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Supporting Information

A SARS-CoV-2 Neutralization Assay Using Single Molecule Arrays

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Supporting Information

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Experimental Section

Plasma samples

COVID-19 positive samples were obtained from patients presenting to Brigham and Women's Hospital with viral respiratory symptoms. SARS-CoV-2 infection was confirmed by a nasopharyngeal RT-qPCR test. Our patient cohort consisted of plasma samples (n = 130) from two patient groups. The first group consisted of patients hospitalized with COVID-19 for less than ten days and not in the ICU (n = 51 samples from 33 patients). The median age for this group was 63 and the average age was 65 years old. There were 14 males and 19 females. The second group consisted of patients who died of COVID-19 (n = 79 samples from 29 patients). The median age for this group was 73 and the average age was 71 years old. There were 15 males and 14 females. Pre-pandemic samples collected before October 1, 2019 from healthy and sick individuals were obtained from the Mass General Brigham Biobank. The median age for this group was 45 and the average age was 46 years old. There were 9 males and 11 females. Samples from vaccinated individuals who have received two doses of Moderna mRNA-1273 were collected at Brigham and Women's Hospital from young individuals. The median age and the average age for this group were both 24 years old. There were 4 males and 4 females. No participant had a previous record of COVID-19 infection. All samples were collected under approval of the Mass General Brigham Institutional Review Board for Human Subjects Research. All plasma was collected in purple top tubes.

Bead conjugation

SARS-CoV-2 spike protein (produced in Bing Chen's lab), nucleocapsid recombinant protein (Ray Biotech 230-30164), and RBD proteins (Sino Biological 40592-V08H, 40592-V08H82, 40592-V08H85, and 40592-V08H90) were conjugated to 647 nm, 488 nm, and 750 nm dye-encoded carboxylated paramagnetic beads (Quanterix), respectively, using EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) chemistry (ThermoFisher Scientific 77149). For the multiplex assay, RBD proteins from wild type SARS-CoV-2 (Sino Biological 40592-V08H), Alpha variant (Sino Biological 40592-V08H82), Beta variant (Sino Biological 40592-V08H85), and Delta variant (Sino Biological 40592-V08H90) were conjugated to 750 nm, 647 nm, 700 nm, and 488 nm dye-encoded carboxylated paramagnetic beads, respectively. Before bead conjugation, 2.8×10^8 beads were washed three times with 200 µL of Bead Wash Buffer (Quanterix), three times with 200 µL of Bead Conjugation Buffer (Quanterix), and resuspended in 300 µL of Bead Conjugation Buffer. Freshly prepared EDC (10 mg/mL in Bead Conjugation Buffer) was immediately added to the beads solution to activate the beads. The beads were agitated on a HulaMixer (ThermoFisher Scientific) for 30 minutes at 4°C and washed once with 200 µL of Bead Conjugation Buffer. The beads were resuspended in 300 µL Bead Conjugation Buffer containing the target protein. The beads and protein solution were agitated on the HulaMixer for two hours at 4°C. After the bead conjugation, the protein-conjugated beads were washed twice with 200 µL of Bead Wash Buffer and blocked with 200 µL of Bead Blocking Buffer (Quanterix) for 30 minutes at room temperature. After blocking, the beads were washed once with 200 µL of Bead Wash Buffer and twice with 200 µL of Bead Diluent (Quanterix) before resuspending in 200 µL of Bead Diluent. The beads were counted using a Beckman Coulter Z1 Particle Counter and stored at 4°C for subsequent use. The amount of protein and EDC used in each reaction were as follows: 20 µg protein and 2 µL EDC for nucleocapsid conjugation, 16.67 µg protein and 6 µL EDC for spike conjugation, and 20 µg protein and 6 µL EDC for RBD conjugation. The bead conjugation was

validated using a biotinylated anti-His tag antibody (ThermoFisher MA121315BTI) using the Simoa HD-X Analyzer (Figure S1).

ACE2 biotinylation

Lyophilized ACE2 recombinant protein was purchased from Sino Biological (10108-H08H) and was resuspended in Biotinylation Reaction Buffer (Quanterix) to a final concentration of 1 mg/mL. 2 mg of EZ-Link NHS-PEG4 Biotin (Thermo Fisher Scientific A39259) was resuspended in deionized water based on the manufacturer's instructions. The NHS-PEG4-Biotin was added in 40-fold molar excess to the protein solution. The solution was incubated for 30 minutes at room temperature. The biotinylated ACE2 was purified using three washes with Biotinylation Reaction Buffer in a 50 KDa Amicon filter.

Simoa neutralization assay

The Simoa assay was performed on the HD-X Analyzer (Quanterix) in an automated two-step assay format according to the manufacturer's instructions. Resorufin β-D-galactopyranoside (RGP), System Wash Buffer 1, System Wash Buffer 2, Simoa Sealing Oil, and plastic consumables for the HD-X were purchased from Quanterix. COVID-19 patient plasma samples were serially diluted in Homebrew Detector/Sample Diluent (Quanterix). The prepared SARS-CoV-2 antigen-conjugated capture beads were mixed and diluted in Bead Diluent, with a total of 500,000 beads per reaction (for the singleplex assays: 125,000 spike-coated beads or nucleocapsid-coated beads or RBD-coated beads and 375,000 helper beads; for the multiplex assay: 125,000 RBD-coated beads for each of the four types of RBD protein). Biotinylated ACE2 was diluted in Homebrew Detector/Sample Diluent to a final concentration of 7,250 pg/mL. This optimal concentration of biotinylated ACE2 was chosen according to Figure S2. Streptavidin-β-galactosidase (SβG) concentrate (Quanterix) was diluted to 150 pM in SβG Diluent (Quanterix). In the first step, 25 μL of the capture beads and 20 μL of the biotinylated ACE2 were incubated with 100 μL of the diluted plasma sample for 35 minutes (the final ACE2 concentration in the reaction was 1,000 pg/mL). After the incubation, six wash steps were performed with System Wash Buffer 1. In the second step, the beads were resuspended in 100 μL of SβG, incubated for 5:15 minutes and washed six times with System Wash Buffer 1. The beads were then resuspended in 25 μL of RGP and loaded into the microwell array. Following the bead loading, the microwell array was sealed with oil and imaged. Average Enzyme per Bead (AEB) values were calculated by the HD-X Analyzer software. We validated the Simoa assay using increasing concentrations of a commercially obtained neutralizing antibody (Sino Biological 40592-R001). All samples were measured in duplicate.

Pseudovirus neutralization assay

For the pseudovirus neutralization assay, the SARS-CoV-2 spike protein was pseudotyped onto a GFP expression-driving lentivirus reporter vector similar to a previously published protocol.[33] Details of the protocol are described online (https://www.protocols.io/private/FA962E30715311EBB0C70A58A9FEAC2A). In order to improve trafficking to the cell membrane for generation of viral-like particles (VLPs), we removed 21 amino acids in the cytoplasmic tail thought to contain a cryptic endoplasmic reticulum (ER) retention signal[34] but left the extracellular domain intact. Plasma samples were then heat-inactivated at 58°C for one hour, and a dilution series was created by performing 5-fold serial dilutions in cell culture medium (DMEM + 10% FBS, 1% P/S) beginning with a 1:50 dilution. Dilutions were then incubated with CoV-2 spike pseudotyped VLPs for 1 hour at 37°C before

seeding modified ACE2-expressing HEK293T cells into the solution. Cells were incubated for 48 hours before trypsinization and fixation (4% paraformaldehyde for 30 minutes) prior to analysis by flow cytometry. ACE2-expressing HEK293Ts were generated by integrating a separate hEf1a-hACE2 lentiviral vector at high multiplicity of infection (MOI) prior to flow activated cell sorting (FACS) for expression of hACE2. An MOI of ~10 infectious particles per 100 cells was used (or MOI of around 0.1). hACE2 expression was found to be stable at 95% for over a dozen passages after sorting.

Neutralization capacities for plasma samples were determined by measuring inhibition of GFP production in ACE2 expressing HEK293T cells as a function of plasma dilutions. Relative quantification of GFP+ cells was determined by flow cytometry, and quantities of GFP expressing cells were normalized to the highest dilution of plasma. NT50 was calculated as the dilution corresponding to a normalized signal of 0.5 (i.e. 50% inhibition of GFP expression).

ELISA neutralization assay

Two commercially available ELISA neutralization assays (ThermoFisher Catalogue #BMS2326, and GenScript SARS-CoV-2 sVNT kit) were performed following manufacturer's instructions. For the ThermoFisher kit, plasma samples were measured at an initial 50x dilution, followed by five additional 3x dilutions. For the GenScript kit, samples were measured at an initial 10x dilution, followed by five additional 4x dilutions. NT50 values were calculated by normalizing signals at each dilution against signals measured at the highest dilution, and then fitting a 4PL-sigmoidal curve and interpolating values corresponding to 50% inhibition of signal.

Simoa antibody assay

Simoa assays for IgG and IgM against four SARS-CoV-2 targets (spike, S1, nucleocapsid and RBD) were performed as previously described.[17] Plasma samples were diluted 4,000-fold in Homebrew Detector/Sample Diluent (Quanterix Corp.). Four antigen-conjugated capture beads were mixed and diluted in Bead Diluent, with a total of 500,000 beads per reaction (125,000 of each bead type). Biotinylated antibodies were diluted in Homebrew Detector/Sample Diluent to final concentrations of: IgG (Bethyl Laboratories A80-148B): 7.73 ng/mL, IgM (Thermo Fisher MII0401): 216 ng/mL: 150 ng/mL. Streptavidin-β-galactosidase (SβG) concentrate (Quanterix) was diluted to 30 pM in SβG Diluent (Quanterix). The serology assay was performed on an HD-X Analyzer (Quanterix) in an automated three-step assay. Average Enzyme per Bead (AEB) values were calculated by the HD-X Analyzer software. AEB values were converted to normalized antibody titers using four calibrators that were included in each HDX run.

Data analysis

Neutralization capacity of plasma samples against SARS-CoV-2 was quantified as follows. Serial dilutions of plasma samples were generated, and measurements for each dilution were normalized to the highest dilution for a given sample. For the pseudovirus-based neutralization assay, percent of GFP+ cells were measured for each dilution, starting at 1:50 and followed by five additional points of 5x dilution. For the Simoa-based neutralization assay, average enzyme per bead (AEB) was measured at a starting dilution of 1:50 followed by 3x dilutions for five additional points. A sigmoidal 4PL curve was fit to these normalized values using Python 3.8, and dilution factors corresponding to 50% loss of signal (NT50) were interpolated from each curve. Antibody kinetics were fit to the Gompertz function. All other data fittings and statistical tests were performed in

GraphPad Prism 7. All figures were plotted in GraphPad Prism 7 and Adobe Illustrator version 2015.

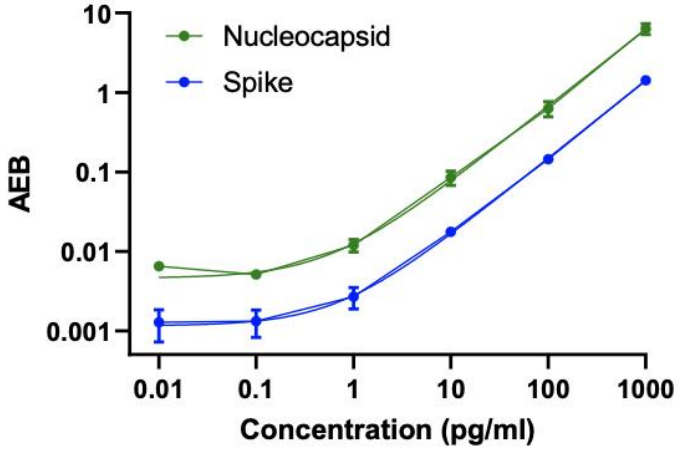

Figure S1. Bead conjugation validation. The spike and nucleocapsid proteins conjugated to the beads were tagged with a His-tag. To confirm that the proteins were conjugated to the beads, different concentrations ranging from 0.01 to 1,000 pg/mL of anti-His-tag antibody were added to the beads. Increasing the concentration of the anti-His-tag antibody resulted in an increase in signal. Mean and standard deviation of two preparations of spike coated beads (blue) and Nucleocapsid coated beads (green) are presented.

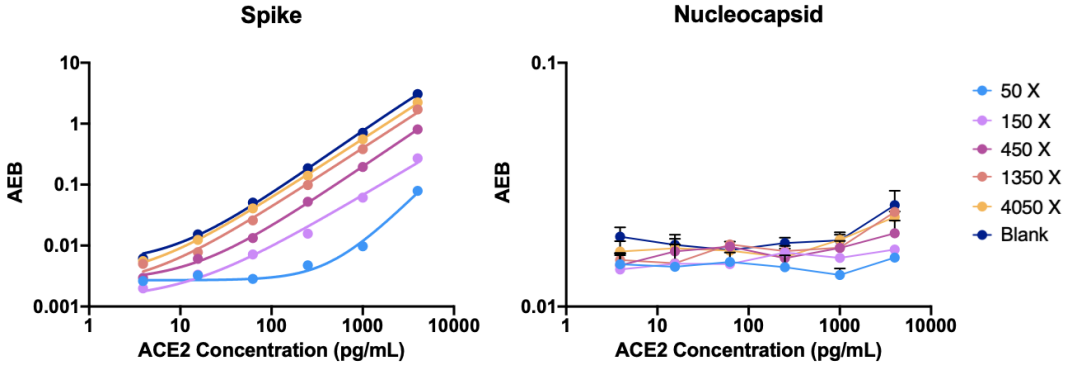

Figure S2. Determination of biotinylated ACE2 concentration. To determine the concentration of biotinylated ACE2 in the Simoa neutralization assay, we obtained plasma samples from one patient with COVID-19 and tested the sample across various plasma dilutions and ACE2 concentrations. The plasma dilution factors ranged from 50X to 4050X and the ACE2 concentrations ranged from 4 pg/mL to 4,000 pg/mL. Higher ACE2 concentrations across the various dilutions led to a higher signal using the spike-coated beads but not the nucleocapsid-coated beads. The optimal ACE2 concentration was determined to be 1000 pg/mL, maximizing the signal for the spike-coated beads while minimizing the signal arising from non-specific binding to the nucleocapsid coated beads.

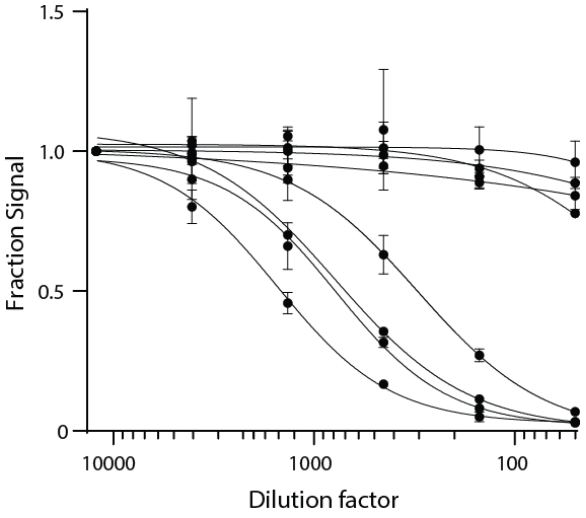

Figure S3: Assay precision across runs. To measure the precision of the ACE2 inhibition Simoa assay, eight plasma samples, either neutralizing or non-neutralizing, were measured in duplicate across two separate runs. Points represent the mean measurements across the two separate runs, while error bars represent measurement ranges.

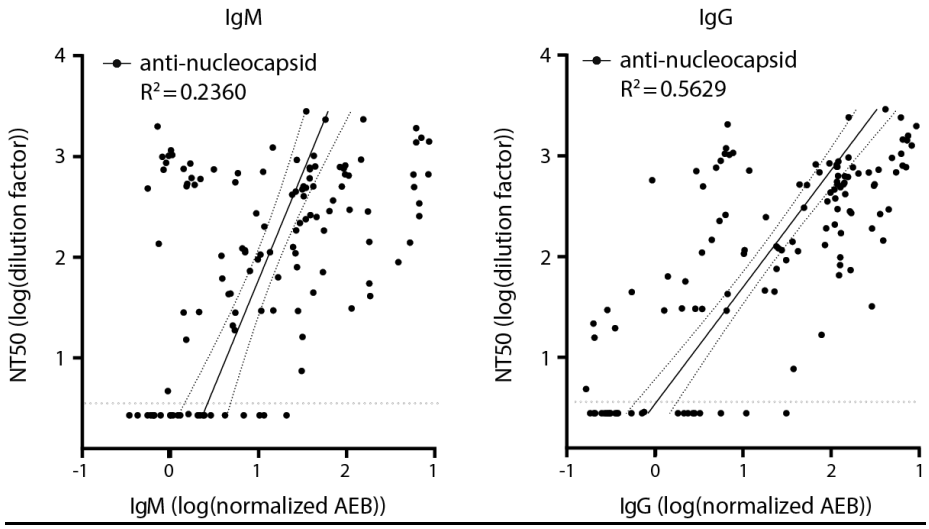

Figure S4: **Correlation between NT50 values and antibodies against nucleocapsid levels.** Correlation between NT50 values and IgM (left) and IgG (right) levels against nucleocapsid.

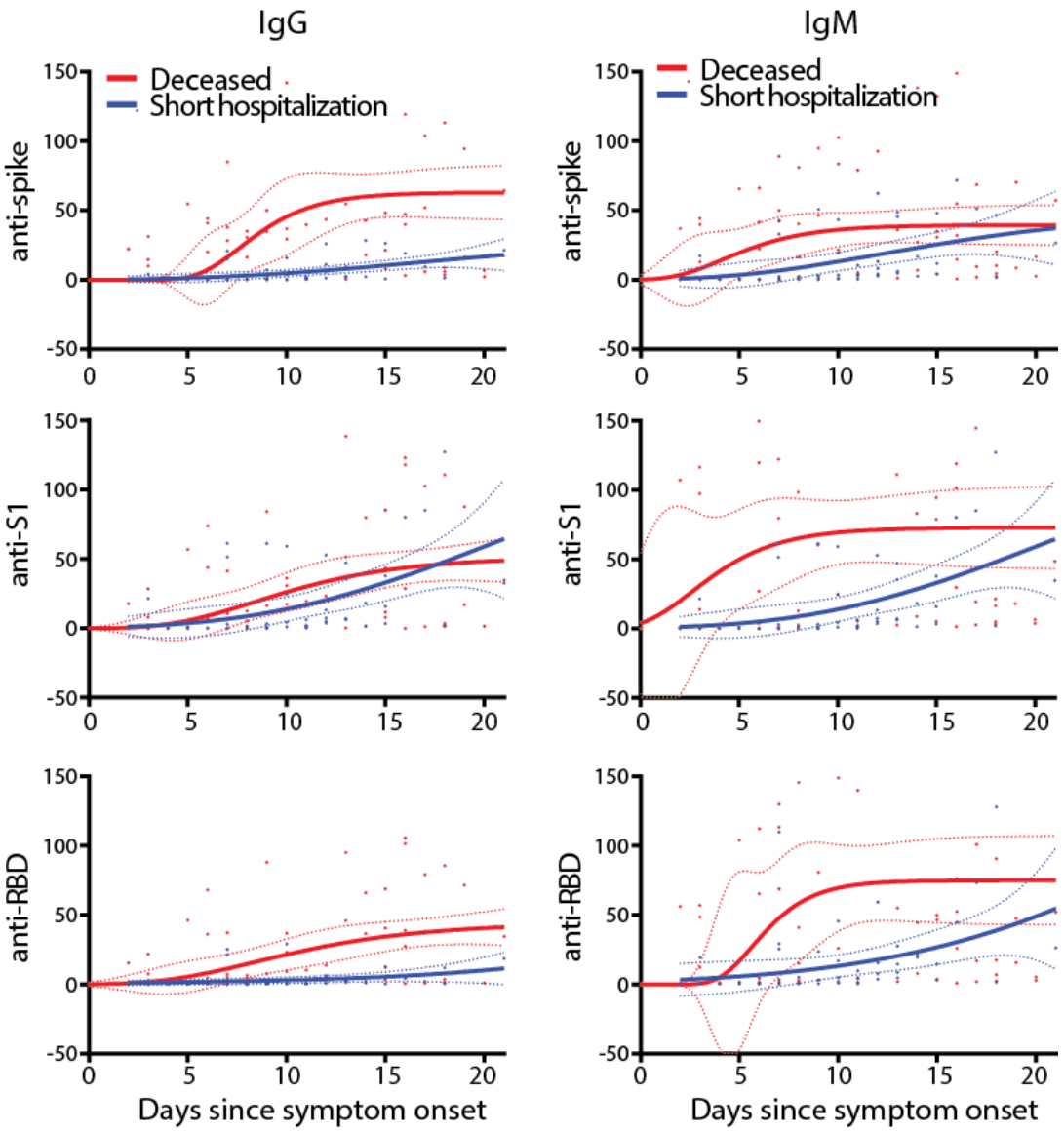

Figure S5: **Antibody levels over time in the two patient groups presented in Figure 5.** Antibody levels over time in the two patient groups: patients hospitalized with COVID-19 for less than ten days who did not require treatment in the intensive care unit (blue), and hospitalized patients who died of COVID-19 (red).

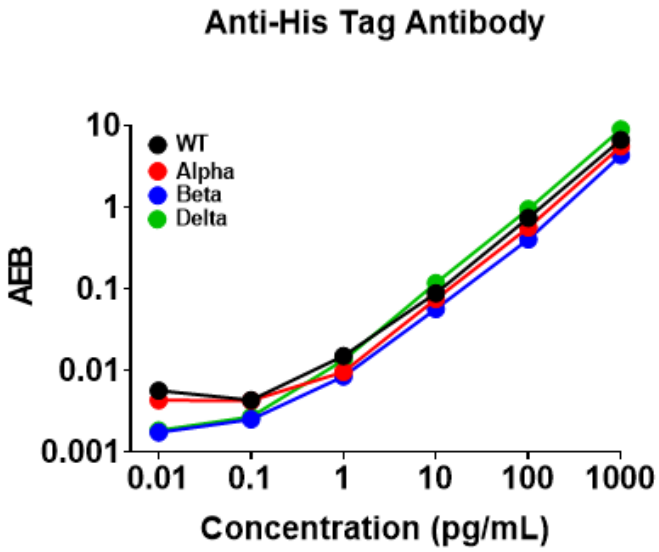

Figure S6: **Variant bead conjugation validation.** The wild type (WT) SARS-CoV-2, SARS-CoV-2 RBD with the N501Y mutation (Alpha variant), SARS-CoV-2 RBD with the K417N, E484K, N501Y mutations (Beta variant), and SARS-CoV-2 RBD with the T478K and L452R mutations (Delta RBD) conjugated to the beads were tagged with a His-tag. To confirm that the proteins were conjugated to the beads, different concentrations ranging from 0.01 to 1,000 pg/mL of anti-His-tag antibody were added to the beads. Increasing the concentration of the anti-His-tag antibody resulted in an increase in signal.

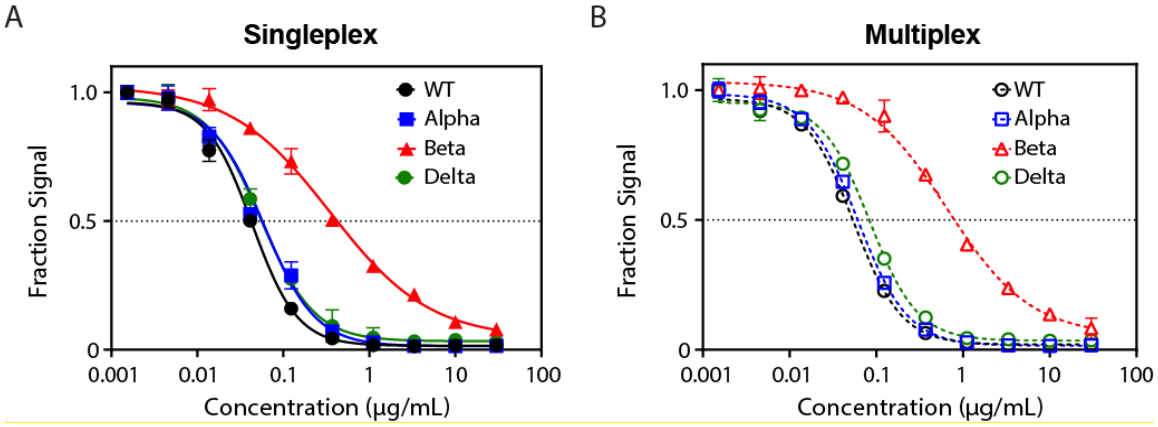

Figure S7: Comparison of singleplex (A) and multiplex (B) Simoa assays using increasing concentrations of a SARS-CoV-2 neutralizing antibody. As the concentration of a neutralizing SARS-CoV-2 antibody increases, the assay signal decreases. Error bars represent the standard deviation of two measurements.

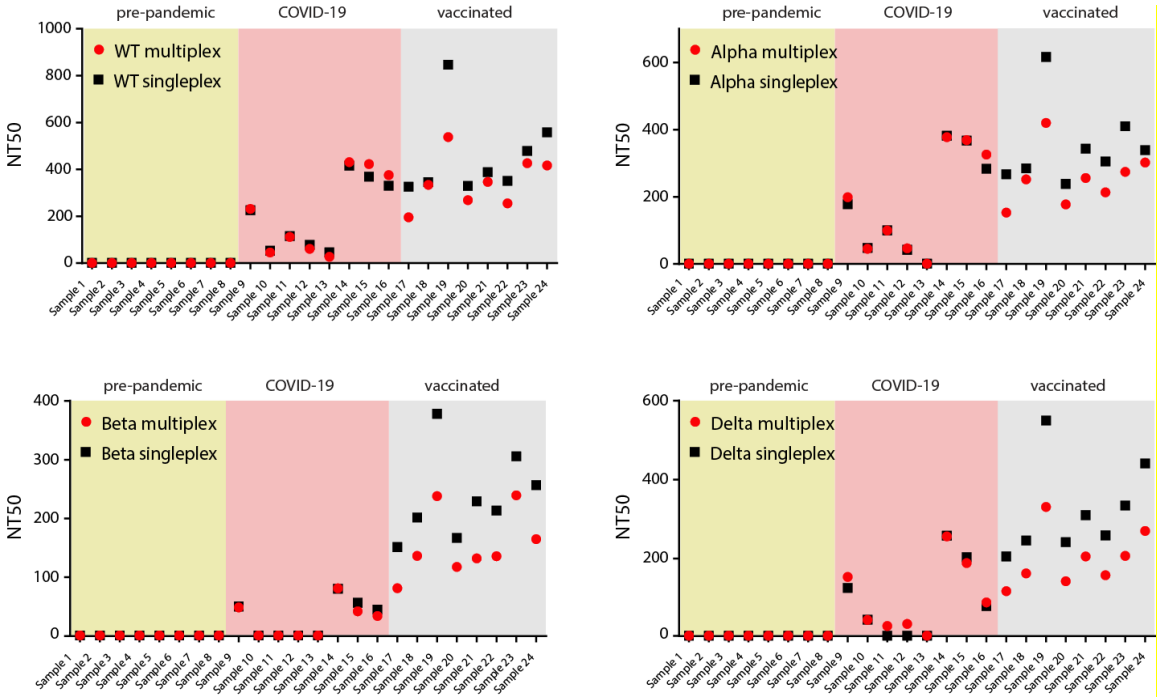

Figure S8: **Validation of the singleplex and multiplex assays in patient samples.** Using both the singleplex and multiplex assays, NT50 values for the SARS-CoV-2 wild-type and the three variants were determined in the samples presented in Figure 6C and 6D

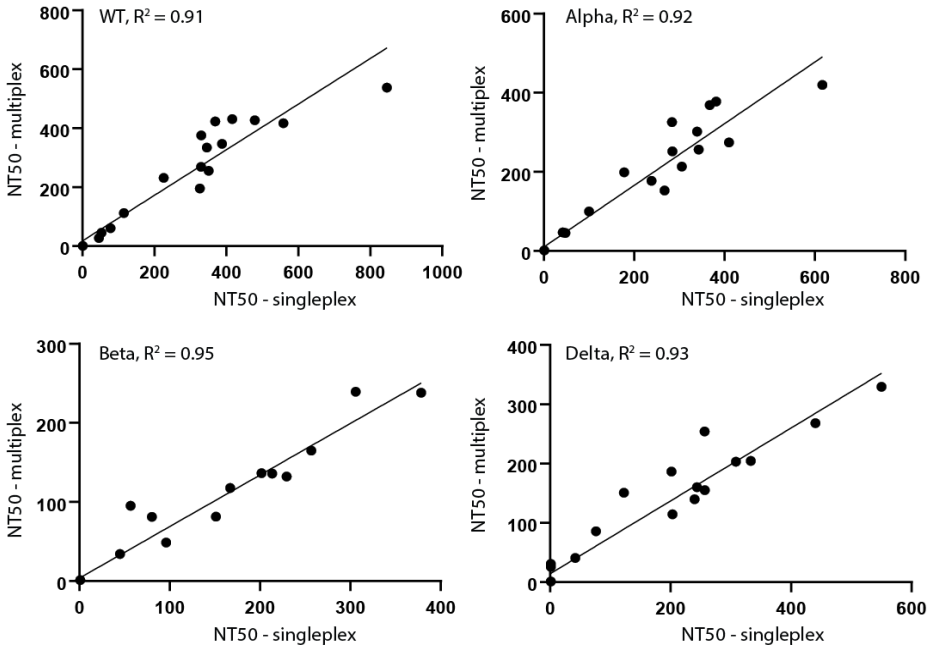

Figure S9: **Correlation between the singleplex and multiplex assays in patient samples.** NT50 values of the singleplex and multiplex assays for the SARS-CoV-2 wild-type and the three variants in the samples presented in Figure 6C and 6D.

Sample Number	NT50
1	51
2	557
3	185
4	73
5	95
6	2337
7	926
8	1017
9	506
10	285

Table S1. IC50 values for the ten COVID19 positive samples presented in Figure 3A.