

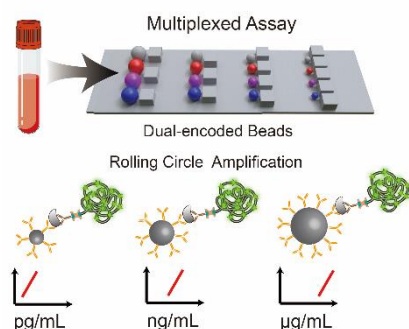


Supporting Information

A Dual-Encoded Bead-Based Immunoassay with Tunable Detection Range for COVID-19 Serum Evaluation

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A multiplexed strategy for chip-based sandwich immunoassays integrated with color/size dual-barcoded beads and rolling circle amplification (RCA) is presented. RCA based on beads of various sizes allows for a tunable detection range from pg mL^{-1} to $\mu\text{g mL}^{-1}$.

Experimental Procedures

Ethical statements. This study was approved by the Institutional Review Board of the Fifth Affiliated Hospital of Sun Yat-sen University (Zhuhai, China) [No. ZDWY (2020) Lunzi No. (K22-1)]. All participants have signed written informed consent.

Materials and reagents. SARS-COV-2 nucleocapsid protein was purchased from Leinco. SARS-COV-2 spike S1 protein, spike RBD protein, biotinylated S1, and N-NTD protein, antibodies targeting Spike S1, spike RBD and nucleocapsid antigens were purchased from Sino Biological. IL-6, capture & biotinylated detection antibody to IL-6 were purchased from PeproTech. IL-1 β was purchased from Beyotime. Capture & biotinylated detection antibody to IL-1 β were purchased from Biolegend. PCT, capture & biotinylated detection antibody to PCT were purchased from RayBio. CRP, capture & biotinylated detection antibody to CRP were purchased from R&D. SU83025 and SU8 developer were from Microchem, Inc. Poly (dimethylsiloxane) (PDMS) Sylgard 184 monomer base and curing agent were purchased from Dow Corning Inc. Carboxylated polystyrene beads (9 - 10 μm in diameter, 12 - 13 μm in diameter, 17 - 18 μm in diameter, 21 - 22 μm in diameter) were purchased from 3A Chemicals (Shanghai, China). CdSe/ZnS Quantum dots (emission peak at 544nm & 620nm) were purchased from BaseLine ChromTech (Tianjin, China). EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride), Sulfo-NHS (Sulfo-N-hydroxysulfosuccinimide) were purchased from Aladdin. MES (2-(N-morpholino) ethanesulfonic acid) buffered saline, bovine serum albumin (BSA), Fluorescein-12-dUTP, Deoxy-nucleotide (dATP/dCTP/dGTP) solution were from Thermo Fisher Scientific. Streptavidin was purchased from Macklin. All DNA oligonucleotides and FITC-labeled streptavidin used in this work were from Sangon Biotech Co., Ltd. (Shanghai, China). The sequences are listed in Supporting Information Table S1. 1 \times TE Buffer, 10 \times TM Buffer, 10 \times TBE Premixed Powder, and DNA marker were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). T4 DNA Ligase and phi29 DNA Polymerase were purchased from New England Biolabs Inc. (Ipswich, MA, USA). YeaRed Nucleic Acid Gel Stain and 6 \times Loading Buffer were purchased from Yeasen Biotech Co., Ltd. (Shanghai, China).

Preparation of quantum dots barcoded beads. The quantum dots barcoded beads were prepared by the swelling-evaporation approach.^[1] 10 mg porous polystyrene microbeads were dispersed in a solvent mixture of 4.5 mL chloroform and isopropyl alcohol (95:5, V/V) with ultrasound for 15 min (100 W). Chloroform solution containing two kinds of CdSe/ZnS quantum dots at precisely controlled ratios was then added to the above solutions and ultrasonic cleaned for 15 min (100 W) and then placed in the vacuum oven for 12 h (30 $^{\circ}\text{C}$, vacuum degree 0.1 MPa). Finally, washed the beads using absolute alcohol three times and dispersed them in alcohol and water (50:50, V/V).

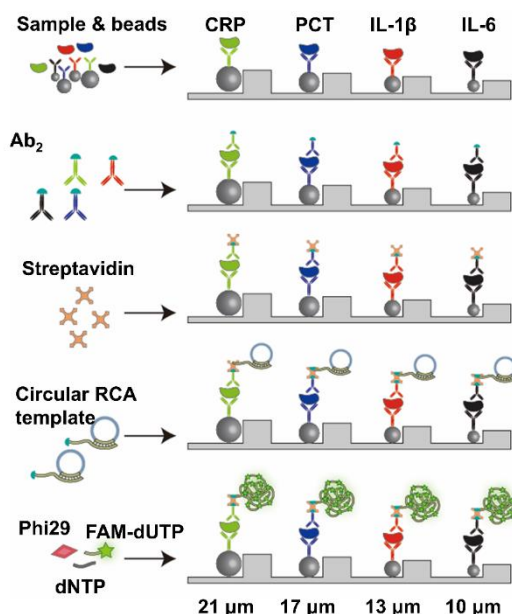
Fabrication of microfluidic chip. Photomask was designed using AutoCAD and silicon molding masters were fabricated by soft photolithography. The process included spin-coating (2,000 rpm, 30 s) of a negative photoresist, soft-baking (2 min at 65 $^{\circ}\text{C}$; 13 min at 95 $^{\circ}\text{C}$), UV-exposing (180 mJ/cm^2), postexposure-baking (2 min at 65 $^{\circ}\text{C}$; 6 min at 95 $^{\circ}\text{C}$), and developing (3 min, SU-8 developer). The mixture of PDMS and curing agent in a 5:1 ratio was poured into the silicon mold and baked at 80 $^{\circ}\text{C}$ for 2 hours. PDMS layer was peeled, punched holes at inlet and outlet, and then bonded to glass layer using oxygen plasma. To prevent nonspecific binding and blood coagulation, bovine serum albumin (1%, v/v) and heparin (10%, v/v) in PBS were injected into the chip and blocked overnight.

Capture antigen & antibody immobilization. QDs-microbeads (2.5 mg) were washed by MES buffer (0.1 M, 200 μL) three times and suspended in MES buffer (1.25 mL) with NHS-sulfo (50 mg) and EDC (50 mg) for 30 min to activate carboxyl. Subsequently, beads were suspended in SARS-COV-2 antigens (5 $\mu\text{g}/\text{mL}$, 750 μL) or capture antibodies to IL-6 (2 $\mu\text{g}/\text{mL}$, 750 μL), IL-1 β (4 $\mu\text{g}/\text{mL}$, 750 μL),

PCT (25 ng/mL, 750 μ L), CRP (2 μ g/mL, 750 μ L). The mixture was briefly vortexed and incubated at 4 $^{\circ}$ C on a rotator overnight, followed by washing by PBST (1 \times PBS + 0.05% Tween 20) three times. For the washing steps, the supernatants were separated from sediment beads by centrifugation at 10000 rpm for 2 min. Protein conjugated beads were blocked by 4% BSA solution for 4 h.

Circular RCA template formation. Padlock template and biotin-primer at a molar ratio of 1:1 were mixed and heated in the PCR thermal cycler as follows: 95 $^{\circ}$ C for 5 min, cooled to 25 $^{\circ}$ C at a rate of 1 $^{\circ}$ C/min, 25 $^{\circ}$ C for 5 min, quickly cooled to 4 $^{\circ}$ C, 4 $^{\circ}$ C for 10 min. The solution was treated with 2.5 μ L of T4 ligase for 2 h at 25 $^{\circ}$ C to ligate the gap of the DNA template and then heated at 65 $^{\circ}$ C for 10 min. Free DNA was removed by adding 100 U exonuclease I (ExoI) to the mixture and incubated at 37 $^{\circ}$ C for 0.5 h. To inactivate the exonuclease, the mixture was kept at 80 $^{\circ}$ C for 15 min and then gradually cooled down to room temperature.

Antibodies & biomarkers detection. The sample/standard solution was mixed with the pre-coated beads (0.25 mg/mL) and introduced into the chip at 2 μ L/min for 10 min. And then channels were washed with PBST solution at 50 μ L/min for 5 min. Next, mixture solution of biotinylated secondary antibody to IL-6 (1 μ g/mL), IL-1 β (2 μ g/mL), PCT (500 ng/mL), CRP (0.5 μ g/mL) or biotinylated detection antigens (2 μ g/mL), PBST, streptavidin (500 nM), mixture of biotinylated primer (50 nM) and circle template (50 nM) were injected into the chip in turn. RCA reaction was carried out at 37 $^{\circ}$ C for 30 min in a premixed solution of phi29 DNA polymerase reaction buffer (10 \times , 2.5 μ L), BSA solution (1 wt%, 0.5 μ L), dA/C/GTP solution (10 mM, 0.1 μ L each), FAM-dUTP (1 mM, 0.5 μ L), phi29 polymerase (10 U/mL, 0.6 μ L) and ultrapure water (20.6 μ L). Finally, EDTA (5 mM, 25 μ L) was used to terminate the RCA reaction, followed by TE buffer washing.



Scheme S1. The procedure of lab-on-a-chip sandwich immunoassay and RCA for simultaneous detection of four biomarkers. Beads were encoded by utilizing four size levels (9 μ m, 13 μ m, 17 μ m, and 21 μ m in diameter). Ab₂: secondary detection antibody.

Agarose gel electrophoresis. The successful cyclization of the DNA template was characterized by running a 3.5% agarose gel electrophoresis analysis with the voltage of 7.5 V/cm for 1 h in 1 \times TAE buffer (40 mmol/L Tris-Acetic, 2 mmol/L EDTA, pH 8.0). The formation of RCA products was analyzed by performing 1% agarose gel electrophoresis at 6 V/cm for 70 min in 1 \times TAE buffer.

Patient sample detection. 11 COVID-19 positive patients and 11 COVID-19 negative patients were from the Fifth Affiliated Hospital of Sun Yat-Sen University. The plasma sample was mixed with beads conjugated antibodies/antigens and loaded into the chip together. The subsequent procedure was the same to describe in "Antibodies & biomarkers detection".

Imaging and analysis. Microscopic images and video were acquired by an inverted microscope (IX 83, Olympus) and confocal laser scanning microscope (FV3000, Olympus). Fluorescent images were obtained with 405 nm, 488 nm, and 560 nm lasers. The beads were monodispersed on the chip through manual repeated perfusion and suction before imaging. We first subtract the noise background signals and measure the mean fluorescence intensity (MFI) of rectangle regions of interest (ROI) on the images according to image J software (National Institutes of Health). The clumped beads would be eliminated during the process of the MFI measurement. Statistical analyses were carried out using GraphPad Prism 7.0 and origin 2021.

Results and Discussion

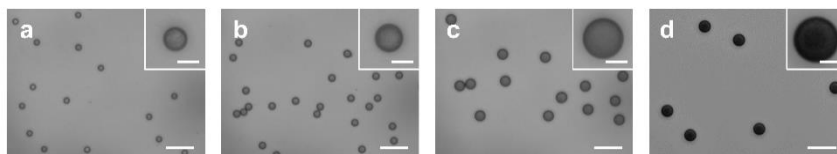


Figure S1. The bright-field images of different polystyrene beads with different sizes (9 μm , 13 μm , 17 μm , 21 μm in diameter from a to d). Scale bars: 50 μm ; 10 μm (magnification boxes).

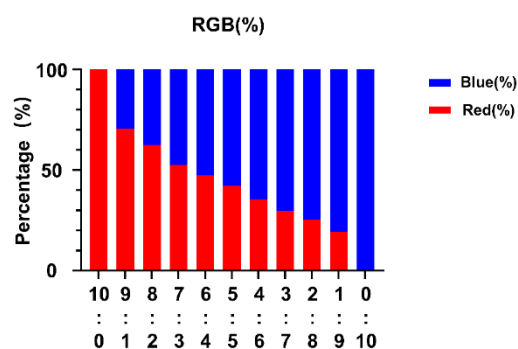


Figure S2. Bar graph showing the percentage of red and blue fluorescence intensity of different color-encoded beads.

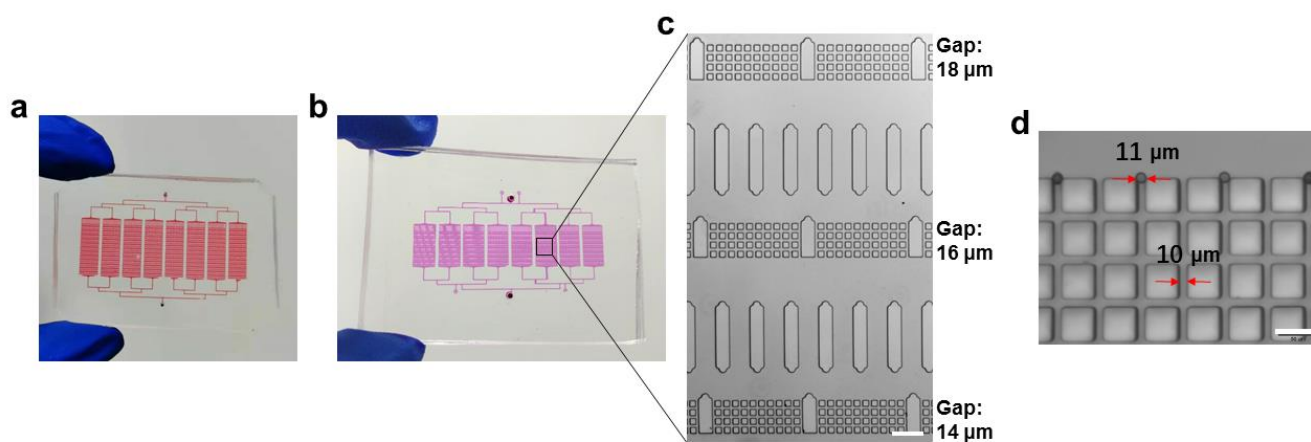


Figure S3. Photographs of the SS-Chips. Gaps ranging from 30 μm to 6 μm (a) and from 20 μm to 6 μm (b) between inlet and outlet. Bright-field images of sorting chambers at 10 \times objective (c) and 40 \times objective (d). Scale bars: 200 μm & 50 μm .

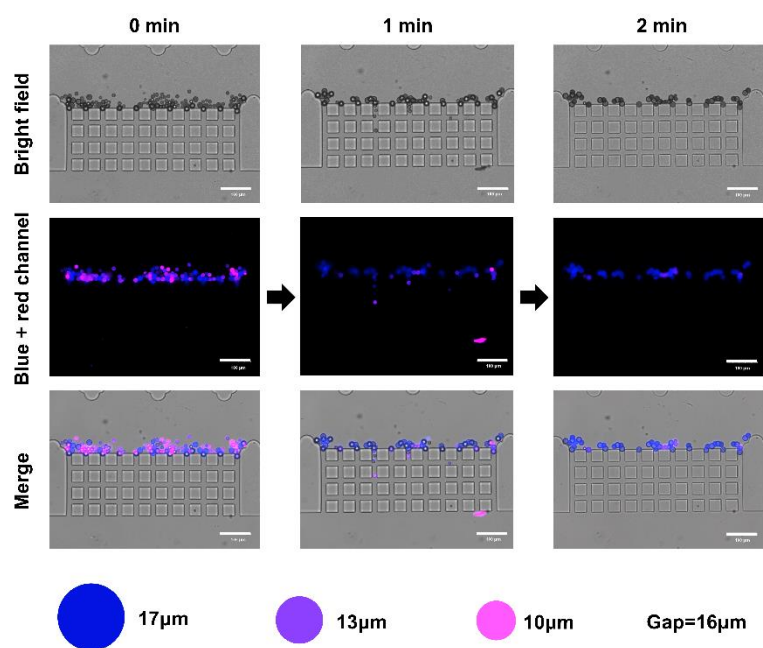


Figure S4. Time-lapse of three kinds of beads of different sizes and colors going through the microbarrier with a gap of 16 μm in diameter. Finally, only beads of 17 μm in diameter were left. Scale bars: 100 μm .

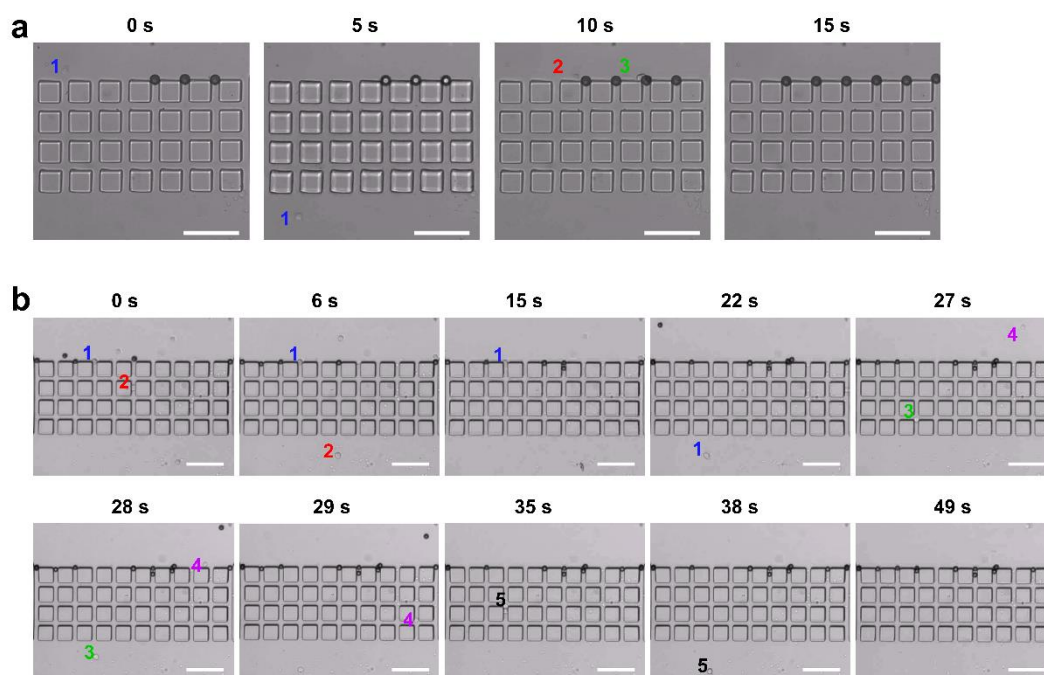


Figure S5. Time-lapse of blood cells and beads of 21 μm in diameter (a) and 13 μm in diameter (b) going through the microbarriers with gaps of 20 μm in diameter (a) and 12 μm in diameter (b). Cells are labeled with numbers. Scale bars: 100 μm .

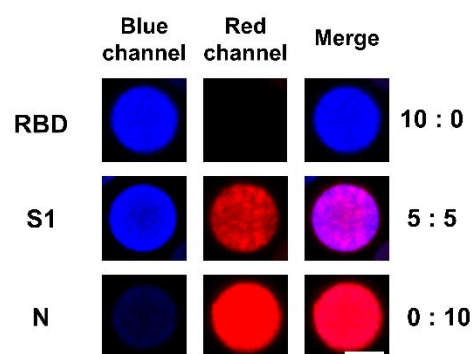


Figure S6. Three color-encoded beads were doped of B-QDs and R-QDs at 10: 0, 5: 5, and 0: 10 ratios for detecting antibodies against RBD, S1, N. Scale bar: 10 μm .

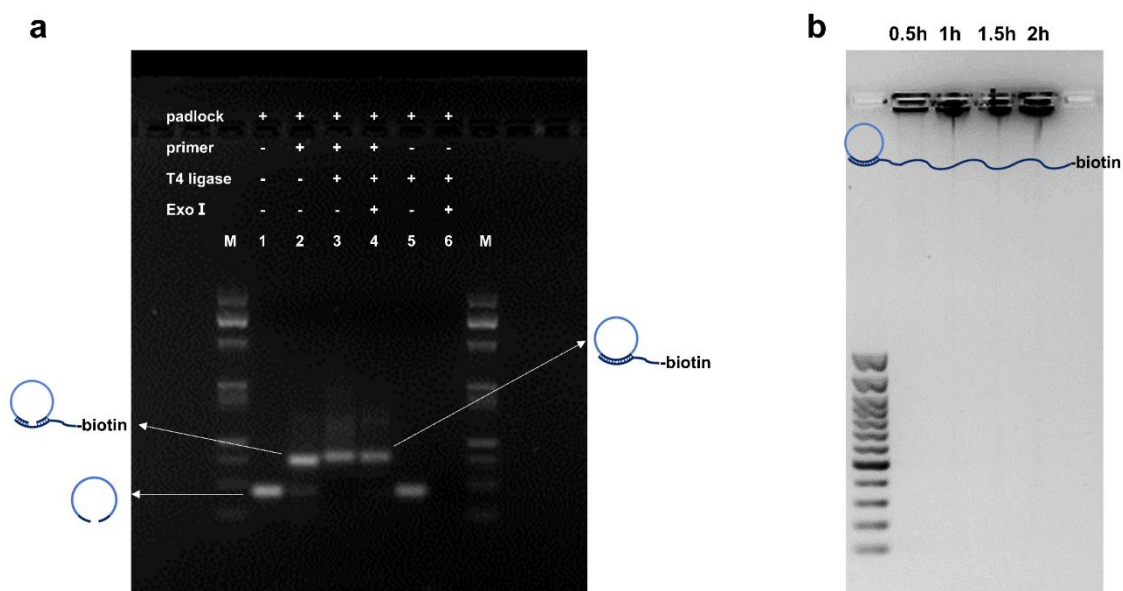


Figure S7. Agarose gel electrophoresis characterization of the RCA template and RCA product. a) Circ-DNA precursor produced only when the template and primer exist at the same time. b) A smeared band for the RCA product indicates the successful formation of the high-molecular-weight RCA product. The lane from left to right shows the RCA products with different reaction times.

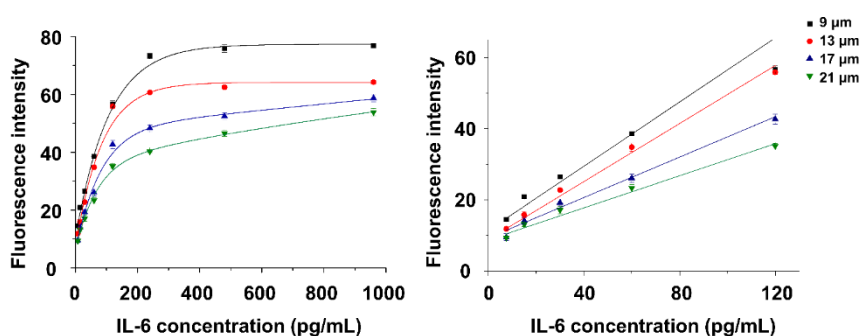


Figure S8. RCA is based on size-barcoded beads for the detection of IL-6 ranging from 0 to 1000 pg mL^{-1} . The error bars represent the standard deviation of three measurements ($n = 3$).

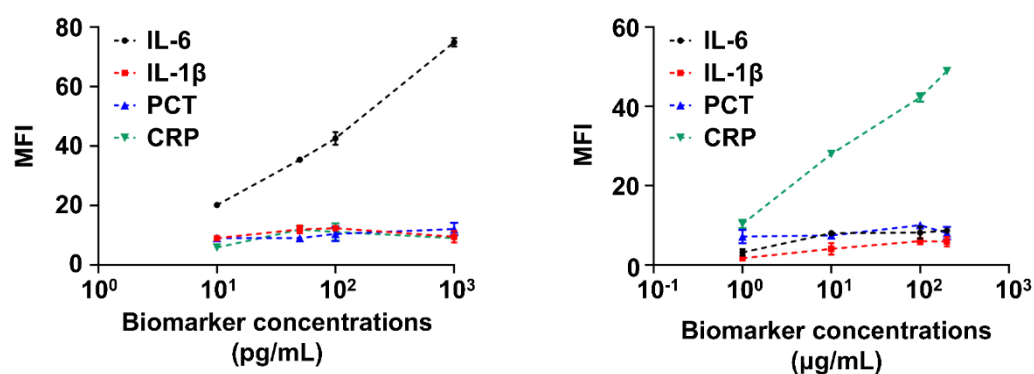


Figure S9. Specificity of SS-Chip immunoassay. Only IL-6 without the other three biomarkers (left) and only CRP without the other three biomarkers (right) were spiked into undiluted serum on SS-Chip immunoassay at different concentrations. The error bars represent the standard deviation of three measurements ($n = 3$).

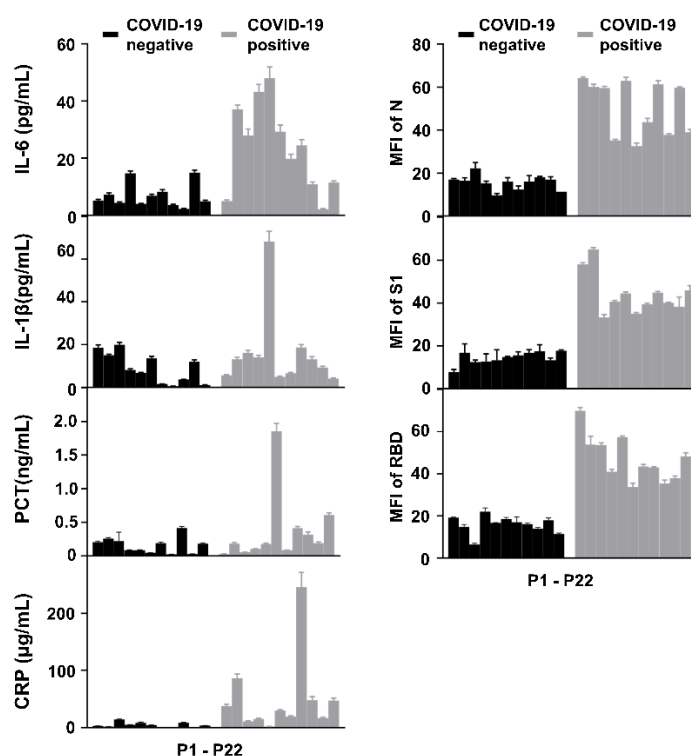


Figure S10. Four biomarkers and three antibodies detection in clinical samples based on SS-Chip immunoassay. The error bars represent the standard deviation of three measurements ($n = 3$).

Table S1. DNA sequences used in RCA.

| sequences | |
|-----------|--|
| primer | 5'-biotin-TTTTTTTTTTTTTTTTGTTCAGTTGCACCACGAATGCAC |
| template | 5'-phosphate-GTGCAACTGAACCTAACGCCATGTGTCTGACTATGTGCATTTCGATACGTATACGGACTCAAGTGGAAACAGTGCATTTCGTG |

[a] Color regions corresponding to the complementary regions in the RCA template and primer.

Movie S1. A whole blood sample mixed with antibody-conjugated beads was introduced into SS-Chip.

References

- [1] G. Wang, P. Zhang, H. Dou, W. Li, K. Sun, X. He, J. Han, H. Xiao, Y. Li, *Langmuir* **2012**, *28*, 6141-6150.