Supporting Information for:

A Serological Aptamer-Assisted Proximity Ligation Assay for COVID-19 Diagnosis and Seeking Neutralizing Aptamers

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Section A: Materials and reagents

All oligonucleotides used in this study were synthesized by Shanghai Sangon Biological Engineering Co., Ltd. (Shanghai, China) and purified by HPLC. For stock solution preparation, all DNA sequences were dissolved and diluted with DPBS buffer to the final concentration of 10 μM. After annealing at 90 °C for 5 min, the resulting solutions were cooled down to room temperature and stored at 4 °C until use. Their sequences are shown in Table S1.

SARS-CoV-2 Nucleocapsid Protein was provided by Cellregen LifeScience Co. (China). His-tagged SARS-CoV-2 Spike S1 Recombinant Protein (40591-V08b1), mFc-tagged human ACE2 (10108-H05H), SARS-CoV-2 Nucleoprotein antibody (Rabbit monoclonal antibody, 40143-R040), SARS-CoV-2 Nucleoprotein antibody (Rabbit monoclonal antibody, 40143-R004), his-tagged Human SARS Coronavirus Nucleoprotein (40143-V08B) and SARS-CoV-2 (2019-nCoV) Nucleoprotein ELISA Kit (KIT40588) were purchased from Sino Biological Inc. (China). Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP, A600974-0001) were purchased from Shanghai Sangon Biological Engineering Co., Ltd. (Shanghai, China). One Step TB Green RT-PCR Kit (RR066A) and TALON Metal Affinity Resins (635504) were purchased from Takara Biomedical Technology (Beijing) Co., Ltd. T4 DNA ligase (M0202S), 10X T4 DNA ligase buffer and 6×gel loading dye (B7025) were purchased from New England Biolabs (Beijing, China) Ltd. SYBR gold nucleic acid gel stain (S11494) and DPBS (14040141) were purchased from Invitrogen (Shanghai, China). SYBR Green I nucleic acid stain (DH395-2) were purchased from Beijing DingGuo Biotechnology Co. (Beijing, China). Normal human serum were purchased from Kejing Biological Technology Co., Ltd.(Jiangsu, China). Other reagents (analytical grade) were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). All solutions were prepared with Millipore water (18.25 $M\Omega \cdot cm^{-1}$).

Buffers used in this work:

DPBS buffer: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, pH 7.4.

1X TBE buffer: 9 mM Tris, 9 mM boric acid, 0.2 mM EDTA, pH 8.2.

1 M PBS buffer: 1370 mM NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 5.0

Section B: Table S1. Sequences of DNA oligonucleotides used in this work[a]

Name	Sequence (5'-3')
Proximity Probe A (PPA)	GCTGGATGTCGCTTACGACAATATTCCTTAGGGGCACCGCTACATTGACACATCCAGC**TTTTT**CCTTCGTTCGTTACTTCTG TTCGTTCTCATGACTGTATGTTAG
Proximity Probe B (PPB)	p-CATTGTAAGAGTATTATCCTAGTTCCTAGACACGACCAAGACAG**TTTTT**GCTGGATGTCACCGGATTGTCGGACATCGGA TTGTCTGAGTCATATGACACATCCAGC**TT**
N48	GCTGGATGTCGCTTACGACAATATTCCTTAGGGGCACCGCTACATTGACACATCCAGC
N58	GCTGGATGTCACCGGATTGTCGGACATCGGATTGTCTGAGTCATATGACACATCCAGC
Forward Primer-N (FP-N)	CGTTCGTTACTTCTGTTCGTTCTC
Reverse Primer-N (RP-N)	CTGTCTTGGTCGTGTCTAGGAAC
Connector-16 (con-16)	AATCTTACAATGCTAACAAA
Connector (con-18)	AATCTTACAATGCTAACATAAA
Connector-20 (con-20)	AATCTTACAATGCTAACATACAAA
Connector-22 (con-22)	AATCTTACAATGCTAACATACAGTAA
mutated PPA-1	GCTGGATGTCGCTTACGACAAT**G**TTCCTTAGG**A**GCACCGCTACATTGACACATCCAGC**TTTTT**CCTTCGTTCGTTACTTCTG TTCGTTCTCATGACTGTATGTTAG
mutated PPA-2	GCTGGATGTCGCTTA**AT**ACAAT**G**TTCCTTAGG**A**GCACCGCTACATTGACACATCCAGC**TTTTT**CCTTCGTTCGTTACTTCTG TTCGTTCTCATGACTGTATGTTAG
mutated PPB-1	p-CATTGTAAGAGTATTATCCTAGTTCCTAGACACGACCAAGACAG**TTTTT**GCTGGATGTCACCGGATTGTCGGACATC**AA**A TTGTCTGAGTCATATGACACATCCAGC**TT**
DNA for MAbA, S1 and N protein	SH-**TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT**CGTTACTTCTGTTCGTTCTCATGACTGTATGTTAG
DNA for MAbB and ACE2	p-CATTGTAAGAGTATGTATCCTAGTTCCTTGTCTGATCTCCACTG**TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT**-SH
Forward Primer-S (FP-S)	CGTTACTTCTGTTCGTTCTCATG
Reverse Primer-S (RP-S)	TGGAGATCAGACAAGGAACTAGG
Apt-S-79s	GTCTTGCGGGGCGGCGGGTTGAGAGGA
Apt-S-268s	GGGGTGGGGTAGTGGTATGGAGCG
Random DNA	CGCCTCGAGTGCAGCC

[a]Proximity Probe A is designed via prolonging the original sequence of aptamer N48 (red region, capable of specifically binding to N protein) by adding spacer region (black), forward primer region (yellow), and a connector region (blue) in the 3'-end. Proximity Probe B is designed via prolonging the original sequence of aptamer N58 (green region, capable of specifically binding to N protein) by adding spacer region (black), reverse primer region (yellow), and a connector region (blue) in the 5'-end. The underlined fragments indicate the mutated bases in proximity probes.

Section C: Experimental details

C1. Procedure for nucleocapsid protein detection

C1.1 Design of the aptamer-assisted proximity ligation assay (Apt-PLA)

The aptamer-assisted proximity ligation assay (Apt-PLA) system contains two key components: (1) two proximity ligation probes (PPA and PPB) that both consists of an aptamer region for target recognition, spacer region to minimize the structural steric hindrance during the assembly, PCR primer region, and ligation region with 5' phosphate modification; (2) an ssDNA connector as the template to assists the ligation by hybridization with two proximity probes at the ligation region.

Two reagent mix was prepared for the Apt-PLA system: (1) Reagent A contains 1.0 μL of PPA (1 nM), 1.0 μL of PPB (1 nM), 1.0 μL of ssDNA connector (2 μM), and 0.5 μL of 10X T4 DNA ligase buffer. (2) Reagent B contains 0.8 units/μL T4 DNA ligase.

In a typical Apt-PLA experiment, 3.5 μL of reagent A was pre-incubated with 1.0 μL of protein target at 25 °C for 20 min to allow aptamer binding, followed by the addition of 0.5 μL of reagent B and incubated at 25 °C for 5 min to enable proximity ligation. After heat inactivation at 65 °C for 10 min, the ligation products were amplified and analyzed by a qPCR using a One Step RT-PCR Kit, in which TB Green was utilized as the fluorescent dye for real-time detection of the PCR products.

All qPCRs were performed on a LightCycler® 96 System (Roche). The PCR reaction mixture contains 5 μL of above ligation products, 0.4 μL of forward primer (10 μM), 0.4 μL of reverse primer (10 μM), and 14.2 μL of reagent mix from the One Step RT-PCR Kit. The PCR reaction mixture was subjected to qPCR according to the following thermal cycle: 45 cycles of 95 °C for 10 s, 65 °C for 60 s. The qPCR curves and Ct values were analyzed using LightCycler® 96 Application Software (Roche).

C1.2 Characterization of the Apt-PLA system

To characterize the Apt-PLA system, a typical Apt-PLA experiment was performed by using 2 ng/mL of nucleocapsid protein in the presence of PPA, PPB and connector. Negative control experiments were performed in the absence of either nucleocapsid protein, PPA, PPB or connector, respectively. All the intermediate qPCR products at 24^{th} cycle were collected, and analyzed using 10% denatured polyacrylamide gel electrophoresis (PAGE), with the following conditions: current (30 mA), buffer (1X TBE), and running time (40 min).

C1.3 Real-time fluorescence monitoring

To real-time monitoring the process of proximity ligation, 70 μL of reagent A was pre-incubated with 10 μL of 50 ng/mL N protein, 10 μL of reagent B and 10 μL of 10X Sybr Green I fluorochrome. The fluorescence measurement of reaction solutions was performed on a SynergyH1M microplate reader (BioTek, USA). Moreover, DPBS without N protein was used as negative control. The parameters were set as follows: excitation wavelength, 488 nm; recording wavelength, 520 nm; interval of time, 15 s.

C1.4 Sensitivity and selectivity test for nucleocapsid protein detection in DPBS buffer

For sensitivity analysis, 1.0 μL of different concentrations of nucleocapsid protein was prepared in DPBS, and tested according to the typical Apt-PLA experiment (Section C1.1).

For selectivity analysis, 1.0 μL of 2 ng/mL nucleocapsid protein and other competing proteins were prepared in DPBS, and tested according to the typical Apt-PLA experiment (Section C1.1).

C1.5 Specificity test of PPA/PPB and other mutated proximity probe toward target N protein.

For specificity analysis, 1.0 μL of 5 ng/mL nucleocapsid protein were prepared in DPBS, and tested by PPA/PPB and other mutated proximity probe pairs according to the typical Apt-PLA experiment (Section C1.1).

C1.6 Design of antibody-assisted proximity ligation assay

The antibody-DNA conjugates were synthesized by the maleimide-thiol reaction using heterobifunctional linker sulfo-SMCC. Briefly, for protein conjugation, 600 μL of 50 μg/mL nucleocapsid Antibody A (MAbA) or nucleocapsid Antibody B (MAbB) in DPBS buffer was mixed with 5 μL of 2 mM sulfo-SMCC. After vortexing for 30 s, the solution was placed on a shaker for 30 min at 37 °C. The mixture was then purified by Amicon-100K using DPBS buffer by 8 times, and dispersed in 200 μL of DPBS. In parallel, a certain amount of thiol-DNA (molar ratio: 10 fold to corresponding protein) was mixed with 36 μL of 1 M PBS buffer (pH 5), and 5 μL of 30 mM TCEP, and incubated at 37 °C for 1 hour. Then, the thiol-DNA was purified by Amicon-3K using DPBS buffer by 8 times, dispersed in 200 μL of DPBS, and then mixed with the above solution of sulfo-SMCC-activated nucleocapsid Antibody. The resulting solution was kept at 4 °C for 2 hours.

To remove un-reacted thiol-DNA, the solution for MAbA-DNA or MAbB-DNA was purified by Amicon-100K 8 times using a DPBS buffer. The concentration of purified antibody-DNA conjugates was

quantified using a NanoDrop Onec (Thermo Fisher), and the final stock solution of MAbA-DNA, and MAbB-DNA were 5 nM. The experimental procedure for the antibody-assisted proximity ligation assay is similar to the protocol described in Section C1.1 except the concentration of MAbA-DNA, and MAbB-DNA was 5 nM.

C1.7 Quantification of nucleocapsid protein in human serum using Apt-PLA system

For nucleocapsid protein detection in human serum, 1.0 μL of different concentrations of nucleocapsid protein was prepared in 100% human serum, and tested according to the typical Apt-PLA experiment (Section C1.1).

C1.8 Methodology comparison with a commercial ELISA kit

For methodology comparison, different amounts of nucleocapsid protein in human serum was detected by a commercial ELISA kit and the procedure was followed by manufacturer's protocol. Briefly, an antibody-coated 96 well plate was first washed three times using 1X washing buffer (300 μL per well). Then, different concentrations of nucleocapsid protein standards or unknown samples were added (100 μL per well), and incubated at 25 °C for 2 hours. Subsequently, the reaction solution in each well was discarded, and the plate was washed three times using 1X washing buffer (300 μL per well). Then, 200 μL of 1X enzyme-labeled antibody was added and incubated at 25 °C for 1 hours. After washing three times using 1X washing buffer (300 μL per well), the enzymatic substrate solution was added (200 μL per well), and incubated for another 20 min at 25 °C. Finally, 50 μL of the stop solution was added to each well, and the absorption of the obtained solution at 450 nm was measured using a SynergyH1M microplate reader (BioTek, USA).

C1.9 Detection of SARS-CoV-2 N protein in clinical serum samples from patients and healthy people

Clinical serum samples were kindly provided by our collaborators, and handled in a local hospital (Hefei, China), following the safety requirements of COVID-19 tests. For nucleocapsid protein detection in patient and healthy people serum, 1.0 μL of serum was tested according to the typical Apt-PLA experiment (Section C1.1). DPBS without N protein was used as negative control.

C1.10 Live subject statement

The authors state that all experiments were performed in compliance with the relevant laws and institutional guidelines. The institutional committee of the Binhu Hospital of Hefei City approved the experiment. The authors also state that the informed consent was obtained for any experimentation with human subjects and the human serum samples used in this study didn't have any identifying information about all the participants. The Binhu Hospital of Hefei City is committed to the protection and safety of human subjects involved in the research. All experimental procedures were completed under biosafety level II conditions.

C2. Procedure for the measurement of protein-protein interactions using an adapted proximity ligation assay

C2.1 Preparation and characterization of the protein-DNA conjugates

The protein-DNA conjugates were synthesized by the maleimide-thiol reaction using heterobifunctional linker sulfo-SMCC. Briefly, for protein conjugation, 600 μL of 50 μg/mL nucleocapsid protein, spike S1 or ACE2 in DPBS buffer was mixed with 5 μL of 2 mM sulfo-SMCC. After vortexing for 30 s, the solution was placed on a shaker for 30 min at 37 °C. The mixture was then purified by Amicon-30K using DPBS buffer by 8 times, and dispersed in 200 μL of DPBS. In parallel, a certain amount of thiol-DNA (molar ratio: 10 fold to corresponding protein) was mixed with 36 μL of 1 M PBS buffer (pH 5), and 5 μL of 30 mM TCEP, and incubated at 37 °C for 1 hour. Then, the thiol-DNA was purified by Amicon-3K using DPBS buffer by 8 times, dispersed in 200 μL of DPBS, and then mixed with the above solution of sulfo-SMCC-activated protein. The resulting solution was kept at 4 °C for 2 hours.

To remove un-reacted thiol-DNA, the solution for spike S1-DNA or ACE2-DNA was purified by Amicon-50K 8 times using a DPBS buffer, while the solution for nucleocapsid protein-DNA was purified using a Co-NTA Resin. The concentration of purified protein-DNA conjugates was quantified using a NanoDrop Onec (Thermo Fisher), and the final stock solution of nucleocapsid protein-DNA, spike S1-DNA, and ACE2-DNA was 2 nM, 2 nM, and 80 nM, respectively.

The protein-DNA conjugates were characterized using 8% native PAGE with the following experimental conditions: voltage (80 V), 0.5X TBE buffer, 90 min.

C2.2 Measure the specific interaction between spike S1 and its receptor ACE2 using an adapted proximity ligation assay

Two reagent mix was prepared for the adapted PLA system: (1) Reagent C contains 2.5 μL of Millipore water (18.25 MΩ·cm), 0.5 μL of ACE2-DNA conjugate (80 nM), 0.5 μL of ssDNA connector (2 μM), and 0.5 μL of 10X T4 DNA ligase buffer. (2) Reagent D contains 0.8 units/μL T4 DNA ligase.

In a typical PLA experiment, 4.0 μL of reagent C was incubated with 0.5 μL of spike S1-DNA conjugate (2 nM), or 0.5 μL of nucleocapsid protein-DNA conjugate (2 nM) as a negative control, at 25 °C for 20 min, followed by the addition of 0.5 μL of reagent D and incubated at 25 °C for 5 min. After

heat inactivation at 65 °C for 10 min, the ligation products were amplified and analyzed by a qPCR. The qPCRs procedure is the same as described in Section C1.1.

C2.3 Measure the neutralization efficiency of free ACE2 to spike S1 using a PLA-qPCR

To measure the neutralization efficiency of free ACE2 to spike S1, 2 μL of different concentrations of ACE2 solution was added to a solution mix containing 5 μL of Millipore water, 1.0 μL of 10X T4 DNA ligase buffer, 2 μL of spike S1-DNA conjugate (2 nM), and incubated at 25 °C for 10 min. Then, the obtained solution was mixed with 3 μL of Millipore water, 1.0 μL of 10X T4 DNA ligase buffer, 2 μL of ACE2-DNA conjugate (80 nM), and 2 μL of connector (2 μM), and incubated at 25 °C for 20 min, followed by the addition of 2 μL of Reagent D and incubated at 25 °C for 5 min. After heat inactivation at 65 °C for 10 min, the ligation products were amplified and analyzed by a qPCR. The qPCRs procedure is the same as described in Section C1.1.

C2.4 Measure the neutralization efficiency of aptamers targeting spike S1

The experimental procedure for the measurement of the neutralization efficiency of aptamers targeting spike S1 is similar to the protocol described in Section C2.3 except that free ACE2 solution with replaced by different concentrations of aptamers or random DNA.

Section D: Supporting Figures

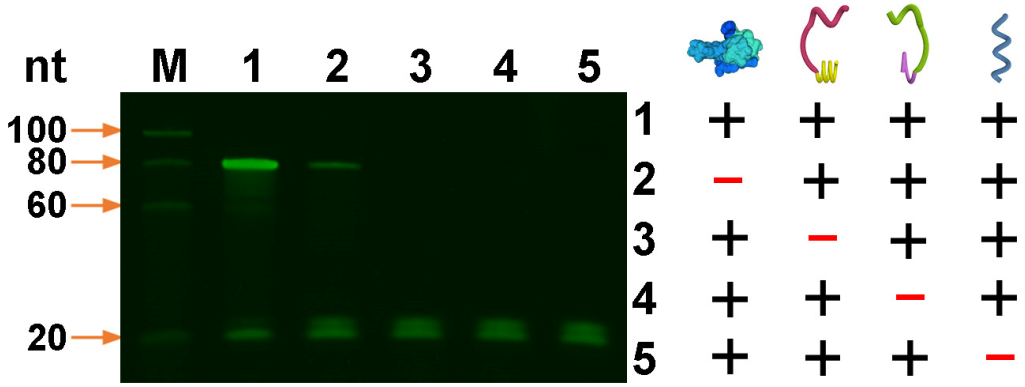

Fig. S1. PAGE analysis of the feasibility of our aptamer-assisted proximity ligation assay for nucleocapsid protein and control samples. Single stranded poly(T) oligonucleotides were used as the marker. The band at high molecular weight (MW) region of approximately 80 nts indicates the formation of ligated DNA products.

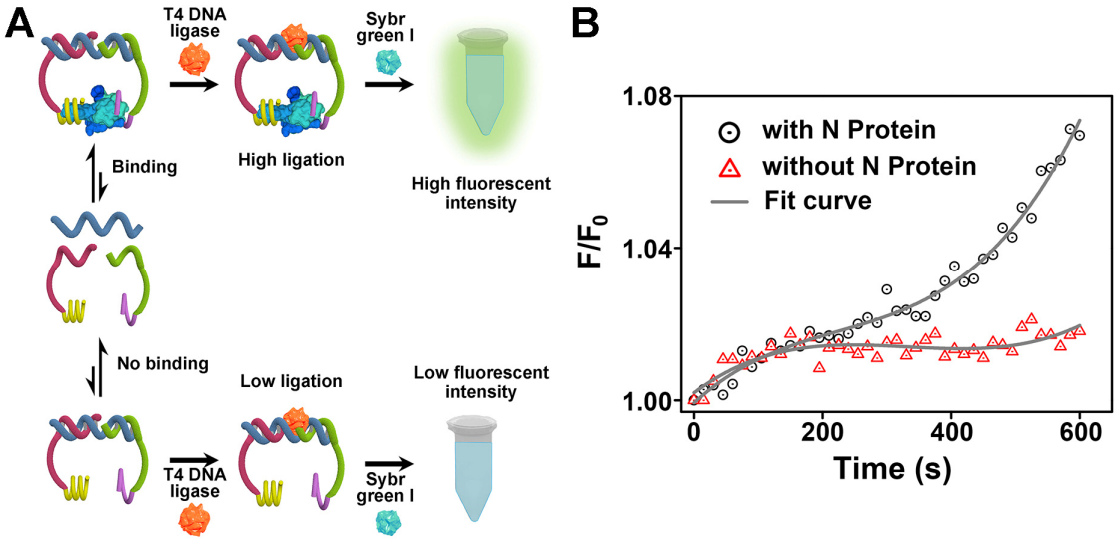

Fig. S2. Real-time fluorescent test for monitoring the rate of strand hybridization. (A) Detection strategy and workflow of the real-time fluorescent test. (B) Comparison of the real-time fluorescence intensity ratio with and without 5 ng/mL of N protein using the Apt-PLA system. The detailed experimental procedure is described in “Section C1.3”.

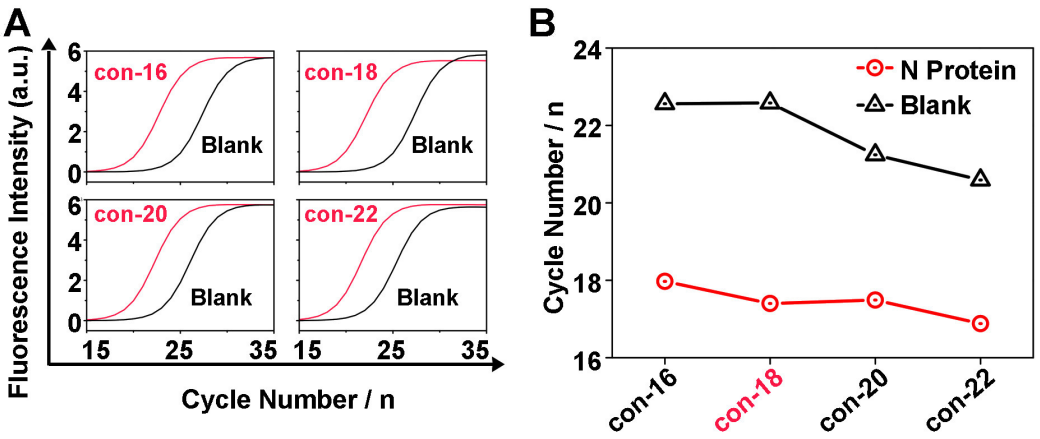

Fig. S3. Optimization of the number of complementary bases between ssDNA connector and the two ligation probes. (A) Comparison of the qPCR curves of N protein and blank samples when using connector with 16, 18, 20, and 22 complementary bases (Con-16, Con-18, Con-20, Con-22) to L1/L2 for proximity hybridization. (B) Comparison of the Ct values.

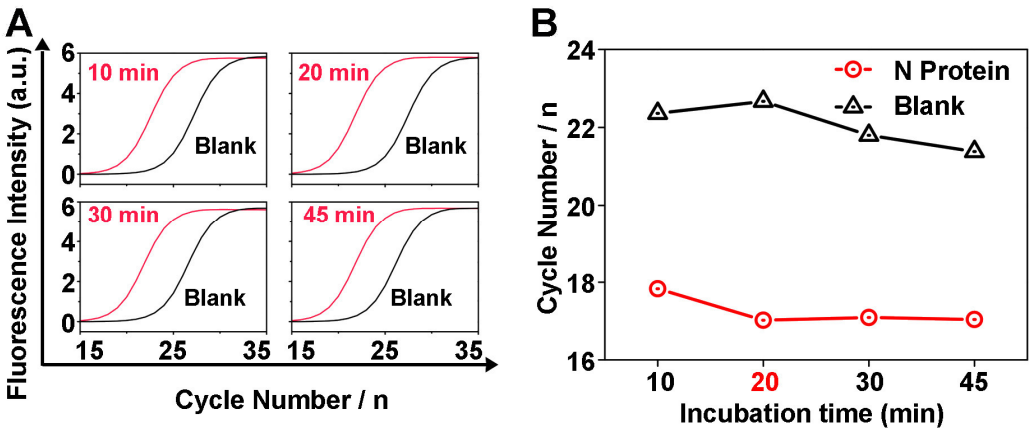

Fig. S4. Optimization of the incubation time between aptamer probes and N protein. (A) Comparison of the qPCR curves of N protein and blank samples with a different incubation time. (B) Comparison of the Ct values.

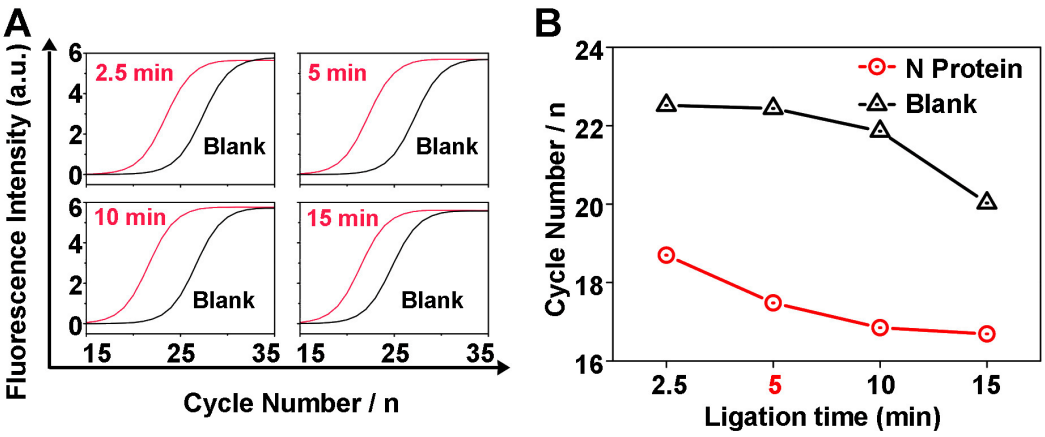

Fig. S5. Optimization of the ligation time using T4 DNA ligase. (A) Comparison of the qPCR curves of 2 ng/mL of N protein and blank samples with a different ligation time. (B) Comparison of the Ct values. According to the maximum ΔCt value between the N protein and the blank, an optimal ligation time of 5 min was obtained. It should be noted that a longer ligation time may cause more non-specific ligation events, thereby decreasing the signal-to-noise ratio.

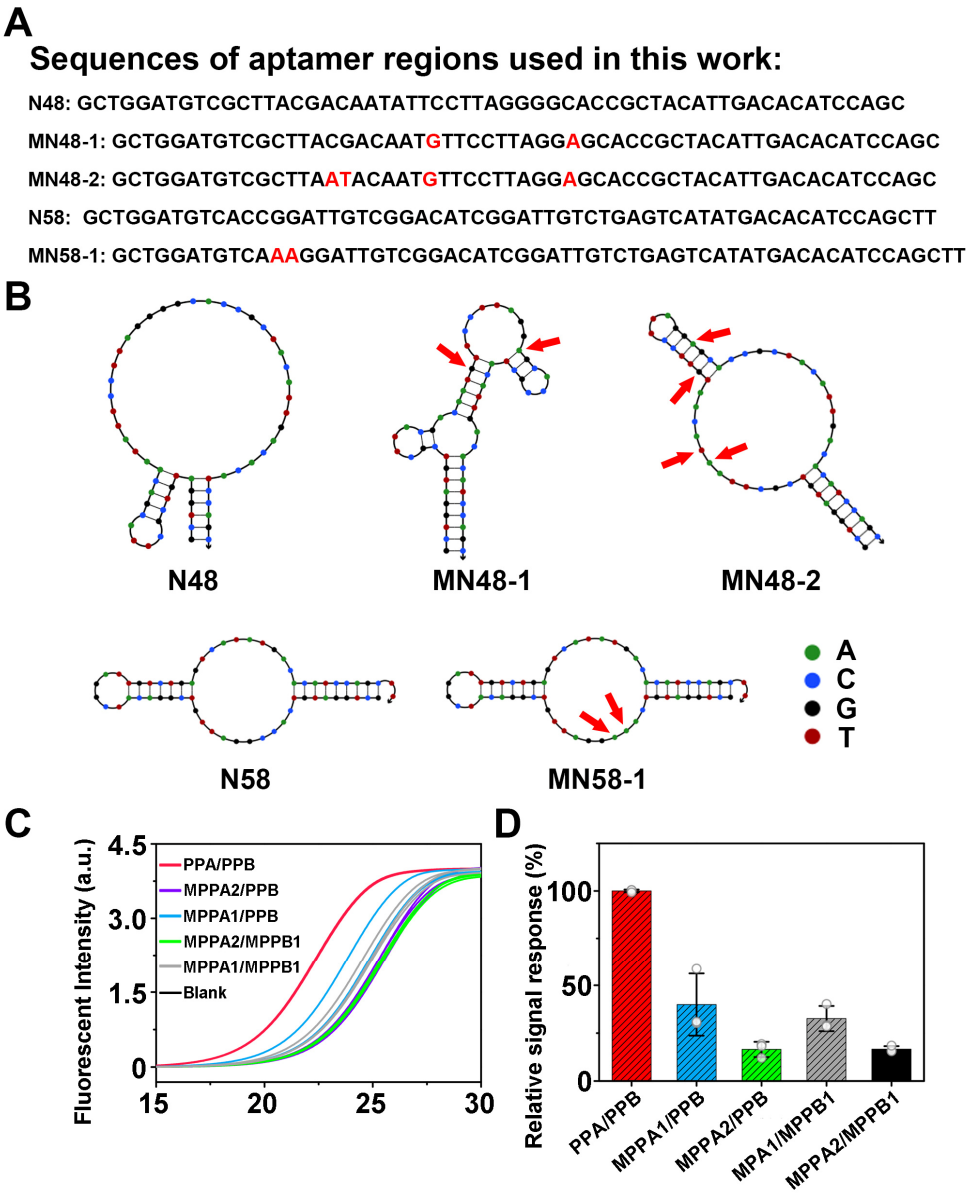

Fig. S6. The detection specificity of PPA and PPB toward target N protein over other mutated proximity probes: mutated proximity probe A-1 (MPPA-1), mutated proximity probe A-2 (MPPA-2), and mutated proximity probe B-1 (MPPB-1). (A) Sequences of aptamer regions used in this work: N48, N58, mutated N48-1 (MN48-1), mutated N48-2 (MN48-2), and mutated N58-1 (MN58-1). (B) The secondary structure of DNA aptamers analysed with NUPACK software. (C) The real-time fluorescence intensity of qPCR signals for proximity probe PPA, PPB and other mutated aptamers of proximity probe:. (D) ΔCt values for PPA/PPB and other mutated proximity probe pairs. ΔCt is the difference between the Ct value of a protein sample and a blank sample. Error bars represent the standard deviations of three parallel tests.

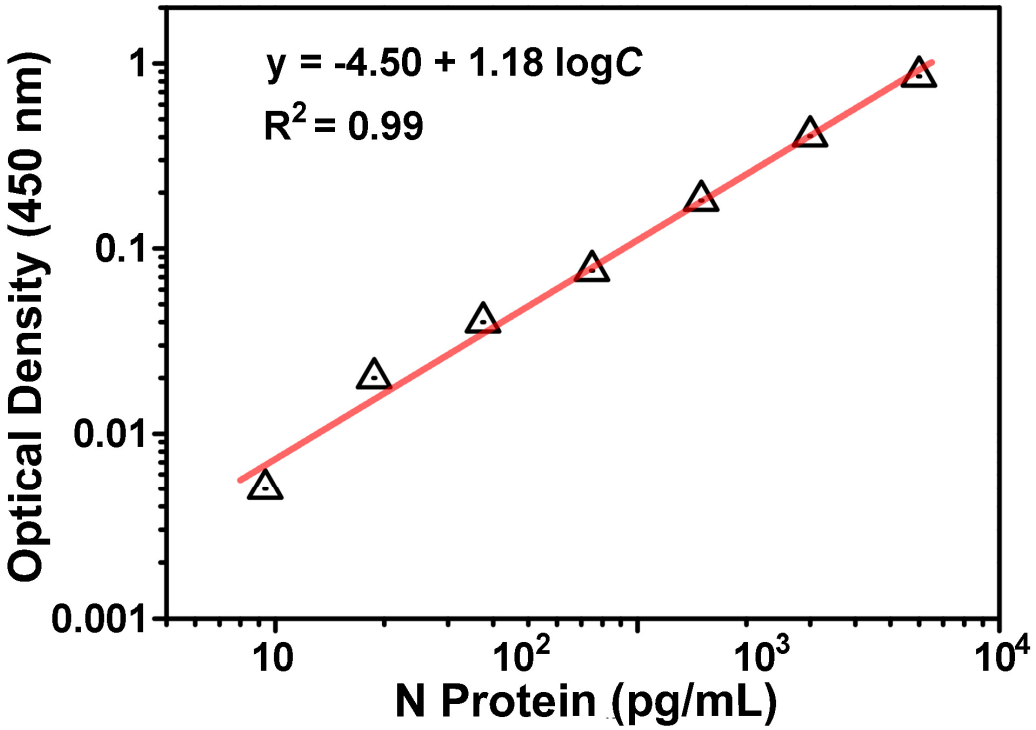

Fig. S7. Calibration curve for nucleocapsid protein detection using a commercial ELISA kit.

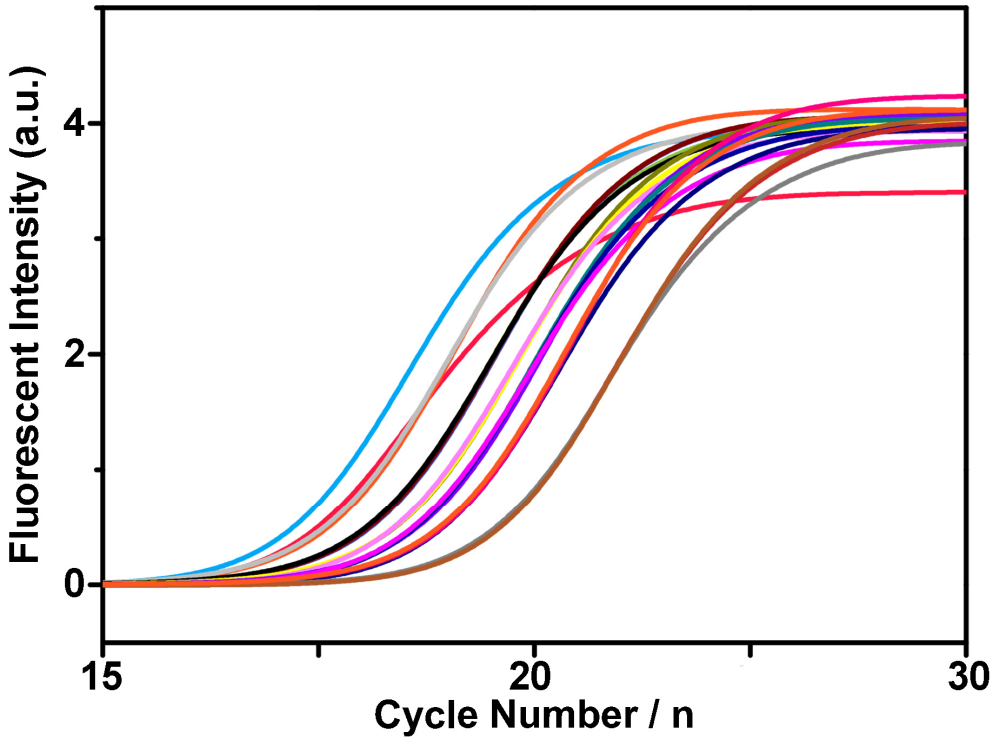

Fig. S8. qPCR curves of our aptamer-assisted proximity ligation assay for nucleocapsid protein detection in human serum. Twenty-one serum samples were prepared by spiking different concentrations of nucleocapsid protein in 100% human serum.

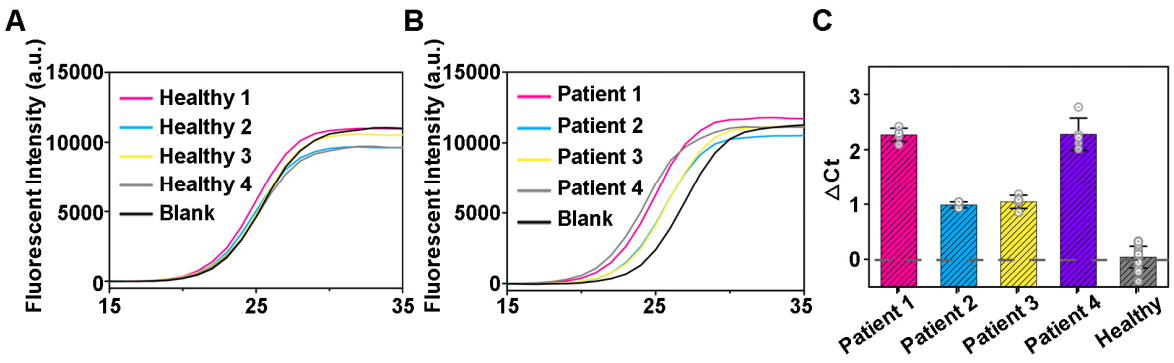

Fig. S9. Performance of the aptamer-assisted proximity ligation assay for N protein detection in healthy people and confirmed patients. The qPCR curves of Apt-PLA results from the serum samples of healthy people (A) and confirmed patients (B). (C) Comparison of ΔCt values for serum samples of healthy people and confirmed patients. ΔCt is the difference between the Ct value of a protein sample and a blank sample. Error bars represent the standard deviations of several parallel tests. The detailed experimental procedure is described in “Section C1.9”.

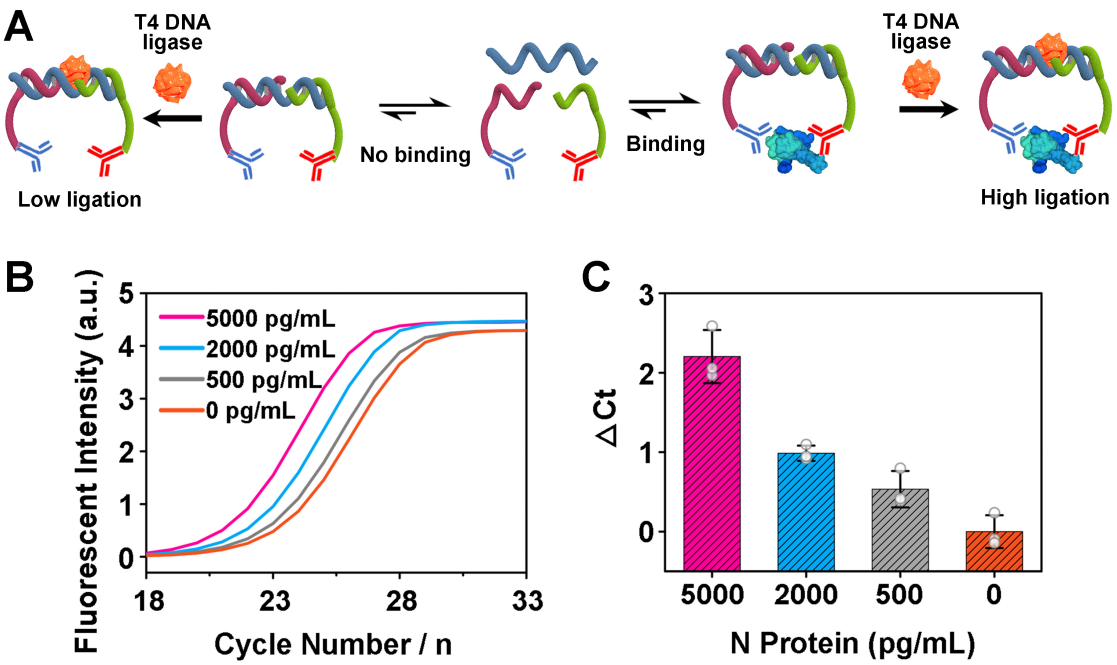

Fig. S10. Performance of the antibody-assisted proximity ligation assay for N protein detection. (A) Detection strategy and workflow of our antibody-assisted proximity ligation assay for nucleocapsid protein. (B) qPCR curves of different concentrations of N protein added in DPBS buffer. (C) ΔCt values for different concentrations of N protein. ΔCt is the difference between the Ct value of a protein sample and a blank sample. Error bars represent the standard deviations of three parallel tests.

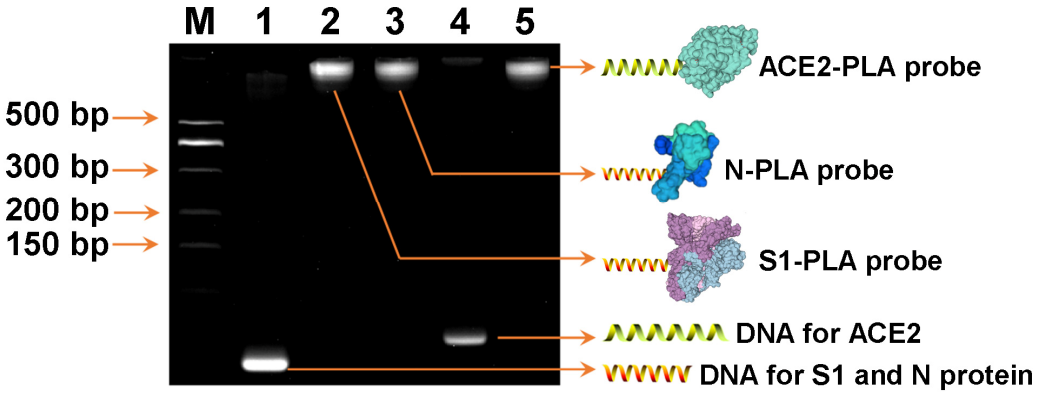

Fig. S11. PAGE analysis of the spike S1-DNA, ACE2-DNA and nucleocapsid protein-DNA conjugates.

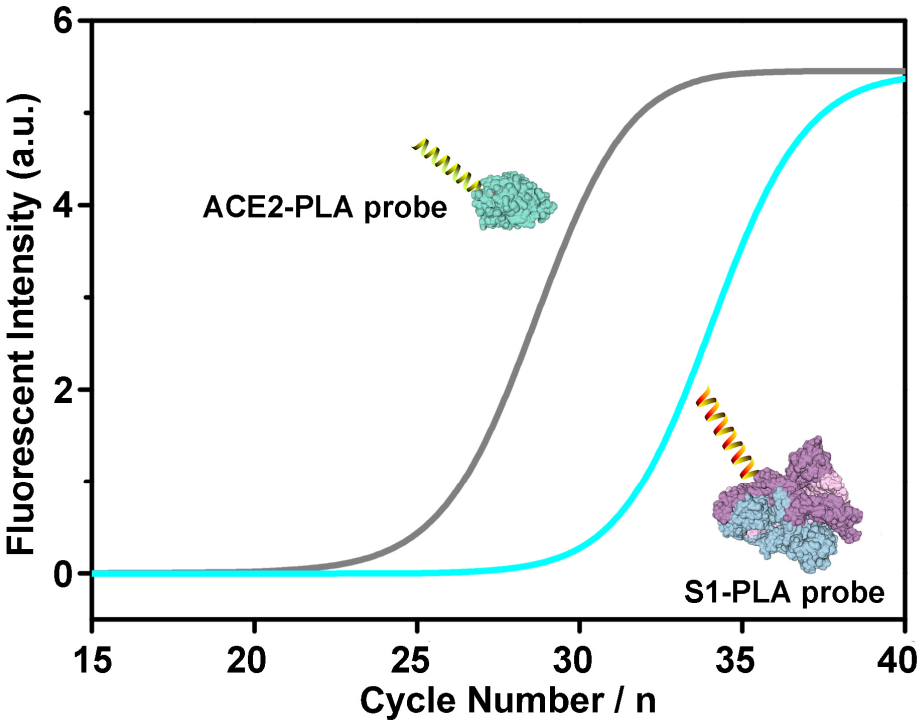

Fig. S12. qPCR curves of our adapted proximity ligation assay in the presence of individual spike S1-DNA probe (200 pM) or ACE2-DNA probe (80 nM).

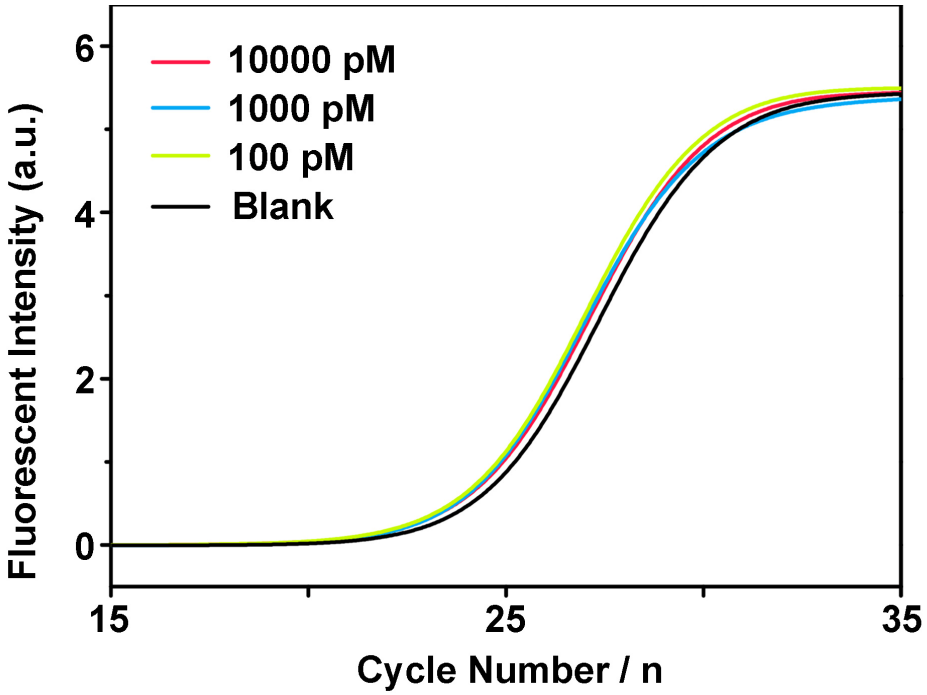

Fig. S13. qPCR curves of our adapted proximity ligation assay with different concentrations of nucleocapsid protein. The spike S1-DNA probe was pretreated with different concentrations of free nucleocapsid protein for 10 min, and then tested using our adapted proximity ligation assay.

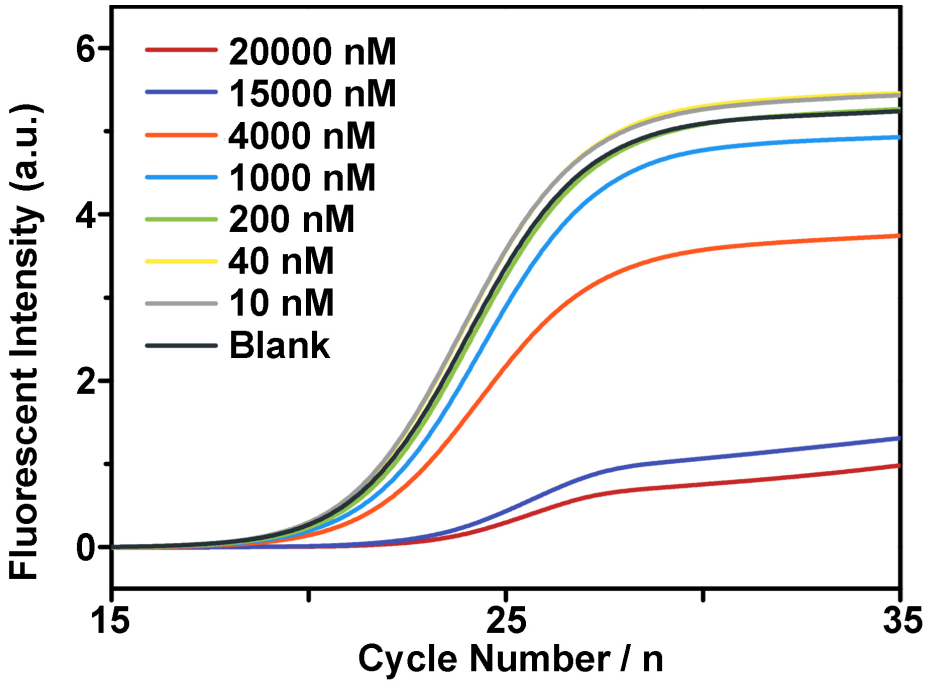

Fig. S14. qPCR curves of our adapted proximity ligation assay with different concentrations of potential neutralizing aptamer (Apt-S-79s) targeting spike S1. The spike S1-DNA probe was pretreated with different concentrations of free Apt-S-79s for 10 min, and then tested using our adapted proximity ligation assay.

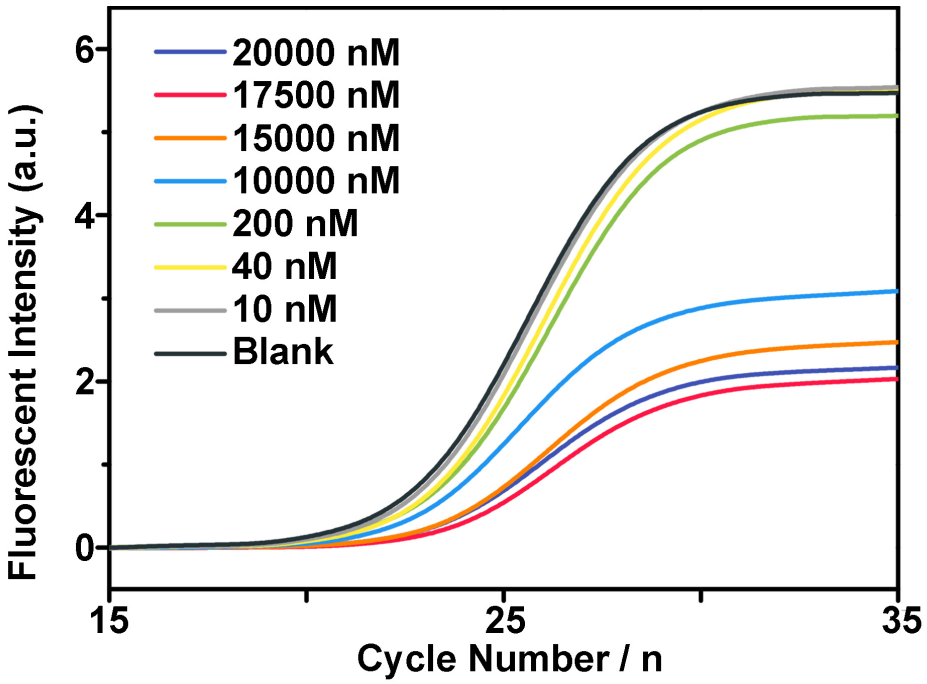

Fig. S15. qPCR curves of our adapted proximity ligation assay with different concentrations of potential neutralizing aptamer (Apt-S-268s) targeting spike S1. The spike S1-DNA probe was pretreated with different concentrations of free Apt-S-268s for 10 min, and then tested using our adapted proximity ligation assay.

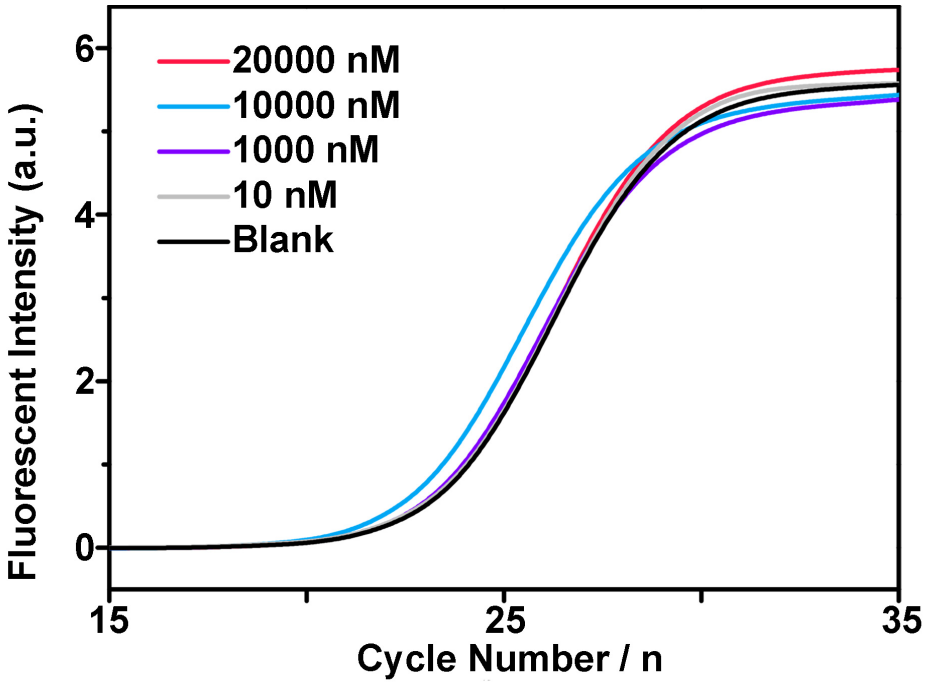

Fig. S16. qPCR curves of our adapted proximity ligation assay with different concentrations of a random DNA. The spike S1-DNA probe was pretreated with different concentrations of free random DNA for 10 min, and then tested using our adapted proximity ligation assay.