Supporting Information

Droplet Cas12a assay enables DNA quantification from unamplified samples at the single-molecule level

Huahua Yue[1,2], Bowen Shu[3,4], Tian Tian[1], Erhu Xiong[1], Mengqi Huang[1], Debin Zhu[5], Jian Sun[6,7,8], Qing Liu[9], Shichan Wang[10], Yirong Li[10] and Xiaoming Zhou[1*]*

1 School of Life Sciences, South China Normal University, Guangzhou 510631, China.

2 College of Biophotonics, South China Normal University, Guangzhou 510631, China.

3 Dermatology Hospital, Southern Medical University, Guangzhou 510091, China.

4 Department of Laboratory Medicine, Guangzhou First People's Hospital, School of Medicine, South China University of Technology, Guangzhou 510180, China.

5 Guangzhou Key Laboratory of analytical Chemistry for Biomedicine, School of Chemistry, South China Normal University, Guangzhou 510006, China.

6 College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, China.

7 National Risk Assessment Laboratory for Antimicrobial Resistance of Animal Original Bacteria, South China Agricultural University, Guangzhou 510642, China.

8 Research Center for African Swine Fever Prevention and Control, South China Agricultural University, Guangzhou 510642, China.

9 Department of Molecular Diagnostics, Sun Yat-sen University Cancer Center; State Key Laboratory of Oncology in South China; Collaborative Innovation Center for Cancer Medicine, Guangzhou 510700, China.

10 Department of Laboratory Medicine, Zhongnan Hospital, Wuhan University, Wuhan 430071, China.

EXPERIMENTAL SECTION

Materials

The LbCas12a, crRNA, and double-labeled (FAM and BHQ1) DNA reporter probes (**Table S1**) were purchased from Guangzhou Bio-Lifesci (Guangzhou, China). ASFV genome DNA was supplied by National Standard Material Platform (Xinyang, China). DEPC-Treated Water (DNase/RNase-Free), Tris-HCl with different pH (pH = 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0), NaCl, $MgCl_2$, and $MnCl_2$, $CaCl_2$, $FeSO_4 \cdot 7H_2O$, $CuCl_2 \cdot 2H_2O$, DTT, PEG-200, Target DNA-F and Target DNA-R (**Table S1**) were purchased from Sangon Biotech (Shanghai, China). Genomic DNAs of *Listeria monocytogenes* (Lm), *Staphylococcus aureus* (Sa), *Neisseria encephalitis* (Ne), *Salmonella typhimurium* (St), *Enterobacter sakazakii* (Es), *Pseudomonas aeruginosa* (Pa) *Haemophilus parasuis* (HPS) and *Streptococcus suis* (SS) were extracted using TIANamp Bacteria DNA Kit (TIANGEN BIOTECH (BEIJING) CO., LTD.) and quantified by Nanodrop 2000 (Thermo Fisher Scientific). *Pseudorabies virus* (PRV) and *Porcine circovirus* (PCV) were extracted by TIANamp Virus DNA/RNA Kit (TIANGEN BIOTECH (BEIJING) CO., LTD.) and quantified by Nanodrop-2000.

Double-stranded DNA activator preparation

For the optimization of Cas12a assay, the dsDNA activator derived from ASFV DNA sequence was formed by gradient annealing of two completely complementary single-stranded DNA. Briefly, 10 μM Target DNA-F and 10 μM Target DNA-R (**Table S1**) were mixed in 1× reaction buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.9). Reactions (volume 50 μL) were incubated in the Mastercycler nexus PCR system (Eppendorf) with the following protocol: 95 °C for 5 min, and then reduce 1 °C every minute until 25 °C. The dsDNA activator was stored at -20 °C for later use.

Report probes optimization

Report probes optimization were conducted by real-time fluorescence detection. 100 nM Cas12a was mixed with 200 nM ASFV-crRNA1, and 400 nM reporter probes of various sequences (**Table S1**) in 1× NEBuffer 2.1 at 25 °C for 10 min (volume 18 μL), respectively. The reaction was then initiated by the addition of 10 nM dsDNA activator

(volume 2 μL). And the reaction without target of interest was employed as a background control (termed as NTC). Reactions (volume 20 μL) were monitored in a Thermal Cycler Dice Real Time System III (TaKaRa) at 37 °C with fluorescence measurements taken every 1 minute (λ_{ex}: 488 nm; λ_{em}: 535 nm), and the total time is 30 min.

Temperature optimization

Temperature optimization were conducted by real-time fluorescence detection. An 18-μL reaction mixture including 100 nM Cas12a, 200 nM ASFV-crRNA1, 400 nM FQ6C reporter probe, and 1× NEBuffer 2.1 was prepared at 25 °C for 10 min. The reaction was then initiated by the addition of 10 nM dsDNA activator (volume 2 μL). And the reaction without target was employed as a background control (termed as NTC). Reactions (volume 20 μL) were monitored in a Thermal Cycler Dice Real Time System III at different temperature (25, 30, 35, 40, 45, and 50 °C) with fluorescence measurements taken every 1 minute (λ_{ex}: 488 nm; λ_{em}: 535 nm), and the total time is 30 min.

Reaction buffer optimization

The optimization of the reaction buffer was conducted by real-time fluorescence detection. For Na^+, 100 nM Cas12a was mixed with 200 nM ASFV-crRNA1, and 400 nM FQ6C reporter probes in 1 × Buffer without Na^+ (**Table S2**) by adding different concentrations of Na^+ (0, 10, 20, 50, and 100 mM) at 25 °C for 10 min (volume 18 μL), respectively. For M^{2+} (divalent metal cations: Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+} and Fe^{2+}), 100 nM Cas12a was mixed with 200 nM crRNA1, and 400 nM FQ6C reporter probes in 1× Buffer-M^{2+} (**Table S2**) by adding different concentrations of Mg^{2+}/Mn^{2+} (0, 5, 10, 15, and 20 mM) or 15 mM different M^{2+} (Mg^{2+}, Mn^{2+}, Ca^{2+}, Cu^{2+} and Fe^{2+}) at 25 °C for 10 min (volume 18 μL), respectively. For DTT, 100 nM Cas12a was mixed with 200 nM crRNA1, and 400 nM FQ6C reporter probes in 1× Buffer-DTT (**Table S2**) by adding different concentrations of DTT (0, 0.5, 1, 2, and 4 mM) at 25 °C for 10 min (volume 18 μL), respectively. For Tris-HCl pH, 100 nM Cas12a was mixed with 200 nM crRNA1, and 400 nM FQ6C reporter probes in 1× Buffer-Tris-HCl (**Table S2**) by

adding 10 mM Tris-HCl with various pH (pH=7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0) at 25 °C for 10 min (volume 18 μL), respectively. For PEG-200, 100 nM Cas12a was mixed with 200 nM crRNA1, and 400 nM FQ6C reporter probes in 1× Buffer-PEG (**Table S2**) by adding various concentration of PEG-200 (0%, 5%, 10%, 15% and 20%) at 25 °C for 10 min (volume 18 μL), respectively. All the above reactions were initiated by the addition of 10 nM dsDNA activator (volume 2 μL). And the reaction without target was employed as a background control (termed as NTC). Reactions (volume 20 μL) were monitored in a Thermal Cycler Dice Real Time System III at 45 °C with fluorescence measurements taken every 1 minute (λ_{ex}: 488 nm; λ_{em}: 535 nm), and the total time is 30 min.

Reaction buffer comparison

The comparison experiments of the reaction buffers were conducted by real-time fluorescence detection. 100 nM Cas12a was mixed with 200 nM ASFV-crRNA1, and 400 nM FQ6C reporter probes in various buffers (Buffer 1, Buffer 2, Buffer 3, Buffer 4 and Enhanced buffer) (**Table S2**) at 25 °C for 10 min (volume 18 μL), respectively. The reaction was then initiated by the addition of 1 nM dsDNA activator (volume 2 μL). And the reaction without target was employed as a background control (termed as NTC). Reactions (volume 20 μL) were monitored in a Thermal Cycler Dice Real Time System III at 45 °C with fluorescence measurements taken every 1 minute (λ_{ex}: 488 nm; λ_{em}: 535 nm), and the total time is 30 min.

Michaelis-Menten analysis

The Michaelis-Menten was analyzed according to previous report.[1] Briefly, the conventional reaction was initiated by 0.1 nM effective complex in 1× Buffer 4 and 0.001, 0.01, 0.1, 0.2, 0.5, 1 or 2 uM of FQ6T substrate at 37°C with fluorescence measurements taken every 30 seconds (λ_{ex}: 488 nm; λ_{ex}: 535 nm), and the total time is 30 min. And the optimal reaction was initiated by 0.1 nM effective complex in 1× Enhanced buffer and 0.001, 0.01, 0.1, 0.2, 0.5, 1 or 2 uM of FQ6C substrate at 45°C with fluorescence measurements taken every 30 seconds (λ_{ex}: 488 nm; λ_{ex}: 535 nm), and the total time is 30 min. The initial velocity (V_0) was calculated by fitting to a linear

regression and plotted against the substrate concentration to determine the Michaelis-Menten constants (Origin Software), according to the following equation: $V_0 = V_{max}$ [S]/(K_m+[S]), where [S] is the substrate concentration, V_{max} is the maximum reaction rate, and K_m is the Michaelis constant. The turnover number (K_{cat}) was determined by the equation: $K_{cat} = V_{max}/[E_t]$, where E_t was 0.1 nM.

crRNA design and activity analysis

After choosing the target genes, several crRNAs (**Table S1**) were designed based on a TTTN PAM site. To evaluate the activity of these crRNAs, dsDNA that can be targeted by different crRNAs were obtained by PCR with specific primer pairs (ASFV-F1/R1, EBV-F1/R1, HBV-S-F/R and HBV-X-F/R),[2, 3] respectively. The target genes are as follows: ASFV-B646L, EBV-EBNA-1, HBV-S/X. After purification, the concentration of targets was quantified by Nanodrop-2000. The activity of these crRNAs was analyzed by real-time fluorescence detection.

***Cis*-cleavage activity analysis**

The cleavage targets used amplification products of ASFV genomic DNA, and PCR primer pairs are listed in **Table S1** (ASFV-F2/R2). The amplification products were purified by E.Z.N.A.® Gel Extraction Kit, and quantified by Nanodrop-2000. Then, Cas12a (100 nM) and crRNA (200 nM) were mixed in 1×NEBuffer 2.1 and 1×Enhanced Buffer and incubated at room temperature for 10 min, respectively. Afterwards, 10 nM cleavage target was added into the mixture and incubated at 37 °C for 10 min, 20 min and 30 min, respectively, followed by heat-inactivation at 75 °C for 10 minutes. Digested products were analyzed by agarose gel electrophoresis.

Definition of V_g ($V_g = \Delta F/\Delta t$)

In real-time fluorescence detection, the fluorescence growth rate was termed as V_g ($V_g = \Delta F/\Delta t$). Wherein the ΔF represents the change of the fluorescence intensity before reaching plateau and Δt is the time frame of 3 min.

Viral DNA detection from clinical serum samples

ASFV DNA preparation from swine serum samples. Swine serum samples were used as a source of the viral DNA templates and were collected from Huangpu District,

Guangzhou City, Guangdong Province, China. All the blood samples were inactivated by 60 °C for 30 min. ASFV DNA in clinical swine serum samples for droplet Cas12a assay were extracted by a heating and chemical reduction method, which can inactivate DNase/RNase in whole blood.[4] Briefly, 100 mM TCEP and 1 mM EDTA were added to swine serum samples. Then the samples were incubated at 50 °C for 5 min followed by heated at 64 °C for another 5 min. The supernatant was obtained by centrifugation (8000 rpm/min) for 5 min and then used for the following experiments.

Real-time quantitative PCR (qPCR) experiments for ASFV detection. QPCR for ASFV detection in swine serum samples was performed using the Diagnostic Kit for African Swine Fever Virus (ZHENGZHOU ZHONGDAO BIOTECHNOLOGY CO., LTD., Zhengzhou, China) according to the instruction manual. Briefly, 25 μL reaction contains 17 μL of PCR reaction premix, 3 μL of enzyme mixture, and 5 μL of DNA samples. The reaction was performed by a Thermal Cycler Dice Real Time System III (TaKaRa). Thermal cycling program: 95 °C for 3 min, followed by 40 cycles of amplification at 95 °C for 8 s, and 55 °C for 30 s. This kit sets a threshold for the detected Ct value: a Ct value ≤ 35 is positive, and a Ct value > 35 is negative.

Viral DNA preparation from human serum samples.

In this study, clinical serum EBV samples were collected from Zhongnan Hospital of Wuhan University (n=7) and Sun Yat-Sen University Cancer Center (n=10), clinical serum HBV samples (n=17) were collected from Zhongnan Hospital of Wuhan University. The HBV and EBV DNAs were extracted using the QIAamp DNA Blood Mini Kit according to the manufacturer's protocol.

qPCR experiments for EBV and HBV detection.

EBNA-1 was the target gene for detection of EBV by qPCR. And S gene was the target region for HBV detection by qPCR. EBV and HBV detection in human serum samples was performed using the TaKaRa TB Green Premix Ex TaqII (Tli RNaseH Plus) according to the instruction manual. Briefly, 20 μL qPCR reaction contained 10 μL of TB Green Premix Ex TaqII (Tli RNaseH Plus) (2×), 500 nM primer pairs (EBV-F2/R2, HBV-S-F/R), [3, 5, 6] and 2 μL of DNA samples. The reaction was performed by a Thermal

Cycler Dice Real Time System III (TaKaRa). The thermal cycling program began with a first stage 95 °C for 5 min, followed by 45 cycles of amplification at 95 °C for 5 s, 59 °C for 30 s, and a dissociation stage at 95 °C for 15 s. The sample was considered positive or negative by the criteria as follows: a Ct value ≤ 40 is positive, and a Ct value > 40 is negative.

Droplet Cas12a assay

The microfluidic chip was prepared according to our previous report. [7] Briefly, after filling the microfluidic chip with oil phase, a single-use plastic syringe is connected to the out inlets of the chip with a length of Teflon tubing. Meanwhile, the Cas12a assay mix (200 nM Cas12a was complexed with 500 nM crRNA1, 500 nM crRNA2, 500 nM FQ6C and target DNA in 1× Enhanced Buffer) was prepared on the Eppendorf PCR-Cooler. Subsequently, 4 μL aliquot of Cas12a assay mix loaded into the sample inlet with a micropipette. Immediately, pulling outward the piston of the syringe and locked in place with one-milliliter volume using a binder clip, droplets were generated using negative pressure. After the imaging chamber is filled with the droplets, the binder clip is removed and the piston is gently withdrew its starting position. And the tubing is cut off and the out inlets are closed with oil phase. After isothermal reaction for 60 min, the microfluidic chip is imaged with an OlympusIX-71 inverted microscope using 10 × magnification objective, to record the bright field and fluorescence images of the droplets. The digital quantification of the droplet Cas12a assay was consistent with our previous report about the droplet Cas13a assay. The signal-to-background ratio (SBR) represented the ratio of the fluorescence intensity of the positive droplet to the negative droplet. The fluorescence intensity of the droplet was measured by Image J.

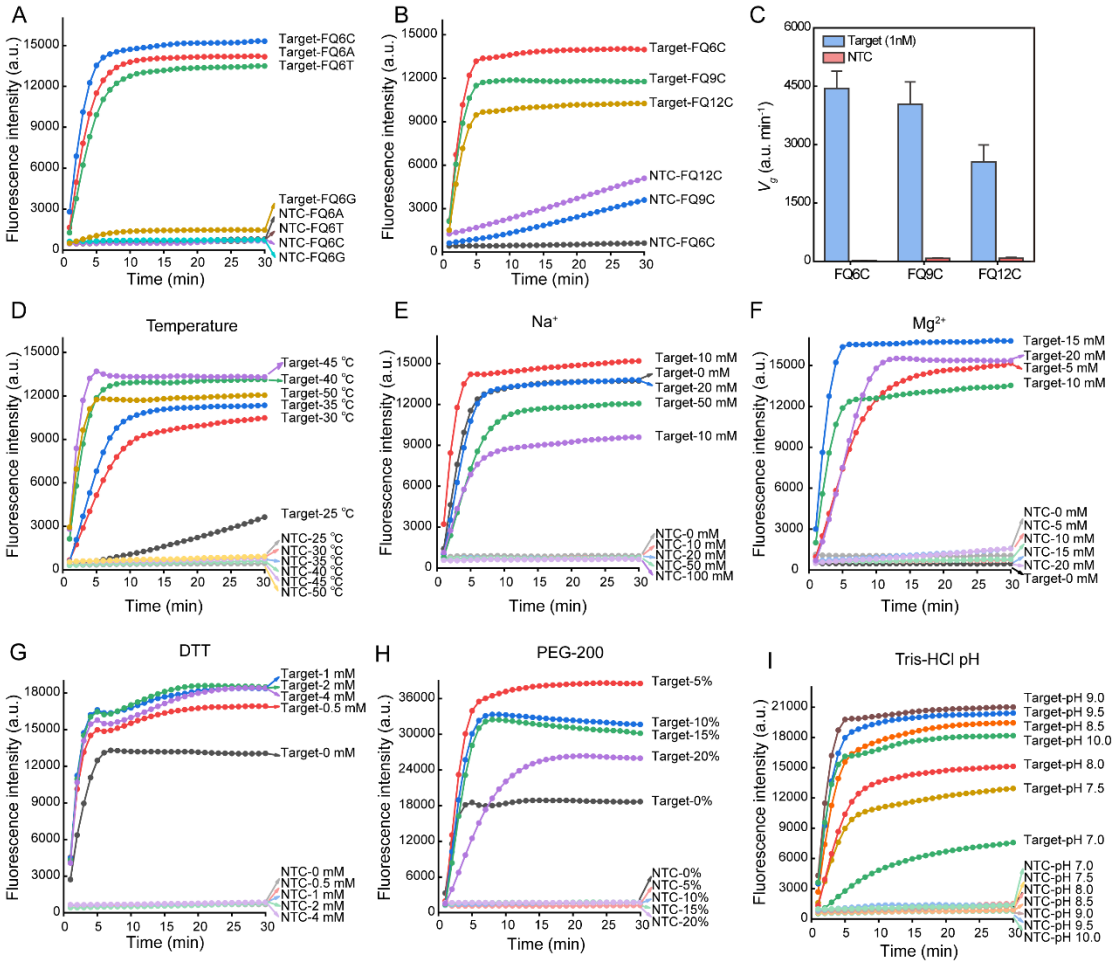

Figure S1 Optimization of the CRISPR-Cas12a detection system. (A) Real-time fluorescent kinetic measurements of Cas12a assay with various reporter probes with different base compositions. (B) Real-time fluorescent kinetic measurements of Cas12a assay with C-rich based reporters with varied base lengths. (C) Comparison of Cas12a assay efficiency using FQ6A, FQ6T, FQ6C, FQ6G. Real-time fluorescent kinetic measurements of the *trans*-cleavage activity of Cas12a with various reaction temperatures (D), Na^+ concentration (E), Mg^{2+} concentration (F), DTT concentration (G), PEG-200 concentration (H), and buffer pH (I).

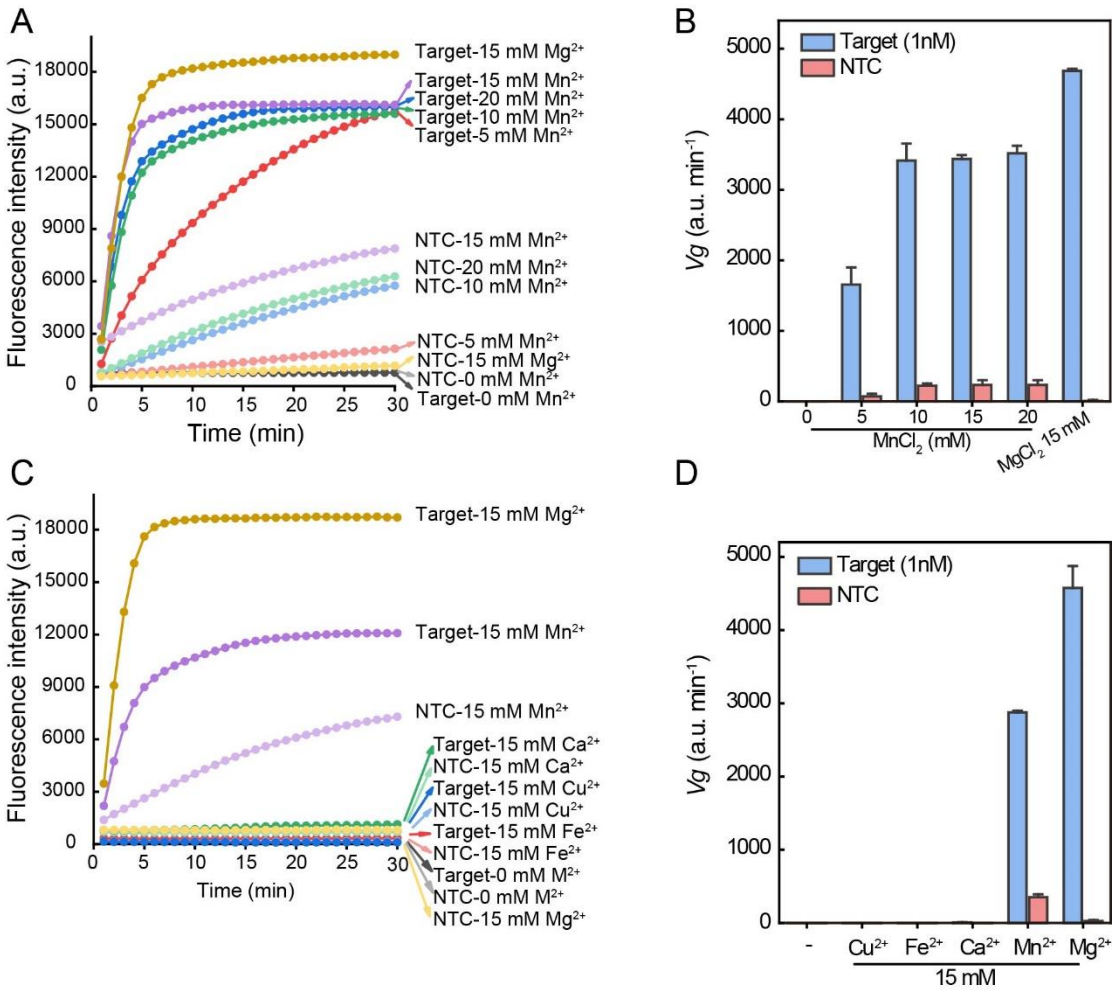

Figure S2 Evaluation of the effect of divalent cation on Cas12a assay efficiency. (A) Real-time fluorescent kinetic measurements of the *trans*-cleavage activity of Cas12a with various concentrations of Mn^{2+} and 15 mM Mg^{2+}. (B) Comparison of the Cas12a assay efficiency under the various concentrations of Mn^{2+} (0, 5, 10, 15, 20 mM) and 15 mM Mg^{2+}. (C) Real-time fluorescent kinetic measurements of the *trans*-cleavage activity of Cas12a with various divalent cation (15 mM Ca^{2+}, Fe^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}). (D) Comparison of the Cas12a assay efficiency under the various divalent cations (15 mM Ca^{2+}, Fe^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}).

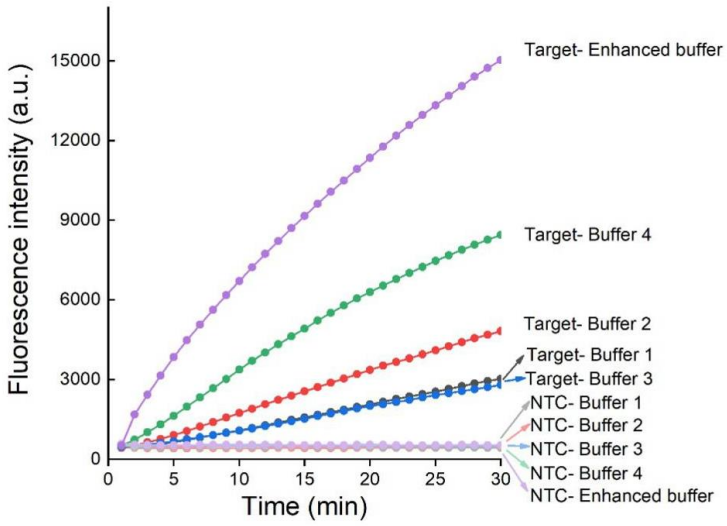

Figure S3 Real-time fluorescent kinetic measurements of the *trans*-cleavage activity of Cas12a with various buffers.

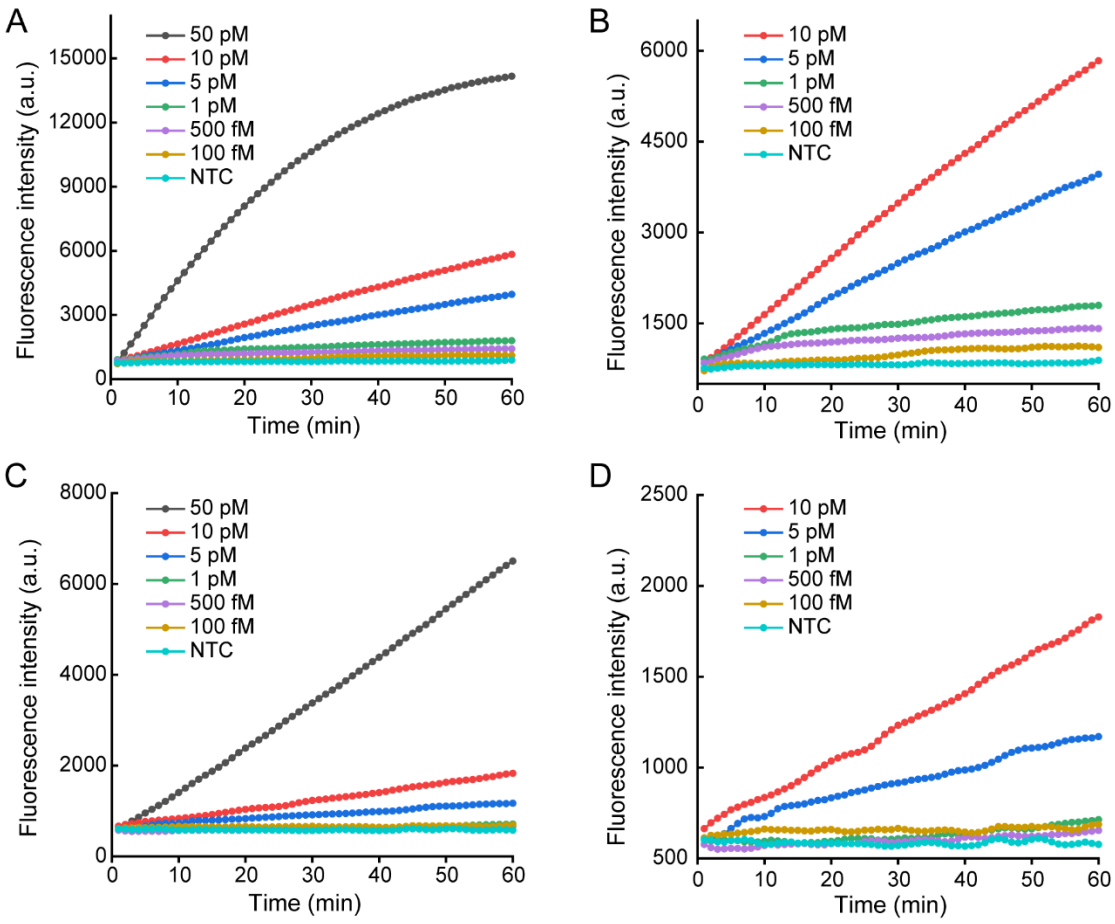

Figure S4 Evaluation of the sensitivity of the Cas12a assay before and after optimization. (A) The sensitivity performance of the Cas12a assay in combination of the enhanced buffer ,45 °C reaction and C6 reporter. 50 pM, 10 pM, 5 pM, 1 pM, 500 fM, and 100 fM of ASFV DNA were tested and NTC represents no target DNA control. (B) An enlarged view of the curves at the low concentrations of 10 pM, 5 pM, 1 pM, 500 fM, and 100 fM of (A). (C) The sensitivity performance of the Cas12a assay in combination of buffer 4, 37 °C reaction and T6 reporter. 50 pM, 10 pM, 5 pM, 1 pM, 500 fM, and 100 fM of ASFV DNA were tested and NTC represents no target DNA control. (D) An enlarged view of the curves at the low concentrations of 10 pM, 5 pM, 1 pM, 500 fM, and 100 fM of (C).

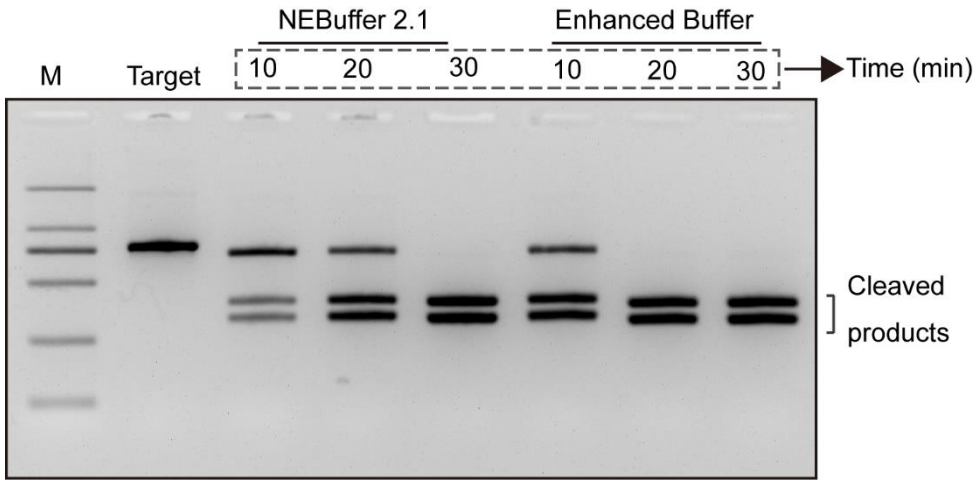

Figure S5 Comparison of the *cis*-cleavage activity of Cas12a assay in NEBuffer 2.1 and Enhanced Buffer.

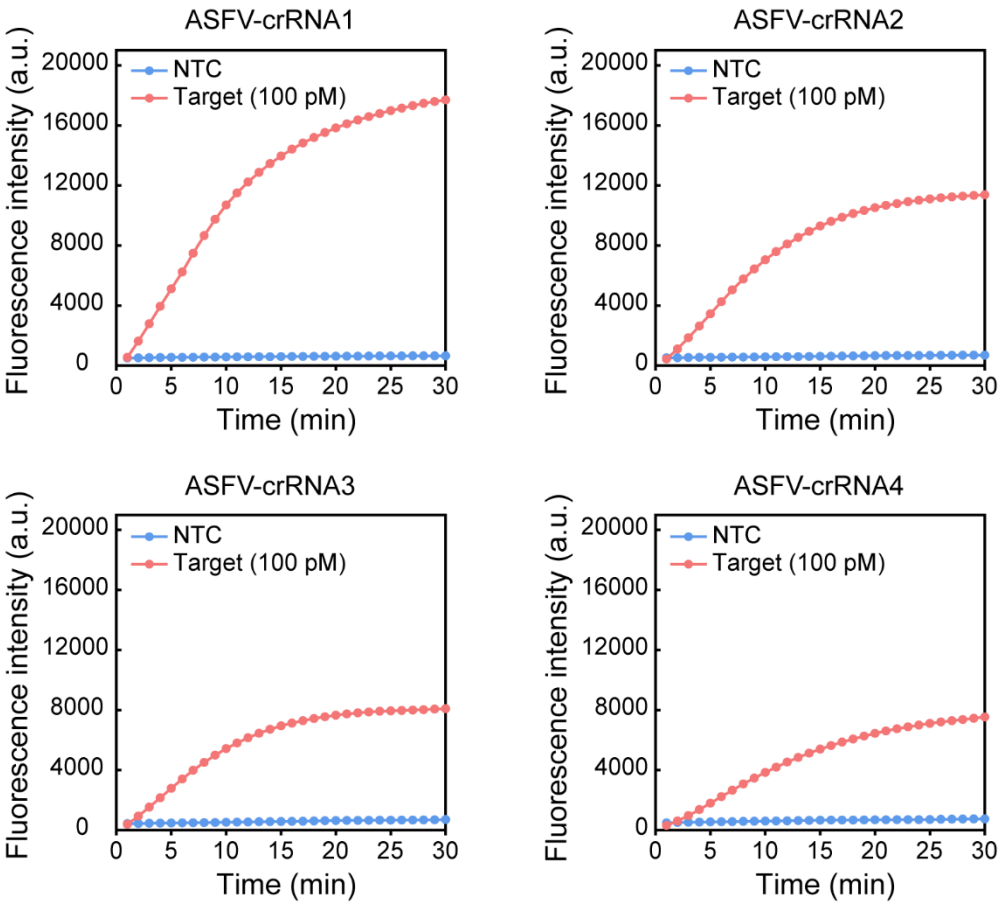

Figure S6 Real-time fluorescent kinetic measurements of the *trans*-cleavage activity of Cas12a with ASFV-crRNA1, ASFV-crRNA2, ASFV-crRNA3, and ASFV-crRNA4 for targeting B646L gene of ASFV.

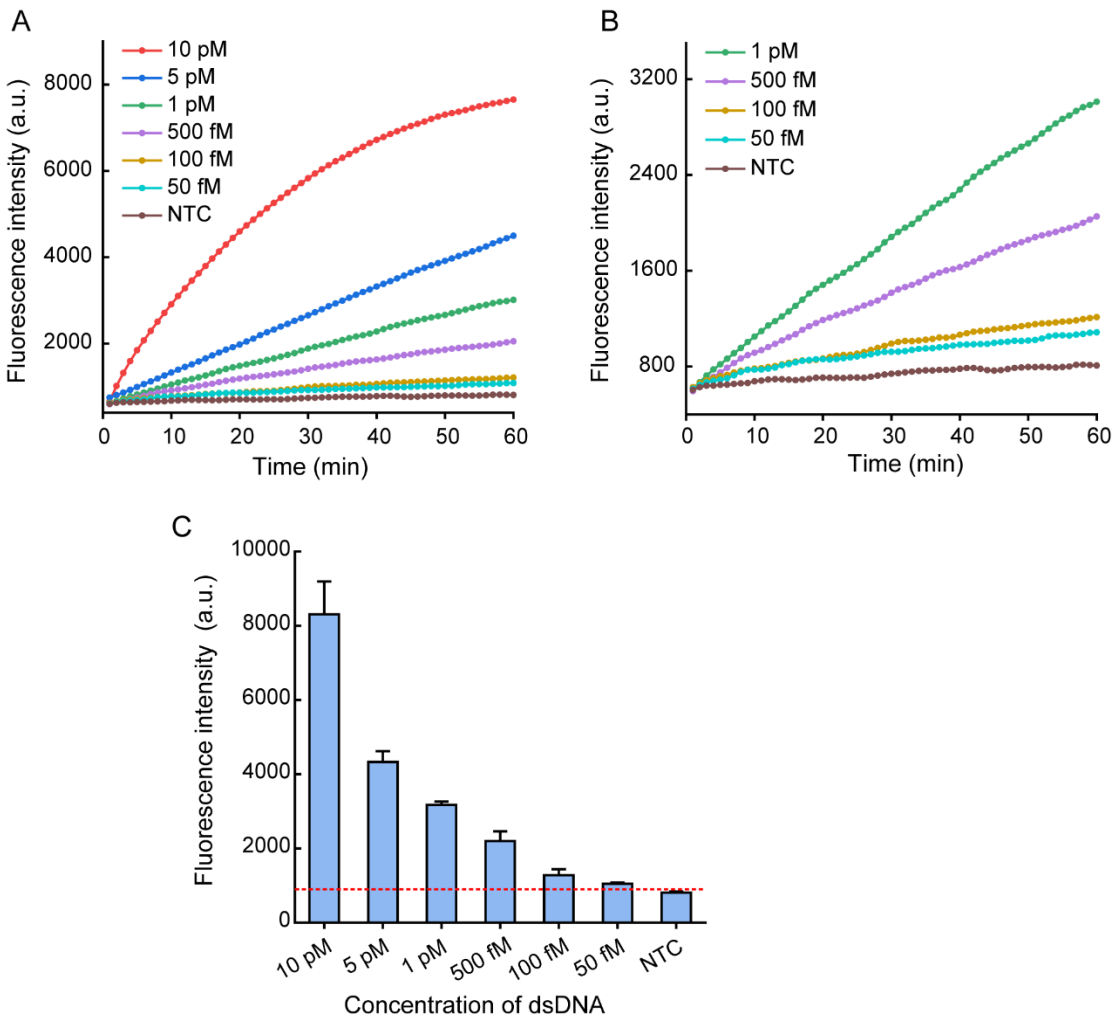

Figure S7 Real-time fluorescent kinetic measurements of Cas12a assay with dual-crRNA targeted strategy. (A) The sensitivity performances of the Cas12a assay with dual crRNAs in combination of the enhanced buffer and 45 °C reaction and C6 reporter. 10 pM, 5 pM, 1 pM, 500 fM, 100 fM and 50 fM of ASFV DNA were used and NTC represents no target DNA control. (B) An enlarged view of the curves at the low concentrations of 1 pM, 500 fM, 100 fM and 50 fM in A. (C) Endpoint fluorescence signals of (A) shows the limit of detection of dual-crRNA-mediated Cas12a assay. Data are represented as mean ± SD of three technical replicates. The red dotted line represents the detection limit, which was determined as mean of NTC plus standard error × 3.

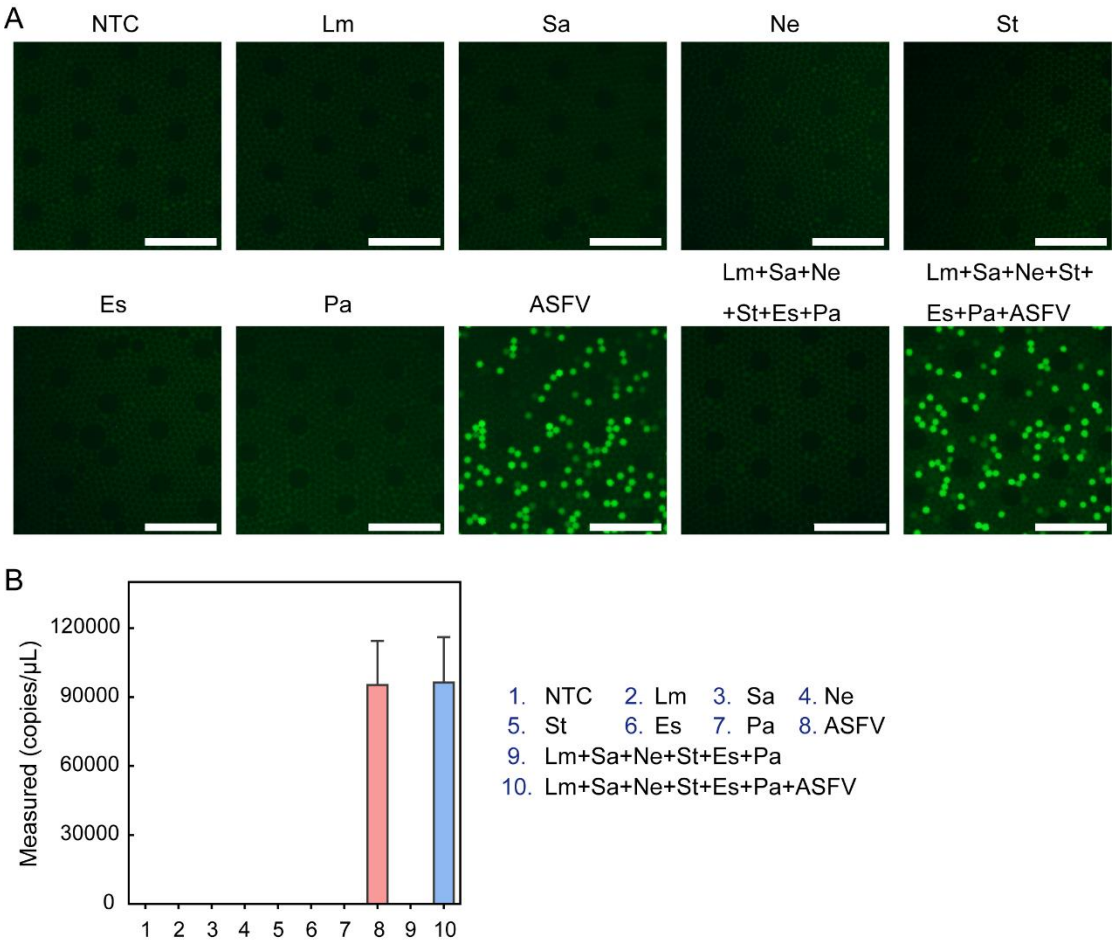

Figure S8 Verification of the specificity of the droplet Cas12a assay. (A) Representative end-point fluorescence images from the droplet Cas12a assay for analyzing Lm, Sa, Ne, St, Es, Pa, ASFV genome DNA, six bacterial DNA mix, and six bacterial DNA+ASFV genome DNA mix by ASFV specific dual-crRNA targeted droplet Cas12a assay. (B) Counting of the DNA copy numbers in Lm, Sa, Ne, St, Es, Pa, ASFV DNA, six bacterial DNA mix, and six bacterial DNA+ASFV genome DNA mix by ASFV-specific dual-crRNA targeting droplet Cas12a assay. Data are represented as mean ± standard error of the difference between means of three technical replicates.

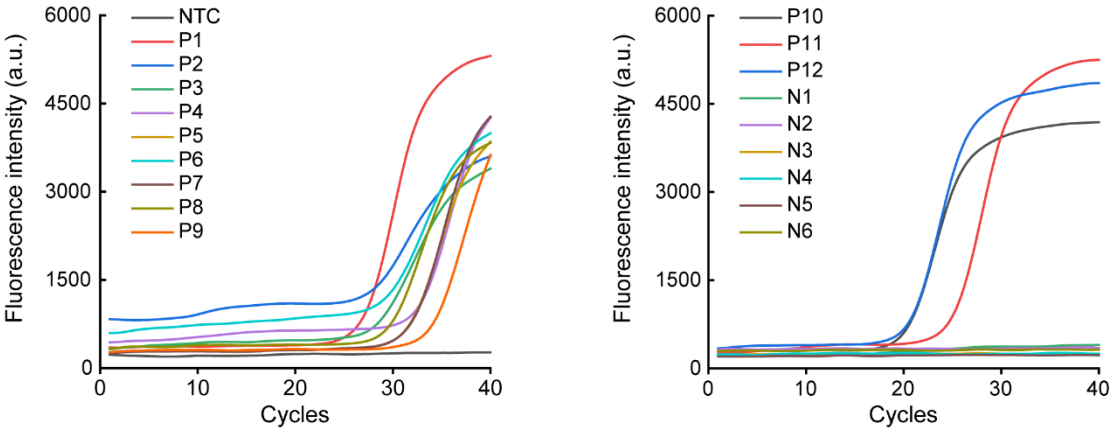

Figure S9 qPCR detection of 12 clinical ASFV-infected positive swine serum samples and 6 negative samples.

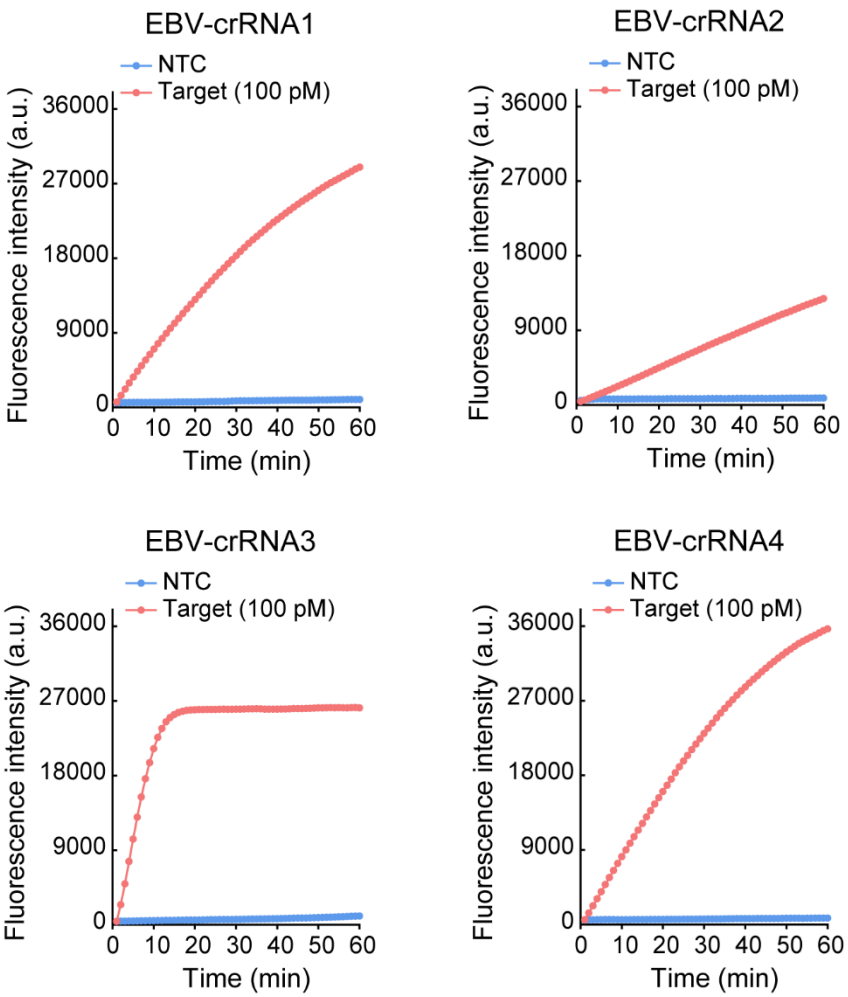

Figure S10 Real-time fluorescent kinetic measurements of the *trans*-cleavage activity of Cas12a with EBV-crRNA1, EBV-crRNA2, EBV-crRNA3 and EBV-crRNA4 for targeting EBNA-1 gene of EBV.

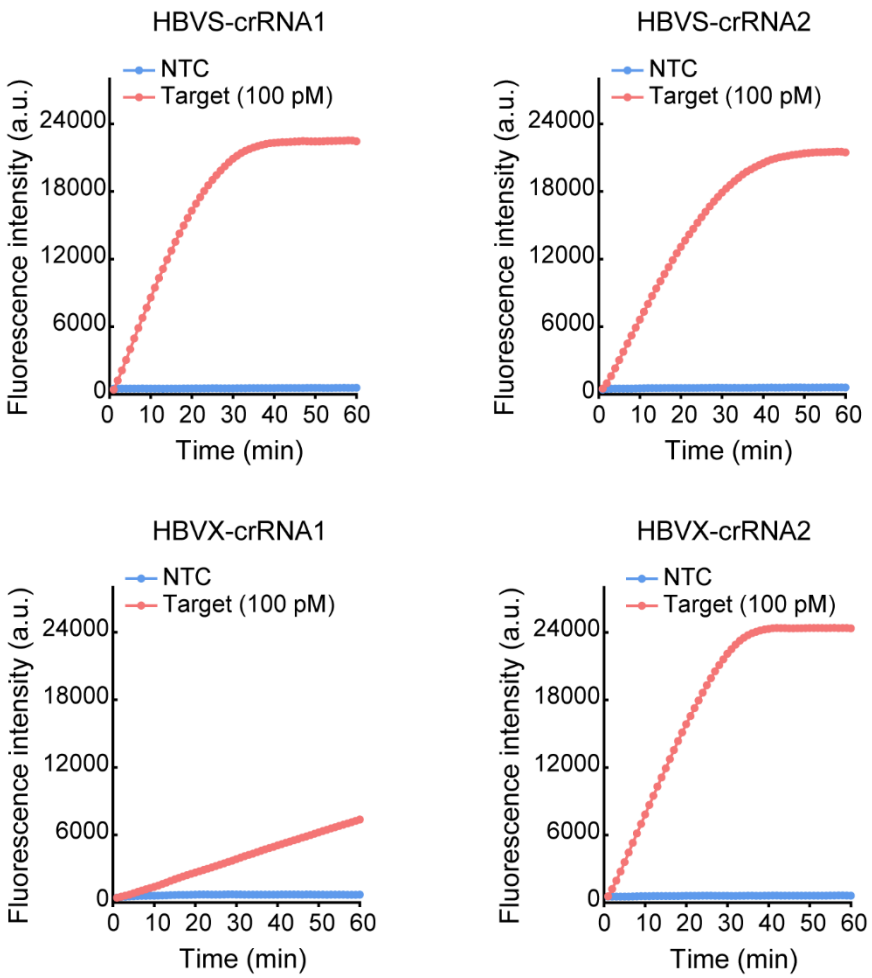

Figure S11 Real-time fluorescent kinetic measurements of the *trans*-cleavage activity of Cas12a with HBV-S-crRNA1 and HBV-S-crRNA2 for targeting S gene, and HBV-X-crRNA1 and HBV-X-crRNA2 for targeting X gene of HBV.

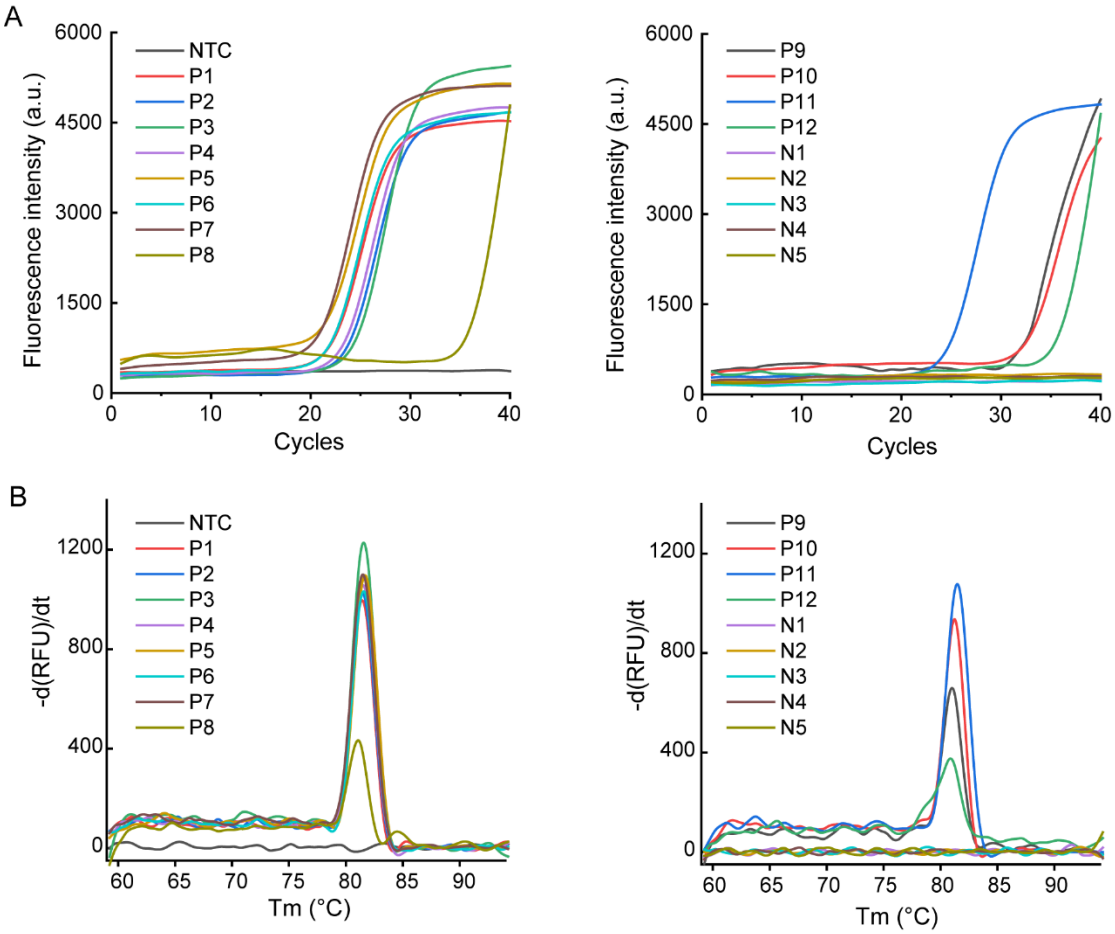

Figure S12 qPCR detection of 12 clinical EBV-infected positive samples and 5 negative samples. (A) Real-time fluorescent curve analysis. (B) Melting curve analysis.

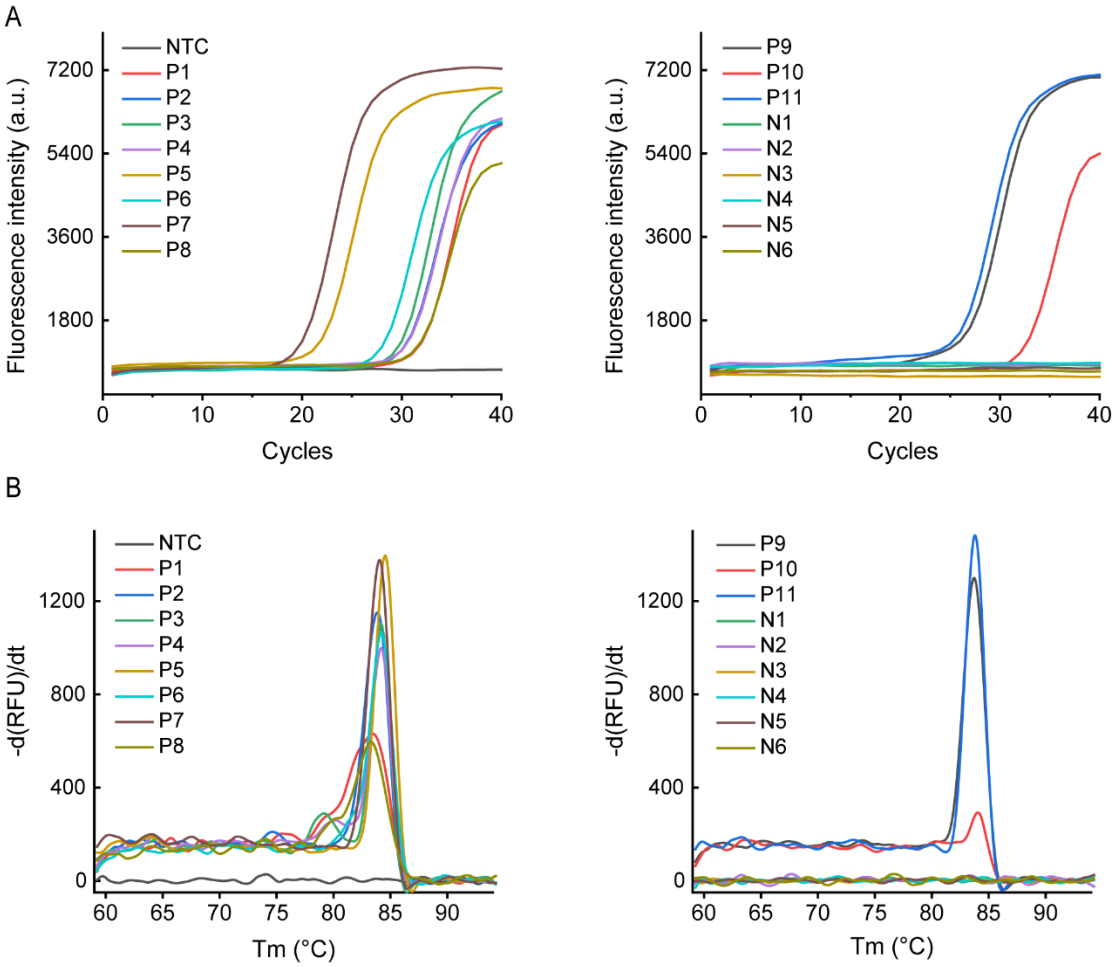

Figure S13 qPCR detection of 11 clinical HBV-infected positive samples and 6 negative samples. (A) Real-time fluorescent curve analysis. (B) Melting curve analysis.

Table S1 Sequences used in this work.

Nucleic Acid ID	Sequences (5'-3')
Target DNA-F	CTGATAGTATTTAGGGGTTTGAGGTCCATTACAGCTGTAATGAAC
Target DNA-R	GTTCATTACAGCTGTAATGGACCTCAAACCCCTAAATACTATCAG
ASFV-F1	GCCGAAGGGAATGGATACTGAGGGAATAGCAA
ASFV-R1	TCCCGAGAACTCTCACAATATCCAAACAGCAG
ASFV-F2	GGACACGTATCAGAGAAAATCG
ASFV-R2	CTTGTGCGCAAATTTTGTATC
ASFV-crRNA1	UAAUUUCUACUAAGUGUAGAU**GGGGUUUGAGGUCCAUUACA**
ASFV-crRNA2	UAAUUUCUACUAAGUGUAGAU**CCUGCUGUUUGGAUAUUGUG**
ASFV-crRNA3	UAAUUUCUACUAAGUGUAGAU**ACGAGAACGUGAACCUUGCU**
ASFV-crRNA4	UAAUUUCUACUAAGUGUAGAU**CGUUCUCGUUAAACCAAAAG**
EBV-F1	GTAGAAGGCCATTTTTCCAC
EBV-R1	CTCCATCGTCAAAGCTGCA
EBV-F2	CCGGTGTGTTCGTATATGGAG
EBV-R2	GGGAGACGACTCAATGGTGTA
EBV-crRNA1	UAAUUUCUACUAAGUGUAGAU**AAUACCACCAAGAAGGUGGC**
EBV-crRNA2	UAAUUUCUACUAAGUGUAGAU**GAAAGCAUCGUGGUCAAGGA**
EBV-crRNA3	UAAUUUCUACUAAGUGUAGAU**CAACCUAAGGCGAGGAACUG**
EBV-crRNA4	UAAUUUCUACUAAGUGUAGAU**CUGAGGUUUUGAAGGAUGCG**
HBV-S-F	CTTCATCCTGCTGCTATGCCT
HBV-S-R	AAAGCCCAGGATGATGGGAT
HBV-X-F	ACGTCCTTTGTTTACGTCCCGT
HBV-X-R	CCCAACTCCTCCCAGTCCTTAA
HBV-S-crRNA1	UAAUUUCUACUAAGUGUAGAU**UCCUCUAAUUCCAGGAUCCU**
HBV-S-crRNA2	UAAUUUCUACUAAGUGUAGAU**CGUCCGAAGGUUUGGUACAG**
HBV-X-crRNA1	UAAUUUCUACUAAGUGUAGAU**CGCGGACUCCCCGUCUGUGC**
HBV-X-crRNA2	UAAUUUCUACUAAGUGUAGAU**GUGGGCGUUCACGGUGGUCU**
FQ6A	FAM-AAAAAA-BHQ1
FQ6T	FAM-TTTTTT-BHQ1
FQ6C	FAM-CCCCCC-BHQ1
FQ6G	FAM-GGGGGG-BHQ1
FQ9C	FAM-CCCCCCCCC-BHQ1
FQ12C	FAM-CCCCCCCCCCCC-BHQ1

Table S2 Buffers used in this work.

	Component
Buffer-Na^+	10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT
Buffer-M^{2+}	10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 1 mM DTT
Buffer-DTT	10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 15 mM $MgCl_2$
Buffer-Tris-HCl	10 mM NaCl, 15 mM $MgCl_2$, 1 mM DTT
Buffer-PEG	10 mM Tris-HCl (pH 9.0), 10 mM NaCl, 15 mM $MgCl_2$, 1 mM DTT
Enhanced Buffer	10 mM Tris-HCl (pH 9.0), 10 mM NaCl, 15 mM $MgCl_2$, 1 mM DTT, 5% PEG-200
Buffer 1	20 mM HEPES (pH 7.5), 150 mM KCl, 10 mM $MgCl_2$, 1% glycerin,0.5 mM DTT
Buffer 2	10 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$,100 μg/mL BSA
Buffer 3	20 mM HEPES (pH 6.8), 60 mM NaCl, 6 mM $MgCl_2$
Buffer 4	10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$,100 μg/mL BSA

Notes:

Buffer-Na^+ indicates that this buffer is used as a basic buffer to optimize sodium concentration.

Buffer-M^{2+} indicates that this buffer is used as a basic buffer to optimize divalent cation concentration.

Buffer-DTT indicates that this buffer is used as a basic buffer to optimize DTT concentration.

Buffer-Tris-HCl indicates that this buffer is used as a basic buffer to optimize pH of Tris-HCl.

Buffer-PEG indicates that this buffer is used as a basic buffer to optimize PEG concentration.

Table S3 Ct values of qPCR for viral DNA detection from real-world samples.

ASFV	NTC	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	N1	N2	N3	N4	N5	N6
Ct	-	27.68	28.62	28.8	33.3	32.22	30.14	32.63	30.23	34.52	20.88	25.68	21.22	-	-	-	-	-	-
EBV	NTC	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	N1	N2	N3	N4	N5	
Ct	-	22.71	24.19	25.08	23.85	22.23	22.48	21.63	37	31.63	33.33	25.24	36.99	-	-	-	-	-	
HBV	NTC	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	N1	N2	N3	N4	N5	N6	
Ct	-	32.72	30.55	31.1	31.27	22.75	28.69	20.82	32.42	27.71	33.13	23.99	-	-	-	-	-	-	

REFERENCES

1. Chen, J. S.; Ma, E.; Harrington, L. B.; Da Costa, M.; Tian, X.; Palefsky, J. M.; Doudna, J. A., CRISPR-Cas12a Target Binding Unleashes Indiscriminate Single-Stranded DNase Activity. *Science* **2018,** *360*, 436-439.

2. Cheung, R. K.; Miyazaki, I.; Dosch, H.-M., Unexpected Patterns of Epstein-Barr Virus Gene Expression During Early Stages of B Cell Transformation. *Int. Immunol.* **1993**, *5*, 707-716.

3. Abe, A.; Inoue, K.; Tanaka, T.; Kato, J.; Kajiyama, N.; Kawaguchi, R.; Tanaka, S.; Yoshiba, M.; Kohara, M., Quantitation of Hepatitis B Virus Genomic DNA by Real-Time Detection PCR. *J. Clin. Microbiol.* **1999**, *37*, 2899-2903.

4. Myhrvold, C.; Freije, C. A.; Gootenberg, J. S.; Abudayyeh, O. O.; Metsky, H. C.; Durbin, A. F.; Kellner, M. J.; Tan, A. L.; Paul, L. M.; Parham, L. A.; Garcia, K. F.; Barnes, K. G.; Chak, B.; Mondini, A.; Nogueira, M. L.; Isern, S.; Michael, S. F.; Lorenzana, I.; Yozwiak, N. L.; MacInnis, B. L.; Bosch, I.; Gehrke, L.; Zhang, F.; Sabeti, P. C., Field-deployable Viral Diagnostics Using CRISPR-Cas13. *Science* **2018,** *360*, 444-448.

5. Gan, Y.-J.; Razzouk, B. I.; Su, T.; Sixbey, J. W., A Defective, Rearranged Epstein-Barr Virus Genome in EBER-Negative and EBER-Positive Hodgkin's Disease. *Am. J. of Pathol.* **2002**, *160*, 781-786.

6. Zhou, Y.; Attygalle, A. D.; Chuang, S.-S.; Diss, T.; Ye, H.; Liu, H.; Hamoudi, R. A.; Munson, P.; Bacon, C. M.; Dogan, A.; Du, M.-Q., Angioimmunoblastic T-Cell Lymphoma: Histological Progression Associates with EBV and HHV6B Viral Load. *Brit. J. Haematol.* **2007**, *138*, 44-53.

7. Tian, T.; Shu, B.; Jiang, Y.; Ye, M.; Liu, L.; Guo, Z.; Han, Z.; Wang, Z.; Zhou, X., An Ultralocalized Cas13a Assay Enables Universal and Nucleic Acid Amplification-Free Single-Molecule RNA Diagnostics. *ACS Nano* **2021**, 15, 1167-1178.