Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

Combination of ultrashort PCR and Pyrococcus furiosus Argonaute for DNA detection

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Supporting Information

1. Experimental Methods

1.1 Primers, enzymes and reagents

All primers, guide DNAs, single-stranded DNA targets and molecular beacons used in this study were synthesized by Sangon (China). T4 polynucleotide kinase (PNK) was purchased from New England Biolabs (USA). 2×Taq PCR MIX was purchased from Yeasen (China). SYBR Gold for resolving urea-denaturing PAGE was purchased from ThermoFisher Scientific, USA. ABScript II cDNA First-Strand Synthesis Kit was purchased from Abclonal.

1.2 Preparation of mocked nucleic acid targets

The VP72 gene of African swine fever virus (ASFV) and the E6 gene of Human papillomavirus 16 were cloned into plasmid pUC19, respectively, and were employed as target. SsDNAs of ORF1ab from SARS-CoV-2, MERS-CoV and SARS-CoV, and ssDNAs of single nucleotide polymorphisms (SNPs) sites from SARS-CoV-2 at nt 8782 (C/T) and nt 28144 (T/C) (8782C, 8782T, 28144T, 28144T) were synthesized, respectively, to be used as targets.

1.3 *Pf*Ago cleavage assay

*Pf*Ago was expressed and purified as previously described1. For PfAgo-mediated cleavage of molecular beacon, a 10 μL reaction system was prepared, containing 20 pmol of purified PfAgo, 10 pmol of molecular beacon, 5 μL usPCR products and 1 μL of 10×reaction buffer (200 mM HEPES (pH7.5), 2.5 mM NaCl, and 5 mM MnCl2). The reaction was carried out at 95 °C for 30 min, followed by fluorescence signals detecting using Bio-rad Real-time fluorescence quantitative PCR instrument (USA).

1.4 Optimization of usPCR

For the cycle temperature optimization, various temperature combinations are as follows: 60°C (denaturation), 30°C or 40°C or 50°C (annealing); 70°C (denaturation), 30°C or 40°C or 50°C (annealing/extension); 80°C (denaturation), 30°C or 40°C or 50°C (annealing/extension); 90°C (denaturation), 30°C or 40°C or 50°C (annealing/extension). The amplification was performed in a 10 μl reaction system, containing a primer pair of primer-g (0.7 μM) and primer-h (0.35 μM), target (1 μl) and 2×Taq PCR Mix, using the

procedure: denaturation for 5 s, annealing/extension for 5 s, 40 cycles. SsDNA of ORF1ab from SARS-CoV-2 was used as targets in this system and products were detected by TBE-denaturing PAGE and mediated *Pf*Ago cleaved molecular beacon.

For optimization of the cycle duration, synthesized ssDNA of SARS-CoV-2 ORF1ab was used as the template and the reaction was carried out under the optimal temperature combination of 70°C /40°C for denaturation/annealing with 40 cycles. Various cycle duration were designed as follows: 70°C 1 s, 40°C (1 s or 2 s or 5 s or 10 s) ; 70°C (1 s or 2 s or 5 s or 10 s), 40°C, 1 s. The reaction system and the detection of products were performed as described in optimization of temperatures.

For the primer ratio optimization, ssDNA of SARS-CoV-2 ORF1ab was used as the template. With various concentrations of primer-g, pg-O, (0 μM, 0.35 μM , 0.7 μM, 1.75 μM , 3.5 μM, 7 μM) and a constant concentration of primer-h, ph-O, (0.35 μM), various primer ratios of pg/ph, that is 0, 1:1, 2:1, 5:1, 10:1, 20:1, were investigated. The procedure employed is as follows: 70°C 1 s, 40°C 1 s, 40 cycles. Except for a different amount of primer-g, the reaction system and the detection of products were performed as described in optimization of temperatures. Following the PCR amplification, both TBE-denaturing PAGE and PfAgo-mediated cleavage of molecular beacon (MB-S2) were carried out as described above.

For optimization of the primer concentrations, various concentrations of pg-O/ph-O (0.7/0.35 μM, 1.4/0.7 μM, 3.5/1.75 μM, 7.0/3.5 μM) were examined. The procedure employed is as follows: 70°C 1 s, 40°C 1 s, various cycles (20 or 30 or 40 or 50 or 60 or 70). Except for different amounts of primers, the reaction system and the detection of products were performed as described in optimization of temperatures.

1.5 Sensitivity assay of USPCRP

To investigate the sensitivity of USPCRP, serial dilutions of ssDNA of SARS-CoV-2 ORF1ab (ssDNA-OS2) (from 1 nM to 1 aM) and dsDNA of HPV16E6 (pUC19-HPV16E6) (from 1 nM to 1 aM) were used as targets. PCR amplification was carried out at 70°C 1 s, 40°C 1 s, 40 cycles with the optimal reaction system of 10 μL containing 1 μL of target DNA, 0.7 μM of primer-g, 0.35 μM of primer-h and 2×Taq PCR Mix. Then, the *Pf*Ago-mediated cleavage of a specific molecular beacon and the fluorescence detection were carried out as described above.

1.6 Specificity assay of USPCRP

To assess the specificity of USPCRP, ssDNA of ORF1ab (ssDNA-O) from SARS-CoV-2 , MERS-CoV (Middle East respiratory syndrome coronavirus) and SARS-CoV (Severe acute respiratory syndrome coronavirus) were synthesized and used as target, respectively. The specificity of PCR amplification was examined by applying each pair of primers specific for one of the three templates to amplify all templates, respectively. PCR amplification was performed under the optimal condition with an optimal reaction system as described above. For measuring the specificity of PfAgo-mediated cleavage of molecular beacon, the specific molecular beacon against ssDNA-O of SARS-CoV-2 (MB-S2) was applied to detecting all the three targets. The PfAgo-mediated cleavage of molecular beacon and the fluorescence detection were carried out as described above. .

1.7 Single-nucleotide polymorphisms discrimination with USPCRP

Two tightly linked Single-nucleotide polymorphisms (SNPs) sites of SARS-CoV-2 at nt 8782 (C/T) and nt 28144 (T/C) were selected as the targets. ssDNAs of partial sequences harboring these SNPs sites within the SARS-CoV-2 genome were synthesized, respectively. The primer-g of 11 nt was designed for localizing the mutation site at 12 or 13 nt from the 5'-end of guide DNAs. PCR amplification was conducted at the determined optimal condition with an optimal reaction system as described above. The following detection of PCR products, PfAgo-mediated cleavage of molecular beacons and fluorescence detection were carried out as described above.

1.8 Detection of viruses from clinical samples

Nucleic acid extracts from suspected high-risk human papillomavirus clinical samples (1, 2, 3, 4, 5, 6) were provided by the School of Medicine of Hubei College of Arts and Sciences. RNA extracts from nasopharyngeal/ oropharyngeal swabs of suspected COVID-19 patients (a1, b1, c1, d1, e1, f1, g1, h1, a2, b2, c2, d2) were provided by Wuhan CDC. HPV16E6 gene and ORF1ab gene were selected as the targets for detecting HPV16 and SARS-CoV-2, respectively. For detection of SARS-CoV-2, reverse transcription of RNA extract was performed to generate the corresponding cDNA using ABScript II cDNA First-Strand Synthesis Kit.

1.9 Analysis of USPCRP fluorescence data

To calculate background subtracted fluorescence data, the initial fluorescence of samples was subtracted to allow for comparisons between different conditions. Fluorescence for background

conditions (no targets) were subtracted from samples to generate background subtracted fluorescence (BKgd-subtracted fluorescence).

2. Supplementary tables

TableS1: DNA sequences used for evaluating the feasibility and reliability of USPCR

Name	Sequences (5'-3')
ssDNA1	AGGCAGCAGTAGGGGAACTTCTCCTGCTAGAATGGCT GGCAATGGCGGTGATGCTGCTC
pg1	℗CCATTGCCAGC
ph1	CTTCTCCTGCTAGA
MB1	6-FAM-cgcaccCTTCTCCTGCTAGAATGGCTGGC AATGGgtgcg-BHQ1

TableS2: DNA sequences used for detecting VP72 gene and HPV16L1 gene

Name	Sequences (5'-3')
VP72-pg	℗TGCCTCCGTAG
VP72-ph	ACATACCCTTCCA
MB-VP72	VIC-cgcaccTTCCACTACGGAGGCAggtgcg-BHQ1
HPV16-pg	℗ACCACAGTTATG
HPV16-ph	TTTGCAGCTCTG
MB-HPV16	6-FAM-cgcaccATGCACAGAGCTGCAAACAAggtgcg-BHQ1
HPV16F	ACTGCAATGTTTCAGGACCCACAGGAG
HPV16R	CCGAAAAGCAAAGTCATATACCTCACGTCGC

TableS3: DNA sequences used for detecting ORF1ab

Name	Sequences (5'-3')
S2-pg	℗GCTTCAGTCAG
S2-ph	ATTGTGCATCAG
MB-S2	6-FAM-cgcaccGTGCATCAGCTGACTGAAGCggtgcg-BHQ1
ssDNA-OS2	ACCCATGCTTCAGTCAGCTGATGCACAATCG
S-pg	GATGCAGTCTG
S-ph	TTGATGCATCCG
ssDNA-OS	ACCCTTGATGCAGTCTGCGGATGCATCAACG
M-pg	ACTGCCCCAAT
M-ph	TGGAATCTTTAG
ssDNA-OM	AGCAGCACTGCCCCAATCTAAAGATTCCAAT

TableS4: DNA sequences used for detecting SNP sites at nt 8782 (C/T) and nt 28144 (T/C)

28144pg	℗CAGTTTCCTGT
28144ph	TTAATTGTAAAAGG

MB-28144T	6-FAM-cgcaccAAGGTAAACAGGAAACTGTAggtgcg-BHQ1
MB-28144C	VIC-cgcaccAAGGTGAACAGGAAACTGTAggtgcg-BHQ1
nt28144C	GCAATTAATTGTAAAAGGTGAACAGGAAACTGTAT
nt28144T	GCAATTAATTGTAAAAGGTAAACAGGAAACTGTAT
8782pg	℗CACATGGTTTAG
8782ph	TACCACCACGCT
MB-8782C	6-FAM-cgcaccCGCTGGCTAAACCATGTGTCggtgcg-BHQ1
MB-8782T	VIC-cgcaccCGCTGACTAAACCATGTGTCggtgcg-BHQ1
nt8782C	ACTACCACCACGCTGGCTAAACCATGTGTCA
nt8782T	ACTACCACCACGCTGACTAAACCATGTGTCA

Bases in red indicate the mutation sites.

3. Supplementary figures

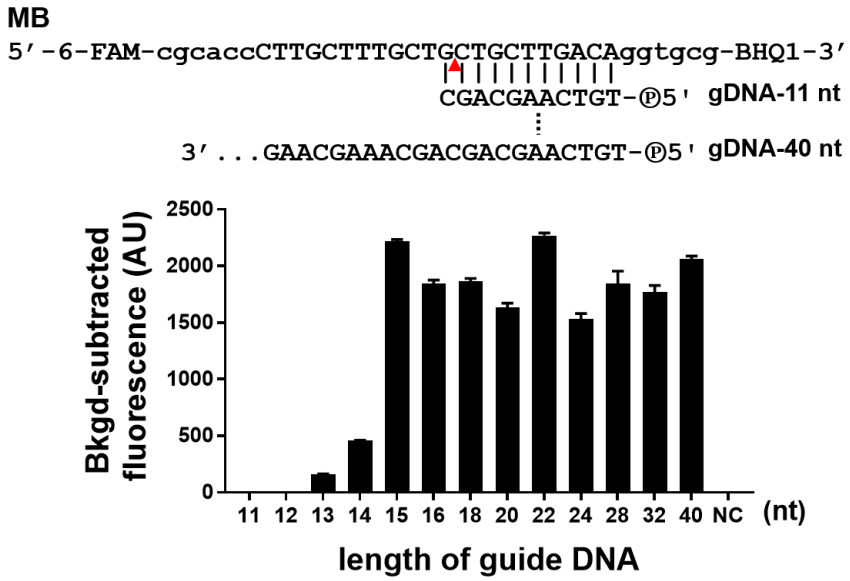

Figure S1 Influences of the length of guide DNA on the cleavage activity of *Pf*Ago. Various lengths of 5'- phosphorylated guide DNAs were generated by T4 polynucleotide kinase treatment of various length of synthesized ssDNAs. Molecular beacons were used as reporter and the cleavage activity of *Pf*Ago was reflected by the observed fluorescence intensity. Data are presented as mean ± standard deviation of three independent replicates. Abbreviations: NC, negative control; MB, molecular beacon; gDNA, guide DNA.

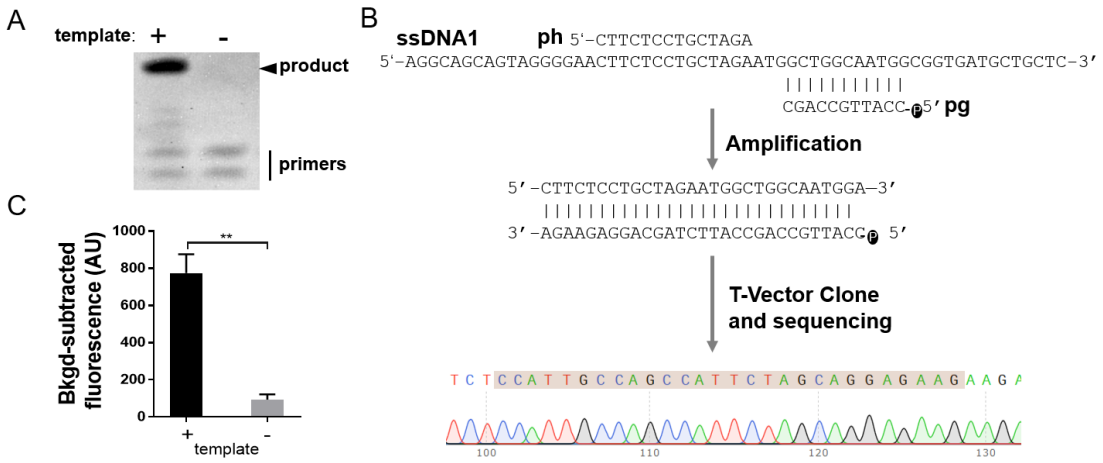

Figure S2 Establishment of usPCR. (A) TBE-PAGE analysis of usPCR products. (B) Result of Sanger sequencing of usPCR products. (C) Detection of the cleavage activity of *Pf*Ago using molecular beacon as reporter. Products of usPCR were directly used to direct *Pf*Ago cleavage of molecular beacon and the fluorescence signal was monitored to indicate the cleavage activity of *Pf*Ago. Data are presented as mean ± standard deviation of three independent replicates. ** $p < 0.01$.

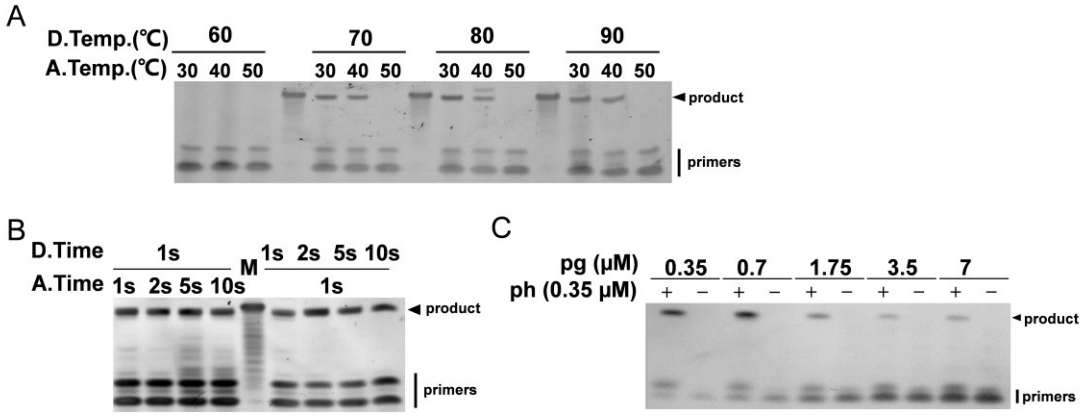

Figure S3 Optimization of USPCRP. Synthesized ssDNA of SARS-CoV-2 ORF1ab gene was used as the template. (A) UsPCR amplification was performed under various temperature combinations as indicated and PCR products were detected with TBE-PAGE. (B) UsPCR amplification was performed under various cycle duration as indicated and PCR products were detected with TBE-PAGE. (C) Influences of various primer ratios on usPCR. PCR amplification was conducted under the determined optimal condition with various primer ratios as indicated, and the resulted products were subjected to TBE-PAGE analysis. D.Temp. : Denaturation Temperature; A.Temp. : Annealing Temperature; D.Time: Denaturation Time; A. Time: Annealing Time. M: standard ssDNA

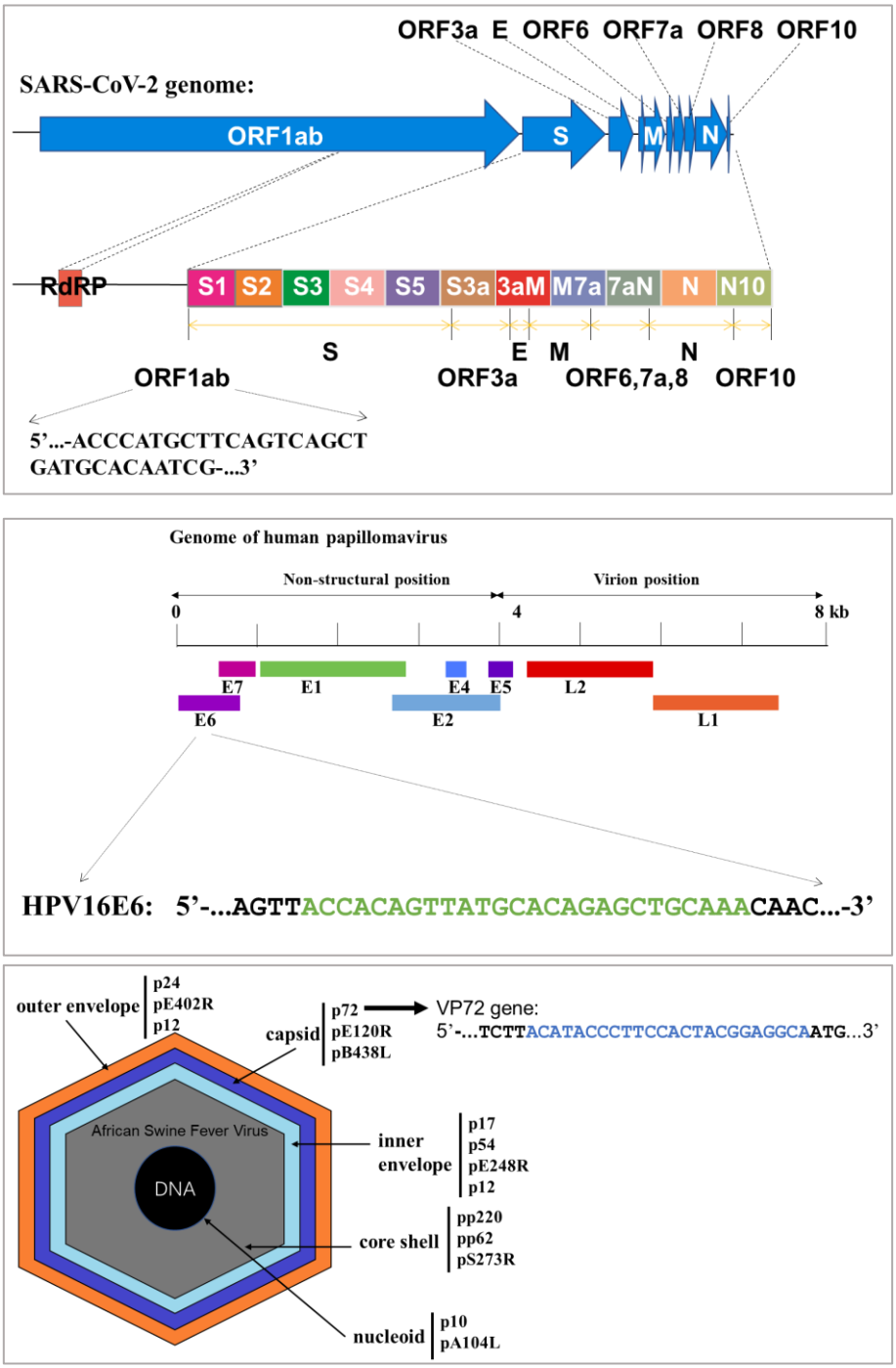

Figure S4 Schematic of the genome of SARS-CoV-2 and HPV16, and the structural domains of the ASFV particle. The target sequence of SARS-CoV-2、HPV16E6 and VP72 gene were marked at the apporpriate loaction.

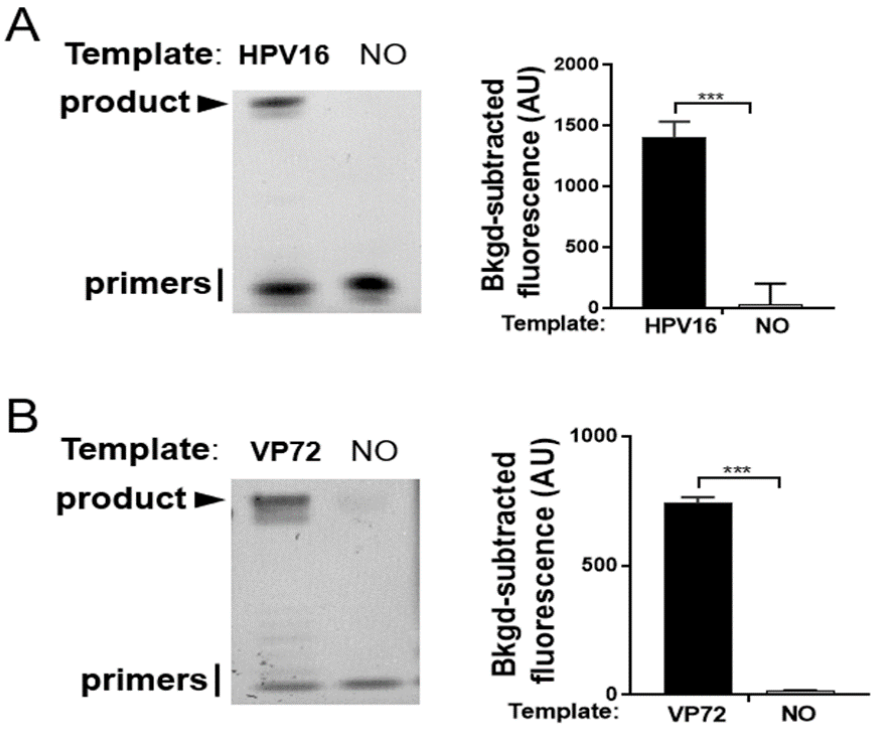

Figure S5 Results of detection of mocked HPV16E6 gene (A) and VP72 gene (B) with USPCRP. Data are presented as mean ± standard deviation of three independent replicates. ***, $p<0.001$. NO: No template

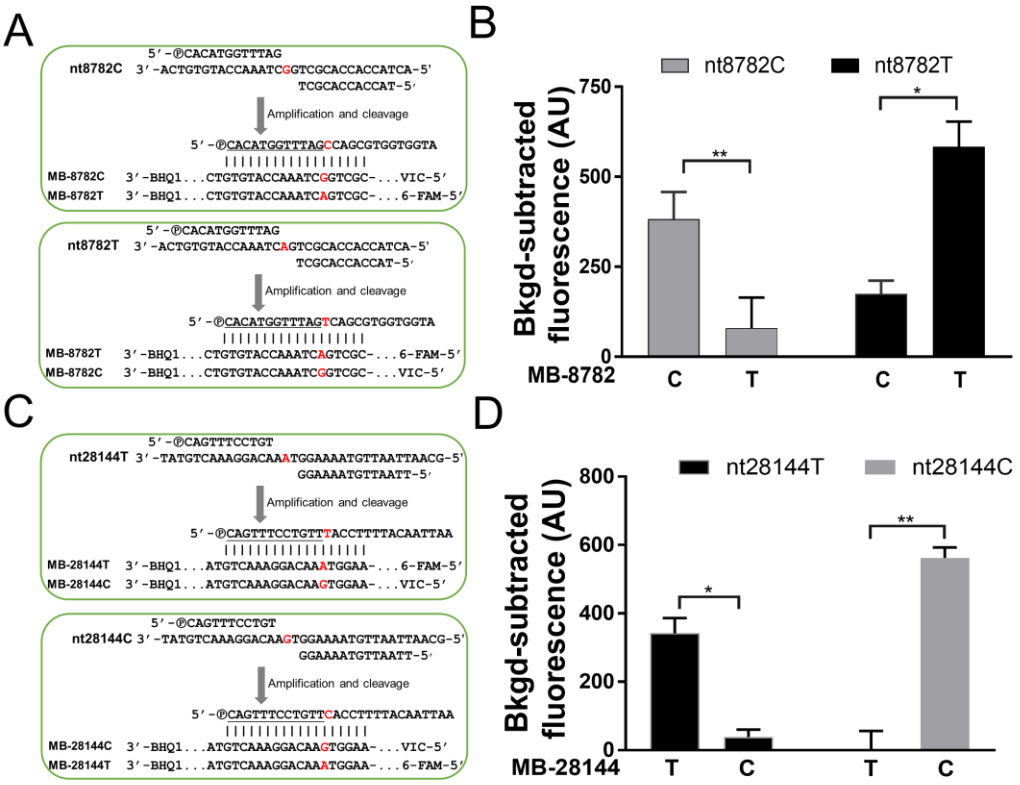

Figure S6 Detection of single-nucleotide polymorphisms (SNPs) with USPCRP. Synthesized ssDNAs of partial sequences harboring the SNPs sites of SARS-CoV-2 genome were used as the target, specific primers were designed to allow the mutation base located at 13 nt from the 5' end of guide DNA. (A) Schematic of detection of SARS-CoV-2 SNPs at nt 8782 (C/T); SNPs were highlighted in red. **(B)** Results of detection of SNPs at nt 8782 (C/T). **(C)** Schematic of detection of SARS-CoV-2 SNPs at nt 28144 (T/C); SNPs were highlighted in red. **(D)** Results of detection of SNPs at nt 28144 (T/C). The corresponding base at the SNPs sites is annotated below each bar, the corresponding base in the molecular beacon are annotated at the top of the bars. Data are presented as mean ± standard deviation of three independent replicates. *p < 0.05, **p < 0.01

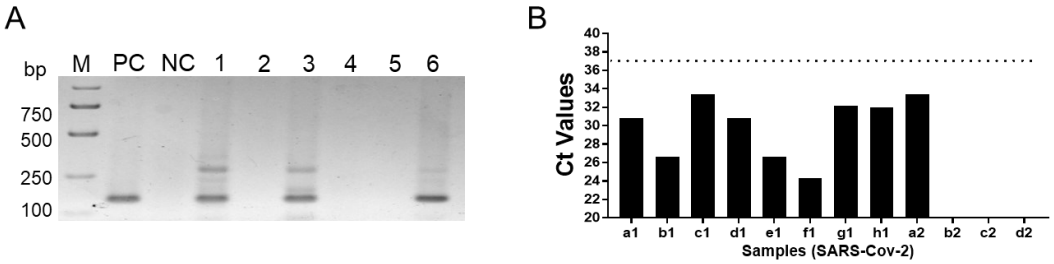

Figure S7 Detection of viruses in clinical samples using PCR or RT-qPCR. (A) Agarose gel electrophoresis analysis of PCR products (150 bp) for HPV16E6 detection from clinical sample (1,2,3,4,5,6); **(B)** Ct-values of RT-qPCR assay of SARS-Cov-2 from clinical samples (a1, b1, c1, d1, e1, f1, g1, h1, a2, b2, c2, d2) performed by Hubei CDC. Abbreviations: PC, positive control, NC, negative control.