Electronic Supplementary Material (ESI) for ChemComm.


Supporting Information

# Mix-and-Read, One-Minute SARS-CoV-2 Diagnostic Assay: Development of PIFE-based Aptasensor†

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## Materials

Four DNA aptamers named PCL-Apto1, PCL-Apto2, PCL-Apto3 and PCL-Apto 4 (Sequences pro-vided in Table S1) were synthesized from SFC Co., Ltd. (Yongin-si, Gyeonggi-do, Korea) with Cyanine 3 (Cy3) or Cyanine 5 (Cy5) conjugated on the 5' end. All experiments were conducted with aptamers dissolved in the optimized binding buffer (2 mM $Na_2HPO_4$, 8 mM $KH_2PO_4$, 5 mM NaCl, 0.5 mM $MgCl_2$, pH 7.4). Prior to all experiments, the aptamers were denatured at 95 ℃ for five minutes and incubated in room temperature to anneal for 30 minutes. All fluorescence measurements were conducted with Synergy H1 Hybrid Multi-Mode Reader (BioTek) at excitation wavelength of 540 nm and emission wavelength of 579 nm. The aptamer (PCL-Apto3) used for our assay was conjugated to Cy3. Concentrations of aptamer and proteins stocks were measured with Nanodrop at 260 nm and 280 nm, respectively, prior to further dilution.

## Methods

*Confirmation of Aptamer-NP Binding.*

To confirm the binding between SARS-CoV-2-N-proteins and aptamers, aptamer-equivalent direct ELISA (Enzyme-Linked Immunosorbent Assay) was performed. N-protein (2 μg/mL in 50 mM NaHCO3, pH 9.4) were dispensed on the black 96-well polystyrene immunoplate with clear bottom (Corning, Cat. No. 3631) for overnight at 4°C to coat the N protein onto the surface. BSA (2 μg/mL) was coated the same way as a negative control. Subsequently, after washing with PBS at pH 7.4 (ThermoFisher, Cat. No. 10010023), StabilBlock® Immunoassay Stabilizer (Surmodics, Product No. ST01-1000) was added for blocking. After incubating at room temperature for one hour and washing with PBS two times, coated N-protein was incubated with various concentrations (25 nM, 50 nM, 100 nM, 200 nM, 400 nM) of aptamers (PCL-Apto1, 2, 3, 4) conjugated with Cy5. Alexa-647 conjugated N-protein antibodies (Fapon Biotech, 2.5 $\mu$g/mL) was used as a positive control. After washing with the binding buffer two times, the bottom of

the plate was scanned (Typhoon FLA 9000 Biomolecular imager) and the fluorescence of the bound aptamers was quantified with ImageQuant TL program.

To confirm the specificity of binding of N Protein towards PCL-Aptos, a scramble aptamer (Table S1) was used as a negative control aptamer. For detection, we utilized a more sensitive, automated PCLOK II IVD instrument (Model Name: ATPI03 PCL, Inc.) that leverages the same working principles of aptamer-equivalent direct ELISA. Cy5-labelled Aptamers were loaded onto a N-protein coated cartridge (PCL, inc.) in 1x Dulbecco's phosphate-buffered saline (ThermoFisher, Cat. No. 14190144) supplemented with 0.5 mM $MgCl_2$ and 1 mg/mL BSA, as per the standard protocol of PCLOKII. The machine incubated NP with the aptamers for 8 minutes on shaking, followed by washing with the same buffer and measurement of a fluorescence signal.

*Detection of Aptamer-N Protein Interaction.*

Aptamer-N Protein detection assay was conducted with black 96-well plates (SPL, Cat. No. 30496) at a final volume of 105 μL. The initial florescence of PCL-Apto3 (1 nM, 100 μL) was measured ~three minutes prior to adding Nucleocapsid protein (Fapon biotech, Cat. No. FPZ0512, 5 μL). After adding target at varying concentrations, the kinetics of the fluorescence signal change was monitored every minute for ten minutes. In a no-target control, 5 μL of water was added instead. All measurements were triplicated and averaged. The concentration of the aptamer probe was optimized using the same system with varied concentrations of aptamer and 40 nM of NP.

*Effect of BSA on Fluorescence Enhancement of Aptamer*

Aptamer-N Protein detection assay was conducted with black 96-well plates at a final volume of 100 μL. PCL-Apto3 (1 nM, 40 μL) was mixed with varying concentrations (0, 1 nM, 3 nM, 5 nM) of BSA (10 μL). The initial florescence of PCL-Apto3 mixed with BSA solution was measured ~three minutes prior to adding Nucleocapsid protein (50 μL). After adding target at

varying concentrations (0, 20 nM, 40 nM), the kinetics of the fluorescence signal change was monitored every minute for ten minutes. In a no-target control, 50 μL of water was added instead. All measurements were triplicated and averaged.

*Virus preparation.*

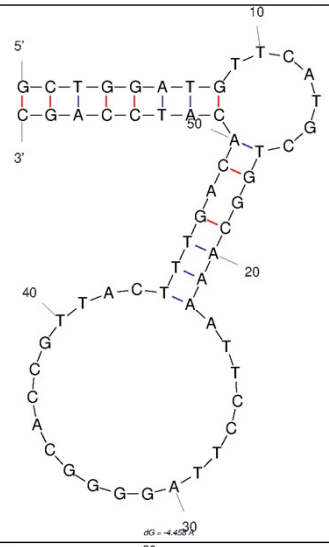
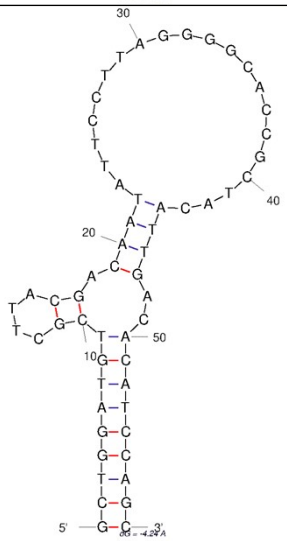
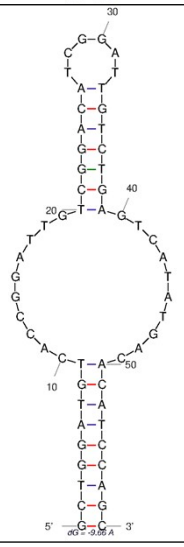
Heat-inactivated SARS-CoV-2 virus was purchased from Zeptometrix (Cat. No. 0810587CFHI), and the reported infectious virus titers ($TCID_{50}$/mL) of the stock solution is $1.15 \times 10^7$. Heat-inactivated Influenza A and B virus were purchased from NIBSC (Cat. No. 01/614, Cat.No. 04/202, respectively). All virus stock solutions were diluted with PBS at pH 7.4 (ThermoFischer, Cat.No.10010023) prior to measurements. No further preparation steps were done before using them for our assay.

*Limit of Detection.*

In order to measure the limit of detection of the assay for N Proteins, aptamer-N Protein interaction assay was conducted with black 384-well plate (SPL, Cat. No. 33384) at a final volume of 50 μL. PCL-Apto3 (1 nM, 25 μL) was mixed with various concentrations of N protein (25 μL) in order to find the lowest amount of N Protein that produces significant signal. All conditions were triplicated and averaged.

*Detection of Aptamer/SARS-CoV-2 Virus Interaction.*

Aptamer-Virus detection assay was conducted with black 384-well plate at a final volume of 30 μL. PCL-Apto3 (1 nM) was mixed in a buffer supplemented with BSA (3 nM) to block nonspecific binding between the aptamer and other proteins in virus lysate. SARS-CoV-2 virus was added in titration (1/4x, 1/40x, 1/200x). Negative controls are Influenza A and Influenza B viruses that have previously been tested to be active. Subsequently, aptamer-virus duplex solution (30 μL) was transferred to each well of the 384 well plate and the fluorescence signal was measured immediately after. All conditions were triplicated and averaged.

| Name | Sequences (5’ to 3’) | Secondary structure |
|---|---|---|
| PCL-Apto1 | GCT GGA TGT TCA TGC TGG CAA AAT TCC TTA GGG GCA CCG TTA CTT TGA CAC ATC CAG C |  |
| PCL-Apto2 | GCT GGA TGT CGC TTA CGA CAA TAT TCC TTA GGG GCA CCG CTA CAT TGA CAC ATC CAG C |  |
| PCL-Apto3 | GCT GGA TGT CAC CGG ATT GTC GGA CAT CGG ATT GTC TGA GTC ATA TGA CAC ATC CAG C |  |

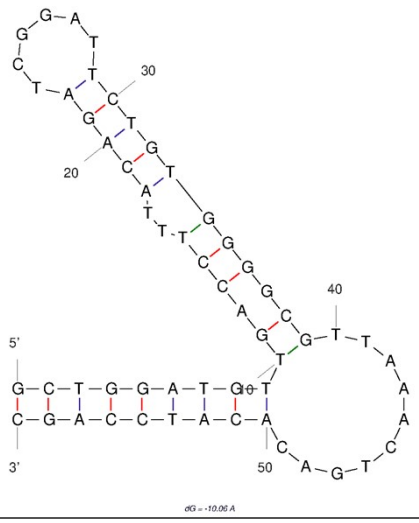
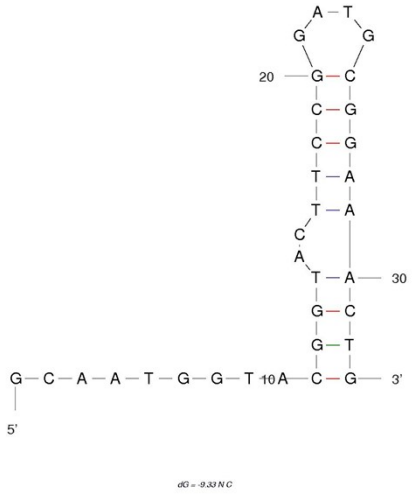
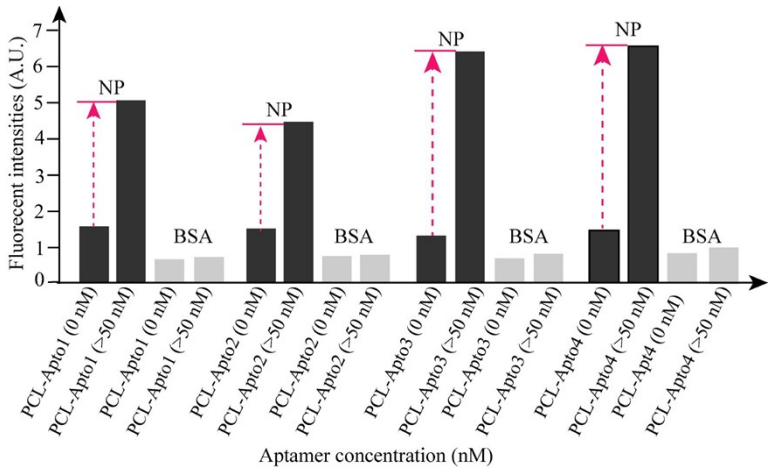
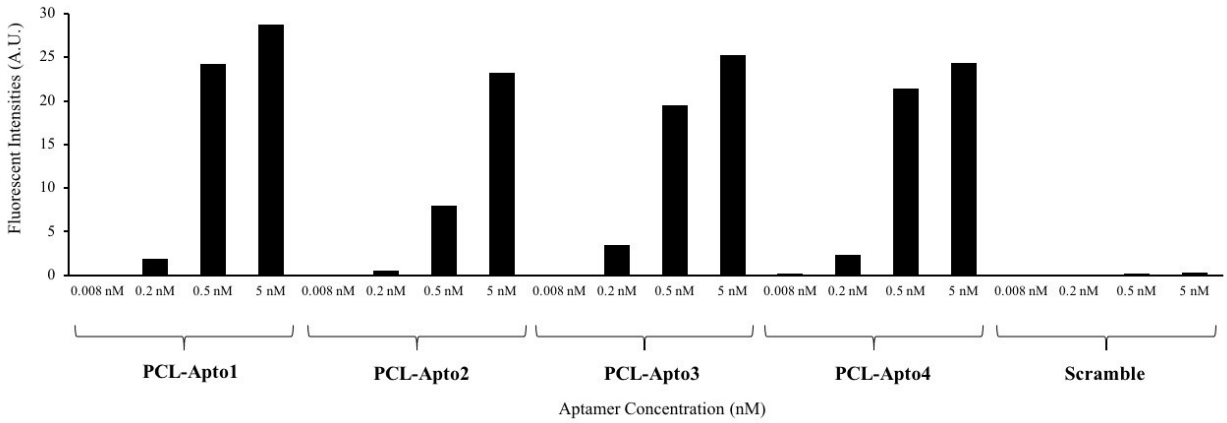
| | | |
|---|---|---|
| PCL-Apto4 | GCT GGA TGT TGA CCT TTA CAG ATC GGA TTC TGT GGG GCG TTA AAC TGA CAC ATC CAG C |  |
| Scramble | GCA ATG GTA CGG TAC TTC CGG ATG CGG AAA CTG |  |

**Table S1.** Sequences and secondary structures of the four aptamers and a scramble aptamer as a negative control. Predicted structures were generated by mFold (37˚C, 0.137 M $Na^{+}$, 0.0005 M $Mg^{++}$) and the displayed structures are the ones predicted to be most stable.[1,2]

**a)**




**b)**




**c)**

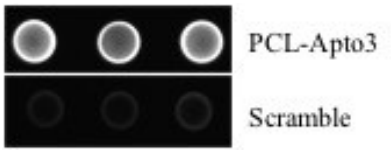



**Fig S1.** Aptamer-equivalent ELISA confirmation of aptamer and NP binding to assess sensitivity and specificity of binding. **a)** Confirmation of the specificity of the aptamer binding towards N Protein only. BSA (2 µg/mL) coated negative control wells were not responsive towards aptamers. **b)** A more sensitive PCL-OK2 Confirmation of the specificity of N protein binding towards PCL-Apto's only. A scramble aptamer used as a negative control was not responsive towards N Proteins. Signal intensities above >15 is saturated and meaningless to compare numerically (0.5 nM, 5 nM). At 0.2 nM when the signals are not saturated, we observe that PCL-Apto3 displays most affinity to NP. **c)** Scanned fluorescence pictures of PCL-Apto3 and scramble aptamer, taken at 5 nM aptamer concentrations for the PCL-OK2 confirmation assay from **b).** The numerical values of **b)** were generated by averaging the fluorescence values detected

on three wells. Saturated PCL-Apto3 signals (>15) indicate their binding to NP, which is in stark contrast to a scramble aptamer's distinctively dark signal that indicates a lack of binding.

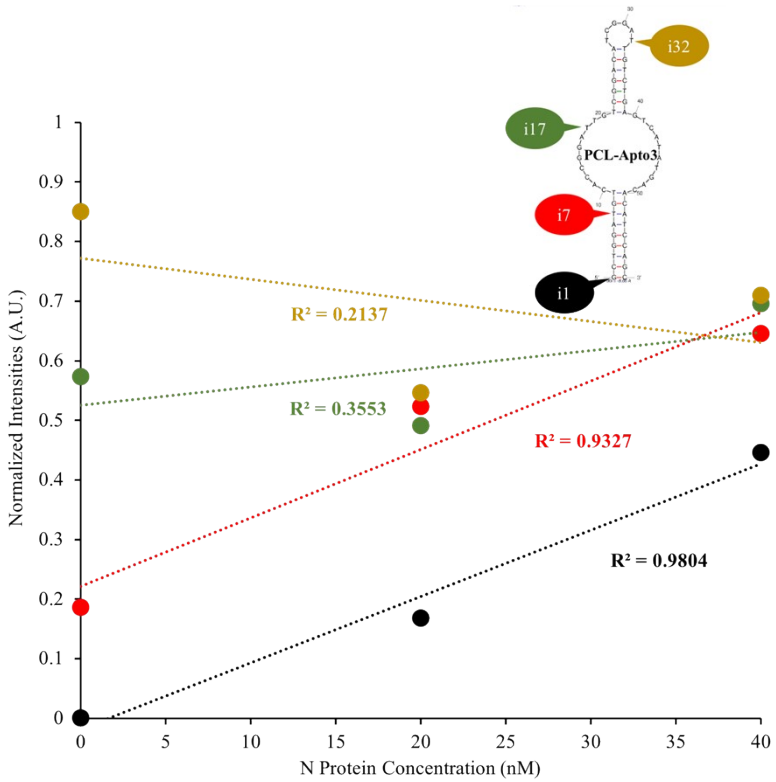
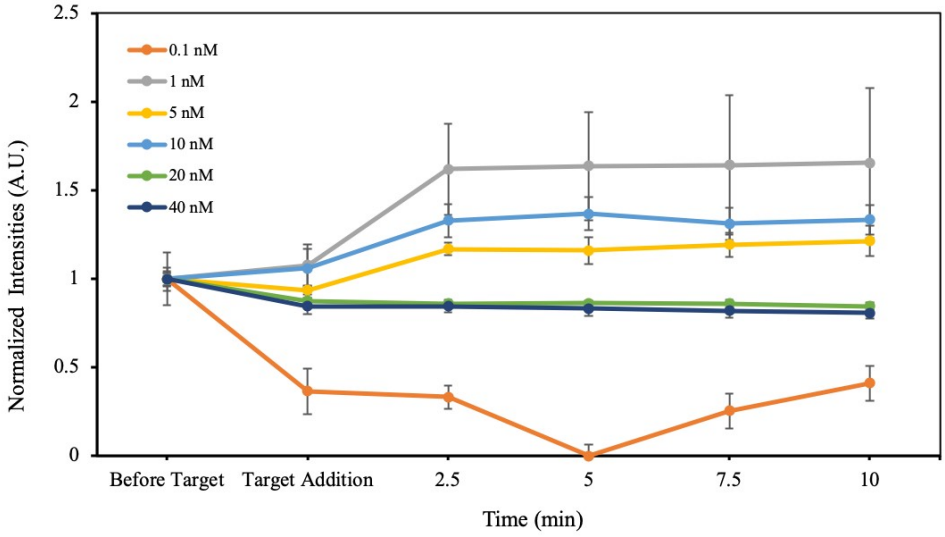



**Fig S2.** Cy3 placement optimization. In order to determine the best placement for Cy3, we performed our standard 96-well plate PIFE assay with four 1 nM PCL-Apto3's where Cy3 is positioned at different places within the aptamer (i1, i7, i17, i32; i1 is the 5' end, and the numbers after i refer to the inclusive number of base pairs from i1 to the position where Cy3 is labelled internally). While i1 and i7 displayed PIFE behavior with excellent linearity and sensitivity to NP, i17 and i32 displayed no linearity. Given this result, we proceeded with i1 (5'-labelled PCL-Apto3) given the ease of labelling. Note that the baseline intensities (at 0 nM NP) of all four aptamers are different despite the same concentration of 1 nM, which likely results from the effect of differential base quenching.[3]

**a)**




**b)**

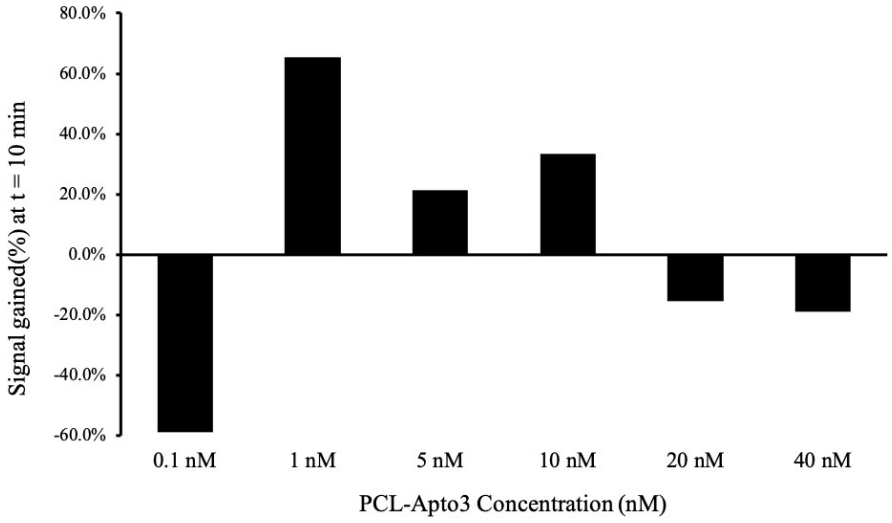



**Fig S3.** Aptamer concentration optimization. In order to optimize the concentration of probes, we performed our standard 96-well plate PIFE assay with varying concentrations (0.1, 1, 5, 10, 20, 40 nM) of PCL-Apto3 and addition of 40 nM NP. **a)** For the ease of comparison of the signal enhancement among different concentrations of aptamers with different baseline signal intensities, the fluorescence signals were normalized individually per each concentration, where the fluorescence value before the addition of the target was normalized to be 1. **b)** 1 nM of aptamer probe showed the highest signal gain %, which is calculated at t = 10 min according the following equation:

$$\frac{(normalized\ signal\ at\ t = 10 - \ normalized\ signal\ before\ adding\ target\ )}{(normalized\ signal\ before\ adding\ target)} \text{ x } 100\ (\%)$$

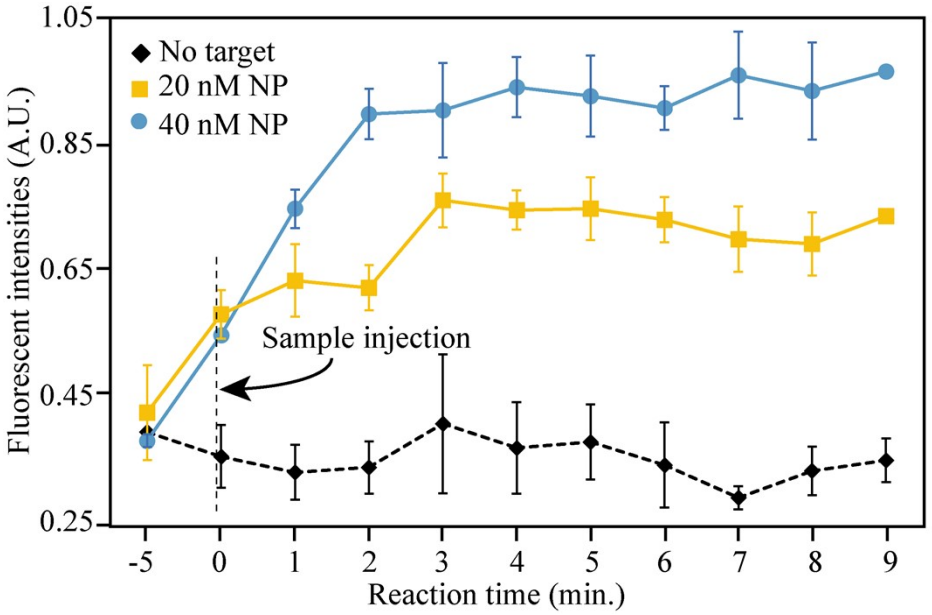



**Fig S4.** Kinetics of the assay after the addition of two different concentration (20 nM and 40 nM) of N protein (t = 0 min) was monitored every minute. Upon adding samples into aptamer solution in the well, the fluorescent signals were measured for 10 minutes at one-minute interval. As similar to 60nM of NP, immediate signal enhancement was observed for all concentrations of NP, and by t = 2 min, maximum fluorescence enhancement is reached, which showed proof-of-concept for PIFE of SARS-CoV-2.

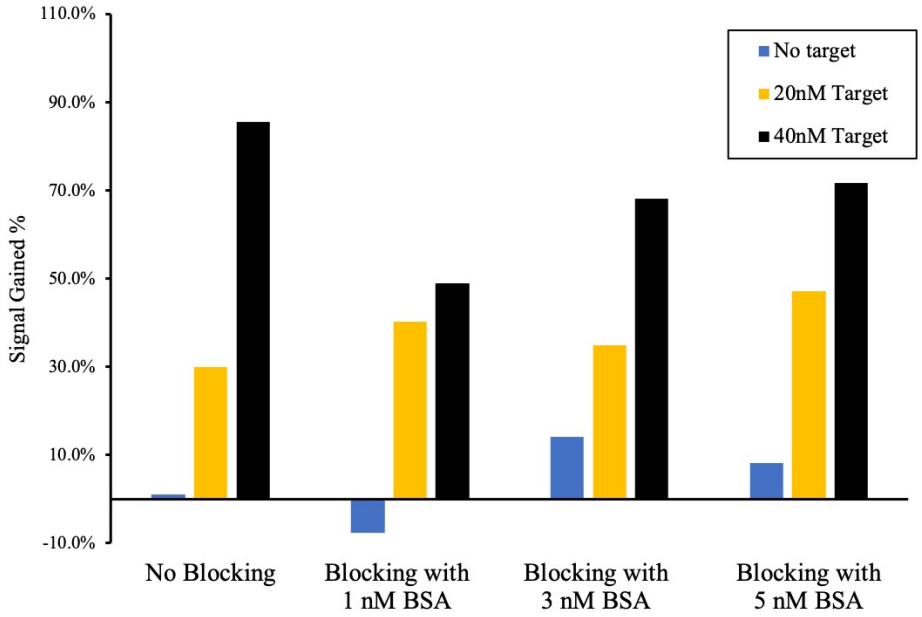



**Fig S5.** Effect of BSA on Fluorescence Enhancement of Aptamer tested against 20, 40 nM NP target. The presence of BSA does not have distinct effect on fluorescence enhancement of probes. Since BSA is a well-known blocking reagent, we used 3nM of BSA to block nonspecific binding in the assay of SARS-CoV-2 virus lysate.

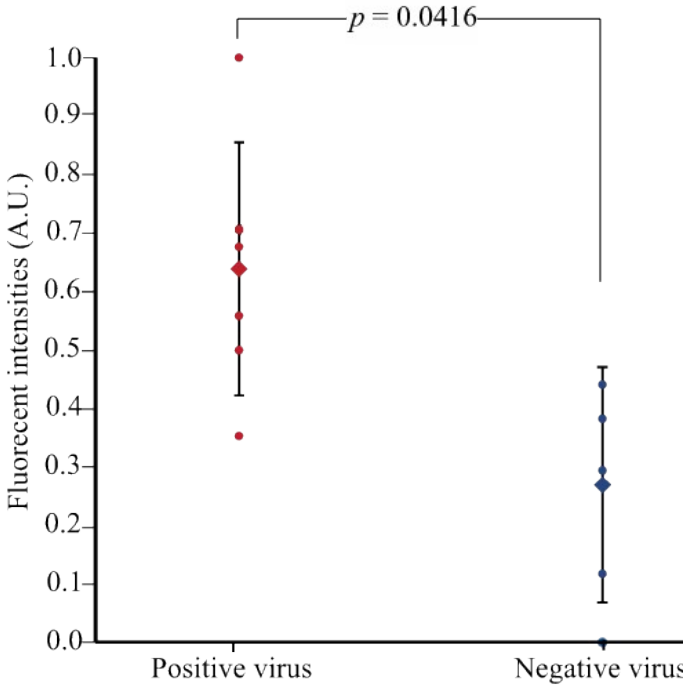



**Fig S6.** Fluorescence values of positive virus samples (SARS-CoV-2, n = 8) and negative virus samples (Influenza A and B, n = 6). The assay discriminated the positive virus with $P = 0.0416$, n = 14. Positive virus samples were 1/4, 1/40, 1/200 dilutions of SARS-CoV-2 stock solution, whereas negative virus samples were Inf A and Inf B.

## References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2 Z. Chen, Q. Wu, J. Chen, X. Ni and J. Dai, Virol. Sin., 2020, 35, 351–354.

3 H. Mao, G. Luo, Y. Zhan, J. Zhang, S. Yao and Y. Yu, *Analyst,* 2018, **143,** 3292-3301