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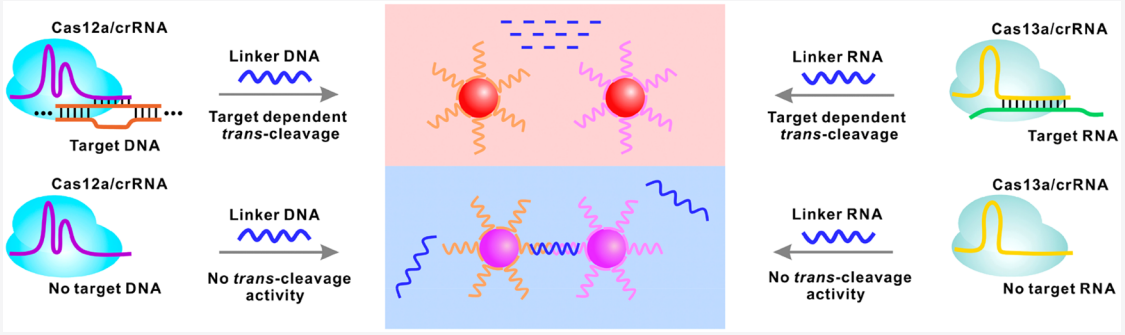
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Universal and Naked-Eye Gene Detection Platform Based on the Clustered Regularly Interspaced Short Palindromic Repeats/Cas12a/13a System

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ABSTRACT: Gold-nanoparticles-based colorimetric assay is an attractive detection format, but is limited by the tedious and ineffective posthybridization manipulations for genomic analysis. Here, we present a new design for a colorimetric gene-sensing platform based on the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system. In this strategy, programmable recognition of DNA by Cas12a/crRNA and RNA by Cas13a/crRNA with a complementary target activates the *trans*-ssDNA or -ssRNA cleavage. Target-induced *trans*-ssDNA or ssRNA cleavage triggers an aggregation behavior change for the designed AuNPs−DNA probes pair, enabling the completion of naked-eye gene detection (transgenic rice, African swine fever virus, and miRNAs as the models) within 1 h. This platform is also showing promise as a fast and inexpensive tool for bacteria identification using *16S rDNA* or *16S rRNA*. A CRISPR/Cas-based colorimetric platform shows superior characteristics, such as probe universality, compatibility with isothermal reaction conditions, on-site detection capability, and high sensitivity, thus, demonstrating its use as a robust next-generation gene detection platform.

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Developing simple, inexpensive, and accurate analytical methods to detect ultralow concentrations of DNA or RNA sequences with high sensitivity and specificity is important for medical molecular diagnosis, agricultural and food testing, and forensic analysis.[1] Ideal diagnostic tests should deliver results quickly and able to be used instantly on a variety of sample types without excessive reliance on a technician or an auxiliary device.[2,3] Colorimetric detection based on gold nanoparticles (AuNPs) is a simple and inexpensive detection format that has attracted considerable interest in the scientific community. Aggregation and dispersion behavior of AuNPs exhibit unique distance-dependent optical properties that can be clearly identified with the naked eye.[4−6] Aggregation of AuNPs shifts the absorption peak to longer wavelengths and changes the color of a colloidal solution from red to purple.[7] Mirkin and colleagues are the pioneers in the development of colorimetric detection strategies based on AuNPs.[7,8] They were the first to invent the DNA-probes-labeled gold nanostructure, which was later termed spherical nucleic acids, for sequence-specific gene detection.[9,10] In the subsequent decades, the academic community has developed a large number of colorimetric gene detection methods on the basis of the cross-linking or non-cross-linking behavior of AuNPs.[11−24]

Some of these methods are coupled with gene amplification technology; thus, the sensitivity is significantly improved and genomic samples can be analyzed. Despite great success, the

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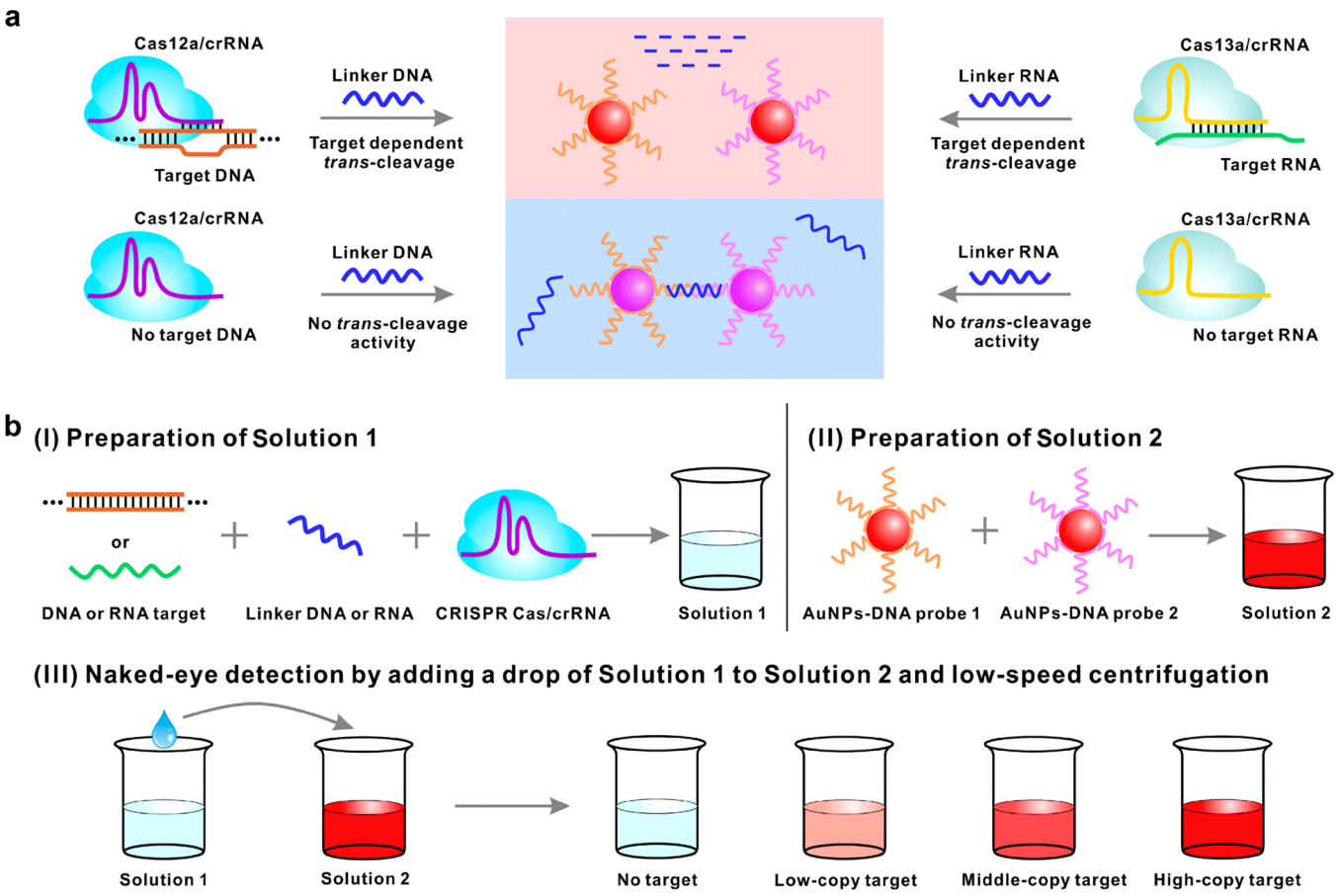


Figure 1. Schematic illustration and workflow of CRISPR/Cas-based colorimetric gene detection. (a) Cas12a/crRNA and Cas13a/crRNA possess target-dependent *trans*-cleavage activity. The *trans*-cleavage activity was used for signal reporting that is based on distance-dependent optical properties of the AuNPs−DNA probes pair. In the presence of a target, linker ssDNA or ssRNA is degraded.The AuNPs−DNA probes pair loses the hybridization linkers and becomes dispersed. In the absence of a target, linker ssDNA and linker ssRNA are intact. A cross-linking reaction of the AuNPs−DNA probes pair with linker ssDNA or ssRNA results in aggregation. (b) Workflow of CRISPR/Cas-based colorimetric gene detection. (I) Target DNA and RNA are added to Cas/crRNA complexes in the presence of linker ssDNA or ssRNA to prepare Solution 1. (II) AuNPs−DNA probes pair is mixed to prepare Solution 2. (III) Naked-eye detection can be completed by adding a drop of Solution 1 to Solution 2 followed by low-speed centrifugation.

following problems still need to be addressed: (a) Most of the existing methods detect a red-to-purple color change, which is similar to a signal-off format. When a low-concentration target is detected, the faint color change makes it difficult to detect and the signal-to-noise ratio is very low. (b) AuNPs−DNA probes are not universal. Probes need to be redesigned when detecting different targets, thus increasing the cost and time spent for optimization of the experimental parameters. Some methods have probe universality by the design of a universal tag sequence in the primer for subsequent AuNPs−DNA probes hybridization;[21] however, this approach may lead to the false-positive problems caused by primer dimer amplification. (c) Detecting genomic amplification products requires denaturation of double-stranded DNA for hybridization.[20] This high-temperature hybridization process is incompatible with the AuNPs−DNA probes stability. Improvement in the stability of AuNPs−DNA probes by time-consuming modification of the nanoparticle surface, such as silicidation, is usually necessary.[22] The problems can be addressed by employing asymmetric amplification to obtain single-stranded products for room-temperature hybridization.[11] However, asymmetric amplification reduces the amplification efficiency and affects the detection sensitivity.

Bacteria have evolved the *clustered regularly interspaced short palindromic repeats* (CRISPR)/Cas immune system to specifically target exogenous threats, such as a phage or exogenous genetic material.[25,26] The sequence-specific recognition capabilities of the CRISPR/Cas system have been applied in a growing variety of fields, such as the pharmaceutical industry, food industry, agricultural industry, and industrial biotechnology;[27−29] these systems are largely developed on the basis of Cas9.[30−32] Recently discovered Cas12a, Cas13a, and Cas14 systems have some new features that are different from the Cas9 system.[33−40] Once Cas12a/crRNA, Cas13a/crRNA, or Cas14/crRNA recognizes their target DNA or RNA, they switch to the active state, wherein the single-stranded DNA (ssDNA) or RNA (ssRNA) substrates are cleaved in a nonspecific way.[33,34,36,39] This collateral cleavage activity is termed *trans*-cleavage. New gene detection technologies using the CRISPR/Cas system, such as DETECTR,[33] HOLMES,[35] and SHERLOCK,[38] with extremely high sensitivity (single-molecule levels) and specificity (single-base resolution), have been developed in combination with gene amplification strategies including polymerase chain reaction (PCR) and recombinase polymerase amplification (RPA). However, most of these strategies involve expensive

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double-labeled (fluorescent and quenching groups) ssDNA or ssRNA substrates and rely on fluorescence detection equipment.[33,35,36,38−40] Zhang and co-workers developed a test strip method for on-site detection.[37] However, similar to the fluorescence detection method, this method requires an expensive dual-labeled fluorescent reporter.

To overcome these limitations, we developed a new genetic detection platform that is based on the CRISPR/Cas technology and AuNPs−DNA probes. This developed gene detection platform has inherently high specificity assisted by CRISPR/Cas recognition and maintains the simplicity of colorimetric detection. Moreover, signal-on detection mode is achieved with the assistance of a low-speed centrifugation method. Successful detection of transgenic DNA, African swine fever virus (ASFV), and miRNA has demonstrated the robust potential of CRISPR/Cas-based colorimetric assay. This platform is also showing promise as a fast and inexpensive tool for bacteria identification using the universal *16S rDNA* amplicons or *16S rRNA*.[41] CRISPR/Cas-based colorimetric platform shows superior characteristics, such as probe universality, compatibility with isothermal reaction conditions, on-site detection capability, and high sensitivity, thus demonstrating its use as a robust next-generation gene detection platform.

EXPERIMENTAL SECTION

Colorimetric DNA Detection Based on the CRISPR/Cas12a System. The Cas12a/crRNA reaction buffer contained 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, and 100 μg/mL bovine serum albumin (BSA), pH 7.9. A 20 μL reaction system contained 100 nM AsCas12a, 200 nM crRNA, and 200 nM linker ssDNA substrate. DNA amplification product (2 μL) was added to the solution, mixed, and reacted in a metal bath for 20 min at 37 °C; then 60 μL of solution was prepared using equal volumes of the two premixed AuNPs−DNA probes solutions (30 μL of AuNPs−DNA probe 1 and 30 μL of AuNPs−DNA probe 2). The samples were incubated at room temperature for 3 min and centrifuged in a tabletop centrifuge at 5000 rpm for 3 min. The supernatant was used for colorimetric detection or absorption spectra analysis.

Colorimetric RNA Detection Based on the CRISPR/Cas13a System. Cas13a/crRNA reaction buffer contained 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, and 100 μg/mL BSA, pH 7.9. A 20 μL reaction system contained 100 nM LbuCas13a, 200 nM crRNA, and 200 nM linker ssRNA substrate. Target RNA (2 μL) was added to the solution, mixed, and reacted in a metal bath for 15 min at 37 °C; then 60 μL of solution was prepared by adding equal volumes of two premixed AuNPs−DNA probes solutions (30 μL of AuNPs−DNA probe 1 and 30 μL of AuNPs−DNA probe 2). The samples were incubated at room temperature for 3 min and centrifuged in a tabletop centrifuge at 5000 rpm for 3 min. The supernatant was used for colorimetric detection or absorption spectra analysis.

RESULTS AND DISCUSSION

Assay Design and Characterization of CRISPR/Cas-Based Colorimetric Gene Detection. Recent studies have shown that Cas12a has both *cis*- and *trans*-ssDNA cleavage activities.[33,34] When Cas12a forms a ternary complex with crRNA and its target DNA, the complex acquires strong "chaotic" activity and cleaves single-stranded DNA into 2−4 nucleotide fragments (called *trans*-cleavage).[34] Studies have shown that both *cis*- and *trans*-cleavage activities of Cas12a are associated with its RuvC pockets.[33] Prior studies have shown that Cas13a has additional collateral cleavage activity after cleavage of its target RNA.[36] Structural studies have analyzed the binary complexes of Cas13a and crRNA in *Leptotrichia shahii* (Lsh) bacteria, revealing that Cas13a functions through two independent active domains.[42] Using this target-dependent *trans*-cleavage by Cas12a and Cas13a, we have developed a new gene detection platform that is based on distance-dependent optical properties of the AuNPs−DNA probes (Figure 1a). In this detection platform, universal linker ssDNA or ssRNA were designed as the *trans*-cleavage substrates for the Cas12a or Cas13a system. Additionally, a pair of universal AuNPs−DNA probes was designed to hybridize to linker ssDNA or ssRNA. In the absence of a target, *trans*-cleavage is not activated, so the linker ssDNA or ssRNA remains intact in the reaction system. The AuNPs−DNA probes pair will undergo a hybridization-induced cross-linking to form an aggregated state. When Cas12a/crRNA and Cas13a/crRNA recognize their target DNA and RNA, respectively, *trans*-cleavage is activated and the linker ssDNA and ssRNA will be degraded (Figure S1, Supporting Information). The AuNPs−DNA probes pair loses the linkers for hybridization and thus becomes dispersed. Visual detection can be performed by estimating the distance-dependent optical properties of the cross-linked and dispersed AuNPs−DNA probes.

After the method was proposed, we proceeded to design a workflow for the gene detection platform (Figure 1b). Because Cas/crRNA complexes, linker ssDNA or ssRNA substrates, and AuNPs−DNA probes can be prepared in advance and are suitable for long-term storage (Figures S2 and S3), the whole detection process can be summarized in three steps: preparation of Solution 1, preparation of Solution 2, and colorimetric detection. The preparation of Solution 1 includes mixing the target genes (or amplicons) with the Cas/crRNA complex and linker substrate; *trans*-cleavage reaction of the CRISPR/Cas system is activated by the targets. The preparation of Solution 2 is very simple and requires simple mixing of the AuNPs−DNA probes pair. Finally, a drop of Solution 1 is added to Solution 2, and after a 3 min incubation period followed by low-speed centrifugation, the test results can be observed with the naked eye. Nucleic acid samples with various numbers of copies can be determined by observing the grades of the color of the AuNPs−DNA probes.

Before performing the detection from actual samples, we executed the optimization experiments using freezing-labeled AuNPs−DNA probes pair to obtain some key parameters for colorimetric detection (Figures S4−S6). Result indicates that the CRISPR/Cas-based detection system requires only 20 min of incubation time, a linker ssDNA concentration of 40 nM, and a linker length of 20 bp (Figure S6). It is worth noting that a ssDNA- or ssRNA-resulting cross-link of AuNPs−DNA probes leads only to a red-to-magenta color change (Figure S4). Such a faint color difference makes naked-eye detection difficult, especially when detecting targets at low concentrations. We thus developed a low-speed centrifugation method where a very sharp color change can be observed (Figure S5).

CRISPR/Cas12a-Based Colorimetric Gene Detection of Genetically Modified Organisms (GMOs) and ASFV. To strengthen the management of genetically modified crops,

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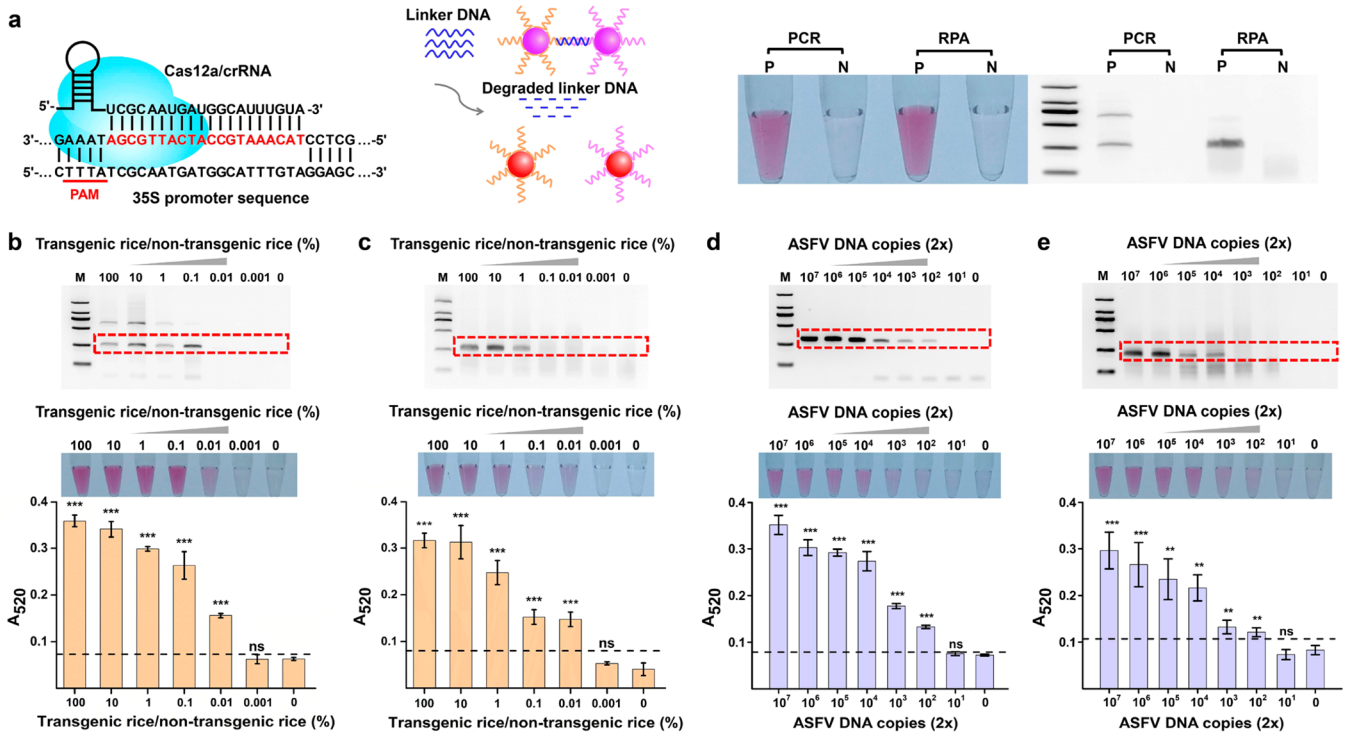


Figure 2. CRISPR/Cas12a-based colorimetric gene detection of GMOs and ASFV. (a) 35S promoter sequence recognition by the Cas12a/crRNA system followed by colorimetric analysis and electrophoretic analysis of the RPA and PCR products of 35S promoter. P and N stand for positive and negative samples, respectively. (b, c) Electrophoretic and colorimetric analysis of (b) PCR products and (c) RPA products with various percentages of transgenic rice DNA. Photograph showing colorimetric responses of the detection system in the presence of various percentages of transgenic rice DNA. The bar chart represents the absorbance (A_{520}) signals of the colorimetric analysis. (d, e) Electrophoretic and colorimetric analysis of (d) PCR products and (e) RPA products with a various number of ASFV DNA copies. Photograph showing colorimetric responses of the detection system in the presence of a various number of ASFV DNA copies. The bar chart represents the absorbance (A_{520}) signals of the colorimetric analysis. The threshold line represents the mean ± 3 standard deviations; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

more than 40 countries and regions including the European Union have enacted the corresponding laws and regulations for management of GMOs and their products.[43] For example, the EU requires a mandatory labeling system when the genetically modified ingredients exceed 1%. The most common components of the transgenic construct, the promoter of the cauliflower mosaic virus (p35S) and the nopaline synthase gene (NOS) from *Agrobacterium tumefaciens*, have become the typical targets for GMOs screening methods.[44] This study is the first to apply the CRISPR/Cas12a-based colorimetric detection system to the detection of 35S promoter from transgenic rice. Figure 2a (left panel) shows that Cas12a/crRNA recognizes a 20 bp target sequence in a 35S promoter sequence adjacent to a TTTA protospacer adjacent motif (PAM) site. *trans*-Cleavage by Cas12a/crRNA is activated by recognition of the 35S promoter sequence with the detection of the signal output using the AuNPs−DNA probes. As shown in Figure 2a (right panel), the straightforward GMOs detection is achieved by the combination with PCR or RPA. In comparison to the results of electrophoretic analysis, the results of CRISPR/Cas12a-based colorimetric analysis are easier to evaluate. In addition, compared to the reported colorimetric detection methods that usually involve a color change from red to magenta to blue, the current method is based on the detection of a colorless-to-red change, thus representing a signal-on detection format. Moreover, CRISPR/Cas12a-based colorimetric detection involves a second step of probe recognition (primer pair amplification represents the first step of recognition), so it can exclude nonspecific signals from the primer dimers or target-independent amplification.

Next, we evaluated the detection ability of the CRISPR/Cas12a-based colorimetric detection system for 35S. We mixed 100 mg of transgenic rice with nontransgenic rice with different mass ratios and milled the rice into a powder for nucleic acid extraction. Subsequently, 20 μL PCR or RPA reaction including 5 ng/μL extracted rice genomic DNA was performed for amplification. After that, 2 μL of the above amplification products were used for colorimetric assay. Combined with PCR or RPA, CRISPR/Cas12a-based colorimetric assays can detect transgenes in rice at an as low as 0.01% level, and the sensitivity of colorimetric analysis is 1 or 2 orders of magnitude higher than that of the electrophoresis analysis. The results show that the sensitivity of the developed method is fully qualified for the needs of the required detection of transgenic GMOs components ranging from 0.1% to 1% worldwide (Figure 2b,c).

African swine fever (ASF) is an acute and potent hemorrhagic infectious disease of the pigs caused by ASFV, which is a double-stranded DNA virus with a capsular membrane belonging to the African porcine virus family. ASFV is transmitted through feces and air and is highly contagious with a 100% mortality rate.[45,46] Since 2018, ASF has spread into more than 14 countries.[47,48] At present, there is no effective treatment or vaccine for ASF. Therefore, early diagnosis of ASF for prevention and control is extremely important. Rapid detection of ASFV infection can be achieved by immunological methods.[49] However, these methods have a

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sensitivity problem because of acute infections; pigs usually die before becoming antibody-positive. This study applied developed colorimetric gene detection to the rapid and sensitive diagnosis of ASFV to help combat the spread of the fatal disease on a global scale. We first used RPA or PCR to amplify the *VP72* gene of ASFV. *VP72* gene encodes the p72 protein, the major capsid protein of ASFV, and the main component of the viral icosahedral capsid, which is highly antigenic and immunogenic. Then we designed Cas12a/crRNA to recognize the *VP72* gene sequence. *VP72* gene binds to the designed Cas12a/crRNA complexes to activate *trans*-cleavage, resulting in disassembly of the AuNPs−DNA probes and subsequent easily detectable color change. Detection sensitivity was evaluated by naked-eye detection or by absorption measurement, and a limit of detection of 200 copies of genome DNA was acquired (Figure 2d,e).

On-Site Detection of Pig Serum Samples. As emphasized previously, rapid detection of ASFV infection is becoming increasingly urgent. Therefore, we future tested whether the CRISPR/Cas12a-based colorimetric assay could be used for on-site detection of nucleic acids from pig body fluid, such as whole blood, serum, and saliva, rather than purified nucleic acids. First, we treated the serum samples using a previously reported heating and chemical reduction method. These treated products from the pig serum samples mentioned above can be directly used as a template for RPA reaction.[40] Moreover, we prepared a toolkit (Figure S7) can be used in a nonlaboratory environment. Combined with the isothermal RPA, the CRISPR/Cas12a-based colorimetric assay can be completed in 1 h, including the pretreatment of samples (Figure 3a,b).

Then we tested 45 pig serum samples by using the proposed CRISPR/Cas12a-based colorimetric assay. For comparison, these samples were also tested by qPCR. As shown in Figure 3c, the detection results of the CRISPR/Cas12a-based colorimetric assay are highly consistent with the results of qPCR (for qPCR results, see Figure S8a−c). These results showed the CRISPR/Cas12a-based colorimetric assay has great potential for rapid field-deployable gene detection while maintaining high accuracy.

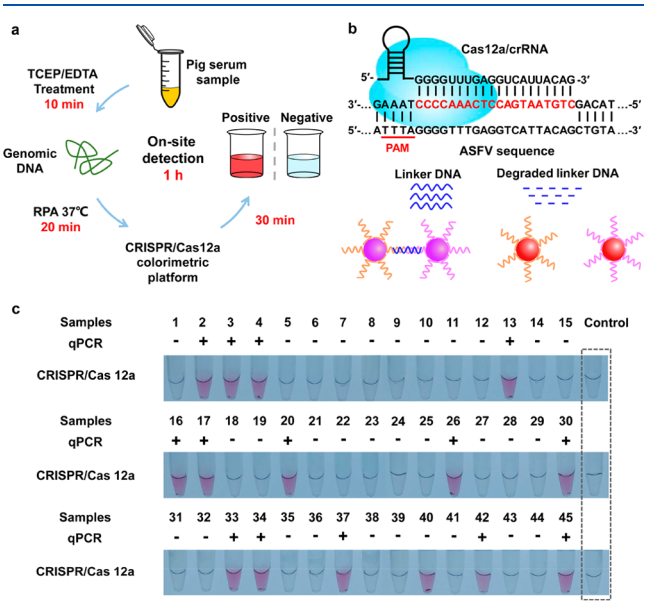


Figure 3. CRISPR/Cas12a-based colorimetric assay for on-site detection of pig serum samples. (a) Schematic illustration of the procedure of the CRISPR/Cas12a-based colorimetric assay for on-site detection of pig serum samples. (b) ASFV sequence recognition by the Cas12a/crRNA system followed by colorimetric analysis. (c) Naked-eye detection results of 45 pig serum samples using CRISPR/Cas12a-based colorimetric assay and with the qPCR assay as the reference.

CRISPR/Cas12a-Based Colorimetric Assay for Bacterial Identification. Bacterial identification based on morphology, isolation culture, and biochemical assays are time-consuming and often inaccurate. On the basis of molecular techniques for *16S rDNA* sequence analysis, bacterial identification without isolation and culture steps can be achieved. *16S rDNA* is a gene encoding prokaryotic *16S rRNA*, approximately 1500 bp in length, and presents in almost all bacterial chromosomes. *16S rDNA* contains conserved regions, which are suitable for designing universal primers. Its variable regions can be employed for molecular identification. Traditionally, molecular techniques based on *16S rDNA* sequence analysis mainly include sequencing or denaturing gradient gel electrophoresis (DGGE). However, both methods are costly and tedious and require a professional technician. Hence, we challenged the CRISPR/Cas12a-based colorimetric assay for bacterial identification.

To perform a proof-of-principle experiment of the current study, we employed a pair of universal primers for amplifying *16S rDNA* from seven common pathogenic bacteria (*Listeria monocytogenes*, *Staphylococcus aureus*, *Neisseria encephalitis*, *Salmonella typhimurium*, *Enterobacter sakazakii*, *Pseudomonas aeruginosa*, and *Vibrio parahemolyticus*) (Figure 4a and Table S1). Electrophoresis analysis indicated that almost the same amplification efficiency for *16S rDNA* from seven bacteria was

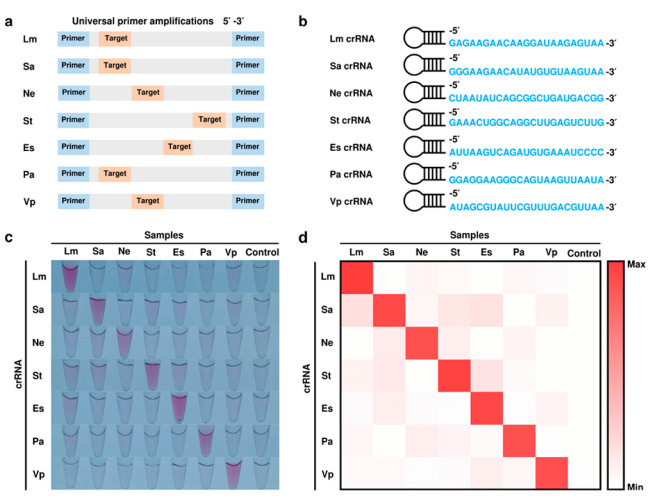


Figure 4. CRISPR/Cas12a-based colorimetric assay for bacterial identification. (a) A pair of universal primers was employed to amplify the *16S rDNA* gene from seven common bacteria. All amplification products have a length of 467 bp. Seven crRNAs were designed to target amplification products according to the PAM site and target sequence specificity. (b) Sequence information on seven crRNAs. The blue letters represent the target region. (c) Typical colorimetric results for orthogonal identification of each bacteria (10 ng/μL). (d) Heat map analysis of the orthogonal identification of seven bacteria based on statistics from three independent experiments: Lm, *Listeria monocytogenes*; Sa, *Staphylococcus aureus*; Ne, *Neisseria encephalitis*; St, *Salmonella typhimurium*; Es, *Enterobacter sakazakii*; Pa, *Pseudomonas aeruginosa*; and Vp, *Vibrio parahemolyticus*, respectively.

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achieved (Figure S9). We then designed seven crRNAs that specifically target sequences within the variable regions of *16S rDNA* from the seven bacteria, respectively, using a customized script program (Figure 4b). The CRISPR/Cas12a-based colorimetric assay and subsequent orthogonal heat map analysis showed that all seven Cas12a/crRNA systems have good detection specificity. No significant cross-reactivity indicated that the current CRISPR/Cas12a-based colorimetric assay provides a robust bacterial identification method (Figure 4c,d).

Extension to Colorimetric miRNA Detection Based on the CRISPR/Cas13a System. Cas13a/crRNA and Cas12a/crRNA have similar target-dependent *trans*-cleavage activity. Therefore, we hypothesize that the developed colorimetric detection platform can be easily extended to RNA detection by employing the same AuNPs−DNA probes pair. The Cas13a/crRNA complex recognizes a target RNA and activates *trans*-cleavage with very high efficiency. For example, a *trans*-cleavage efficiency of at least 10^4 turnovers per target RNA recognition was observed in the case of LbuCas13a.[36] Our previous studies have shown that miRNAs at femtomolar concentrations can be detected using the dual-labeled fluorescent ssRNA reporter.[50] This potent detection ability prompted direct miRNA detection without an amplification step. This feature is very important because miRNAs are very short and some indirect steps, such as reverse transcription and PCR, will have an adverse effect on the detection of their true expression levels.

In this study, we applied the CRISPR/Cas13a-based colorimetric method to RNA detection. RNA detection based on this system needs only minor modifications compared to that of the CRISPR/Cas12a-based strategy. As shown in Figure 5a, with use of *miRNA-17* as a model, the Cas13a/crRNA complex is designed to target the *miRNA-17* sequence. A linker ssRNA with the same sequence as the linker ssDNA used in the CRISPR/Cas12a-based colorimetric detection system was employed as the substrate for target-dependent *trans*-cleavage. The sensitivity of *miRNA-17* detection was as low as 500 fM, which is comparable or even superior to that of the methods based on dual-labeled fluorescent ssRNA reporter (Figure 5b and Figure S10a,b). We attribute this high sensitivity to the clean background signal obtained by low-speed centrifugation. Moreover, the colorimetric method had good specificity for evaluation of 1 nM target *miRNA-17* compared to that of other interfering agents at the same concentration such as *miRNA-10b*, *miRNA-21*, and *miRNA-155* (Figure 5c). Although good specificity of the analysis of different gene families of miRNAs is obtained (Figure 5d), that from the analysis of miRNA members with highly related sequences in the same gene family remains a challenge. We thus applied this CRISPR/Cas-based colorimetric assay to distinguish highly similar miRNAs, such as *miRNA-17*, *miRNA-20a*, *miRNA-20b*, and *miRNA-106a*, with single- or double-nucleotide differences at the same concentration of 1 nM (Figure 5e). We employed the *miRNA-17*-targeted Cas13a/crRNA system to analyze four miRNA members in the same gene family as *miRNA-17*, whereas unsatisfactory detection specificity was achieved, especially for *miRNA-106a*, which has a single-nucleotide difference at the terminal position (Figure S10c,d). We then set a mismatched base in the "mismatch hypersensitive" region (9−14 nt) of the crRNA of the CRISPR/Cas13a system, resulting in the specificity improving substantially. All four miRNAs were

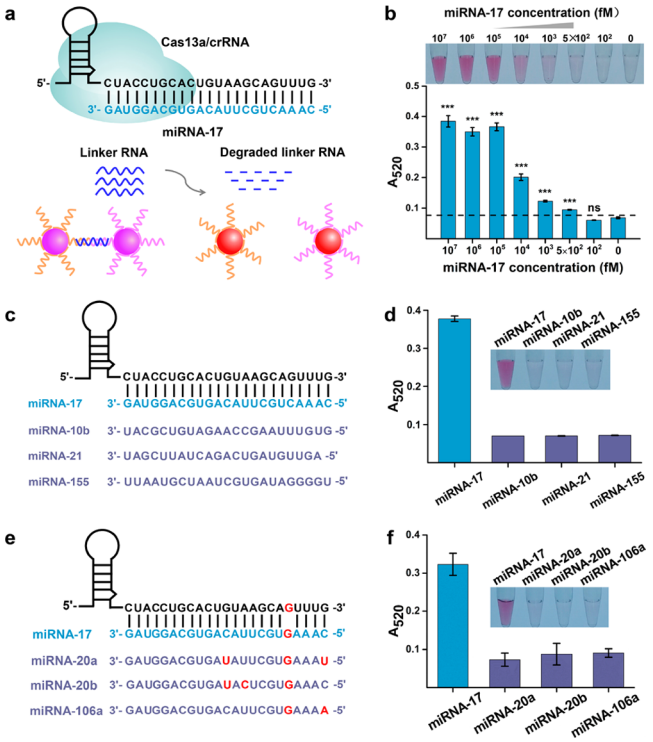


Figure 5. Colorimetric RNA detection based on CRISPR/Cas13a. (a) *miRNA-17* recognition by the Cas13a/crRNA system and a scheme of colorimetric analysis. (b) Cas13a/crRNA for colorimetric analysis of *miRNA-17*. Photograph showing colorimetric responses of the detection system in the presence of different concentrations of *miRNA-17*. The bar chart represents the absorbance (A_{520}) signals of colorimetric analysis. (c) Sequences of crRNA with 23 nt spacer and miRNAs in a different gene family. (d) Cas13a/crRNA for colorimetric analysis of 1 nM *miRNA-17* and miRNAs in different gene families. The bar chart represents the absorbance (A_{520}) signals of colorimetric analysis. (e) Sequences of mutant crRNA with 23 nt spacer and *miRNA-17* family members. Mismatched nucleotides in crRNA and miRNAs are marked in red. (f) Cas13a/crRNA for colorimetric analysis of 1 nM *miRNA-17* family members. The bar chart represents the absorbance (A_{520}) signals of colorimetric analysis. The threshold line represents the mean ± 3 standard deviations; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

discriminated with high specificity (Figure 5f). This indicates that, with an optimized crRNA design, CRISPR/Cas13a-based colorimetric method enables RNA detection with single-base resolution. This is particularly meaningful for the analysis of a miRNA family with a single-base sequence difference at the terminal position, which is usually extremely difficult to achieve by the conventional RT-PCR method.

CRISPR/Cas13a-Based Colorimetric Assay for Bacterial Identification. From the above results, it is clearly confirmed that the CRISPR/Cas13a-based colorimetric assay had satisfactory sensitivity (fM levels) and discriminability (single-base resolution) for miRNAs from the different and same gene families; we therefore envision the use of a CRISPR/Cas13a system to identify bacteria on the basis of *16S rRNA* targets. Compared to the *16S rDNA* detection, direct detection of *16S rRNA* has some obvious advantages, including avoiding amplification and thus saving time and costs. In addition, because RNA has a short half-life, detection of RNA is an indicator for identifying live and dead bacteria. In the proof-of-principle experiment, six bacteria (*L. monocyto-*

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genes, S. aureus, S. typhimurium, E. sakazakii, P. aeruginosa, and *V. parahemolyticus*) were selected as the models. We designed six crRNAs to specifically target sequences from a variable region of *16S rRNA* from six bacteria, respectively (Figure 6a).

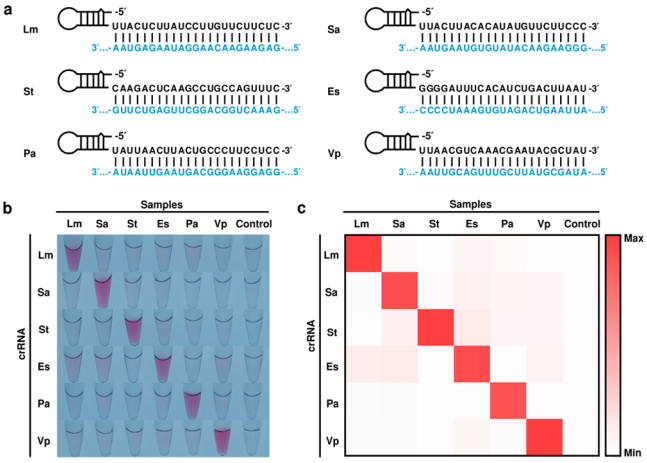


Figure 6. CRISPR/Cas13a-based colorimetric assay for bacterial identification based on *16S rRNA* targets. (a) Six crRNAs sequences and corresponding *16S rRNA* target regions. (b) Typical colorimetric results for orthogonal identification of six bacteria (25 ng/μL). (c) Heat map analysis of the orthogonal identification of six bacteria (25 ng/μL) based on statistics from three independent experiments: Lm, *L. monocytogenes*; Sa, *S. aureus*; St, *S. typhimurium*; Es, *E. sakazakii*; Pa, *P. aeruginosa*; and Vp, *V. parahemolyticus*, respectively.

The CRISPR/Cas13a-based colorimetric assay and orthogonal heat map analysis visually displayed that the designed Cas13a/crRNA systems have excellent specificity for all six bacteria, demonstrating that the developed CRISPR/Cas13a-based colorimetric assay has potential application value in bacterial identification (Figure 6b,c).

CONCLUSION

Colorimetric gene detection based on AuNPs has been developed for more than 20 years. However, certain limitations, such as signal-off detection format, low signal-to-noise ratio, requirements of high hybridization temperature, lack of probe universality, and low discrimination of color changes, are encountered in the use of AuNPs-based colorimetric systems for genomic samples detection and hinder their applicability. Here, we presented a new colorimetric gene detection platform that is based on the CRISPR/Cas12a/13a system that has achieved several critical advances compared to the existing methods: (a) No high-temperature hybridization process is required; (b) specificity is significantly improved with the introduction of isothermal CRISPR/Cas recognition; (c) use of low-speed centrifugation can assist in developing a signal-on colorimetric gene detection platform with improved detection sensitivity. Detection sensitivity of this colorimetric assay is comparable to that of the fluorescence methods (Figure S11). (d) Universality of the current colorimetric gene detection platform was demonstrated by successful detection of transgenic rice, ASFV, miRNAs, and bacterial identification using *16S rDNA*, and *16S rRNA* with the same AuNPs−DNA probes pair. A comparison of current CRISPR/Cas-based colorimetric assay with conventional gene detection strategies and other AuNPs-based colorimetric detection platforms provide further evidence of the advantage of this method (Tables S2 and S3). Although this study provides only a preliminary experimental result, successful detection of ASFV from serum samples using a toolkit in nonlaboratory environments indicated that in the future the CRISPR/Cas-based colorimetric assay can be applied to medical diagnostics, such as the identification of respiratory pathogens, or bacterial typing in agriculture and environmental applications in the field.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.9b05597.

Materials and apparatus; sequence information for oligonucleotides used in this work; synthesis and characterization of AuNPs; expression and purification of AsCas12a and LbuCas13a proteins; preparation of crRNA; sample collection and preparation; optimization of the experimental conditions; additional figures and tables (PDF)

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Notes

The authors declare no competing financial interest.

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