Electronic Supplementary Information

Colorimetric detection of RNA fragments based on associated toehold-mediated reaction and gold nanoparticles

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Experimental Section

Materials and reagents. Oligonucleotides (Table S1) were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China). Agarose and 4-(2-hydroxyerhyl)piperazine-1-erhanesulfonic acid (HEPES) were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Tris base, 40% acrylamide/bisacrylamide (19:1) solution, ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), ethylenediaminetetraacetic acid tetrasodium (EDTA), boric acid were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). SYBR gold nucleic acid gel stain was bought from Thermo Fisher Scientific. Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$) and sodium citrate were purchased from Sigma-Alrich (St. Louis, MO, USA). All other reagents were of analytical grade and used as received. Ultrapure water (18.25 MΩ•cm) obtained from a UP water purification system was used throughout the experiment. 500 bp DNA marker was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). 50 bp DNA ladder was purchased from Takara Biomedical Technology (Beijing) Co., Ltd. Sequences of ORF1ab and N genes were synthesized and constructed on pUC57 vector to form recombinant plasmid by Sangon Biotech Co., Ltd. (Shanghai, China). The recombinant plasmid, with a strand length over 2900 bases containing ORF1ab and N sequences, was dissolved in 1 × TE buffer and stored at −80 °C until further use. The ORF1ab sequence embedded in the recombinant plasmid is 5'-CTACAGCCATAACCTTTCCACATACCGCAGACGGTACAGACTGTGTTTTTA

AGTGTAAAACCCACAGG-3', and the N sequence embedded in the recombinant plasmid is 5'- GTTGTTGTTGGCCTTTACCAGACATTTTGCTCTCAAGCTGGTT CAATCTGTCAAGCAGCAGCAAAGCAAGAGCAGCATCACCGCCATTGCCAG CCATTCTAGCAGGAGAACTTCCCC-3'. T7 exonuclease was purchased from New England Biolabs Ltd. (Ipswich, MA, USA). TwistAmp Basic Kit was bought from TwistDX Inc. (England).

Table S1. Sequences of oligonucleotides used in this study.[a]

Name	Sequence (5' to 3')
ORF1ab	*CCAUAACCUUUC*CACAUACCGCAGACGG
N	CAUUGCCAGCCAUUCU*AGCAGGAGAAGU*
Connector	CCGTCTGCGGTATGTGAGAATGGCTGGCAATG
Initiator	CCATAACCTTTCAGCAGGAGAACT
H1[13]	AGTTCTCCTGCTGAAAGGTTATGGTATCAAAGCGTCCATAA CCTTTCA
H2[13]	CCATAACCTTTCAGCAGGAGAACTTGAAAGGTTATGGACG CTTTGATA
H1[15]	AGTTCTCCTGCTGAAAGGTTATGGCCCTAACTACCATAACC TTTCAGC
H2[15]	CCATAACCTTTCAGCAGGAGAACTGCTGAAAGGTTATGGT AGTTAGGG
H1[17]	AGTTCTCCTGCTGAAAGGTTATGGTCAATTCCCATAACCTT TCAGCAG
H2[17]	CCATAACCTTTCAGCAGGAGAACTCTGCTGAAAGGTTATGG GAATTGA
ORF1ab-Mismatched	CCAUAACCUUUCUACAUACCGCAGACGG
ORF1ab-Deleted	CCAUAACCUUUC_ACAUACCGCAGACGG
ORF1ab-Inserted	CCAUAACCUUUCCUACAUACCGCAGACGG
F-primer	T*T*T*T*T*T*TGTTGTTGTTGGCCTTTACCAGACATTTTGCT CT
R-primer	CCTGTGGGTTTTACACTTAAAAACACAGTCTGTA

[a] The two parts of associated toehold are italicized in ORF1ab and N sequences. The complementary bases between ORF1ab and connector are marked in light blue, while

the complementary bases between N and connector are marked in orange. The superscripts 13, 15 17 represents different stem lengths of H1 and H2 hairpins. All the complementary bases between H1 and H2 are marked with same color. The stem regions are all underlined. The mismatched base in ORF1ab is marked in purple. The deleted base in ORF1ab is marked with dash. The inserted base in ORF1ab is marked in purple and the original base is marked bold. "*" denotes phosphorothioate bonds for exonuclease protection.

Instrumentation. UV–vis absorption spectra were recorded on a TU-1901 spectrometer (Persee, China). Stained polyacrylamide gel was imaged on GelDocTM XR^+ imaging system (Bio-RAD Laboratories Inc. USA). Dynamic light scattering (DLS) measurements were performed on a Zetasizer Nano ZS (Malvern, UK). 50 μL of 3 nM AuNPs was transferred to 50-μL disposable cuvettes (Sarstedt, Germany) for measurements. Transmission electron microscopy (TEM) measurements were made on a JSM-6700F transmission electron microscope operated at an accelerating voltage of 200 kV (JEOL, Japan). The sample for TEM characterization was prepared by adding a drop of colloidal solution on a carbon-coated copper grid and then drying.

Synthesis of AuNPs. Gold nanoparticles (13 nm diameter) were synthesized by reduction of $HAuCl_4$ by sodium citrate.[1] Briefly, a solution of sodium citrate (10 mL, 38.8 mM) was rapidly added to a vigorously stirred boiling aqueous solution of $HAuCl_4$ (100 mL, 1 mM). After a continuous boiling for 10 min, the mixed solution was stirred for additional 15 min. The solution was then cooled to room temperature, filtered through a 0.22 μm filter. The synthesized gold nanoparticles were aliquoted in 2 mL centrifuge tubes (Axygen, U.S.) and stored at 4 °C in the refrigerator before use. The concentration of synthesized AuNPs was determined according to the absorbance at 520 nm and the corresponding molar extinction coefficient.[2]

Agarose gel electrophoresis. 2% agarose gel was prepared by dissolving 0.6 g agarose in 30 mL 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) through microwave heating for about 40 s. The gel was injected into gel tray and cooled down at room temperature for 30 min before use. 6 μL of DNA samples were mixed with 4 μL of HEPES buffer (10 mM HEPES, 300 mM NaCl, pH 7.0) and 2 μL

of 6× loading buffer, and 5 μL of the mixture was loaded into the well of the gel. Agarose gels were run at 100 V for 50 min. The gel was then stained with 1× SYBR gold for 40 min and photographed with the fluorescence imaging system.

Polyacrylamide gel electrophoresis. 10% polyacrylamide gel was prepared by mixing 6.25 mL of 40% acrylamide/bisacrylamide solution (19:1), 5 mL of 5× Tris-borate-EDTA (TBE) buffer, 13.75 mL of deionized water, 180 μL of 0.1 g/mL APS and 18 μL of TEMED. 6 μL of DNA samples were mixed with 4 μL of HEPES buffer (10 mM HEPES, 300 mM NaCl, pH 7.0) and 2 μL of 6× loading buffer, and 2 μL of the mixture was loaded into the well of the gel. Electrophoresis was run at 150 V for 5 h in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0), followed by gel staining and imaging.

Investigation of reaction condition. Concentrated DNA stock solutions were prepared in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and diluted to reaction concentration by HEPES buffer (10 mM HEPES, 300 mM NaCl, pH 7.0). H1 and H2 were heated at 95 °C for 2 min and cooled down slowly to room temperature. The annealed H1 and H2 were stored at 4 °C for further use.

To optimize the hairpin stem, annealed H1 and H2 (400 nM) with stem lengths of 13, 15, 17 base pairs were respectively mixed with connector (100 nM), ORF1ab and N (100 nM) in HEPES buffer, and the reaction was allowed to perform at 37 °C for 3 h. Control experiments were performed by incubating H1 and H2 under the same condition. The samples were loaded on agarose gel.

To confirm the formation of DNA triplex, equal concentration of connector, ORF1ab and N (100 nM) were mixed in HEPES buffer and incubated for 30 min at room temperature, followed by polyacrylamide gel characterization.

To compare the triggering ability of associated toehold and intact initiator, annealed H1 and H2 (400 nM) with stem lengths of 15, 17 base pairs were respectively reacted with DNA triplex or initiator (100 nM) at 37 °C for 3 h, followed by agarose gel characterization.

To optimize the reaction time, annealed $H1^{15}$ and $H2^{15}$ (400 nM) were mixed with connector (100 nM), ORF1ab and N (100 nM) in HEPES buffer and incubated at 37

°C for 1 h, 2 h, 3 h and 4 h, followed by agarose gel characterization.

To optimize the reaction pH, annealed $H1^{15}$ and $H2^{15}$ (400 nM) were mixed with connector (100 nM), ORF1ab and N (100 nM) in HEPES buffer (10 mM HEPES, 300 mM NaCl) with various pH of 5.0, 6.0, 7.0, 8.0 and 9.0, and incubated at room temperature or 37 °C for 3 h, followed by agarose gel characterization.

To optimize the reaction ionic strength, annealed $H1^{15}$ and $H2^{15}$ (400 nM) were mixed with connector (100 nM), ORF1ab and N (100 nM) in HEPES buffer (10 mM HEPES, pH 7.0) with NaCl concentrations of 150 mM, 300 mM and 500 mM, and incubated at room temperature or 37 °C for 3 h, followed by agarose gel characterization.

To optimize the reaction temperature, annealed $H1^{15}$ and $H2^{15}$ (400 nM) were mixed with connector (100 nM), ORF1ab and N (100 nM) in HEPES buffer (10 mM HEPES, 300 mM NaCl, pH 7.0) and incubated at room temperature or 37 °C for 3 h, followed by agarose gel characterization.

To investigate the polymerized products along with the increasing concentration of ORF1ab and N, the reaction was performed by mixing annealed $H1^{15}$ and $H2^{15}$ (400 nM), connector (100 nM) with different concentrations of ORF1ab and N (0 nM, 60 nM, 80 nM and 100 nM) at 37 °C for 3 h, followed by agarose gel characterization.

To test HCR reaction triggered by different existence of viral gene fragments, annealed $H1^{15}$ and $H2^{15}$ (400 nM), connector (100 nM) were respectively mixed with HEPES buffer, ORF1ab (100 nM), N (100 nM), and ORF1ab and N (100 nM). After the reaction at 37 °C for 3 h, the samples were loaded on agarose gel.

To optimize the final salt concentration that induces a color change between the absence of RNA sequences and the presence of RNA sequences, a final concentration of 30 mM, 40 mM, 50 mM, 60 mM and 70 mM NaCl were tested, followed by measurement of absorption spectra.

Colorimetric detection of viral gene fragments. Annealed $H1^{15}$ and $H2^{15}$ (400 nM), connector (100 nM) were mixed with different concentrations of ORF1ab and N, and the mixture was incubated at 37 °C for 3 h. 4 μL of above mixture was added into 44 μL of 3 nM AuNPs solution. After an incubation time of 5 min, a final concentration

of 50 mM NaCl was introduced, followed by UV-vis spectra measurements or visual observation. For testing responses to single viral gene fragment or single-base mutated sequence, the procedures are performed as mentioned above.

Colorimetric detection of long recombinant plasmid containing ORF1ab and N sequences. Annealed $H1^{15}$ and $H2^{15}$ (400 nM), connector (100 nM) were mixed with 20 nM recombinant plasmid or pUC57 plasmid, and the mixture was incubated at 37 °C for 3 h. The above mixture was mixed with 3 nM AuNPs, and after an incubation time of 5 min, a final concentration of 50 mM NaCl was introduced, followed by UV-vis spectra measurement. For the upstream amplification of sample, a mixture of 2.4 μL of 10 μM primers, 29.5 μL TwistAmp Basic RPA rehydration buffer, 8.2 μL DNase/RNase-free water, and 5 μL of 200 copies/mL recombinant plasmid were vortexed briefly and added to the TwistAmp lyophilized powder mix. After addition of 2.5 μL of 280 mM magnesium acetate to the reaction tube, the obtained 50 μL mixture was incubated at 37 °C for 30 min. 5 μL above recombinase polymerase amplification (RPA) product was added to the mixture of 5 μL NEB buffer, 2 μL of 10 units/μL T7 exonuclease and 29 μL DNase/RNase-free water, followed by addition of 7.5 μL of 10% SDS. The above solution was mixed well and incubated at room temperature for 10 min, and then 15 mM EDTA was added to terminate the reaction. The pretreated sample was then subjected to colorimetric detection as described above.

Additional Figures.

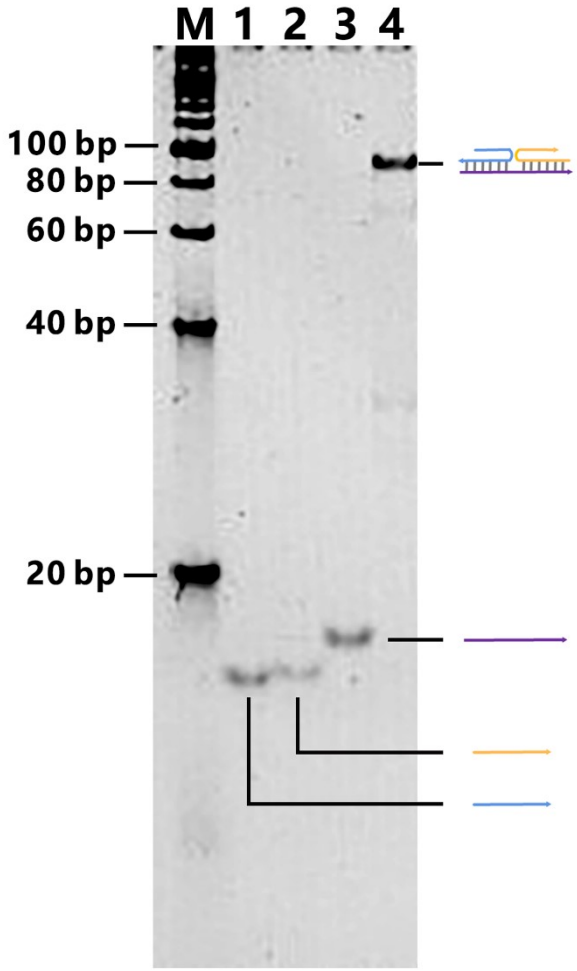

Fig. S1 Characterization of the DNA triplex formation by native polyacrylamide gel electrophoresis. Lane 1: ORF1ab; lane 2: N; lane 3: connector; lane 4: ORF1ab + N + connector; lane M: 500 bp DNA Marker

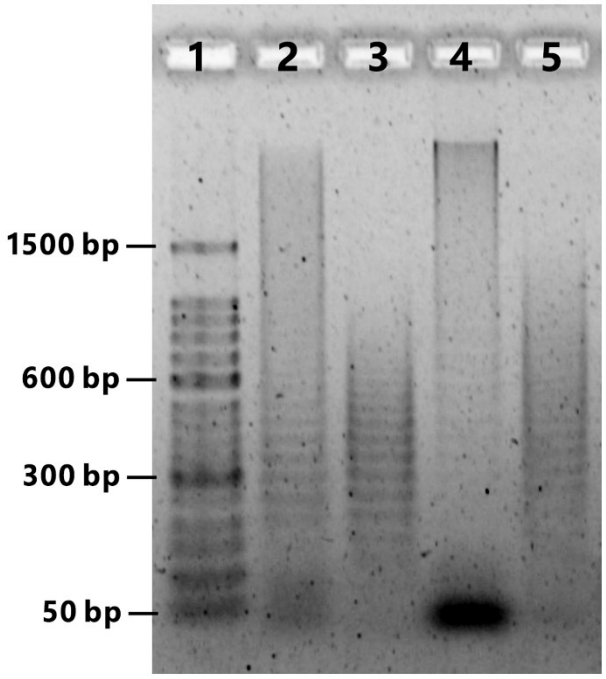

Fig. S2 Comparison of the triggering ability between associated toehold and intact initiator for initiating HCR reaction by agarose gel electrophoresis. Lane 1: 50 bp DNA ladder; lane 2: $H1^{15}$ + $H2^{15}$ + 0.25× (connector + ORF1ab + N); lane 3: $H1^{15}$ + $H2^{15}$ + 0.25× initiator; lane 4: $H1^{17}$ + $H2^{17}$ + 0.25× (connector + ORF1ab + N); lane 5: $H1^{17}$ + $H2^{17}$ + 0.25× initiator.

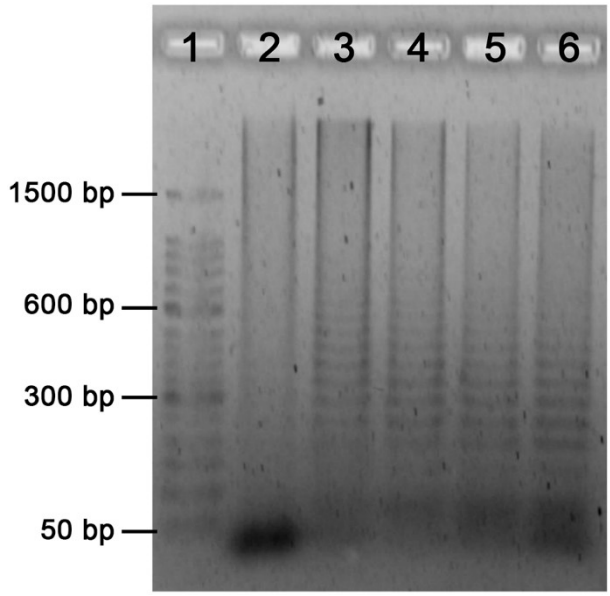

Fig. S3 Investigation of pH influence to HCR reaction by agarose gel electrophoresis. Lane 1: 50 bp DNA ladder; lane 2 to lane 5: HCR reaction results of $H1^{15}$ + $H2^{15}$ + 0.25× (connector + ORF1ab + N) performed in HEPES buffer (10 mM HEPES, 300 mM NaCl) with various pH 5.0, 6.0, 7.0, 8.0 and 9.0.

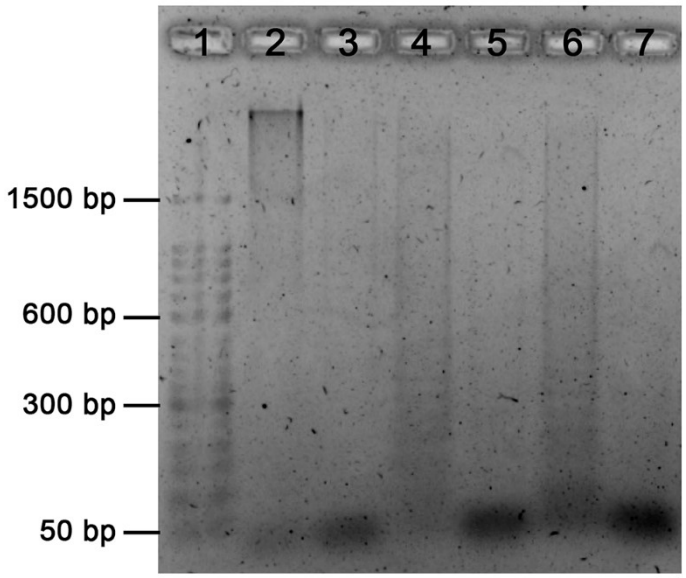

Fig. S4 Investigation of ionic strength influence to HCR reaction by agarose gel electrophoresis. Lane 1: 50 bp DNA ladder; lane 2 to lane 7: HCR reaction results of $H1^{15}$ + $H2^{15}$ + 0.25×connector in the presence or the absence of ORF1ab and N performed in HEPES buffer (10 mM HEPES, pH 7.0) with NaCl concentrations of 150 mM, 300 mM and 500 mM.

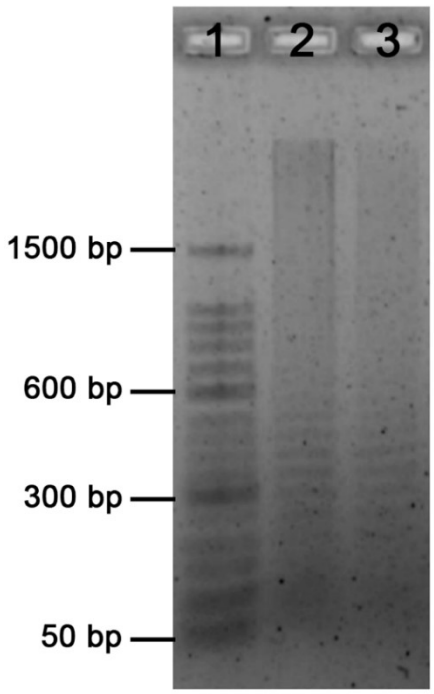

Fig. S5 Investigation of temperature influence to HCR reaction by agarose gel electrophoresis. Lane 1: 50 bp DNA ladder; lane 2 to lane 3: HCR reaction results of $H1^{15}$ + $H2^{15}$ + 0.25× (connector + ORF1ab + N) performed at 37 °C and at room temperature.

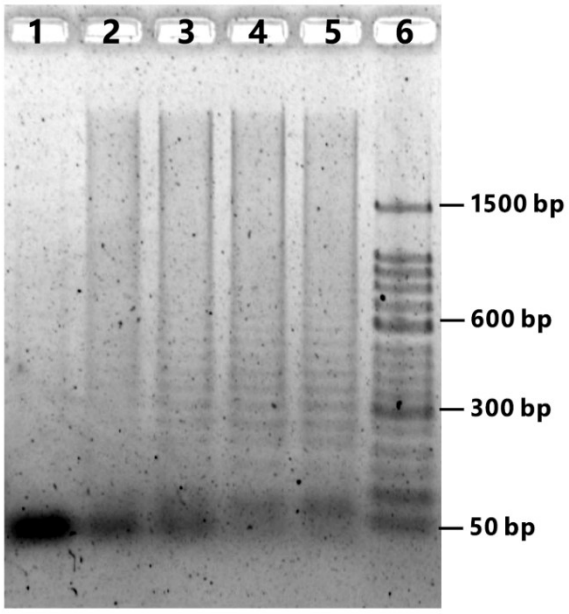

Fig. S6 Investigation of the HCR reaction time by agarose gel electrophoresis. Lane 1: H1 + H2; lanes 2 to 5: HCR reaction results with periods of reaction time for 1 h, 2 h, 3 h and 4 h [H1 + H2 + 0.25× (connector + ORF1ab + N)]; lane 6: 50 bp DNA ladder.

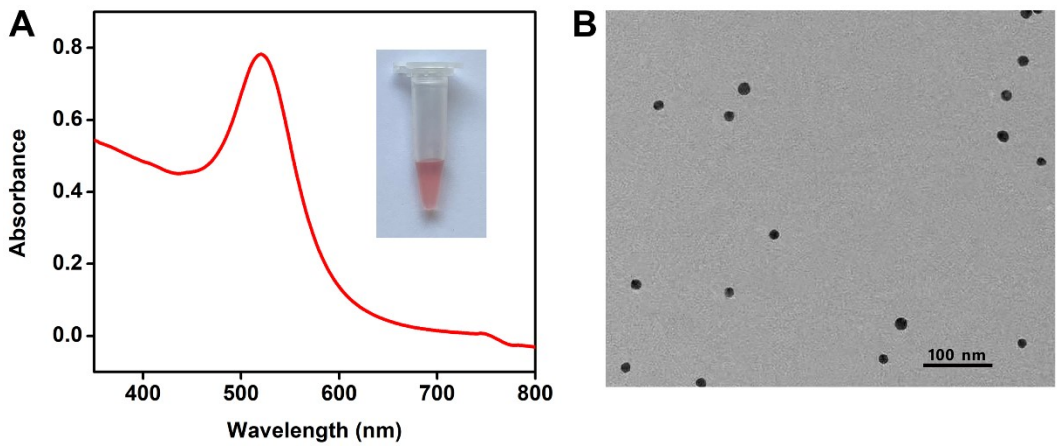

Fig. S7 UV-vis absorption spectra (A) and transmission electron microscopy (TEM) images of synthesized AuNPs. Inset (A): photograph of the AuNP solution.

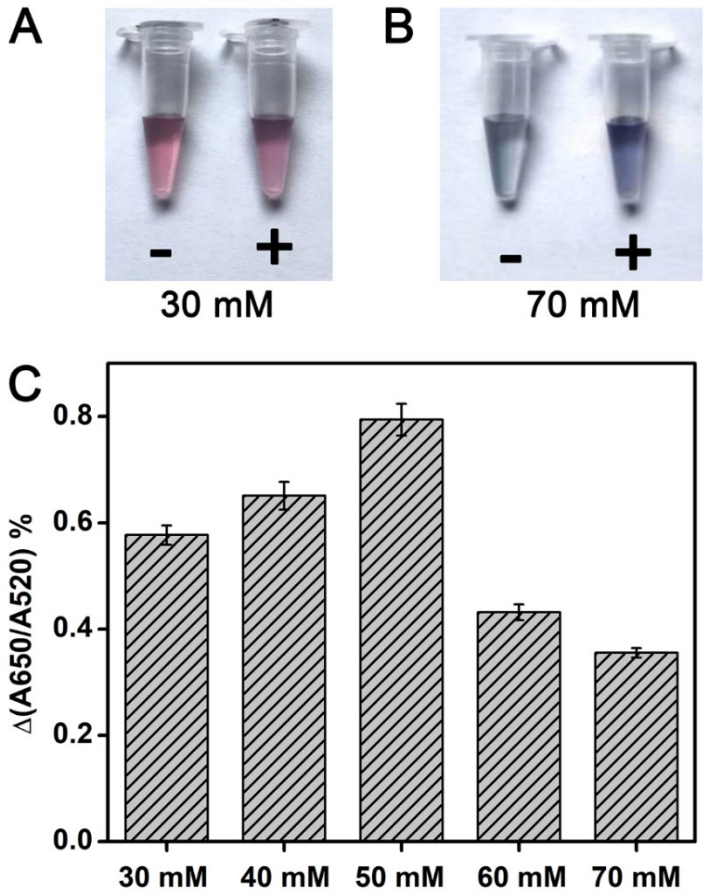

Fig. S8 Visual observation of the colorimetric detection system in the absence and the presence of dual RNA fragments with a final NaCl concentration of 30 mM (A) and 70 mM (B), and relative increase of absorption ratio (A650/A520) in the presence of ORF1ab and N versus that in the absence of the dual RNA fragments with different final salt concentrations (C).

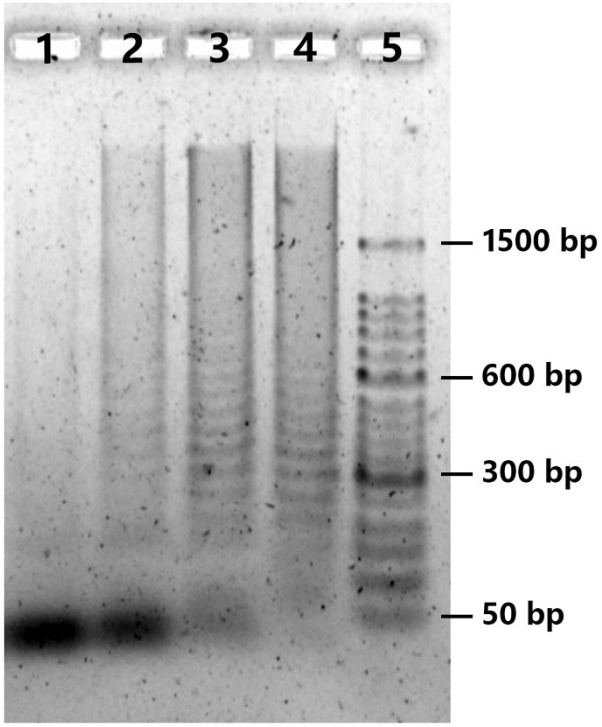

Fig. S9 Investigation of HCR reaction products along with the increasing concentration of ORF1ab and N. Lane 1: H1 + H2 + 0.25× connector; lane 2: H1 + H2 + 0.25× connector + 0.15× (ORF1ab + N); lane 3: H1 + H2 + 0.25× connector + 0.20× (ORF1ab + N); lane 4: lane 3: H1 + H2 + 0.25× connector + 0.25× (ORF1ab + N); lane 5: 50 bp DNA ladder.

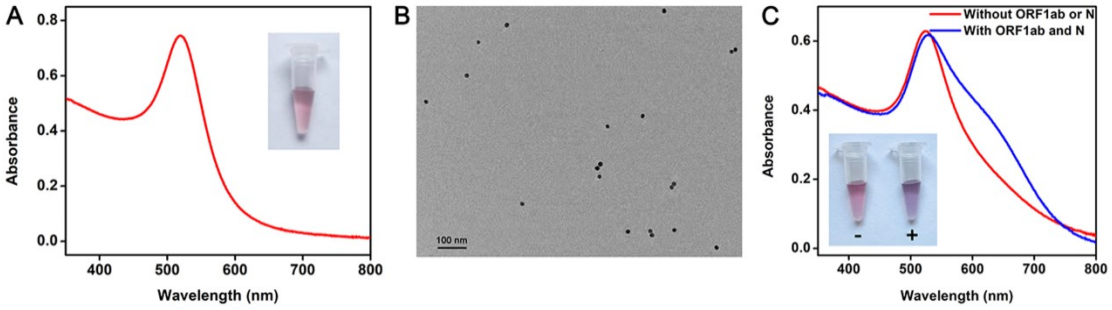

Fig. S10 UV-vis absorption spectra (A), transmission electron microscopy image (B), and the colorimetric detection of ORF1ab and N by using synthesized AuNPs after a storage time of six months in centrifuge tubes at 4 °C in the refrigerator.

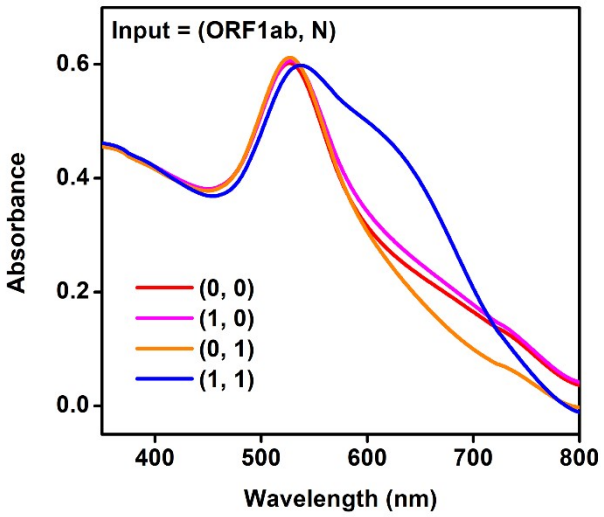

Fig. S11 UV-vis absorption spectra of the colorimetric detection system treated with no viral gene fragment, single viral gene fragment (ORF1ab or N), and dual viral gene fragments (ORF1ab and N).

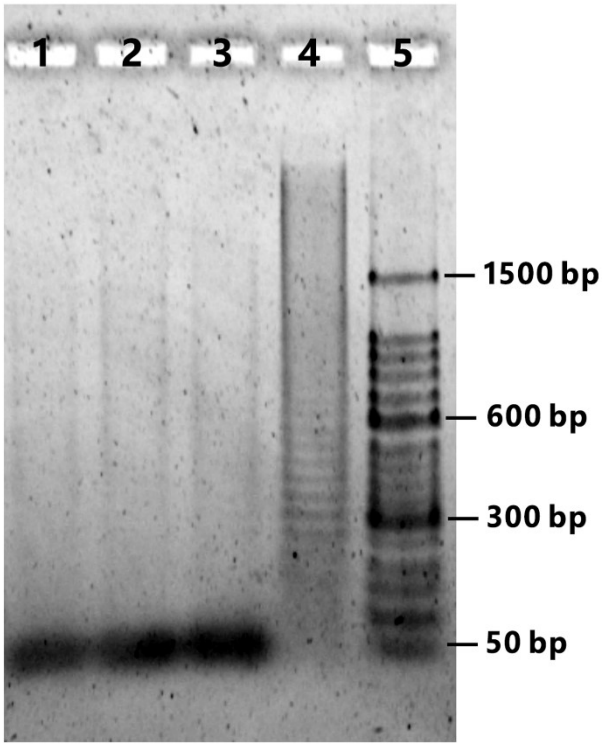

Fig. S12 Investigation of the HCR reaction triggered by different existence of viral gene fragments. Lane 1: H1 + H2 + 0.25× connector; lane 2: H1 + H2 + 0.25× (connector + ORF1ab); lane 3: H1 + H2 + 0.25× (connector + N); lane 4: H1 + H2 + 0.25× (connector + ORF1ab + N); lane 5: 50 bp DNA ladder.

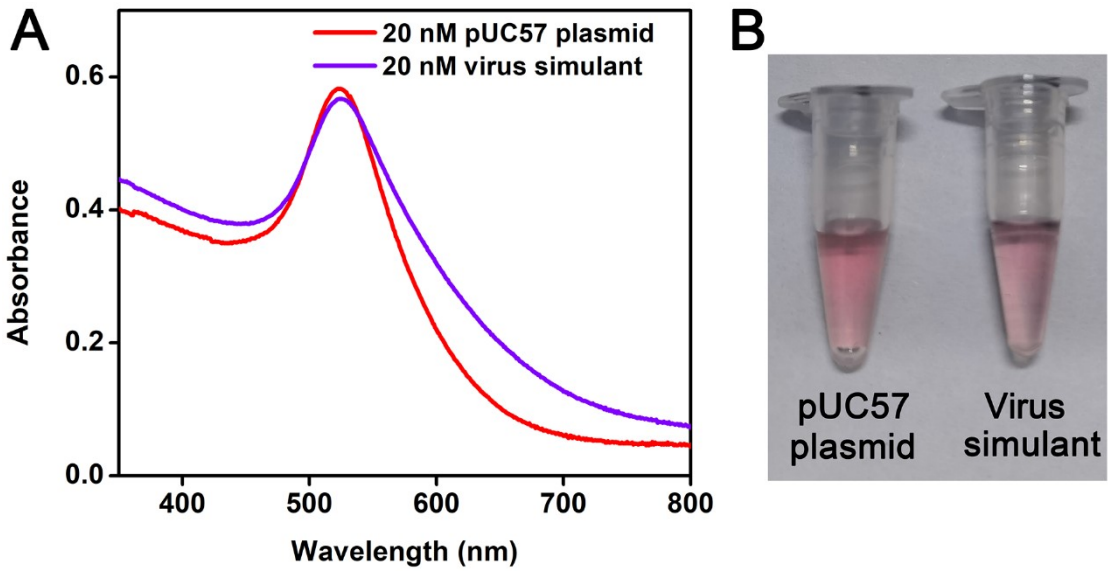

Fig. S13 UV-vis absorption spectra (A) and visual color change (B) of the colorimetric system in the presence of 20 nM pUC57 plasmid or 20 nM virus simulant.

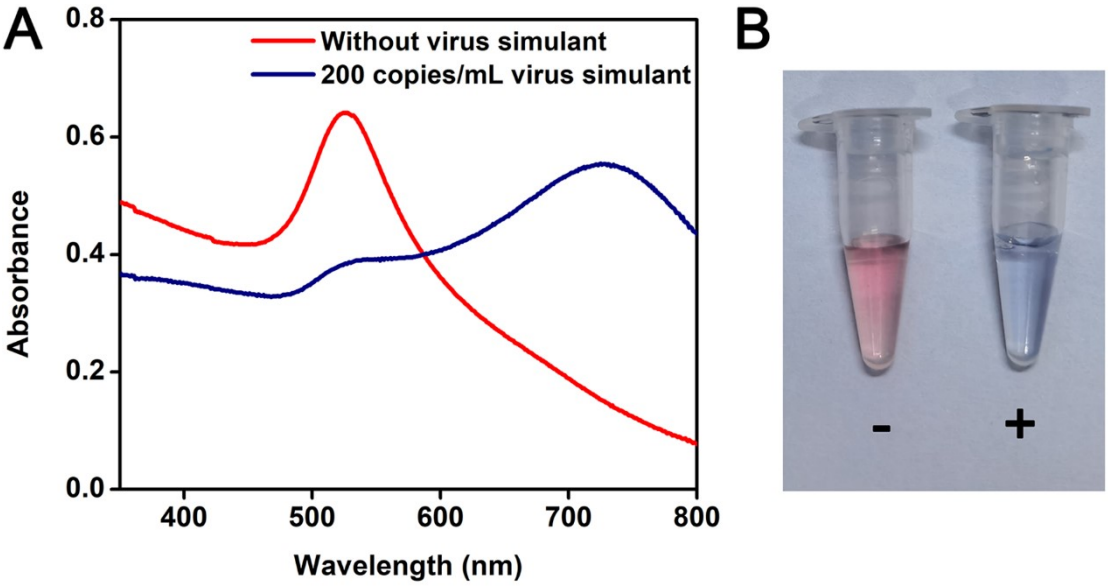

Fig. S14 UV-vis absorption spectra (A) and visual color change (B) by coupling an upstream recombinase polymerase amplification of sample with the colorimetric system for detection of 200 copies/mL virus simulant.

References:

1 K. C. Grabar, R. G. Freeman, M. B. Hommer and M. J. Natan, *Anal. Chem.*, 1995, **67**, 735.

2 H. D. Hill and C. A. Mirkin, *Nat. Protoc.*, 2006, **1**, 324.