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IMMUNO-COV™ v2.0: Development and Validation of a High-Throughput Clinical Assay for Measuring SARS-CoV-2-Neutralizing Antibody Titers — Source link

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1 # IMMUNO-COV™ v2.0: Development and Validation of a High-Throughput
2 # Clinical Assay for Measuring SARS-CoV-2-Neutralizing Antibody Titers

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17 Running title: Development and Validation of IMMUNO-COV™ v2.0

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24 **ABSTRACT**

25 Neutralizing antibodies are key determinants of protection from future infection, yet well-
26 validated high-throughput assays for measuring titers of SARS-CoV-2-neutralizing antibodies
27 are not generally available. Here we describe the development and validation of IMMUNO-
28 COV^{TM} v2.0 a scalable surrogate virus assay, which titrates antibodies that block infection of
29 Vero-ACE2 cells by a luciferase-encoding vesicular stomatitis virus displaying SARS-CoV-2
30 spike glycoproteins (VSV-SARS2-Fluc). Antibody titers, calculated using a standard curve
31 consisting of stepped concentrations of SARS-CoV-2 spike monoclonal antibody, correlated
32 closely ($p < 0.0001$) with titers obtained from a gold-standard PRNT50% assay performed using
33 a clinical isolate of SARS-CoV-2. IMMUNO-COV^{TM} v2.0 was comprehensively validated using
34 data acquired from 242 assay runs performed over seven days by five analysts, utilizing two
35 separate virus lots, and 176 blood samples. Assay performance was acceptable for clinical use in
36 human serum and plasma based on parameters including linearity, dynamic range, limit of blank
37 and limit of detection, dilutional linearity and parallelism, precision, clinical agreement, matrix
38 equivalence, clinical specificity and sensitivity, and robustness. Sufficient VSV-SARS2-Fluc
39 virus reagent has been banked to test 5 million clinical samples. Notably, a significant drop in
40 IMMUNO-COV^{TM} v2.0 neutralizing antibody titers was observed over a six-month period in
41 people recovered from SARS-CoV-2 infection. Together, our results demonstrate the feasibility
42 and utility of IMMUNO-COV^{TM} v2.0 for measuring SARS-CoV-2-neutralizing antibodies in
43 vaccinated individuals and those recovering from natural infections. Such monitoring can be
44 used to better understand what levels of neutralizing antibodies are required for protection from
45 SARS-CoV-2, and what booster dosing schedules are needed to sustain vaccine-induced
46 immunity.

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47 **INTRODUCTION**

48 On March 11, 2020, the World Health Organization declared COVID-19, caused by SARS-
49 CoV-2, a pandemic. Since then, the coordinated efforts of numerous researchers, biotechnology
50 and pharmaceutical companies, contract manufacturers, healthcare organizations, and
51 governmental agencies have resulted in the approval and initial distribution of the first SARS-
52 CoV-2 vaccines. Clinical trial data indicate that the vaccines currently approved in the US are
53 approximately 95% effective at preventing COVID-19 (1,2). However, the durability of this
54 protection is unknown. Neutralizing antibody responses following vaccination correlate with
55 protective immunity (3–6), yet an increasing number of studies, including this one, demonstrate
56 that neutralizing antibody levels fall steadily in the months following natural SARS-CoV-2
57 infection or vaccination (7–11). Thus, protective antibody responses, including those elicited by
58 vaccination, may be relatively short-lived, and repeat vaccine dosing over several years may be
59 necessary to achieve and maintain herd immunity. It is not currently known what titer of
60 neutralizing antibodies confers protection from SARS-CoV-2 infection or COVID-19. Studies to
61 monitor neutralizing antibody responses and the associated risk of infection at various timepoints
62 post-vaccination are needed to inform decisions on the appropriate timing of booster vaccine
63 doses. To facilitate these studies, a reliable, high-throughput method for quantitatively measuring
64 neutralizing antibody titers is critically needed.

65 Over the course of the past year, numerous rapid serological tests have been developed, and
66 many have received Emergency Use Authorization (EUA) approvals for the detection of
67 antibodies against SARS-CoV-2. These tests, which are primarily enzyme-linked
68 immunosorbent assay (ELISA)-based, provide a convenient way to identify individuals
69 previously infected with SARS-CoV-2. However, it is well-known that only a small subset of

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70 virus-specific antibodies are capable of neutralizing virus infectivity, and thereby protecting
71 against future viral infection and disease (12). Importantly, the rapid serological assays for which
72 EUA approvals have been granted are not able to discriminate between neutralizing and non-
73 neutralizing antibodies. Available evidence also suggests that post-vaccination and post-infection
74 neutralizing antibody titers do not correlate strongly with total antibody titers (10,13–16), and it
75 is unknown whether neutralizing antibody titers decay over time more rapidly than non-
76 neutralizing antibodies. Thus, for reliable assessment of the level of protection against SARS-
77 CoV-2 infection in vaccinated or previously infected individuals, neutralizing antibody assays
78 are preferred.

79 The gold standard assay for the quantitation of virus neutralizing antibodies is the plaque-
80 reduction neutralization test (PRNT). While providing a reasonable measure of the blood
81 concentration of antibodies capable of neutralizing the SARS-CoV-2 virus, PRNT is labor
82 intensive and requires use of a clinical virus isolate, such that the test can only be performed
83 under biosafety level 3 containment. Safer alternative neutralization assays have been developed
84 using non-replicating lentiviral vectors (10,14,17,18) or vesicular stomatitis viruses (VSVs)(19)
85 pseudotyped with the SARS-CoV-2 spike glycoprotein. However, due to technical factors
86 impacting the manufacture of these pseudotyped viruses, they are generally produced in small
87 batches of variable titer, which significantly limits the scalability of these assays. The use of
88 fully replication competent VSVs expressing the SARS-CoV-2 spike protein provides an
89 attractive alternative for the development of neutralizing assays (20–22), as they can be
90 propagated extensively to generate much larger reagent stocks. Moreover, because the natural
91 VSV glycoprotein (G) is replaced with the SARS-CoV-2 spike protein, these recombinant
92 viruses mimic SARS-CoV-2 entry, which is initiated by binding of the spike protein to its

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93 receptor angiotensin-converting enzyme 2 (ACE2) on the cell surface (23–25). Once bound to
94 ACE2 via its receptor binding domain (RBD), the spike protein is proteolytically cleaved by the
95 cell surface transmembrane serine protease TMPRSS2 or by endosomal cysteine proteases
96 cathepsin B/L, providing a critical trigger for subsequent membrane fusion and virus entry into
97 the cell (23,26). Studies have mapped the targets of SARS-CoV-2-neutralizing antibodies to
98 diverse epitopes within the spike protein, and antibodies that block ACE2 receptor binding, spike
99 protein cleavage, or subsequent conformational rearrangements of the spike protein that lead to
100 membrane fusion are all strongly neutralizing (27–31).

101 Here, we describe the development, optimization, and validation of IMMUNO-COV™ v2.0,
102 a fully scalable neutralization assay that uses a replication competent G cistron-deleted
103 recombinant VSV encoding both the SARS-CoV-2 spike protein and firefly luciferase (Fluc)
104 (Fig. 1). Over 23,000 vials of this virus were prepared and cryopreserved from a single large-
105 production run, providing sufficient material to assay more than 5 million serum or plasma
106 samples. Anti-SARS-CoV-2-neutralizing antibody titers determined using IMMUNO-COV™
107 v2.0 demonstrated strong linear correlation with titers obtained using the classical PRNT under
108 BSL-3. IMMUNO-COV™ v2.0 assay performance has remained robust and accurate for at least
109 three months, during which time we have conducted extensive validation testing and subsequent
110 verification studies. In keeping with the observations of other groups (7,8,16,28,32), higher titers
111 of neutralizing antibodies were observed in subjects recovering from more severe SARS-CoV-2
112 infections, though strong responses were also seen in several subjects who had only mild disease
113 symptoms. Importantly, a substantial decline in neutralizing antibody levels was observed in
114 most COVID-19 convalescent subjects who were tested repeatedly over a six-month period,
115 regardless of the initial antibody titer. Taken together, our results underscore the importance of

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116 monitoring neutralizing antibody titers of over time, and demonstrate how IMMUNO-COV^{TM}

117 v2.0 can be used to accurately quantify these responses at scale.

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118 ## Results

119 ***Generation of VSV-SARS2-Fluc***. Our previously published SARS-CoV-2 neutralization
120 assay relied upon virus-induced fusion of two dual split protein (DSP) reporter cell lines to
121 generate a luciferase signal (21). To further improve assay throughput and eliminate the need for
122 two cell lines we generated a recombinant VSV (VSV-SARS2-Fluc) encoding SARS-CoV-2
123 spike-Δ19CT (S-Δ19CT) in place of VSV-G, and firefly luciferase (Fluc) as an additional
124 transcriptional unit located between the S-Δ19CT and VSV-L genes (Fig. 2A). Cells infected
125 with VSV-SARS2-Fluc express the virus-encoded luciferase, which is used to measure the level
126 of virus infection. Incorporation of SARS-CoV-2 spike protein into VSV-SARS2-Fluc virions
127 was confirmed by immunoblot (Fig. 2B). VSV-SARS2-Fluc infection and replication were also
128 dependent on cellular ACE2 expression. Robust VSV-SARS2-Fluc replication and virus-induced
129 cell death were observed in Vero-ACE2 cells, which overexpress the SARS-CoV-2 receptor
130 ACE2 (Fig. 2C and D), but not in hamster BHK-21 cells (Fig. 2C and E), which do not express
131 human ACE2. The control virus VSV-Fluc, which encodes VSV-G, but not S-Δ19CT, efficiently
132 infected and replicated in Vero-ACE2 and BHK-21 cells. Cellular luciferase activity specifically
133 correlated to replication of the Fluc-expressing viruses (Fig. 2F and G), with loss of luciferase
134 signal at later timepoints coinciding with the death of infected cultures. Together, these data
135 confirmed functional VSV-G replacement with S-Δ19CT and efficient Fluc expression from the
136 VSV-SARS2-Fluc virus.

137 ***Vero-ACE2 cells are an optimal cell substrate for detecting virus neutralization.*** VSV-
138 SARS2-Fluc infects Vero cells via endogenously expressed ACE2 receptors (21). We
139 hypothesized that ACE2 overexpression could enhance Vero cell susceptibility to VSV-SARS2-
140 Fluc and thereby improve assay sensitivity. To this end, we tested Vero-ACE2 cells, which

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141 stably overexpress human ACE2 as confirmed by flow cytometry (Fig. 2H and I), in the assay.
142 While Vero and Vero-ACE2 cells naturally express relatively high levels of TMPRSS2 (Fig. 2J
143 and K), we also generated a stable cell line overexpressing both ACE2 and TMPRSS2 to
144 elucidate the effect of TMPRSS2 on assay performance. VSV-SARS2-Fluc infection induced
145 higher luciferase expression in Vero-ACE2 cells compared to Vero cells (Fig. 3A). Luciferase
146 expression was not further enhanced by overexpression of TMPRSS2, and notably, VSV-
147 SARS2-Fluc neutralization by the well-characterized neutralizing anti-SARS-CoV-2 spike
148 monoclonal antibody mAb10914 was less apparent on Vero-ACE2/TMPRSS2 cells compared to
149 Vero-ACE2 cells (Fig. 3A). Since Vero-ACE2 cells provided for more sensitive detection of
150 viral neutralization, these cells were selected as the cell substrate for assay development.

151 We also examined the effect of Vero-ACE2 cell seeding density on assay performance.
152 Higher luciferase activity was detected when cell density was increased from 5,000 to 10,000
153 cells/well (96 well plate; Fig. 3B), but further increasing the cell density to 20,000 cells/well led
154 to only a modest additional incremental increase in luciferase signal. Moreover, the higher cell
155 density of 20,000 cells/well was associated with a less effective neutralization of luciferase
156 signal when the virus was exposed to the neutralizing antibody mAb10914. We concluded that
157 10,000 cells/well was the optimal seeding density for detection of virus neutralization.

158 To demonstrate the detection of neutralizing antibodies in patient samples, we used serum
159 samples confirmed as seronegative or seropositive by the commercial EUROIMMUN Anti-
160 SARS-CoV-2 ELISA (IgG), which detects anti-SARS-CoV-2 spike antibodies. Serum samples
161 were incubated with VSV-SARS2-Fluc for 30 minutes at room temperature then added to culture
162 wells containing pre-plated Vero-ACE2 cells. All five of the seropositive samples substantially
163 inhibited virus infection, resulting in suppression of luciferase activity (Fig. 3C). No reduction in

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164 luciferase activity was observed when the VSV-SARS2-Fluc virus was pre-incubated with
165 seronegative samples, confirming that neutralizing antibodies were detected only in seropositive
166 donor samples.

167 ***Consistency of different VSV-SARS2-Fluc production lots***. To determine the optimal
168 quantity of virus to add to each assay well, we tested the capacity of mAb10914 and seropositive
169 plasma to neutralize increasing amounts of VSV-SARS2-Fluc. Highly neutralizing seropositive
170 plasma and mAb10914 at a concentration of 2 μg/mL inhibited infectivity by at least 90%,
171 independent of the amount of virus added to the well (Fig. 4A). In contrast, mAb10914 at a
172 concentration of 0.2 μg/mL noticeably blocked infectivity in this assay only when less than 900
173 plaque forming units (pfu) of virus were added to each well. Based on this experiment, the
174 optimal quantity of VSV-SARS2-Fluc virus to be added to each well to ensure sensitive
175 detection of low-levels of neutralizing antibodies is between 200 and 400 pfu. Consistency of
176 virus lots was confirmed by comparing mAb10914 inhibition of two independent lots of VSV-
177 SARS2-Fluc (produced at different times and representing subsequent virus passages).
178 Luciferase activity over a range of concentrations of mAb10914 was nearly indistinguishable
179 between the two different virus lots (Fig. 4B). Comparing the mAb10914 inhibition curve with
180 200, 300, and 400 pfu of virus per well, the linear range was slightly wider when 300 pfu/well of
181 VSV-SARS2-Fluc was used. Therefore, we used 300 pfu for all future assay runs. We also tested
182 the stability of the thawed VSV-SARS2-Fluc virus when stored on ice or at room temperature
183 prior to being used in the assay. No significant reduction in virus infectivity or neutralization
184 occurred following an 8-hour incubation on ice (Fig. 4C). Likewise, the virus was stable for up
185 to an hour at room-temperature, with only a modest titer decrease noted after two hours (Fig.
186 4D).

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187 ***Heat-inactivation of serum samples is not necessary for assay compatibility.*** In cellular
188 assays, heat-inactivation of plasma and serum samples is often necessary to limit matrix
189 interference that can affect cell or virus viability. To determine whether heat-inactivation was
190 required for IMMUNO-COV^{TM} v2.0, twenty matched serum and plasma samples were thawed
191 and aliquoted, with one aliquot kept on ice, while the other aliquot was heat-inactivated at 56°C
192 for 30 minutes. Both aliquots were then tested in the assay. Overall, heat-inactivation had little
193 effect on neutralizing activity. All seronegative samples remained negative and all seropositive
194 samples remained positive in the assay, regardless of whether the samples had been heat-
195 inactivated (Fig. 5A and 5B). Importantly, heat-inactivated samples did not exhibit diminished
196 virus neutralizing capacity, suggesting that complement proteins do not enhance the
197 neutralization of VSV-SARS2-Fluc in this assay format. For plasma samples, heat-inactivation
198 and subsequent clarification prevented thermal coagulation and sample loss during the assay,
199 thereby improving assay performance. We therefore continued to use heat-inactivation for all
200 subsequent assays with plasma samples, while using non-heat-inactivated serum samples.

201 ***Serum and plasma demonstrate low matrix interference.*** In our original cell fusion-based
202 IMMUNO-COV^{TM} assay we observed significant matrix interference at high concentrations of
203 serum and plasma (21). To determine whether IMMUNO-COV^{TM} v2.0, which provides a more
204 direct measure of virus infection, is similarly hampered by matrix interference, we ran numerous
205 seronegative samples in the assay at 2-fold serial dilutions ranging from 1:20 through 1:640.
206 Minimal matrix interference was observed with serum, sodium-heparin plasma, ACD plasma,
207 and K2-EDTA plasma (Fig. 5C, D, E, and F). In fact, higher concentrations of plasma appeared
208 to have a stabilizing effect on the virus relative to cell culture medium alone and were associated
209 with higher levels of luciferase activity at assay readout. Likewise, serum appeared to increase

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210 virus stability relative to medium alone, though some matrix interference was observed at the
211 1:20 dilution. Thus, the IMMUNO-COV^{TM} v2.0 assay is compatible with testing at low sample
212 dilutions, which may be of importance if higher detection sensitivities are desired.

213 ***Quantification of neutralizing antibody titers using a standard curve.*** To determine the titer
214 of neutralizing antibodies in a test sample without the need for serial two-fold sample dilutions,
215 we developed an assay format in which just one or two dilutions of a test sample are read against
216 a standard calibration curve included on every assay plate. For the development of a calibration
217 standard and assay controls, we used two well-characterized neutralizing anti-spike monoclonal
218 antibodies, mAb10914 and mAb10922. Both antibodies neutralized VSV-SARS2-Fluc in a dose-
219 dependent manner (Fig. 6A), whereas no virus inhibition was observed using isotype antibody at
220 any of the concentrations tested. Based on these findings we established a six-point standard
221 curve using two-fold dilutions of mAb10914 in tissue culture medium at concentrations ranging
222 from 0.8 μg/mL to 0.025 μg/mL (Fig. 6B). To quantify the viral neutralizing titers of test
223 samples, each antibody concentration in the standard curve was converted to a virus neutralizing
224 titer (VNT) by multiplying the antibody concentration by 400. The correction factor of 400 was
225 chosen as it produced VNT values that approximated PRNT50% values obtained for samples
226 assayed at a 1:80 dilution (see below). The final standard curve range for the assay therefore
227 gives a VNT readout of 10-320 for a sample assayed at a 1:80 dilution. In numerous tests (n=242
228 assay runs), the 160, 80, 40, and 20 VNT standards fell within the linear range >99% of the time
229 (Table 1). In most runs (87.6%), either the 320 or 10 VNT standard was also within the linear
230 range. Thus, the standard curve effectively spanned the assay linear range. To quantitate
231 antibody titers above 320 VNT, additional sample dilutions above 1:80 were employed in the
232 assays described below.

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233 ***Under standard conditions the assay limit of detection is 32 VNT.*** To determine the limit of
234 detection (LOD) of the assay we first determined the assay limit of blank (LOB), representing
235 the background signal from seronegative serum. To this end, we assayed seven known
236 seronegative serum samples at a 1:80 dilution on 12 assay runs and calculated the luciferase
237 signal as a percentage of the signal in media only controls. As observed previously (Fig. 5C),
238 seronegative samples stabilized virus, and the LOB was a luciferase response of 124.5%
239 compared to media alone. Seronegative serum samples were subsequently spiked with low
240 concentrations of standard mAb10914 (0.01, 0.02, 0.04, 0.06, 0.08, and 0.1 μg/mL,
241 corresponding to VNTs of 4, 8, 16, 24, 32, and 40) and assayed side-by-side with unspiked
242 samples (Fig. 6C). Based on a total of 60 values obtained for each spike level, the lowest
243 concentration of mAb10914 at which ≥95% of the luciferase response values were below the
244 LOB was 0.08 μg/mL. This concentration corresponded to a VNT of 32, which was accepted as
245 the LOD for the assay.

246 ***The assay exhibits high specificity and sensitivity.*** To evaluate the sensitivity and specificity
247 of IMMUNO-COV^{TM} v2.0 when used to discriminate between positive and negative results, we
248 performed blinded testing of 176 serum samples that were categorized as either positive or
249 negative for SARS-CoV-2-neutralizing antibodies based on the readouts from ELISA and gold
250 standard PRNT. All samples that tested positive for SARS-CoV-2 spike binding antibodies by
251 ELISA were subsequently analyzed by PRNT, with only those samples that were positive by
252 PRNT considered positive for neutralizing antibodies. Samples that tested negative by ELISA
253 but positive in the IMMUNO-COV^{TM} v2.0 assay were also tested by PRNT to confirm the
254 presence or absence of neutralizing antibodies. In these analyses, our assay demonstrated 100%
255 specificity when compared to both PRNT50% and PRNT80% results, as all PRNT-negative

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256 samples tested negative in IMMUNO-COV^{TM} v2.0 (Table 2). Assay sensitivity was 93.7%
257 relative to PRNT50% and 98.4% relative to PRNT80%. Moreover, 140 serum samples acquired
258 prior to March 2020 (134 acquired from 2017-2019, 5 acquired in early 2020) from donors
259 recovered from infection with endemic human coronaviruses HKU1 (n=35), NL63 (n=32), OC43
260 (n=35), or 229E (n=35) were all negative for neutralizing antibodies when tested using the
261 IMMUNO-COV^{TM} v2.0 assay. Thus, the assay specifically detected neutralizing antibodies to
262 SARS-CoV-2 and most likely does not cross react to the four common human coronaviruses.

263 We also assessed assay variability. Each of the blinded serum samples was assayed on five
264 distinct runs performed by four different operators over a period of five days. Perfect consensus
265 of positive and negative results between all five runs was observed for 174 (98.9%) of the
266 samples. Antibody titers of positive samples were consistent between operators and assay runs,
267 with titers across five different runs exhibiting 27.9% CV (n=59), which compared favorably to a
268 CV of 65.1% for the PRNT (n=8 samples, two separate runs). Inter-assay precision was also
269 evaluated based on the performance of the standard curve and assay controls. For this purpose,
270 we included quality control (QC) high (0.154 µg/mL) and QC low (0.031 µg/mL) controls
271 consisting of mAb10922 spiked into negative pooled sera on each assay plate. From 207 assay
272 runs, QC high and QC low VNT readouts both demonstrated less than 30% inter-assay
273 variability (Table 3 and Fig. 7). Intra-assay variability, which was assessed by running the same
274 controls in 24 wells of the same plate, was below 20% for both controls (QC high = 8.6%, QC
275 low = 19.1%). Collectively, these data demonstrate that the IMMUNO-COV^{TM} v2.0 assay has
276 acceptably low levels of intra- and inter-assay variability.

277 ***Assay equivalence of serum and plasma samples.*** While most of our assay validation studies
278 were conducted using serum samples, we also performed matrix equivalency testing to confirm

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279 assay compatibility with different plasma matrices. To this end, we acquired matched serum,
280 sodium heparin plasma, ACD plasma, and K2/EDTA plasma samples from 26 of the 176
281 subjects whose serum samples were used to evaluate assay specificity and sensitivity, and tested
282 the matched samples side-by-side in the assay. The consensus results and VNT antibody titers of
283 positive samples from five assay runs were compared for each matrix. The average percentage
284 relative error for each matrix was within ±30% for all plasma matrices (Table 4). Although all
285 three plasma matrices demonstrated equivalency in this experiment, in other experiments (data
286 not shown) the sodium heparin plasma samples did not exhibit dilutional linearity. Thus, only
287 ACD plasma and K2/EDTA plasma are currently considered acceptable matrices for clinical
288 testing.

289 ***IMMUNO-COV™ v2.0 VNT antibody titers correlate closely to PRNT50% titers.*** The BSL-
290 3 PRNT with wild-type SARS-CoV-2 remains the gold standard for detection of neutralizing
291 antibodies. Therefore, we compared the titers (VNT) measured using IMMUNO-COV™ v2.0
292 with those determined by PRNT. A strong correlation (Pearson's $R = 0.8870$, $p < 0.0001$) was
293 observed between VNTs and PRNT50% titers (Fig. 8). Therefore, neutralization of VSV-
294 SARS2-Fluc in our assay closely mirrors the neutralization of SARS-CoV-2, and IMMUNO-
295 COV™ v2.0 titers provide an accurate measure of an individual's level of neutralizing
296 antibodies. Moreover, VNTs can be quickly compared to PRNT50% titers using a conversion
297 table (Table 5), which we generated based on our data obtained using the two different assays.

298 ***Individuals with more severe disease symptoms tend to develop higher titers of neutralizing***
299 ***antibodies.*** Increasing evidence indicates that disease severity influences the strength of the
300 neutralizing antibody response (7,8,16,28,32). To examine whether individuals in our study with
301 more severe disease developed higher titers of neutralizing antibodies, we correlated antibody

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302 titers with self-reported disease symptoms from 46 previously infected donors who had tested
303 positive for SARS-CoV-2-neutralizing antibodies. Samples used for this analysis were collected
304 within the time window of two weeks to two months post confirmation of COVID-19 diagnosis.
305 A wide range of neutralizing antibody titers was observed among these donors with significant
306 overlap between the disease severity groupings (Fig. 9). Mean neutralizing antibody titers
307 increased with increasing disease severity, though differences were not statistically significant.
308 Our data, therefore, support previous findings that strong neutralizing antibody responses are
309 more likely in individuals who have recovered from severe disease, but wide variation in
310 neutralizing titers occur within all disease severity groupings.

311 ***SARS-CoV-2-neutralizing antibody titers fall steadily after recovery from infection***. To
312 provide long-term protection from COVID-19, neutralizing antibodies must persist at sufficiently
313 high levels to block infection or mitigate pathogenesis. To examine the durability of SARS-CoV-
314 2-neutralizing antibodies after recovery from natural infection, we determined the change in
315 neutralizing antibody titers from 13 subjects between April and October 2020. In April, all 13 of
316 these subjects had been diagnosed with COVID-19 within the previous two months and had
317 measurable levels of SARS-CoV-2-neutralizing antibodies. Samples collected in April were
318 stored at $\leq$ -65°C and assayed side-by-side with new samples collected in October from the same
319 subjects. A two- to five-fold drop in neutralizing antibody titers was observed in all but one
320 subject (Fig. 10A and Table 6). The outlier showed a 3-fold increase, suggesting possible
321 asymptomatic re-exposure to the virus. In three subjects, the VNT from October dropped below
322 the limit of detection in serum, though neutralizing antibodies could still be detected at very low
323 levels in ACD-plasma from two of these subjects. Together, these data indicate that SARS-CoV-
324 2 neutralizing antibody titers fall quite rapidly over time following natural infection. Importantly,

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325 while the PRNT confirmed the substantial decrement in SARS-CoV-2-neutralizing antibody
326 titers over six months (Fig. 10B), a similar trend was not observed using a "neutralization" assay
327 that measures binding of the spike RBD to immobilized ACE2 receptor (Fig. 10C and Table 6).
328 When samples were tested using this SARS-CoV-2-spike RBD binding assay, antibody levels in
329 several subjects were similar in April and October. This finding highlights the importance of
330 quantifying neutralizing antibodies by inhibition of live virus rather than relying on a surrogate
331 receptor binding assay.

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332

DISCUSSION

333 With vaccine roll-out ongoing and critical questions still unanswered regarding the durability
334 of protective immune responses, the need for an accurate, scalable test that can quantitatively
335 measure SARS-CoV-2-neutralizing antibodies remains a priority. Only a small subset of
336 antibodies capable of binding to the spike glycoprotein have neutralizing activity and are most
337 likely to afford protection against SARS-CoV-2 infection (16,27,29,33). Commercially available
338 monoclonal antibodies proven to be of benefit for the treatment of COVID-19 were selected
339 based on their potent virus neutralizing activity (34–38). Yet, most serological tests currently in
340 use detect total spike-binding antibodies but do not measure the capacity of these antibodies to
341 neutralize virus infectivity. The traditional assay for detection and quantification of neutralizing
342 antibodies, the PRNT, is low-throughput and for SARS-CoV-2 must be performed under high
343 biocontainment (BSL-3), making it impractical for widespread use. Here, we describe the
344 development and clinical validation of a novel assay, IMMUNO-COV^{TM} v2.0, which is now
345 available as a scalable laboratory developed test for quantitatively measuring SARS-CoV-2-
346 neutralizing antibody titers. Our data show that IMMUNO-COV^{TM} v2.0 can be used for accurate
347 tracking of neutralizing antibody titers over time in individuals following natural infection or
348 vaccination (Fig. 10). Such information will be needed to better define what constitutes a
349 protective immune response, and what is the durability of the protective immune response
350 following natural infection or vaccination. Answers to these questions will be important to better
351 inform vaccine dosing schedules and other public health initiatives aimed at controlling the
352 pandemic.

353 The IMMUNO-COV^{TM} v2.0 assay measures the concentration of antibodies in serum or
354 plasma that can neutralize the infectivity of the VSV-SARS2-Fluc virus in Vero-ACE2 cells, as

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355 detected by a reduction in luciferase activity compared to cells that have been infected in the
356 absence of neutralizing antibodies (Fig. 1). Importantly, results from IMMUNO-COV^{TM} v2.0
357 correlate closely with PRNT50% titers determined using a clinical isolate of SARS-CoV-2 (Fig.
358 8), indicating that neutralization of VSV-SARS2-Fluc accurately mirrors SARS-CoV-2
359 neutralization. Other groups have likewise observed strong correlation between the readouts of
360 virus neutralization assays using VSV and lentiviral pseudotypes displaying the SARS-CoV-2
361 spike glycoprotein and readouts of classical PRNT conducted under BSL-3 using clinical isolates
362 of SARS-CoV-2 (18,22,39). Given the strong correlations between titers determined using
363 IMMUNO-COV^{TM} v2.0 and those determined using classical PRNT50% and PRNT80% assays,
364 we generated a conversion table that facilitates the rapid conversion of IMMUNO-COV^{TM} v2.0
365 titers to corresponding PRNT50% titers (Table 5). Moreover, the VNT scale for IMMUNO-
366 COV^{TM} v2.0 was designed to yield numerical values roughly equivalent to the PRNT50% titers
367 obtained for a given sample.

368 The currently available spectrum of tests for determining titers of SARS-CoV-2-neutralizing
369 antibodies are based on clinical isolates of SARS-CoV-2 (PRNT) (40–42), replicating surrogate
370 viruses (typically VSV-derived) (20–22), non-replicating spike protein pseudotyped viruses
371 (primarily using VSV or lentiviruses) (10,14,17–19), or entirely nonviral platforms (RBD-ACE2
372 binding assays) (43,44). Binding assays using spike receptor binding domain (RBD) are
373 attractive due to the speed at which results can be obtained. However, they measure only that
374 subset of neutralizing antibodies capable of blocking the binding of the SARS-CoV-2 spike
375 protein RBD to its immobilized ACE2 receptor. They do not functionally measure virus
376 neutralization, and since only a portion of SARS-CoV-2-neutralizing antibodies binds to the
377 RBD (27,29), the relevance of these assays relative to those that directly measure the inhibition

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378 of virus infection remains an open question. In relation to this important question, we observed a
379 strong correlation between IMMUNO-COV™ v2.0 and PRNT50% titers in samples acquired at
380 different times following SARS-CoV-2 infection. In contrast, we observed a much less robust
381 correlation between PRNT50% titers and the c-Pass SARS-CoV-2 surrogate virus neutralization
382 test kit, which is a spike RBD binding assay (Fig. 10).

383 In addition to comparing our assay to the gold standard PRNT assay, we performed full
384 clinical validation of IMMUNO-COV2™ v2.0, which included evaluating the parameters of
385 linearity, assay dynamic range, sensitivity, determination of the limit of blank (LOB) and limit of
386 detection (LOD), dilutional linearity and parallelism, precision, clinical agreement, matrix
387 equivalence, clinical specificity and sensitivity, and assay robustness. IMMUNO-COV™ v2.0
388 exhibited excellent clinical agreement with 100% assay specificity (Table 2). We also tested
389 samples obtained predominately before 2019 from individuals recovered from infection with one
390 of the four common human coronaviruses (HKU1, NL63, OC43, or 229E). All these samples
391 tested negative for neutralizing antibodies, suggesting that IMMUNO-COV™ v2.0 is specific to
392 SARS-CoV-2-neutralizing antibodies and most likely will not detect neutralizing antibodies
393 directed against other human coronaviruses.

394 As has been reported by others (7,8,16,28,32), we observed that donors recovering from
395 more severe COVID-19 disease generally developed higher-titer neutralizing antibody responses
396 (Fig. 9). However, several individuals with only mild COVID-19 symptoms developed strong
397 neutralizing antibody responses, and two individuals with severe disease developed relatively
398 weak neutralizing antibody responses. Thus, SARS-CoV-2-neutralizing antibody titers cannot be
399 accurately predicted based on the severity of the disease manifestations that an individual
400 experiences, highlighting the importance of neutralizing antibody testing to determine anti-

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401 SARS-CoV-2 immune status. Irrespective of the initial magnitude of the neutralizing antibody
402 response, repeat IMMUNO-COV^{TM} v2.0 testing demonstrated a relatively steep decline in
403 SARS-CoV-2-neutralizing antibody titers over six months (Fig. 10). This finding is in keeping
404 with those of other investigators (7–11), and highlights the importance of tracking neutralizing
405 antibodies over time. It should be noted that some other studies suggest that SARS-CoV-2-
406 neutralizing antibody titers are relatively stable (45,46). More research is needed to better
407 understand the durability of neutralizing antibody responses to SARS-CoV-2 and their
408 relationship to cell-mediated responses. Further investigation is also needed to determine
409 whether vaccination provides immunity against SARS-CoV-2 viral variants, and we are
410 conducting ongoing studies to confirm that IMMUNO-COV^{TM} v2.0 can detect immunity against
411 SARS-CoV-2 variants.

412 It is not currently known what minimum titer of SARS-CoV-2-neutralizing antibodies is
413 necessary to assure protection against future infection. Likely there will be considerable variation
414 between individuals because of the multiple additional factors impacting susceptibility to
415 infection, including age, sex, race, ethnicity, and various comorbid conditions. Nevertheless, it is
416 widely accepted that higher levels of neutralizing antibodies afford a higher degree of protection
417 from future infection. Large, coordinated studies following SARS-CoV-2-neutralizing antibody
418 titers in various cohorts of vaccinated and previously infected individuals will be needed to
419 understand immune correlates of protection, the durability of the protective response, and the
420 appropriate frequency for administration of booster doses of the approved SARS-CoV-2
421 vaccines. With the advent of IMMUNO-COV^{TM} v2.0, a fully validated, high throughput
422 laboratory developed test that accurately and robustly determines neutralizing antibody titers, we
423 can now move forward with these much-needed population studies. We have generated and

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424 cryopreserved sufficient VSV-SARS2-Fluc virus to perform over 5 million assays, and the assay
425 is accurate and reproducible even between different virus lots (Fig. 4). Moreover, during
426 validation testing, the IMMUNO-COV™ v2.0 assay exhibited favorable precision compared to
427 the PRNT, with acceptable levels of intra- and inter-assay variability (Table 3) and low run-to-
428 run variability in quantitative VNT readouts. Therefore, we believe that IMMUNO-COV™ v2.0
429 will provide a useful and lasting standardized assay that can be used to normalize and harmonize
430 neutralizing antibody titers for consistent monitoring of neutralizing antibody levels over time
431 and in large study populations.

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432 **MATERIALS AND METHODS**

433 *Cells*: African green monkey Vero cells (ATCC® CCL-81™), Vero-αHis (47), and baby
434 hamster kidney BHK-21 cells (ATCC® CCL-10™) were maintained in high-glucose DMEM
435 supplemented with 5% fetal bovine serum and 1X penicillin/streptomycin (complete media) at
436 37°C/5% CO_2. Vero-ACE2-Puro (Vero-ACE2) cells were generated by transducing Vero cells
437 with lentiviral vector LV-SFFV-ACE2-Puro, encoding the human ACE2 cDNA (GenBank
438 BC039902) under control of the spleen focus forming virus (SFFV) promoter and linked to the
439 puromycin resistance gene via a P2A cleavage peptide. Vero-ACE2-Puro/TMPRSS2-Puro
440 (Vero-ACE2/TMPRSS2) cells were generated by transducing Vero-ACE2-Puro cells with
441 lentiviral vector SFFV-TMPRSS2-Puro encoding human TMPRSS2 cDNA (GenBank:
442 BC051839) under control of the SFFV promoter and linked to the puromycin resistance gene via
443 a P2A cleavage peptide. Vectors used for stable-cell generation were verified by whole plasmid
444 sequencing performed by MGH CCIB DNA Core (Cambridge, MA). Transduced cells were
445 selected using 10 μg/mL puromycin. Following selection, Vero-ACE2 cells were maintained in
446 complete media supplemented with 5 μg/mL puromycin. Puromycin was excluded when cells
447 were seeded for assays.

448 *Generation of VSV-SARS2-Fluc*: Full-length Luc2 (Fluc) was PCR-amplified from pLV-
449 SFFV-Luc2-P2A-Puro (Imanis #DNA1034) with a 5' NheI and 3' AscI restriction site. To
450 generate the viral genome, the amplified PCR product was cloned into pVSV-SARS-CoV-2-S-
451 Δ19CT (21) between the SΔ19CT and L genes (Figure 2A) using the NheI and AscI restriction
452 sites. Plasmid was sequence verified and used for infectious virus rescue on BHK-21 cells as
453 previously described (48). VSV-G was co-transfected into the BHK-21 cells to facilitate rescue
454 but was not present in subsequent passages of the virus. For initial amplification, the virus was

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455 propagated in Vero-αHis cells by inoculating 80% confluent monolayers in 10-cm plates with 1
456 mL of virus. Virus was harvested 48 h after inoculation, aliquoted, and stored at ≤ -65°C until
457 use. For further amplifications and generation of large-scale stocks, the virus was propagated in
458 Vero-ACE2 cells by inoculating 90% confluent monolayers at an MOI of 0.02 or 0.03 plaque
459 forming units per cell. Virus was harvested after 48 h, aliquoted, and stored at ≤ -65°C until use.
460 Aliquots were used to determine viral titers by plaque assay on Vero-αHis cells.

461 *Replication Curves:* Vero-ACE2 or BHK-21 monolayers in 10-cm plates were inoculated in
462 duplicate with OptiMEM alone (mock), VSV-Fluc (MOI=0.01), or VSV-SARS2-Fluc
463 (MOI=0.01). After 2 h at 37°C/5% CO_2, complete media was added to a total volume of 6 mL/
464 plate. At 2, 12, 24, 36, and 48 h, 0.25 mL aliquots of culture supernatant were removed from
465 plates and replaced with 0.25 mL of fresh media. Aliquots were stored at ≤ -65°C immediately
466 after collection until the time of titering. To determine viral titers, aliquots were thawed and
467 assayed by plaque assay on Vero-αHis cells. Throughout the infection time course, cell photos
468 were taken from the 10-cm plates at a 100x magnification using an inverted microscope.

469 *Reagents*: D-luciferin potassium salt (Gold Biotechnology #LUCK-1G) was diluted in DPBS
470 to generate 15 mg/mL stocks. For initial studies, 20 µL/well of stocks were used for assays. For
471 later studies (starting with validation studies), stocks were diluted 1:10 in DPBS and 50 µL/well
472 were used for assays. mAb10914 and mAb10922 are human α-SARS-CoV-2 spike neutralizing
473 monoclonal antibodies. mAb10914 was prepared and scaled up using methods previously
474 described by Regeneron Pharmaceuticals, Inc. (35), and mAb10922 was purchased from
475 GenScript (#U314YFG090_1).

476 *Luciferase Assay Time Course:* Vero-ACE2 or BHK-21 cell monolayers in 96-well black-
477 walled plates with clear bottoms were infected with VSV-Fluc or VSV-SARS2-Fluc at a

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478 multiplicity of infection of 0.03 plaque forming units per cell. Media only wells were used as
479 mock controls. For each condition, 24 wells were prepared to facilitate 8 time points done in
480 triplicate. At 2, 12, 16, 20, 24, 28, 36, and 48 h after inoculation, d-luciferin was added to one set
481 of triplicate wells and bioluminescence was immediately measured using a Tecan Infinite II
482 instrument (100 ms integration, 100 ms settle time per well).

483 *Collection of Plasma and Sera Samples*: A clinical protocol to collect blood samples for
484 assay validation was reviewed and approved by Western IRB on April 1, 2020 (study ID: VYR-
485 COV-001). Samples were obtained with informed consent and the protocol was conducted under
486 ICH-GCP and all applicable sections of the Code of Federal Regulations. Serum and plasma
487 samples were collected in April 2020 from patients who had previously tested positive for
488 SARS-CoV-2 infection by a PCR test, patients who had known exposure to individuals infected
489 with SARS-CoV-2 and symptoms of COVID-19, and a cohort of patients with no known
490 exposure to or symptoms of COVID-19 and presumed to be seronegative. Clinical information
491 was self-reported. A total of 150 adult volunteers were enrolled and provided blood samples at
492 BioTrial in Newark, New Jersey and Olmsted Medical Center in Rochester, Minnesota in April
493 2020. A subset of 26 participants returned and volunteered a second blood sample 6 months later
494 in October 2020.

495 Geisinger provided 140 frozen sera samples comprising the endemic human coronavirus
496 panel. These samples were collected from subjects who had tested positive for the presence
497 of coronavirus HKU1, coronavirus NL63, coronavirus OC43, coronavirus 229E using the
498 Geisinger Respiratory Pathogen Panel PCR test (Geisinger Medical Labs) on average 282.5 days
499 before the collection date (median: 129.3 days; range 1171.3 - 29.1 days).

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500 *IMMUNO-COV^{TM} v2.0 Neutralization Assays*: Except where noted during initial
501 optimization experiments, Vero-ACE2 cells were seeded at 1×10^4 cells/well in 96-well black-
502 walled plates with clear bottoms 16-24 h before being used for neutralization assays. On the day
503 of assay, test samples and controls were prepared and mixed with VSV-SARS2-Fluc in U-
504 bottom suspension cell culture plates to a final volume of 240 µL/well. Any indicated antibody
505 concentrations or sample dilutions represent the antibody concentration or sample dilution
506 following mix with virus. Except when noted otherwise, serum samples were thawed and used
507 for assay without additional processing, while plasma samples were prepared by heat-
508 inactivation for 30 min at 56°C, followed by clarification at 12,000 × g for 5 min and transfer of
509 the liquid supernatant to fresh tubes. During initial optimization experiments, various
510 concentrations of virus were tested, but for all subsequent assays, virus was used at 300 pfu/well
511 (300 pfu/100 µL in U-well mixtures). Virus, test samples, and controls were all diluted as
512 appropriate in OptiMEM to generate final concentrations. For each plate, a standard curve
513 consisting of 0.8, 0.4, 0.2, 0.1, 0.05, and 0.025 µg/mL mAb10914 in OptiMEM, and controls NC
514 (pooled negative matrix at 1:80), QC High (0.154 µg/mL mAb1022 in pooled negative matrix at
515 1:80), and QC Low (0.031 µg/mL mAb10922 in pooled negative matrix at 1:80) were included.
516 Virus mixes in U-well plates were incubated at room temperature for 30-45 min, and then 100
517 µL of mixes were overlaid onto the Vero-ACE2 monolayers in duplicate. Plates were returned to
518 a 37°C/5% CO_2 incubator for 24-28 hours. D-luciferin was then manually added to wells using a
519 multi-channel pipet, and luminescence was read immediately (30-90 seconds) after d-luciferin
520 addition using a Tecan M Plex or Tecan Lume instrument (100 ms integration, 100 ms settle
521 time per well).

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522 *Determination of Virus Titers*: Virus neutralizing titers (VNTs) were determined based on a
523 calibration curve. The calibration curve was run on each plate and consisted of mAb10914
524 spiked into pooled SARS-CoV-2 seronegative sera at 0.8, 0.4, 0.2, 0.1, 0.05, and 0.025 μg/mL.
525 From the calibration curve, the equivalent concentration of neutralizing antibody for a given
526 luciferase signal was determined. To convert to VNT, the antibody equivalent concentration was
527 multiplied by 400, a correction factor chosen to yield VNT values similar to PRNT50% values.

528 *Determinant of Limit of Blank (LOB) and Limit of Detection (LOD)*: Seven known
529 seronegative samples were analyzed at 1:80 dilution on 12 different assay runs, performed on
530 three consecutive days, by six different analysts, using two separate virus lots. Luciferase signal
531 relative to a media control was determined for each sample. The datasets were non-normal by
532 Anderson-Darling and Shapiro-Wilk test, so the LOB was established using a non-parametric
533 model with the 5th percentile value of relative luciferase response obtained for each dataset. From
534 this analysis, the LOB was a response level of 124.5%. To determine the LOD, five seronegative
535 samples (at 1:80 dilution) were spiked with low levels of calibrator material (mAb10914) at 0.01,
536 0.02, 0.04, 0.06, 0.08, or 0.1 μg/mL and assayed on 12 different assay runs, performed on three
537 consecutive days, by six different analysts, using two separate virus lots. Every run also included
538 unspiked negative samples and media control. Datasets were evaluated for the titer that resulted
539 in a response level below the corresponding LOB for each of the dilutions. From these analyses,
540 the LOD was determined to be 32 VNT.

541 *Blinded Sample Testing*: Sera and plasma samples were randomized by independent
542 operators prior to being given to analysts for testing. Samples were assayed in batches, with an
543 unknown number of positive and negative samples in each batch. All samples were assayed at
544 1:80, 1:160, 1:320, 1:640, 1:1280, and 1:2560 dilutions. For specificity and sensitivity studies,

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545 each blinded sample was tested by four different analysts, on at least three different days, in a
546 total of five separate assay runs, using two different virus lots. For comparison studies, samples
547 were tested using the EUROIMMUN anti-SARS-CoV-2 ELISA (IgG) according to the
548 manufacturer's directions.

549 *Assay Variability Assessment*: QC High (0.154 µg/mL), QC Low (0.031 µg/mL), and matrix
550 blank (0 µg/mL) controls consisting of mAb10922 diluted in pooled negative serum (at 1:80)
551 were used along with the standard curve to assess assay variability. For inter-assay variability
552 studies, controls were tested in duplicate on a total of 207 assay runs performed by five different
553 analysts across a span of five days using two different lots of virus. For intra-assay variability
554 studies, each control was assayed in 24 wells in the same assay run performed by the same
555 analyst.

556 *Matrix Equivalency Assessment*: Matched serum, sodium heparin plasma, ACD plasma, and
557 K2/EDTA plasma samples were obtained (see Collection of plasma and sera samples). Samples
558 were blinded and assayed as described for blinded sample testing, using appropriate pooled
559 negative matrix controls.

560 *PRNT*: Serum samples were heat-inactivated for 30 min at 56°C and serially 2-fold diluted in
561 Dulbecco's minimal essential medium supplemented with 2% heat-inactivated fetal bovine
562 serum. SARS-CoV-2 (USA-WA1/2020) (49) was diluted to approximately 200 PFU/mL and
563 mixed with an equal volume of diluted serum (final dilutions of serum with virus were 1:20,
564 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120, 1:10240, 1:20480, 1:40960). Virus
565 mixed with an equal volume of medium alone was used as a control. After a 1 h incubation at
566 37°C, 250 µL of virus/serum or virus/media mixes were used to inoculate Vero-E6 monolayers
567 in 6-well plates. Absorption proceeded for 1 h at 37°C with occasional rocking, before

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568 monolayers were overlaid with 4 mL of 1.6% low-melting agarose in Minimal Essential Media
569 supplemented with 4% fetal bovine serum and antibiotics. Plates were incubated at 37ºC for two
570 days when plaques appeared, then fixed with 10% formaldehyde, and stained with 2 mL of
571 0.05% neutral red, followed by incubation for 6 h at 37ºC. Plaques were counted and the
572 PRNT50% and PRNT80% titers were determined as the lowest dilution at which the number of
573 plaques was reduced by 50% or 80%, respectively, compared to the virus/medium control.
574 Plaque counts greater than 30 were too numerous to count and were considered as equivalent to
575 the virus/media control.

576 *sVNT Binding Assay*: Serum samples were tested using the SARS-CoV-2 Surrogate Virus
577 Neutralization Test (sVNT) Kit (GenScript #L00847) according to the manufacturer's directions.

578 *Flow Cytometry*: Vero-αHis (Vero) or Vero-ACE2 cells were dislodged using Versene,
579 counted, and transferred to microcentrifuge tubes ($5x10^5$ cells/tube was used for ACE2 staining
580 and $1.5x10^6$ cells/tube was used for TMPRSS2 staining). For ACE2 staining, cells were pelleted
581 and resuspended in 100 µL FACS buffer (2% FBS in DPBS) containing 0.2 µg goat-α-human
582 ACE2 (R&D Systems #AF933). After 30 min on ice, cells were rinsed with 1 mL FACS buffer
583 and resuspended in 100 µL FACS buffer containing 5 µL donkey-α-goat IgG-PE secondary
584 antibody. After 30 min on ice, cells were rinsed with 1 mL FACS buffer and fixed with 1%
585 paraformaldehyde for 15 min on ice. Cells were washed twice with FACS buffer, resuspended in
586 500 µL FACS buffer and analyzed on a CYTOFLEX flow cytometer (Beckman Coulter). For
587 TMPRSS2 staining cells were resuspended in 1 mL ice-cold 70% ethanol in DPBS and incubated
588 on ice for 10 min. Cells were centrifuged, washed once with 1 mL FACS buffer, and
589 resuspended in 100 µL of a 0.5% saponin solution containing 4 µg rabbit α-TMPRSS2
590 (Invitrogen #PA5-14264). After 30 min on ice, samples were washed twice with 1 mL FACS

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591 buffer and resuspended in 100 μL of a 0.5% saponin solution containing 2 μL goat α-rabbit IgG-
592 AF647 secondary antibody. After 30 min on ice, cells were washed twice with FACS buffer and
593 fixed with 1% paraformaldehyde for 15 min on ice. Cells were washed twice with FACS buffer,
594 resuspended in 500 μL FACS buffer and analyzed on a CYTOFLEX flow cytometer (Beckman
595 Coulter). For both ACE2 and TMPRSS2 staining, positive staining was compared against a
596 control sample stained with secondary antibody only.

597 *Immunoblot*: Viruses were concentrated by high-speed centrifugation, and 5×10^5 pfu (VSV-
598 SARS2-Fluc) or 5×10^5 TCID50 units (VSV-GFP) were diluted in LDS sample buffer
599 (Invitrogen #B0007) and reducing agent (Invitrogen #B0009) according to the manufacturer's
600 directions. Cell lysates from HEK-293T cells stably expressing SARS-CoV-2 spike protein were
601 also prepared as controls. All samples were incubated at 70ºC for 10 min and 40 μL of each
602 sample was run in duplicate on 4-12% Bis-Tris gels (Invitrogen #NW04125Box) along with
603 precision plus protein dual color standard (Bio-Rad #161-0374). Proteins were transferred to
604 nitrocellulose membranes using a Power Blotter XL. Membranes were blocked in 5% non-fat dry
605 milk in TBST, washed three times with TBST, and incubated for 1 h at room temperature with
606 primary antibody mouse α-SARS-CoV-2 Spike (1:1000, GeneTex #GTX632604) or mouse
607 monoclonal α-VSV-G clone 8G5F11 (1:10,000, Absolute Antibody #Ab01401-2.3). Membranes
608 were washed three times with TBST and incubated for 1 h at room temperature with secondary
609 antibody goat α-mouse IgG-HRP (Prometheus #20-304) at 1:20,000. Membranes were washed
610 three times with TBST, and protein bands were developed for 2 min at room temperature using
611 ProSignal® Dura ECL Reagent (Prometheus #20-301). Protein bands were imaged using a
612 BioRad ChemiDoc Imaging System.

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613 *Statistical Analyses:* Descriptive statistics, comparisons, and regression analyses were
614 performed in Graph Pad Prism, v9.0.0 (San Diego, CA). Tests for normality of variance were
615 conducted, and whenever possible parametric comparisons were used. For non-normal datasets,
616 non-parametric approaches were used. A four-parameter non-linear regression was used for the
617 calibration curve of the virus neutralizing units within the assay. For correlation analyses,
618 Spearman's correlation analysis was conducted.
619

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631 ## AUTHOR CONTRIBUTIONS

632 Designed and planned experiments: RV, TC, SR, CL

633 Performed experiments: RV, SR, CL, RN, L. Schnebeck, AR, KS, SW, GR

634 Analyzed data: RV, TC, SR, CL

635 Cloned and rescued virus: PL, CG, MH, SR

636 Generated critical reagents (cells, mAbs): JB, SR, A. Baum, CAK

637 Clinical trial implementation and test sample acquisition: A. Bexon, SN, BB, L. Suksanpaisan

638 Wrote the manuscript: RV, SJR

639 Contributed intellectually to assay development: RV, TC, SJR, L. Suksanpaisan, KWP, ST

640

641 ## CONFLICTS OF INTEREST

642 Vyriad, Imanis Life Sciences, and Regeneron are collaborating in the commercial development
643 of this assay. Most coauthors of this manuscript are employees of at least one of the above
644 organizations as noted in the author affiliations. SJR and KWP are co-founding scientists,
645 officers, and stockholders both in Vyriad and Imanis Life Sciences.

646

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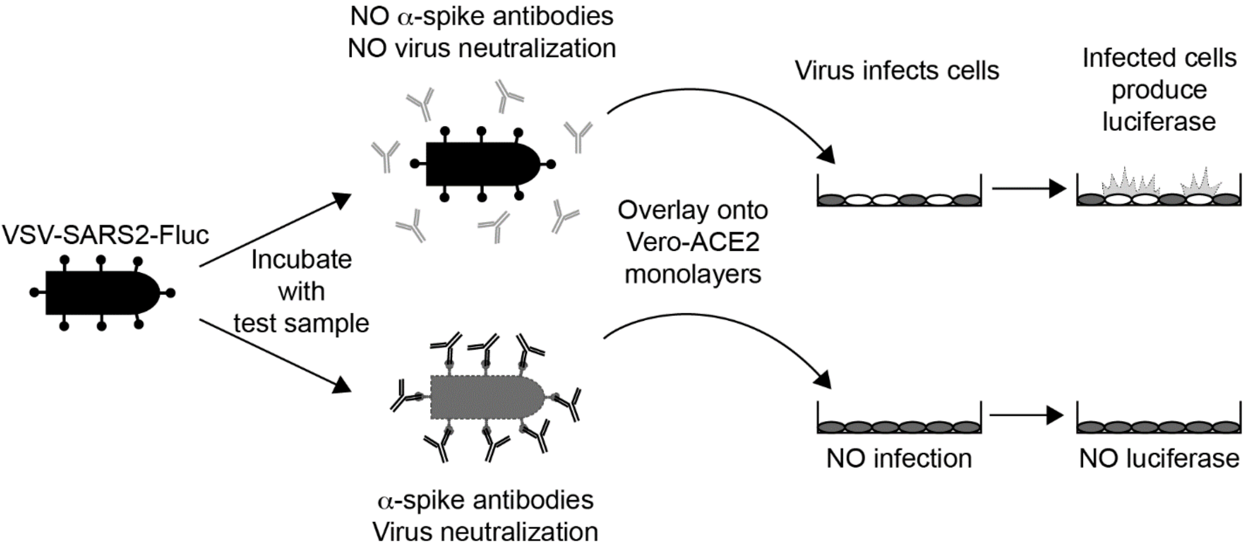
788 **FIGURES**



789

790 **Figure 1:** *Overview of the IMMUNO-COV^{TM} v2.0 Assay.* A VSV expressing SARS-CoV-2 spike
791 and firefly luciferase (VSV-SARS2-Fluc) is incubated with test sera or plasma. In the absence of
792 SARS-CoV-2-neutralizing antibodies (top) the virus retains infectivity and infects Vero-ACE2
793 monolayers. If the test sample contains SARS-CoV-2-neutralizing antibodies (bottom), the
794 antibodies inhibit infection by blocking cell entry. As virus replication proceeds, infected cells
795 express luciferase, which is used to quantitate virus-infection. High luciferase signal means the
796 test sample did not neutralize the virus, while decreased luciferase indicates the presence of SARS-
797 CoV-2-neutralizing antibodies.

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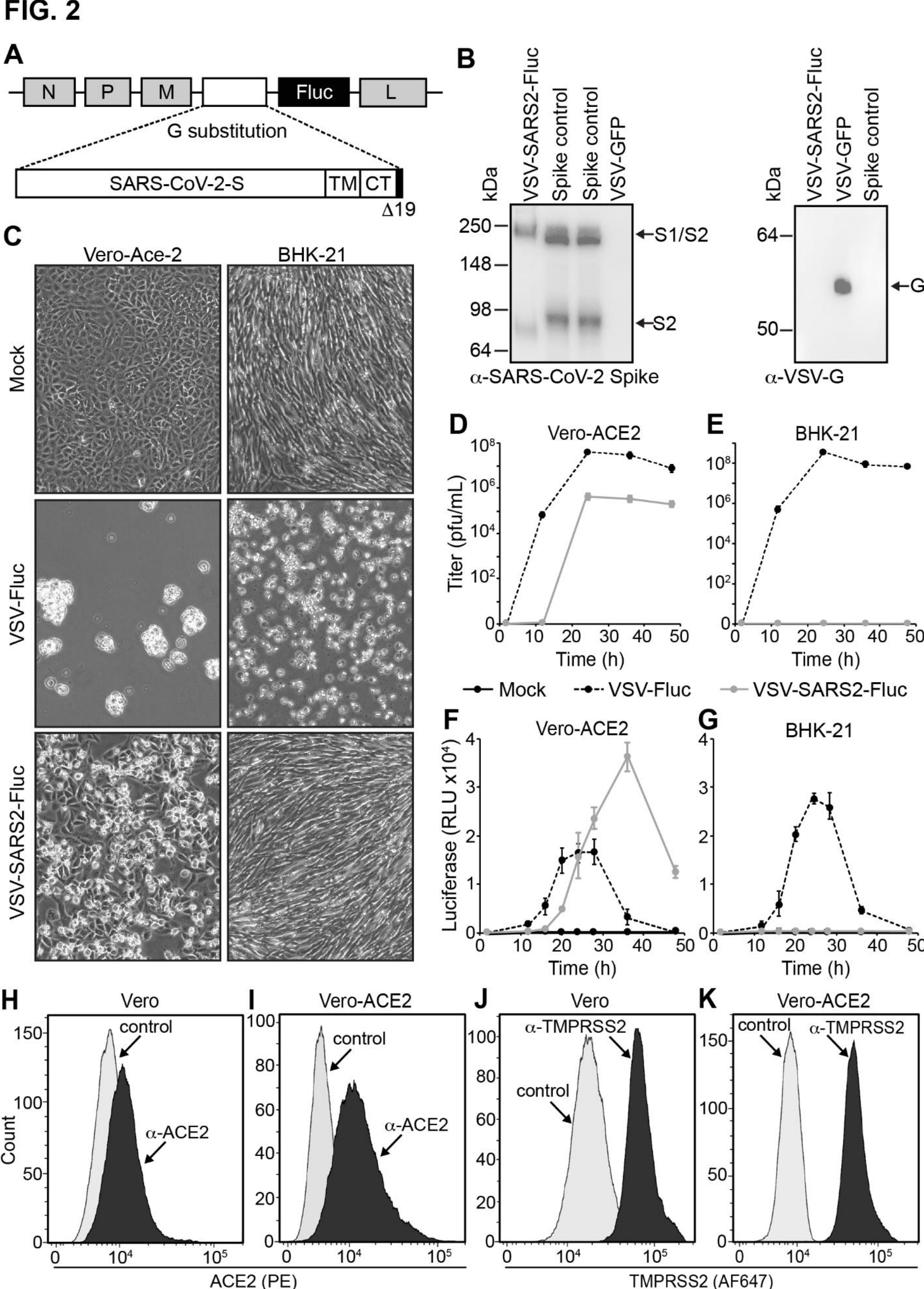

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799 **Figure 2:** *Generation and Characterization of VSV-SARS2-Fluc.* A) Schematic Representation of
800 the VSV-SARS2-Fluc Genome. The location of the VSV N, P, M (M51R), and L genes are shown.
801 In place of VSV-G a codon optimized SARS-CoV-2 spike gene with a 19 amino acid C-terminal
802 (CT) deletion (Δ19CT) is substituted. TM is transmembrane domain. Firefly luciferase (Fluc) is
803 inserted as an additional transcriptional element between S-Δ19CT and L. Not drawn to scale. B)
804 Immunoblot Analysis. VSV-SARS2-Fluc or VSV-GFP control virus (5×10^5 total pfu) or spike
805 control from lysates of cells overexpressing SARS-CoV-2 spike were subjected to immunoblot
806 analysis using α-SARS-CoV-2 spike antibody (left) and α-VSV-G antiserum (right). Arrows
807 indicate the full-length S1/S2 variant and cleaved S2 variant of spike and the VSV-G proteins. C)
808 Infection of Cell Monolayers. Vero-ACE2 or BHK-21 cell monolayers were infected with VSV-
809 SARS2-Fluc, control VSV-Fluc, or mock-infected. Images were taken 48 h post infection at 100X
810 magnification. D-E) Replication Curves. Vero-ACE2 or BHK-21 cell monolayers were infected
811 as in panel C and the virus titers from culture supernatants collected at the indicated times post
812 inoculation were determined. F-G) Luciferase Activity. Vero-ACE2 or BHK-21 cells were
813 infected with VSV-SARS2-Fluc, control VSV-Fluc, or mock-infected in 96-well plates, and at the
814 indicated times luciferase activity was measured. H-K) Flow Cytometry. Expression of ACE2 (H
815 and I) and TMPRSS2 (J and K) were measured in Vero and Vero-ACE2 cells by flow cytometry
816 using α-ACE2 or α-TMPRSS2, respectively. Controls were secondary antibody only.

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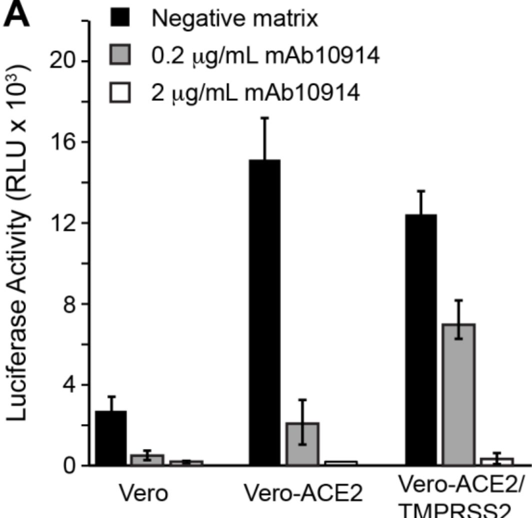
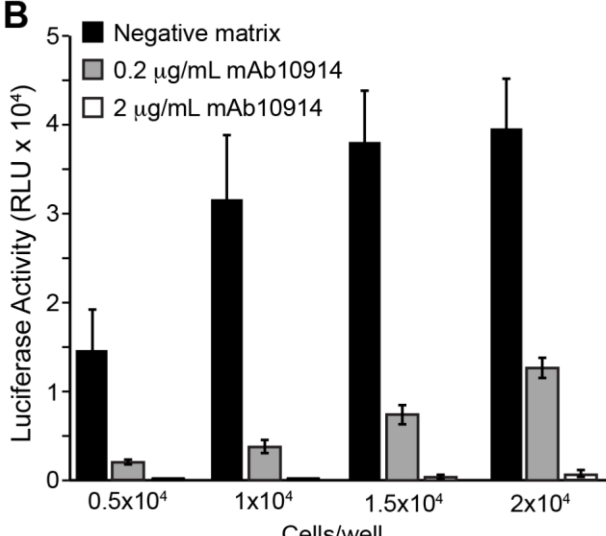
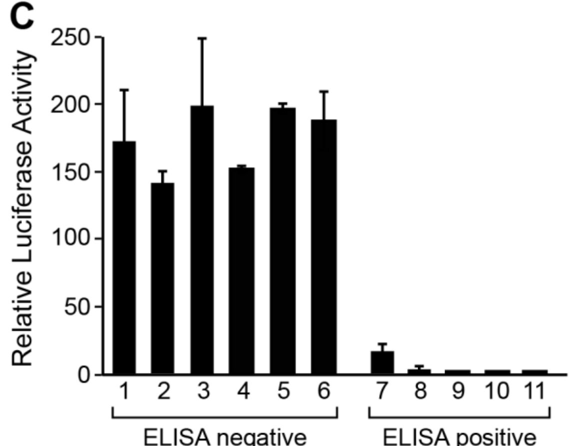
FIG. 3







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818 **Figure 3:** *Inhibition of VSV-SARS2-Fluc by Monoclonal Antibodies and Convalescent Sera.* A)
819 Infectivity of Different Vero Cell Lines. VSV-SARS2-Fluc was incubated with 2 or 0.2 µg/mL of
820 monoclonal anti-SARS-CoV-2 spike antibody mAb10914 in pooled seronegative sera, or pooled
821 seronegative sera alone (negative matrix). After 30 min, virus mixes were overlaid onto Vero,
822 Vero-ACE2, or Vero-ACE2/TMPRSS2 cells. Luciferase activity was measured after an additional
823 24 h. Values represent the average (mean) RLU ± standard deviation. B) Optimization of Cell
824 Density. The indicated numbers of Vero-ACE2 cells were seeded in 96-well plates. The following
825 day, virus mixes as described in panel A were overlaid onto the cell monolayers. Luciferase
826 activity was measured after an additional 24 h. Values represent the average (mean) RLU ±
827 standard deviation. C) Neutralization by Convalescent Sera. VSV-SARS2-Fluc was incubated
828 with pooled seronegative sera at 1:80 dilution or sera samples from 11 donors (6 seronegative, 5
829 seropositive for anti-SARS-CoV-2 antibodies by ELISA assay) at 1:80 dilution. After 30 min,
830 virus/sera mixes were overlaid onto Vero-ACE2 cells. Luciferase activity was measured after an
831 additional 24 h. Values represent average (mean) luciferase activity relative to the pooled
832 seronegative sera sample control ± standard deviation.

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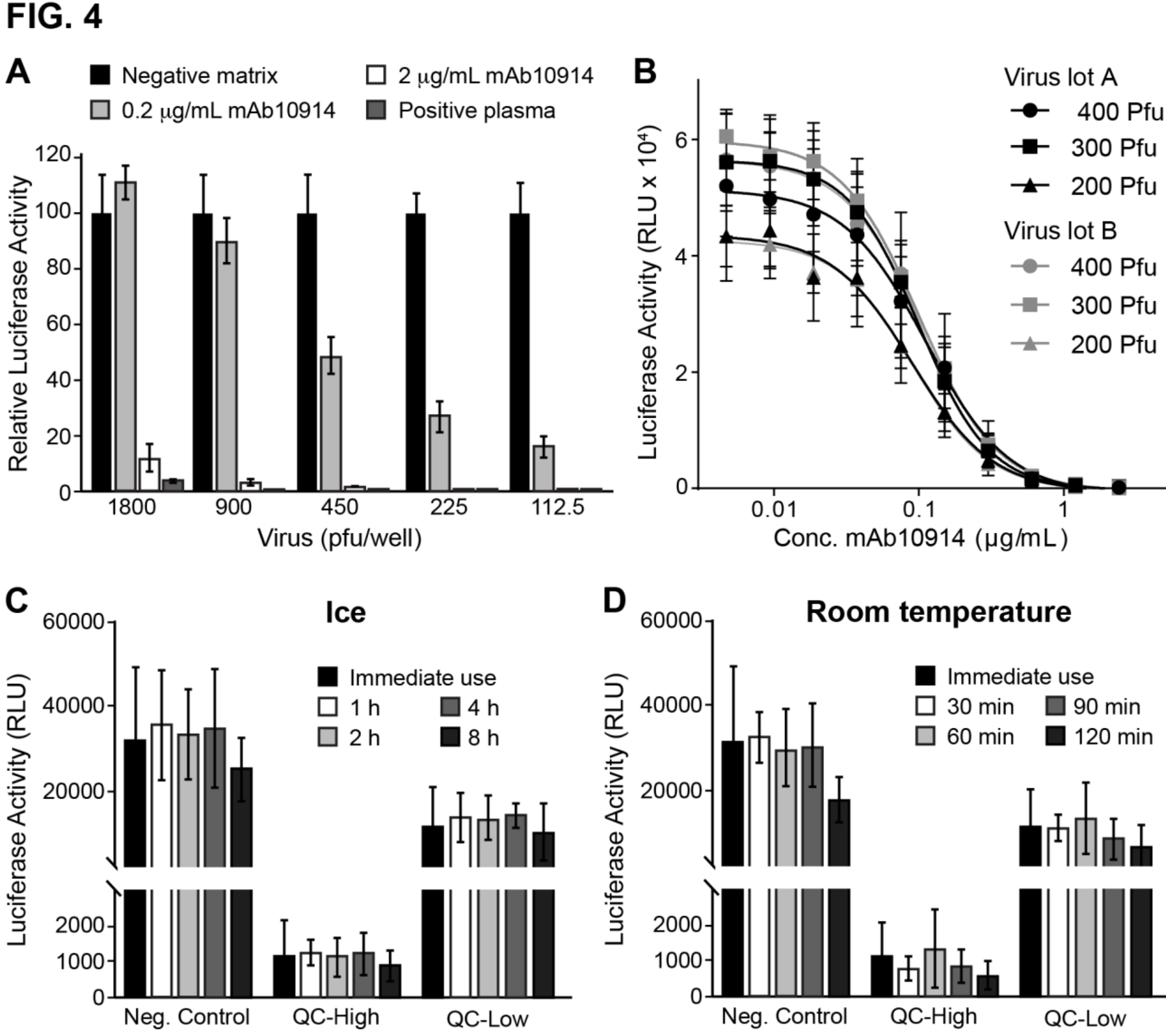
FIG. 4



833

834 **Figure 4**: *Assay Performance of VSV-SARS2-Fluc.* A) Susceptibility of Virus to Antibody
835 Neutralization. The indicated amounts (plaque forming units; pfu) of VSV-SARS2-Fluc were
836 incubated with 2 or 0.2 μg/mL anti-SARS-CoV-2 spike monoclonal antibody mAb10914, a SARS-
837 CoV-2 seropositive plasma sample at 1:80 dilution, or pooled seronegative serum (negative matrix,
838 1:80). After 30 min, virus mixes were overlaid onto Vero-ACE2 cells, and luciferase activity was
839 measured after an additional 24 h. Values represent the average (mean) luciferase activity relative
840 to the negative matrix control ± standard deviation. B) Consistency of Virus Lots. Varying amounts
841 (pfu) of two different lots (A and B) of VSV-SARS2-Fluc were incubated with the indicated

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842 concentrations of mAb10914. Luciferase activity was measured after an additional 24 h. Values
843 represent the average (mean) RLU ± standard deviation. C-D) Virus Stability. Aliquots of VSV-
844 SARS2-Fluc were removed from the freezer, thawed, and either used immediately for assay
845 (Immediate use) or stored at either room temperature or on ice for the indicated time (h). For assay,
846 300 pfu of VSV-SARS2-Fluc was incubated with 0.154 (QC-High) or 0.031 (QC-Low) µg/mL of
847 anti-SARS-CoV-2 spike monoclonal antibody mAb10922 in pooled seronegative sera, or in
848 pooled seronegative sera alone (Neg. Control). After 30 min, virus mixes were overlaid onto Vero-
849 ACE2 cells and luciferase activity was measured after an additional 24 hours. Values represent the
850 average (mean) RLU ± standard deviation.

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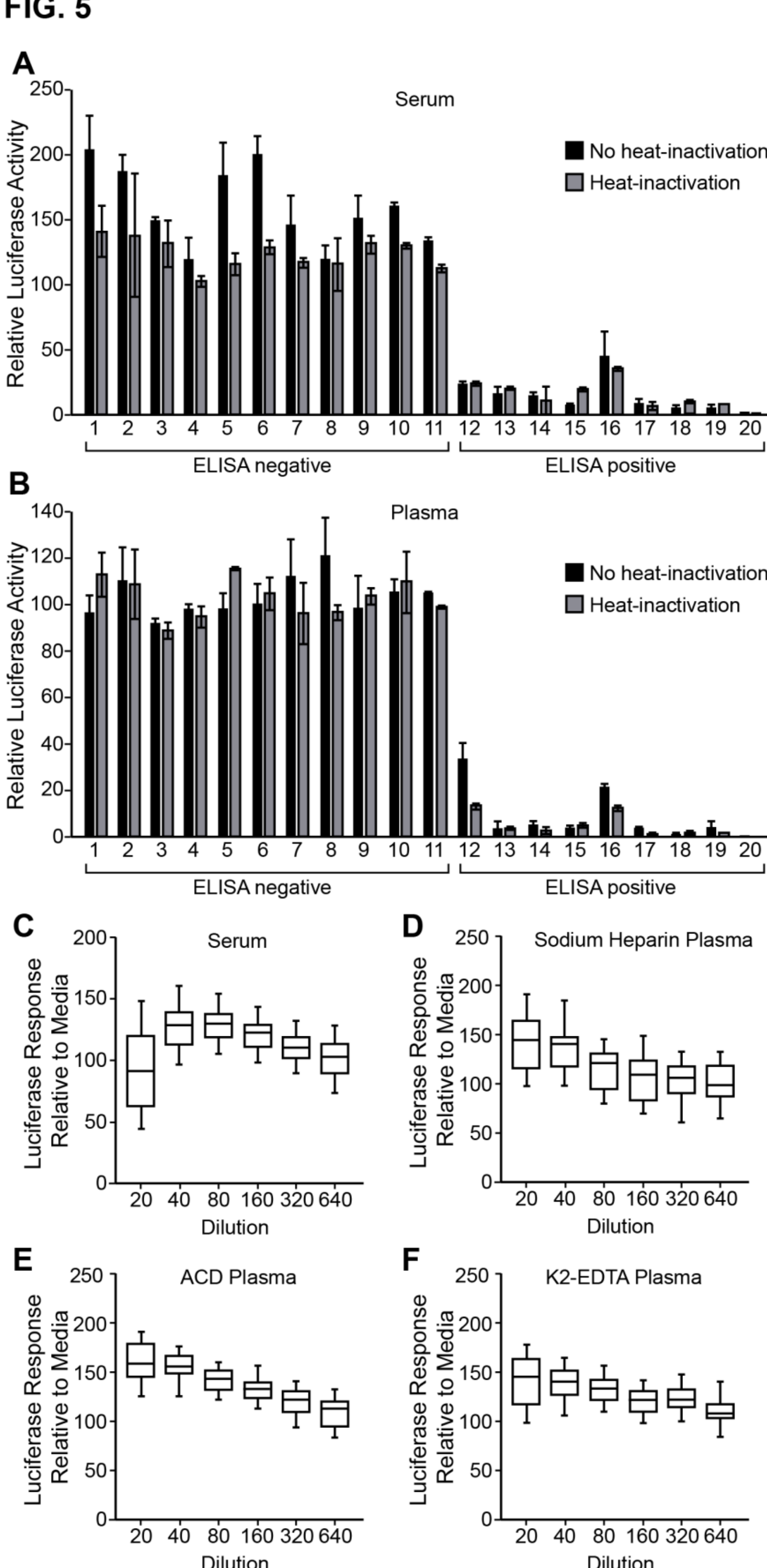

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852 **Figure 5:** *Effect of Sample Matrix on Assay Performance*. A-B) Effect of Heat-Inactivation of Sera
853 or Plasma. Matched sera (A) and sodium-heparin plasma (B) samples from 20 donors (11
854 seronegative, 9 seropositive for anti-SARS-CoV-2 antibodies by ELISA assay) were split and
855 either incubated on ice or at 56°C for 30 min. Following incubation, plasma samples were clarified
856 by centrifugation. Samples were then incubated at 1:80 dilution with VSV-SARS2-Fluc. Pooled
857 seronegative sera or plasma were used as assay controls. After 30 min, virus mixes were overlaid
858 onto Vero-ACE2 cells, and after an additional 24 h, luciferase activity was measured. Values
859 represent the average (mean) luciferase activity relative to the pooled seronegative matrix control
860 ± standard deviation. C-F) Characterization of Matrix Interference. Seronegative sera (C, n=40),
861 sodium-heparin plasma (D, n=40), ACD plasma (E, n=26), or K2-EDTA plasma (F, n=49) samples
862 were serially diluted as indicated and incubated with VSV-SARS2-Fluc. Virus mixed with media
863 only was used as a control. After 30 min, virus mixes were overlaid onto Vero-ACE2 cells, and
864 after an additional 24 h, luciferase activity was measured. Values represent the average (mean)
865 luciferase activity relative to the media control ± standard deviation.

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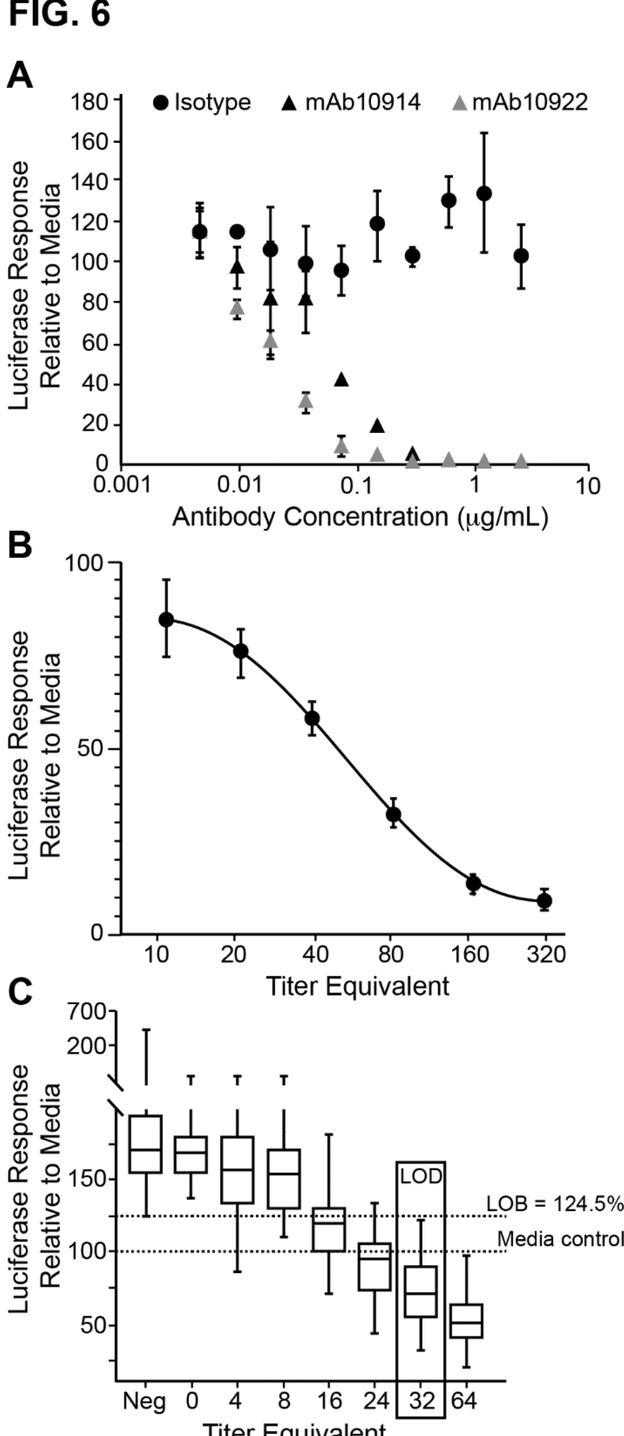
FIG. 6



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867 **Figure 6:** *Establishment of a Standard Curve for Titer Calculations.* A) Antibody-Specific
868 Neutralization of VSV-SARS2-Fluc. The indicated concentrations of anti-SARS-CoV-2 spike
869 monoclonal antibodies mAb10914 or mAb10922 or isotype control antibody were incubated with
870 VSV-SARS2-Fluc. After 30 min, virus mixes were overlaid onto Vero-ACE2 cells, and luciferase
871 activity was measured after an additional 24 h. Values represent the average (mean) luciferase
872 activity relative to the media control ± standard deviation. B) Standard Curve Performance. VSV-
873 SARS2-Fluc was incubated with 0.8, 0.4, 0.2, 0.1, 0.05, or 0.025 μg/mL (corresponding to the
874 indicated equivalent VNTs) of mAb10914 or negative pooled sera alone. After 30 min, virus mixes
875 were overlaid onto Vero-ACE2 cells, and luciferase activity was measured after an additional 24
876 h. Values represent average (mean) luciferase activity relative to the pooled negative sera control
877 ± standard deviation from 242 unique assay runs. C) Limit of Detection. Five different
878 seronegative sera samples (at 1:80 dilution) were spiked with anti-SARS-CoV-2 spike monoclonal
879 antibody mAb10914 at 0.01, 0.02, 0.04, 0.06, 0.08, and 0.1 μg/mL (corresponding to the indicated
880 equivalent VNTs), and incubated with VSV-SARS2-Fluc. VSV-SARS2-Fluc incubated with
881 unspiked sera samples (Neg) or media alone were included as controls. After the 30 min
882 incubation, virus mixes were overlaid onto Vero-ACE2 cells, and luciferase activity was measured
883 after an additional 24 h. Box and whisker diagrams display the interquartile range in the box, with
884 the center line representing the median for the data set and whiskers representing the lower 5%
885 and upper 95% value. Values are based on a total of 12 different assay runs performed on three
886 separate days by six analysts using two different virus lots.

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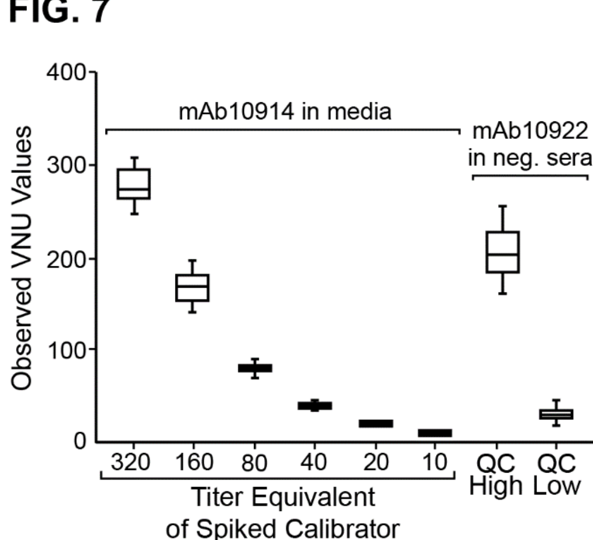

887

888 **Figure 7:** *Inter-Assay Variability of Standards and Controls.* Standards consisting of monoclonal
889 anti-SARS-CoV-2 spike antibody mAb10914 at 0.8, 0.4, 0.2, 0.1, 0.05, and 0.025 µg/mL in media,
890 and QC High and QC Low controls consisting of 0.154 and 0.031 µg/mL antibody mAb10922 in
891 pooled seronegative sera were incubated with VSV-SARS2-Fluc. Pooled seronegative sera alone
892 was used as a negative control. After 30 min, virus mixes were overlaid onto Vero-ACE2 cells,
893 and luciferase activity was read after an additional 24 h. A total of 207 assay runs were performed
894 over five days, by five analysts, using two different virus lots. Box plot represents the 25th to 75th
895 percentile of the data with the line representing the media titer equivalent (VNT) value. Whiskers
896 display the minimum and maximum values.

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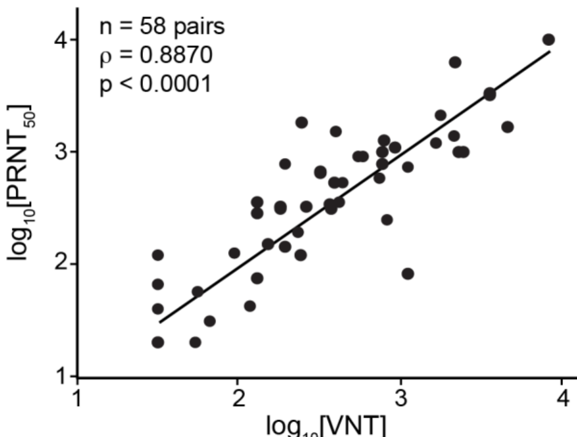
FIG. 8



897

898 **Figure 8:** *Correlation of Virus Neutralizing Units to PRNT50%.* 58 SARS-CoV-2 seropositive
899 sera samples were assayed using IMMUNO-COV^{TM} v2.0 starting at a 1:80 dilution. Established
900 controls, including a standard curve (0.8, 0.4, 0.2, 0.1, 0.05, and 0.025 µg/mL mAb10914 in
901 media), were included on each assay plate. The IMMUNO-COV^{TM} v2.0 titer (VNT) was
902 determined using the standard curve, where one VNT equals the concentration of mAb10914
903 multiplied by 400. All samples were subjected to PRNT using a clinical isolate of SARS-CoV-2.
904 Statistical comparison of VNT relative to PRNT50% was performed using Spearman's rank order
905 correlation analysis as both datasets had a non-gaussian distribution ($p < 0.0001$).

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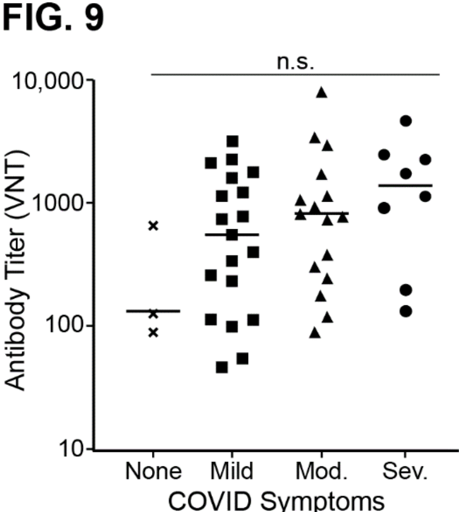

906

907 **Figure 9:** *The Strength of Neutralizing Antibody Responses Correlates to Disease Severity*. As
908 part of assay validation (Table 2), neutralizing antibody titers were determined for 46 donors who
909 self-reported COVID-19 disease symptoms at least two weeks prior to sample donation. Disease
910 symptoms were classified as severe (acute respiratory distress or pneumonia), moderate (shortness
911 of breath), mild (fever, feverish, cough, chills, myalgia, rhinorrhea, sore throat, nausea/vomiting,
912 headache, abdominal pain, or diarrhea), or none (asymptomatic). The graph indicates the titer value
913 (VNT) for each donor grouped based on disease symptoms. Bars represent the average (mean) titer
914 for each group. Differences in antibody titers based on disease severity were not statistically
915 significant (n.s.) by one-way ANOVA ($p = 0.1904$).

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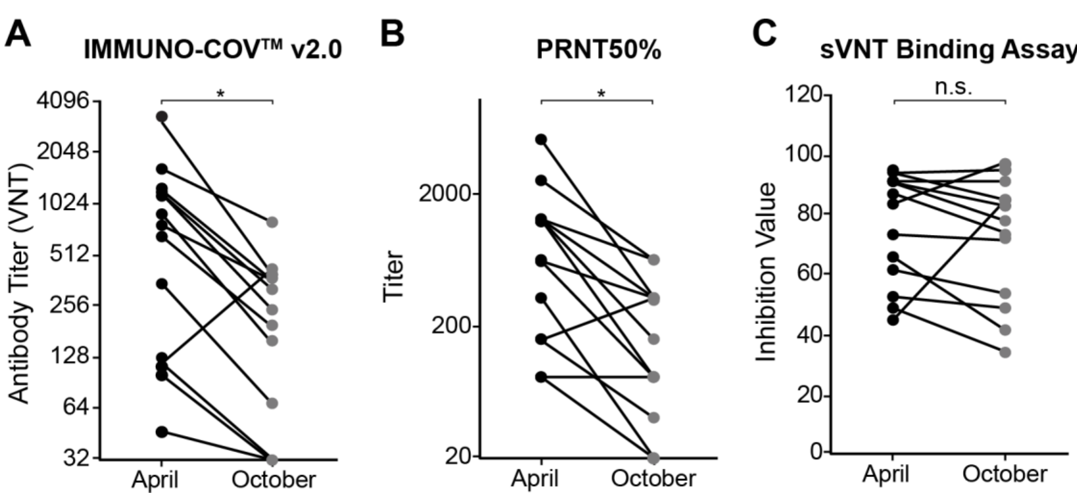

916

917 **Figure 10:** *Durability of neutralizing antibody responses*. A-C) Samples were collected from
918 donors in April and October 2020 (n=13). Neutralizing antibody levels were measured using
919 IMMUNO-COV^{TM} v2.0 (A), PRNT assay (B), or the c-PASS SARS-CoV-2 neutralization
920 antibody detection kit (C), which is a binding assay that utilizes the SARS-CoV-2 spike RBD
921 domain. The reductions in antibody titers were statistically significant for IMMUNO-COV^{TM} v2.0
922 and PRNT assay, but not for the sVNT binding assay ($p = 0.0007$, 0.0004, and 0.4669, respectively,
923 from paired T test).

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TABLES

Table 1: Assay Linearity

Standard	**ST1**	**ST2**	**ST3**	**ST4**	**ST5**	**ST6**
Nominal Value[1]	**320**	**160**	**80**	**40**	**20**	**10**
Mean Value	278.0	167.3	80.3	39.3	20.4	10.7
SD	23.7	21.4	8.7	3.1	1.7	1.3
%CV	8.5	12.8	10.8	8.2	8.1	12.0
%RE	-13.1	4.6	0.4	-1.9	1.9	7.3
% in range[2]	59.5	99.2	99.6	100.0	99.4	39.4
Number in range[2]	144	240	241	242	226	95

SD = standard deviation
CV = co-efficient of variation
RE = relative error
[1]Expected VNT value based on concentration of mAb10914 in each standard.
[2]Total *n* from all runs is 242.

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Table 2: Assay Specificity and Sensitivity

	Specificity[1]	**Sensitivity[2]**
PRNT50%		
Percentage	100%	93.7%
Sample Agreement	113/113	59/63
PRNT80%		
Percentage	100%	98.4%
Sample Agreement	116/116	59/60

[1]IMMUNO-COV™ v2.0 negative results relative to PRNT/ EUROIMMUN IgG ELISA negative results (samples positive by ELISA but negative by PRNT were considered negative).

[2]IMMUNO-COV™ v2.0 positive results relative to PRNT positive results. Any samples collected from donors previously PCR-positive for SARS-CoV-2, or positive for SARS-CoV-2 antibodies by IMMUNO-COV™ v2.0 or EUROIMMUN IgG ELISA were tested by PRNT assay.

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Table 3: Intra- and Inter-Assay Variability

QC Level	**QC High**			**QC Low**			**Matrix Blank**
Predicted VNT[1]	**160**			**32**			**0**
Precision Criteria	**% Response**	**VNT**	**Intra-Assay %CV**	**% Response**	**VNT**	**Intra-Assay %CV**	**Intra-Assay %CV**
Mean Value	2.8	208.6	13.3	37.7	29.8	21.4	9.8
SD	1	37.7		11.5	8.6		
%CV	37.8	18.1		30.6	28.8		

SD = standard deviation
CV = co-efficient of variation
n = 207
[1]Predicted VNT of QC samples based on concentration of mAb10922 spiked into matrix blank.

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Table 4: Matrix Equivalency

	Sodium heparin-plasma	**ACD-plasma**	**K2/EDTA-plasma**
%RE (relative to sera)[1]	+9.3	-9.8	+24.6

%RE = percent relative error
[1]Consensus VNT titers from the indicated plasma samples were compared to the consensus VNT titer for the matched serum sample. n=12.

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Table 5: VNT to PRNT50% Conversion

VNT	PRNT50%
< 32	< 1:40
32 to 40	1:40
41 to 80	1:80
81 to 180	1:160
181 to 400	1:320
401 to 800	1:640
801 to 1600	1:1280
1601 to 2400	1:2560
> 2400	> 1:2560

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Table 6: Longevity of Neutralizing Antibodies

Donor	IMMUNO-COV™ v2.0 Titer (VNT)			c-PASS Value[1]		
	April[2]	October[3]	Relative titer	April[2]	October[3]	Relative value
1	1652	784	0.47	94	95	1.01
2	1187	320	0.27	84	96	1.14
3	47	< LOD	≤ 0.68	49	35	0.71
4	769	376	0.49	92	92	1.00
5	3030	378	0.12	88	74	0.84
6	1179	246	0.21	91	79	0.87
7	1219	378	0.31	95	84	0.88
8	124	< LOD[4]	≤ 0.26	67	42	0.63
9	894	156	0.17	74	73	0.99
10	102	< LOD[4]	≤ 0.31	62	54	0.87
11	660	195	0.30	91	83	0.91
12	350	68	0.19	52	49	0.94
13	114	418	3.67	45	85	1.89

[1]Samples analyzed using the c-PASS SARS-CoV-2 Surrogate Virus Neutralization Test (sVNT) Kit
[2]April samples acquired from donors 2 to 8 weeks following COVID-19 symptoms or diagnosis.
[3]October samples acquired from same donors approximately 6 months after April samples acquired.
[4]Matched ACD-plasma samples were also analyzed and exhibited low-levels of neutralizing antibodies in some assay runs.

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