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CRISPR-Cas12-Based Rapid Authentication of Halal Food

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ABSTRACT: The halal food market is globally growing along with the increased risk of adulteration. We proposed an amplification-free and mix-to-read CRISPR-Cas12-based nucleic acid analytical strategy allowing rapid identification and analysis of pork components, thus enriching the toolbox for ensuring halal food authenticity. We designed and optimized guide RNA (gRNA) targeting the pork cytochrome b (Cyt b) gene. gRNA allowed specific identification of the target Cyt b gene from pork components followed by activation of Cas12 protein to abundantly cleave single-stranded DNA probes with terminally labeled fluorophore and quencher groups, thus turning on fluorescence. The presence of the pork Cyt b gene thus can be mix-and-read- and only-one-step-detected, which may indicate the risk of halal food adulteration. The method allowed specific discrimination of pork meat from beef, mutton, and chicken and yielded a detection limit of 2.7 ng/μL of total DNA from pork meat. The reliability of the method was tested using the following processed meat products: halal foods beef luncheon meat and spiced beef and non-halal foods sausage and dried pork slices. The CRISPR-Cas12-based nucleic acid test strategy is promising for rapid food authentication.

KEYWORDS: *food authentication, CRISPR-Cas12, halal food, gene analysis, Cyt b gene*

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■ INTRODUCTION

The world's Muslim population is currently estimated at 1.6 billion and is predicted to be 2.2 billion by 2030. The halal food market is growing, with an estimated value of $1.6 trillion per year at present.[1,2] Halal food emphasizes health, which also attracts many non-Muslim consumers. As a result, the global halal food industry is currently one of the fastest growing consumer sectors in the world. Although halal food is properly labeled, some manufacturers mix pork with beef and mutton to make higher profits, in order to reduce the cost of food production because pork is cheaper than beef and mutton. In addition, once the meat is treated and purified, it is difficult to ensure its material source or whether it is mixed with pork and its derivatives in processing.[3] A mass of scientific research has been done to defend consumer's rights and ensure the implementation of food labeling laws;[4,5] rapid methods for detecting pork contamination are important to ensure the authenticity of halal products.

Analytical methods that can be used to detect pork contaminations in meat food are by targeting either their chemical elements, components, or biomolecules.[6−9] Measurement of the stable isotope of the bioelements (H, C, N, O, and S), the Warner−Bratzler shear force method, optical techniques (color measurements and fluorescence), electrical probing or electromagnetic wave probing, and ultrasonic measurements can indicate the species of meats.[10] However, the data analysis can be complex, and these methods are not stable and specific when foods are processed. Particularly, biomolecules, mainly protein and DNA, are used as highly specific biomarkers for identifying different meat species.[11,12] The most common techniques in protein analysis are the use of antibodies or aptamers to identify specific protein epitopes based on an enzyme-linked immunosorbent assay, the use of immunosensors, or separation strategies for proteins using an electric field, such as isoelectric focusing, electrophoresis, and chromatography.[10,13] However, protein-based methods have limitations in detecting animal species in cooked, baked, or heat-treated food because proteins tend to denature at high temperatures, limiting their availability in downstream recognition steps. In addition, the use of monoclonal antibodies requires cumbersome procedures and a high budget, and polyclonal antibodies are a cheaper option, but they often recognize multiple epitopes, resulting in non-specific binding.[14] DNA is stable and is found in most cells with species specificity, which helps to extract and identify the sources of food components from various tissues. At present, the main techniques for analyzing meat varieties founded on DNA are the polymerase chain reaction (PCR) and real-time quantitative PCR (qPCR).[15] They are highly sensitive and can detect pork contamination in processed food, but they also have some limitations. The PCR method requires expensive instruments, skilled operators, and a lengthy electrophoresis process. Using a lateral flow immunoassay to substitute the electrophoresis process to detect the amplified DNA products could increased the availability of PCR for on-site detection.[16] qPCR uses a real-time fluorescence monitoring process to quantify the DNA copies, with a dramatic increment of the cost of PCR instruments.[17,18] The amplification process still complicates

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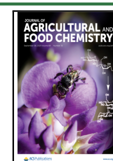

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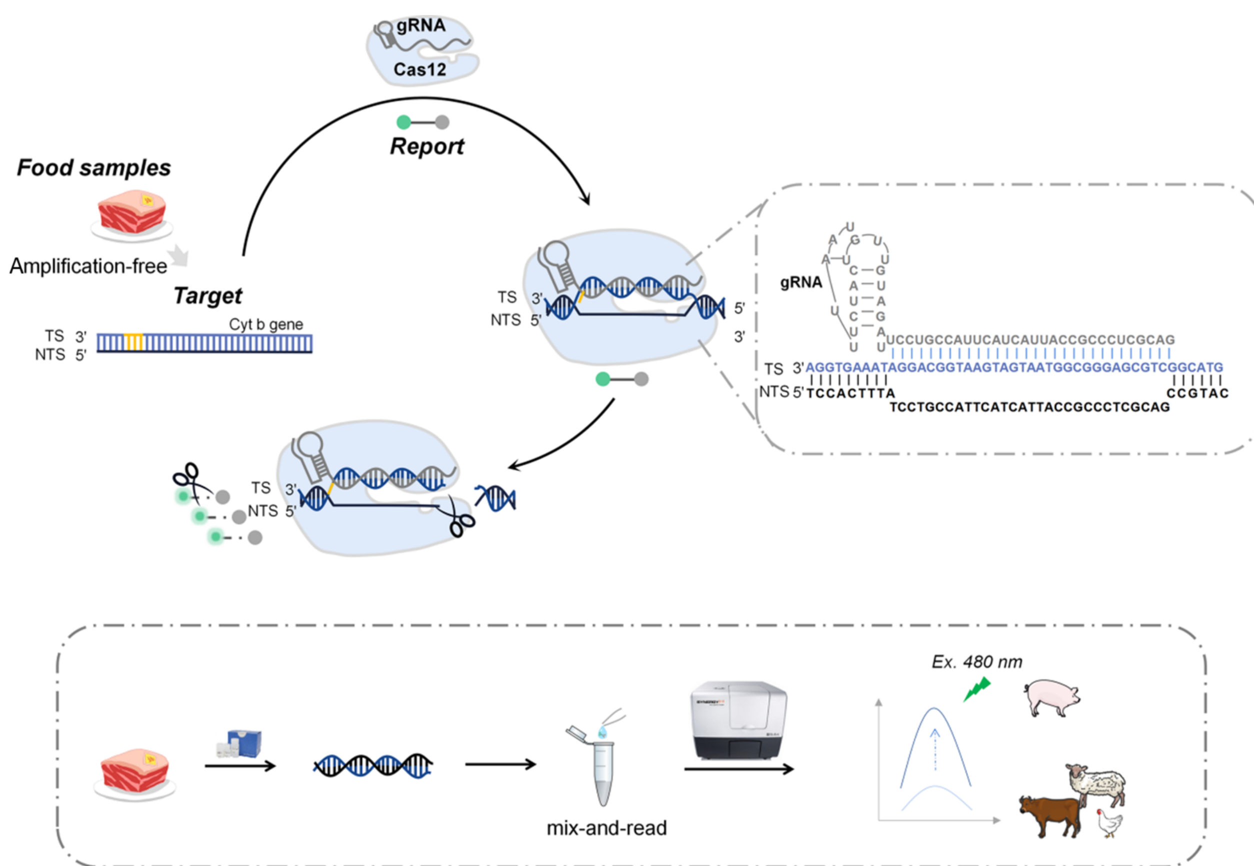
Scheme 1. Schematic Illustration of the Working Principle of CRISPR-Cas12-Based Nucleic Acid Tests for Halal Food Authentication



the detection process and increases the test costs, hindering their widespread use in resource-limited regions.

Clustered regularly interspaced short palindromic repeats (CRISPR) along with corresponding CRISPR-associated Cas protein are a kind of acquired programmable immune system against foreign invading nucleic acids, which uses CRISPR RNA (crRNA, also known as guide RNA, gRNA) to recognize and target invasive nucleic acids.[19] The CRISPR-Cas12 system belongs to the class 2 type V-A CRISPR-Cas system, which is an endonuclease guided by RNA.[20] The binding of gRNA with target DNA will activate the trans-cleavage activity of Cas12 to cleave single-stranded DNA.[21] The CRISPR-Cas12 system has been utilized to construct nucleic acid test strategies and mainly to detect pathogenic microorganisms such as papillomavirus and coronavirus.[22−24] Genes of target pathogenic microorganisms are first amplified by nucleic acid amplification techniques, such as PCR, loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA), and CRISPR-Cas12 is utilized to specifically identify the amplified DNA sequences.[25] Involvement of CRISPR-Cas12 can dramatically increase the specificity for identifying target genes, along with enhanced sensitivity induced by the trans-cleavage activity of Cas12a.[21] Further adaption of lateral flow strips permitted the detection of pathogenic microorganisms by the naked eye, thus facilitating on-site detection.[26] While CRISPR-Cas12-based nucleic acid tests are promising, they have not yet been utilized for food authenticity.

Herein, we proposed a CRISPR-Cas12-based amplification-free, mix-and-read nucleic acid analytical strategy allowing us to rapidly identify and analyze pork components. All of the detection processes can be incubated in one test tube at 37 °C, eliminating costly PCR instruments and sharply shortening the sample-to-answer turnaround time. The method allowed specific discrimination of pork meat from beef, mutton, and chicken meat. The reliability of the method has been tested using the following processed meat products: halal foods beef luncheon meat and spiced beef and non-halal foods sausage and dried pork slices. The CRISPR-Cas12-based nucleic acid test strategy is promising for rapid authentication of halal food.

■ MATERIALS AND METHODS

Reagents and Equipment. Phi29 DNA polymerase (20 U/μL) with 10× reaction buffers and T7 RNA polymerase (20 U/μL) with 5× T7 RNA polymerase buffers were purchased from Thermo Fisher Scientific (Waltham, USA). Cas12a protein with 10× 2.1 buffer, DNase I with 10× reaction buffer, ribonucleotide solution mix (rNTPs), and deoxynucleotide solution mix (dNTPs) were obtained from New England Biolabs (Beijing, China). Platinum SYBR Green qPCR SuperMix-UDG w/ROX was provided by Thermo Fisher Scientific (Waltham, USA). Molecular biology-grade water was purchased from Corning Inc. (New York, USA). All the reagents were dissolved in molecular biology-grade water. All the oligonucleotide sequences used were custom-prepared by Sangon Biotechnology Co., Ltd. (Shanghai, China); the sequences are listed in Table S1. The DNA reporter was purified by high-performance liquid chromatography (HPLC), and its 5′ and 3′ terminals were modified with 6-carboxyfluorescein (FAM) fluorophores and black hole quencher 1 (BHQ1), respectively. DNA concentration was estimated by measuring the absorption using the fluorescence microplate reader Synergy H1 (BioTek, USA). Fluorescence spectra were measured using the microplate reader Synergy H1 with an excitation/emission

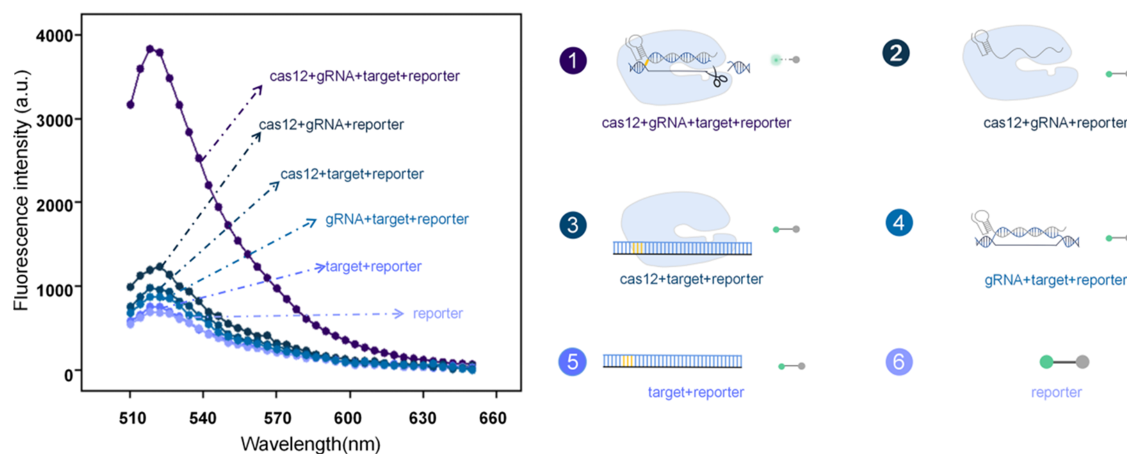


Figure 1. Investigation of the working principle of CRISPR-Cas12-based nucleic acid tests. Fluorescence curves of CRISPR-Cas12 in each reaction step.

wavelength of 480/510 nm. qPCR was performed in 96-well plates using the Real-Time System CFX connect (Bio-Rad, USA). Mitochondrial cytochrome b gene (Cyt b gene) sequences of pork and three other animal species (beef, mutton, and chicken) were retrieved from the NCBI database (pork: NC_000845.1, beef: EU807948.1, mutton: EU365990.1, and chicken: EU839454.1).

Sample Preparation and DNA Extraction. Fresh meat samples of pork, beef, mutton, and chicken were purchased from local markets. For the retail survey, four processed meat samples (beef luncheon meat, spiced beef, sausage, and dried pork slices) were obtained from retail markets in Chengdu. Samples were stored at −20 °C until they are required for analysis. In each case, 25−50 mg of raw meat and processed meat samples were cut into pieces and then ground in a pestle and mortar into powder by adding liquid nitrogen. The Rapid Animal Genomic DNA Isolation Kit (Sangon Biotechnology Co., Ltd., Shanghai, China) was utilized to extract all kinds of meat genomic DNA according to its instructions. Briefly, animal tissue was added to Buffer Digestion to completely lyse the cells; then, Buffer PA, isopropanol, and 75% ethanol were sequentially added to extract the DNA; and finally the residual ethanol was completely volatilized. The obtained DNA was dissolved in 50 μL of H_2O. The extracted DNA was immediately carried over to the next experiment or preserved at −20 °C.

Preparation of gRNA. The transcription reaction of gRNA was carried out in a 200 μL centrifuge tube containing 5 μL of the DNA nucleotide sequence of gRNA (10 μM), 5 μL of promoter (10 μM), 5 μL of Phi29 buffer (10×), and 21 μL of H_2O, and the mixture was incubated at 90 °C for 3 min and then at room temperature for 30 min. The next step was the addition of 0.5 μL of Phi29 DNA polymerase (20 U/μL) and 1 μL of dNTPs (10 mM each for dATP, dGTP, dCTP, and dTTP), letting them react at 30 °C for 30 min, and afterward incubation at 65 °C for 10 min for inactivation of Phi29 DNA polymerase. Then, 1 μL of T7 RNA polymerase (20 U/μL), 2 μL of rNTPs (25 mM each for ATP, GTP, CTP, and TTP), and 10 μL of T7 RNA polymerase buffer (5×) were added to the mixture, and the mixed solution was incubated at 37 °C for 12 h to generate gRNA. Lastly, 2 μL of DNase I and 6 μL of buffer (10×) were added to the solution, incubated for 2 h at 37 °C to remove the remaining DNA, and then heated at 65 °C for 15 min for enzyme inactivation.

Process of Pork Gene Detection. Pork gene detection was performed at 37 °C, containing 4 μL of New England Biolabs (NEB) buffer 3.1, 4 μL of Cas12 (1 μM), 4 μL of synthesized gRNA (2 μM), and 20 μL of H_2O, incubated for10 min to form Cas12/gRNA duplex, then added 500 ng/μL of extracted DNA and 4 μL of reporter (5 μM) incubated at 37 °C for 2.5 h. Fluorescence emission was excited at 480 nm and detected at 510 nm using the fluorescence microplate reader Synergy H1 (BioTek, USA). All samples were analyzed three times.

qPCR Detection of Pork Genes. Beef, mutton, and chicken were chosen as the control for the specificity test of this assay. Additionally, halal foods beef luncheon meat and spiced beef and non-halal foods sausage and dried pork slices were used to test the reliability of the method. The extracted total DNA from these samples was analyzed by CRISPR-Cas 12-based nucleic acid tests and qPCR. qPCR was conducted in 96-well plates using the Real-Time System CFX connect (Bio-Rad, Singapore). A total of 20 μL of qPCR mixture containing 2 μL of extracted total DNA (10 ng/μL), 4 μL of amplification forward primer (2.5 μM), 4 μL of amplification reverse primer (2.5 μM), 10 μL of Platinum SYBR Green qPCR SuperMix-UDG w/ROX was prepared. PCR was held at 95 °C for 10 min, and then 40 amplification cycles were carried out at 95 °C for 15 s and 60 °C for 1 min.[27]

RESULTS AND DISCUSSION

Working Principle. CRISPR-Cas12-based nucleic acid tests for detecting pork contaminant lies in the direct activation of the ribonuclease of CRISPR-Cas12 via the target pork gene (Scheme 1);[28] the structure of gRNA can be referred to in previous works.[29] Mitochondrial gene Cyt b was chosen as the target gene as it was specific enough to discriminate the different meat species.[1] In addition, it can be copied multiple times in cells, and thus the detection of mitochondrial genes would increase the sensitivity for target meat components unlike the detection of nuclear genes. The presence of target pork Cyt b gene DNA activates the catalytic domains of Cas12 protein via specific hybridization with the gRNA locus to cleave the nearby single-stranded DNA (ssDNA) reporters.[26,30−32] A random reporter ssDNA with a terminally labeled 6-FAM (F) fluorophore and a BHQ1 (B) quencher was adopted to monitor the recognition event of CRISPR-Cas12/gRNA complex binding with target DNA via recovery of the fluorescence of the 6-FAM fluorophore.[33] If no target existed, the CRISPR-Cas12/gRNA complex did not yield cleavage activity, resulting in low fluorescence as the 6-FAM fluorophore was quenched by BHQ1. In this way, the presence of the pork Cyt b gene DNA was induced a fluorescence change, which was conveniently measured using a fluorometer.[29,34]

The CRISPR-Cas12/gRNA system employs reagents that are easily available and equipment that is found in most routine laboratories; thus, it has the potential for achieving a rapid and specific method for halal food authentication analysis. Notably,

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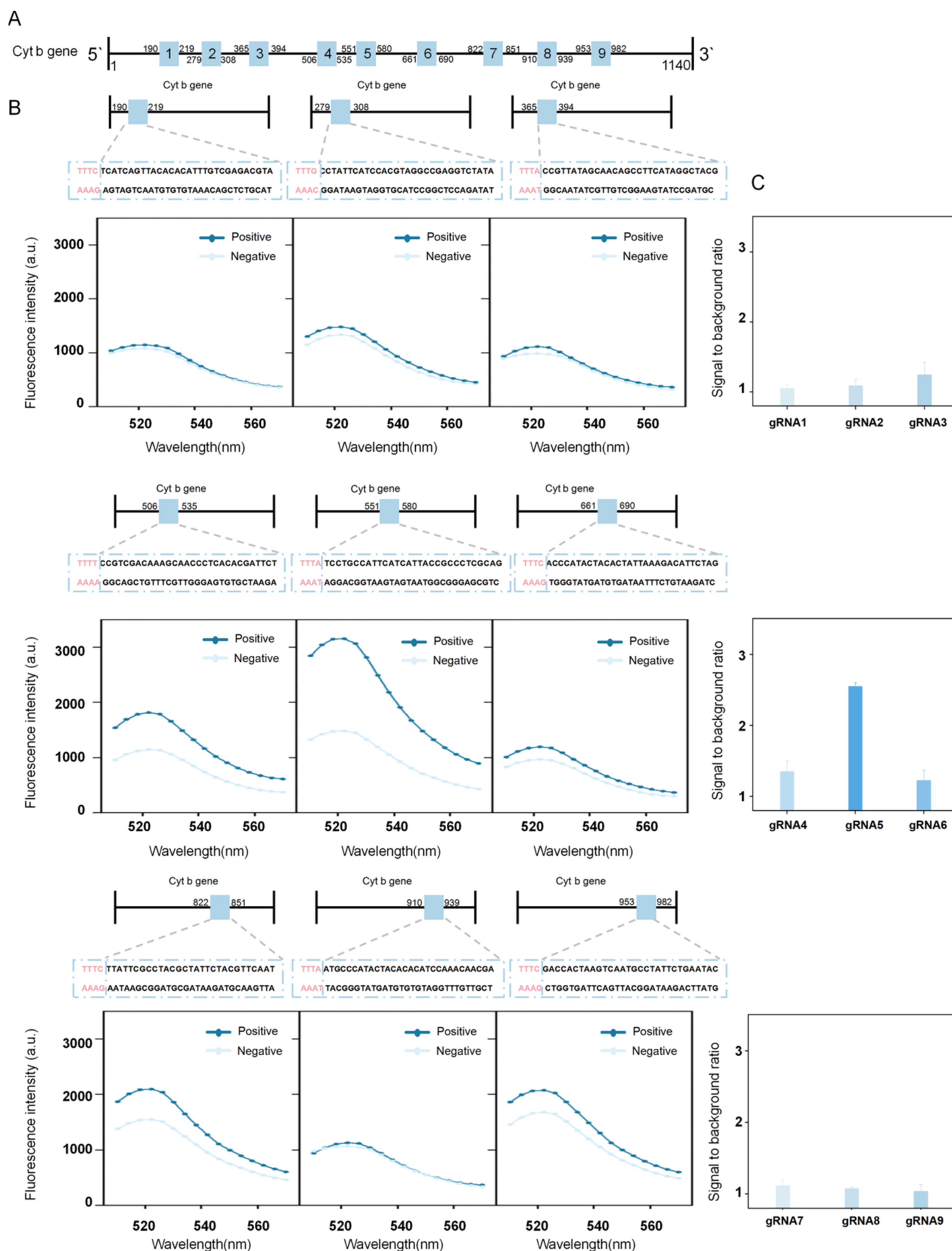

Figure 2. Optimization of the location of gRNA. (A) Sites of gRNA in the Cyt b gene. (B) Fluorescence curves of CRISPR-Cas12 system using different gRNAs. (C) Signal to background ratio of CRISPR-Cas12 system to detect the Cyt b gene using different gRNAs.

all detection processes could be carried out in one test tube at 37 °C, avoiding the complex temperature-control process.

To confirm the working principle of CRISPR-Cas12-based nucleic acid tests, fluorescence analysis of each step of the assay was conducted (Figure 1). The presence of reporters led to initially low fluorescence because fluorescence of 6-FAM is quenched by the BHQ1 group. The addition of target Cyt b gene DNA barely enhances the intensity of the fluorescence, indicating that using only the target does not cleave the reporter. Further addition of gRNA or Cas12 to the target still led to a low fluorescence intensity, illustrating that either Cas12 or gRNA alone does not initiate the cleavage of reporters. Meanwhile, the presence of Cas12 and gRNA without target DNA still exhibited a low fluorescence intensity. Upon the addition of target DNA, a dramatic enhancement of the fluorescence intensity from 1562 to 4471 occurred, indicating the activation of cleavage activity of the Cas12/gRNA system by target DNA. The results elucidated that the

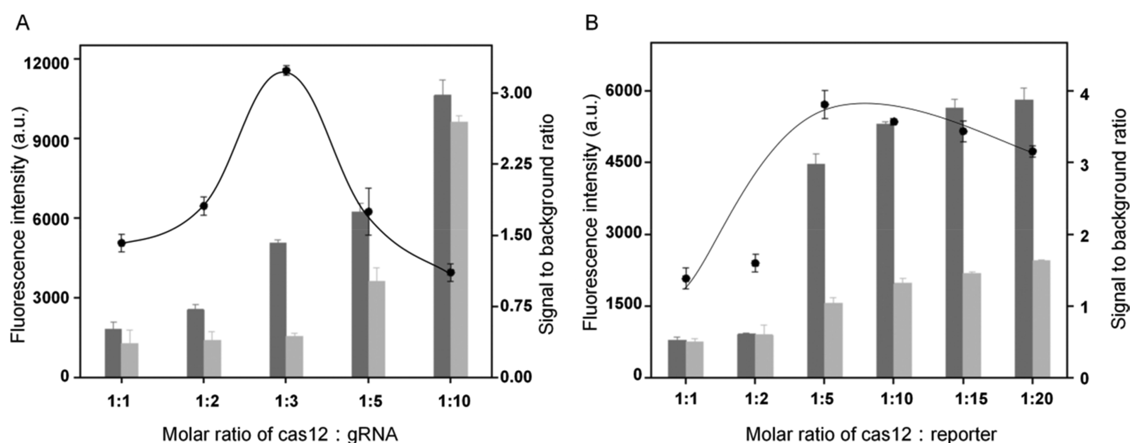

Figure 3. Optimization of the sensing conditions. (A) Fluorescence intensity and S/B ratio of different Cas12 protein-to-gRNA molar ratio. (B) Fluorescence intensity and S/B ratio of different Cas12 protein-to-reporter molar ratio.

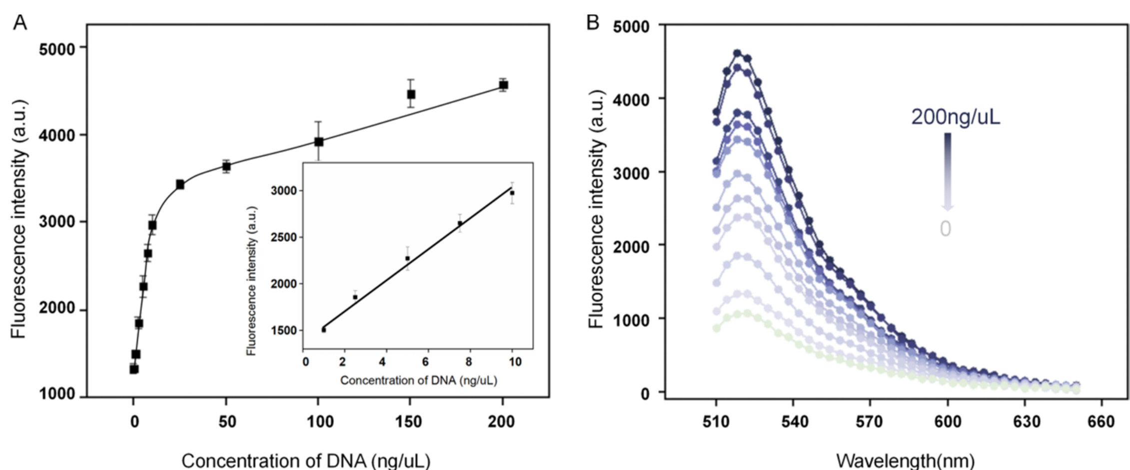

Figure 4. Quantification performance of CRISPR-Cas12-based nucleic acid detection platform. (A) Relationship between the concentration of total DNA from pork meat and fluorescence intensity; (B) Fluorescence curves of the method corresponding to the addition of the concentrations of total DNA ranging from 0 to 200 ng/μL (0, 1, 2.5, 5, 7.5, 10, 25, 50, 100,150, and 200 ng/μL).

target Cyt b gene DNA could efficiently activate the cleavage capacity of Cas12/gRNA system toward reporters. Thus, the CRISPR-Cas12 system may be used to construct nucleic acid tests for halal food authentication.

Optimization of the Sensing Conditions. The selection of a gRNA locus could substantially affect the fluorescence response of the CRISPR-Cas12 system and thus was first optimized (Figure 2). The gRNAs were screened to eliminate potential non-specific gRNAs binding to the Cyt b gene of other animal species using the online BLAST local alignment tool in the NCBI database (http://www.ncbi.nlm.nih.gov/blast). The proposed gRNA held no significant similarity in terms of the Cyt b gene with the other tested species. We moved the gRNA binding sites by designing gRNA with different recognition sequences and chose a 30 nt nucleic acid sequence after TTTN (N: A, G, T, C) in pork Cyt b gene DNA as the gRNA locus, the gRNA region within the Cyt b gene from 190 to 982.[33] The results indicated that the highest signal-to-background (S/B) ratio was achieved when selected the location of gRNA 5 as the identify loci. Compared to other gRNA loci, gRNA 5 dramatically elevated its S/B ratio to 2.51, while the S/B ratio of other loci was approximately 1 (Figure 2B). The other loci gave low S/B ratios maybe because of the complex secondary structure of the genes, which may have resulted in the difficulty of gRNA to bind.

The concentration of gRNA and fluorescent reporter could significantly affect the fluorescence response in CRISPR-Cas12-mediated nucleic acid tests (Figure 3). The fluorescence intensity was recorded under varied molar ratios of Cas12 protein to gRNA. A higher concentration of gRNA would contribute to enhanced fluorescence. In addition, the maximum S/B ratio reached was 3.25 in a Cas12 protein to gRNA ratio of 1:3; subsequently, the background fluorescence intensity sharply enhanced, resulting in a drop of the S/B ratio (Figure 3A). For the Cas12 protein to reporter molar ratio, the fluorescence intensity of negative background samples (without the addition of pork Cyt b gene DNA) and positive samples (with the addition of the pork Cyt b gene DNA) both rose up with the increase in the Cas12 protein to reporter ratio (Figure 3B), and the maximum S/B ratio (3.76) was achieved when the Cas12 protein-to-reporter ratio was 1:5.

Quantification Performance. Under the above optimal experimental conditions, we tested the detection performance of the CRISPR-Cas12 system, and a series of different amounts of total DNA (0−200 ng/μL) extracted from pork meat were added to the reaction system (Figure 4). The fluorescence intensity gradually increased with enhanced concentration of

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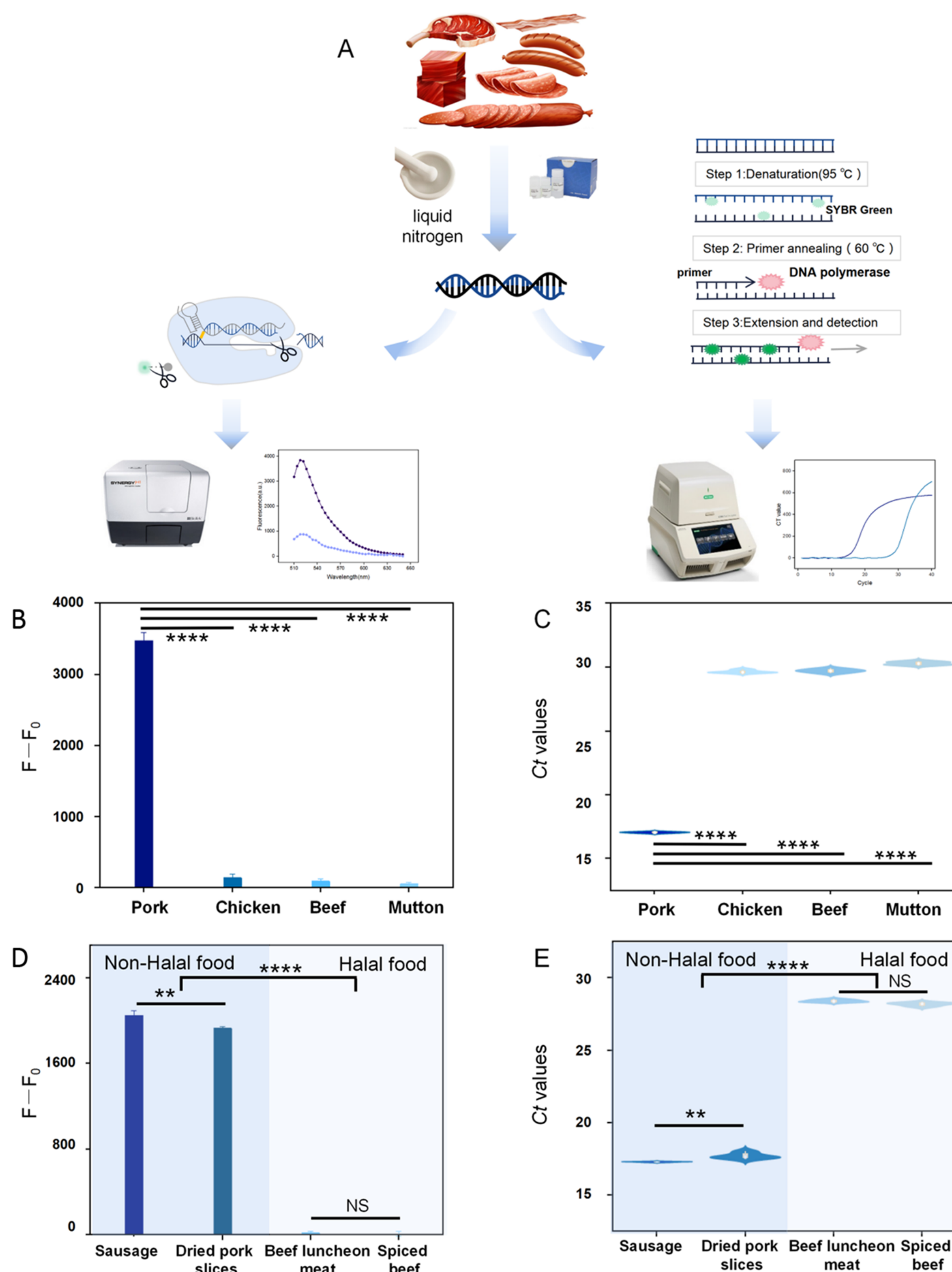

Figure 5. CRISPR-Cas12-based nucleic acid tests for halal food authentication. (A) Schematic diagram of CRISPR-Cas12-based and qPCR-based nucleic acid tests for halal food authentication; (B) fluorescence intensity change of CRISPR-Cas12-based nucleic acid tests responding to pork, chicken, beef, and mutton DNA. (C) *Ct* value of qPCR corresponding to pork, chicken, beef, and mutton DNA; (D) fluorescence intensity of CRISPR-Cas12-based nucleic acid tests responding to non-halal food of sausage and dried pork slices samples and halal food of beef luncheon meat and spiced beef; and (E) *Ct* value of qPCR corresponding to non-halal food of sausage, dried pork slices samples, and the halal food of beef luncheon meat and spiced beef DNA. Statistical significances tested were obtained by two-tailed unpaired Student's *t*-test: NS, $P > 0.05$; **, $P < 0.01$; ****, $P < 0.0001$. F was defined as the fluorescence intensity in the presence of the sample DNA, and F_0 was the fluorescence signal without the addition of any sample DNA.

total DNA. The addition of total DNA with the concentration range from 1 to 10 ng/μL showed its good linear relationship with the fluorescence intensity (Figure 4A). The linear regression equation for total DNA detection was $y = 171.3x + 1345.6$ ($R^2 = 0.9909$), where x and y represent the concentration of total DNA and fluorescence intensity of the CRISPR-Cas12 system, respectively. The assay yielded a detection limit (LOD) of 2.7 ng/μL, which was calculated by the concentration corresponding to the fluorescence intensity at three times standard deviation of the control group that had no DNA. Particularly, the pork DNA can be mix-and-read-detected in one test tube at 37 °C, avoiding the complex operation process.

Specific Tests. The identification of meat species and halal food authentication is becoming a major concern in the globe. We tested the ability of the CRISPR-Cas12-based nucleic acid

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tests to discriminate different meat species. Three common meat species (beef, mutton, and chicken) were chosen as control groups. Total DNA was extracted from a 50 mg sample of each meat species (Figure 5A). When the target strain pork Cyt b gene DNA was introduced in the system, the $F-F_0$ value sharply increased (Figure 5B and Figure S1A). F is defined as the fluorescence intensity in the presence of each meat DNA, and F_0 is the fluorescence signal without the addition of any meat DNA. Among these meat species, only the pork Cyt b gene DNA can lead to a remarkably distinguishable fluorescence change compared to the background. We proceeded with RT-qPCR in parallel to verified the result, the *Ct* value of pork meat is dramatically decreased compare with other meat species (Figure 5C and Figure S1B). Both CRISPR-Cas12-based and qPCR-based nucleic acid tests allowed identification of pork meat from beef, mutton, and chicken, while the former can proceed in one-test tube at a constant temperature, yielding a promising rapid assay for meat species identification.

Food Authentication of the Processed Meat Products. The reliability of the assay was tested with the following processed meat products: halal foods beef luncheon meat and spiced beef and non-halal foods sausage and dried pork slices (Figure 5D). Samples (50 mg) of each processed meat products were used for DNA extraction. The presence of total DNA extracted from the two halal food samples induced fluorescence close to that of the blank group, indicating that the two halal food samples were both correctly labeled as having no pork. The two processed pork foods provided total DNAs that generated a remarkable fluorescence signal enhancement compared to that of the blank group (Figure S2A), indicating pork content. qPCR was used to confirm the results (Figure 5E and Figure S1B). CRISPR-Cas12-based nucleic acid tests exhibits potential in food authentication of the processed meat products.

Mitochondrial gene Cyt b was chosen as the target gene, which can be copied multiple times in cells, and a large amount of the Cyt b gene DNA can be obtained even when the DNA is severely broken due to intense processing conditions.[35] Meanwhile, studies have shown that the degree of damage to the sample DNA to be tested is closely related to the processing temperature, such as heating at 100 °C where the fragment length of the DNA will decrease to 1100 bp and at 120 °C where it will decrease to below 600 bp.[36] In our proposed CRISPR-Cas12-based nucleic acid tests, the length of gRNA was identified to be only 30 bp. Thus, even if DNA is partially damaged, it had a negligible effect on the results of the authentication of halal food using the CRISPR-Cas12-based nucleic acid tests.

In summary, we constructed a rapid, simple, and low-cost detection platform for halal food authentication based on a CRISPR-Cas12 system. The detection platform allowed amplification-free and mix-to-read detection of target genes, thus enabling rapid detection of pork gene DNA in complex samples, and presented excellent selectivity in the authentication of halal food against other processed meat products. All the detection processes could be achieved in one test tube at 37 °C. Thus, CRISPR-Cas12-based nucleic acid tests are promising for rapid and on-site authentication of halal food as well as other high-value foods.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c03078.

Oligonucleotide sequences (Table S1); DNA sequences of pork, chicken, beef, and mutton Cyt b genes (Figure S1); fluorescence intensity of CRISPR-Cas12-based nucleic acid tests for the pork Cyt b gene with respect to reaction time (Figure S2); fluorescence curves and qPCR amplification curves corresponding to different raw meat DNA (Figure S3); and fluorescence curves and qPCR amplification curves corresponding to different processed meat product DNA (Figure S4) PDF

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Notes

The authors declare no competing financial interest.

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