Appendix A. Supplementary data

All-in-one in situ colorimetric RT-LAMP assay for point-of-care testing of SARS-CoV-2

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S1. Materials and Methods

S1-1. Materials

Certified Reference Material of 2019 Novel Corona Virus (2019-nCoV) Ribonucleic Acid Genome and Reference Material of 2019-nCoV Pseudovirus RNA were both purchased from National Institute Metrology, China (Beijing, China). Carrier RNA with a concentration of 1 μg/μL was obtained from the QIAamp Viral RNA Mini Kit purchased from QIAGEN (Hilden, Germany).

S1-2. Screening of the real-time PCR primers for detecting GX/P2V RNA

As the amount of viral RNA captured on aGBs was determined by using RT-qPCR assay, we need to screen the optimal primers for GX/P2V detection. 6 primer sets were designed and tested using 2.93×10^5 copies/μL GX/P2V RNA according to the protocol described in Section 2.5 in the main text (**Tables S1** and **S2**).

S1-3 Using carrier RNA to enhance the viral RNA capturing on aGBs

5 μL of 1 μg/μL carrier RNA and 195 μL lysis buffer were mixed thoroughly prior to performing viral RNA extraction on aGBs, and then various concentrations of GX/P2V RNA were purified using our developed protocol described in Section 2.3 in the main text. For comparison, various concentrations of GX/P2V RNA were also extracted following our developed method without the addition of carrier RNA in the lysis buffer. After the viral RNA was captured on aGBs, the aGBs/RNA composite was directly used as the template to perform RT-qPCR testing as described in Section 2.5 in the main text.

S1-3 Concentrating viral RNA using aGBs

To further increase the amount of viral RNA captured on aGBs, we increased the initial sample input volume. Following the aGBs-based viral RNA extraction protocol described in Section 2.3 in the main text, 100 μL of sample input and an equal volume of lysis buffer were used to perform viral RNA extraction. For the sample input volume was 1000 μL, increase the amount of lysis buffer proportionally to 1000 μL and keep the other steps the same with what we described in Section 2.3, followed by performing RT-qPCR test as described in Section 2.5 in the main text.

S1-4 The tolerance of LAMP reaction to aGBs

As the aGBs were introduced to the LAMP reaction, we need to investigate whether they inhibit DNA amplification. A 50 μL fluorescence-based LAMP reaction, which includes 25 μL WarmStart LAMP 2× Master Mix, 5 μL 10× primer mix, 5 μL of template, 1 μL fluorescent dye, 14 μL nuclease-free water, and 3 aGBs, was set up in a real-time thermolcycler through SYBR® channel. The reaction was incubated at 65℃ for 32 s for 120 cycles and the fluorescence signal was collected in each cycle. For comparison, LAMP reactions without the addition of aGBs were performed as well. When the input template was plasmid DNA

with a concentration of 1.91×10^4 copies/μL, primer set SORF1a was used; while the template was 2.93×10^4 copies/μL of GX/P2V RNA, primer set PM was used. Negative control reactions were performed by using nuclease-free water instead of nucleic acid templates.

S1-5 Optimizing of the LAMP reaction temperature

The reaction temperature of the LAMP assay was optimized using the fluorescence-based RT-LAMP method as described in Section 2.6 in the main text. 2.93×10^5 copies/μL of GX/P2V RNA was used as templates to set up the reaction at different incubation temperatures: 50, 55, 60, 65, 68, 70℃.

S1-6 Optimizing of the LAMP primers

Four LAMP primer sets, including two targeting the ORF1ab gene, one targeting the M gene and the other one targeting the N gene of the GX/P2V genome, were designed for detecting GX/P2V and then tested using fluorescence-based RT-LAMP assay. 2.93×10^5 copies/μL of the purified GX/P2V RNA was used as the template to set up the reaction by following the protocol described in Section 2.6 in the main text. In addition, five primer sets, including two targeting the ORF1ab gene, one targeting the M gene and the other two targeting the N gene of the SARS-CoV-2 genome, were screened for SARS-CoV-2 detection by using a purified RNA clinical specimen which had been identified as COVID-19 positive by RT-qPCR. The primer sequences used in this work are listed in **Table S1**.

S1-7 Determination of the analytical sensitivity for detecting GX/P2V RNA

Primer set PM was used in the following experiments (**Table S1**).

The GX/P2V virus particles were 10-fold serially diluted using 1× PBS solution, and then 50 μL viral RNA was extracted from each sample with 200 μL of input volume by using QIAamp Viral RNA Mini Kit. Next, a 2 μL aliquot of the purified viral RNA was used to perform the RT-qPCR test, fluorescence-based RT-LAMP test, and conventional colorimetric RT-LAMP test, both with two replicates, according to the protocols described in Sections 2.5 and 2.6 in the main text. The results were shown in **Figures 5A**, **B** and **C**.

GX/P2V samples at various concentrations were also detected using our developed all-in-one in situ colorimetric RT-LAMP assays. The detailed test procedures followed the protocols we described in Sections 2.3 and 2.7 in the main text, with one exception that the incubation time lasted for 50 min. The results were read by photographing reaction tubes every 10 min using an iPhone 6s Plus cellphone with default settings. The results were shown in Figure 5D.

Furthermore, the all-in-one in situ colorimetric RT-LAMP was carried out using nine 2-fold serial diluted GX/P2V samples with 10 technical replicates at each concentration. The samples were diluted utilizing 1× PBS solution and then a 200 μL aliquot of each sample was used to set up the reactions following the protocols described in Sections 2.3 and 2.7 in the main text. Then, a probit analysis was performed based on the all-in-one in situ LAMP results, using GraphPad Prism 9 software. The results were shown in **Figures 5E** and **F**.

Our assay was further validated in simulated GX/P2V samples. 20 oropharyngeal swab specimens from the healthy donors were collected and were stored in a 3-mL viral transport medium (VTM) solution at -70℃. Pipette 500 μL aliquot from each sample and mix them thoroughly to form a 10 mL pooled VTM solution. Then, GX/P2V virus particles were spiked into the pooled VTM solution to the final concentrations of 4 and 15 copies/μL respectively, followed by performing our developed assay with 20 replicates at each concentration, according to the protocols described in Sections 2.3 and 2.7 in the main text. The results were shown in **Figure S6**.

S1-8 Determination of the analytical sensitivity for detecting SARS-CoV-2 RNA

SARS-CoV-2 reference materials were 10-fold serially diluted with nuclease-free water, and then 2 μL

of the diluted samples (2 replicates) were used to set up the conventional colorimetric RT-LAMP reaction utilizing primer set SORF1a-A1, according to the protocol described in Section 2.6 in the main text. The results were then read by photographing reaction tubes every 10 min using an iPhone 6s Plus cellphone with default settings. To get a stable LOD, samples SR1 and SR2 with 5 replicates were tested for a second time, and the results were photographed after incubation for 40 min. The results were shown in **Figure S7D** and **Table S4**.

The LOD of the all-in-one assay for detecting SARS-CoV-2 RNA was further determined using simulated pseudovirus samples. Reference materials of SARS-CoV-2 pseudovirus samples were 2-fold serially diluted using pooled VTM solution and then a 200 μL aliquot of each sample (10 replicates) was used to set up the reactions following the protocols described in Sections 2.3 and 2.7 in the main text. A published primer set was utilized in this experiment.[1] Then, probit analysis was performed based on the LAMP results, using GraphPad Prism 9 software. The results were shown in **Figure S9** and **Table S5**.

S1-9 Determination of the analytical specificity for detecting SARS-CoV-2 RNA

Compared with some common human coronaviruses, such as SARS-CoV, MERS-CoV, and HCoV-229E, SARS-CoV-2 shares a much higher nucleotide identity with GX/P2V (**Figure S1**). Therefore, the analytical specificity of the LMAP assay for SARS-CoV-2 detection was firstly evaluated by using GX/P2V RNA. GX/P2V virus particles were spiked into the pooled VTM solution to final a concentration of 10^4 copies/μL and then the 50 μL genome RNA was extracted from a 200 μL spiked sample using QIAamp Viral RNA Mini Kit. Next, a 2 μL aliquot of the purified GX/P2V RNA was used to perform the fluorescence-based RT-LAMP test (3 replicates), using either SORF1a-A1 or SN-A primer set, according to the protocols described in Sections 2.5 and 2.6 in the main text. In addition, tests using SARS-CoV-2 RNA as templates were performed as well. The results were shown in **Figure S7B**.

The assay specificity was further validated by using 20 negative clinical specimens. We acquired 20 oropharyngeal swab specimens in VTM solution from the healthy donors and then 200 μL of each sample was used to perform our assay according to the protocols described in Sections 2.5 and 2.6 in the main text. Herein, a published primer set was utilized to set up the reactions. Positive control was performed as well using SARS-CoV-2 pseudovirus. The results were shown in **Figure S8**.

S2. Figures

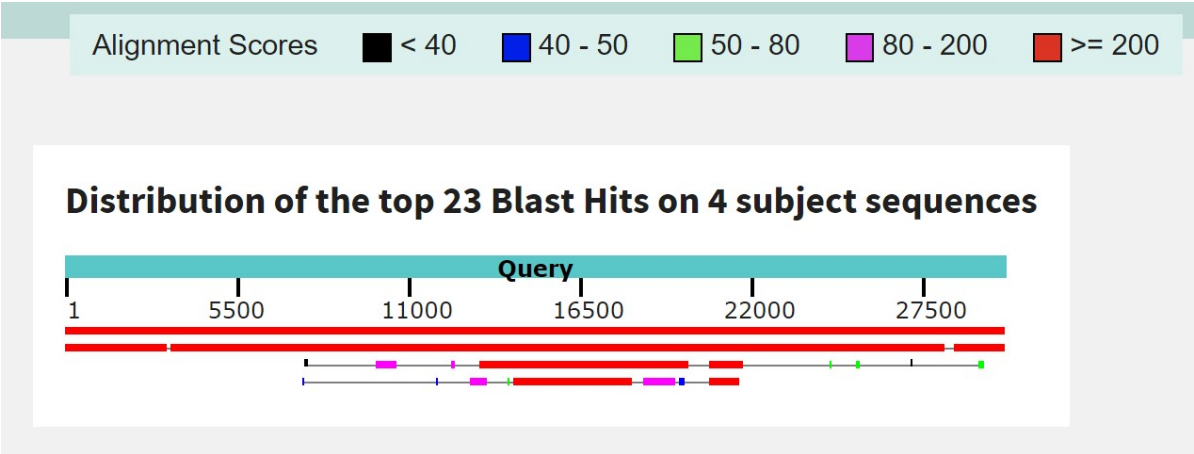

Figure S1. Whole genome sequence alignment of SARS-CoV-2 with that of other coronaviruses using the online BLASTN software. The Query is SARS-CoV-2 (NC_045512) sequence, and the other sequences from top to down are GX/P2V (MT072864), SARS-CoV (DQ497008), MERS-CoV (KP209310), HCoV-229E (KU291448).

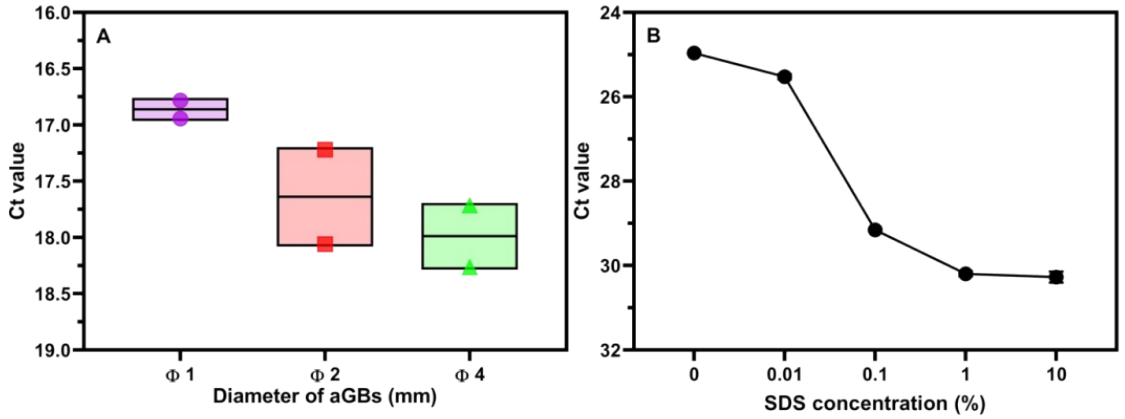

Figure S2. The effect of aGBs diameter on viral RNA extraction efficiency (A), the effect of detergent SDS on viral RNA extraction efficiency (B).

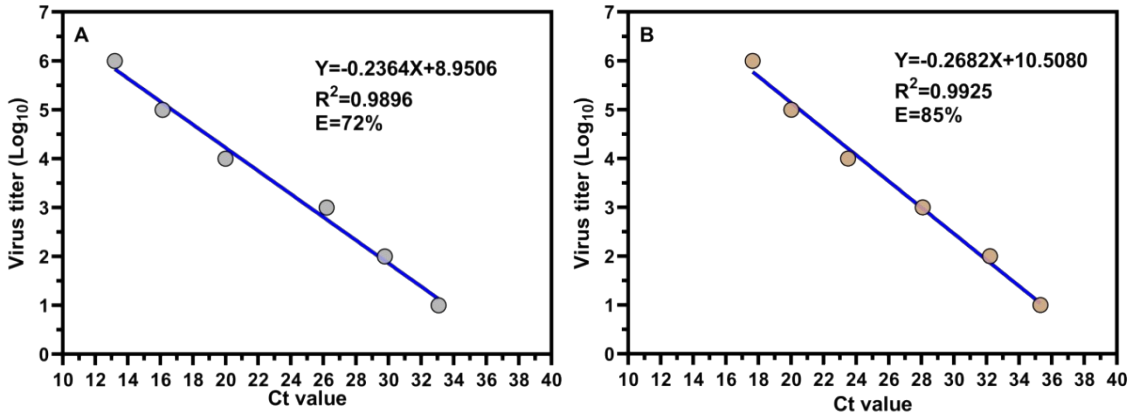

Figure S3. The PCR amplification efficiency obtained by using GX/P2V RNA as template. The RNA template was extracted either by AXYGEN Kit (A) or our developed aGBs-based method (B). The amplification efficiency is calculated as: $E = (10^{(-slope)} - 1) \times 100\%$, and the slope values were showed on above figures.

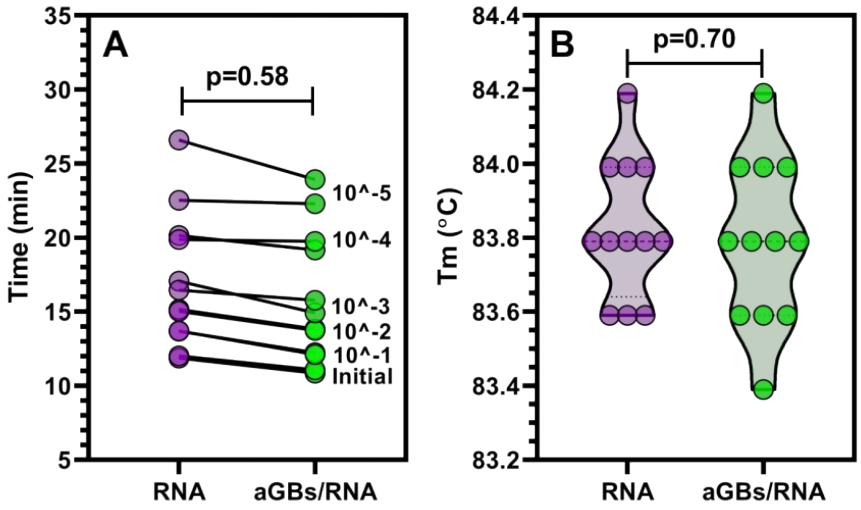

Figure S4. Comparison of the amplification efficiency of RT-LAMP reaction by using either viral RNA or aGBs/RNA composite as templates. The time-to-positive results of six 10-fold serially diluted samples (A), and the corresponding Tm values (B). The above fluorescence RT-LAMP reactions were performed in a real-time qPCR instrumentation through the SYRB® channel at 65℃ for 90 cycles with each cycle for 30 s to collect fluorescence data.

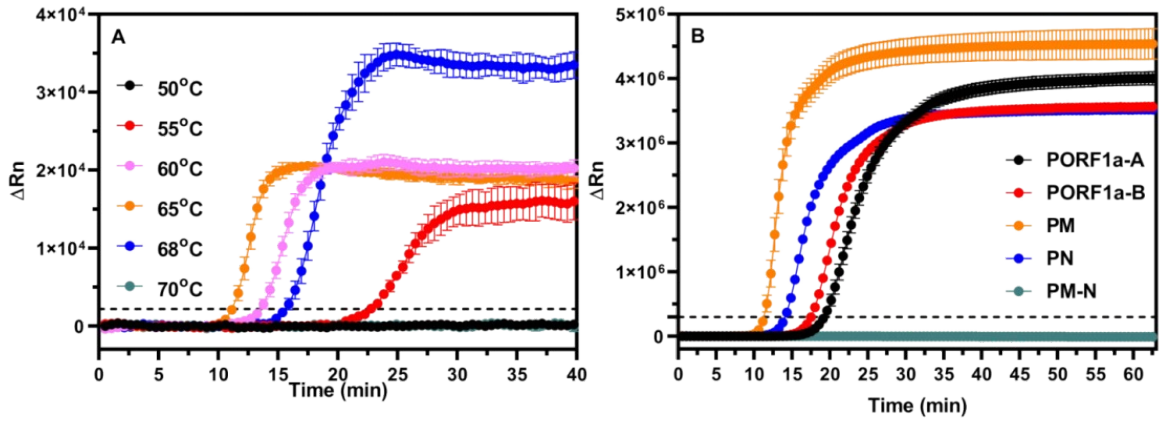

Figure S5. Optimization of the LAMP reaction temperature by amplifying GX/P2V M gene (B), and screening of the LAMP primers for GX/P2V (A). These tests were ran by employing the fluorescent RT-LAMP assay in a real-time qPCR instrument through SYBR® channel (65℃ for 30 s, 120 cycles). Reaction time, which is corresponding to the intersection of the threshold line and amplification curve in *x* axis, were used here to evaluate amplification efficiency of the RT-LAMP reaction.

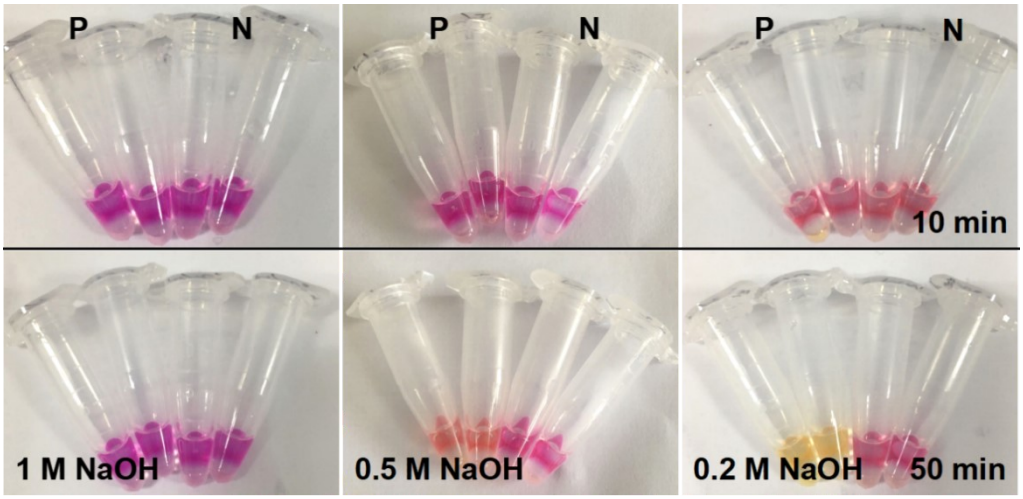

Figure S6. Optimization of the sensitivity of the color reaction of the all-in-one in situ RT-LAMP assay using various concentrations of sodium hydroxide solution. P indicates the positive reactions using GX/P2V virus particles as the target, while N indicates the negative reactions using 1× PBS as the no-template control. When 1 μL 1 M NaOH was added to 50 μL LAMP reaction buffer, the pH of reaction solution increased shown by a dark purple coloration. After incubation for 50 min, no color change was observed for the positive sample. This might be due to the denaturation of DNA polymerase in the high concentration of NaOH solution. When 1 μL 0.5 M NaOH was added to the LAMP system, the positive reactions were changed from purple to red after incubation for 50 min. While 1 μL 0.2 M NaOH was added to the LAMP buffer, the positive reactions exhibited a distinct color change from dark red to yellow after incubation for 50 min. In those tests, all negative reactions showed negative results without color change.

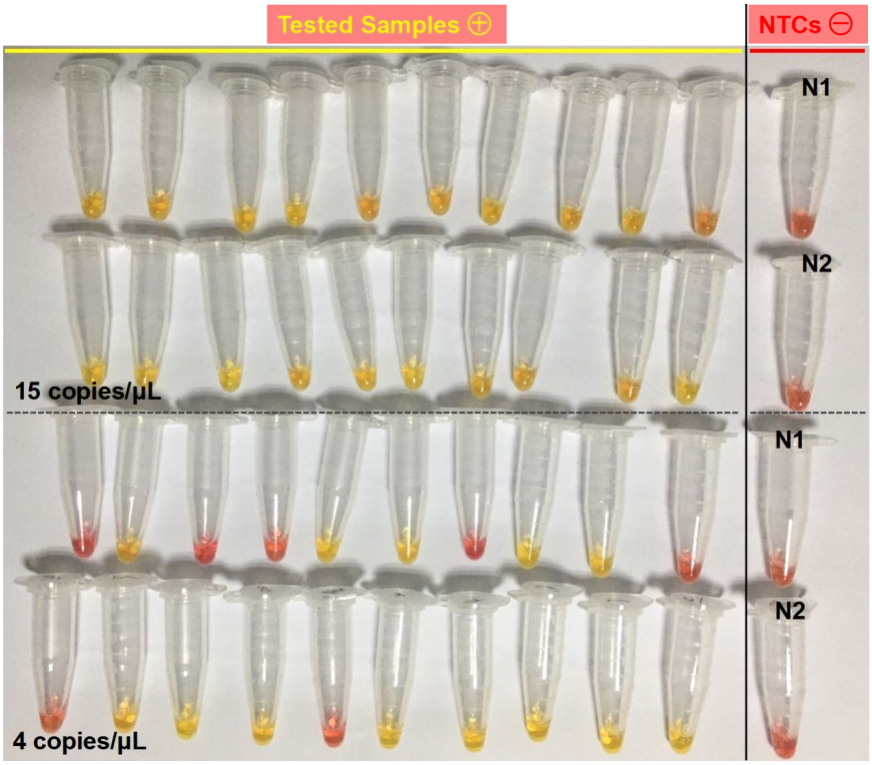

Figure S7. All-in-one in situ colorimetric RT-LAMP results of simulated GX/P2V samples.

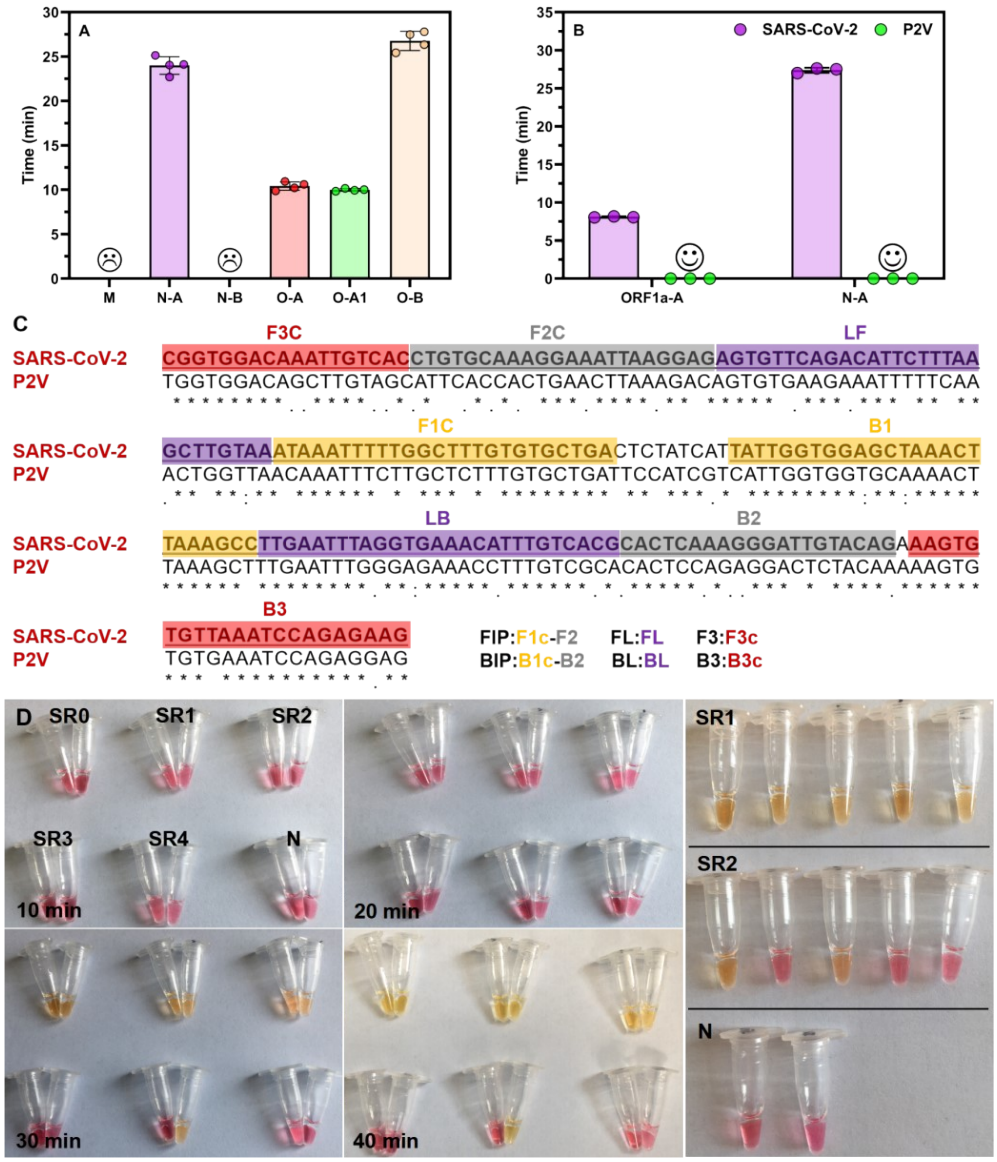

Figure S8. Screening of the LAMP primers for SARS-CoV-2 testing (A), analytical specificity of the LAMP assay using primer sets O-A1 and N-A, respectively (B), a sequence alignment of the LAMP amplicon between the O-A1 region of SARS-CoV-2 and GX/P2V (C), and the LOD of the LAMP assay (D).

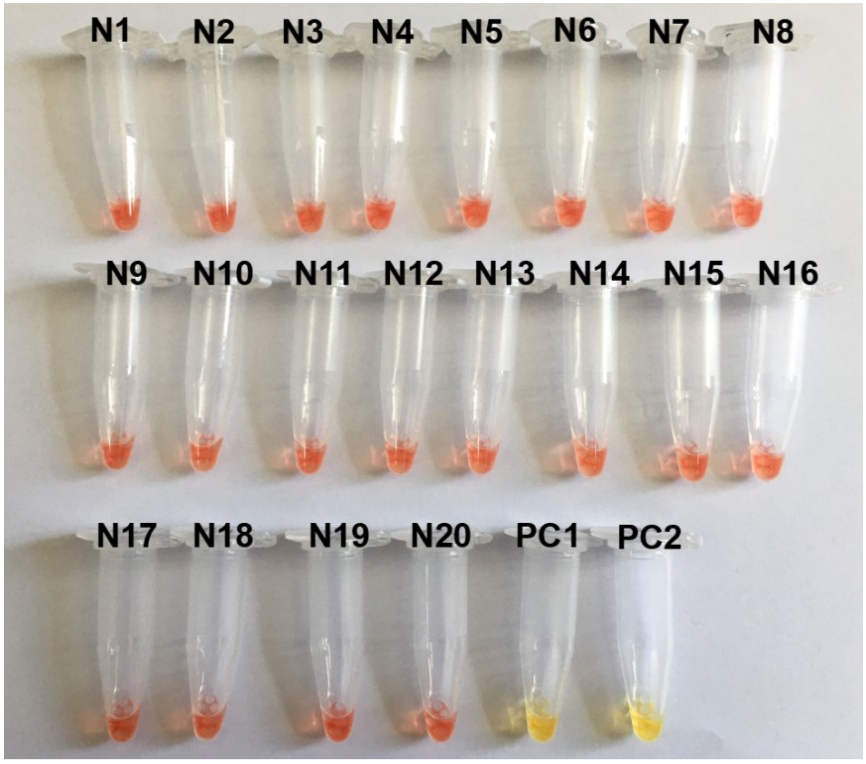

Figure S9. Determining the specificity of the all-in-one in situ colorimetric RT-LAMP assay for detecting SARS-CoV-2 RNA by using 20 negative samples. PC1 and PC2 indicate the positive control reaction.

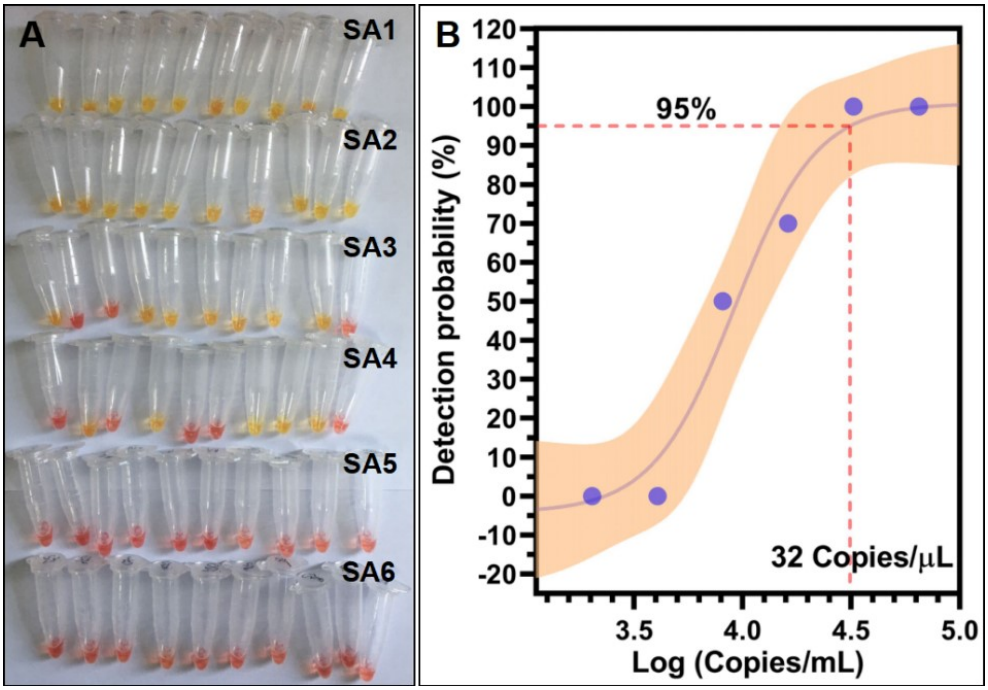

Figure S10. LOD determination of the all-in-one in situ colorimetric RT-LAMP assay for detecting simulated SARS-CoV-2 pseudovirus samples using a probit analysis. The all-in-one RT-LAMP results (A), and the plot of a sigmoidal concentration-response curve where the detection probability is plotted relative to the concentration of SARS-CoV-2 pseudovirus (B). Six 2-fold serially diluted samples with 10 replicates at each concentration were used to assess the LOD of our developed assay, the concentrations of simulated SARS-CoV-2 pseudovirus samples decreased from top to down (See Table S4 for more details). The green region surrounded the plot indicates the 95% confidence interval.

S3. Tables

Table S1. The LAMP and RT-qPCR primer sequences used in this work.

Name	Sequence (5'→3')	Length (bp)
SORF1a-A-F3	CGGTGGACAAATTGTCAC	18
SORF1a-A-B3	CTTCTCTGGATTTAACACACTT	22
SORF1a-A-LF	TTACAAGCTTAAAGAATGTCTGAACACT	28
SORF1a-A-LB	TTGAATTTAGGTGAAACATTTGTCACG	27
SORF1a-A-FIP	TCAGCACACAAAGCCAAAAATTTATCTGTGCAAAGGAAATTAAGGAG	47
SORF1a-A-BIP	TATTGGTGGAGCTAAACTTAAAGCCCTGTACAATCCCTTTGAGTG	45
SORF1a-A1-FIP	TCAGCACACAAAGCCAAAAATTTAT**TTTT**CTGTGCAAAGGAAATTAAGGAG	51
SORF1a-A1-BIP	TATTGGTGGAGCTAAACTTAAAGCC**TTTT**CTGTACAATCCCTTTGAGTG	49
SORF1a-B-F3	CTGCACCTCATGGTCATGTT	20
SORF1a-B-B3	GATCAGTGCCAAGCTCGTC	19
SORF1a-B-FIP	GAGGGACAAGGACACCAAGTGTGGTAGCAGAACTCGAAGGC	41
SORF1a-B-BIP	CCAGTGGCTTACCGCAAGGTTTTAGATCGGCGCCGTAAC	39
SORF1a-B-LF	ACCACTACGACCGTACTGAAT	21
SORF1a-B-LB	TTCGTAAGAACGGTAATAAAGGAGC	25
SN-A-F3	TGGCTACTACCGAAGAGCT	19
SN-A-B3	TGCAGCATTGTTAGCAGGAT	20
SN-A-FIP	TCTGGCCCAGTTCCTAGGTAGTCCAGACGAATTCGTGGTGG	41
SN-A-BIP	AGACGGCATCATATGGGTTGCACGGGTGCCAATGTGATCT	40
SN-A-LF	GGACTGAGATCTTTCATTTTACCGT	25
SN-A-LB	ACTGAGGGAGCCTTGAATACA	21
SN-B-F3	CCAGAATGGAGAACGCAGTG	20
SN-B-B3	CCGTCACCACCACGAATT	18
SN-B-FIP	AGCGGTGAACCAAGACGCAGGGCGCGATCAAAACAACG	38
SN-B-BIP	AATTCCCTCGAGGACAAGGCGAGCTCTTCGGTAGTAGCCAA	41
SN-B-LF	TTATTGGGTAAACCTTGGGGC	21
SN-B-LB	TTCCAATTAACACCAATAGCAGTCC	25
SM-F3	GGTTTCCTATTCCTTACATGGA	22
SM-B3	TGAAGTAGCTGAGCCACATC	20
SM-FIP	GGCCATAACAGCCAGAGGAAAATTTGTCTTCTACAATTTGCCTA	44
SM-BIP	TTTTGTGCTTGCTGCTGTTTACAAGCCTACAAGACAAGCC	40
SM-LF	ATACAAAAACCTATTCCTGTTGGCA	25
SM-LB	AGAATAAATTGGATCACCGGTGGAA	25
PORF1a-A-F3	CAGGTACTGGTCAGGCAA	18
PORF1a-A-B3	ACAGACTGTGTTTTTAAGTGTA	22
PORF1a-A-FIP	CTATGTGGCATCTACAGTACAAGCACCGGAAGCCAATATGGATC	44
PORF1a-A-BIP	TAACCCTAAAGGTTACTGTGAGCTCCCACTGGGTCATTAGCA	42
PORF1a-A-FL	CACCACCAAAGGATTCTT	18
PORF1a-A-BL	GTACAAATACCTACCACTTG	20
PORF1a-B-F3	TGGTGGACAGCTTGTAGC	18
PORF1a-B-B3	CTCCTCTGGATTTCACACACTT	22
PORF1a-B-FIP	TCAGCACAAAGAGCAAGAAATTTGTATTCACCACTGAACTTAAAGAC	47
PORF1a-B-BIP	CATTGGTGGTGCAAAACTTAAAGCTTTGTAGAGTCCTCTGGAGTG	45
PORF1a-B-LF	TAACCAGTTTGAAAAATTTCTTCACACT	28

PORF1a-B-LB	TTGAATTTGGGAGAAACCTTTGTCGCA	27
PM-F3	GGTTTCCTATTTCTAACATGGA	22
PM-B3	TGAAGTAGCTAAGCCACATC	20
PM-FIP	GGCCAAAGTAGCCAGAGGAAAATTTGTCTTTTACAGTTCGCCTA	44
PM-BIP	CTTTGTGCTTGCTGCTGTTTACAAGCCCACAAGACAAGTC	40
PM-FL	GTACAGAAACCTATTCCTGTTAGCA	25
PM-BL	AGAATCAATTGGATTACCGGTGGAA	25
PN-F3	TGACCAAATTGGCTACTACCG	21
PN-B3	TCCTTGAGGAAGTTGTAGCAC	21
PN-FIP	CCAGCTTCTGGCCCAGTTCCTTCGTGGTGGTGACGGTA	38
PN-BIP	TATGGGTTGCAACTGAGGGAGCTGTTGTTTGGATTGCGGGT	41
PN-FL	CGTGGACTGAGATCTTTCATTT	22
PN-BL	ACACCAAAAGATCACATTGG	20
M-P2VF1	TTCGCCTATGCTAACAGGAATAG	23
M-P2VR1	TAAACAGCAGCAAGCACAAAG	21
M-P2VF2	GGTCCTTCAATCCAGAAACAAAC	23
M-P2VR2	TCACAGCACCGATGACAAG	19
M-P2VF3	CTTTGTGCTTGCTGCTGTTTA	21
M-P2VR3	GCCTGAATGAAGCAATGAAGTAG	23
rdrp-P2VF1	GATCTCAATGGGAACTGGTATGA	23
rdrp-P2VR1	GCCCTCGTAAGTGTGAGAATAG	22
rdrp-P2VF2	CACACCTTATGGGTTGGGATTA	22
rdrp-P2VR2	GTTTGCGAGCAAGAACAAGAG	21
rdrp-P2VF3	GCTGTAGTTTGTCACATCGTTTC	23
rdrp-P2VR3	TGTACCACCTGGTTTCACATATAG	24

Table S2 Screening of the real-time PCR primers for GX/P2V RNA detection.

Name	Ct				Tm (℃)			
	With template		No-template		With template		No-template	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
M-P2V1	17.24	0.17	UD	/	76.90	0.00	/	/
M-P2V2	17.51	0.06	UD	/	78.12	0.00	/	/
M-P2V3	**16.78**	**0.26**	**UD**	**/**	**79.81**	**0.00**	**/**	**/**
rdrp-P2V1	15.57	0.09	35.29	0.07	76.74	0.00	73.98	0.22
rdrp-P2V2	15.15	0.12	UD	/	77.05	0.00	/	/
rdrp-P2V3	18.08	0.01	39.16	/	77.20	0.00	74.13	/

“SD” is the abbreviation of “Standard Deviation”, and the “UD” indicates undetermined. M-P2V3 primer set was here used to detect GX/P2V RNA by RT-qPCR through SYBR® channel in our study.

Table S3. Comparison of the LODs of various methods for GX/P2V RNA detection.

Sample	Copy/μL	qPCR		LAMP				in situ LAMP	
				Fluorescence		Colorimetric		Colorimetric	
		Ct	Result	Time	Result	Color	Result	Color	Result
S0	2.93×10^5	17.94	+[#2]	11.82	+	Yellow	+	Yellow	+
S1	2.93×10^4	21.17	+	13.15	+	Yellow	+	Yellow	+
S2	2.93×10^3	24.56	+	15.99	+	Yellow	+	Yellow	+
S3	2.93×10^2	28.31	+	17.40	+	Yellow	+	Yellow	+
S4	2.93×10^1	32.48	+	24.61	+/−	Yellow/Red	+/−	Yellow	+
S5	**2.93×10^0**	**35.76**	**+**	**UD**	**−**	**Red**	**−**	**Yellow**	**+**
S6	2.93×10^{-1}	/	/	/	/	Red	−	Yellow/Red	+/−
N	0	UD[#1]	−[#3]	UD	−	Red	−	Red	−

[#1], represents undetermined; [#2], represents positive result; [#3], represents negative result; [#4], represents inconclusive result. All the tests were performed with 2 technical replicates.

Table S4. LOD of the all-in-one in situ RT-LAMP assay for detecting SARS-CoV-2 RNA samples.

Name	Copy/mL	Copy/μL	Copy/reaction (2 μL)	Detection probability (%)
SR0	400000.0	400.0	800.0	2/2
SR1	40000.0	40.0	80.0	7/7
SR2	4000.0	4.0	8.0	4/7
SR3	400.0	0.4	0.8	0/2
SR4	40.0	0.04	0.08	1/2
N	0.0	0.0	0.0	0/4

Table S5. LOD of the all-in-one in situ RT-LAMP assay for detecting simulated SARS-CoV-2 pseudovirus samples.

Name	Copy/mL	Copy/μL	Copy/reaction (200 μL)	Detection probability (%)	
SA1	65000.0	65.0	13000.0	10/10	100
SA2	32500.0	32.5	6500.0	10/10	100
SA3	16250.0	16.3	3250.0	7/10	70
SA4	8125.0	8.1	1625.0	5/10	50
SA5	4062.5	4.1	812.5	0/10	0
SA6	2031.3	2.0	406.3	0/10	0

Reference

[1] Nawattanapaiboon K, Pasomsub E, Prombun P, et al. Colorimetric reverse transcription loop-mediated isothermal amplification (RT-LAMP) as a visual diagnostic platform for detection of the emerging coronavirus SARS-CoV-2. *Analyst*, 2021, 146 (471).