Denaturation bubble-mediated two-stage isothermal nucleic acid amplification in a single closed tube

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Supplementary Information

Materials and reagents

Staphylococcus aureus (ATCC 25923), *Escherichia coli* O157:H7 (ATCC 35150), *Salmonella typhimurium* (ATCC 14028), and *Vibrio Parahemolyticus* (ATCC 17802) strains, purified RNA of SARS-CoV-2, influenza A (H1N1) virus, influenza A (H3N2) virus, and influenza B (Victoria) virus, as well as Colorimetric/Fluorescence Iso Buffer, were provided by Navid Biotechnology Co., Ltd (Qingdao, China). *Bst* 2.0 WarmStart DNA Polymerase, Extreme Thermostable Single Strand Binding (ET-SSB), 10× Isothermal Amplification Buffer II, ThermoPol Reaction Buffer (10×), Exonuclease III, and Exonuclease I were purchased from New England Biolabs Inc. (Beijing, China). PEG-200 and neutral red staining solution (1 g/L) were purchased from Sangon Biotech Co., Ltd (Shanghai, China). HiScript II Reverse Transcriptase, T4 DNA Ligase, and 10× Ligase Buffer were purchased from Vazyme Biotechnology Co., Ltd (Nanjing, China). 31000 EvaGreen Dye (20× in water) was purchased from Biotium, Inc. (CA, USA). TIANamp Bacteria DNA Kit and dNTPs were purchased from Tiangen Biotech Co., Ltd (Beijing, China). All the other chemicals and reagents were of analytical grade.

The padlock probes (PPs) for preparing dumbbell probes (DPs), as well as oligonucleotides primers involved in this work were designed and optimized by NUPACK software (http://www.nupack.org/) and NCBI primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast), and synthesized by Sangon Biotech Co., Ltd (Shanghai, China), whose sequences were shown in Table S1. All the padlock probes are chemically 5'-phosphorylated.

The procedure of amplification reactions

SEA was conducted in a 20 μL reaction mixture containing 2 μL sample, 1.6 μL dNTPs (10 mM each), 2 μL forward and reverse primer (10 μM each), 0.4 μL *Bst* 2.0 WarmStart DNA Polymerase (8 U/μL), 2 μL 10× Isothermal Amplification Buffer II, 0.5 μL EvaGreen Dye, 0.5 μL PEG-200, 0.2 μL ET-SSB (0.5 μg/μL), 8.8 μL nuclease-free water and 0.3 μL HiScript II Reverse Transcriptase (200 U/μL, for RNA targets only). The reaction mixture of RC-SEA was designed based on SEA with some modifications. Specifically, 2 μL of the prepared DP was added to the reaction mixture. Besides, for ssRNA targets detection, the amount of forward primer (10 μM) was altered to 1 μL. Correspondingly, the amount of nuclease-free water was adjusted to keep the total volume constant. SEA and RC-SEA were performed by a CFX Connect™ Real-Time PCR System (Bio-Rad, CA, USA) at 61℃ for 60 min, and the fluorescence signal was monitored at 1-min intervals.

LAMP was employed as a comparison to evaluate the performance of RC-SEA, which was performed by adding 2.5 μL sample, 4 μL dNTPs (10 mM each), 0.5 μL F3 and B3 (10 μM each), 0.4 μL FIP and BIP (100 μM each), 0.2 μL LF and LB (100 μM each), 0.5 μL *Bst* 2.0 WarmStart DNA polymerase (8 U/μL), 2.5 μL 10× ThermoPol reaction buffer, 0.5 μL $MgSO_4$ solution (100 mM), 0.625 μL EvaGreen Dye, 0.3 μL HiScript II Reverse Transcriptase (200 U/μL) and 11.875 μL nuclease-free water into a total volume of 25 μL at 65℃ for 60 min. LAMP was performed by a CFX Connect™ Real-Time PCR System (Bio-Rad, CA, USA), and the fluorescence signal was monitored at 1-min intervals.

The reaction mixture of colorimetric SEA, RC-SEA, and LAMP assays were

prepared by replacing the reaction buffer in each fluorescence reaction mixture with an equal volume of Colorimetric/Fluorescence Iso Buffer and adding 2 μL neutral red stain solution (1g/L). The amount of nuclease-free water was also adjusted to keep the total volume constant. The colorimetric assays were conducted in a dry bath module at 61℃ (SEA and RC-SEA) or 65℃ (LAMP) for 60 min, and the results were directly read by the naked eye after placing the tubes on white cardboard.

Dumbbell probe preparation

The DPs were prepared by ligating padlock probes with T4 DNA ligase. Briefly, the padlock probe of 1 μM was first heated at 95℃ for 5 min and then cooled to room temperature to ensure the self-complementary of the probe. Subsequently, 10 μL padlock probe was mixed with 2 μL T4 DNA Ligase (800 U), 2 μL 10× Ligase Buffer, and 6 μL nuclease-free water to comprise the reaction mixture with a total volume of 20 μL, which was incubated at 16℃ overnight for self-ligation of the padlock probe. The T4 DNA ligase was inactivated by incubating the reaction mixture at 65℃ for 10 min and thereby terminated the self-ligation reaction. Afterward, 0.5 μL Exonuclease III (50 U) and 0.5 μL Exonuclease I (10 U) were added to the reaction mixture to digest the padlock probes that failed to self-ligate. Finally, the reaction mixture was incubated at 80℃ for 20 min to inactivate the two exonucleases. The prepared DP was stored at 4℃ for subsequent use.

To check whether the padlock probes were successfully sealed, we designed two primers with the sequences complementary to the two loops. Two microliters of each

primer (10 μM each) and 4 μL ligation products were added into the SEA reaction system, which was then incubated at 61℃ for 60 min. The same amount of untreated padlock probes replaced the ligation products to add into the SEA reaction system for comparison. The amplification products were subjected to native PAGE analysis. In the presence of the sealed DPs, the RCA reaction will be triggered to generate amplicons with different lengths, and thereby multiple bands will appear in the native PAGE image. In contrast to the sealed DPs, the padlock probes cannot trigger exponential amplification, therefore only the bands representing padlock probes and primers can be observed in the native PAGE image.

Multiparametric optimization

Six parameters of RC-SEA, including SEA primer concentration, the DP stem length, DP concentration, *Bst* DNA polymerase dosage, dNTPs concentration, and reaction temperature were optimized by keeping all the parameters constant except the one to be optimized. DNA extract of 1.0×10^6 CFU/mL *S. aureus* and 1.0×10^5 copies/mL SARS-CoV-2 RNA were employed as the templates. In SEA primer set concentration optimization for DNA target detection, the parameters to be optimized were SEA primer set concentration: 0.5, 1.0, 1.5, 2.0, 2.5 μM; DP stem length: 24-nt; DP concentration: 1.0 nM; *Bst* DNA polymerase dosage: 3.2 U; dNTPs concentration: 0.8 mM; reaction temperature: 61℃. In SEA primer concentration optimization for RNA target detection, the forward primer concentration was fixed to 1.0 μM, while the forward primer concentration was set to 1.0, 0.5, 0.25, 0.125, 0.0625 μM. The other

parameters were the same as mentioned above. In DP stem length optimization, corresponding conditions were SEA primer set concentration: 1.0 μM; DP stem length: 12-, 16-, 20-, 24-, 28-bp; DP concentration: 1.0 nM; *Bst* DNA polymerase dosage: 3.2 U; dNTPs concentration: 0.8 mM; reaction temperature: 61℃. In DP concentration optimization, corresponding conditions were SEA primer set concentration: 1.0 μM; DP stem length: 24-nt; DP concentration: 100, 10, 1, 0.1, 0.01 nM; *Bst* DNA polymerase dosage: 3.2 U; dNTPs concentration: 0.8 mM; reaction temperature: 61℃. In *Bst* DNA polymerase dosage optimization, corresponding conditions were SEA primer set concentration: 1.0 μM; DP stem length: 24-nt; DP concentration: 1 nM; *Bst* DNA polymerase dosage: 2.4, 3.2, 4.0, 4.8, 5.6 U; dNTPs concentration: 0.8 mM; reaction temperature: 61℃. In dNTPs concentration optimization, corresponding conditions were SEA primer set concentration: 1.0 μM; DP stem length: 24-nt; DP concentration: 1 nM; *Bst* DNA polymerase dosage: 3.2 dNTPs concentration: 0.4, 0.6, 0.8, 1.0, 1.2 mM; reaction temperature: 61℃. In reaction temperature optimization, corresponding conditions were SEA primer set concentration: 1.0 μM; DP stem length: 24-nt; DP concentration: 1 nM; *Bst* DNA polymerase dosage: 3.2 dNTPs concentration: 0.8 mM; reaction temperature: 60℃, 61℃, 62℃, 63℃ and 64℃.

Feasibility, specificity, and sensitivity evaluation

S. aureus suspension was ten-fold serially diluted from 1.0×10^8 to 1.0×10^2 CFU/mL, whose genomic DNA was extracted by a TIANamp Bacteria DNA Kit

according to the manufacturer's instruction for comparison. SARS-CoV-2 RNA was ten-fold serially diluted from 1.0×10^8 to 1.0×10^2 copies/mL. The prepared DNA and RNA solution was used as templates for feasibility, specificity, and sensitivity evaluation. Specifically, DNA extract of 1.0×10^8 CFU/mL *S. aureus* and 1.0×10^8 copies/mL SARS-CoV-2 RNA were employed as the templates for feasibility evaluation of RC-SEA on DNA and RNA targets, respectively. Besides, DNA extract of 1.0×10^7 CFU/mL *S. aureus* and 1.0×10^8 copies/mL SARS-CoV-2 RNA, as well as the DNA or RNA from the same amount of the pathogens that may cause similar symptoms, including *E. coli* O157:H7, *S. typhimurium* and *V. Parahemolyticus*, as well as influenza A (H1N1) virus, influenza A (H3N2) virus, and influenza B (Victoria) virus were employed as the templates for specificity evaluation of RC-SEA on DNA and RNA targets, respectively. Moreover, DNA and RNA mixtures with various ratios of the target nucleic acid (100%, 10%, 1%, 0.1%, 0.01% and 0%) were prepared by mixing different amount of target nucleic acid with other irrelevant DNA or RNA from above non-target pathogens, which were used as the templates to assess the anti-jamming capability of RC-SEA. The irrelevant nucleic acid from different non-target pathogens in the mixtures were of equal ratio. The gradient-diluted DNA and RNA solutions were used as templates for sensitivity evaluation of the RC-SEA on DNA and RNA targets, respectively.

Table S1. Sequences of nucleic acids used in this work

		Sequence (5'-3')
***S. aureus* 16S rRNA ([a] D83356.1)**		
[b] RC-SEA primer set	Primer F	GAATTCCATGTGTAGCGGTGAA
	Primer R	TTCCTCCATATCTCTGCGCAT
	[c] PP-12bp	[d] CACTAGGAATTCCATGTGTAGCGGTGAACTAGTGGCACACTTCCTCCATATCTCTGCGCATGTGTGC
	PP-16bp	CACTAGTTGAATTCCATGTGTAGCGGTGAAAACTAGTGGCACACCATTCCTCCATATCTCTGCGCATTGGTGTGC
	PP-20bp	CACTAGTTCCGAATTCCATGTGTAGCGGTGAAGGAACTAGTGGCACACCAACTTCCTCCATATCTCTGCGCATGTTGGTGTGC
	PP-24bp	CACTAGTTCCAGGAATTCCATGTGTAGCGGTGAACTGGAACTAGTGGCACACCAACAATTCCTCCATATCTCTGCGCATTTGTTGGTGTGC
	PP-28bp	CACTAGTTCCAGCTGAATTCCATGTGTAGCGGTGAAAGCTGGAACTAGTGGCACACCAACAAGTTTCCTCCATATCTCTGCGCATACTTGTTGGTGTGC
LAMP primer set [1]	F3	CGTGGGGATCAAACAGGATT
	B3	CATGCTCCACCGCTTGTG
	FIP	TAGCTGCAGCACTAAGGGGC-CCACGCCGTAAACGATGAG
	BIP	ACGCATTAAGCACTCCGCCT-GGGTCCCCGTCAATTCCT
	LF	GGAAACCCCCTAACACT
	LB	GGGGAGTACGACCGCAAGGT
SARS-COV-2 N gene ([a] KF149982.1)		
[b] RC-SEA primer set	Primer F	AAGCCTCTTCTCGTTCCTCAT
	Primer R	TTGAACTGTTGCGACTACGTG
	PP	CACTAGTTCCAGAAGCCTCTTCTCGTTCCTCATCTGGAACTAGTGGCACACCAACAATTGAACTGTTGCGACTACGTGTTGTTGGTGTGC
LAMP primer set [2]	F3	AGATCACATTGGCACCCG
	B3	CCATTGCCAGCCATTCTAGC
	FIP	TGCTCCCTTCTGCGTAGAAGCCAATGCTGCAATCGTGCTAC
	BIP	GGCGGCAGTCAAGCCTCTTCCCTACTGCTGCCTGGAGTT
	LF	GCAATGTTGTTCCTTGAGGAAGTT
	LB	GTTCCTCATCACGTAGTCGCAACA

[a] GenBank accession number.

[b] Primers F and R of RC-SEA were also employed for SEA reaction.

[c] PP represented the padlock probe. The numbers of base pairs represented the length of the stem of the dumbbell primer.

[d] The double underline sequence was the same with Primer F in RC-SEA primer set. The wavy line sequence was with Primer R in the RC-SEA primer set.

1. Z. Xu, L. Li, J. Chu, B. M. Peters, M. L. Harris, B. Li, L. Shi and M. E. Shirtliff, Food Res Int, 2012, 47, 166-173.

2. W. E. Huang, B. Lim, C. C. Hsu, D. Xiong, W. Wu, Y. Yu, H. Jia, Y. Wang, Y. Zeng, M. Ji, H. Chang, X. Zhang, H. Wang and Z. Cui, Microb Biotechnol, 2020, 13, 950-961.

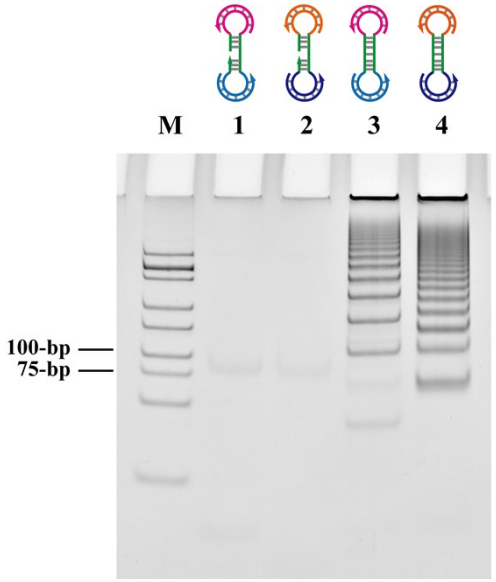

Fig.S1. Native PAGE image of the products of the amplification reactions with the untreated padlock probes and those treated with T4 DNA ligase. M represented DNA marker (25-500 bp). Lanes 1 and 2: amplicons of the reaction with untreated padlock probes for *S. aureus* 16S rDNA and SARS-CoV-2 N gene detection; lanes 3 and 4: amplicons of the reaction with the corresponding padlock probes treated with T4 DNA ligase.

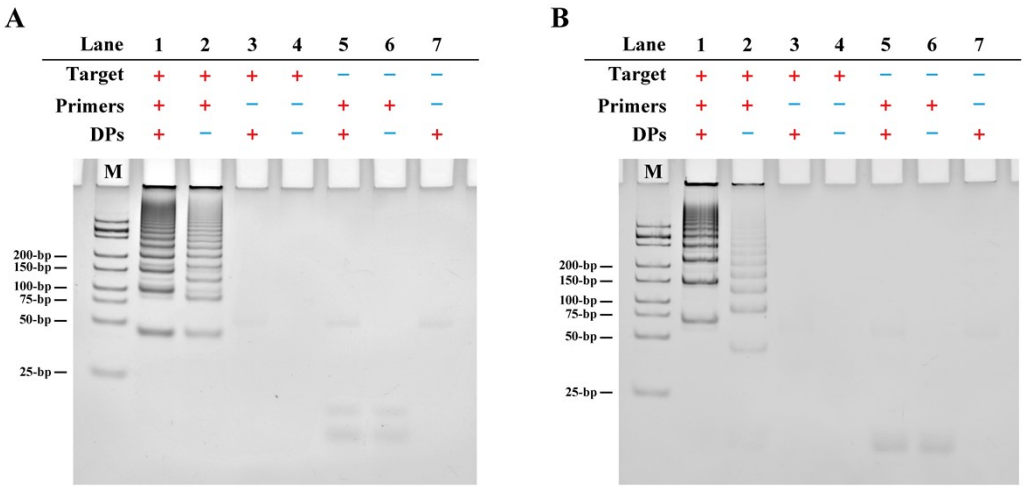

Fig.S2. Validation of the RC-SEA principle. Native PAGE analysis image of RC-SEA process for (A) *S. aureus* 16S rDNA and (B) SARS-CoV-2 N gene detection. Lane 1: target + primers + DPs; lane 2: target + primers; lane 3: target + DPs; lane 4: target; lane 5: primers + DPs; lane 6: primers; lane 7: DPs. M represented DNA marker (25-500 bp).

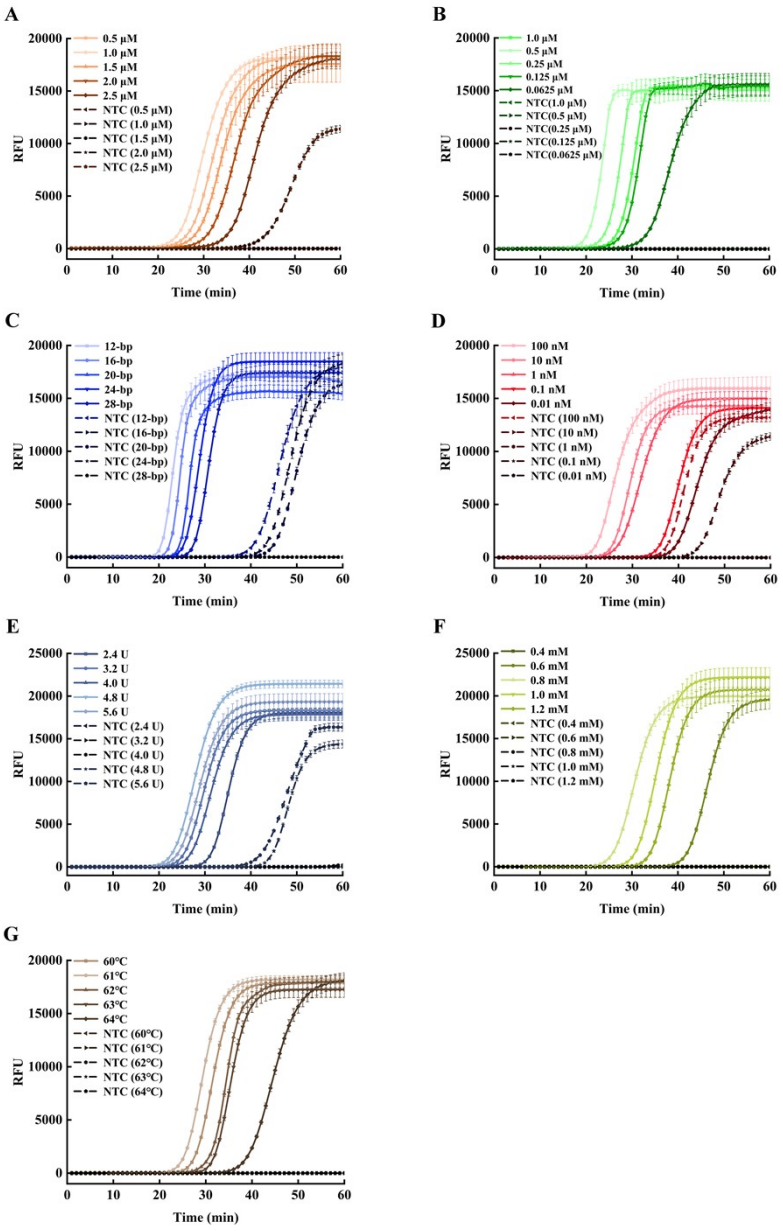

Fig.S3. Changes of fluorescence curves of RC-SEA with (A) different amounts of SEA primers, (B) different primer ratios, (C) DPs of different lengths, (D) different amounts of DPs, (E) different dosages of *Bst* DNA polymerase, (F) different amount of dNTPs, as well as (G) those performed at different temperature. NTC represented the no template control. All parameters remained the same except that which was to be optimized. Error bars represent the standard deviations of three independent measurements.

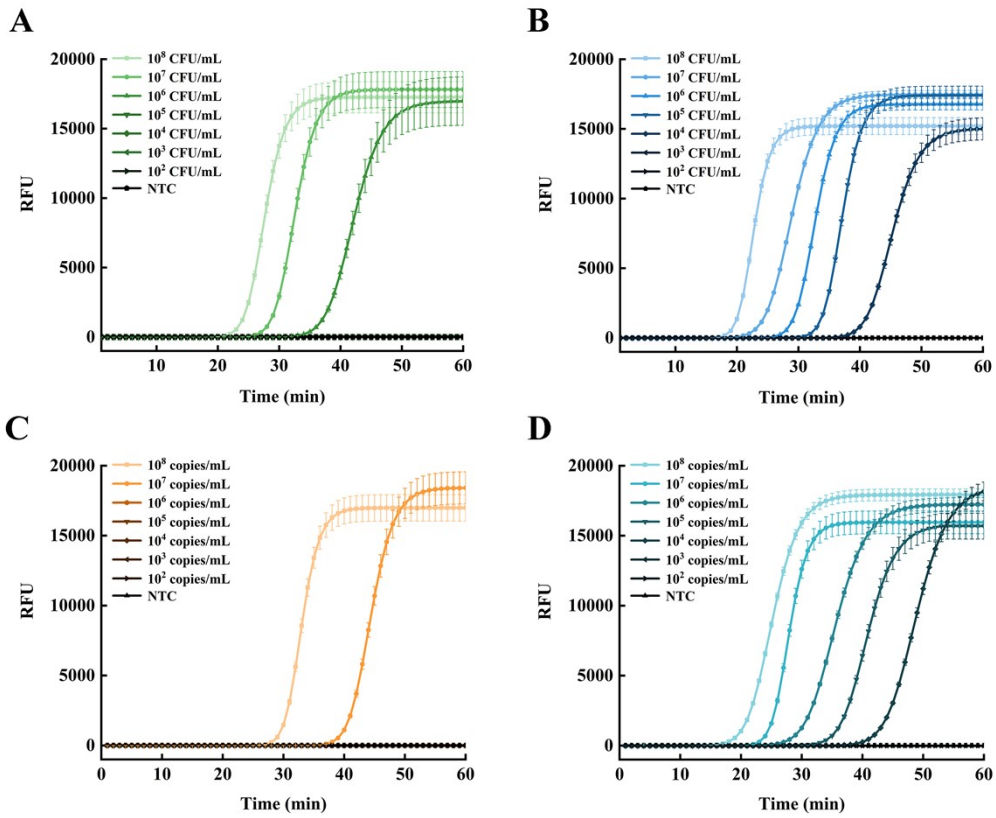

Fig.S4. Fluorescence curves of (A) SEA and (B) LAMP for *S. aureus* 16S rDNA detection in DNA extracts from 10-fold serial dilutions of the *S. aureus* suspension from 10^8 to 10^2 CFU/mL. Fluorescence curves of (C) SEA and (D) LAMP for SARS-CoV-2 N gene detection in 10-fold serial dilutions of SARS-CoV-2 RNA from 10^8 to 10^2 copies/mL. NTC represented no template control. Error bars represent the standard deviations of three independent measurements.

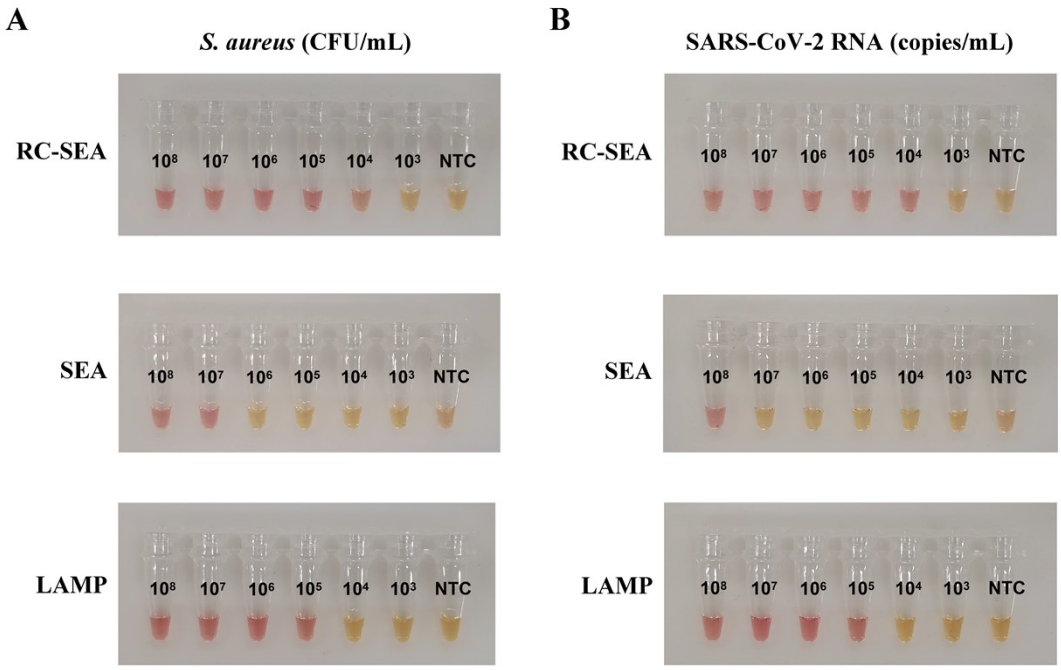

Fig.S5. Sensitivity of RC-SEA based colorimetric assays to DNA and RNA targets. RC-SEA, SEA, and LAMP based colorimetric assays for (A) *S. aureus* 16S rDNA detection in DNA extracts of 10-fold serial dilutions of the *S. aureus* suspension from 10^8 to 10^3 CFU/mL, and (B) SARS-CoV-2 N gene detection in 10-fold serial dilutions of SARS-CoV-2 RNA from 10^8 to 10^3 copies/mL. NTC represented no template control.