# Quantitating SARS-CoV-2 Neutralizing Antibodies from Human Dried Blood Spots

Katherine Berman, Greta Van Slyke, Hayley Novak, Jean M. Rock, Rachel Bievenue, Amanda Damjanovic, Kate DeRosa, Gianna Mirabile, Roxie C. Girardin, Alan P. Dupuis, Kathleen A. McDonough, Monica M. Parker, Linda M. Styer*, and Nicholas J. Mantis*

Division of Infectious Diseases, Wadsworth Center, New York State Department of Health, Albany, NY 12208

**Running title:** Neutralizing antibodies in dried blood spots

*co-corresponding authors: Linda Styer
Nicholas J Mantis, Division of Infectious Diseases, Wadsworth Center, Albany, NY 12208,
email: Linda.Styer@health.ny.gov
email: nicholas.mantis@health.ny.gov

## ABSTRACT

**Background:** In the earliest days of COVID-19 pandemic, the collection of dried blood spots (DBS) enabled public health laboratories to undertake population-scale seroprevalence studies to estimate rates of SARS-CoV-2 exposure. With SARS-CoV-2 seropositivity levels now estimated to exceed 94% in the United States, attention has turned to using DBS to assess functional (neutralizing) antibodies within cohorts of interest.

**Methods:** Contrived DBS eluates from convalescent, fully vaccinated and pre-COVID-19 serum samples were evaluated in SARS-CoV-2 plaque reduction neutralization titer (PRNT) assays, a SARS-CoV-2 specific 8-plex microsphere immunoassay, a cell-based pseudovirus assay, and two different spike-ACE2 inhibition assays.

**Results:** DBS eluates from convalescent individuals were compatible with RBD-ACE2 inhibition assays, an in-house Luminex-based RBD-ACE2 inhibition assay, and commercial real-time PCR-based neutralization assay (NAB-Sure™) but not cell-based pseudovirus assays or PRNT. The insensitivity of cell-based pseudovirus assays was overcome with DBS eluates from vaccinated individuals with high SARS-CoV-2 antibody titers.

**Conclusion:** SARS-CoV-2 neutralizing titers can be derived with confidence from DBS eluates, thereby opening the door to the use of these biospecimens for the analysis of vulnerable populations and normally hard to reach communities.



**Keywords:** Serology; Neutralizing; COVID-19; Antibody; Human

## 1. INTRODUCTION

The collection and serologic analysis of dried blood spots (DBS) from population-based serosurveys proved integral to understanding the dynamics of SARS-CoV-2 infection in the first months of the COVID-19 pandemic. In New York State, for example, a statewide seroprevalence study among ~15,000 individuals in public settings (e.g., grocery store fronts) conducted in April 2020 afforded some of the first insights into overall disease incidence and demographics of virus exposure in urban and rural communities (1). Similarly, a cross-sectional serosurvey of ~400,000 newborns in New York State that employed heel stick DBS collected between November 2019 and November 2021 afforded unprecedent insight into COVID-19 exposures and vaccination status among a diverse population of reproductive-aged females (2). Other groups have leveraged clinician-assisted and self-collected DBS to conduct large scale serosurveys of vulnerable cohorts, school age children, and otherwise hard to reach communities (3-10). In addition to ease of collection, once collected onto Whatman filter paper, DBS samples are stable for long periods of time under refrigeration (4-8$^{o}$C) or frozen (up to 20 $^{o}$C) in the presence of desiccants (11, 12).

Within the COVID-19 pandemic, DBS have largely been employed in serological studies aimed at determining SARS-CoV-2 S and N reactivity as measures of infection and vaccination. However, with SARS-CoV-2 seropositivity levels now estimated to exceed 94% in the United States, the value of such serosurveys has diminished (13). Furthermore, the emergence of omicron subvariants with highly mutated spike proteins confounds one's ability to extrapolate neutralizing antibody titers from binding antibody units (BAUs) (14, 15). For those and other reasons, there is considerable interest in developing a method to assess SARS-CoV-2 neutralizing titers directly from DBS eluates.

Indeed, several reports have already described the use of DBS eluates to assess functional activity of SARS-COV-2 antibodies. For example, Itell and colleagues examined paired plasma and fingerstick (FS) DBS from convalescent individuals in SARS-COV-2 spike-pseudotyped lentiviral particle assay in 96- and 384-well format (16). In another report, Sancilio and colleagues successfully used an ACE2-RBD

89 binding assay on the Mesoscale Diagnostics (MSD) platform to estimate neutralizing
90 titers in contrived DBS (cDBS) eluates (17) and in self-collected DBS from a community-
91 based serology survey (18). However, neither study benchmarked their results against
92 plaque reduction neutralization titer (PRNT) values, nor did they perform cross
93 comparisons of the different *in vitro* assays (e.g., pseudovirus versus ACE2-RBD).
94 Therefore, to further advance the utility and validity of DBS for the purpose of
95 functional SARS-COV-2 serology, we investigated the compatibility of contrived DBS
96 eluates with different SARS-COV-2 neutralization assays and compared their outputs to
97 PRNT values derived from matched serum. While DBS eluates proved incompatible
98 with cell-based SARS-COV-2 pseudovirus neutralization assays, we found that two
99 different ACE2-RBD inhibition assays, an in-house Luminex-based assay and a
100 commercial real-time PCR-based assay (NAB-Sure™), yielded neutralization values
101 that strongly correlated with PRNT. An 8-plex MIA based solely on binding antibodies to
102 spike antigens also served as a proxy for PRNT. As the two RBD-ACE2 inhibition
103 assays employed in this study are amenable to high-throughput testing, we propose to
104 integrate one or both into our DBS workflow for analysis of cohort specific studies going
105 forward.
106
107 **2. METHODS**
108
109 **2.1 SARS-CoV-2 reagents**. The source and descriptions of SARS-CoV-2 reagents
110 used in this study is provided in **Table 1**.
111
112 **2.2 Human serum samples.** A set of serum samples from 30 PCR positive donors was
113 purchased from Access Biologicals, LLC, (Vista, CA), herein referred to as "Panel
114 D". Positive test results were reported in the initial U.S. wave of infections between
115 3/15/2020 and 4/25/2020; and samples were drawn between 6/3/2020-6/4/2020,
116 indicating that all donors were infected with the Washington variant of SARS-CoV-
117 2. The panel consists of 21 female and 9 male donors; ages ranged from 25- to 66-
118 years-old; all donors were symptomatic. A set of 87 COVID-19 negative serum samples
119 collected between 3/24/2017 and 11/9/2018 was also purchased from Access

120 Biologicals, LLC. This set is referred to as “Panel E”, which consists of pre-pandemic
121 SARS-CoV-2 sero-negative donors (73 females and 14 males between 18 and 64-
122 years-old). Upon receipt, Panel D and E serum samples were stored at -80ºC.
123
124 **2.3 Contrived DBS.** Contrived DBS were created by mixing an equal volume of Panel
125 D or E serum with washed human red blood cells (RBC). Fifty microliters (50 μL) of the
126 RBC-serum mixture were spotted onto Whatman 903 filter paper (SigmaAldrich, St.
127 Louis, MO) and allowed to air dry at room temperature for 4 h before being packaged in
128 semi-permeable bags with desiccant and stored at -20ºC. Red blood cells (RBC) were
129 collected by centrifugation (3,000 RPM at 4ºC) from commercially obtained fresh human
130 whole blood [Type O EDTA ] (ZenBio, Durham, NC).
131
132 **2.4 Plaque reduction neutralization titer (PRNT).** PRNT was determined as described
133 (19, 20). Briefly, 2-fold serially diluted serum (100 µl) was mixed with an equal volume of
134 150-200 plaque forming units (PFUs) of SARS-CoV-2 (isolate USA-WA1/2020, BEI
135 Resources, NR-52281) and incubated for 1 h at 37°C, 5% $CO_2$. The virus:serum mixture
136 (100 µl) was applied to VeroE6 cells grown to 95%–100% confluency in 6 well plates.
137 Adsorption of the virus:serum mixture was allowed to proceed for 1 h at 37°C, 5% $CO_2$.
138 Following the adsorption period, the wells were overlaid with 0.6% agar prepared in cell
139 culture medium (Eagle’s Minimal Essential Medium, 2% heat inactivated FBS,
140 100 µg/ml penicillin G, 100 U/ml streptomycin). Two days post-infection, a second agar
141 overlay containing 0.2% neutral red prepared in cell culture medium was applied.
142 Plaque determinations were conducted 1-2 days later. Neutralizing titers were defined
143 as the inverse of the highest dilution of serum providing 50% (PRNT50) or 90%
144 (PRNT90) viral plaque reduction relative to a virus only control.
145
146 **2.5 Microsphere Immunoassay (MIA)**. The development and validation of an 8-plex
147 MIA consisting of three different nucleocapsid N antigens, RBD, Spike S1 (S1), full
148 length Spike (FLS), and full length Trimer (FLT) is described elsewhere (J. Rock,
149 L.Styer, *manuscript in preparation*). Magplex-C microspheres (Luminex Corp) were
150 coupled with 7 different SARS-CoV-2 antigens and 1 internal control antibody. Each

151 antigen/antibody was coupled to a different bead region allowing the beads to be
152 multiplexed in the same well. A 3.3 mm DBS punch from each sample was combined
153 with 250 µl elution buffer (Tris-buffered saline, 1% casein blocker) (Bio-Rad
154 Laboratories) in a 96 well plate. The DBS punches were eluted for 1 hour at room
155 temperature. Antigen-coated beads (diluted to 1,250 beads/bead set/well with assay
156 buffer (PBS, 2% BSA, pH 7.4)) and 25 µl DBS eluate were combined in a 384 well plate
157 and incubated at 37°C for 30 minutes while shaking (300 RPM) in the dark. Plates were
158 washed for two cycles using the BioTek 405TS microplate washer and 50 µl wash buffer
159 (PBS, 2% BSA, 0.02% Tween-20, 0.05% sodium azide, pH 7.4). 50 µl phycoerythrin-
160 tagged goat anti-human IgG was added to each well. Plates were incubated at 37°C for
161 30 minutes while shaking (300 RPM) in the dark and washed as described above.
162 Antibody-bead complexes were resuspended with 90 µl xMap sheath fluid (Luminex),
163 plates were incubated for 1 minute at room temperature while shaking (300 RPM), and
164 70 µl from each well was read on a Luminex FlexMAP 3D instrument. Results were
165 presented in median fluorescence intensity (MFI) for each well. Positive, negative, and
166 background controls were included with every plate.
167
168 **2.6 Reporter virus particle (RVP) neutralization assays.** Pseudo-typed lentiviral
169 reporter virus particles (RVP) tagged with Renilla luciferase (Luc) and 293T-hsACE2
170 cells were obtained from Integral Molecular, Inc. (Philadelphia, PA). To determine the
171 SARS-CoV-2 neutralizing activity in human serum and paired cDBS eluates, serum and
172 DBS eluates were diluted 1:50 in DMEM containing 10mM HEPES [pH 7.4], 10% fetal
173 bovine serum and 100 U/mL penicillin and 0.1 mg/mL streptomycin (Sigma-Aldrich).
174 Paired sample types were serially diluted in duplicate in cell medium. RVPs were
175 thawed at 37°C and immediately diluted per the manufacturer's specification in cell
176 medium and then 50 µl was added to the assay plate. A total of 50 µl of diluted human
177 serum or DBS eluate were then transferred from the dilution plate and mixed with RVP
178 solution. Serum and DBS eluates were incubated with RVPs for 1 hour at 37°C with 5%
179 $CO_2$. Positive and negative control wells were included on each assay plate and
180 consisted of RVPs only (100% infectivity control), and no RVPs (0% infectivity control).
181 During RVP-serum incubation 80-90% confluent 293T-hsACE2 cells were harvested

182 and cell density was determined using a hemocytometer. Cells were diluted to a density
183 of 2 x $10^5$ cells/mL in cell culture media and 100 µl was added to each well of the RVP +
184 specimen plate and mixed with gentle pipetting. Plates were incubated at 37°C with 5%
185 $CO_2$ for 72 h. Quantification of SARS-CoV-2 RVP infection was determined using the
186 *Renilla*-Glo® luciferase assay system (Promega Corp., Madison, WI). Microtiter assay
187 plates were centrifuged for 5 min at 2000 rpm to prevent cell loss, then supernatant was
188 removed with a pipet and 30 µl of PBS was added to each well. *Renilla*-Glo® substrate
189 diluted 1:100 (v/v) into buffer was prepared fresh; 30 µl added per well and plates were
190 then incubated for 10 min at room temperature. Relative light units (RLU) were detected
191 on a SpectroMax iD3 (Molecular Devices, LLC, San Jose, CA) and analyzed with
192 SoftMax Pro 7.0 software. A Z' value was calculated for each plate for assay accuracy
193 and comparability, plates with a Z'<0.5 were repeated. The RLU mean and standard
194 deviation (SD) of 100% infected wells was determined, and a minimum limit of detection
195 was calculated for each plate as = $(\text{Mean} - (3\text{xSD}))_{100\%\text{Infected}}$. Samples that failed to
196 reach the LOD cut-off value at the most concentrated dilution were classified as non-
197 neutralizing (<LOD) and assigned a value of 0 for statistical analysis purposes. For each
198 plate the RLU mean value for 0% infected wells was subtracted from the mean of 100%
199 infected wells resulting in a normalized infection value (NIV); % normalized RVP
200 neutralization was calculated as = $100-((S_{AVE}/\text{NIV})*100)$, where $S_{AVE}$= the average of
201 duplicate sample RLU values.
202
203 **2.7 Human ACE2 inhibition (iACE2) assay.** Recombinant soluble human ACE2 was
204 generated by the Wadsworth Center's Protein Expression core facility. The coding
205 region for human ACE2 (residues 1-615; Genbank #) was cloned into pcDNA3-
206 sACE2(WT)-8his [(https://www.addgene.org/browse/sequence/298420/] and transfected
207 into ExpiCHO-S cells using ExpiFectamine, as per manufacturer's instructions
208 (ThermoFisher (https://www.thermofisher.com/order/catalog/product/A29133). The
209 recombinant protein was affinity purified on 2 x 1ml HisTrap™ Excel (Cytiva) columns
210 connected in series. ACE2 was eluted with a linear gradient of 20 mM Tris-HCl, 500 mM
211 NaCl, 500 mM imidazole pH 8.0 over 20 column volumes. Fractions containing ACE2,
212 as assessed by SDS-PAGE, were pooled and dialyzed twice against PBS [pH 7.4].

213 Protein concentration assessed by Bradford assay with bovine albumin as a standard
214 curve. Biotinylated using EZ-Link NHS Biotin (Thermo Fisher Scientific).
215 Magplex-C microspheres (Luminex) from separate bead regions were coupled
216 with RBD and FLT from SARS-CoV-2 isolate USA-WA1/2020 ("wild type") and
217 resuspended to 2500 beads/region/well. Diluted beads were transferred to wells of a
218 non-binding 96 well plate; 50 µl of serum or DBS eluate (1:138) was then added to each
219 well and incubated for 1 h RT with agitation in the dark. Plates were washed twice
220 manually with wash buffer (190 µl) and then biotinylated-hACE2 (see below; 4 µg/mL)
221 was added to each well, plates were incubated for an additional 45 min at RT, shaking
222 in the dark. Plates were washed, probed with streptavidin-PE (1 µg/mL) and incubated
223 at RT for 30 min. The plates were washed to removed unbound streptavidin-PE then
224 analyzed using FlexMap3D. The MFI for ACE2 binding (in the absence of antibody) was
225 derived from a single column of wells containing diluted beads. Inhibition of ACE2
226 (iACE2) binding calculated as: 190 µl % ACE2 binding-inhibition (iACE2), per sample
227 well, where % iACE2 = 100-[Raw value/(Average of wells with specific VoC beads and
228 no serum or DBS eluate) x 100].
229
230 **2.8 NAB-Sure™**
231 The NAB-Sure™ SARS-CoV-2 Neutralizing Antibody Test (Spear Bio, MA, USA) was
232 performed according to the manufacturer's instructions (www.spear.bio/nabsure). NAB-
233 Sure™ is a competitive binding assay that measures the ability of antibodies in DBS
234 samples to block SARS-CoV-2 Spike Protein (S1) from binding to recombinant hACE2
235 in solution. The assay was validated at the Wadsworth Center using eluates (n=11) from
236 SARS-CoV-2 infected individuals with range of Spike-specific titers provided by
237 SpearBio. Contrived Panel D DBS punches (6mm) were incubated in NAB-Sure™
238 elution buffer at 37$^{o}$C for 1 h. Afterwards, eluates (15µL) were processed according to
239 the manufacturer's instructions. The test was read using a 7500 Fast Real-Time PCR
240 System (Thermo Fisher Scientific) in the Wadsworth Center's Bloodborne Virus
241 Laboratory. CT values were converted to $NT_{50}$ values relative to the negative control
242 sample that is devoid of SARS-CoV-2 neutralizing antibodies. The cycle difference
243 between the sample CT value and the negative control CT value was calculated.

244 Inhibition % was calculated as = $1-1/2^x$, where x= cycle difference. An interpolated NT50
245 value was determined using GraphPad Prism software. The final NT50 value= y*50,
246 where y=interpolated NT50 value.
247
248 **2.9 Statistical Analysis.**
249 Statistical analyses were performed using GraphPad Prism 9 software. Correlations
250 were assessed by the Pearson correlation coefficient. An unpaired, two-tailed Welch's t-
251 test was performed to determine significance of differences in ACE2 inhibition between
252 the two groups. Statistical significance of differences in RVP dilutions was assessed
253 using a 2-way ANOVA. In all cases, the following apply: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq$
254 $0.001$, ****$p < 0.0001$.
255
256 **3. RESULTS**
257 **3.1 Generation of standard SARS-CoV-2 DBS panel.** The primary objective of this
258 study was to assess SARS-CoV-2 virus neutralizing activity from DBS eluates
259 benchmarked against PRNT assays, since performing actual PRNT assays on DBS
260 eluates is neither practical nor technically feasible. For this purpose, we generated
261 contrived DBS (cDBS) from a commercial panel ("Panel D") consisting of 30 individuals
262 with a defined history of SARS-CoV-2 infection during or prior to June 2020. The
263 participants ranged in age from 25 to 63 (average 45) with 21 females and 9 males. Of
264 the 30 COVID-19 positive samples, 26 were confirmed positive via SARS-CoV-2 PCR
265 and four strictly through medical diagnosis$PRNT_{50}$ values for 15 of the 30 samples were
266 determined using SARS-CoV-2 Washington (WA1), as described (19). The average
267 $PRNT_{50}$ for the cohort was 351 (+/-271). In addition, the percent SARS-CoV-2
268 neutralization at a fixed 1:100 serum dilution (average 55 +/- 22%) was extrapolated
269 based on PRNT curves to enable direct comparison with values derived from DBS
270 eluates (see below). A summary of the 15 Panel D participants is provided in **Table 2.**
271 We also generated cDBS samples from Panel D. As detailed in the Materials and
272 Methods, serum was combined with an equal volume of human RBCs then spotted (50
273 μl) onto Whatman 903 filter paper. The spots were allowed to dry at room temperature
274 for 4 h then stored with desiccant at -20ºC. Recovery of 90% ($\pm$0.04%) total IgG was

275 achieved from a single 3 mm punch (1/8'') by incubating the punch in 250 μl in TBS with
276 1% casein for 1 h at room temperature (**Figure 1**). The cDBS eluates were prepared
277 fresh for each assay described below. In addition, we also generated cDBS spots from
278 a commercially available panel of serum samples from a healthy cohort (**Panel E)** as
279 well as a fully vaccinated cohort (**Panel H**).
280
281 **3.2 Assessment of SARS-CoV-2 S- and N-specific IgG in DBS eluates by MIA.**
282 Using a recently optimized 8-plex MIA targeting different nucleocapsid (N) and Spike
283 antigens, we examined the relationship between MFIs derived from cDBS eluates and
284 PRNT values. Specifically, the 8-plex MIA includes full length Trimer (FLT), full length
285 spike (FLS), S1, RBD and three N antigens as shown in **Table 1**. As expected, Panel D
286 cDBS eluates had higher median fluorescent intensity (MFI) than Panel E cDBS eluates
287 for all seven SARS-CoV-2 antigens (**Figure S1**). In terms of correlation with PRNT (%
288 neutralization @ 1:100 dilution), the values ranged from 0.76 for FLT (p=0.001) and S1
289 (p=0.001) to 0.82-0.83 for FLS (p =0.0002) and RBD (p=0.0001), respectively (**Figure**
290 **2**). Summing the MFIs for all four spike antigens ("sum Spike") did not greatly increase r
291 values (r = 0.81) (**Figure 2E**). As expected, the correlation between N MFI and PRNT
292 was not significant (r =0.39; p>0.1; **Figure 2F**). We conclude that MFI derived from DBS
293 eluates to a given SARS-CoV-2 spike antigen correlates with virus neutralizing activity
294 as defined by PRNT.
295
296 **3.3. Pseudovirus neutralization activity in cDBS eluates.** Non-replicating, lentiviral-
297 based pseudoviruses expressing SARS-CoV-2 trimeric spike proteins are employed
298 widely to assess SARS and SARS-CoV-2 neutralizing antibody titers in plasma and
299 serum (21-24). However, it is unclear whether DBS eluates are amenable with these
300 cell-based assays. To investigate this, we employed a commercially available non-
301 replicating Reporter Virus Particles (RVP) expressing the SARS-CoV-2 spike protein
302 (D648G) and encoding *Renilla* luciferase (**Figure 3**) (25). We confirmed RVP efficacy
303 using the human monoclonal antibody CC12.3, which neutralizes SARS-CoV-2 with a
304 reported $IC_{50}$ of 0.026 μg/ml (24). In our hands, CC12.3 had an $IC_{50}$ of 0.04 μg/ml
305 (**Figure 3A**). Moreover, the 15 Panel D serum samples had neutralization activities that

306 ranged from 15% to 98% with high concordance with PRNT (r=0.67; p=0.007) (**Figure**
307 **3B**). Thus, RVP serves as reliable measure of SARS-CoV-2 activity in the context of
308 serum.
309 To determine if the RVP assay is compatible with cDBS, we collected eluates
310 from three 1/8" (3 mm) punches from each of the 15 cDBS Panel D spots and 7 Panel E
311 spots as negative controls. Eluates were generated by incubating the punches in
312 DMEM-HEPES at room temperature for 2 h with agitation. Eluates were then diluted
313 1:50 into DMEM-HEPES medium and then mixed with RVPs prior to application to cells
314 293T cells constitutively expressing human ACE2 (see **Materials and Methods**).
315 Panel D eluates had 60-90% neutralization activity [average 68%] that were
316 modestly correlated with PRNT (r value of 0.61; p=0.02) (**Figure 3C**). However, the
317 results were confounded because Panel E eluates (negative controls) had
318 "neutralization" (interfering) activity that was essentially equivalent to Panel D (**Figure**
319 **3D**). Thus, RVP assay was not able to distinguish between Panel D and Panel E
320 eluates. Efforts to improve the sensitivity of the assay through dilution (>1:3200),
321 alternative elution buffers, and removal of hemoglobin using reagents designed for this
322 purpose (e.g., HemogloBind™) were unsuccessful (**Figure 3D**; **data not shown**). We
323 conclude that cDBS eluates from convalescent (unvaccinated) individuals have
324 insufficient virus neutralizing titers to distinguish them from unvaccinated controls in the
325 pseudovirus (RVP) platform.
326
327 **3.4 Relationship between PRNT and iACE2.** We next tested cDBS eluates for the
328 ability to inhibit ACE2-RBD interactions in using a Luminex-based assay (iACE2). In the
329 assay, RBD- and FLT-coupled microspheres were incubated with soluble biotinylated-
330 ACE2 in the absence or presence of competitor antibodies (e.g., monoclonal, antisera
331 or DBS eluates), then probed with streptavidin-PE and analyzed by using FlexMap3D
332 (Luminex). A reduction in MFI was interpreted as antibody-mediated inhibition of ACE2
333 binding to RBD- and FLT. The utility of the in-house iACE2 assay was confirmed using
334 the well characterized monoclonal antibody CC12.3, which neutralizes SARS-CoV-2 by
335 interfering with ACE2-RBD interactions (24). In the iACE2 assay, CC12.3 had an $IC_{50}$

336 value for WT FLT and RBD of 0.04 ug/mL and 0.02 ug/mL, respectively (**Figure S2**).
337 These values are consistent with those reported by Rogers and colleagues (24).
338 We examined Panel D DBS eluates in the iACE2 assay, with the expectation that
339 Panel D eluates would have greater inhibition activity than Panel E. Indeed, Panel D
340 had significantly higher iACE2 values (23% average; range 10%-50%) as compared to
341 Panel E (9% average; range 0%-15%) (**Figure 4A**). In terms of the correlation with
342 PRNT, Panel D iACE2 values had an r-value of 0.77 ($p<0.001$) for RBD-coated beads
343 and 0.72 ($p=0.003$) for FLT-coated beads. iACE2 (%) values for FLT-coated beads
344 ranged from 25% to 40% (avg 31%) (**Figure 4; Figure S2**). Panel D DBS eluates
345 performed slightly less well than the matched serum samples from which they were
346 derived, at the same dilution, indicating that a degree of ACE2-RBD inhibitory activity is
347 lost during the collection of cDBS eluates. Panel D serum samples (1:100 dilution) had
348 iACE2 activity that correlated with PRNT with $r= 0.81$ ($p<0.001$; **Figure S2**). These
349 results demonstrate that iACE2 assay affords a reliable estimate of SARS-CoV-2
350 neutralization potential using cDBS eluates.
351
352 **3.5. NAB-Sure™.** NAB-Sure™ is a commercially available cell-free assay based on
353 successive proximity extension amplification reaction (SPEAR) technology with qPCR
354 as a readout. Disruption of a proximity extension amplification reaction primed by
355 recombinant SARS-CoV-2 S1 protein and human ACE2 by neutralizing antibodies in
356 DBS eluates results in increased CT values. To investigate this technology, we first
357 evaluated the NAB-Sure™ assay using a set of 11 previously authenticated DBS
358 eluates provided by the manufacture and which were subjected to five 3-fold serial
359 dilutions (1 to 243) and tested in duplicate using in-house instrumentation. The NAB-
360 Sure™ assay revealed $NT_{50}$ values that ranged from 210 to 9500 (avg. 2333) with 17.7
361 %CV (**Figure 5A**). There was a significant correlation between $NT_{50}$ values from DBS
362 eluates tested by NAB-Sure™ and paired serum samples tested by PRNT [Pearson's r
363 = 0.99; $p<0.0001$] (**Figure 5A**). Thus, NAB-Sure™ was compatible with DBS eluates
364 and had a high concordance with PRNT.
365 We then used the NAB-Sure™ assay to evaluate cDBS eluates from Panel D
366 (#1-14) and E (#1-7). The samples were tested 3 times in independent reactions under

367 identical assay conditions. Concordance across the different reactions was high, as
368 evidenced by r values between 0.85-0.92 **(Figure 5B-D)**. The average %CV across
369 samples was 20.3%. All reactions reached significance when compared to PRNT. From
370 these results, we conclude that NAB-Sure™ is sufficiently sensitive to detect SARS-
371 CoV-2 neutralizing titers in DBS eluates, based on high degree of concordance with
372 PRNT.
373
374 **3.6 Assessing SARS-CoV-2 neutralizing activity in vaccinated individuals.** Studies
375 prior to this point relied on DBS eluates derived from SARS-CoV-2 infected individuals
376 with relatively low overall Spike-specific antibody titers as well as SARS-CoV-2
377 neutralizing levels. With an estimated >90% of the United States population seropositive
378 for SARS-CoV-2 due to vaccination and/or natural infection at this point in time (13), we
379 wished to revisit the utility of DBS eluates in assessing virus-specific antibody titers and
380 neutralizing activity in a vaccinated cohort. For this purpose, we generated cDBS from a
381 commercially available panel (**Panel H**) of serum samples collected from individuals
382 who had received two doses of the original Moderna (mRNA 1273) vaccine. As shown
383 in **Figure 6**, Panel H DBS eluates (n=15; 15746 $\pm$ 1790 MFI) had RBD-specific antibody
384 levels (MFI) what were 1.5 times higher than those in Panel D (10392 $\pm$ 3325 MFI) and
385 44 times higher than Panel E (351 $\pm$ 313 MFI). DBS eluates were evaluated in the
386 iACE2 assay (**Figure 6C, D**), RVP (pseudovirus) neutralization assay (**Figure 6E, F**)
387 and NAB-Sure™ (**Figure 6G, H**). Unfortunately, insufficient volumes of this panel were
388 available for PRNT assessment. Nonetheless, the results of these analyses
389 demonstrated that all three platforms (iACE2, RVP, NAB-Sure™) were compatible with
390 DBS eluates as evidenced by ACE2 inhibition values approaching 100% (**Figure 6C),**
391 RVP $NT_{50}$ values > 5000 (**Figure 6E**), and NAB-Sure™ $NT_{50}$ values approaching 18,000
392 (**Figure 6G**). There were statistically significant correlations between RBD binding
393 antibody levels (MFI; y-axis) and iACE2 values (x-axis; **Figure 6D),** as well as RBD
394 binding antibody levels (MFI; y-axis) and RVP $NT_{50}$ values (x-axis; **Figure 6F**). This
395 observation is consistent with the earliest observations serology-based correlates of
396 immunity elicited by the spike-encoding mRNA vaccines (14). We did not, however,
397 observe a significant correlation between RBD antibody levels (MFI) and NAB-Sure™

398 $NT_{50}$ values (**Figure 6H**) or RVP $NT_{50}$ values and NAB-Sure™ $NT_{50}$ values (r = 0.17;
399 **data not shown**). These results suggest that NAB-Sure™ may perform optimally with
400 DBS eluates samples with lower Spike-specific antibody titers, whereas the iACE2 and
401 RVP assays are better suited for samples with higher Spike-specific antibody titers.
402 Thus, specific assays may be tailored to specific cohorts and their anticipated SARS-
403 CoV-2 titers.
404
405 **4.0 DISCUSSION**
406 As the World Health Organization (WHO) and other public health agencies
407 integrate long term COVID-19 disease management strategies into the broader
408 landscape of infectious disease, DBS have the potential to serve as a centralized
409 biospecimen type from which to estimate levels of natural and vaccine-induced
410 immunity at population scale (11). Indeed, DBS-based seroprevalence and
411 neutralization assays have been established and deployed to monitor vaccine-induced
412 immunity for the likes of polio (26), human papillomavirus (HPV) (27) and several
413 bacterial pathogens and parasites (28). However, with SARS-COV-2 seroprevalence
414 nearing universality as a result of natural infections/reinfections alongside widespread
415 vaccination, the future utility of DBS to COVID-19 will depend on the development of
416 robust methods to assess functional antibody titers alongside established BAU
417 determinations (14, 29). With this objective in mind, we investigated the compatibility of
418 DBS eluates with different SARS-COV-2 neutralization, pseudovirus neutralization, and
419 ACE2-RBD inhibition assays. While neither the SARS-CoV-2 PRNT nor the pseudovirus
420 (RVP) neutralization assays proved compatible with DBS eluates from convalescent
421 individuals due to high background, values from ACE2-RBD inhibition assays and NAB-
422 Sure™ correlated neutralization titers ($NT_{50}$ and %) from paired serum. In a cohort of
423 SARS-CoV-2 vaccinated individuals, the ACE2-RBD inhibition and RVP neutralization
424 assays proved more robust than NAB-Sure™, at least under these experimental
425 conditions and with this limited data set. With this in mind, we would recommend
426 tailoring SARS-CoV-2 antibody assessments based on cohorts under investigation. In
427 summary, we conclude that DBS eluates are indeed compatible with multiple SARS-

428 CoV-2 surrogate neutralizing assays and can be applied in parallel with existing and
429 forecasted community and population-based serosurveillance efforts.
430 Itell and colleagues were the first to investigate the use of DBS to access
431 functional (neutralizing) antibodies from COVID-19 convalescents and SARS-CoV-2
432 vaccinees (16). In that report, a cell-based SARS-CoV-2 pseudovirus assay proved to
433 be a reliable measure of neutralizing titers with both mock DBS (venous blood spotted
434 on Whatman filter paper) and self-collected fingerprick DBS from vaccinated individuals.
435 In a larger scale study, Roper and colleagues reported success "estimating" SARS-
436 CoV-2 pseudovirus neutralizing titers from fingerprick DBS eluates but noted that
437 multiple samples of the 12 tested were eliminated due to values below limit of detection
438 (30). In their study, fingerprick DBS eluates were not reported at dilutions less than 256,
439 suggesting to us the possibility of interference at low dilutions. They also used eluates
440 from a single 19 mm sample disc into PBS, compared to ~9 mm (3 x 3 mm) in our study
441 eluted into DMEM and 6 mm by Itell into PBS with 0.1 % Tween-20 (16). Collectively,
442 our results are consistent with DBS eluates being compatible with certain SARS-CoV-2
443 pseuodovirus assays but only when dilutions exceed ~256 to eliminate blood-based
444 interference.
445 McDade and colleagues reported the compatibility of DBS eluates with an ACE2-
446 RBD inhibition (iACE2) assay on the Mesoscale Diagnostics platform (17). We now
447 extend those results and demonstrate that both an in-house Luminex-based assay and
448 a commercial real-time PCR-based assay (NAB-Sure™), give rise to inhibition values
449 that strongly correlate with neutralization titers derived from PRNT. While we recognize
450 that the iACE2 assay is strictly a surrogate neutralization assay, both platforms
451 described herein have the potential to be integrated alongside existing high-throughput
452 SARS-CoV-2 multiplexed serologic assays. At present, we employ an 8-plex SARS-
453 CoV-2 microsphere immunoassay (MIA) run in 384 well format on the Luminex platform
454 with automated liquid handling (12). The in-house iACE2 assay described here is also
455 run on the Luminex platform using the same magnetic microspheres as the MIA. The
456 overall costs of the iACE2 assay are at least an order or magnitude less expensive than
457 cell-based pseudovirus assay with commercial RVPs. Moreover, the in-house iACE2
458 assay is easily multiplexed to include microspheres coated with RBD from circulating

459 VoCs and Omicron subvariants side-by-side with ancestral RBD. Indeed, we have
460 applied a 4-plex RBD assay to several thousand primary DBS samples collected from
461 an elderly cohort (G. Mirabelle, K. Berman, N. Mantis, unpublished results).

462 The NAB-Sure™ assay has the advantage of being ultrasensitive and amenable
463 to scale-up and automation and provided $NT_{50}$ titers similar to PRNT. Moreover, as
464 NAB-Sure™ is a commercial kit, there were no in-house costs associated with reagent
465 quality assurance or validation making the kit comparable (cost wise) with most
466 pseudovirus assays. In our hands, NAB-Sure™ proved most robust with DBS eluates in
467 which SARS-CoV-2 levels were on the low end (e.g. convalescent samples), while RVP
468 and iACE2 performed well with high titer samples associated with vaccination. It is
469 unclear whether this distinction will apply to DBS samples aimed at evaluating antibody
470 titers against SARS-CoV-2 variants of concern (VoC) like the highly divergent JN.1. In
471 fact, a tiered approach to interrogating DBS eluates may be in order where MFI values
472 alone provide a rough idea of neutralizing titers within a given sample especially when
473 dealing with thousands of samples from diverse cohorts (31). In terms of functional
474 antibodies, the Luminex-based iACE2 assay is both affordable and adaptable to any
475 given RBD. In fact, we have performed iACE2 multiplexing with four different RBD
476 variants simultaneously on hundreds of primary DBS samples from multiple different
477 cohorts (G. Mirabile,K. Berman, N. Mantis, *manuscript in preparation*). Ultimately, both
478 the NAB-Sure™ and pseudovirus assays can be employed as a means to establish
479 quantitative $NT_{50}$ equivalents not achievable with MFI or iACE2 alone.

480 The use of DBS for infectious disease serology and functional antibody testing is
481 not without intrinsic challenges, including variability in blood collection by fingerpick,
482 uniformity of antibody recovery in eluates, and limited sample volumes (11).
483 Nonetheless, the benefits of DBS as a biospecimen type for surveillance of hard-to-
484 reach communities are well recognized. For example, in work spearheaded by the CDC,
485 DBS from ~1,000 children in Bangladesh were sampled for neutralizing antibodies to
486 polio types 1, 2, and 3 using a modified poliovirus microneutralization assay (26). In the
487 case of polio, a neutralizing antibody titer of 1:8 correlated with protection and was used
488 as the threshold definition of seropositivity. Similarly, in a study examining natural
489 versus vaccine-induced antibody responses to human papillomavirus (HPV),

490 neutralizing activity was detected in 2 mm DBS punches eluted into 0.2 mL saline (27).
491 Another consideration is that for SARS-CoV-2 at least there is a strong congruence
492 between oral fluids and DBS (K. DeRosa, N. Pasaic, C. Heaney, L. Styer, and N.
493 Mantis, *manuscript in preparation*). Thus, DBS and oral fluids might potentially be used
494 interchangeably as means to survey large cohorts for immunity and vaccine
495 responsiveness (32).

496

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508
509 **CRediT**
510

| Author | Conceptualization | Curation | Formal analysis | Funding acquisition | Investigation | Methodology | Admin | Resources | Software | Supervision | Validation | Visualization | Writing-original | Writing-review |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| KB | | | x | | x | x | | | | | x | x | x | x |
| GVS | | | | | x | x | | | | | | | | |
| HN | | | | | x | x | | | | | | | | |
| JRM | | | x | | x | x | | | | | x | x | | |
| RB | | | x | | x | x | | | | | x | x | x | x |
| AD | | x | x | | | | | | | | x | | x | x |
| KD | | x | | | x | x | | | | | | | | x |
| GM | | | | | x | x | | | | | | | | |
| RCG | | | x | | x | x | | | | | x | | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| APD | | | | | x | x | | | | | x | | | |
| KAM | | | | | | | x | x | | x | | | | x |
| MMP | x | | x | | | | x | x | | x | | | | |
| LMS | x | | x | | | | x | x | | x | | | x | x |
| NJM | x | | x | x | | | x | x | | x | | | x | x |

**Conflicts of Interests (COI).** The authors have no conflicts of interest to declare.

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688

689 **FIGURE LEGENDS**

690 **Figure 1. Generation of contrived DBS eluates.** (**Top**) Schematic showing generation
691 of contrived DBS (cDBS), DBS punches, and eluate collection. (**Bottom**) Summary of
692 Panel D and Panel E samples used in this study. RT = room temperature, TBS = tris
693 buffered saline

694

695 **Figure 2. Correlation between PRNT and MIA with cDBS eluates.** (**Top**) Workflow
696 for the 8-plex MIA. (**Bottom**) Subset of Panel D cDBS eluates (n=15) underwent testing
697 via 8-plex MIA and were compared to paired serum tested by PRNT. Pearson
698 correlation of MFI values and SARS-CoV-2 neutralizing activity (%) is shown for the
699 following antigens: (A) FLT; (B) FLS; (C) S1; (D) RBD; (E) sum spike (see text for
700 details); and (F) N. The Pearson r value and p-values are shown as insets within each
701 panel.

702

703 **Figure 3. Compatibility of cDBS eluates with RVP neutralizing assay. (Top)**
704 Workflow for the RVP neutralizing assay. (**Bottom**) (A) Neutralizing titers associated
705 with CC 12.3 with $IC_{50}$ shown as text. (B) Correlation between PRNT and RVP with
706 matched Panel D serum samples; (C) Correlation between PRNT (% neutralization)
707 generated with Panel D serum versus RVP generated from matched cDBS eluates; (D)
708 Comparison of RVP neutralization of Panel D and Panel E cDBS eluates during serial
709 dilutions. Asterisks indicate a significant difference between groups by 2-way ANOVA,
710 where *p=0.02, **p=0.009, ***p=0.0002, ****p<0.0001. For (B) and (C), Pearson r and p-
711 value are shown as a panel inset.

712

713 **Figure 4. Correlation between Luminex-based ACE2 inhibition and SARS-CoV-2**
714 **neutralization activity.** (**Top**) Workflow for the ACE2 inhibition (iACE2) assay. (A)
715 Examination of iACE2 activity in Panel D cDBS eluates (n=15) versus Panel E eluates
716 (n=7). In (A), asterisks indicate a significant difference between groups by Welch's t-
717 test, where ***p=0.0006. (B) Correlation of iACE2 values from Panel D cDBS eluates,
718 as shown in (A), to SARS-CoV-2 neutralizing activity (%) from paired serum analyzed

719 via PRNT. cDBS eluates were evaluated in triplicate. The Pearson r and p-value are
720 shown as a panel inset for (B). The dotted line on (B) represents the average iACE2
721 activity for SARS-CoV-2 negative cohort (Panel E; n=7).
722
723 **Figure 5. NAB-Sure exhibits high concordance with PRNT.** (**Top**) Workflow for the
724 NAB-Sure™ assay. (A) Pearson correlation of NAB-Sure ™ $NT_{50}$ from authentic DBS
725 sample eluates compared to PRNT paired serum. DBS samples were evaluated in
726 duplicate. (B-D) Pearson correlation comparing NAB-Sure ™ $NT_{50}$ for cDBS eluates and
727 paired serum $NT_{50}$ from PRNT for 14 Panel D samples. (B-D) were exact replicates of
728 each other. For (A)-(D), Pearson r and p values are shown as a panel insets.
729
730 **Figure 6. Assessment of SAR-CoV-2 neutralizing titers in cDBS eluates from**
731 **vaccinated individuals (Panel H). (A)** Panel H, D and E cDBS eluates were rank
732 ordered from high to low (left to right) based on $RBD_{WT}$ MFI determined in the 8-plex
733 MIA. (**B**) Correlation plot between Panel H $RBD_{WT}$ MFI and Diasorin Liaison values
734 provided from Access Biologics LLC. Panel H cDBS eluate activities in (C) Luminex-
735 based iACE2 assay, (E) RVP neutralization assay and (G ) NAB-Sure ™ with
736 corresponding correlation plots shown in right panels (D, F, H). Pearson r and p values
737 are shown as a panel insets.
738

| Table 1. SARS-CoV-3 Reagents and Sources | | | | |
|---|---|---|---|---|
| **Reagent** | **Description** | **Source** | **Catalogue Number** | **Usage** |
| TBS | Tris Buffered Saline with Casein | BioRad | 1610782 | DBS Elution |
| FLT | Full Length Trimeric SARS-CoV-2 Spike | MassBiologics | N/A | 8-Plex MIA/iACE2 |
| FLS | Full length SARS-CoV-2 Spike, His-Tag | Native Antigen | REC31868-100 | 8-Plex MIA |
| Spike Subunit 1 (S1) | SARS-CoV-2 (2019-nCoV) Spike S1, His-Tag | Sino Biological | 40591-V08H | 8-Plex MIA |
| RBD | SARS-CoV-2 Stable RBD, Thrombin-His | MassBiologics | N/A | 8-Plex MIA |
| Nucleocapsid (N) | 2019-nCoV Nucleocapsid Protein, His tag | SinoBiological | 40588-V08B | 8-Plex MIA |
| Nucleocapsid (N-NA) | SARS-CoV-2 Nucleoprotein, His-Tag (E. coli) | Native Antigen | REC31812-100 | 8-Plex MIA |
| Nucleocapsid (NHT) | SARS-CoV-2 (2019-nCoV) Nucleocapsid Protein (His tag) | Sino Biological | 0588-V07E | 8-Plex MIA |
| Internal Control (IC) | Mouse anti-Human IgG3 Secondary Antibody | ThermoFisher | MA1-83242 | 8-Plex MIA |
| WT sRBD | SARS (WA1/2020) RBD, Thrombin-His | MassBiologics | N/A | iACE2 |
| hACE2 | Biotinylated ACE2 | Wadsworth Center Protein Core | N/A | iACE2 |
| CC12.3 | Human Mab RBD-A WT D614G | Scripps | N/A | iACE2/RVP |
| RVP-702L | WT D614G Strain Reporter Virus Particles | Integral Molecular | CL-275A | RVP |
| 293-hsACE2 Cells | 293T cells expressing human ACE2 receptor | Integral Molecular | TA-060520-MC | RVP |

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**Table 2. Panel D Summary**

| | | | Serum (PRNT) | | MFI in 8-plex MIA by Antigen | | | | | | iACE2 | RVP | | NAb |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| # | Age | Gender | $NT_{50}$ | %Neut | N[a] | S1[b] | FLS[c] | FLT[d] | RBD[e] | Sum[f] | iACE2[g] | NT50 | %Neut | $NT_{50}$ |
| 1 | 38 | F | >832 | 93 | 6121.0 | 4915 | 10178 | 14238 | 11185 | 40516 | 43 +/- 5.3 | 379 | 89 | 712 |
| 2 | 45 | F | 104 | 51 | 3283.5 | 521 | 1514 | 2557 | 1509 | 6100 | 17 +/- 4.3 | 183 | 65 | 90 |
| 3 | 44 | F | 208 | 42 | 2925.5 | 2377 | 4880 | 6843 | 5306 | 19406 | 22 +/- 2.2 | 151 | 58 | 244 |
| 4 | 48 | F | >832 | 93 | 3396.0 | 4295 | 9836 | 15808 | 11335 | 41274 | 53 +/- 2.7 | 203 | 79 | 557 |
| 5 | 55 | F | 416 | 33 | 544.0 | 872 | 2779 | 4562 | 2868 | 11081 | 14 +/- 3.4 | 435 | 67 | 196 |
| 6 | 65 | F | 208 | 60 | 4560.0 | 3221 | 6926 | 7929 | 5972 | 24047 | 14 +/- 3.4 | 395 | 64 | 270 |
| 7 | 40 | F | 208 | 77 | 7424.0 | 1714 | 4967 | 6497 | 5496 | 18674 | 18 +/- 2.3 | 513 | 67 | 189 |
| 8 | 39 | M | 832 | 70 | 6465.0 | 4561 | 8677 | 16991 | 10578 | 40806 | 42 +/- 7.1 | 262 | 71 | 899 |
| 9 | 34 | F | IND | 30 | 1434.0 | 1147 | 2983 | 3948 | 2873 | 10951 | 17 +/- 1.8 | 239 | 68 | 249 |
| 10 | 46 | F | 208 | 40 | 11565.0 | 433 | 2941 | 9150 | 2051 | 14575 | 15 +/- 4.3 | 229 | 68 | 176 |
| 11 | 30 | M | 208 | 47 | 6989.5 | 3387 | 7843 | 9969 | 6513 | 27711 | 16 +/- 3.4 | 141 | 64 | 356 |
| 12 | 47 | F | 208 | 40 | 2468.0 | 1432 | 3145 | 5014 | 4503 | 14093 | 17 +/- 4.2 | 188 | 65 | 249 |
| 13 | 57 | F | 26 | 21 | 1455.5 | 736 | 1907 | 2948 | 2176 | 7766 | 12 +/- 1.2 | 280 | 68 | 114 |
| 14 | 36 | M | 416 | 60 | 5371.5 | 2682 | 6136 | 9272 | 5869 | 23958 | 24 +/- 5.1 | 209 | 62 | 364 |
| 15 | 30 | F | 208 | 72 | 7691.0 | 2296 | 6687 | 9218 | 5867 | 24068 | 21 +/- 2.9 | 290 | 67 | 321 |

Footnotes: a: N, SARS-CoV-2 Nucleocapsid; b: S1, SARS-CoV-2 Spike subunit 1; c: FLS, SARS-CoV-2 Full Length Spike; d: FLT, SARS-CoV-2 Full length Trimer; e: RBD, SARS-CoV-2 Receptor Binding Domain; f: Sum, represents the total of all Spike antigens ; g: iACE2 indicates inhibition of biotinylated ACE2 binding to RBD or FLT antigen complexed to MagPlex beads. add MIA = microsphere immunoassay; MFI = median fluorescence intensity; NAb = NabSure neutralizing antibody; RVP = reporter virus assay

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746 **Supplemental Figure Legends**

747

748 **Figure S1. Comparison of Panel D and Panel E cDBS.** The full cohort for Panel D
749 (n=30) and Panel E (n=86) samples underwent testing via 8-plex MIA. MFI values for
750 the following antigens are compared: N, N NA, NHT, RBD, S1, FLS and FLT. Asterisks
751 indicate a significant difference between groups by 2-way ANOVA, where ****$p<0.0001$.

752

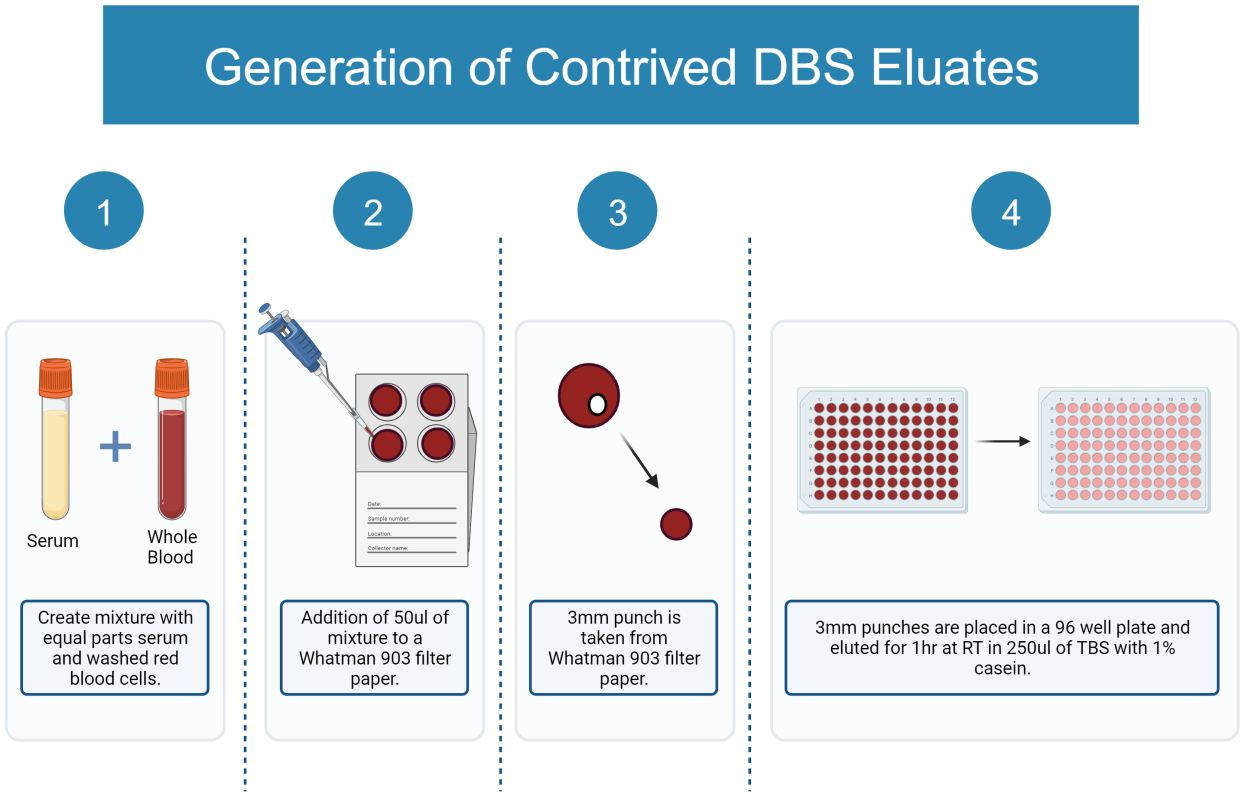
753 **Figure S2. Validation of Luminex-based ACE2 inhibition assay.** (A) MFI values
754 (binding) was assessed in the context of either FLT or RBD with varying concentrations
755 of biotinylated hACE2. (B) ACE2 inhibition titers associated with CC 12.3 in the context
756 of FLT or RBD. $IC_{50}$ is shown as text. (C) Pearson correlation of Panel D serum
757 analyzed by ACE2 inhibition and SARS-CoV-2 neutralizing activity (%), r-value and p-
758 value shown as text. (D) Pearson correlation of Panel D DBS eluates analyzed by ACE2
759 inhibition and SARS-CoV-2 neutralizing activity (%), in the context of FLT. The r-value
760 and p-value are shown as text. The dotted line on (D) represents the average iACE2
761 activity for SARS-CoV-2 negative cohort (Panel E; n=7).

762

763

Figure 1

**A.**


Generation of Contrived DBS Eluates
1
2
3
4
Serum
Whole Blood
Create mixture with equal parts serum and washed red blood cells.
Addition of 50ul of mixture to a Whatman 903 filter paper.
3mm punch is taken from Whatman 903 filter paper.
3mm punches are placed in a 96 well plate and eluted for 1hr at RT in 250ul of TBS with 1% casein.

**B.**

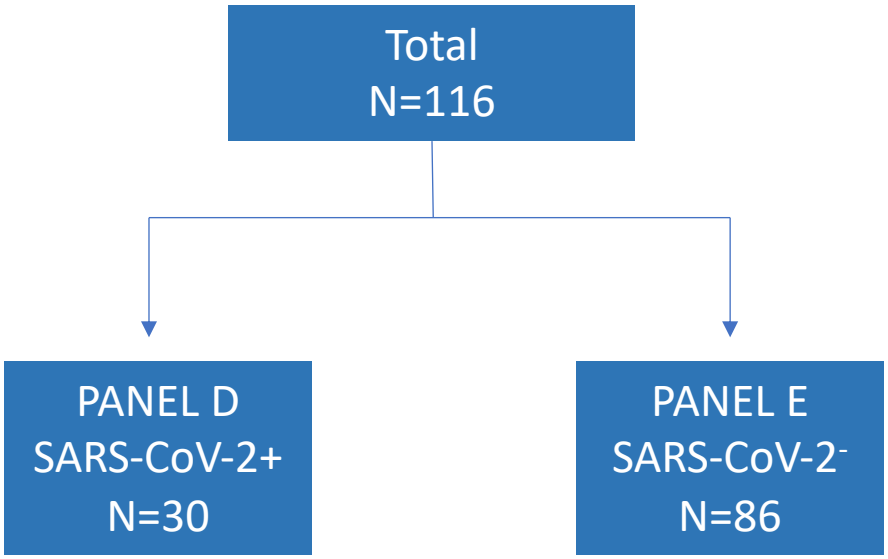
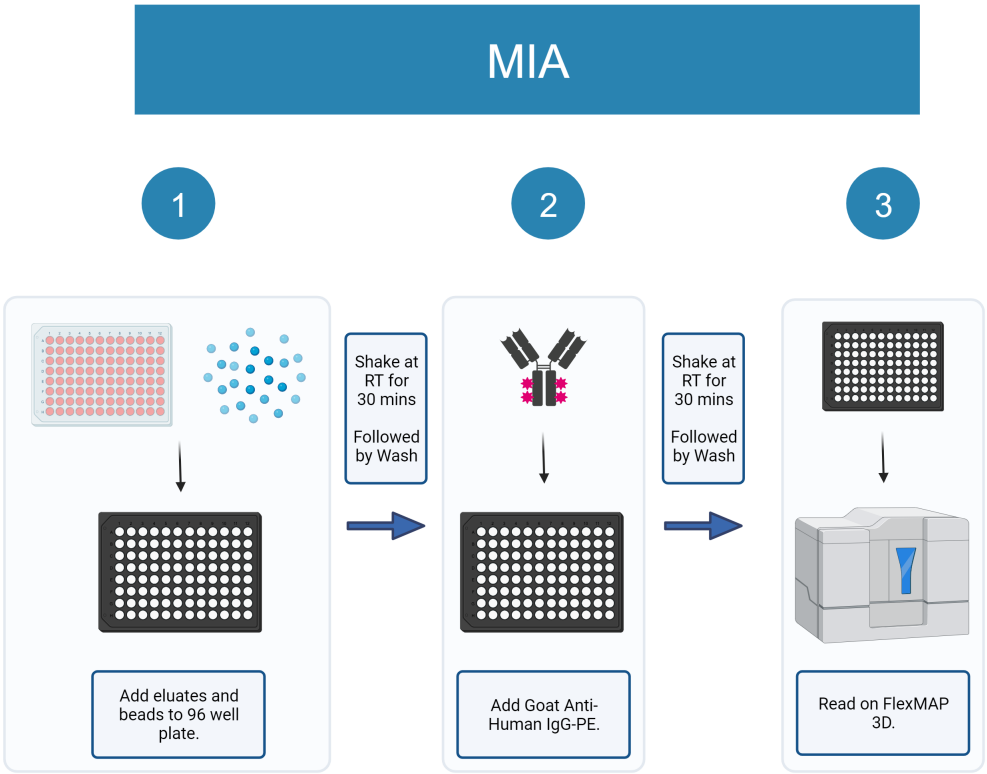

Total
N=116
PANEL D
SARS-CoV-2+
N=30
PANEL E
SARS-CoV-2-
N=86

Figure 2


MIA
1
2
3
Shake at RT for 30 mins
Followed by Wash
Shake at RT for 30 mins
Followed by Wash
Add eluates and beads to 96 well plate.
Add Goat Anti-Human IgG-PE.
Read on FlexMAP 3D.

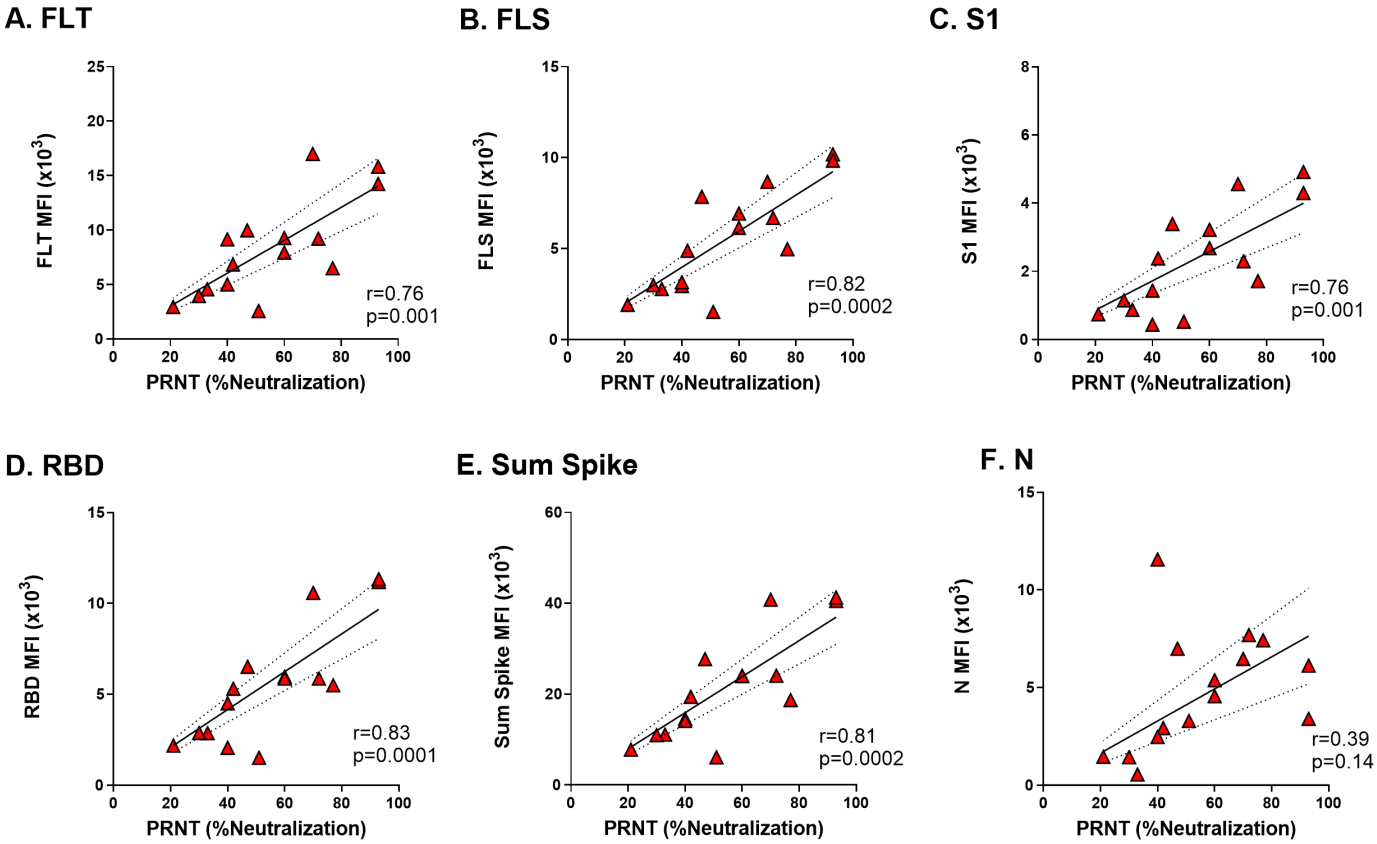
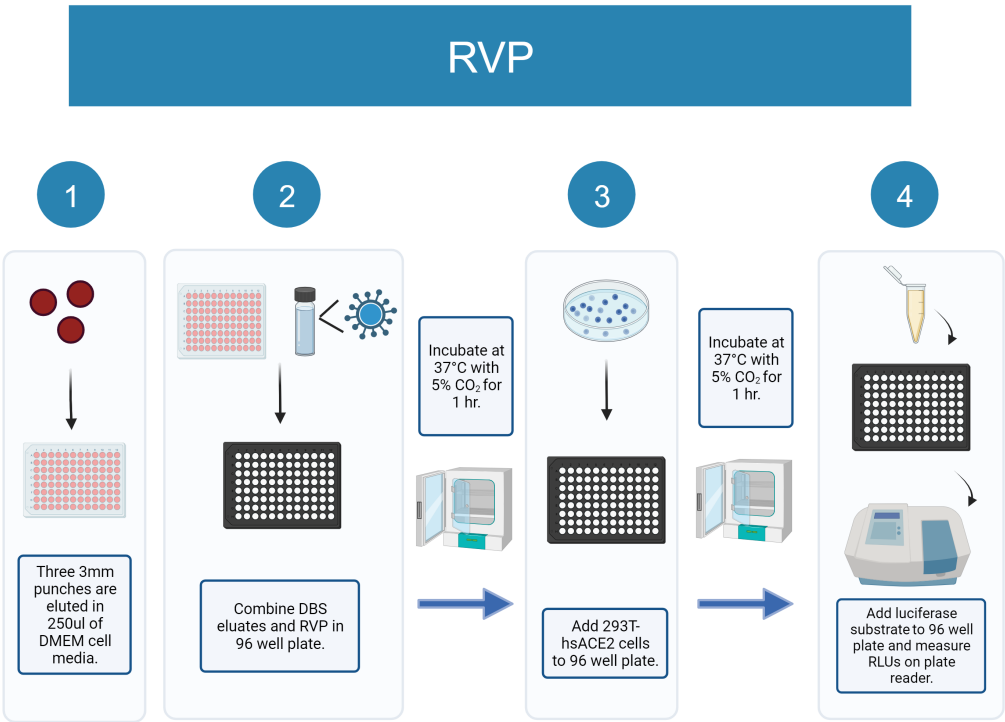
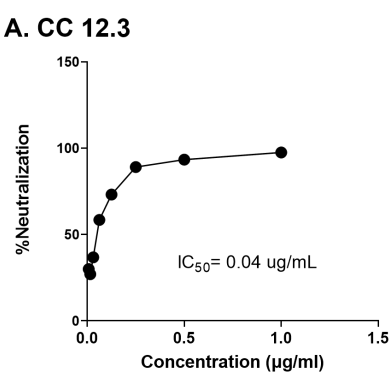
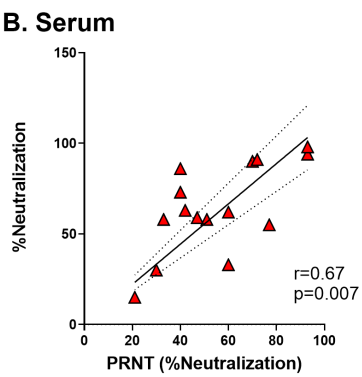
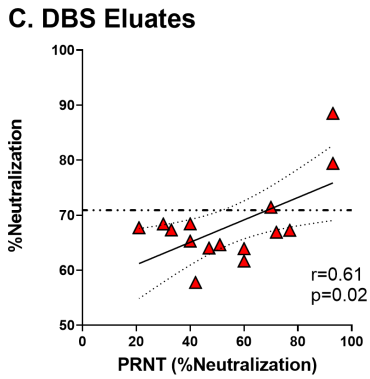

A. FLT
FLT MFI (x10^3)
PRNT (%Neutralization)
r=0.76
p=0.001
B. FLS
FLS MFI (x10^3)
PRNT (%Neutralization)
r=0.82
p=0.0002
C. S1
S1 MFI (x10^3)
PRNT (%Neutralization)
r=0.76
p=0.001
D. RBD
RBD MFI (x10^3)
PRNT (%Neutralization)
r=0.83
p=0.0001
E. Sum Spike
Sum Spike MFI (x10^3)
PRNT (%Neutralization)
r=0.81
p=0.0002
F. N
N MFI (x10^3)
PRNT (%Neutralization)
r=0.39
p=0.14

Figure 3


RVP
1
2
3
4
Three 3mm punches are eluted in 250ul of DMEM cell media.
Combine DBS eluates and RVP in 96 well plate.
Incubate at 37°C with 5% $CO_2$ for 1 hr.
Add 293T-hsACE2 cells to 96 well plate.
Incubate at 37°C with 5% $CO_2$ for 1 hr.
Add luciferase substrate to 96 well plate and measure RLUs on plate reader.


A. CC 12.3
%Neutralization
Concentration (µg/ml)
$IC_{50}$= 0.04 ug/mL
0
50
100
150
0.0
0.5
1.0
1.5


B. Serum
%Neutralization
PRNT (%Neutralization)
r=0.67
p=0.007
0
50
100
150
0
20
40
60
80
100


C. DBS Eluates
%Neutralization
PRNT (%Neutralization)
r=0.61
p=0.02
50
60
70
80
90
100
0
20
40
60
80
100

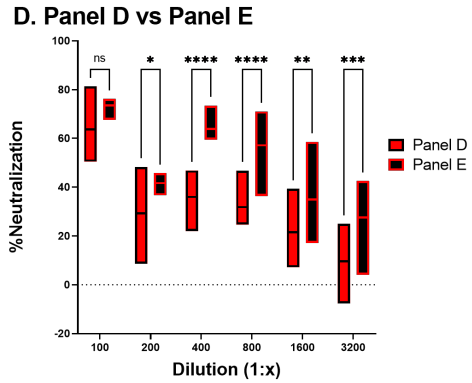
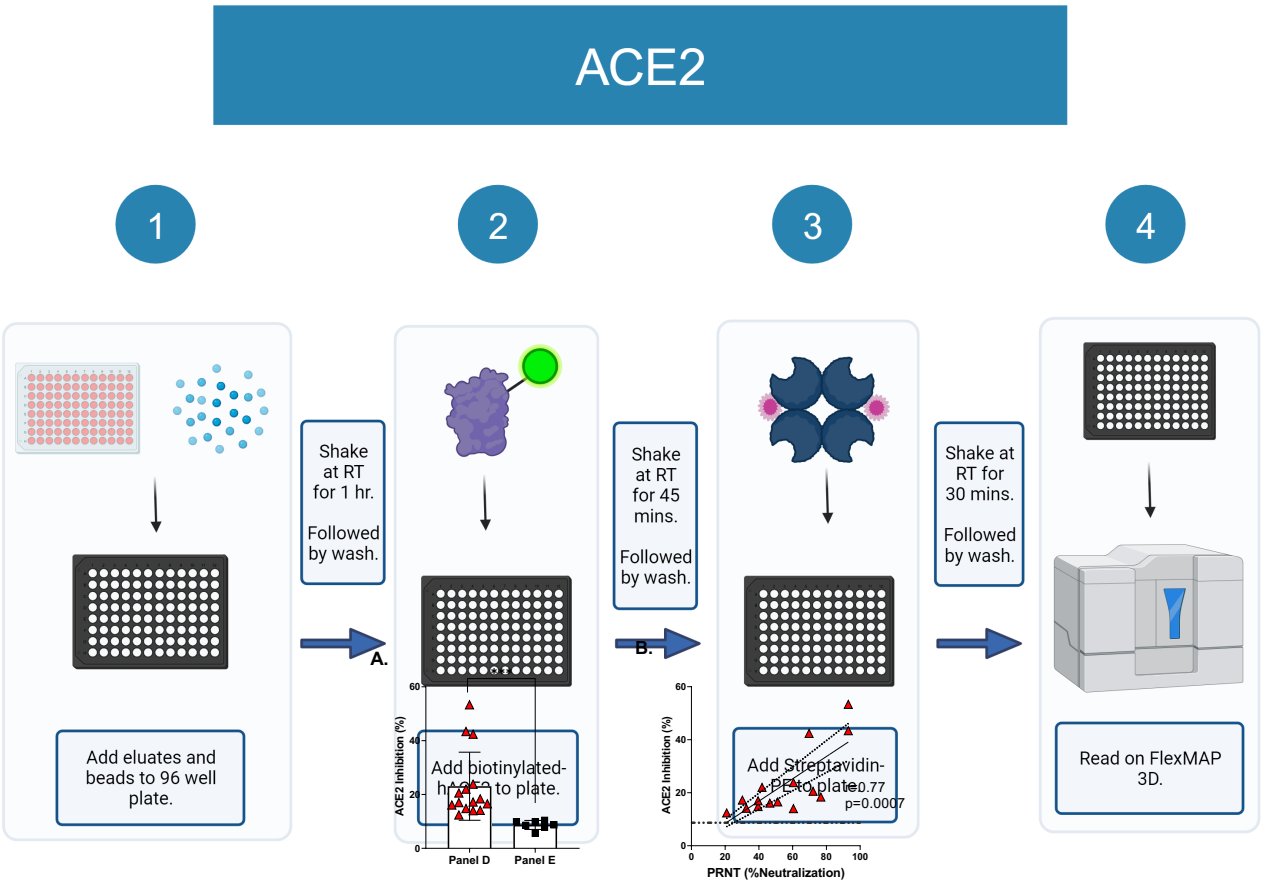

D. Panel D vs Panel E
%Neutralization
Dilution (1:x)
ns
*
****
****
**
***
Panel D
Panel E
-20
0
20
40
60
80
100
100
200
400
800
1600
3200

Figure 4


ACE2
1
2
3
4
Add eluates and beads to 96 well plate.
Shake at RT for 1 hr.
Followed by wash.
Add biotinylated-
to plate.
Shake at RT for 45 mins.
Followed by wash.
Add Streptavidin-
to plate.
Shake at RT for 30 mins.
Followed by wash.
Read on FlexMAP 3D.
A.
B.
ACE2 Inhibition (%)
Panel D
Panel E
PRNT (%Neutralization)
r=0.77
p=0.0007

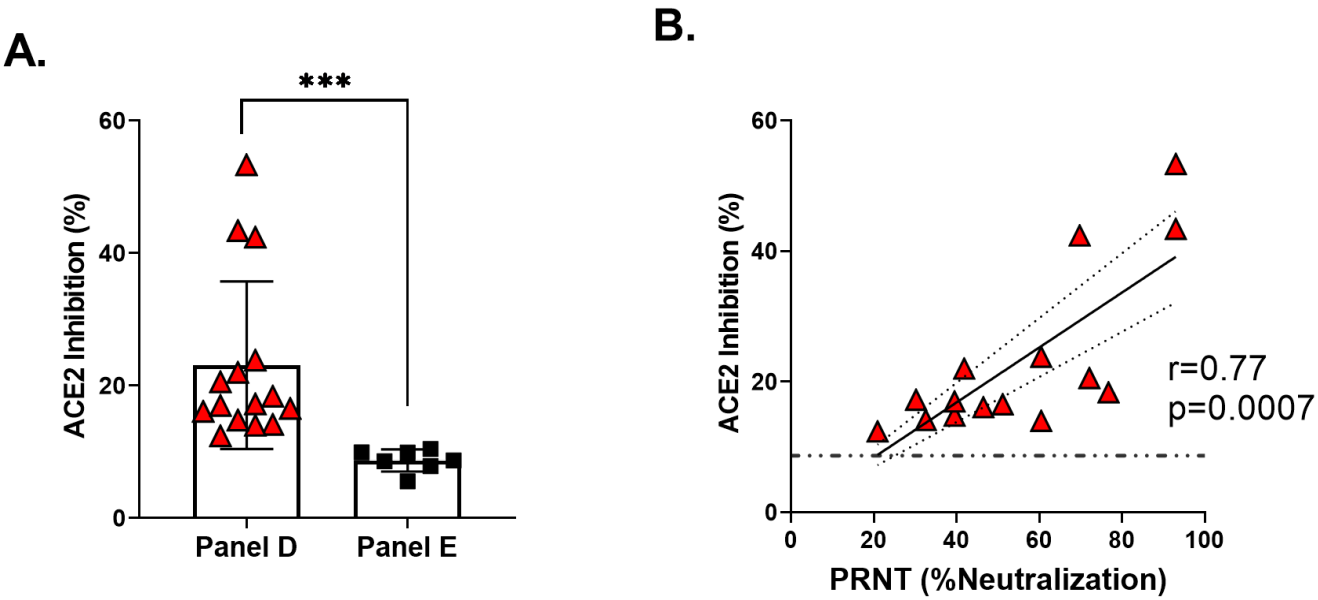
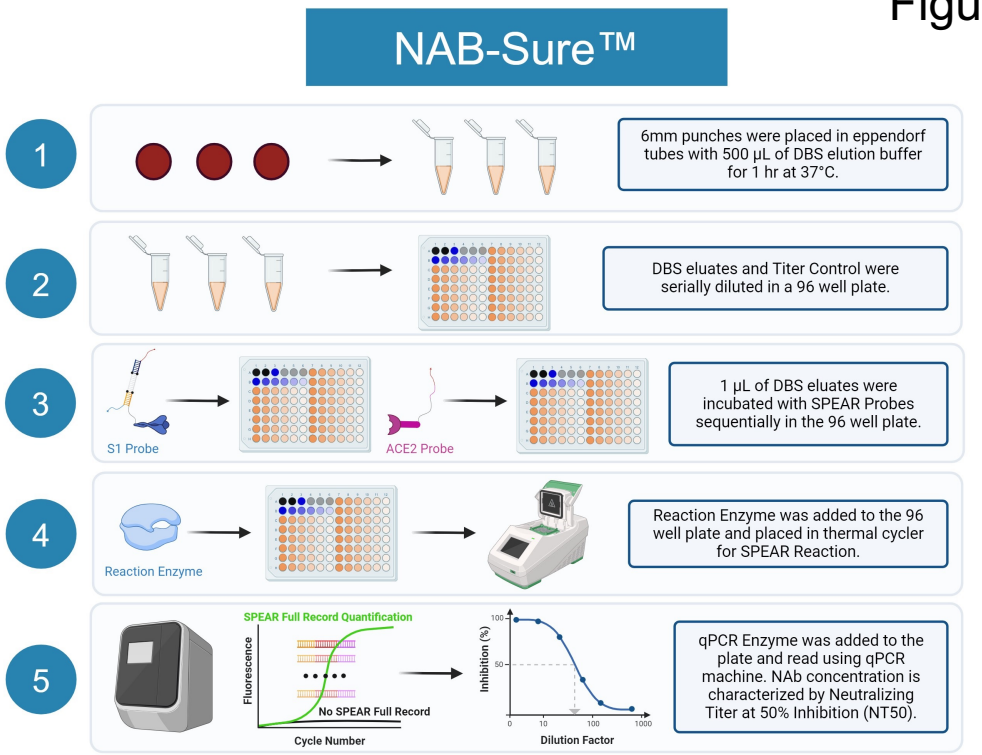

A.
***
ACE2 Inhibition (%)
60
40
20
0
Panel D
Panel E
B.
ACE2 Inhibition (%)
60
40
20
0
0
20
40
60
80
100
PRNT (%Neutralization)
r=0.77
p=0.0007

Figure 5


NAB-Sure™
1
6mm punches were placed in eppendorf tubes with 500 µL of DBS elution buffer for 1 hr at 37°C.
2
DBS eluates and Titer Control were serially diluted in a 96 well plate.
3
S1 Probe
ACE2 Probe
1 µL of DBS eluates were incubated with SPEAR Probes sequentially in the 96 well plate.
4
Reaction Enzyme
Reaction Enzyme was added to the 96 well plate and placed in thermal cycler for SPEAR Reaction.
5
SPEAR Full Record Quantification
Fluorescence
No SPEAR Full Record
Cycle Number
Inhibition (%)
Dilution Factor
qPCR Enzyme was added to the plate and read using qPCR machine. NAb concentration is characterized by Neutralizing Titer at 50% Inhibition (NT50).

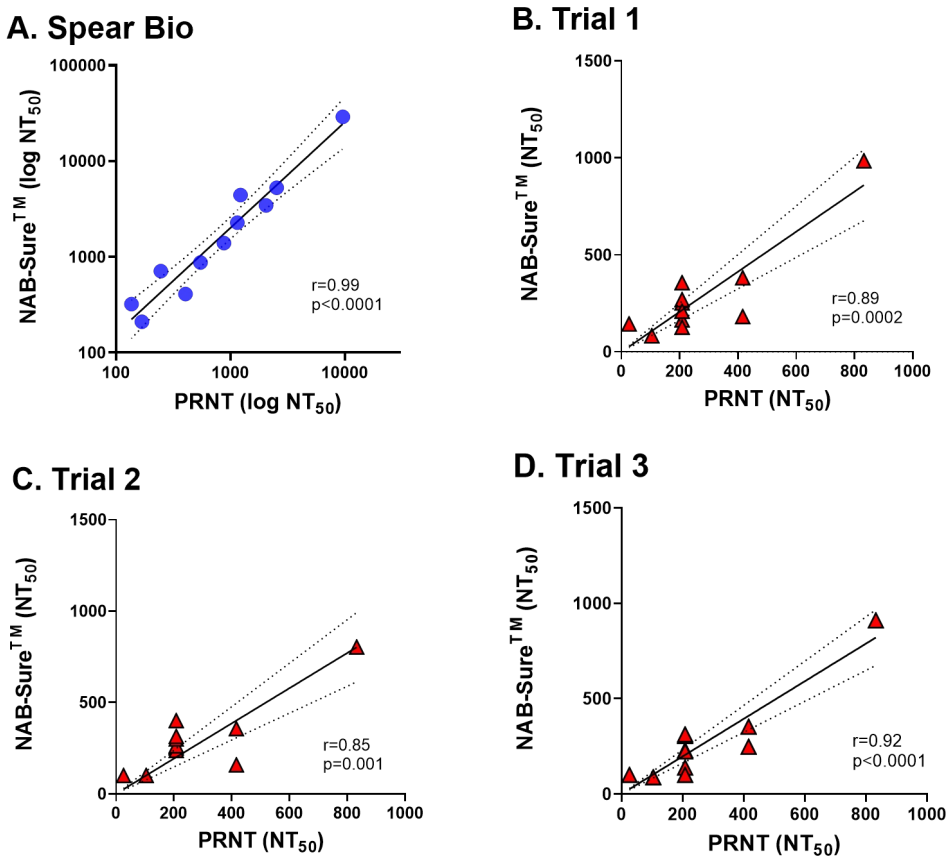

A. Spear Bio
NAB-Sure™ (log NT50)
PRNT (log NT50)
r=0.99
p<0.0001
B. Trial 1
NAB-Sure™ (NT50)
PRNT (NT50)
r=0.89
p=0.0002
C. Trial 2
NAB-Sure™ (NT50)
PRNT (NT50)
r=0.85
p=0.001
D. Trial 3
NAB-Sure™ (NT50)
PRNT (NT50)
r=0.92
p<0.0001

Figure 6

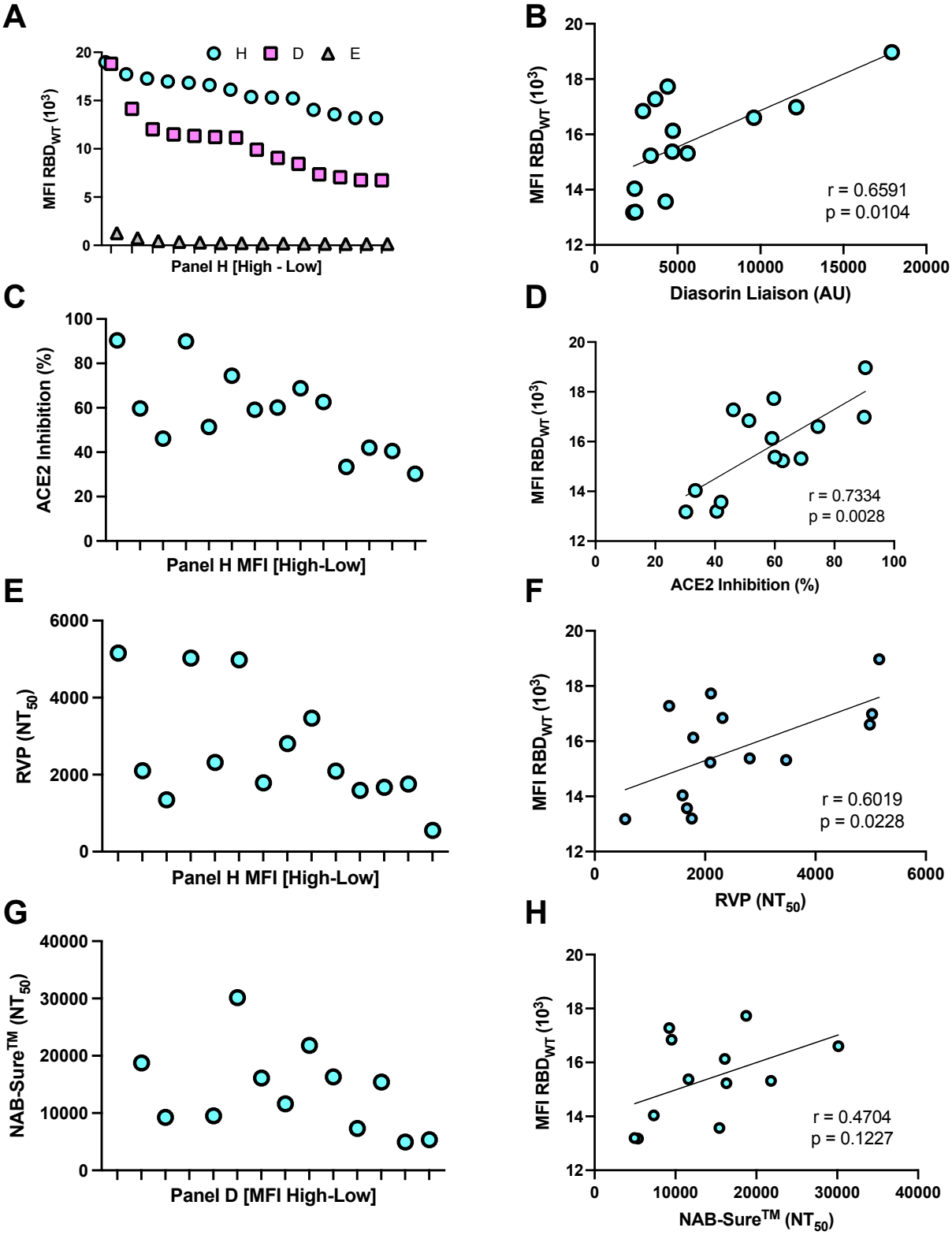

A
H
D
E
MFI RBD$_{WT}$ ($10^3$)
Panel H [High - Low]
B
MFI RBD$_{WT}$ ($10^3$)
r = 0.6591
p = 0.0104
Diasorin Liaison (AU)
C
ACE2 Inhibition (%)
Panel H MFI [High-Low]
D
MFI RBD$_{WT}$ ($10^3$)
r = 0.7334
p = 0.0028
ACE2 Inhibition (%)
E
RVP ($NT_{50}$)
Panel H MFI [High-Low]
F
MFI RBD$_{WT}$ ($10^3$)
r = 0.6019
p = 0.0228
RVP ($NT_{50}$)
G
NAB-Sure$^{TM}$ ($NT_{50}$)
Panel D [MFI High-Low]
H
MFI RBD$_{WT}$ ($10^3$)
r = 0.4704
p = 0.1227
NAB-Sure$^{TM}$ ($NT_{50}$)

Figure S1

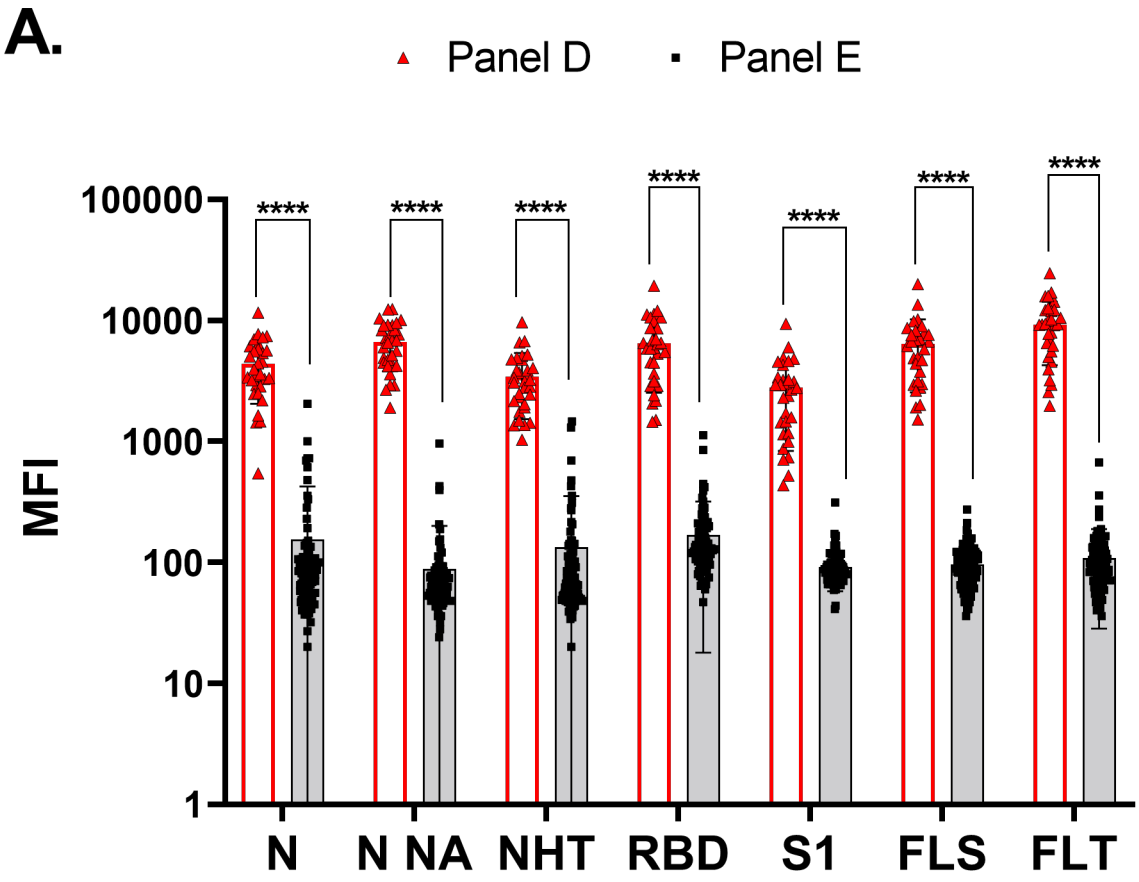
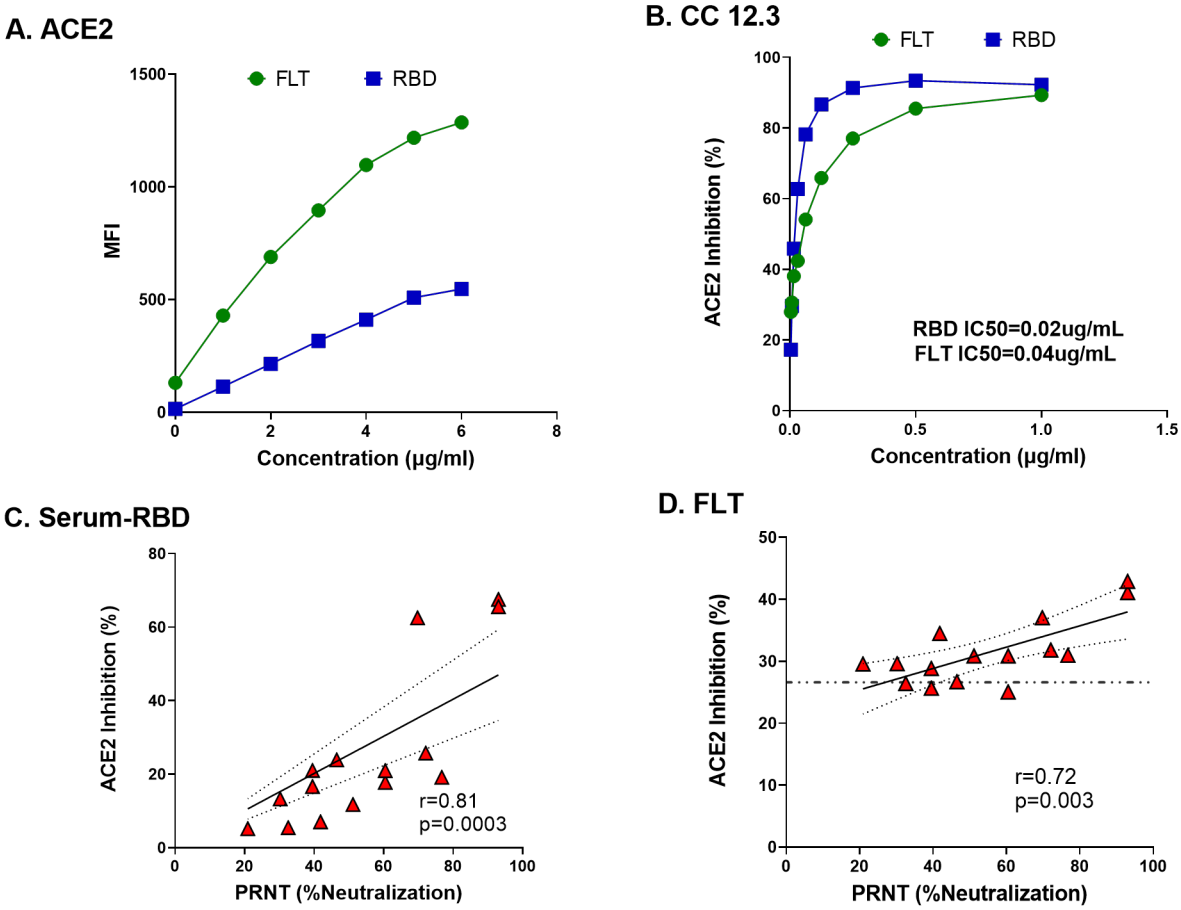

A.
Panel D
Panel E
MFI
100000
10000
1000
100
10
1
****
N
N NA
NHT
RBD
S1
FLS
FLT

Figure S2


A. ACE2
FLT
RBD
MFI
Concentration (µg/ml)
B. CC 12.3
FLT
RBD
ACE2 Inhibition (%)
RBD IC50=0.02ug/mL
FLT IC50=0.04ug/mL
Concentration (µg/ml)
C. Serum-RBD
ACE2 Inhibition (%)
r=0.81
p=0.0003
PRNT (%Neutralization)
D. FLT
ACE2 Inhibition (%)
r=0.72
p=0.003
PRNT (%Neutralization)