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African swine fever virus

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ACS Synth. Biol., **Just Accepted Manuscript** • DOI: 10.1021/acssynbio.0c00057 • Publication Date (Web): 12 Aug 2020

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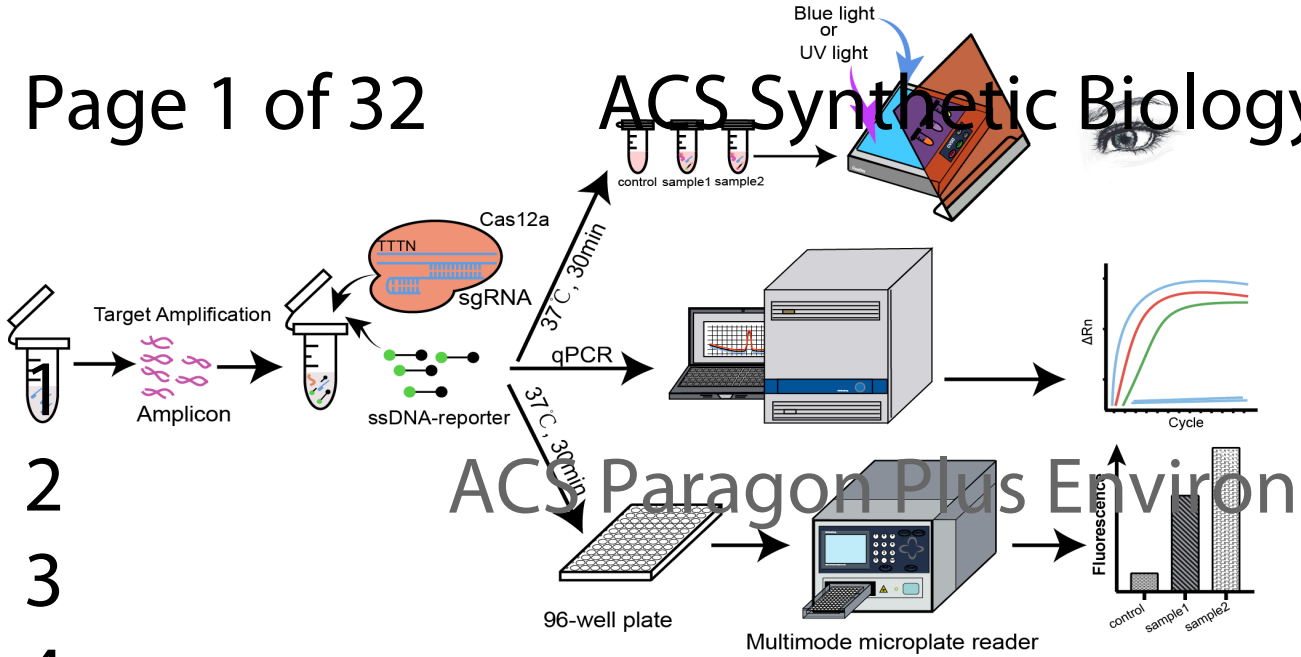
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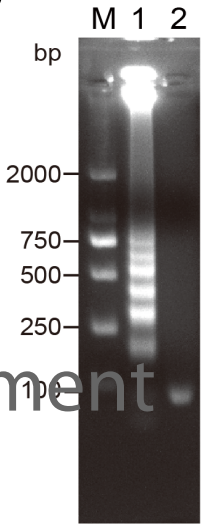
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CRISPR-Cas12a enhanced fluoresence assay





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Application of CRISPR-Cas12a enhanced fluorescence assay coupled with nucleic acid amplification for the sensitive detection of African swine fever virus

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ABSTRACT

African swine fever (ASF) is one of the most severe diseases of pigs. In this study, CRISPR-Cas12a (also known as Cpf1) system coupled with nucleic acid amplification was optimized for the detection of ASV virus (ASFV). Two novel single-stranded DNA-fluorophore-quencher (ssDNA-FQ) reporters were developed to increase the brightness of the fluorescent signal for the visualization of nucleic acid detection. CRISPR-Cas12a system was used to simultaneously cleave the polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP) amplicons and the newly developed ssDNA-FQ reporter, resulting in fluorescence that could be easily detected in multiple platforms, especially on cheap and portable blue or UV light transilluminator. This specific cleavage with fluorescence reveals the presence of the amplicon and confirms its identity, thereby preventing false-positive test results from non-specific amplicons. This method is also un-interfered by the presence of large amounts of irrelevant background DNA and displays no cross-reactivity with other porcine DNA or RNA viruses. When coupled with LAMP, the Cas12a platform can detect a plasmid containing p72 with as few as 2 copies/μL reaction. Our results indicate that the CRISPR-Cas12a enhanced fluorescence assay coupled with nucleic acid amplification is robust, convenient, specific, confirmatory, affordable, and potentially adaptable for ASF diagnosis.

KEYWORDS:

Nucleic acid detection, CRISPR-Cas12a enhanced fluorescence assay, African swine fever virus, pig

African Swine fever (ASF) is an acute, hemorrhagic, and severe infectious disease in domestic pigs caused by the African Swine fever virus (ASFV) with up to 100% fatality rate.[1] The World Organization for Animal Health (OIE) lists ASF as a statutory report animal disease (https://www.oie.int). This infectious disease can be transmitted through alive or dead pigs, domestic or wild pigs, pork products, feeds, and fly.[2-6] Outbreaks have been reported in Africa[1] and parts of Europe, South America, and the Caribbean.[7,8] Since 2018, massive ASF outbreaks leaving numerous pig deaths have been reported in China.[9-11] Although ASF is not a risk to human health, it causes major economic losses, threatens food security, and limits pig production in affected countries with an estimated economic loss of $2 billion for swine production worldwide.[12]

The ASFV genome is a double-stranded DNA molecule of 170 to 190 kbp length that contains between 151 and 167 open reading frames (ORFs) depending on the virus strain.[13] Virions are complex in structure with multiple membrane and protein layers. Its outermost protein shell is an icosahedral capsid mainly composed of the protein p72 encoded by the viral gene B646L.[14] According to the p72 sequences, ASFVs have been divided into 24 different genotypes.[15] Sequencing analysis showed that the 417 bp of p72 in the ASFV in China shared 100% identity with the Georgian strain (Georgia 2007) currently spreading in Russia and Eastern Europe.[16] The major capsid protein p72 is the most important structural component of the virion accounts for 31%–33% of the total mass of the virion [14,17] and thus is one of the major antigens detected in infected pigs.[18] Thus, the ASFV p72 gene is frequently chosen for ASFV detection assay based on its high conservation level.[19,20]

To date, no vaccine and effective drug against ASF have been approved;[21] therefore, efficient disease control depends on early detection and diagnosis. Various laboratory assays, including virus isolation assay,[22] antigen ELISA,[23] direct immunofluorescence test,[24] chimeric DNA/LNA-based biosensor,[25] droplet digital polymerase chain reaction (ddPCR),[26] recombinase polymerase amplification (RPA),[27,28] and loop-mediated isothermal amplification (LAMP),[29] were developed to detect ASFV. Among them, nucleic acid-based diagnosis is the top choice, and real-time PCR assay has been recommended by the OIE as the main diagnosis assay for ASF due to its high sensitivity and low cross-contamination rate. However, these assays are expensive and labor-intensive for the routine detection of ASFV in large numbers of specimens. In addition, quantitative PCR (qPCR) requires expensive instrument and

highly trained personnel.

A low-cost and sensitive method is crucial for nucleic acid detection. CRISPR-based diagnostic has been recently established based on the collateral effect of an RNA-guided and RNA-targeting CRISPR effector, Cas12a or Cas12b.[30-32] CRISPR-Cas12a/b is highly sensitivity, specific and is convenient to use in the detection of target dsDNA or ssDNA.[33, 34] A CRISPR-Cas12a-based platform could be used for rapid and visual nucleic acid detection.[35, 36] Although this technology is suitable for ASFV detection,[37-39] further improvements are still needed to reduce its costs and optimize its performance. Here, a flexible CRISPR-Cas12a enhanced fluorescence assay was developed for nucleic acid detection, especially for early ASF diagnosis. A highly sensitive, specific, and visual method was established by combining CRISPR-Cas12-assisted nucleic acid detection technology with low-cost PCR or convenient LAMP reaction.

MATERIALS AND METHODS

Exonuclease I cleavage assay

Seven different types of ssDNA-FQ reporters, namely, HEX-N12-BHQ1, JOE-N12-BHQ1, TET-N12-BHQ1, ROX-N12-BHQ2, TAMRA-N12-BHQ2, FAM-N12-BHQ1, and FAM-N14-MGBNFQ were synthesized by TSINGKE (China) to identify ssDNA-FQ reporter suitable for CRISPR-Cas12a reaction. All these ssDNA-FQ reporters contain a fluorophore at the 5′ end, and a matched non-fluorescent quencher at the 3′ end are listed in Table S1. Exonuclease I (M0293S, NEB) cleavage assay was then conducted for ssDNA-FQ reporters at 37 °C for 10 min on NaCha Multi-Temp Platform (Monad, China). The fluorescence signal of degraded ssDNA-FQ reporters cleaved by exonuclease I was detected by a visible light gel viewer (EP2020, BioTeke) and an automatic gel imaging analysis system (Peiqing Science and Technology) with blue and UV light transilluminators, respectively. The fluorescence intensity and background signals of these ssDNA-FQ reporters before or after cleavage by exonuclease I were further detected by the microplate reader (EnSpire Multimode Plate Reader, PerkinElmer). Fluorescence excitation and emission wavelengths were arranged to 490–580 and 520–610 nm, respectively, corresponding to the excitation and emission profile of ssDNA-FQ reporters.

Polymerase chain reaction

Primers were designed by Primer Premier 5.0 (PREMIER Biosoft) based on the highly conserved region of the p72 sequences of all ASFV strains available in GenBank, and the gene sequence of ASFV from Pig/HLJ/2018 strain (MK333180.1) was used as reference.[2] The corresponding partial fragment of the p72 gene was synthesized (TSINGKE, China) and cloned into the pMD18T vector (pMD18T-p72) (Table S2). 2 × PCR Master Mix (Takara) was used for all PCR reactions. Each amplification template (10 ng) containing the appropriate copy number of p72 gene was added to the master mix (5 μL) containing buffer, primers, dNTPs, and *Taq* polymerase in a PCR microfuge tube. PCR reactions were performed in a Biometra T-Gradient Thermoblock Thermal Cycler PCR System with an amplification reaction as follows: 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, 72 °C for 36 s, and final extension at 72 °C for 5 min. Upon completion, 5 μL of each sample was isolated for agarose gel electrophoresis analysis, and the remaining portion was set aside for the Cas12a nuclease assay. For ASFV p72, a positive PCR reaction yielded a 597 bp amplicon. The primers used for PCR reaction are listed in Table S1.

Preparation of Cas12a single-guide RNAs (sgRNAs)

Five sgRNAs targeting ASFV p72 were designed by CRISPR-offinder software (Table S2)[40] and complemented to the target sites with a 5′ TTTN protospacer adjacent motif (PAM) in the DNA strand opposite the target sequence (Table S2). ClustalW program (https://www.genome.jp/tools-bin/clustalw) was used for the sequence conservative analysis of sgRNAs. The sgRNA templates were amplified from a pUC57-T7-sgRNA (Table S3) using a forward primer containing a T7 promoter and a reverse primer containing different lengths of nucleotide target sequence (Table S1). The PCR product was purified using the PCR purification kit (Beijing Tianmo Sci&Tech Development), and its amplified form served as the DNA template for *in vitro* transcription reactions using HiScribe™ T7 High Yield RNA Synthesis Kit (E2040S, NEB) following the manufacturer's instructions. The resulting sgRNAs were purified through acid-phenol-chloroform (NEB) extraction and resuspended in RNase-free water following isopropanol precipitation. sgRNA concentration was quantified by NanoDrop One™ spectrophotometer (Thermo Fisher Scientific).

Generation of CRISPR-Cas12a-based polymerase chain reaction (PCR) and quantitative real-time PCR (qPCR) assays

Lba Cas12a (M0653T) and NEBuffer 2.1 (B7203S) were purchased from New England Biolabs (NEB, UK). The sgRNA was synthesized using the same method for Cas12a sgRNA. Each Cas12a reaction contained 250 nM Cas12a, 500 nM sgRNA, 300 nM ssDNA-FQ reporter, 3 μL of PCR product, and 2 μL of NEBuffer 2.1. The total volume was adjusted to 20 μL with nuclease-free water. Afterward, 3 μL of the unpurified PCR production was added into each tube, and the reactions were incubated at 37 °C for 30 min and inactivated at 98 °C for 2 min. Reverse primers of *in vitro* transcription containing sgRNA complementary sequences were used as ssDNA activators (Table S1).

After completion, the reaction products were immediately transferred to a 96-well black polystyrene microplate (Costar) containing 80 μL of nuclease-free water and then measured by the microplate reader (EnSpire Multimode Plate Reader, PerkinElmer). Fluorescence excitation and emission wavelengths were arranged to 526 and 566 nm, respectively, corresponding to the excitation and emission profile of ssDNA-FQ reporter. For direct visualization under blue (470 nM) or UV illumination, PCR tubes containing 20 μL of Cas12a reactions were placed on a blue or UV light transilluminator. The results were recorded by using a smartphone camera or an automatic gel imaging analysis system (Peiqing Science and Technology) with exposure time set at 15 ms.

CRISPR-Cas12a-based qPCR assay was conducted. The Cas12a cleavage reaction was immediately transferred to a 7500 real-time PCR System (Applied Biosystems) at 37 °C with reading cycles every 90 s. The result was displayed as amplification curves.

Evaluation of the sensitivity and fluorescence intensity of Cas12a detection

The copy number of pMD18T-p72 plasmid containing a 597 bp ASFV p72 sequence was calculated based on the plasmid and insert molecular weight: number of copies = (amount × 6.022 × 10^{23})/(length × 1 × 10^{9} × 650) (https://toptipbio.com/dna-copy-number-qpcr/). The pMD18T-p72 DNA was serially diluted from 8 × 10^{7} to 8 × 10^{0} copies/μL. PCR was performed on the dilution series, and the products were analyzed by gel electrophoresis. Additional fluorescence

intensity tests were conducted to compare HEX-N12-BHQ1 and JOE-N12-BHQ1 reporters using the dilutions of p72 segment at concentrations from 8×10^3 to 8×10^0 copies/μL reaction. Parallel samples were analyzed using the Cas12a cleavage reaction, and the fluorescence was read using a blue or UV light transilluminator, a microplate reader, or a 7500 real-time PCR system.

Generation of a CRISPR-Cas12a-based loop-mediated isothermal amplification (LAMP) assay and comparison of its sensitivity to traditional qPCR

The corresponding partial fragment of p72 gene was synthesized (TSINGKE, China) and cloned into the pEASY vector (pEASY-T1-P72) for LAMP assay and traditional qPCR assay. The copy number of the pEASY-T1-P72 plasmid containing a 780 bp ASFV p72 sequence was calculated using the above method. The pEASY-T1-P72 DNA was serially diluted from 2×10^5 to 2×10^{-1} copies/μL. LAMP primers were designed using PrimerExplorer V5 (http://primerexplorer.jp/lampv5e/). The traditional qPCR technology and related primer pairs recommended by OIE were used to detect the ASFV p72 gene (https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.08.01_ASF.pdf).

LAMP reaction was performed on a series of dilution, and the products were analyzed by gel electrophoresis. Each amplification template (10 ng) containing the appropriate copy number of p72 gene was added into the master mix containing 1 μL of Bst 3.0 DNA polymerase (NEB), 2.5 μL of 10 × isothermal amplification buffer, 6 mM $MgSO_4$, 14 mM each of dNTP Mix, and 2.5 μL of primer mix (10 × primer: 1.6 μM of Asfv-p72-FIP/BIP; 0.2 μM of Asfv-p72-F3/B3, 0.4 μM of Asfv-p72-LF/LB). LAMP reactions were performed in a NaCha Multi-Temp Platform (Monad, China) with the following amplification program: 65 °C for 50 min, and 80 °C for 10 min. Afterward, 3 μL of each sample was used for 2% agarose gel electrophoresis, and 3 μL of the remaining portion was set aside for Cas12a nuclease assay. LAMP reaction efficiencies were compared among different reaction conditions, namely, reaction temperature, reaction time, and primers.

Sensitivity to detect the ASFV p72 gene was compared between CRISPR-Cas12a enhanced fluorescence assay and traditional qPCR. The qPCR assay for the detection of ASFV p72 followed the reaction conditions recommended by OIE. Each amplification template containing the appropriate copy number of p72 gene was added into the master mix containing 10 μL of SYBR Green Realtime PCR Master

Mix (TOYOBO) and 0.5 μM concentration for each qPCR primer. The qPCR reaction was performed in CFX96™ Real-Time PCR System (BIO-RAD) with the amplification program as follows: 40 cycles of 94 °C for 10 s, 60 °C for 30 s. Amplification and cycle threshold (Ct) value were observed and calculated. The following experiment was conducted to further evaluate whether the CRISPR-Cas12a enhanced fluorescence assay can be used to directly detect ASFV from the serum of healthy pigs. In brief, 20 μL of unprocessed serum containing pEASY-T1-P72 (100 ng) and 20 μL QE buffer (Lucigen QuickExtract™ DNA Extraction Solution) were incubated at 95 °C for 5 min, and 1 μL of chilled reaction product was added into the LAMP reaction. Finally, 3 μL of each sample was used for 2% agarose gel electrophoresis, and 3 μL of the remaining portion was set aside for Cas12a nuclease assay. The primers used for LAMP and qPCR are listed in Table S1. All primers were synthesized by TSINGKE (China).

Evaluation of specificity of Cas12a detection

Porcine DNA was extracted from a healthy pig. Porcine genomic DNA and pMD18T-p72 plasmid (1:1) were mixed and used as template to evaluate whether the method of detecting ASFV-p72 gene with Cas12a is interfered by the background DNA. The DNA of PRV-Eα was extracted using a DNA extraction kit. The RNAs of PRRSV, H1N1-PR8, JEV, and PDCoV were extracted using a total RNA extraction kit (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized from an RNA template through reverse transcription using M-MLV reverse transcriptase (TOYOBO). The primer pairs were used in PCR reaction were as follows: PRV-Eα (PRV-gE-F and PRV-gE-R), PRRSV (PRRSV-M-F and PRRSV-M-R), H1N1-PR8 (H1N1-PR8-F and H1N1-PR8-R), JEV (JEV-C-F and JEV-C-R), and PDCoV (PDcoV-N-F and PDcoV-N-R) (Table S1). Templates containing DNA or RNA were extracted from appropriately infected pig or cells containing 10 ng per reaction. No template DNA was used as the negative control. PCR reactions were performed, and the products were verified by agarose gel electrophoresis. Finally, the amplicons were assayed using the Cas12a detection method with the ASFV specific sgRNA as described above.

Whether Cas12a nuclease assay could distinguish target DNA with single-nucleotide mismatch was determined to further check the target specificity of CRISPR-Cas12a enhanced fluorescence assay. The target of ASFV p72 gene was employed for single

nucleotide mutation analysis and point-mutated in regions including the PAM sequence and the 1st– 21st base positions of the guide sequence. These point-mutated sequences were contained in the forward primers and introduced into the target sequences through PCR amplification. The experimental process was adopted from Li et al. (2018).[41] The pEASY-T1-P72 (10 ng) was added to the 2 × PCR Master Mix (5 μL) containing buffer, 1 μM Target-P72-F/Target-P72-R. The primers from Target-PAM1A-F to Target-P72-F-21T can be selected to replace Target-PAM1A-F to construct the point mutant template (Table S1). Thermoblock thermal cycler PCR system had the following amplification reactions: 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, 72 °C for 12 s, and final extension at 72 °C for 5 min. Afterward, 3 μL of unpurified PCR product was set aside for the Cas12a nuclease assay. After completion, the parallel samples were analyzed using Cas12a cleavage reaction, and fluorescence was read using a blue or UV light transilluminator and a microplate reader.

Statistical analysis

Statistical analysis was performed using R programming language. Means ± S.E.M (standard errors of the mean) was determined for each treatment group in the separated experiments. Two-tailed Student's t-test was used to determine significant differences between treatment and control groups (*** $P < 0.001$, ns: no significant).

RESULTS

Identification of two novel ssDNA-FQ reporters to improve the brightness of CRISPR-Cas12a-assisted nucleic acid visualization/detection

Seven different types of ssDNA-FQ reporters were designed to improve the brightness of the fluorescence signal from CRISPR-Cas12a-assisted nucleic acid detection (Table S1). The fluorescence properties of these ssDNA-FQ reporters were first compared using an exonuclease I cleavage assay because the ssDNA-FQ reporter can be easily cleaved by exonuclease I. As shown in Figure 1A, the degrade ssDNA-FQ reporters can emit different fluorescence colors under the blue or UV lights, such as green, orange, and yellow green. The fluorescence characteristics of these ssDNA-FQ reporters were further measured by the multi-functional microplate reader. The results showed that different ssDNA-FQ reporter modifications affected the fluorescence intensity and background signals (Figure 1B). In particular, a

relatively high background fluorescence signal was observed for three ssDNA-FQ reporters, namely, TET-N12-BHQ1, FAM-N12-BHQ1, and FAM-N14-MGBNFQ (Figure 1A, B). The fluorescence intensities of the degraded JOE-N12-BHQ1 and ROX-N12-BHQ2 reporters were higher than those of the HEX-N12-BHQ1 and TAMRA-N12-BHQ2 reporters under blue or UV lights (Figure 1A, B). Prior to exonuclease I cleavage, the background fluorescence signal of the JOE-N12-BHQ1 and ROX-N12-BHQ2 reporters was relatively low under blue or UV lights (Figure 1A, B). Therefore, the JOE-N12-BHQ1 and ROX-N12-BHQ2 reporters could potentially enhance the fluorescent signal brightness of the CRISPR-Cas12a-assisted nucleic acid detection. Subsequent experiments used only JOE-N12-BHQ1 as the ssDNA-FQ reporter.

Establishing a CRISPR-Cas12a enhanced fluorescence assay for the detection of ASFV

A CRISPR-Cas12a enhanced fluorescence assay coupled with PCR amplification was established for the detection of ASFV. A negative control and pMD18T-p72 DNA were separately used as templates to evaluate its validity. Blue and UV light transilluminators were used to read out the results and determine whether they could be visualized for detecting p72 with the naked eye under fluorescence condition. The blue or UV light readout was based on the destruction of the newly developed JOE-N12-BHQ1 reporter. Up to five sgRNAs of the p72 were designed and synthesized by *in vitro* transcript. CRISPR-Cas12a fluorescence assays were performed, and the result showed different detection activities of the selected five sgRNAs (Figure 2). Among them, a sgRNA named p72-sgR-5 showed the highest detection activity for the target PCR product (dsDNA) and the ssDNA activator (Figure 2). Furthermore, sequence conservative analysis of sgRNAs using the ClustalW program showed that only the sequence of p72-sgR-5 was highly conservative in different ASFV strains (Figure S1). Therefore, p72-sgR-5 is the optimal sgRNA that could be used to enhance CRISPR-Cas12a-assisted nucleic acid detection fluorescence intensity for various ASFV strains.

Sensitivity of the CRISPR-Cas12a enhanced fluorescence assay coupled with PCR amplification

The pMD18T-p72 DNA was diluted from 8×10^7 to 8×10^0 copies/μL to evaluate the

sensitivity of CRISPR-Cas12a enhanced fluorescence assay. According to the concentration gradient test in Figure 3A, the limit of detection (LoD) of the general PCR assay was 8×10^2 copies/μL (lane 6). The PCR amplification product of p72 was further digested by Cas12a and then detected by the blue or UV light transilluminator. The result showed that the sensitivity of this technique reached 4×10^1 copies/μL of plasmid containing p72 (tube 8) (Figure 3B). CRISPR-Cas12a-based qPCR assay was also conducted, and the result was consistent with the LoD using blue or UV light transilluminator (Figure 3C). Determining the Ct value for samples with extremely low or high template concentrations by setting an artificial threshold is difficult because the amplification curve changes sharply between different concentrations of target template. Therefore, this detection strategy should be used only for monitoring the Cas12a digestion reaction in real-time.

The fluorescence intensities of JOE-N12-BHQ1 and HEX-N12-BHQ1 reporters were compared using four different DNA template concentrations. As shown in Figure S2A, the detection sensitivity using JOE-N12-BHQ1 reporter was similar to that using HEX-N12-BHQ1 reporter. However, the fluorescence intensity of JOE-N12-BHQ1 reporter was significantly higher ($P < 0.001$) than that of HEX-N12-BHQ1 reporter after cleavage by Cas12a (Figure S2B). This result was consistent with Figure 1 and further showed the relatively high fluorescence intensity of the CRISPR-Cas12a enhanced fluorescence assay using JOE-N12-BHQ1 as the ssDNA-FQ reporter.

Establishing a convenient and highly sensitive CRISPR-Cas12a enhanced fluorescence assay coupled with LAMP amplification

CRISPR-Cas12a enhanced fluorescence assay coupled with LAMP amplification assay was developed to facilitate the development of the highly sensitive point-of-care testing strategy for the early diagnosis of infectious diseases especially ASF. Three sets of LAMP primers were designed (Figure S3A) with different relative positions for the sgRNA and primer pairs in the LAMP amplification region. Gel electrophoresis of the LAMP products generated by different primer pairs revealed that all LAMP reactions detected ASF (Figure S3B). However, Cas12a did not cleave the product amplified by the first pair of LAMP primers (LAMP-1) potentially due to the inappropriate sgRNA position (Figure S3C). Therefore, a third pair of LAMP primers (LAMP-3) was randomly selected for further experiments. Concentration gradient test (lane 6) (Figure 4A) revealed that the LoD of the general LAMP assay

was 2×10^0 copies/μL. The LAMP amplification product of p72 was digested by Cas12a and then detected by blue or UV light transilluminator. The result confirmed that the sensitivity of this technique reached 2×10^0 copies/μL of plasmid containing p72 (tube 6) (Figure 4B).

The sensitivity of the ASF assay was further measured using a multi-functional microplate reader and traditional qPCR assay, and the results showed consistency between the sensitivity of ASFV detection by the CRISPR-Cas12a enhanced fluorescence assay and that of qPCR (Figure 4C, D). For example, the Ct values observed in the qPCR assays of samples 7 and 8 (Figure 4D) were ≥ 35, which can be considered as negative and were consistent with detection by the CRISPR-Cas12a enhanced fluorescence assay. Furthermore, ASFV detection using the CRISPR-Cas12a enhanced fluorescence assay required only approximately 1 h, which is shorter than that for traditional qPCR detection (Figure S4A, B). In addition, the temperature of LAMP reaction also affected the efficiency of ASF detection. Therefore, the optimal annealing temperatures for LAMP reactions used in combination with the Cas12a cleavage assay are between 57 °C and 67 °C (Figure S5A, B).

The capability of the CRISPR-Cas12a enhanced fluorescence assay to directly amplify ASFV nucleic acid from the serum from healthy pigs was assessed. The sample was processed for DNA extraction using the DNA QuickExtract kit. The ready-to-use DNA in the supernatant was then directly transferred to the LAMP reaction tube (Figure S6A). The results showed that unprocessed serum samples could be used to detect ASF with this CRISPR-Cas12a enhanced fluorescence assay (Figure S6B, C). Owing to its high sensitivity for ASFV detection, this method can serve as a useful field diagnostic tool to monitor for ASFV outbreaks and rapid quarantine control of the disease.

Non Cas12a visual detection interference from background porcine DNA

In practical applications, DNA viral samples are usually extracted directly from infected pig tissues and thus could be included background porcine DNA. This process may interfere with certain molecular diagnostic techniques. The direct PCR production of p72 into the Cas12a assay was subjected to the presence or absence of 100 ng of background DNA from healthy pigs to assess the interference from background porcine DNA. Agarose gel electrophoresis and Cas12a detection using

different platforms showed no difference between these treatments (Figure 5A, B). In addition, the Cas12a detection of pMD18T-p72 plasmid with or without porcine genomic DNA was also performed using the multi-functional microplate reader and quantitative real-time PCR machine. The results indicated that Cas12a did not engage to substantial off-target cleavage against porcine DNA and was not inhibited by the abundance of non-target DNA (Figure 5C, D). Therefore, the CRISPR-Cas12a enhanced fluorescence assay coupled with nucleic acid amplification protocol could be applied to DNA containing critical levels of porcine background DNA without requiring additional steps to purify the target ASFV DNA.

Specificity of the CRISPR-Cas12a enhanced fluorescence assay

In actual molecular testing for viral infection, the co-infection of the pig with ASFV and other common porcine DNA or RNA viruses might occur. The CRISPR-Cas12a method was used for specific PCR amplicons from the DNA of PRV-Eα and the cDNA of PRRSV, H1N1-PR8, JEV, and PDCoV to determine whether these viruses could be falsely detected. Agarose gel electrophoresis confirmed that all PCR reactions generated the expected products, whereas the negative controls without viral DNA or cDNA yielded no amplicon (Figure 6A). When these positive amplicon solutions were subjected to CRISPR-Cas12a detection, only the ASFV p72 gene-derived sample emitted fluorescence under blue or UV lights, and no fluorescence was observed for its negative control and other viral DNA or cDNA (Figure 6B). Thus, the high concentrations of amplicons from the DNA of PRV-Eα, cDNA of PRRSV, H1N1-PR8, JEV, and PDCoV did not lead to false-positive fluorescence, thereby highlighting the stringent specificity of Cas12a detection for its intended target. The specificity of the primer pair for the detection of ASFV p72 was also evaluated, and the result showed that ASFV p72 could be specifically detected using combined one-step PCR and Cas12a assay (Figure S7A, B). Point mutations at different positions in the target ssDNA sequence, including the PAM region and the guide sequences, were induced to test whether the CRISPR-Cas12a enhanced fluorescence assay could discriminate single-base differences (Figure 7A). Only the mutations in either the PAM sequences (T<u>A</u>TN or <u>AAA</u>N, underlined letters indicate mutation bases) or the 5th, 10th, and 13th bases of the guide sequences resulted in a declined fluorescence signal; however, no remarkably difference was observed when the mutation was within other regions (Figure 7B, C). These results suggest that this

assay can be applied when single base resolution is required to identify mismatches at a known position that can be targeted by the PAM sequence or the indicated guide sequence bases.

DISCUSSION

ASF is a notifiable, highly contagious disease that can cause serious economic losses.[9,12] Rapid, reliable ASFV diagnosis is critical to guiding the cull sick pigs and preventing the spread of ASFV infection.[22] Nonetheless, further simplification of ASFV detection procedures to reduce the detection cost and labor requirement of conventional methods remains a challenge. Here, a CRISPR-Cas12a enhanced fluorescence assay coupled with nucleic acid amplification was established for the simple, convenient, rapid and highly sensitive detection of ASFV. This method applies Cas12a/sgRNA complex as a targeting material and JOE-N12-BHQ1 as a fluorescent reporter that could be easily detected in multiple platforms (Figure 8), such as automatic gel imaging analysis system, microplate reader, real-time PCR system, and especially the cheap and portable blue light transilluminator.

The fluorescence reporter is essential for visualizing nucleic acids in Cas12a reaction. ssDNA-FQ reporters are currently mainly modified by HEX and FAM.[33-35] Another study used gold nanoparticles for naked eye visualization.[36] Here, a test for seven ssDNA-FQ reporters revealed that the JOE and ROX-modified reporters were highly suitable for enhanced fluorescence visualization assay. More new types of ssDNA-FQ reporters are currently being explored in our laboratory. Other unknown ssDNA-FQ reporters with different modifications maybe also visualize nucleic acids with the naked eye, excite fluorescent signals with different colors, improve detection sensitivity, and reduce background fluorescent signals. Selecting highly active sgRNAs is critical for a successful Cas12a reaction. This work and previous reports showed that sgRNA activity is related to sequence characteristics, sequence length, and stem-loop structures within the sgRNA scaffold.[40] Here, five sgRNAs targeting to ASFV p72 were designed by CRISPR-offinder, one of which exhibited high activity. This high performance may be related to the relatively low GC% content of this sgRNA (Table S2). However, further study is necessary to definitively identify the factors most strongly affecting the sgRNA detection activity. Future optimization of sgRNAs will entail a combination of experimental data with machine learning for the refined analysis of the sequence-related factors contributing to sgRNA activity to

improve the sensitivity of nucleic acid detection by this CRISPR-Cas12-based system through enhanced sgRNA activity.

CRISPR-Cas12a combined with LAMP assay was developed for directly visualizing DNA detection at the single-copy level. The LoD of ASFV using CRISPR-Cas12a enhanced fluorescence assay coupled with LAMP amplification was better than that using PCR reaction, and this finding is consistent with the qPCR detection sensitivity recommended by OIE. Different from gene diagnosis methods using quantitative real-time PCR for ASFV DNA,[42] the LAMP assay has simple operation and low requirements for equipment. The reaction could be achieved by using a water bath or an incubator, and the detection of the results is also straightforward. Furthermore, the total turnaround time for combing PCR amplification with CRISPR-Cas12a fluorescence assay was approximately 2 h, which could be shortened to approximately 1 h by combining with LAMP amplification. This finding implies that the latter strategy could rapidly and reliably detect ASFV DNA. Our results revealed that LAMP and Cas12a combined reaction shortens the detection and satisfies three aims: convenience, immediacy, and reliance.

The CRISPR-Cas12a-based detection method has advantages over monoclonal antibody in conventional immunoassay systems because of its ability to interact with target genes in a sequence-specific manner. Compared with that of PCR, qPCR, RPA, or LAMP reactions alone, the specificity of the CRISPR-Cas12a-based nucleic acid detection methods is remarkably higher. These nucleic acid amplification methods sometimes produce nonspecific products through mispriming or primer-dimer formation. The true and false positives should migrate differently on an agarose gel, but this step is not achieved by the above single techniques.[43] The Cas12a detection assay emits only fluorescence when the nucleic acid amplification products contain a matching sequence. This activity differs from that in existing methods where a 1 bp mismatch between sgRNA and target sequence can eliminate Cas12a-mediated cleavage.[32] However, Cas12a nuclease can recognize single-base differences at specific positions in the target sequence. Although the proposed detection method may not accurately distinguish all single-base differences, it is potentially applicable for identifying the presence or absence of SNPs at known positions. Such high specificity could be used as an additional check for false positives from PCR or LAMP amplification products. Furthermore, the specificity of CRISPR-Cas12a-based nucleic acid detection methods was determined by the primer pairs of amplification

and sgRNA. The use of suitable software to design specific primer pairs and sgRNAs is necessary to apply this technology to detect nucleic acids. Nucleic mutations in the p72 genes encoded by different ASFV strains were induced by multiple sequence alignments analysis. Therefore, specific or highly conservative sgRNAs should be designed for specific or wide-spectrum strains depending on the purpose of the test.

In nucleic acid detection experiments, a major concern are the false positives due to aerosol contamination. Given the reaction temperature is 37 °C for RPA and Cas12a, both reagents can be kept in a single test tube. The RPA reaction can be first performed in a centrifuge tube, and while it progresses, the Cas12a and sgRNA reagents will remain stable within the cap of the tube. Thus, when the RPA reaction is completed, the Cas12a and sgRNA reagents can be spun down into the tube for nucleic acid detection, and the resultant reaction mixture can be imaged for fluorescence. Recent reports confirmed that this strategy can effectively reduce aerosol pollution.[44] However, because the optimal temperature for the LAMP reaction is between 57 °C and 67 °C, further study is needed to develop a method for keeping the reagents of both reactions in a single test tube.

In conclusion, a CRISPR-Cas12a enhanced fluorescence assay coupled with PCR or LAMP amplification was successfully established for the sensitive detection of ASFV. This approach could be applied for any other targets by simply changing the primer pairs and sgRNA sequence and thus is able to detect various pathogens and ASFV by using cheap and portable blue light transilluminator.

DATA AVAILABILITY

The authors declare that the main data supporting the findings of this study are available within the manuscript or the material. Extra data are available from the corresponding author upon request.

SUPPORTING INFORMATION

Additional information on the features of different sgRNAs and ssDNA-FQ reporters, the amplification efficiencies of specific primers, reaction time and temperatures on LAMP in the CRISPR-Cas12a enhanced fluorescence assays. The ability and specificity of the CRISPR-Cas12a enhanced fluorescence assay to amplify ASFV. Additional list of primers sequences used for target amplification, sgRNAs, and partial vector sequence used for *in vitro* transcription of sgRNAs.

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ABBREVIATIONS

CRISPR, clustered regularly interspaced short palindromic repeats; sgRNA, single-guide RNA; PAM, protospacer adjacent motif; ASF, African swine fever; ASFV, African swine fever virus; ssDNA, single-strand DNA; ssDNA-FQ reporter, single-stranded DNA-fluorophore-quencher reporter; LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reactions; qPCR, quantitative PCR; SNP, single nucleotide polymorphism; ORFs, open reading frames; UV, Ultraviolet; Ct, cycle threshold; PRV, Pseudorabies virus; PRRSV, Porcine Reproductive and

Respiratory Syndrome Virus; H1N1, influenza A virus subtype H1N1; JEV, Japanese encephalitis virus; PDCoV, Porcine deltacoronavirus.

ACKNOWLEDGMENTS

This work was supported by the National Transgenic Project of China (grant numbers: 2018ZX08009-26B and 2016ZX08006003-004), the NSFC Major Research Plan – Major Scientific Problems of African Swine Fever virus (grant numbers: 31941008), the Fundamental Research Funds for the Central Universities (grant numbers: 2662018JC002), and the National Natural Science Foundation Youth Fund of China (grant numbers: 31702102).

AUTHOR CONTRIBUTIONS

Most of the experimental work was co-conducted by D.T and J.L. with minor contributions from B.X., L.N., X.L., J.R., X.L., Y.M., X.L. and C.L. X.N. helped in analyzing the data and drawing figures, G.P. and L.S. provided the ASFV DNA template and technical support. S.X. conceived the project, designed the experiments and wrote the manuscript. T-N.T-T. helped in revising the manuscript. S.Z. provided support and supervised the project. All authors read and approved the final manuscript.

COMPETING INTERESTS

S.Z., S.Z., X.L., X.L. D.T., J.L., B.X. and X.N. have filed a patent application relating to the technology.

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Figures and Legends

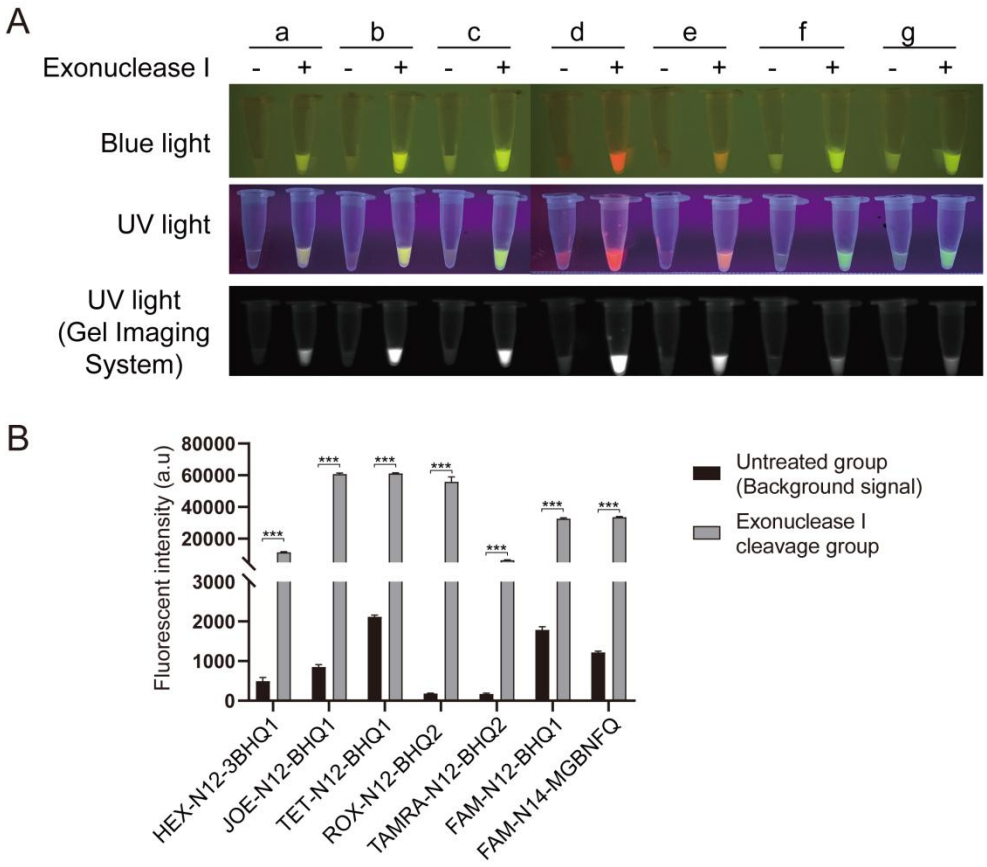


Figure 1. Comparison of the fluorescence intensity and background signals of seven different types of single-stranded DNA-fluorophore-quencher (ssDNA-FQ) reporters. A. Determination of the fluorescence characteristics of seven different types of ssDNA-FQ reporters by using exonuclease I cleavage assay. B. Detection of the fluorescence intensity and background noise of seven different types of ssDNA-FQ reporters by a multi-functional microplate reader. (a) HEX-N12-3BHQ1 reporter, (b) JOE-N12-BHQ1 reporter, (c) TET-N12-BHQ1 reporter, (d) ROX-N12-BHQ2 reporter, (e) TAMRA-N12-BHQ2 reporter, (f) FAM-N12-BHQ1 reporter, and (g) FAM-N14-MGBNFQ reporter. "N": adenine (A), thymine (T), cytosine (C) or guanine (G). Blue or UV lights: The pictures were captured under blue (470 nM) and UV lights by a smartphone camera and a gel imaging system using UV light. Error bars represent standard errors of the mean (S.E.M.); $n = 3$ (B), *** $P <$ 0.001.

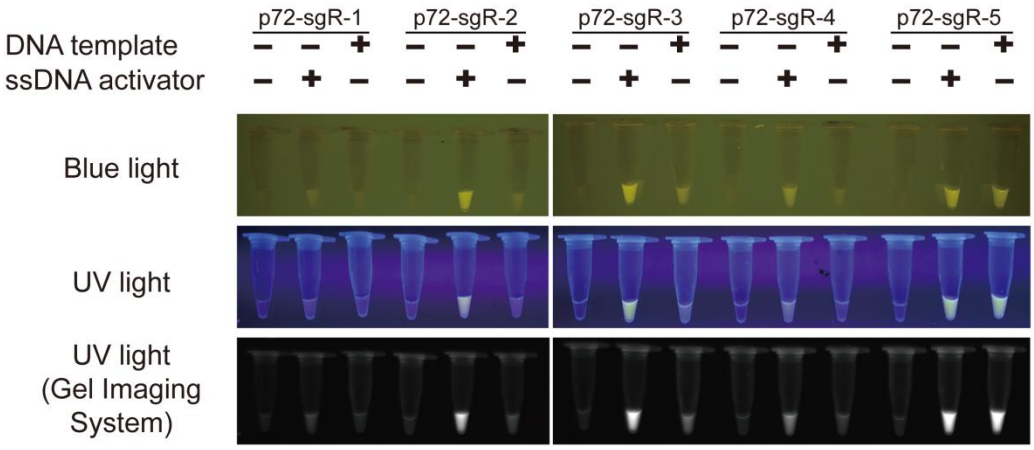


Figure 2. Identification of the highly active sgRNA for African swine fever virus (ASFV) p72 gene using CRISPR-Cas12a enhanced fluorescence assay. p72-sgR-1, or 2, or 3, or 4, or 5 represent different sgRNAs targeting to ASFV p72 gene. DNA template represents PCR production of p72 gene fragment. ssDNA activator is a sgRNA-complementary ssDNA. Blue or UV lights: The pictures were captured under blue (470 nM) and UV lights by a smartphone camera and gel imaging system using UV light.

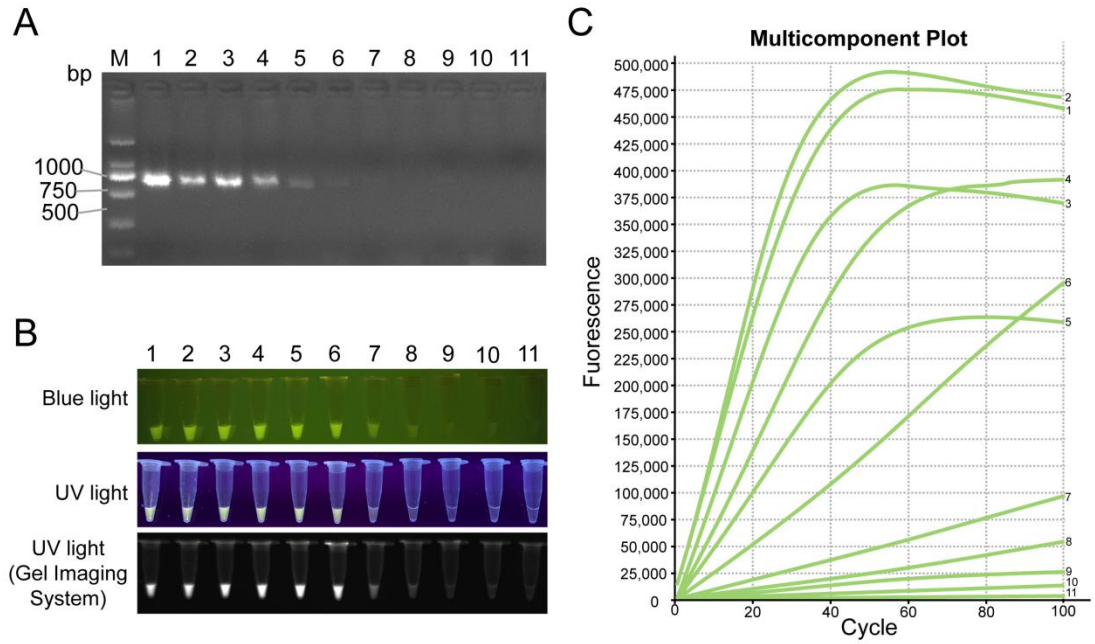
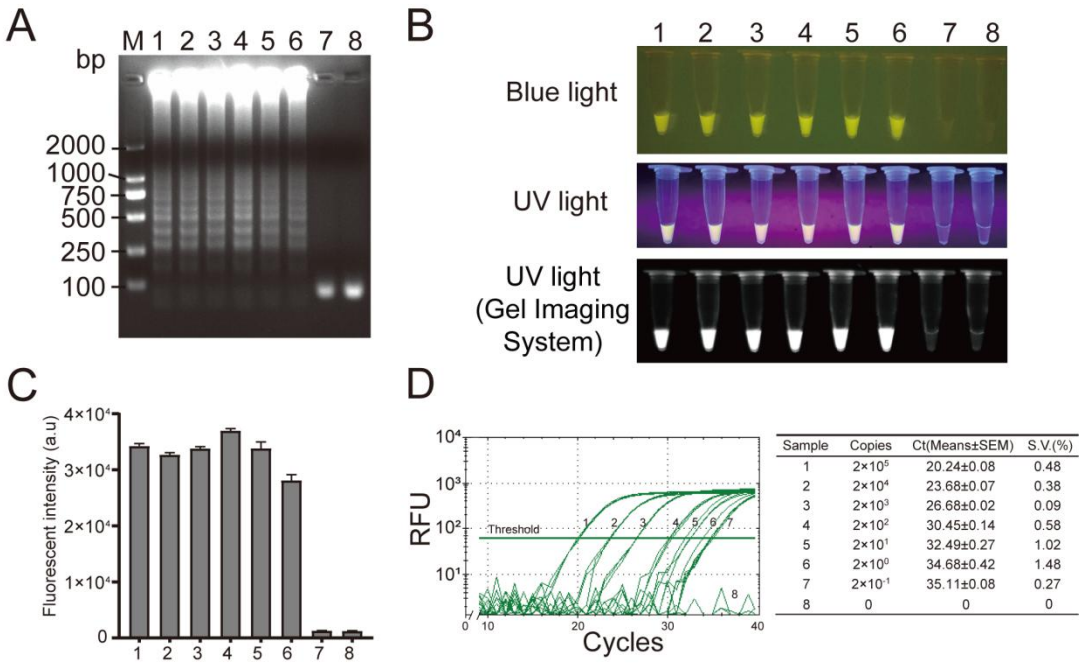


Figure 3. Comparison of the detection limit of CRISPR-Cas12a enhanced fluorescence assay coupled with polymerase chain reaction (PCR) amplification and general PCR. A. Products of the PCR reaction by using agarose gel electrophoresis analysis; B. Fluorescent signals from a series of 10-fold dilutions of pMD18T-p72 plasmid DNA using the CRISPR-Cas12a enhanced fluorescent assay coupled with PCR amplification. C. Sensitivity of the CRISPR-Cas12a enhanced fluorescence assay coupled with PCR amplification as monitored in a 7500 real-time PCR system. Lane M: DNA ladder; bp: base pairs. Lanes or tubes: (1) 8×10^7 copies/μL; (2) 8×10^6 copies/μL; (3) 8×10^5 copies/μL; (4) 8×10^4 copies/μL; (5) 8×10^3 copies/μL; (6) 8×10^2 copies/μL; (7) 8×10^1 copies/μL; (8) 4×10^1 copies/μL; (9) 2×10^1 copies/μL; (10) 8×10^0 copies/μL; (11) non-template control group. Blue or UV lights: The pictures were captured under blue (470 nM) and UV lights by a smartphone camera and gel imaging system using UV light.



Sample	Copies	Ct(Means±SEM)	S.V.(%)
1	2×10^{5}	20.24±0.08	0.48
2	2×10^{4}	23.68±0.07	0.38
3	2×10^{3}	26.68±0.02	0.09
4	2×10^{2}	30.45±0.14	0.58
5	2×10^{1}	32.49±0.27	1.02
6	2×10^{0}	34.68±0.42	1.48
7	2×10^{-1}	35.11±0.08	0.27
8	0	0	0

Figure 4. Comparison of the detection limit of CRISPR-Cas12a enhanced fluorescence assay coupled with loop-mediated isothermal amplification (LAMP) amplification and general LAMP. A. Products of the LAMP reaction analyzed through agarose gel electrophoresis. B. Fluorescent signals from a series of 10-fold dilutions of pEASY-T1-P72 plasmid DNA using the CRISPR-Cas12a enhanced fluorescent assay coupled with LAMP amplification. C. Sensitivity of the CRISPR-Cas12a enhanced fluorescence assay coupled with LAMP amplification as monitored in a multi-functional microplate reader. D. Sensitivity of the detection of ASFV by quantitative real-time PCR as monitored in a 7500 real-time PCR system. Lane M: DNA ladder; Lanes or tubes: (1) 2×10^5 copies/μL; (2) 2×10^4 copies/μL; (3) 2×10^3 copies/μL; (4) 2×10^2 copies/μL; (5) 2×10^1 copies/μL; (6) 2×10^0 copies/μL; (7) 2×10^{-1} copies/μL; (8) non-template control. bp: base pairs, Ct: Cycles; RFU: Relative fluorescence units. Blue or UV lights: The pictures were captured under blue (470 nM) and UV lights by a smartphone camera and gel imaging system using UV light.

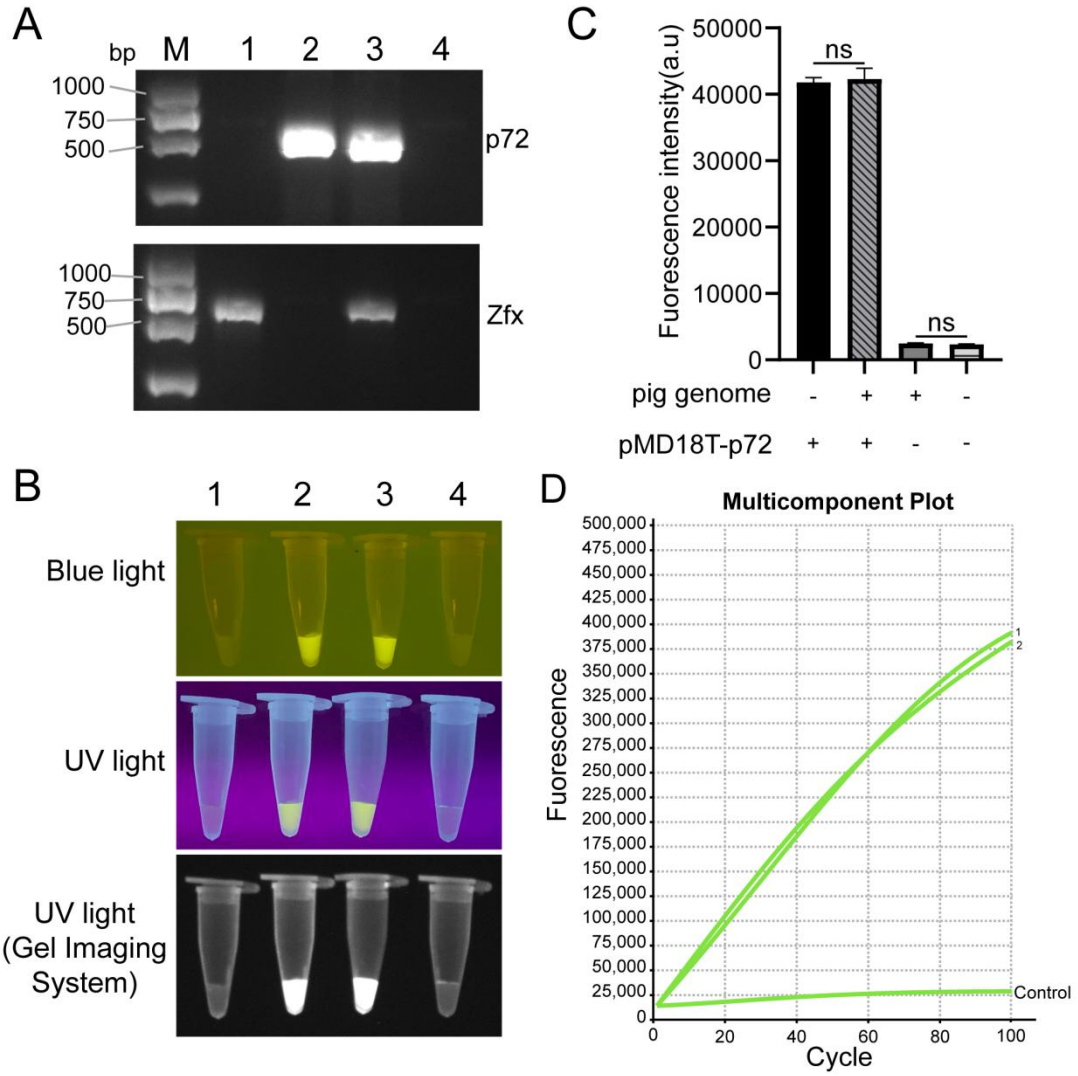


Figure 5. Evaluation of background porcine genomic DNA interference with the detection of ASFV-p72 gene in CRISPR-Cas12a enhanced fluorescence assay. A. PCR products of p72 and Zfx genes by gel electrophoresis analysis in porcine genomic DNA and pMD18T-p72 plasmid templates. B. Fluorescent signals from PCR products of p72 and Zfx genes in porcine genomic DNA and pMD18T-p72 plasmid templates by the CRISPR-Cas12a enhanced fluorescent assay using the Blue or UV Transilluminator. C and D, Detection of pMD18T-p72 plasmid with or without porcine genomic DNA by the CRISPR-Cas12a enhanced fluorescent assay using a multi-function microplate reader and CRISPR-Cas12a-based quantitative real-time PCR assay, respectively. Lanes or tubes (A – B): 1 – 4 correspond to PCR amplification of porcine genomic DNA, pMD18T-p72 plasmid, porcine genomic DNA and pMD18T-p72 plasmid (1:1) mixed template, its negative control, respectively; Lane M: DL2000 marker, bp: base pairs. Blue or UV lights: The pictures

were captured under blue (470 nM) and UV lights by a smartphone camera and gel imaging system using UV light. Error bars indicate standard errors of the mean (S.E.M); $n = 3$ (C). ns: no significant.

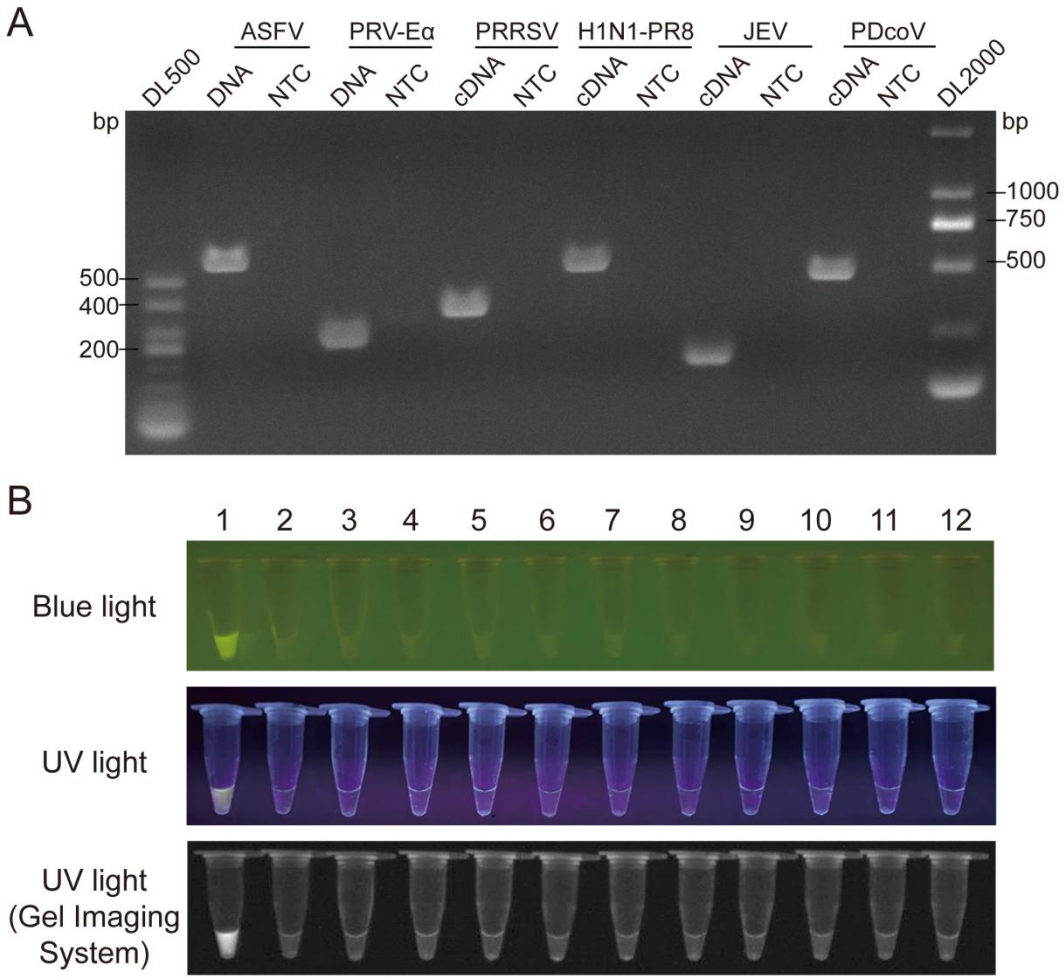


Figure 6. Specificity of the CRISPR-Cas12a enhanced fluorescent assay for detection of ASFV. A. Agarose gel electrophoresis for detection of specific PCR amplicons. B. Fluorescence signal detection under the blue (470 nM) and UV lights. Lanes or tubes: (1) pMD18T-p72 DNA; (3) DNA of PRV-Eα; (5, 7, 9 and 11) cDNA of PRRSV, H1N1-PR8, JEV, PDCoV, respectively; sample numbers: (2, 4, 6, 8,10 and 12) non-template control; bp: base pairs. Blue or UV lights: The pictures were captured under blue (470 nM) and UV lights by a smartphone camera and gel imaging system using UV light.

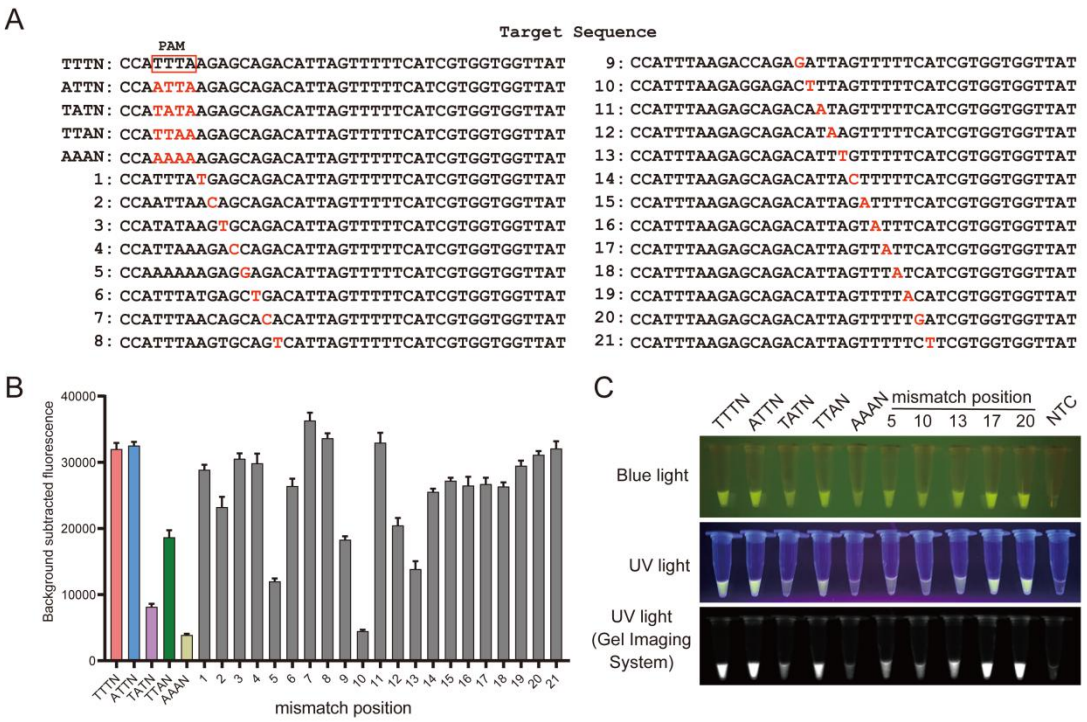


Figure 7. Evaluation of the specificity of the CRISPR-Cas12a enhanced fluorescent assay by distinguishing the single-base mismatches. A. Schematics showing mismatched position in the targeting sequence. PAM sequences are colored in red box, SNPs are colored in red. B. Fluorescence signal of the CRISPR-Cas12a enhanced fluorescent assay was obtained by the multi-functional microplate reader. Error bars represent standard errors of the mean (S.E.M.); $n = 3$. C. The fluorescence signal detection under the blue (470 nM) and UV lights. Blue or UV lights: The pictures were captured under blue (470 nM) and UV lights by a smartphone camera and gel imaging system using UV light.

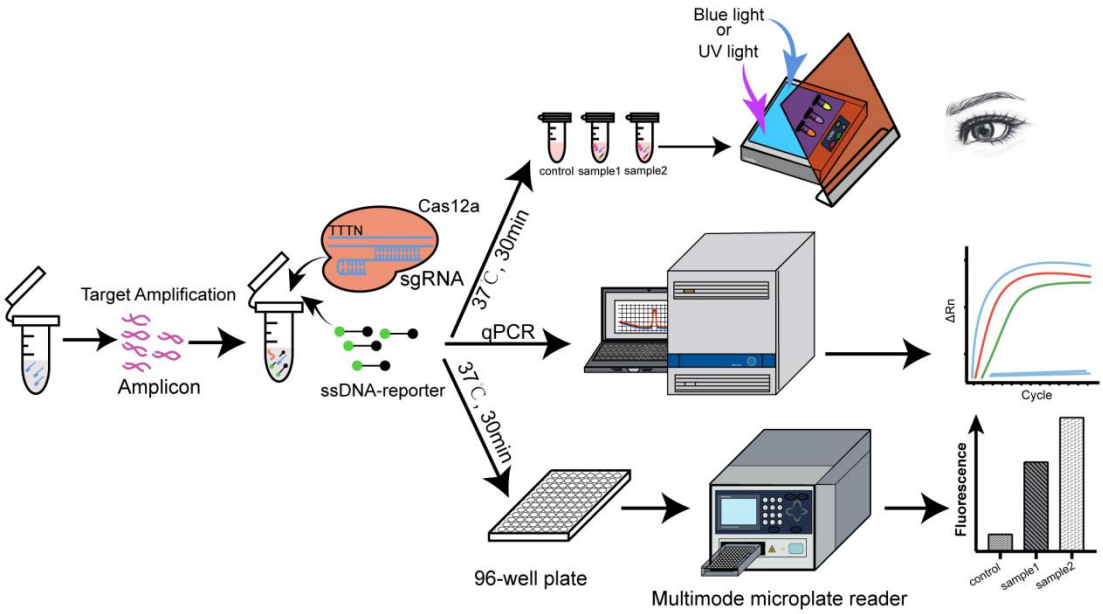


Figure 8. Schematic of the CRISPR-Cas12a enhanced fluorescence assay coupled with nucleic acid amplification. The newly identified JOE-N12-BHQ1 and ROX-N12-BHQ1 are fluorescent reporters that could be easily detected in multiple platforms, such as automatic gel imaging analysis system, microplate reader, or Real-Time PCR system, especially the cheap and portable Blue Light Transilluminator. sgRNA: single-guide RNA, TTTN: A typical PAM sequence for Cas12, ssDNA: single-strand DNA, qPCR: quantitative polymerase chain reaction. Blue or UV lights: The pictures were captured under blue (470 nM) and UV lights by a smartphone camera and gel imaging system using UV light.