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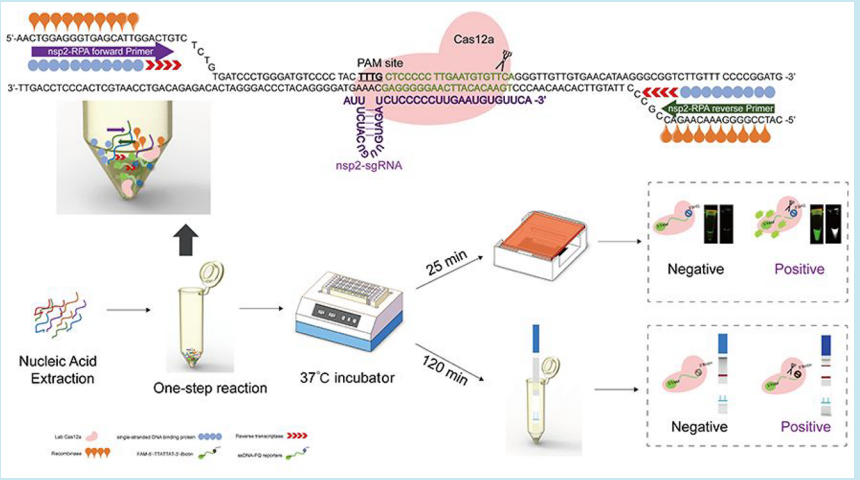
Highly Sensitive CRISPR/Cas12a-Based Fluorescence Detection of Porcine Reproductive and Respiratory Syndrome Virus

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ABSTRACT: Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease of swine that is caused by PRRS virus (PRRSV). In this study, we established a fluorescence assay for highly sensitive detection of PRRSV through integration of the reverse transcription−recombinase polymerase amplification (RT-RPA)-coupled Cas12a system with an optical property of single stranded DNA−fluorescently quenched (ssDNA-FQ) reporter. This technique can achieve isothermal and visual detection of PRRSV in 25 min. In particular, the assay reaction can be completed in a single tube. The limit of sensitivity for PRRSV detection was single copy without cross-reactivity of other porcine viruses. Correlation between 11 PRRSV clinical samples measured by the quantitative reverse transcription polymerase chain reaction (RT-qPCR) and CRISPR/Cas12a assay was determined; the result showed that our results were highly accurate. To sum up, this study developed a visual, sensitive, and specific method of nucleic acid detection based on a CRISPR-Cas12a technique for the on-site detection of PRRSV.

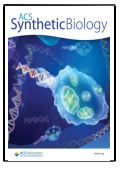
KEYWORDS: *PRRSV, CRISPR-Cas12a, recombinase polymerase amplification, visualization, detection*

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Porcine reproductive and respiratory syndrome (PRRS) is caused by PRRS virus (PRRSV) infection, is one of the most severe swine infectious diseases, and is responsible for substantial economic losses.[1−5] PRRSV is an enveloped single-strand positive-sense RNA virus belonging to the family *Arteriviridae* and the order *Nidovirales*. High variability and recombination among different PRRSV strains further strengthen the prevention and control of PRRS.[6,7] Under a transmission electron microscope, the PRRS virion appears to be a spherical or oval-shaped particle of 50−60 nm in diameter.[8,9] The PRRSV genome is approximately 15 kb long, with 11 open reading frames (ORFs) encoding 14 non-structural and 8 structural proteins.[3,10−12] They are processed into at least 14 nonstructural proteins (NSPs) by four ORF1a encoded proteinases called NSP1α, NSP1β, NSP2, and NSP4.[1,12−14] Nonstructural protein 2 (NSP2) is encoded by *nsp2*; it is the largest product and one of the most important viral proteins in the PRRSV life cycle.[13,15−17]

Currently, several molecular diagnostic methods for PRRSV detection have been developed, such as Loop-mediated isothermal amplification assay (LAMP), quantitative real-time PCR (qPCR), enzyme linked immunosorbent assay (ELISA), and Droplet Digital PCR (ddPCR).[18−22] However, the LAMP reaction depends on multiplexing primer pairs and can easily

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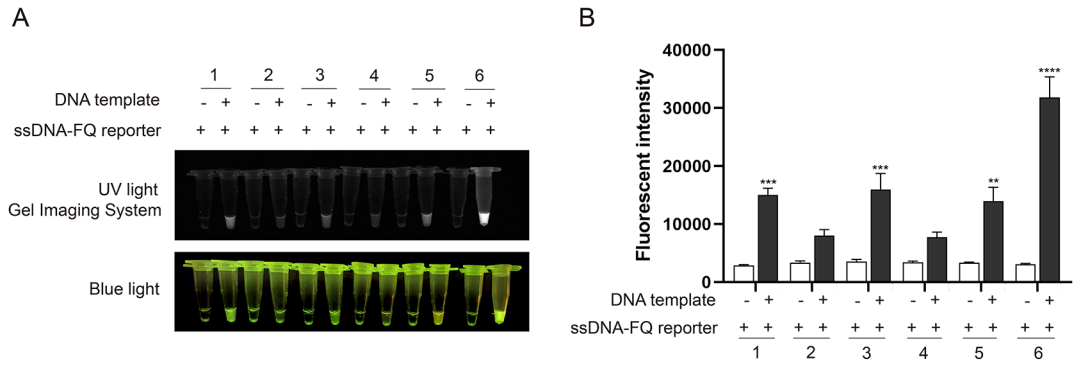


Figure 1. Identification of the optimum ssDNA-FQ reporters for detecting PRRSV by CRISPR/Cas12a-based fluorescence assay. (A) Determination of the fluorescence intensity of 6 types of ssDNA-FQ reporters using Cas12a-based detection assay. DNA template represents conserved sequence of *nsp2* in pUC57 nsp2 plasmid (100 ng/μL); Nsp2-sgRNA02 was used for the reaction. No.1, JOE-BHQ2 reporter; No.2, JOE-BHQ1 reporter; No.3, JOE-N-BHQ2 reporter; No.4, JOE-CY3-BHQ2 reporter; No.5, JOE-CY3-N-BHQ2 reporter and No.6, FAM-BHQ2 reporter. (B) Detection of the fluorescence intensity and background noise of 6 ssDNA-FQ reporters of Cas12a-based detection assay *via* ImageJ software. Error bars represent the SEM of the fluorescent intensity calculated from three individual reactions. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$. Blue or UV lights: The pictures were captured under blue and UV lights.

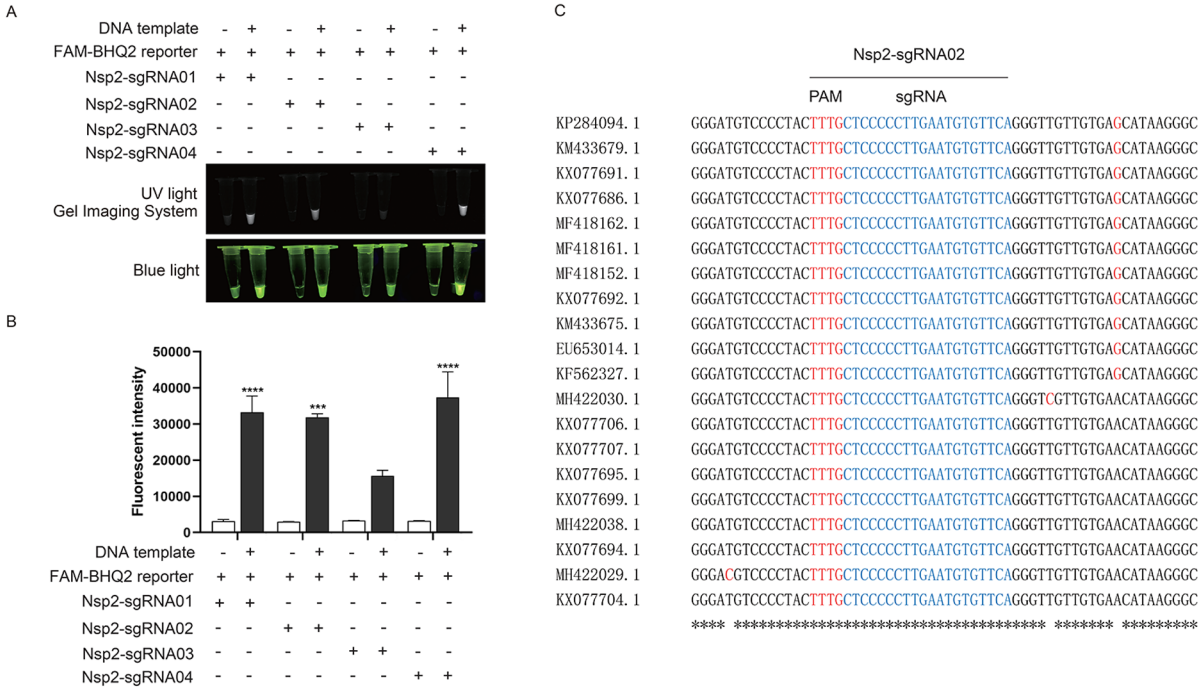


Figure 2. Identification of the highly activity sgRNAs for detecting PRRSV by CRISPR/Cas12a-based fluorescence assay. (A) Determination of the highly active sgRNAs of 4 sgRNAs *via* Cas12a-based fluorescence assay under blue and UV lights. DNA template: pUC57 nsp2 plasmid (100 ng/μL). (B) Quantification of fluorescence intensity was performed using ImageJ software. (C) Analysis of conserved sequences of Nsp2-sgRNA02 among 20 different PRRSV strains. Error bars represent the SEM of the fluorescent intensity calculated from three individual reactions. ****$P < 0.0001$, ***$P < 0.001$. Blue or UV lights: The pictures were captured under blue and UV lights.

generate false-positive results.[23] Some studies show that qPCR has already been used for detection of PRRSV, though it requires long testing times and skilled operators.[23−26] Although ddPCR has much higher technical sensitivity compared to qPCR, it also requires sophisticated and expensive equipment and high-level personnel expertise.[19]

To achieve a feasible, specific, sensitive, and reliable on-site diagnostic strategy for virus detection, CRISPR-associated protein (Cas) based methods were developed.[23,27−30] The CRISPR/Cas12a system, which belongs to the class 2 type V-A CRISPR-Cas system, performed collateral cleavage on the nontargeted single stranded DNA-fluorescently quenched (ssDNA-FQ) reporter.[31−33] A previous study demonstrated that LAMP assay coupled with CRISPR/Cas12a system can be used to detect African Swine Fever Virus (ASFV).[34] Here, a highly sensitive CRISPR/Cas12a-based fluorescence detection method requiring no expensive reagents or special instruments was established for PRRSV.

RESULTS AND DISCUSSION

Optimizing the ssDNA-FQ Reporter for CRISPR/Cas12a-Based PRRSV Detection Platform. In order to enhance the brightness of the fluorescent signal based on Cas12a nucleic acid detection, six different types of ssDNA-FQ

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reporters, such as JOE-BHQ2, JOE-BHQ1, JOE-N-BHQ2, JOE-CY3-BHQ2, JOE-CY3-N-BHQ2, and FAM-BHQ2, were designed and synthesized (Table S1). Next, the plasmid containing the PRRSV *nsp2* gene (pUC57 nsp2) was used as a template, and a sgRNA targeting this gene was selected to guide the Cas12a cleavage reaction. As shown in Figure 1A and B, only 4 ssDNA-FQ reporters emitted strong fluorescence lights after being cleaved by Cas12a, and they can be observed with the naked eye under blue light. After comparing the fluorescence intensity of these ssDNA-FQ reporter, we found that the fluorescence intensity of the FAM-BHQ2 ssDNA reporter was the highest (Figure 1B, $P < 0.0001$). Therefore, FAM-BHQ2 ssDNA reporter was chosen for subsequent experiments.

Identification of Conserved and High-Activity sgRNAs for Detection of PRRSV. To select the appropriate sgRNAs for detection of PRRSV, a set of conserved sequence regions of 4 sgRNAs were designed and analyzed (Table S1). As shown in Figure 2A and B, we found that the activities of 4 sgRNAs differed, with Nsp2-sgRNA04 showing the highest fluorescence signal ($P < 0.0001$), and Nsp2-sgRNA03 showing the lowest activity using pUC57 nsp2 plasmid as template (Figure 2B). Furthermore, a consensus sequences analysis of these sgRNAs using the ClustalW program was conducted, which demonstrated that Nsp2-sgRNA02 was the only highly conserved sgRNA for targeting all PRRSV strains (Figure 2C and Figure S1). The fluorescence intensities of the degraded FAM-BHQ2 reporters, coupled with Nsp2-sgRNA02, demonstrated no significant difference with Nsp2-sgRNA04 or Nsp2-sgRNA01 under UV light. Furthermore, the sequence of Nsp2-sgRNA02 was highly conserved among different PRRSV strains. Thus, subsequent experiments only used Nsp2-sgRNA02 along with the FAM-BHQ2 reporter.

Detection Sensitivity of CRISPR/Cas12a Combined with PCR Amplification. To evaluate the detection sensitivity of CRISPR/Cas12a combined with PCR amplification, the pUC57 nsp2 plasmid DNA was diluted from 3×10^{10} to $3 \times 10^{\circ}$ copies/μL, which was used as a template. The detection sensitivity of PCR and Cas12a cleavage assays was compared. We found that the limit of detection for general PCR assay for PRRSV *nsp2* gene was only 3×10^{6} copies (Figure 3A). Next, the PCR amplification product of *nsp2* was further digested by Cas12a. As shown in Figures 3B and C, the detection sensitivity of CRISPR/Cas12a coupled with PCR amplification for PRRSV *nsp2* gene can reach 3×10^{4} copies (Figure 3C, $P < 0.0001$). The result indicates that CRISPR/Cas12a combined with PCR amplification assay was more sensitive for detection of PRRSV than the general PCR assay.

CRISPR/Cas12a Combined with RPA Amplification for Detection of PRRSV. To facilitate on-site detection of PRRSV, CRISPR/Cas12a combined with RPA or RT-RPA amplification was performed. As shown in Figure 4A and Figure S2, only the sgRNA specific targeting to *nsp2* gene can emit strong fluorescence under blue or UV lights, with no fluorescent readouts in the nontemplate control or other viral DNA (Figure S2). Next, we found that the sensitivity of CRISPR/Cas12a combined with RPA amplification can reach to $3 \times 10^{\circ}$ copies using positive plasmid containing *nsp2* as an amplification template (Figure 4B and C, $P < 0.05$). In addition, to improve amplification efficiency of RPA or RT-RPA, the optimal primer pair was selected (Figure S3). Samples of the nontemplate control above a cycle threshold (Ct) of 35 were considered negative in the qPCR assay, while

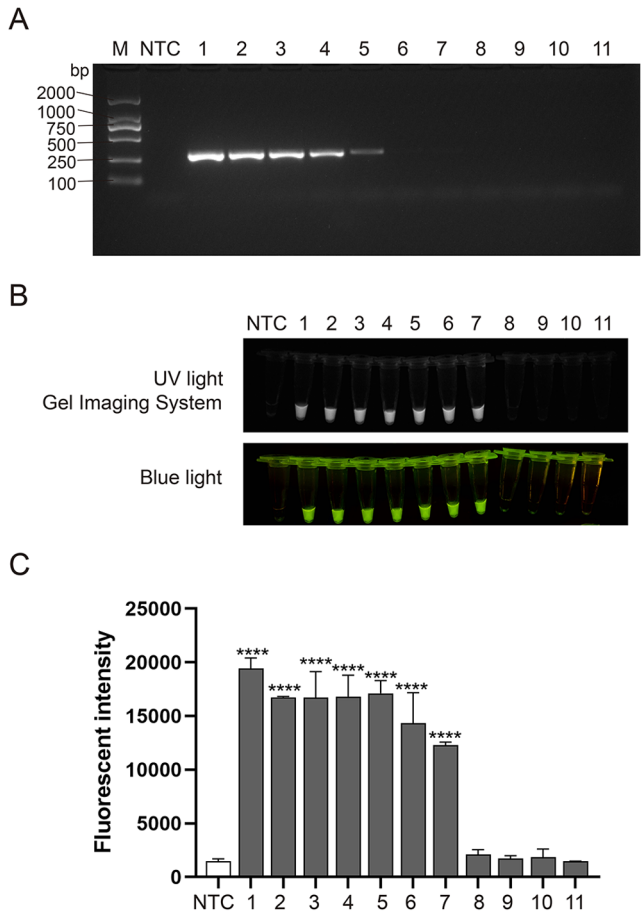
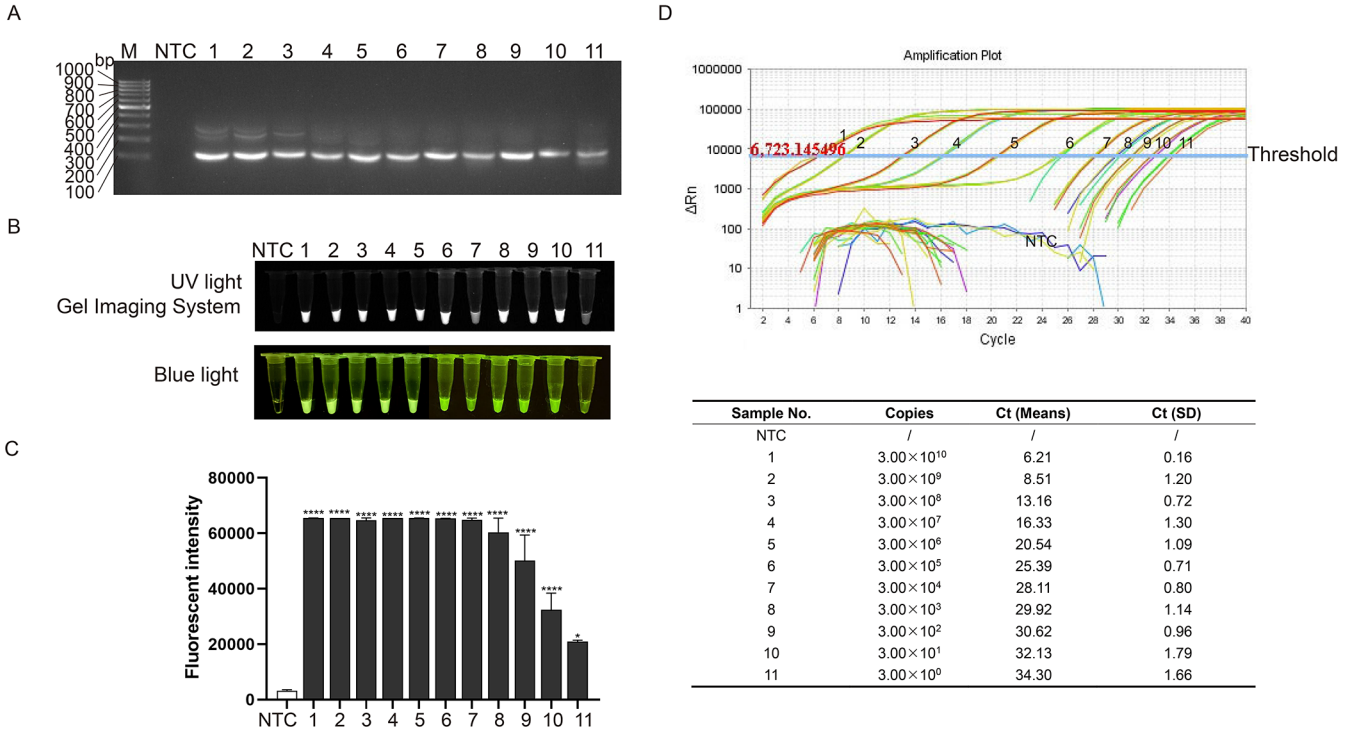


Figure 3. Evaluation of the detection limit of CRISPR/Cas12a combined with PCR amplification. (A) Agarose gel electrophoresis of PCR products. (B) Detection of CRISPR/Cas12a combined with PCR amplification using a series of 10-fold dilutions pUC57 nsp2 plasmid (from 10^{10} to 1 copies per reaction) as reaction template. (C) Quantification of fluorescence intensity was performed using ImageJ software. Lane M, DNA DL2000 marker ladder; Lane NTC, Nontemplate control group; bp, base pairs. Lanes or tubes: (1) 3×10^{10} copies/μL; (2) 3×10^{9} copies/μL; (3) 3×10^{8} copies/μL; (4) 3×10^{7} copies/ μL; (5) 3×10^{6} copies/μL; (6) 3×10^{5} copies/μL; (7) 3×10^{4} copies/μL; (8) 3×10^{3} copies/μL; (9) 3×10^{2} copies/μL; (10) 3×10^{1} copies/μL; (11) $3 \times 10^{\circ}$ copies/μL. Blue or UV lights: The pictures were captured under blue (470 nM) and UV lights by a smart phone camera and Gel Imaging System using UV light. Error bars represent SEM; $n = 3$, $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

samples with Ct values <35 were considered positive. By comparing CRISPR/Cas12a combined with RPA amplification and conditional qPCR assay, we found that the limit of detection for PRRSV was consistent (Figure 4D). Therefore, combining RPA amplification with the conserved and highly active sgRNA makes the CRISPR/Cas12a combined with RPA amplification assay more suitable for field sensitive detection of PRRSV.

Establishment of One-Step CRISPR/Cas12a Combined with RPA Amplification for Detection of PRRSV in a Single Tube. To simplify the CRISPR/Cas12a-based method for on-site detection of PRRSV, one step of CRISPR/Cas12a combined with RPA amplification for detection of PRRSV in a single tube was developed. We determined that all reagents, such as Cas12a, Nsp2-sgRNA02, RPA enzymes, and

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Sample No.	Copies	Ct (Means)	Ct (SD)
NTC	/	/	/
1	3.00×10^{10}	6.21	0.16
2	3.00×10^{9}	8.51	1.20
3	3.00×10^{8}	13.16	0.72
4	3.00×10^{7}	16.33	1.30
5	3.00×10^{6}	20.54	1.09
6	3.00×10^{5}	25.39	0.71
7	3.00×10^{4}	28.11	0.80
8	3.00×10^{3}	29.92	1.14
9	3.00×10^{2}	30.62	0.96
10	3.00×10^{1}	32.13	1.79
11	3.00×10^{0}	34.30	1.66

Figure 4. Comparison of the detection limit of two-step CRISPR/Cas12a combined with RPA amplification and qPCR. (A) Agarose gel electrophoresis of RPA purification products. (B) Detection of two-step CRISPR/Cas12a combined with RPA amplification using series of 10-fold dilutions pUC57 nsp2 plasmid (from 10^{10} to 1 copies per RPA mixture) as amplification templates. (C) Quantification of fluorescence intensity was performed using ImageJ software. (D) Sensitivity of the qPCR amplification in a 96-Well Thermal Cycler PCR System. (A−C): Lane N, nontemplate control group; (1) 3×10^{10} copies/μL; (2) 3×10^{9} copies/μL; (3) 3×10^{8} copies/μL; (4) 3×10^{7} copies/ μL; (5) 3×10^{6} copies/ μL; (6) 3×10^{5} copies/μL; (7) 3×10^{4} copies/μL; (8) 3×10^{3} copies/μL; (9) 3×10^{2} copies/μL; (10) 3×10^{1} copies/μL; (11) $3 \times 10^{\circ}$ copies/ μL, Lane NTC, nontemplate Control group. Blue or UV lights: The pictures were captured under blue (470 nM) and UV lights with a smart phone camera and Gel Imaging System using UV light. Error bars represent SEM; $n = 3$, ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

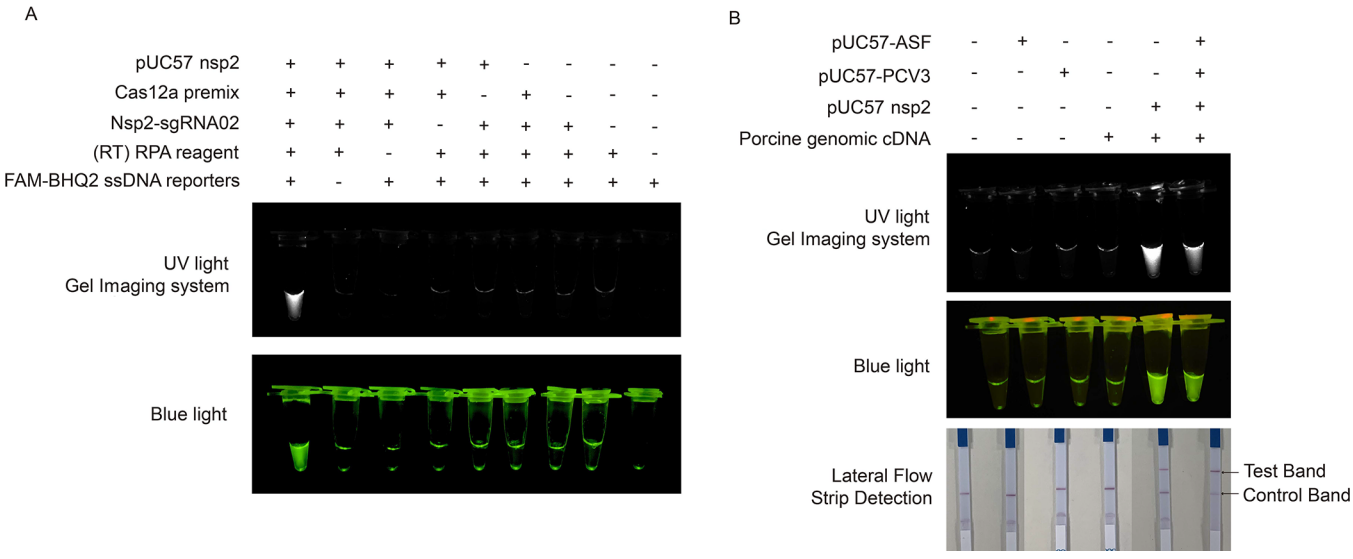
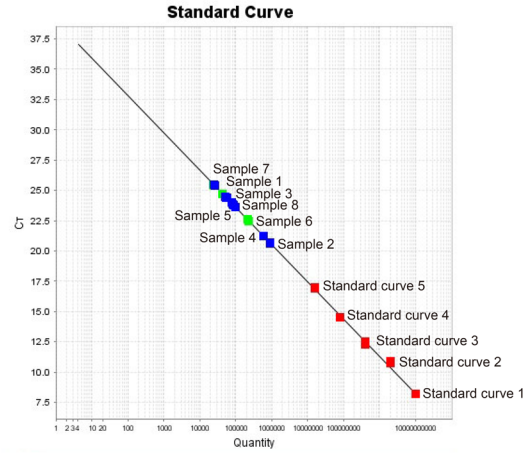


Figure 5. Specificity of the one-step CRISPR/Cas12a combined with RPA amplification for detecting PRRSV. (A) Specificity evaluation of detection of PRRSV. pUC57 nsp2: pUC57 nsp2 plasmid containing the PRRSV *nsp2* gene sequence (3×10^{3} copies); Cas12a premix: 250 nM Cas12a and 5 μL of NEBuffer 2.1; nsp2-sgRNA02:1 μM nsp2-sgRNA02; RPA regent: 20 μM of RPA primers, recombinase, single-stranded DNA binding protein (SSB), strand-displacement DNA polymerase; FAM-BHQ2 reporters: 300 nM FAM-BHQ2 reporters. (B) Specificity of the one-step CRISPR/Cas12a combined with RPA amplification. pUC57 nsp2: pUC57 nsp2 plasmid containing partial PRRSV *nsp2* gene (3×10^{3} copies); pUC57-ASF: pUC57-ASF plasmid containing partial ASFV *p17* gene (3×10^{3} copies); pUC57-PCV3: pUC57-PCV3 plasmid containing partial PCV3 *cap* gene (3×10^{3} copies); Porcine genomic cDNA: genomic cDNA from a healthy pig; detection of pUC57 nsp2 and healthy porcine cDNA mixed template (1:1); detection of pUC57 nsp2, pUC57-ASF, pUC57-PCV3, and healthy porcine cDNA mixed template (1:1:1:1).

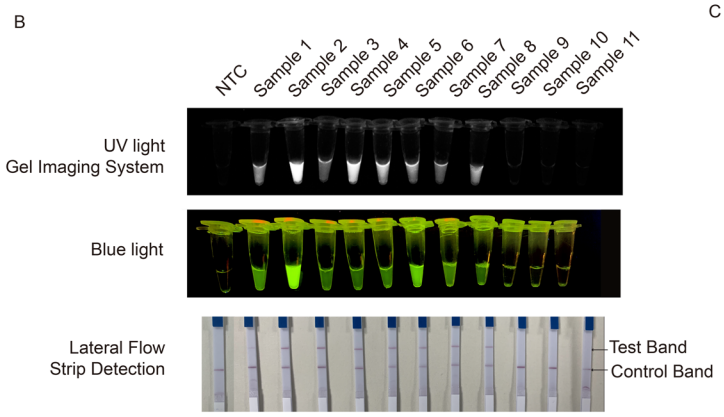
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A



Item	Copies	Ct (Means)	Ct (SD)
NTC	/	/	/
Standard curve 1	1.00×10^{10}	7.88	0.37
Standard curve 2	2.00×10^{9}	10.93	0.16
Standard curve 3	4.00×10^{8}	12.42	0.10
Standard curve 4	8.00×10^{7}	14.53	0.02
Standard curve 5	1.60×10^{7}	16.83	0.13
Sample 1	12375.50	24.95	0.97
Sample 2	794478.47	20.67	0.02
Sample 3	11838.52	24.86	1.35
Sample 4	333416.34	21.12	0.19
Sample 5	28482.95	23.64	0.17
Sample 6	38934.03	23.11	1.08
Sample 7	11116.84	25.36	0.11
Sample 8	20199.06	24.02	0.06
Sample 9	/	/	/
Sample 10	/	/	/
Sample 11	/	/	/

B



C

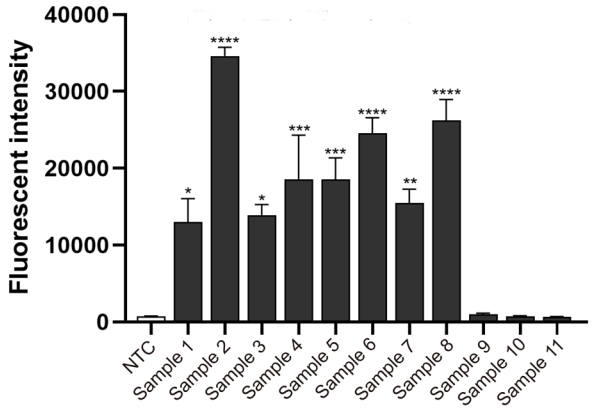


Figure 6. Evaluation of the reliability of using one-step CRISPR/Cas12a combined with RPA amplification to detect PRRSV clinical samples. (A) Detection of a total 11 PRRSV infected and uninfected samples by RT-qPCR. (B) Detection of PRRSV clinical samples by one-step CRISPR/Cas12a combined with RPA amplification and combination with the lateral flow strip method. RNA samples were diluted to 100 ng/μL. (C) The fluorescence intensity of detection image was quantified using ImageJ software. Pictures were captured under blue and UV lights. SEM standard error of the mean. Statistically significant differences are given compared to the control group. $n = 3$ technical replicates, ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Blue or UV lights: The pictures were captured under blue and UV lights.

FAM-BHQ2 ssDNA reporters can be mixed into one reaction tube (Figure 5A). The genome cDNA from healthy pigs, pUC57-ASF, pUC57-PCV3, and pUC57 nsp2 were used to test the specificity of the one-step CRISPR/Cas12a combined with RT-RPA amplification assay. As shown in Figure 5, we found that one-step assay can highly specifically detect of PRRSV, even combined with the lateral flow strip assay (Figure 5B). Next, we found that when using one-step CRISPR/Cas12a combined with the RT-RPA amplification method, the single copy of PRRSV can be directly detected from *nsp2* RNA within 25 min (Figure S4), while in combination with the lateral flow strip method, it requires more than 120 min for complete the detection reaction (Figure S5). Thus, the one-step CRISPR/Cas12a assay can be used for rapid detection of PRRSV.

Detection of PRRSV in Clinical Samples by the One-Step CRISPR/Cas12a Combined with RT-RPA Amplification. In order to evaluate whether one-step CRISPR/Cas12a combined with RT-RPA amplification can be accurately used for detecting clinical samples, a total of 11 positive and negative PRRSV infected or uninfected samples were used as test samples. These samples were first detected and confirmed by RT-qPCR assay (Figure 6A). For the Ct values of RT-qPCR results for the nontemplate control, >35 were considered as a negative sample. As shown in Figure 6A, Sample 2 contained 794 478 copies of PRRSV, while Sample 7 contained 11 116 copies of PRRSV. Samples 1−8 were all defined as positive and 9−11 were considered negative by both RT-qPCR and one-step CRISPR/Cas12a combined with RT-RPA amplification assay (Figure 6B). As shown in Figure 6C, the fluorescence signal of samples 2, 6, and 8 ($P < 0.0001$) were significantly brighter than the negative samples 9, 10, and 11. Therefore, one-step CRISPR/Cas12a combined with RT-RPA amplification can be used for accurate detection of PRRSV. This can be also confirmed by CRISPR/Cas12a combined with lateral flow strip assay (Figure 6B).

After comparing one-step CRISPR/Cas12a combined with RT-RPA amplification from a previous study,[20] we found that the detection limit of our method can reach to single copy (Figure S4), while CRISPR-Cas13a assay for detecting PRRSV was only 172 copies/reaction. This can be compared to the recently reported novel detection of SARS-CoV-2,[28,35,36] which showed that STOPCovid.v2 assay[28] and SHERLOCK[37] require an open reaction tube for a two step reaction, which may result in aerosol pollution. Compared to the CASLFA[37] and DETECTR,[36] we found that our method can achieve

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detection limitation of single copy virus in 25 min, while the detection limitation of two methods up to 10 copies, and required more than 30 min. The one-step CRISPR/Cas12a combined with RT-RPA amplification assay is a closed detection system: it requires nuclei acid extracted from the clinical sample, one more step than the STOPCovid.v2 assay;[28] thus, our method still needs further improvement in extraction-free detection. In conclusion, one-step CRISPR/Cas12a combined with RT-RPA amplification can provide a highly accurate, sensitive, and specific method for on-site detecting PRRSV.

MATERIALS AND METHODS

Sample Collection and Preparation of Viral Nucleic Acid. Spleen and lung samples from healthy pigs were collected from farms and were quickly deposited in RNAlater (DP408, TIANGEN) for storage. All animal procedures were performed according to the protocols of the Institutional Animal Care and Use Committee of the Chinese Academy of Agricultural Sciences. The experiment process was as follows: 0.1 g of spleen or lung tissue was collected into a clean Rnase-free microcentrifuge tube and 1 mL of TRIzol (15596−026, Invitrogen) was added. Samples were then vigorously centrifuged and incubated at room temperature for 5 min. We then added 200 μL of chloroform to the samples, mixed it by inversion, incubated it on ice for 10 min, and then centrifuged it at 12 000 rpm/min for 10 min at 4 °C. After centrifugation, 600 μL of the top layer aqueous phase was then mixed with 600 μL of isopropanol in a clean microcentrifuge tube, and incubated on ice for 10 min before the mixture centrifugation at 12 000 rpm for 7 min. Supernatants were discarded and 800 μL of 75% ice-cold ethanol was added. After centrifugation at 800 rpm/min for 5 min, the supernatant was discarded and the pellet was resuspended in 40 μL RNase-free water and stored at −80 °C. Total RNA was reverse-transcribed into complementary DNA (cDNA) through reverse transcription using HiScript II Reverse Transcriptase (R223−01, Vazyme). Additionally, the partial *P17* nucleic acid sequences of ASFV (Accession No. MH681419.1) and partial *cap* sequences of PCV3 (Accession No. KY418606.1) were synthesized, and ligated into a pUC57 vector (Sangon Biotech).

Polymerase Chain Reaction (PCR) and Quantitative Real-Time PCR (qPCR). Primers were designed by NCBI-Primer blast online (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) based on the target sequences of all the PRRSV strains available in GenBank. DNA sequences of PRRSV (accession No. KX077704.1), ASFV (accession No. MH681419.1), PCV3 (accession No. KY418606.1) were amplified from pUC57 nsp2, pUC57-ASF, and pUC57-PCV3, respectively. One μL of pUC57 nsp2 (containing different copy numbers of the *nsp2* gene, from 100 ng to 10^{-8} ng) was added as DNA template mixed with 12.5 μL Two × LAmp Master Mix (*P*312−01, Vazyme), 10 μM for each primer, and sterilized water to 50 μL in a PCR tube. All the general PCR reactions were performed in a 96-Well Thermal Cycler PCR System (Thermo Fisher Scientific) according to the following amplification protocols: initial denaturation at 96 °C for 5 min, 30 cycles of denaturation at 96 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 20 s, and final extension at 72 °C for 7 min. The primer pairs used in the PCR reaction are listed in Table S1. Amplification was confirmed by analytical agarose gel electrophoresis with 8 μL of each sample, and the remaining sample was used for the Cas12a-based fluorescence detection.

The qPCR and RT-qPCR assay used to detect *nsp2* was performed according to the recommended reaction conditions. For qPCR, each amplification template was serially diluted from 3 × 10^{10} to 3 × 10° copies of the pUC57 nsp2 plasmid, and mixed with 10 μL of SYBR Green qPCR Master Mix (Q711−03, Vazyme), 0.5 μM for each qPCR primer, and water to the 20 μL final reaction volume. For RT-qPCR, PRRSV copy number in RNA samples was determined by RT-qPCR using five points of the serially diluted *nsp2* RNA sample with a known copy number as standard curve (from 1.00 × 10^{10} copies to 1.60 × 10^{7} copies), and added to the mixed reaction containing 10 μL of Luna Universal One-Step Reaction Mix (E3005, NEB), 1 μL Luna WarmStart RT Enzyme Mix (E3005, NEB), 0.4 μM concentration of each RT-qPCR primer, and water to the 20 μL final reaction volume. The qPCR and RT-qPCR reactions were performed in a Step-One Plus Real-Time PCR Instrument Applied Biosystems (Life Technologies) according to their recommended amplification protocol and each amplification template were diluted to 100 ng/μL. Upon completion, the Ct values were calculated based on the threshold. Primers were all synthesized by Sangon Biotech and are listed in Table S1.

Preparation of RNAs. Nsp2-sgRNA01, Nsp2-sgRNA02, Nsp2-sgRNA03, and Nsp2-sgRNA04 targeting PRRSV-Nsp2 were designed using CRISPR-offinder software[34] (Table S2). These sgRNAs and the PRRSV-Nsp2 transcription template were amplified from a pUC57-T7-sgRNA plasmid and pUC57 nsp2 plasmid, respectively, with primer pairs listed in Table S2. Purified PCR amplicon of these sgRNAs and PRRSV-Nsp2 then served as the DNA template for *in vitro* transcription reactions with a HiScribe T7 High Yield RNA Synthesis Kit (E2040S, NEB). The product of sgRNAs and *nsp2* RNA fragments were purified using a Monarch RNA Cleanup Kit (T2040L, NEB). The concentration of each RNA was quantified by gel image and NanoDrop One spectrophotometer (Thermo Fisher Scientific).

CRISPR/Cas12a-Based Fluorescence Detection Assay. For each CRISPR/Cas12a-based assay, 250 nM Lba Cas12a (M0653T, NEB), 1 μM sgRNA, 300 nM ssDNA-FQ reporter, 3 μL of nucleic acid sample, and 2 μL of NEBuffer 2.1 (B7203S, NEB) were adjusted to 20 μL using nuclease-free water in each Cas12a reaction. After the reactions were incubated at 37 °C for 25 min, the reaction tubes were then photographed and recorded by smart phone under the blue light or analysis by Gel Imaging System (BioRad) after an exposure time of 15 ms.

The normalized copy number for pUC57 nsp2 was determined as follows: pUC57 nsp2 plasmid was serially diluted from 100 ng to 10^{-8} ng/μL and then calculated based on different molecular weight. The PCR and RPA assays were all performed on the same dilution series, and the amplification product was analyzed by gel electrophoresis. Parallel samples were all analyzed using the Cas12a-based fluorescence detection, and the intensity of fluorescence captured by gel electrophoresis was then measured using ImageJ software. A Cas12a-based fluorescence intensity test was conducted to compare 6 ssDNA-FQ reporters using dilutions of the *nsp2* at concentrations of 3 × 10^{9} copies/μL reactions.

RPA Reaction and Cas12a Combined with RPA Amplification. Two pairs of RPA primers (Table S1) were designed using Primer Premier 5.0 (PREMIER Biosoft)

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according to the targeting sequences containing nsp2-sgRNA02. The RPA single reaction was performed as previously described[35] using the kit (WLRB8201KIT, AMP-Future Biotech) to amplify the DNA template. The RT-RPA reaction was performed using the kit (WLRB8204KIT) and an RNA template. Primes of ASF-RPAF and ASF-RPAR were designed to amplify ASFV, while PCV3-RPAF and PCV3-RPAR were used for PCV3 (Table S1). Each RPA amplification template containing the different copy number of *nsp2* gene (from 100 ng/μL to 10^{-8} ng/μL) was mixed with 20 μM of RPA primer mix (10 × primer: 100 μM nsp2-RPAF, 100 μM nsp2-RPA2R), recombinase, single-stranded DNA binding protein (SSB), strand-displacement DNA polymerase, or reverse transcriptase for RT-RPA, 1 μL total RNA, after which Rnase-free water was added to the 25 μL final reaction volume. Before purification, each RPA or RT-RPA reaction was performed in a Dry Bath incubator (TIANGEN) according to the following amplification protocol: 37 °C for 20 min. Subsequently, the RPA or RT-RPA amplicon was then purified using a PCR purification Kit (28106, QIAGEN) before gel electrophoresis, while 3 μL of the remaining sample was set aside for the two-step Cas12a-based fluorescence assay. The RPA amplification reaction efficiencies were compared based on different reaction temperature, incubation time, and primers.

A one-step CRISPR/Cas12a combined with RT-RPA amplification assay was tested. A single RT-RPA reaction solution was mixed with a Cas12a-reaction premix (250 nM Cas12a, 1 μM sgRNA, 300 nM ssDNA-FQ reporter, 5 μL of NEBuffer 2.1, and added Rnase-free water to 25 μL) without purification in one tube to produce a final reaction volume of 50 μL. The mixture solution was incubated in one dry bath pot at ∼37 °C, after which the RT-RPA amplification was initiated and the binding sites of the Cas12a-crRNA complexes were exposed due to strand displacement.

Detection of Genomic DNA and Clinical PRRSV RNA Samples by CRISPR/Cas12a-Based Assay. Genomic DNA was extracted from a healthy pig using a DNA extraction kit (DP315, TIANGEN). The cDNA from a healthy pig and pUC57 nsp2 plasmid (1:1) were then well-mixed and used to evaluate interference by the porcine cDNA. The DNA of PRRSV, ASFV, and PCV3 detection were all based on a Cas12a-based assay coupled with PCR or RPA amplification, which used pUC57 nsp2, pUC57-ASF, and pUC57-PCV3 plasmids as template, respectively. To evaluate whether detecting the *nsp2* gene with Cas12a can be interfered with the other viruses, the PCR and RPA amplicons of pUC57 nsp2, pUC57-ASF, and pUC57-PCV3 were mixed (1:1:1) and used as a template for the Cas12a-based detection assay. All RPA and PCR amplicons were verified *via* agarose gel electrophoresis.

A total of 11 clinical PRRSV infection or uninfected samples were collected from pigs farm and virus RNA was extracted according to the methods described above. Then, these RNA samples were diluted to 100 ng /μL per for conducting RT-RPA reaction. All clinical PRRSV samples were verified by RT-qPCR prior to detection with the RT-RPA reaction. The parallel samples were analyzed *via* Cas12a-based detection assay.

CRISPR/Cas12a-Based Lateral Flow Strip Assay. To perform the CRISPR/Cas12a-based Lateral flow strip assay, FAM-Biotin ssDNA reporter (FAM-5′-TTATTAT-3′-Biotin) was used. The detection efficiency of the lateral flow strip was then tested using 100 ng pUC57 nsp2 plasmids as a positive sample, with a Cas12a-based mixture reagent (250 nM of Cas12a, 1 μM sgRNA, 5 μM FAM-Biotin ssDNA reporter, and 2 μL of NEBuffer 2.1). The reaction time of the lateral flow strip was calculated from 20 to 120 min at 37 °C. The visual detection was performed according to the manufacturer's instructions (JY0301, Tiosbio). The RPA or RT-RPA reaction mixture was mixed with 250 nM of Cas12a, 1 μM sgRNA, 5 μM FAM-Biotin ssDNA reporter, and 5 μL of NEBuffer 2.1. After 120 min of incubation at 37 °C, 50 μL of sterilized water was added for mixing after which the strips were inserted.

Statistical Analysis. Statistical analysis was performed using a two-way ANOVA (mixed model). Values are means ± SEM. Statistically significant differences are given compared to the control group. $N = 3$ technical replicates, $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. SEM standard error of the mean.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00103.

Figure S1: A comparative sequence analysis of Nsp2-sgRNA01, Nsp2-sgRNA03, and Nsp2-sgRNA04 in 20 different PRRSV strains; Figure S2: Specificity of two-step Cas12a-based fluorescence assay; Figure S3: Detection of PRRSV nsp2 gene by RPA with two RPA primer pairs; Figure S4: Visual sensitivity detection of one-step CRISPR/Cas12a combined with RT-RPA amplification; Figure S5: Visual detection of PRRSV by CRISPR/Cas12a-based lateral flow strip assay; Table S1: Primer pairs and sgRNAs targeting sequences used in this study; Table S2: Synthetic sequence fragment in pUC57 vector; Table S3: Partial sequence of *nsp2* in 20 different PRRSV strains (PDF)

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Author Contributions

Zhonglin Tang designed and managed the project. Shengsong Xie provided the help in CRISPR experiment. Siyuan Liu conducted the experiments and wrote the manuscript. Dagang Tao designed sgRNA and assisted in the establishment of an *in vitro* transcription method. Yuying Liao, Shouzhang Sun, Yulan Zhao, Peng Yang, Yonggang Liu, and Yijie Tang provided help in the qPCR, RT-qPCR PCR detection, plasmid generation, and clinical sample collection. Zhonglin Tang, Shengsong Xie, Yalan Yang, and Bin Chen revised the manuscript. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CRISPR; clustered regularly interspaced short palindromic repeats; sgRNA; single-guided RNA; PAM; protospacer adjacent motif; PRRS; porcine reproductive and respiratory syndrome; PRRSV; porcine reproductive and respiratory syndrome virus; ASF; African swine fever; ASFV; African swine fever virus; PCV3; Porcine circovirus type 3; ssDNA; single-stranded DNA; ssDNA-FQ reporter; single-stranded DNA-fluorophore-quencher reporter; RPA; recombinase polymerase amplification; LAMP; loop-mediated isothermal amplification; PCR; polymerase chain reactions; qPCR; quantitative real-time PCR; RT-qPCR; quantitative reverse transcription polymerase chain reaction; ddPCR; droplet digital PCR; ORFs; open reading frames; UV; ultraviolet; Ct; cycle threshold.

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