of fluorescence intensity at 1-min intervals (Fig. S2). As can be seen from Fig. S2, the entire RPA-Cas12a-FS system reaction was completed within 20 min, as indicated by the plateau phase of the fluorescence signal. Thus, we had confirmed that the entire cleavage process only required 20 min. In addition, Fig. S2 also shows that the longer the ssDNA length, the lower the fluorescence intensity and the longer the time required to reach the plateau phase.

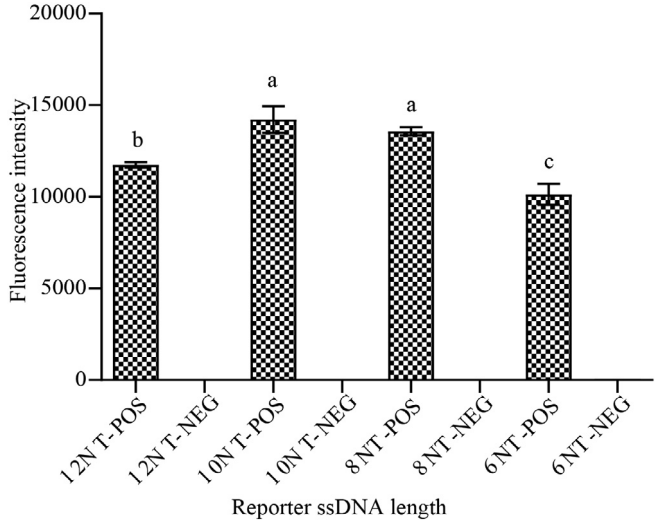


Fig. 2. RPA-Cas12a-FS Optimized Measurement of ssDNA Length. Different lowercase letters indicate significant differences between members of each group. POS: positive samples, NEG: negative samples.

It may be that the closer the BHQ1 and HEX, the better the quenching effect, thus, the lower the background value and the higher the fluorescence intensity. Moreover, the lower the repetitive cleavage rate, the shorter the ssDNA and the catalytic site of Cas12a, meaning that the probability of being cut off is reduced. The average fluorescence intensity (from highest to lowest) from the four ssDNA lengths was 10 NT > 8 NT > 12 NT > 6 NT. ANOVA classified all of these recoveries into three groups (a, b and c in Fig. 2), with $p = 0.05$, meaning that there was a statistically significant difference. The 10 NT and 8 NT fluorescence intensity had no significant difference, both being group a in Fig. 2. Considering that 8NT could effectively reduce the detection cost, we choose this as the reporter ssDNA length. The length of ssDNA was optimized to 8 NT through a series of experiments, which not only reduced costs but also allowed on-site rapid testing using portable equipment.

We next compared CrRNA length and designed two different lengths of specific CrRNA for fluorescence specificity testing (shown in Fig. S3). Results showed that the fluorescence intensity using a 19 bp CrRNA was significantly superior to fluorescence intensity using a 24 bp CrRNA for *P-CamV35S* ($P < 0.0001$). The fluorescence intensity of a 24 bp CrRNA was superior to that of a 19 bp CrRNA for *T-NOS* ($P < 0.01$). Additionally, the fluorescence intensity of a 24 bp CrRNA was superior to that of a 19 bp CrRNA for *E. coli* DNA ($P < 0.0001$), and the fluorescence intensity of a 24 bp CrRNA was superior to that of a 19 bp CrRNA using beef DNA ($P < 0.05$). This may require more testing. This experiment combined RPA with ancillary cleavage of CrRNA, improving the specificity of rapid detection, and providing technical support for food safety.

3.3. Enhanced RPA-Cas12a-FS fluorescence detection in food safety

We then tested our RPA-Cas12a-FS system in terms of the detection of transgenic products, foodborne pathogens, or animal components and carried out product and sensitivity validation tests using various characterization factors.

3.3.1. RPA-Cas12a-FS system applications to GMOs

The characterization factors for transgenic products were the *P-CaMV35S* promoter and the *T-nos* terminator. According to reports, these two parameters cover 81.4% of all global commercial transgenic products. Therefore, the detection of these two parameters was extremely representative. To determine the sensitivity of the RPA-Cas12a-FS system for *P-CaMV35S* and *T-nos*, a diluted plasmid DNA of *P-CaMV35S* and *T-nos* was added to RPA reaction to detect the fluorescence intensity (Fig. 3). We found that the fluorescence intensity gradually decreased as the diluted plasmid DNA of *P-CaMV35S* and *T-nos* was scaled down (Fig. 3 A-4 and Fig. 3 B-4). ANOVA classified all of the diluted plasmid DNA of *P-CaMV35S* and *T-nos* into six groups (a, b, c, d, e and f in Fig. 3 A-4 and Fig. 3 B-4) with $p = 0.05$, meaning that there was a statistically significant difference. As is shown in Fig. 3 A-4 and Fig. 3 B-4, the sensitivity of *P-CaMV35S* and *T-NOS* fluorescence detection was 10 copies.

For product validation, we selected common transgenic products as shown in Table S1 for verification of our RPA-Cas12a-FS system on *P-CaMV35S* and *T-nos* (Fig. 2 A-1, Fig. 3 A-2, Fig. 3 B-1 and Fig. 3 B-2). We found that the RPA-Cas12a-FS system had extremely good performance in the on-site rapid detection of transgenic products. From these test results, we saw that the RPA-Cas12a-FS system had good reproducibility and stability, meaning it was suitable for use in seed farms with deficient experiment facilities, as well as for the on-site testing of routine foodstuffs. To make this detection system feasible in actual application, we employed the RPA-Cas12a-FS system for on-site rapid detection in fields, and results showed good reproducibility. An on-site test was performed at the Songjiang seed farm and no transgenic

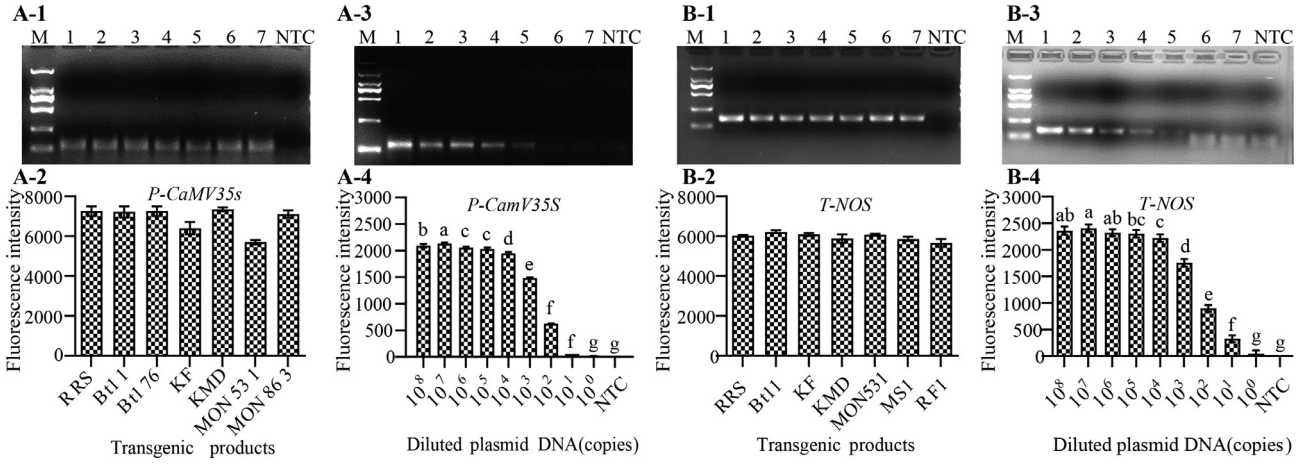


Fig. 3. RPA-Cas12a-FS application in GMO. A-1, A-2, A-3, and A-4 show molecular specificity and sensitivity verification for *P-CamV35S*. B-1, B-2, B-3, and B-4 show molecular specificity and sensitivity verification for *T-nos*. M: DL2000 Marker; NTC: No template added as negative control. A-1 and B-1: 1: RRS; 2: Bt11; 3: Bt176; 4: KF; 5: KMD; 6: MON531; 7: MON863. A-3 (diluted *P-CamV35S* plasmid DNA) and B-3 (diluted *T-NOS* plasmid DNA); 1: 10^6 copies; 2: 10^5 copies; 3: 10^4 copies; 4: 10^3 copies; 5: 10^2 copies; 6: 10^1 copies; 7: 10^0 copies. Different lowercase letters indicate significant differences between members of each group.

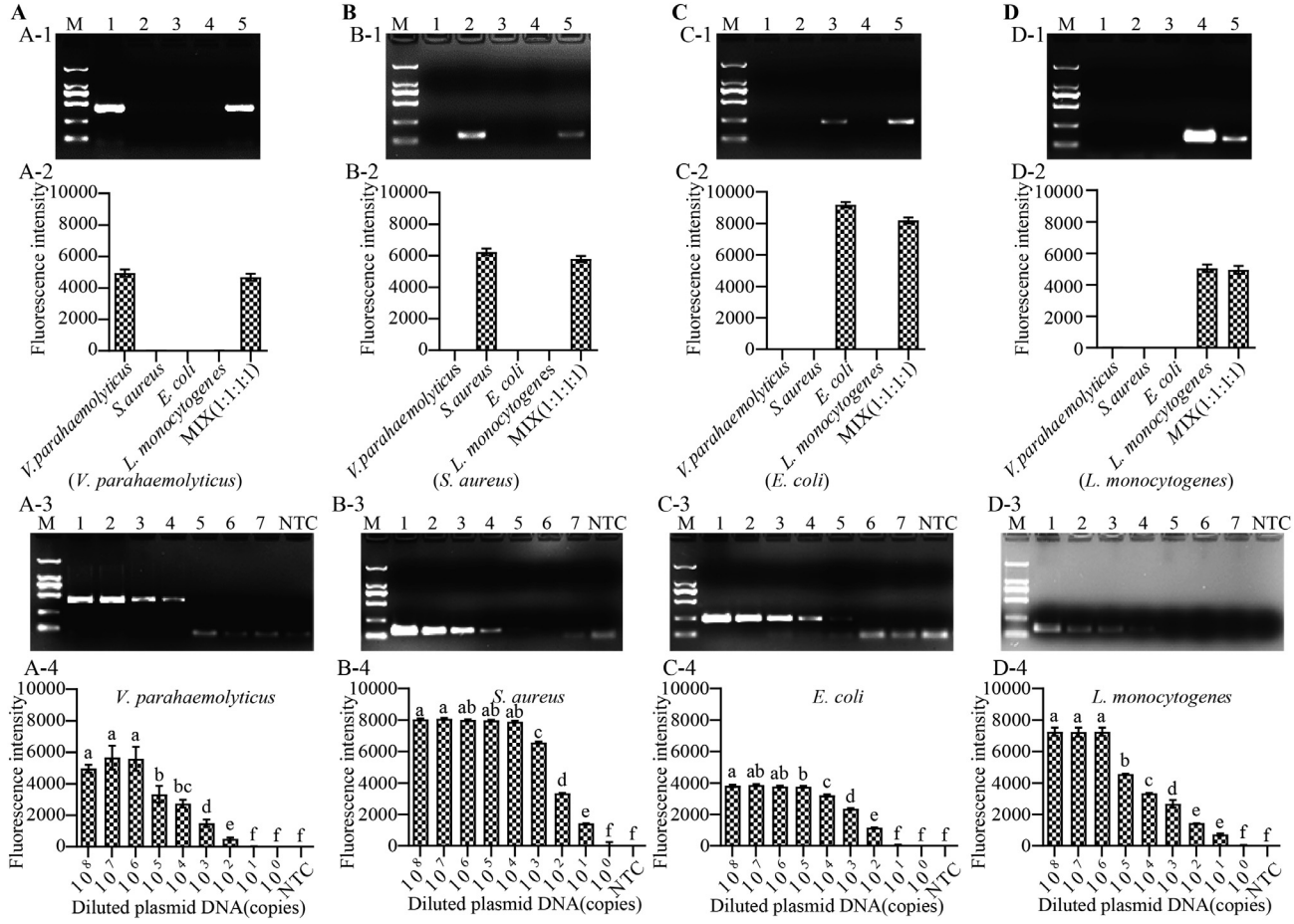


Fig. 4. RPA-Cas12a-FS system application to foodborne pathogens. A, B, C, and D show the molecular specificity and sensitivity verification for *V. parahaemolyticus, S. aureus*, *E. coli*, and *L. monocytogenes.* M: DL2000 Marker; NTC: No template added as a negative control. A-1, B-1, C-1, and D-1: 1: *V. parahaemolyticus*; 2: *S. aureus*; 3: *E. coli*; 4: *L. monocytogenes*; 5: MIX (*V. parahaemolyticus*: *S. aureus*: *E. coli*: *L. monocytogenes* = 1:1:1:1). A-3, B-3, C-3, and D-3: 1: 10^6 copies; 2: 10^5 copies; 3: 10^4 copies; 4: 10^3 copies; 5: 10^2 copies; 6: 10^1 copies; 7: 10^0 copies. Different lowercase letters indicate significant differences between members of each group.

products were detected in 30 tested samples (Fig. S4).

3.3.2. RPA-Cas12a-FS system applications in foodborne pathogens

To enable the RPA-Cas12a-FS system for various other applications, we attempted to employ it in foodborne pathogen detection. We selected *V. parahaemolyticus*, *S. aureus*, *E. coli,* and *L. monocytogenes* for detection, as these are common foodborne pathogens. The designed CrRNA had high specificity, high sensitivity, and stable fluorescence intensity for each foodborne pathogen. The RPA-Cas12a-FS system also specifically detected the genes of different foodborne pathogens, enabling the identification of foodborne pathogens at the nucleic acid level (shown in Fig. 4 A-2, B-2, C-2, and D-2).

To determine the sensitivity of the RPA-Cas12a-FS system for each foodborne pathogen, diluted plasmid DNAs with the target gene fragments from *V. parahaemolyticus*, *S. aureus*, *E. coli*, and *L. monocytogenes* were added to RPA reactions to detect the fluorescence intensity (Fig. 4 A-4, B-4, C-4, and D-4). We found that the fluorescence intensity gradually decreased as the diluted plasmid DNAs with the target gene fragments from *V. parahaemolyticus*, *S. aureus*, *E. coli*, and *L. monocytogenes* decreased. ANOVA classified all of this diluted plasmid DNA with the target gene fragments from *V. parahaemolyticus, S. aureus, E. coli,* and *L. monocytogenes* into seven groups (a, b, c, d, e, and f in Fig. 4 A-4, B-4, C-4, and D-4) with $p = 0.05$, meaning that there was a statistically significant difference. As is shown in Fig. 4 A-4, the sensitivity of *V. parahaemolyticus* fluorescence detection was 100 copies. As is shown in Fig. 4 B-4, C-4, and D-4, the sensitivity of *S. aureus, E. coli,* and *L. monocytogenes* fluorescence detection was 10 copies. Thus, this RPA-Cas12a-FS system could be used to detect foodborne pathogens.

3.3.3. RPA-Cas12a-FS system applications in the traceability of meat products

To validate the RPA-Cas12a-FS system for various other applications, we attempted to employ it in animal-derived-ingredient detection. In animal-derived-ingredient detection, the main problem is often meat adulteration, such as adulterating expensive beef with relatively cheap duck or pork. Therefore, we selected pork, beef, and duck meat as our animal-derived ingredients factors. The designed CrRNAs had high specificity, high sensitivity, and stable fluorescence intensity for each animal-derived species. The RPA-Cas12a-FS system also specifically detected the genes of different species, enabling the identification of animal-derived species at the nucleic acid level (shown in Fig. S5 A-2, B-2, and C-2).

To determine the sensitivity of the RPA-Cas12a-FS system on each animal-derived species, the diluted plasmid DNA with the target gene fragments from pork, beef, and duck was added to RPA reaction to detect the fluorescence intensity (Fig. S5 A-4, B-4, and C-4). We found that the fluorescence intensity gradually decreased as the diluted plasmid DNA with the target gene fragments from pork, beef, and duck decreased. ANOVA classified all of these diluted plasmid DNA with the target gene fragments from pork into eight groups (a, b, c, d, e, f, and g in Fig. S5 A-4) with $p = 0.05$, meaning that there was a statistically significant difference. As is shown in Fig. S5 A-4, the sensitivity of pork fluorescence detection was 100 copies. ANOVA classified all of these diluted plasmid DNA with the target gene fragments from beef and duck

into eight groups (a, b, c, d, e, f, g, and h in Fig. S5 B-4 and Fig. S5 C-4) with $p = 0.05$, meaning that there was a statistically significant difference. As is shown in Fig. S5 B-4 and Fig. S5 C-4, the sensitivity of beef and duck fluorescence detection was 10 copies. The RPA-Cas12a-FS system was then verified using 11 kinds of meat samples (as blind sample) from a local super market. The verification results showed that this method was consistent with issued current standards (Table S3). This RPA-Cas12a-FS system could be used, then, to detect adulteration of meat products.

4. Discussion

Food analysis is necessary to ensure the authenticity of foods, which helps people avoid foods containing specific ingredients (Ahmed, Hasan, Hossain, Saito, & Tamiya, 2010). Assays based on nucleic acids, such as PCR, RT-qPCR, and ddPCR can identify ingredients with high sensitivity. A brief summary of these methods is shown in Table S4. Each test method has its own significance and value towards making final conclusions about food quality and safety (Nazir & Iqbal, 2019). PCR is a primary method at the DNA level. Qualitative analysis is comprised of specific detection of target DNA sequence in test samples. In this method, target gene elements are multiplied to millions or billions using thermal cycling. This amplified DNA can be visualized by gel electrophoresis techniques (Cao et al., 2018). Quantitative PCR is an up-to-date method, principally based on RT-PCR and is generally known as qRT-PCR. It is more robust, specific and sensitive, and provides good quantitative results. The process of amplification is presented in real-time by capturing a fluorescent signal in more sophisticated way (Yao, Li, Cao, Liu, & Fan et al., 2018; Yao, Li, Cao Liu, & Qi et al., 2018). The principle of ddPCR is to divide the reaction system containing nucleic acid molecules into tens of thousands of droplets. After PCR amplification, the fluorescence signal is detected for each droplet, and accurate absolute quantification is achieved according to a Poisson distribution (Bogožalec Košir et al., 2019). However, these assays require expensive equipment and are not suitable for poorly equipped laboratories or field diagnosis (Chai et al., 2013). Therefore, it is necessary to develop a simple, relatively low-cost, rapid, specific, and highly sensitive detection method requiring minimal equipment to detect the presence of targeted DNA-segments in samples. The RPA-Cas12a-FS systems proposed in this study are based on RNA-mediated, DNA-targeting CRISPR effector protein Cas12a collateral cleavage as a starting point and used Cas12a non-targeting ssDNA (one end contained a fluorescent group and the other a quencher group) for collateral target cleavage. Fluorescence intensity was used to characterize various factors. The entire system completes a full run within 40 min, delivering rapid and high-sensitivity detection.

From Table S3, the method in this article is shown to have high detection sensitivity and is more suitable for rapid on-site detection of samples than other methods. Unlike conventional analysis methods, RPA-Cas12a-FS does not rely on sophisticated equipment, so in some cases, it should be more suitable than conventional methods. Only one handheld centrifuge and portable fluorescence analyzer are needed to complete rapid on-site detection, while traditional detection methods still need to be completed in the laboratory. Previously, we combined the RPA technique and test strip LFD and obtained a sensitivity of 100 and 50 copies for *P-CaMV35S* and *T-nos*, respectively (Liu et al., 2020). Although the RPA-LFD detection method can also achieve on-site quick measurement, its sensitivity is 10 times lower than that of RPA-Cas12a-FS. Compared with a previous method detection method, the detection sensitivity of RPA-Cas12a-FS was higher, and the operations could be completed by persons with minimal training, which greatly improved the detection efficiency and intensity, improving the effect for food safety early warning systems, and potentially saving more. This system can be used at the bottom of the food inspection industry chain, such as at food testing stations.

This system was named RPA-Cas12a-FS and the schematic diagram is shown in Fig. 1. This detection system was mainly divided into 2 parts. First, the factors in various foodstuffs were classified and specific RPA primers were designed to amplify the target nucleic acid fragment. Second, CrRNAs that target nucleic acids were designed and the property of collateral cleavage of target nucleic acids by Cas12a in the CRISPR system was utilized to characterize various potential factors based on changes in fluorescence intensity in the designed ssDNA. The development of the RPA-Cas12a-FS system was to enable high-quality and rapid detection of various potential factors in foodstuffs. This method was then applied to samples with foodborne pathogens, transgenic products, and meat products traceability and provided good reliability for the on-site rapid detection of various nucleic acid levels, saving a significant amount of time and effort. Therefore, this method is convenient for laboratory workstations, grassroots local law enforcement agencies, and inspection and quarantine departments, where experimental equipment is relatively lacking.

This novel method has several characteristics. First, the RPA system was optimized to a reaction volume of 20 μL, which can ensure RPA amplification efficiency and a reduction in reaction cost. This system is simple to operate and only requires 15 min at room temperature before 1 μL of RPA product is added to the RPA-Cas12a-FS system and fluorescence intensity is measured after 20 min. This is a rapid and sensitive on-site detection technique that can be used for on-site rapid detection of various nucleic acid levels. Second, specific sequence identification of CrRNAs of 19 nucleotides or 24 nucleotides in length was designed, which makes more specific combining with RPA specific amplification. Third, the length of the ssDNA was optimized to 8 NT, which saved detection cost. Forth, the enhanced Cas12a detection method is carried out at 37 °C in a portable device and the LOD was 10 copies, making it suitable for use with foodborne pathogens, transgenic products, and meat products in field or in under-equipped laboratories.

5. Conclusion

In conclusion, a simple, relatively low-cost, rapid, specific, and highly sensitive on-site detection method based on CRISPR-Cas12a was established for molecular identification of foodborne pathogenic bacteria, genetically modified crops, and meat adulteration. This novel method can provide an alternative tool for detection at the DNA level using a portable thermostatic heater and handheld fluorescence analyzer, making it amenable to a wide number of geographical locations, particularly in resource-poor settings.

CRediT authorship contribution statement

Hua Liu: Investigation, Writing - original draft. **Jinbin Wang:** Writing - review & editing. **Haijuan Zeng:** Investigation. **Xiaofeng Liu:** Visualization. **Wei Jiang:** Investigation. **Yu Wang:** Investigation. **Wanbao Ouyang:** Investigation. **Xueming Tang:** Conceptualization, Methodology, Funding acquisition.

Declaration of Competing Interest

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

Acknowledgments

This study was supported by the Natural Science Foundation of Shanghai No. 19ZR1436800, Shanghai Agricultural Science Committee Foundation of China [No. 2-1 (2020)], the SAAS Program for Excellent Research Team No. 2017 (B-07), Shanghai Sailing Program No. 20YF1443000, Run-up Program of Shanghai Academy of Agricultural Sciences No. ZP19212, Xinjiang High-level Talent Development Plan.

No. 2017-669, and Shanghai Fresh Corn Technology System Project No. 10, 2017.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127608.

References

Ahmed, M. U., Hasan, Q., Hossain, M. M., Saito, M., & Tamiya, E. (2010). Meat species identification based on the loop mediated isothermal amplification and electrochemical DNA sensor. *Food Control, 21*(5), 599–605. https://doi.org/10.1016/j.foodcont.2009.09.001.

Bai, J., Lin, H., Li, H., Zhou, Y., Liu, J., Zhong, G., ... Yang, J. (2019). Cas12a-based on-site and rapid nucleic acid detection of African swine fever. *Frontiers in Microbiology, 10*, 2830. https://doi.org/10.3389/fmicb.2019.02830.

Bogožalec Košir, A., Demšar, T., Štebih, D., Žel, J., & Milavec, M. (2019). Digital PCR as an effective tool for GMO quantification in complex matrices. *Food Chemistry, 294*, 73–78. https://doi.org/10.1016/j.foodchem.2019.05.029.

Cao, J., Yao, Y., Fan, K., Tan, G., Xiang, W., Xia, X., ... Zhang, L. (2018). Harnessing a previously unidentified capability of bacterial allosteric transcription factors for sensing diverse small molecules in vitro. *Science Advances, 4*(11), eaau4602. https://doi.org/10.1126/sciadv.aau4602.

Chai, Z., Ma, W., Fu, F., Lang, Y., Wang, W., Tong, G., ... Li, X. (2013). A SYBR Green-based real-time RT-PCR assay for simple and rapid detection and differentiation of highly pathogenic and classical type 2 porcine reproductive and respiratory syndrome virus circulating in China. *Archives of Virology, 158*(2), 407–415. https://doi.org/10.1007/s00705-012-1504-7.

Chaijarasphong, T., Thammachai, T., Itsathitphaisarn, O., Sritunyalucksana, K., & Suebsing, R. (2019). Potential application of CRISPR-Cas12a fluorescence assay coupled with rapid nucleic acid amplification for detection of white spot syndrome virus in shrimp. *Aquaculture, 512*, Article 734340. https://doi.org/10.1016/j.aquaculture.2019.734340.

Chang, Y., Deng, Y., Li, T., Wang, J., Wang, T., Tan, F., ... Tian, K. (2020). Visual detection of porcine reproductive and respiratory syndrome virus using CRISPR-Cas13a. *Transboundary and Emerging Diseases, 67*(2), 564–571. https://doi.org/10.1111/tbed.13368.

Chen, J. S., Ma, E., Harrington, L. B., Da Costa, M., Tian, X., Palefsky, J. M., & Doudna, J. A. (2018). CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. *Science, 360*(6387), 436–439. https://doi.org/10.1126/science.aar6245.

Creutzburg, S. C. A., Swartjes, T., & van der Oost, J. (2020). Medium-throughput in vitro detection of DNA cleavage by CRISPR-Cas12a. *Methods, 172*, 27–31. https://doi.org/10.1016/j.ymeth.2019.11.005.

Delong, R. K., & Zhou, Q. (2015). Experiment 6 – Polymerase chain reaction (PCR). In R. K. Delong & Q. Zhou (Eds.), Introductory Experiments on Biomolecules and their Interactions, (pp. 59–66). Boston: Academic Press.

Fonfara, I., Richter, H., Bratovič, M., Le Rhun, A., & Charpentier, E. (2016). The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA. *Nature, 532*(7600), 517–521. https://doi.org/10.1038/nature17945.

General Administration of Quality Supervision and Inspection of the People's Republic of China. (2016). Method for detection of pathogens in food for export-real-time PCR method SN/T 1870-2016, China Standard Press. http://down.foodmate.net/standard/sort/4/48614.html.

Gootenberg, J. S., Abudayyeh, O. O., Kellner, M. J., Joung, J., Collins, J. J., & Zhang, F. (2018). Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. *Science, 360*(6387), 439–444. https://doi.org/10.1126/science.aaq0179.

Gootenberg, J. S., Abudayyeh, O. O., Lee, J. W., Essletzbichler, P., Dy, A. J., Joung, J., ... Freije, C. A. (2017). Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science, 356*(6336), 438–442. https://doi.org/10.1126/science.aam9321.

Huang, C.-H., Lee, K.-C., & Doudna, J. A. (2018). Applications of CRISPR-Cas Enzymes in Cancer Therapeutics and Detection. *Trends in Cancer, 4*(7), 499–512. https://doi.org/10.1016/j.trecan.2018.05.006.

Lei, C., Li, S.-Y., Liu, J.-K., Zheng, X., Zhao, G.-P., & Wang, J. (2017). The CCTL (Cpf1-assisted Cutting and Taq DNA ligase-assisted Ligation) method for efficient editing of large DNA constructs in vitro. *Nucleic Acids Research, 45*(9), Article e74. https://doi.org/10.1093/nar/gkx01.

Li, L., Li, S., Wu, N., Wu, J., Wang, G., Zhao, G., & Wang, J. (2019). HOLMESv2: A CRISPR-Cas12b-assisted platform for nucleic acid detection and DNA methylation quantitation. *ACS Synthetic Biology, 8*(10), 2228–2237. https://doi.org/10.1021/acssynbio.9b00209.

Li, S.-Y., Cheng, Q.-X., Liu, J.-K., Nie, X.-Q., Zhao, G.-P., & Wang, J. (2018). CRISPR-Cas12a has both cis-and trans-cleavage activities on single-stranded DNA. *Cell Research, 28*(4), 491–493. https://doi.org/10.1038/s41422-018-0022-x.

Li, S.-Y., Cheng, Q.-X., Wang, J.-M., Li, X.-Y., Zhang, Z.-L., Gao, S., ... Wang, J. (2018). CRISPR-Cas12a-assisted nucleic acid detection. *Cell Discovery, 4*(1), 1–4. https://doi.org/10.1038/s41421-018-0028-z.

Li, S.-Y., Liu, J.-K., Zhao, G.-P., & Wang, J. (2018). CADS: CRISPR/Cas12a-assisted DNA steganography for securing the storage and transfer of DNA-encoded information. *ACS Synthetic Biology, 7*(4), 1174–1178. https://doi.org/10.1021/acssynbio.8b0007410.1021/acssynbio.8b00074.s001.

Liu, H., Wang, J., Li, P., Bai, L., Jia, J., Pan, A., ... Tang, X. (2020). Rapid detection of P–35S and T-nos in genetically modified organisms by recombinase polymerase amplification combined with a lateral flow strip. *Food Control, 107*, Article 106775. https://doi.org/10.1016/j.foodcont.2019.106775.

Ma, E., Harrington, L. B., O'Connell, M. R., Zhou, K., & Doudna, J. A. (2015). Single-stranded DNA cleavage by divergent CRISPR-Cas9 enzymes. *Molecular Cell, 60*(3), 398–407. https://doi.org/10.1016/j.molcel.2015.10.030.

Ministry of Agriculture and Rural Areas of the People's Republic of China. (2012). Detection of genetically modified plants and derived products Qualitative PCR methods for the regulatory elements CaMV 35S promoter, FMV 35S promoter, NOS promoter, NOS terminator and CaMV 35S terminator Announcement No.1782 of the Ministry of Agriculture-3-2012, http://down.foodmate.net/standard/sort/3/38254.html.

Ministry of Agriculture and Rural Areas of the People's Republic of China. (2018). Identification of animal-derived materials in meat Qualitative realtime PCR method. NYT3309-2018, China Agricultural Press. http://down.foodmate.net/standard/sort/5/54422.html.

National Standard of the People's Republic of China. (2018). Detection of genetically modified organisms and derived products Qualitative real-time polymerase chain reaction (PCR) methods GB/T19495.5-2018. https://www.spc.org.cn/online/GB%252FT%252019495.4-2018/.

Nazir, S., & Iqbal, M. Z. (2019). *Molecular identification of genetically modified crops for biosafety and legitimacy of transgenes. Gene editing-technologies and applications.* IntechOpen https://doi.org/10.5772/intechopen.81079.

Rath, D., Amlinger, L., Rath, A., & Lundgren, M. (2015). The CRISPR-Cas immune system: Biology, mechanisms and applications. *Biochimie, 117*, 119–128.

Świat, M. A., Dashko, S., den Ridder, M., Wijsman, M., van der Oost, J., Daran, J.-M., & Daran-Lapujade, P. (2017). FnCpf1: A novel and efficient genome editing tool for Saccharomyces cerevisiae. *Nucleic Acids Research, 45*(21), 12585–12598. https://doi.org/10.1093/nar/gkx1007.

Tsou, J.-H., Leng, Q., & Jiang, F. (2019). A CRISPR test for detection of circulating nuclei acids. *Translational Oncology, 12*(12), 1566–1573. https://doi.org/10.1016/j.tranon.2019.08.011.

Waiblinger, H.-U., Ernst, B., Anderson, A., & Pietsch, K. (2008). Validation and collaborative study of a P35S and T-nos duplex real-time PCR screening method to detect genetically modified organisms in food products. *European Food Research and Technology, 226*(5), 1221–1228. https://doi.org/10.1007/s00217-007-0748-z.

Xiao, G., Liu, S., Liu, H., He, X., Zhang, S., Liang, Z., ... Zhang, G. (2020). CRISPR/Cas12a-based biosensing platform for precise and efficient screening of CRISPR/Cas9-induced biallelic mutants. *Talanta, 210*, Article 120613. https://doi.org/10.1016/j.talanta.2019.120613.

Yao, Y., Li, S., Cao, J., Liu, W., Fan, K., Xiang, W., ... Wang, W. (2018). Development of small molecule biosensors by coupling the recognition of the bacterial allosteric transcription factor with isothermal strand displacement amplification. *Chemical Communications, 54*(38), 4774–4777. https://doi.org/10.1039/C8CC01764F.

Yao, Y., Li, S., Cao, J., Liu, W., Qi, F., Xiang, W., ... Zhang, L. (2018). A novel signal transduction system for development of uric acid biosensors. *Applied Microbiology and Biotechnology, 102*(17), 7489–7497. https://doi.org/10.1007/s00253-018-9056-8.

Zalatan, J. G., Lee, M. E., Almeida, R., Gilbert, L. A., Whitehead, E. H., La Russa, M., ... Qi, L. S. (2015). Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. *Cell, 160*(1–2), 339–350. https://doi.org/10.1016/j.cell.2014.11.052.

Zetsche, B., Gootenberg, J. S., Abudayyeh, O. O., Slaymaker, I. M., Makarova, K. S., Essletzbichler, P., ... Regev, A. (2015). Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell, 163*(3), 759–771. https://doi.org/10.1016/j.cell.2015.09.038.