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3 **Evaluation of neutralizing antibodies against SARS-CoV-2 variants after infection and**  
4 **vaccination using a multiplexed surrogate virus neutralization test**  
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28 **Running head:** Neutralizing antibodies against SARS-CoV-2 variants  
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30 **Keywords:** surrogate virus neutralization test, SARS-CoV-2 variants, COVID-19, antibody,  
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33 diagnostics  
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35 **Nonstandard Abbreviations:** sVNT, surrogate virus neutralization test; plex-sVNT, multiplex  
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37 surrogate virus neutralization test; ACE2, angiotensin converting enzyme-2 receptor protein;  
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39 PRNT, plaque reduction neutralization test; VOC, variant of concern; VOI, variant of interest;  
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41 ICU, intensive care unit; RFI, relative fluorescence intensity; RBD, receptor binding domain  
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## 1 **Abstract**

2 **BACKGROUND:** The SARS-CoV-2 virus has mutated and evolved since the inception of the  
3 COVID-19 pandemic bringing into question the future effectiveness of current vaccines and  
4 antibody therapeutics. With evolution of the virus updated methods for the evaluation of the  
5 immune response in infected and vaccinated individuals are required to determine the durability  
6 of the immune response to SARS-CoV-2 variants.

7 **METHODS:** We developed a multiplexed surrogate virus neutralization test (plex-sVNT) that  
8 simultaneously measures the ability of antibodies in serum to inhibit binding between  
9 angiotensin converting enzyme-2 (ACE2) and 7 SARS-CoV-2 trimeric spike protein variants,  
10 including wild type, B.1.1.7( $\alpha$ ), B.1.351( $\beta$ ), P.1( $\gamma$ ), B.1.617.2( $\delta$ ), B.1.617.1( $\kappa$ ), and B.1.429( $\epsilon$ ).  
11 The assay was validated against a plaque reduction neutralization test (PRNT).  
12 We evaluated 170 samples from 97 COVID-19 patients and 281 samples from 188 individuals  
13 that received the Pfizer-BioNTech or Moderna mRNA vaccines.

14 **RESULTS:** The plex-sVNT demonstrated >96% concordance with PRNT. Antibody  
15 neutralization activity was significantly reduced for all SARS-CoV-2 variants compared to wild  
16 type in both the infected and vaccinated cohorts. There was a decline in overall antibody  
17 neutralization activity, within both cohorts, out to 5 months post infection or vaccination, with  
18 the rate of decline being more significant for the vaccinated.

19 **CONCLUSIONS:** The plex-sVNT provides a correlative measure to PRNT and a convenient  
20 approach for evaluating antibody neutralization against SARS-CoV-2 variants. Neutralization of  
21 SARS-CoV-2 variants is reduced compared to wild type and declines over the ensuing months  
22 after exposure or vaccination within each cohort, however it is still unknown what degree of  
23 neutralizing capacity is protective.

## 24 INTRODUCTION

25 SARS-CoV-2 has infected nearly 200 million people with 4 million deaths worldwide in  
26 the first year and a half of the COVID-19 pandemic. The release of highly effective coronavirus  
27 vaccines has greatly reduced the rate of severe disease and death in areas where vaccination rates  
28 are high. Correlates of immunity in an individual are still not well defined. SARS-CoV-2  
29 continues to mutate which raises uncertainty regarding the effectiveness and duration of the  
30 immune response following natural infection and vaccination. Gain-of-function viral variants  
31 can result in improved replication capacity, viral infectivity, transmissibility, and neutralizing  
32 antibody escape. SARS-CoV-2 Variants of Concern (VOCs) have been identified and currently  
33 include; B.1.1.7 (UK, 201/501Y.V1, Alpha, or  $\alpha$ ), B.1.351 (South Africa, 20H/501Y.V2, Beta,  
34 or  $\beta$ ), P.1 (Brazil, Gamma, or  $\gamma$ ), and B.1.617.2 (India, Delta, or  $\delta$ ). Additional Variants of  
35 Interest (VOIs) currently include; B.1.525 (Eta, or  $\eta$ ), B.1.526 (Iota, or  $\iota$ ), B.1.617.1 (India,  
36 Kappa, or  $\kappa$ ), C.37 (Peru, Lambda, or  $\lambda$ ), and B.1.621 (Columbia, Mu, or  $\mu$ ). Other variants such  
37 as B.1.429 (California, Cal20, Epsilon, or  $\epsilon$ ), were once listed as VOCs or VOIs and have since  
38 been removed from the list as others have emerged and become more dominant. Emerging data  
39 suggests that these VOCs/VOIs are susceptible to neutralization by SARS-CoV-2 antibodies in  
40 convalescent and vaccinee serum, however, to a lesser degree than wild-type virus (1-4). Most of  
41 these studies evaluated one time-point soon after vaccination with one specific vaccine for a  
42 limited number of variants, limiting the ability to compare: 1) neutralizing capacity against  
43 multiple variants within a specific group (e.g.  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  after infection) 2) neutralizing  
44 activity in different groups (e.g. natural infection vs. vaccination), and 3) the rate of decline  
45 between time-points (e.g. 1 vs. 5 months after onset of symptoms/first vaccination) for all  
46 variants in different groups (e.g. natural infection vs. vaccination).

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3 47 Various methods used to measure the SARS-CoV-2 humoral response have been  
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5 48 described including qualitative and quantitative methods for total antibody or antibody  
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7 49 subclasses (IgG, IgM, IgA) (1, 4), IgG avidity (5), and antibody neutralization activity. Plaque  
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9 50 reduction neutralization tests (PRNT) measure SARS-CoV-2 neutralizing antibody titer and  
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11 51 involve the use of live pathogens and target cells (6, 7). These laborious tests require a high  
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13 52 degree of expertise and expose laboratory personnel to infection risks, limiting the widespread  
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15 53 availability of testing. Surrogate virus neutralization tests (sVNTs) based on antibody-mediated  
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17 54 blockage of molecular interactions have been described (8-10). An sVNT measures the  
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19 55 competitive inhibition of the interaction between a viral structural protein and angiotensin-  
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21 56 converting enzyme 2 (ACE2), the receptor of SARS-CoV-2 on host cells. Like PRNT, sVNTs  
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23 57 detect neutralizing antibodies in an isotype-independent manner, offering a key advantage over  
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25 58 antibody concentration assays. Here we present a multiplexed sVNT (plex-sVNT) method for the  
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27 59 simultaneous evaluation of the ability of antibodies produced after infection or vaccination to  
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29 60 inhibit the interaction between ACE2 and SARS-CoV-2 trimeric spike protein containing current  
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31 61 VOCs. No studies to date have evaluated neutralizing antibodies from infected and vaccinated  
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33 62 individuals for all major variants of concern using the same analytical method for comparison.  
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## 64 **MATERIALS AND METHODS**

### 65 **Sample Collection**

66 **Ethical review.** Two separate protocols, one for remnant specimens from patients who had  
67 COVID-19 natural infection (IRB #20-30387) and the other for COVID-19 vaccinated healthy  
68 individuals (IRB #20-33062), were approved by the Institutional Review Board of the University  
69 of California, San Francisco. The committee judged that written consent was not required for use

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3 70 of remnant specimens. Written informed consent was obtained for blood collections from  
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5 71 COVID-19 vaccinated individuals.  
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10 73 **Subjects and specimens.** The COVID-19 infection cohort utilized remnant serum or plasma  
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12 74 samples (n=170) from routine clinical hospital laboratory testing. All samples were collected at  
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14 75 least 12 days from symptom onset with an attempt to collect samples across the range of days out  
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17 76 to 6 months. When two or more samples were collected for a patient, they were separated by at  
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19 77 least 7 days. The median number of specimens per subject was 1 for the non-ICU patients and 2  
20  
21 78 for ICU patients. All patients (n=97) had positive results by SARS-CoV-2 real-time polymerase  
22  
23 79 chain reaction (RT-PCR) in nasopharyngeal swabs between March and July 2020. Clinical data  
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25 80 were extracted from electronic health records and included demographic information, patient-  
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27 81 reported symptom onset date and indicators of disease severity. Patients were categorized based  
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29 82 on their level of care; patients admitted to an intensive care unit at any time were classified as  
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31 83 ICU patients, whereas those admitted to a hospital or managed as outpatients were considered  
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33 84 non-ICU patients. The criteria for ICU admission at the hospital remained the same throughout  
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35 85 the course of the study. No patients received convalescent plasma or monoclonal antibody drugs.  
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40 86 The COVID-19 vaccination cohort (n=188) utilized serum samples (n=281) collected via  
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42 87 phlebotomy from healthcare workers. All individuals received either the Pfizer-BioNTech  
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44 88 (n=150) or Moderna (n=38) vaccine between December 2020 and February 2021. No one had a  
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46 89 previous COVID-19 infection as determined by self-report and all had a negative test for  
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48 90 antibodies to the SARS-CoV-2 nucleocapsid protein. All samples were collected at least 7 days  
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50 91 after second vaccination and 28 days (Pfizer-BioNTech) or 35 days (Moderna) from first  
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52 92 vaccination with an attempt to collect samples across the range of days out to 6 months. When  
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93 two or more samples were collected from a subject, they were separated by at least 7 days. The  
94 median number of specimens per subject was 1 for the Pfizer-BioNTech and 2 for Moderna.  
95 Demographic data and dates of vaccination were collected following informed consent.

96 All samples were maintained at -20 °C for the duration of the study. After thawing at  
97 room temperature, samples were briefly vortexed before testing in singlicate.

### 99 **Recombinant trimeric spike proteins and ACE-2-FC fusion proteins**

100 Recombinant trimeric spike protein variants comprising wild-type Wuhan strain, UK  
101 B.1.1.7 ( $\alpha$ ), South African B.1.351 ( $\beta$ ), Brazilian P.1 ( $\gamma$ ), two Indian B.1.617.1 and B.1.617.2 ( $\kappa$   
102 and  $\delta$ ) and California B.1.429 ( $\epsilon$ , receptor binding domain only) were purchased from Icosagen,  
103 Estonia. Recombinant trimeric spike protein for the WA1 strain was not commercially available.  
104 Besides variant specific mutations, each trimeric protein contains aa 14-1211, plus two extra  
105 amino acids (AS) in the N-terminus, trimerization domain and His-6 tag at its C-terminus. Also  
106 added is a GS linker between the protein and trimerization domain as well as a GSG linker  
107 between the trimerization domain and His-tag. The furin cleavage site RRAR between Spike S1  
108 and S2 is mutated to GSAS.

109 Variant specific mutations to trimeric proteins include HV 69-70 del, Y144 del, N501Y,  
110 A570D, D614G, P681H, T716I, S982A, D1118H for B.1.1.7 ( $\alpha$ ), L18F, D80A, D215G, LAL  
111 242-244 del, R246I, K417N, E484K, N501Y, D614G, A701V for B.1.351 ( $\beta$ ), L18F, T20N,  
112 P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I, V1176F for P.1 ( $\gamma$ ),  
113 T19R, G142D, E156G, del\_F157-R158, L452R, T478K, D614G, P681R for B.1.617.2 ( $\delta$ ) and  
114 G142D, E154K, L452R, E484Q, D614G, P681R for B.1.617.1 ( $\kappa$ ). All recombinant proteins

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3 115 were expressed in Chinese hamster ovary-based cell line and cell free supernatant used to purify  
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5 116 proteins by Ni-affinity chromatography and filtration.  
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8 117 The gene encoding the recombinant human ACE2-Fc fusion protein comprising  
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10 118 extracellular 18-741 amino acid domain of human ACE-2 receptor protein (NP\_068576.1) fused  
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12 119 to 225 amino acid human Fc fragment at the carboxyl terminus via a 9-residue glycine spacer  
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14 120 was transfected into HEK-293\_sus cells. The expressed soluble fusion ACE2-Fc protein was  
15  
16 121 purified over Protein A resin column. The eluted protein was buffer exchanged into PBS, PH 7.0  
17  
18 122 and biotinylated using EZ-link sulfo NHS-biotin at 50 molar excess of biotin reagent to ACE2-  
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20 123 Fc fusion protein (Thermo Scientific). Unbound biotin was removed via desalting over Thermo  
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22 124 Fisher Zebra spin column.  
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### 27 28 126 **Multiplexed SARS-CoV-2 surrogate virus neutralization test (plex-sVNT)**

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30 127 Trimeric spike protein variants were coupled separately to spectrally distinct  
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32 128 paramagnetic beads. Briefly, SARS-CoV2 neutralizing antibodies present in serum/plasma  
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34 129 compete with biotinylated-human ACE2-Fc protein for binding to trimeric proteins that are  
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36 130 coupled to the beads (**Fig. 1A and 1B**). After washing, the beads are incubated with streptavidin-  
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38 131 phycoerythrin (SA-PE; Agilent) conjugate followed by additional washes. Washed beads are  
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40 132 suspended in buffer followed by passage through the detector that measures the relative  
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42 133 fluorescent signal (RFI) associated with beads on the BioPlex 2200 platform (Bio-Rad  
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44 134 Laboratories). The identity of the dyed beads is determined by the fluorescence of the dyes, and  
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46 135 the amount of antibody captured by the antigen is determined by the fluorescence of the attached  
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48 136 PE. Raw data are calculated in relative fluorescence intensity (RFI). The RFI signal is inversely  
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50 137 proportional to the concentration of SARS-CoV-2 neutralizing antibodies in the sample. An  
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3 138 optimal signal was observed using 1 ug/mL biotinylated ACE2-Fc protein and 6 ug/mL SA-PE  
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5 139 conjugate.

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8 140 Direct binding of the recombinant ACE2 receptor protein to trimeric proteins was  
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10 141 demonstrated using fluorescence of the attached PE to the biotinylated ACE2 receptor protein in  
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12 142 the presence of normal healthy pre-pandemic serum. The observed RFI signal was normalized to  
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14 143 calculate average fluorescent intensity/ $\mu$ g variant specific protein. Dose dependent inhibition of  
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16 144 ACE2 binding to trimeric protein by neutralizing antibodies present in the infected and  
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18 145 vaccinated cohort samples was calculated by subtracting the fluorescence of the test sample from  
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20 146 the mean fluorescence of the normal healthy samples while % inhibition was calculated as  
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22 147 follows:  $1 - (\text{RFI of the test sample} / \text{RFI of the normal sample})$ .

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26 148 The assay cutoff of 25% inhibition for the ACE2 - trimeric protein binding was  
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28 149 established based on 99th percentile cutoff using commercially available healthy normal,  
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30 150 pregnancy and potential cross reactant samples (N = 866) collected prior to November 2019.  
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32 151 Precision was determined for low (34%), medium (65%) and high (91%) percent inhibition.  
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34 152 Within-run, between-run, and total imprecision ranged from 0.5% to 3.9%, 1.1% to 3.4%, and  
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36 153 1.2% - 7.1%, respectively. Linearity was assessed using the WHO international standard for  
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38 154 SARS-CoV-2 antibody (NIBSC code 20/136). The assay was log-log linear ( $r=0.99$ ) from 80%  
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40 155 (corresponding to 1000 IU/mL) to 21% (corresponding to 31.5 IU/mL). A high sample  
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42 156 corresponding to 100% inhibition was used to demonstrate extended linearity from 80%-100%.

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49 158 **PRNT SARS-CoV2 neutralization assay**

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51 159 The PRNT assay was conducted in a biosafety level 3 (BSL-3) laboratory at the Colorado  
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53 160 State University Infectious Disease Research Center (Fort Collins, CO). Samples were heat



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3 161 inactivated for 30 min at 56 °C, and serial twofold dilutions were prepared in a 96-well plate  
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5 162 (Greiner Bio One). Viral stock (strain hCoV-19/USA/WA1/2020, BEI Resources) containing  
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8 163 approximately 200 plaque-forming units (pfu) per 0.1 ml was added to each well containing  
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10 164 plasma dilutions. Following an incubation period at 37°C in a 5% CO<sub>2</sub> incubator, 6-well plates  
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12 165 (Greiner Bio One) containing recently confluent Vero cells (ATCC) were inoculated with the  
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14 166 virus–plasma mixtures. After a second incubation period at 37°C, 2 ml of overlay (2× MEM with  
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16 167 4% FBS [Peak Serum] and agarose) was added to each well. After 24 h incubation at 37°C, a  
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18 168 second overlay containing neutral red (Millipore Sigma) was dispensed into each well and the  
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20 169 number of plaques was counted 48–72 h after initial inoculation. The highest dilution of plasma  
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22 170 that inhibited plaque formation by 50% (PRNT<sub>50</sub>) and 90% (PRNT<sub>90</sub>) was determined based  
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24 171 upon the titer of the viral stock and the number of plaques present at each dilution. Samples with  
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26 172 PRNT<sub>50</sub> titers of <1:20 are considered negative for neutralizing antibodies.  
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### 33 174 **Statistical analysis**

35 175 All data were entered into the Research Electronic Data Capture (REDCap) database.  
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37 176 Comparisons for parameters passing the Shapiro–Wilk test for normality were performed using a  
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39 177 paired, two-tailed *t*-test where statistical significance was defined as  $P < 0.05$ . Correlation  
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41 178 between the plex-sVNT and PRNT was determined using Spearman coefficients. Positive and  
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43 179 negative percent agreement (PPA, NPA) were performed using multiple cutoffs for neutralizing  
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45 180 titers since the true protective titer has not been established. Ideal cutoffs for the plex-sVNT  
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47 181 method that maximized PPA and NPA were calculated using the Youden Index. Neutralizing  
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49 182 antibody (% inhibition or %I) measurements for the COVID-19 variants compared to wild type  
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51 183 were analyzed with-in each group (ICU, non-ICU, Pfizer-BioNTech, Moderna) using one-way  
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3 184 repeated measures ANOVA, followed by the Fisher Least Significant Difference test. The  
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5 185 percent inhibition for SARS-CoV-2 variants was compared to wild-type using Deming  
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7 186 regression and Bland-Altman analysis with determination of the Spearman correlation. The rate  
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10 187 of decline of percent inhibition versus days since symptom onset or first vaccination was  
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12 188 determined using simple linear regression analysis and calculation of the 95% confidence  
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14 189 interval (95% CI). Statistical analysis was performed using Analyse-it software (method  
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17 190 validation edition, version 4.95) and GraphPad Prism (version 9.2.0).  
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## 192 **RESULTS**

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24 193 The COVID-19 infection cohort included 97 SARS-CoV-2 RT-PCR positive patients, 41  
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26 194 (42%) admitted to the hospital and 56 outpatients (**Table 1**). Of the hospitalized patients, 28  
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28 195 (68%) were admitted to the ICU and 22 (54%) required mechanical ventilation. The ICU patients  
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31 196 were older (median age 54.7 y) and a higher percentage were male (64.3%) compared to non-  
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33 197 ICU patients (43.5 y and 43.5%). The median number of days from symptom onset to sample  
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35 198 collection was significantly shorter for ICU patients (29) compared to non-ICU patients (55).  
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38 199 The COVID-19 vaccination cohort included 188 individuals that received two doses of an  
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40 200 mRNA vaccine, 150 Pfizer-BioNTech and 38 Moderna (**Table 1**). The median age for those  
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42 201 receiving the Pfizer (41.5 y) and Moderna (39.5 y) vaccination were similar. Both groups were  
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44 202 predominately female, with a higher percentage of females in the Pfizer (78.7%) compared to  
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46 203 Moderna (60.5%) group. The median time between vaccine doses differed by 7 days for Pfizer  
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48 204 (21) and Moderna (28), while the median number of days from first vaccine dose to sample  
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51 205 collection was not statistically different.  
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3 206 The performance of the plex-sVNT using wild-type trimeric spike protein was compared  
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5 207 against the PRNT virus neutralization assay for 76 samples from the COVID-19 infection cohort  
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8 208 and 102 commercially available healthy normal, pregnancy and potential cross reactant samples.  
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10 209 The Spearman correlation between the plex-sVNT assay (cut-off, 25% inhibition) and PRNT<sub>50</sub>  
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12 210 (cut-off, 1:10) and PRNT<sub>90</sub> (cut-off, 1:20) was 0.80 (95%CI: 0.69-0.88,  $P < 0.001$ ), and 0.88  
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14 211 (95%CI: 0.81-0.93,  $P < 0.0001$ ), respectively (**Fig. 2, A and B**).  
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17 212 The positive agreement and negative agreement for 178 samples was 96% (95%CI: 88.9-98.6%)  
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19 213 and 99% (95%CI: 97.4%-99.8%) , respectively, for both PRNT<sub>50</sub> and PRNT<sub>90</sub> using the plex-  
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21 214 sVNT upper 99<sup>th</sup> percentile cut-off of 25%. PPA and NPA using optimized sVNT cut-offs  
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23 215 compared to increasing PRNT<sub>50</sub> titers are shown in **Figure 2C**. The PPA ranged from 100%  
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25 216 (PRNT<sub>50</sub> 1:20 and plex-sVNT 19%) to 93.9% (PRNT<sub>50</sub> 1:640 and plex-sVNT 54%). The NPA  
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27 217 ranged from 99.0% (PRNT<sub>50</sub> 1:20 and plex-sVNT 19%) to 93.0% (PRNT<sub>50</sub> 1:640 and plex-  
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29 218 sVNT 54%).  
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33 219 Antibodies capable of neutralizing the interaction between SARS-CoV-2 variants and  
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35 220 ACE2 were present in all individuals tested from all groups, however, there was a high degree of  
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37 221 variability in neutralizing capacity between subjects likely given the diverse timing of sample  
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39 222 collection relative to infection or vaccination (**Figure 3**). Overall, the median percent inhibition  
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41 223 was lower for all variants compared to wild type in all four groups. The difference reached  
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43 224 statistical significance for all variants in all groups compared to wild type ( $P < 0.0001$ ), except  
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45 225 epsilon for the COVID-19 non-ICU group ( $P = 0.31$ ). The decrease in percent inhibition towards  
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47 226 the different variants followed the same trend for all 4 groups. Neutralizing antibodies ranking  
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49 227 from lowest percent inhibition to highest compared to wild type were beta, gamma, alpha,  
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51 228 delta/kappa, and then epsilon. The decrease in neutralizing capacity towards the variants  
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3 229 compared to wild type was less pronounced for the COVID-19 ICU group. The heterogeneity  
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5 230 between the four groups (**Table 1**) does not allow for further comparison of results between  
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8 231 groups. For the entire dataset (all samples in all groups), Deming regression for SARS-CoV-2  
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10 232 variants compared to wild-type demonstrated slopes ranging from 1.21 (delta) to 1.33 (beta) and  
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12 233 Spearman correlations ranging from  $r_s=.960$  (kappa) to  $r_s=.993$  (alpha) (Figure 4). Bland-Altman  
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14 234 biases for percent inhibition were -16 (alpha), -21 (beta), -19 (gamma), -8 (delta), and -8 (kappa)  
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17 235 (**Figure 4**) and -3 (epsilon – data not shown).

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19 236 **Figure 5** shows the percent inhibition of neutralizing antibodies for wild type SARS-  
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21 237 CoV-2 versus days since symptom onset or first vaccination for the COVID-19 infection and  
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24 238 vaccination cohorts. There was an inverse correlation between percent inhibition and days since  
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26 239 symptom onset/vaccination out to 6 months in all groups except the non-ICU infected group.  
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28 240 This may be due to the limited number of data points for the non-ICU group between 120 and  
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30 241 160 days or the large inter-individual variation in % inhibition prior to 120 days. For wild type,  
31  
32 242 the decline was more pronounced ( $P<0.0001$ ) in the Pfizer-BioNTech group (slope -0.27, 95%  
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34 243 CI: -0.32 - -0.23)) compared to the Moderna group (slope -0.15, 95% CI: -0.20 - -0.11) and both  
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36 244 vaccine groups ( $P<0.0001$ ) compared to the COVID-19 ICU group (slope -0.04, 95% CI: -0.07 -  
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38 245 -0.01). The results were similar for the variants compared to wild type in the vaccination groups  
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40 246 with slightly lower percent inhibition overall (**Figure 5 E and F**).

## 41 42 43 44 45 247 46 47 248 **DISCUSSION**

48  
49 249 This paper describes a bead-based multiplexed method for the analysis of neutralizing  
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51 250 antibodies against SARS-CoV-2 variants. More specifically it measures the ability of infection-  
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54 251 or vaccine-induced antibodies to inhibit binding between the SARS-CoV-2 trimeric spike protein

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3 252 (wild-type and variant) and ACE2, serving as an in-vitro surrogate for in-vivo viral-host  
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5 253 invasion. The use of trimeric recombinant spike protein variants offers advantages over isolated  
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8 254 protein subunits or domains, such as the receptor binding domain (RBD) since it mimics the  
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10 255 natural conformation of the native spike protein. Emerging SARS-CoV-2 variants exhibit  
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12 256 mutations or deletions in the spike structural domain other than the RBD or S1 subunit;  
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15 257 therefore, use of the trimeric spike protein has significant advantages for evaluating antibody  
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17 258 activity against variants.

19 259 To date, only one sVNT method has received emergency use authorization from the US  
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21 260 FDA (cPASS, GeneScript) for the analysis of neutralizing antibodies to wild-type virus,  
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24 261 however, this method is an enzyme-linked immunoassay designed for batch testing in a high  
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26 262 complexity laboratory. The plex-sVNT method is a high throughput assay that can measure  
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28 263 neutralizing antibodies to wild type and emerging variants in one test simultaneously on an  
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31 264 automated analyzer with all results obtained in 52 minutes. The high concordance and correlation  
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33 265 with a gold-standard PRNT supports its utility as a viable alternative to time-consuming  
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35 266 conventional cell-based assays which are limited in availability, and only capable of testing one  
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38 267 variant at a time. The PRNT and plex-sVNT methods were compared using the respective assay  
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40 268 cut-offs (titer and % inhibition). A more clinically relevant comparison would use the titer and %  
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42 269 inhibition that signify viral protection, which are likely higher than the cut-offs, however, these  
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45 270 are not yet defined. Assay agreement remained high (>93%) between PRNT<sub>50</sub>, at increasing  
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47 271 titers from 1:20 to 1:640, compared to plex-sVNT using optimized cutoffs, suggesting that the  
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49 272 plex-sVNT would be a viable surrogate to PRNT regardless of the cut-off used. Limitations in  
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51 273 sample volume prohibited comparisons between PRNT and plex-sVNT for the variants. As  
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54 274 variants continue to emerge, such as the most recent omicron variant, the plex-sVNT described

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3 275 here can be adapted to monitor neutralizing antibody responses after infection and vaccination,  
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5 276 however the process required for regulatory approval would likely hamper timely widespread  
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8 277 clinical implementation.

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10 278 This study identified neutralizing activity of infection- and vaccine- elicited antibodies  
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12 279 against 6 SARS-CoV-2 variants, suggesting that some immunity is retained against the current  
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14 280 variants. The ability of antibodies to neutralize binding of variant trimeric spike protein to ACE2  
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16 281 was reduced compared to wild type, for both the COVID-19 infection and vaccination cohorts.  
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18 282 Neutralizing antibodies ranking from lowest to highest bias in percent inhibition compared to  
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20 283 wild type were beta, gamma, alpha, delta/kappa, and then epsilon. The decrease in neutralizing  
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22 284 capacity towards the variants was less pronounced for the COVID-19 ICU group. There was an  
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24 285 inverse correlation between percent inhibition and days since symptom onset/vaccination out to 6  
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26 286 months in all groups except the non-ICU infected group. Within the vaccine cohort, the rate of  
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28 287 decline was significantly faster for Pfizer-BioNTech compared to Moderna for wild type and all  
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30 288 variants. The lack of timed longitudinal samples in the study, resulting from the use of remnant  
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32 289 samples, prohibited further analysis of the durability of the immune response.

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37 290 The significance of these finding with regards to an individual's immune response is  
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39 291 currently unknown. Data on the rate of re-infection or vaccine breakthrough for these variants,  
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41 292 will be the subject of future epidemiologic research. Other mechanisms within the human  
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43 293 immune system, such as the existence of T-cell function and memory B-cells may be sufficient  
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45 294 to protect the vaccinated and those previously infected with SARS-CoV-2. A recent study  
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47 295 examined the breakthrough infection rate for individuals who received the Pfizer vaccine for  
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49 296 SARS-COV-2 variants and reported a higher incidence for the  $\alpha$  strain after the first dose, and a  
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51 297 higher incidence for the  $\beta$  strain after the second dose (12). In another study, Bergwerk et al.  
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3 298 characterized 39 healthcare workers who developed a COVID-19 infection after receiving a  
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5 299 SARS-CoV-2 vaccine. During the peri-infection period, they found lower neutralizing antibody  
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8 300 titers in blood compared with non-infected matched controls (13). They also showed that higher  
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10 301 antibody neutralizing capacity was associated with lower viral loads. The plex-sVNT would be  
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12 302 an appropriate test should evaluation of a larger population prove to be warranted for research  
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15 303 and clinical investigations.

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17 304 It is still unknown what degree of neutralizing capacity of SARS-CoV-2 antibodies  
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19 305 correlates with significant immunity or complete protection from COVID-19 infection. The  
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21 306 heterogeneity in methodologies used to evaluate the humoral immune response complicates  
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24 307 interpretation across studies. Estimates of neutralization capacity against variants compared to  
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26 308 wild-type vary substantially between studies likely due to variations in assay design and  
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28 309 populations studied (1-4). To our knowledge this is the first study to evaluate neutralizing  
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31 310 antibodies in both infected and vaccinated individuals for multiple variants using the same  
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33 311 analytical method for comparison. More studies are needed to determine the true utility of  
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35 312 measuring the SARS-CoV-2 antibody response over-time to inform clinical and population  
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38 313 health outcomes.

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3 321 **Author Contributions:** *All authors confirmed they have contributed to the intellectual content of this*  
4 322 *paper and have met the following 4 requirements: (a) significant contributions to the conception and*  
5 323 *design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for*  
6 324 *intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for*  
7 325 *all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of*  
8 326 *the article are appropriately investigated and resolved.*

9 327  
10 328 **Authors' Disclosures or Potential Conflicts of Interest:** *Upon manuscript submission, all authors*  
11 329 *completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

12 330  
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14 332 **Consultant or Advisory Role:** None declared.

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24 342 Rad.

25 343  
26 344  
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398 Table 1. Demographics, clinical characteristics and sample information for the COVID-19 infection and  
 399 SARS-CoV-2 vaccinated cohorts.

Characteristic <sup>1</sup>	COVID-19 Non-ICU patients	COVID-19 ICU patients	Pfizer- BioNTech Vaccination	Moderna Vaccination
Number of subjects	69	28	150	38
Age				
Median [IQR]	48 [32 - 60]	57.5 [43 - 63]	41.5 [34 - 50]	39.5 [32 - 56]
Mean (SD)	47.0 (17.4)	54.7 (12.0)	43.1 (10.6)	43.7 (14.6)
Male sex	30 (43.5)	18 (64.3)	32 (21.3)	15 (39.5)
Admitted to hospital	13 (18.8)	28 (100)	N/A	N/A
Mechanical ventilation	0 (0)	22 (78.6)	N/A	N/A
Days between doses	N/A	N/A	21 [18 – 35]	28 [21 – 33]
Median [min – max]				
Median specimens per subject	1 [1 – 1]	2 [2 – 4]	1 [1 – 2]	2 [2 – 2]
Total number of specimens	77	93	208	73
Days from:				
Onset of symptoms*	55 [12 - 174]	29 [12 - 225]	N/A	N/A
First vaccine dose*	N/A	N/A	91.5 [28 - 160]	108 [36 - 157]

400 <sup>1</sup>Medians [interquartile range], mean (standard deviation), N (%), or \*median [minimum – maximum] are reported.  
 401 N/A – not applicable.

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3 **403 Figure captions**  
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8 **405 Fig. 1. Multiplex SARS-CoV-2 surrogate virus neutralization test (plex-sVNT).** (A) Binding  
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10 **406** of biotinylated ACE2 to spectrally distinct paramagnetic beads coupled with trimeric spike  
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12 **407** protein (wild type or variant) in the absence of neutralizing antibodies. The amount of  
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14 **408** biotinylated ACE2 bound to the beads is measured by the fluorescence of the attached  
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16 **409** Phycoerythrin (PE). (B) Competition between biotinylated ACE2 and neutralizing antibodies in  
17  
18 **410** the sample for binding to trimeric spike proteins. The presence of neutralizing antibody in patient  
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20 **411** sample blocks ACE2 binding thereby reducing the signal.  
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24 **412**

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26 **413 Fig. 2. Correlation between the plex-sVNT and PRNT<sub>50</sub> (A) and PRNT<sub>90</sub> (B).** Dashed lines  
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28 **414** represent the discriminative line between positive and negative for each assay. (C) Concordance  
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30 **415** between PRNT and the multiplex surrogate virus neutralization test.  
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33 **416**

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35 **417 Fig. 3. Percent inhibition of neutralizing antibodies against SARS-CoV-2 variants.** (A)  
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37 **418** COVID-19 non-ICU patients (n=77). (B) COVID-19 ICU patients (n=93). (C) Pfizer/BioNTech  
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39 **419** vaccinated individuals (n=208). (D) Moderna vaccinated individuals (n=73). Box and whisker  
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41 **420** plots represent the median and upper and lower quartile (box) with min and max (whiskers) and  
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43 **421** mean (+). All variants were statistically different ( $P < 0.0001$ ) from wild-type for all groups (A-D)  
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45 **422** except epsilon compared to wild-type for the COVID-19 non-ICU group ( $P = .31$ )  
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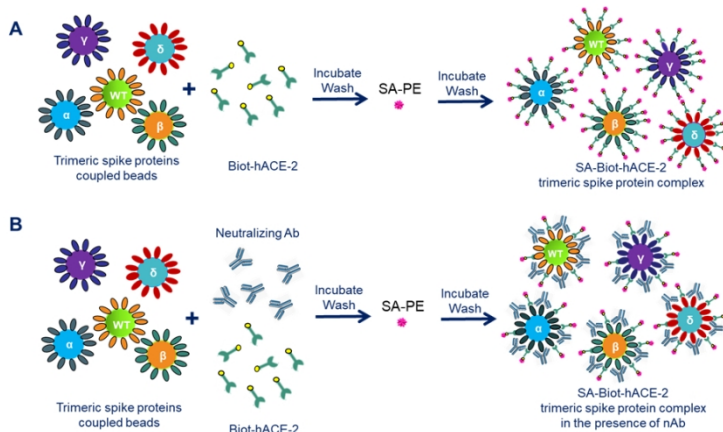
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3 424 **Fig. 4. Deming regression (A-E) and Bland-Altman (F-J) analysis for SARS-CoV-2 variants**  
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5 425 **compared to wild-type.** Slope and spearman correlation is shown for each SARS-CoV-2 variant  
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8 426 compared to wild-type.  
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10 427  
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12 428 **Fig. 5. Plot of percent inhibition by neutralizing antibodies vs. days since symptoms onset**  
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14 **or first vaccination.** Percent inhibition of ACE2 binding to wild type trimeric spike protein for  
15 429  
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17 430 (A) COVID-19 ICU (n=93, slope -0.04, 95% CI: -0.07 - -0.01), (B) COVID-19 non-ICU (n=77,  
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19 431 slope – undetermined), (C) Pfizer-BioNTech (n=208, slope -0.27, 95% CI: -0.32- -0.23), and (D)  
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21 432 Moderna (n=73, slope -0.15, 95% CI: -0.20 - -0.11). Decline in percent inhibition of ACE2  
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24 433 binding to variant trimeric spike protein for (E) Pfizer/BioNTech (slopes range from -0.25 - -  
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26 434 0.33) and (F) Moderna (slopes range from -0.15 - -0.30).  
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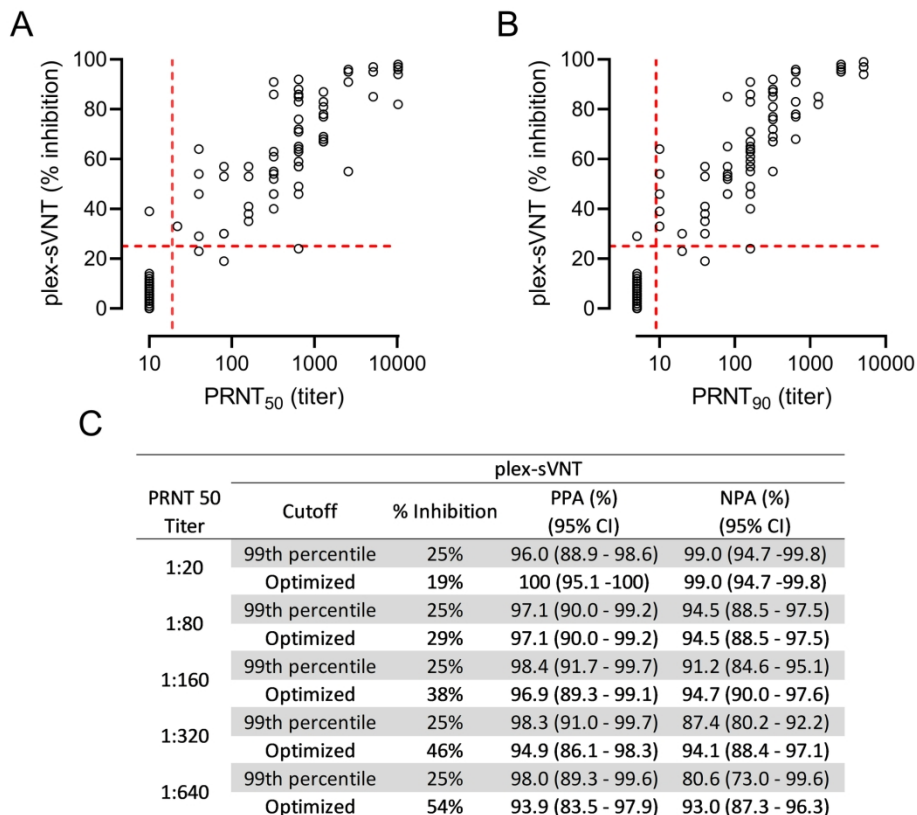
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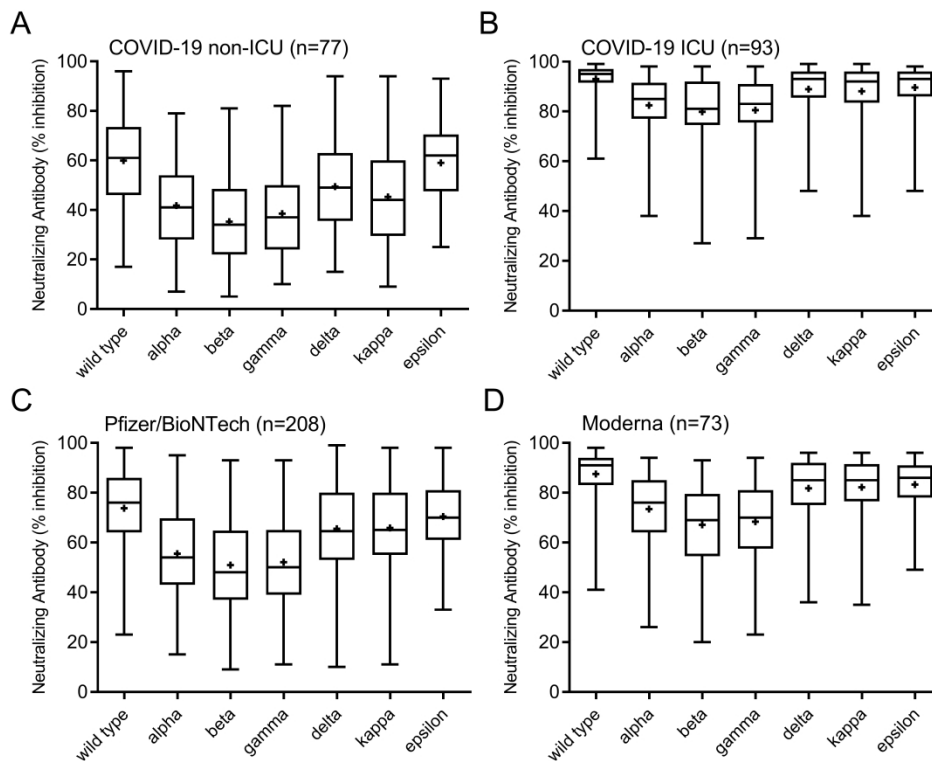
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Figure 2



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Figure 3

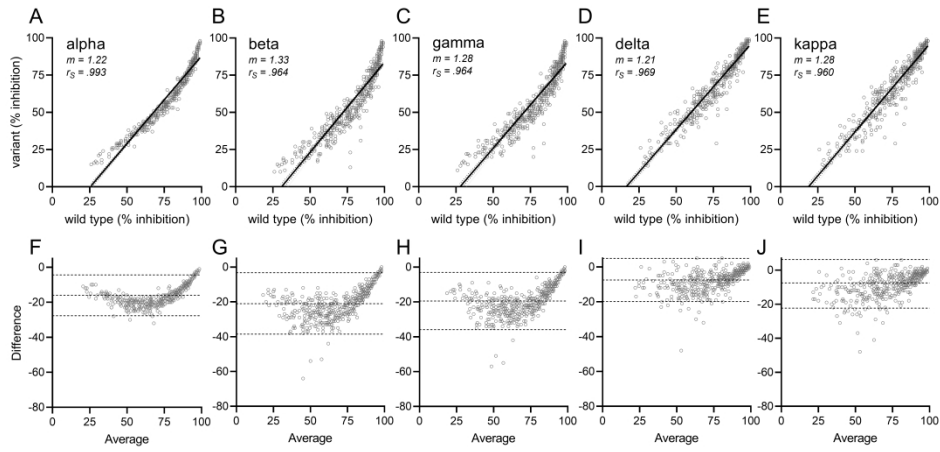


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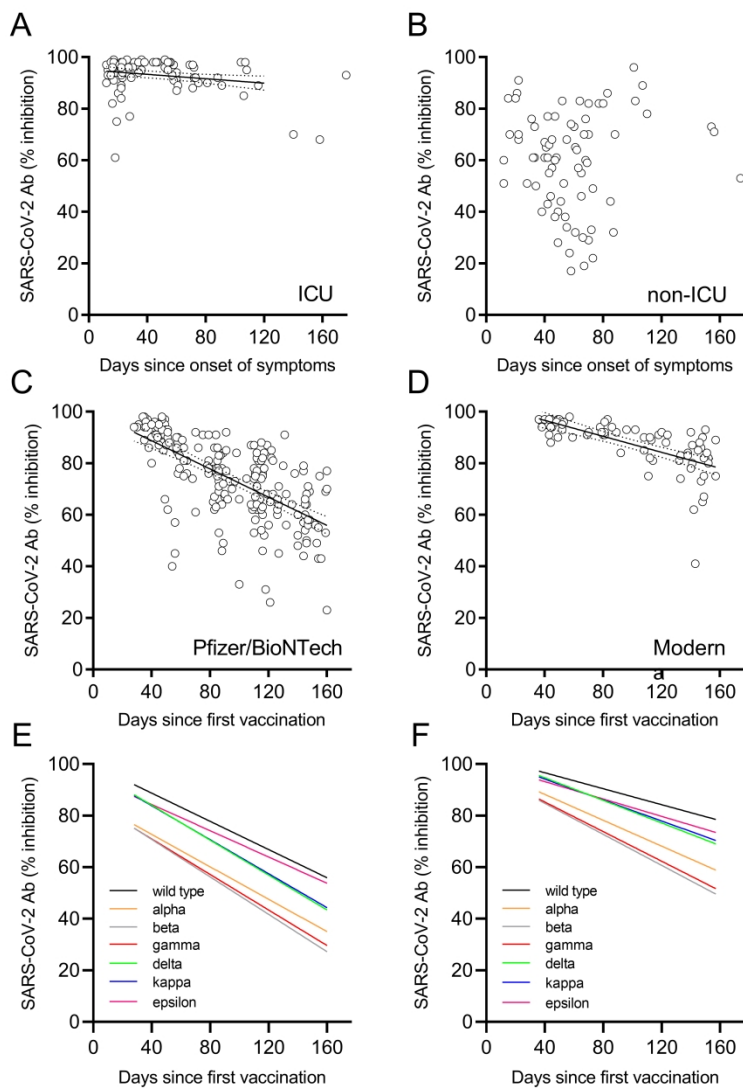
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Figure 4



270x137mm (600 x 600 DPI)

Figure 5



175x235mm (600 x 600 DPI)