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Supporting Information For

RAA-CRISPR-HCR Cascade Amplification for Ultrasensitive Visual Detection of African Swine Fever Virus DNA

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1. Experimental Section

1.1 Chemicals and apparatus

Materials. All the oligonucleotides were synthesized and purified by Sangon Biotechnology Co., Ltd. (Shanghai, China) (Table S1). Tris(hydroxymethyl)-aminomethane (Tris), MgCl2, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES), dimethyl sulfoxide (DMSO), KCl, ethylenediaminetetraacetic acid (EDTA), and NaCl were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Hemin, H2O2, and 2,2-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid (ABTS) were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). A hemin stock solution (1 mM) was prepared using DMSO and was stored at -20 °C, strictly avoiding any light exposure. EnGen® Lba Cas12a （Cpf1）and NEBuffer2.1 were purchased from New England Biotechnology (Beijing) Co., Ltd (Beijing, China). The RAA kit was purchased from Jiangsu Qitian Gene Biotechnology Co., Ltd. (Jiangsu, China) The buffer solutions used in the experiments were prepared as follows: Reaction buffer: 50 mM Tris-HCl, 140 mM NaCl, 1 mM $MgCl_2$, pH = 7.4; NEBuffer 2.1: 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 100 μg/ml BSA, pH = 7.9; 2× HEPES buffer: 50 mM HEPES, 400 mM NaCl, 40 mM KCl, 2% dimethyl sulfoxide (DMSO), 0.1% Triton X-100, pH = 8.0; 1× TBE buffer: 88 mM Tris-HCl, 2 mM EDTA, pH = 8.0. All chemicals were directly used without other purification. The solutions were prepared with ultra-pure Milli-Q water, exhibiting a resistivity of 18.2 $M\Omega \cdot cm^{-1}$, sourced from a Millipore system. ASFV DNA in porcine tissue fluid samples was obtained from the Harbin Veterinary Research Institute.

Instruments. The Ultraviolet-visible (UV-Vis) absorption spectra were collected using a Shimadzu UV-3600 spectrophotometer (Kyoto, Japan). pH measurements were performed utilizing a Sartorius PB-10 digital pH-meter (Beijing, China). Documentation of gel images was carried out with the assistance of a GBOX-F3-E gel imager system (Gene Company Limited, England).

1.2 Polyacrylamide Gel Electrophoresis (PAGE) Experiment.

HCR reaction and the protein cleavage ability were analyzed by 12% natural

polyacrylamide gel electrophoresis (PAGE). The electrophoresis parameters were maintained at 70 V for a duration of 90 min in 1× TBE buffer. After the reaction was completed, the polyacrylamide gel was stained in a 0.4× Gel-Red dye in a dark environment at 25 °C for 10 minutes and visualized using a GBOX-F3-E gel imager.

1.3 Detection of ASFV DNA.

The genetic target at various concentrations was diluted in 2 μL of the reaction buffer, followed by adding 4 μL of Cas12a (1 μM), 4 μL of crRNA (1 μM), 1.5 μL of trigger DNA (10 μM), and 8.5 μL of reaction buffer. The mixture was subsequently incubated at 37 °C and shaken for 30 min. After that, 3 μL of H1 (10 μM), 3μL of H2 (10 μM), 4 μL of hemin (1mM) and 63 μL of 2× HEPES buffer were added to the mixture and the reaction was allowed to proceed at 25 °C (300 rpm shaking) for another 20 min. Then, 5 μL of ABTS (40 mM) and 2 μL of H_2O_2 (50 mM) were added to the above mixture and retained at room temperature for 10 min. The color of the reaction tube was photographed, and the absorbance of the solution was measured by UV-Vis spectrometer. All experiments were repeated at least three times.

1.4 RAA amplification.

The RAA assays were carried out with a commercial recombinase-aided amplification (RAA) kit at 50 μL reaction volumes. According to the operating instructions, 2 μL of ASFV3 at a certain concentration, 2 μL of the forward primer F (10 μM) and the reverse primer R (10 μM) of ASFV, 25 μL of A buffer, 16.5 μL of DNase-free water and 2.5 μL of magnesium acetate (280 mM) were added to the mixture. Subsequently, the above solution was incubated at 37 °C and 300 rpm shaking for 30 min. The resulting RAA products were stored at 4 °C for further use.

1.5 Specificity Experiment.

Equal volumes of ASFV1 (1 μM) and ASFV2 (1 μM) were mixed in a reaction buffer and incubated at 37 °C (300 rpm shaking) for 120 min, serving as ASFV DNA target for subsequent experiments. Then, 2 μL (10 copies/μL) of different types of viral nucleic acid targets (Ebola, Zika, DENGUE, H7N9, ASFV) was mixed with 4 μL of Cas12a (1 μM), 4 μL of crRNA (1 μM), 1.5 μL of trigger DNA (10 μM) and 8.5 μL of

reaction buffer. This mixture was subsequently incubated at 37 °C (300 rpm shaking) for 30 min. Following that, 3 μL of H1 (10 μM), 3 μL of H2 (10 μM), 4 μL of hemin (1mM), and 63 μL of 2× HEPES buffer was added. After reacting at 25 °C (300 rpm shaking) for 20 min, 5 μL of ABTS (40 mM) and 2 μL of H_2O_2 were introduced to the mixture for 10 min incubation at 25 °C. To measure the absorption of the solution, the color of the reaction tube was then collected using a UV-Vis spectrometer.

1.6 Sensitivity Experiment.

According to the operating instructions, the standard material of the ASFV B646L Gene Plasmid was diluted to 0, 1, 10, 50, 100, 1000 and 5000 copies/μL and used as targets for subsequent experiments. 10 μL of the targets, 2 μL of the forward primer F (10 μM) and the reverse primer R (10 μM), 25 μL of A buffer, 8.5 μL of DNase-free water and 2.5 μL of magnesium acetate (280 mM) were evenly mixed. Subsequently, the above solution was incubated at 37 °C and 300 rpm shaking for 30 min. Then, 2 μL of the resulting RAA products, 4 μL of Cas12a (1 μM), 4 μL of crRNA (1 μM), 1.5 μL of trigger DNA (10 μM), and an extra 8.5 μL of reaction buffer were evenly mixed. The mixture was subsequently incubated at 37 °C and shaken for 30 min. After that, 3 μL of H1 (10 μM), 3μL of H2 (10 μM), 4 μL of hemin (1mM) and 63 μL of 2× HEPES buffer were added to the mixture and the reaction was allowed to proceed at 25 °C (300 rpm shaking) for another 20 min. Then, 5 μL of ABTS (40 mM) and 2 μL of H_2O_2 (50 mM) were added to the above mixture and retained at room temperature for 10 min. The color of the reaction tube was photographed, and the absorbance of the solution was measured by UV-Vis spectrometer. All experiments were repeated at least three times.

1.7 ASFV Detection in Swine Tissue Fluid Samples.

Inactivated swine tissue fluid samples from the Harbin Veterinary Research Institute were used. Virus DNA was extracted from 30 μL samples using the ZK-01 automatic nucleic acid extractor (Nanjing Zhongkebio Medical Technology CO., Ltd.) and directly used for RAA or PCR analysis. qPCR was conducted with the Diagnostic Kit (BTN15-23000) following the manual. Each 20 μL reaction included 10 μL PCR

premix, 3 μL primer/enzyme mix, and 7 μL DNA. Reactions ran on an ABI 7500 PCR Instrument (Thermo Fisher) with a thermal cycle of 95 °C for 3 min, then 40 cycles at 95 °C for 15 s and 60 °C for 1 min. A Ct value below 35 was considered positive. The detection steps are the same as those used for ASFV DNA detection described above. The threshold was determined as the mean ΔA value of the negative controls plus three standard deviations (mean + 3SD).

2. Supplementary Figures and Tables

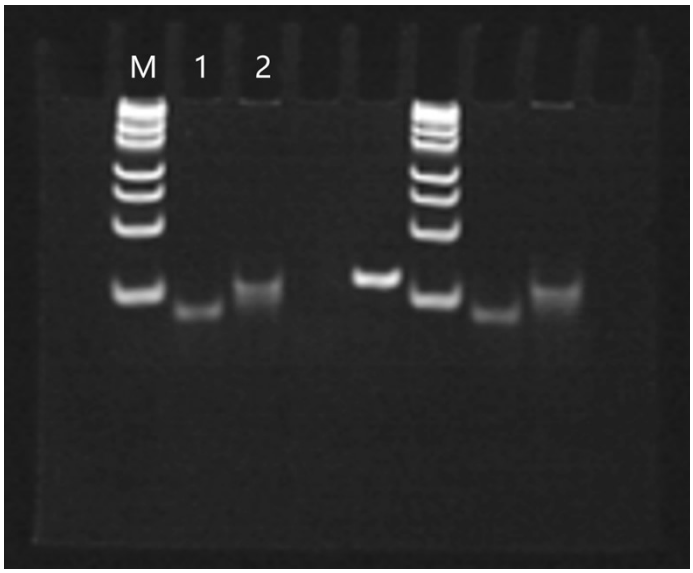

Fig. S1 The original, full-length images of the gel in Fig. 2b. The first three lanes correspond to the three lanes in the manuscript. Other bands are irrelevant bands. Lane M: 25-500 bp marker; Lane 1: Cas12a/crRNA + Trigger; Lane 2: Cas12a/crRNA + Trigger + ASFV DNA.

Fig. S2 The original, full-length images of the gel in Fig. 2d. Lanes 1, 2, 4, 6, and 8 correspond to the five bands in the manuscript. (Lane 1: 25-500 bp marker; Lane 2-3: H1; Lane 4-5: H2; Lane 6-7: H1 + H2; Lane 8-9: H1 + H2 + Trigger). [H1] = [H2] = 1 μM; [Trigger] = 500 nM.

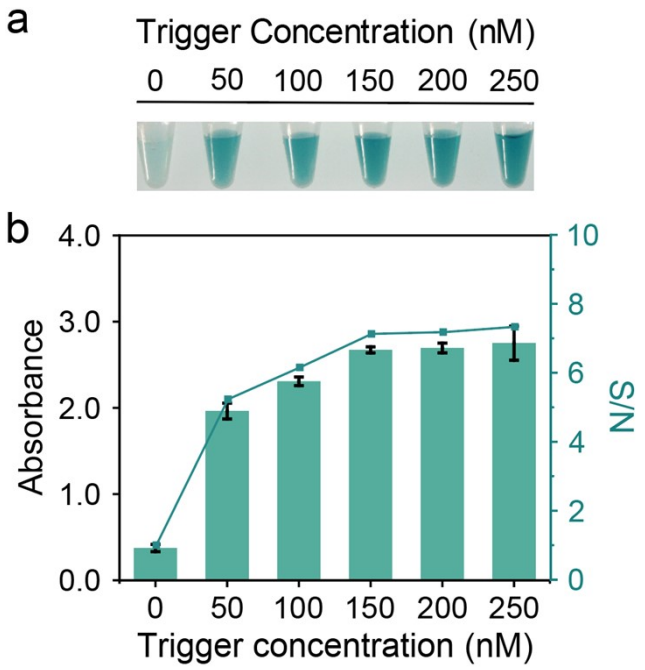

Fig. S3 Detection of ASFV DNA using the CRISPR-HCR system: Changes in UV absorbance at Trigger concentrations of 0 nM, 50 nM, 100 nM, 150 nM, 200 nM, and 250 nM. The absorbance reached its peak at a Trigger concentration of 150 nM (n=3 technical replicates; bars represent mean ± SD). The signal-to-noise ratio (S/N) was calculated as the ratio of the absorbance at a given Trigger concentration (signal) to the background absorbance in the absence of the Trigger (noise).

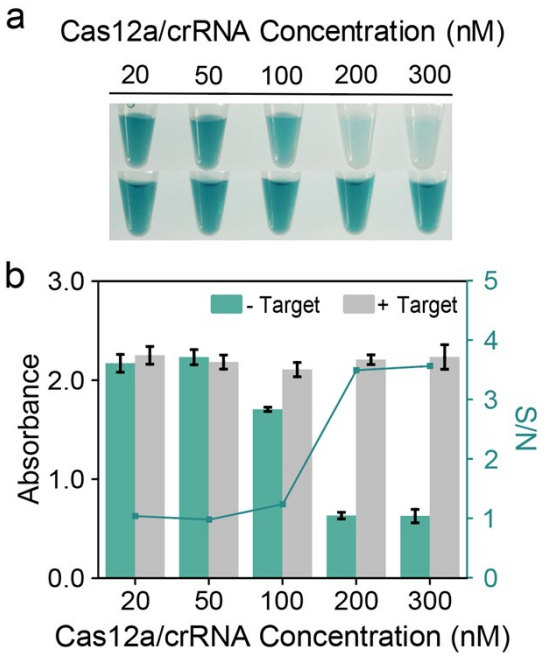

Fig. S4 Detection of ASFV DNA using the CRISPR-HCR system: Changes in UV absorbance at Cas12a and crRNA concentrations of 20 nM, 50 nM, 100 nM, 200 nM, and 300 nM, in the presence or absence of ASFV DNA. The signal-to-noise ratio peaked at a Cas12a and crRNA concentration of 200 nM (n=3 technical replicates; bars represent mean ± SD). The signal-to-noise ratio (S/N) was calculated as the ratio of the absorbance in the presence of ASFV DNA (signal) to the absorbance in the absence of ASFV DNA (noise) at a given Cas12a/crRNA concentration.

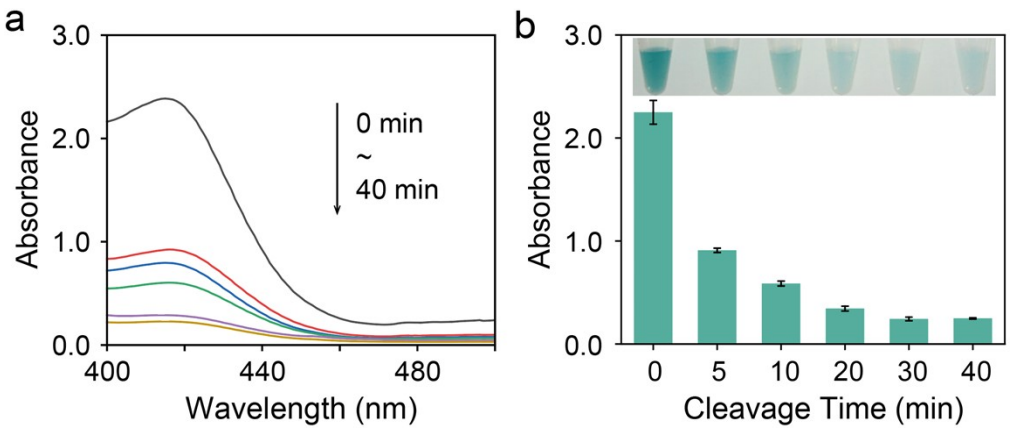

Fig. S5 (a) UV absorption spectra showing the cleavage kinetics of the CRISPR-Cas12a system complex. (b) Detection of ASFV DNA (100 nM) using the CRISPR-HCR system: Changes in UV absorbance after Trigger cleavage by the CRISPR-Cas12a system for 0 min, 5 min, 10 min, 20 min, 30 min, and 40 min. The decrease in absorbance reached a plateau after a reaction time of 30 min (n=3 technical replicates; bars represent mean ± SD).

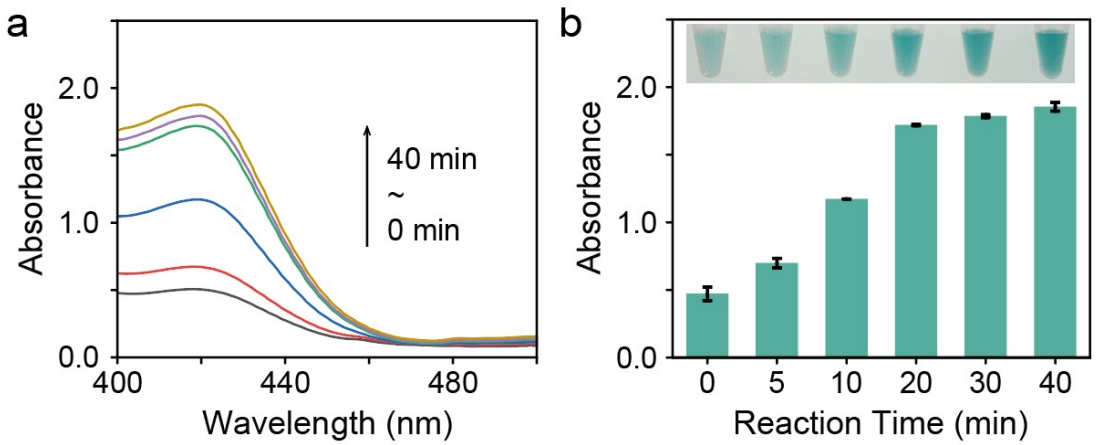

Fig. S6 (a) UV absorption spectra showing the amplification kinetics of the HCR system coupled with the CRISPR-Cas12a system. (b) Detection of ASFV DNA (100 nM) using the CRISPR-HCR system: Changes in UV absorbance after the HCR reaction proceeded for 0 min, 5 min, 10 min, 20 min, 30 min, and 40 min. The absorbance reached its peak after a reaction time of 20 min (n=3 technical replicates; bars represent mean ± SD).

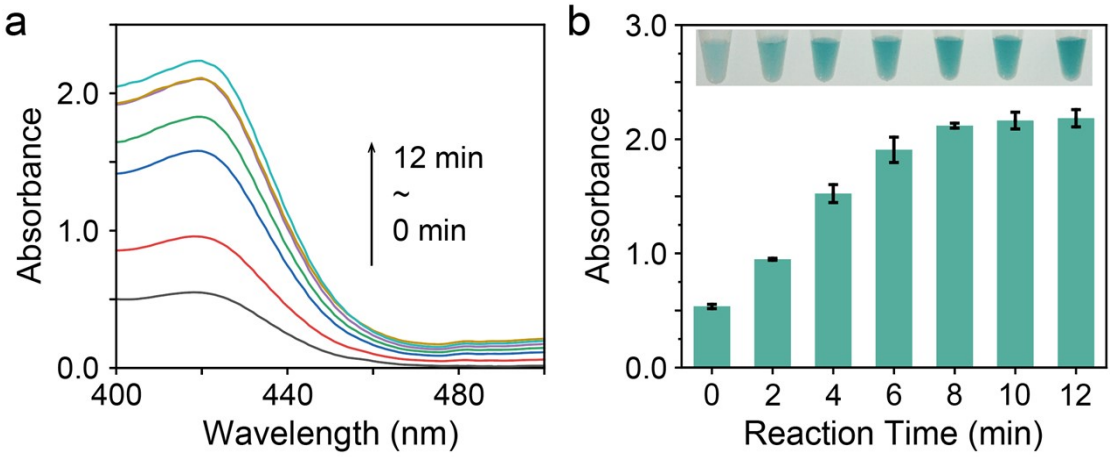

Fig. S7 (a) UV absorption spectra showing the catalysis of ABTS/H_2O_2 by the G-quadruplex/hemin complex in the CRISPR-Cas12a system. (b) Detection of ASFV DNA (100 nM) using the CRISPR-HCR system: Changes in UV absorbance after ABTS/H_2O_2 catalysis for 0 min, 2 min, 4 min, 6 min, 8 min, 10 min, and 12 min. The absorbance reached its peak at a catalysis time of 8 min (n=3 technical replicates; bars represent mean ± SD).

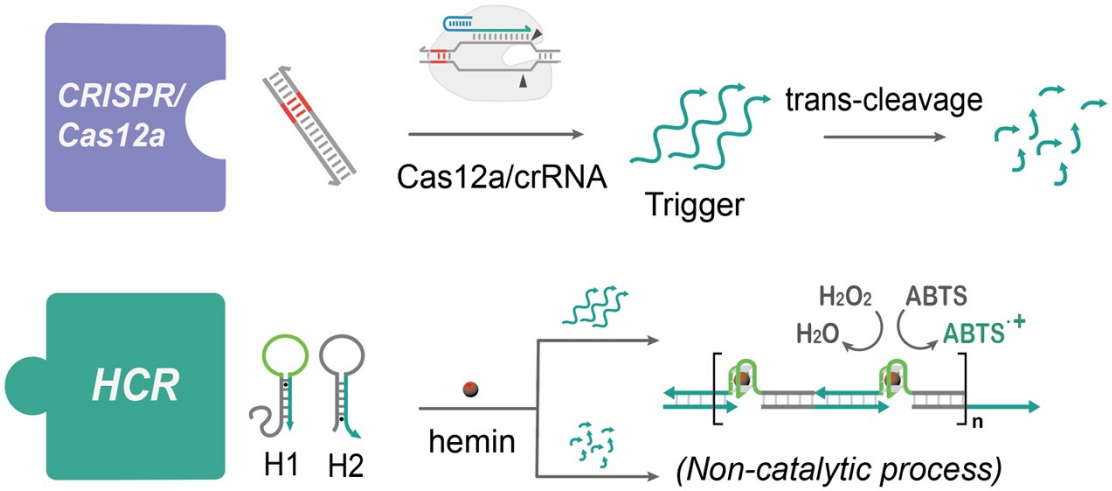

Fig. S8 Schematic diagram of the visual detection of ASFV DNA using the CRISPR-HCR system.

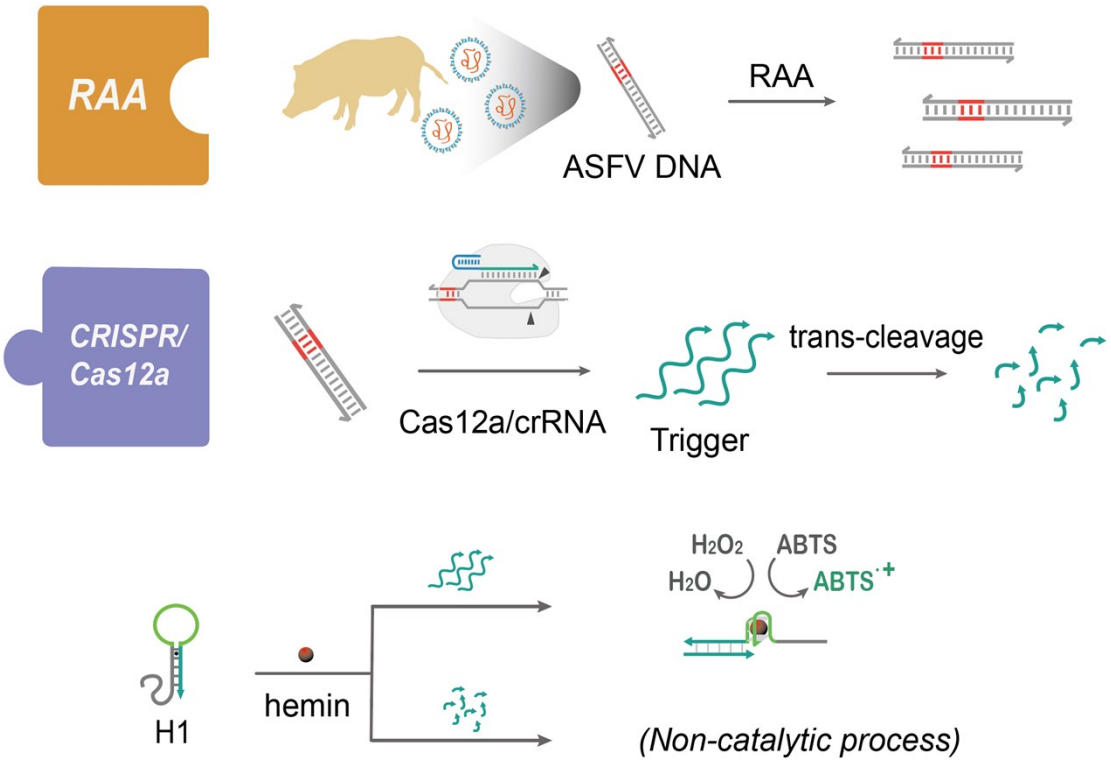

Fig. S9 Schematic diagram of the visual detection of ASFV DNA using the RAA-CRISPR system.

Table S1. All DNA and RNA sequences used in this work.

Name	Sequence（5’-3’）
Trigger	AGAACAAGGTGTTTAAGTACCCTACCAGCGCTTCAGCG
H1	TACTTAAACACCTTGTTCTTGGGTAGGGCGGGTTGGGAA ATTACCCAACAACAAGGTGTT
H2	AGAACAAGGTGTTTAAGTATAACACCTTGTTGTTGGGTA
crRNA	UAAUUUCUACUAAGUGUAGAUGUGCGGCUUGUGCAAA UCAUGAA
ASFV1	TTCATGATTTGCACAAGCCGCACCAAAGCAA
ASFV2	TTGCTTTGGTGCGGCTTGTGCAAATCATGAA
ASFV3	CGCTGCGTATCATTTTCATCGGTAAGAATAGGTTTGCTT TGGTGCGGCTTGTGCAAATCATGAATGTTGCATAGGAG AGGGCCACTGGTTCCCTCCA
F	GAAATTAATACGACTCACTATAGGGTAATTTCTACTAAG TGTAGATGTGCGGCTTGTGCAAATCATGAA
R	TTCATGATTTGCACAAGCCGCACATCTACACTTAGTAGA AATTACCCTATAGTGAGTCGTATTAATTTC
H7N9 DNA	CTCCACAGCAAGCTCATGGTC
Ebola DNA	GTCTTTTCCCTCAACTATCGGC
ZIKA DNA	CTGGCATCATGAAGAATCCCG
DENGUE DNA	CAACACACCAGAAGGGATTAT

ASFV1 and ASFV2: Complementary oligonucleotide sequences that, upon annealing, form a double-stranded DNA fragment mimicking the ASFV B646L gene target. ASFV3: A longer DNA template containing the target sequence, used for RAA amplification. F and R: RAA amplification primers designed for the ASFV B646L gene.