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

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Running title: Neutralizing antibodies in dried blood spots

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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, an in-house Luminex-based RBD-ACE2 inhibition assay and a commercial real-time PCR-based inhibition assay (NAB-Sure™).

Results: DBS eluates from convalescent individuals were compatible with the spike-ACE2 inhibition assays, but not cell-based pseudovirus assays or PRNT. However, 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°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

binding assay on the Mesoscale Diagnostics (MSD) platform to estimate neutralizing titers in contrived DBS (cDBS) eluates (17) and in self-collected DBS from a community-based serology survey (18). However, neither study benchmarked their results against plaque reduction neutralization titer (PRNT) values, nor did they perform cross comparisons of the different *in vitro* assays (e.g., pseudovirus versus ACE2-RBD).

Therefore, to further advance the utility and validity of DBS for the purpose of functional SARS-COV-2 serology, we investigated the compatibility of contrived DBS eluates with different SARS-COV-2 neutralization assays and compared their outputs to PRNT values derived from matched serum. While DBS eluates proved incompatible with cell-based SARS-COV-2 pseudovirus neutralization assays, we found that two different ACE2 inhibition assays, an in-house Luminex-based assay, and a commercial real-time PCR-based assay (NAB-Sure™), yielded neutralization values that strongly correlated with PRNT. An 8-plex MIA based solely on binding antibodies to spike antigens also served as a proxy for PRNT. As the two RBD-ACE2 inhibition assays employed in this study are amenable to high-throughput testing, we propose to integrate one or both into our DBS workflow for analysis of cohort specific studies going forward.

2. METHODS

2.1 SARS-CoV-2 reagents. The source and descriptions of SARS-CoV-2 reagents used in this study is provided in **Table 1**.

2.2 Human serum samples. A set of serum samples from 30 PCR positive donors was purchased from Access Biologicals, LLC, (Vista, CA), herein referred to as “Panel D”. Positive test results were reported in the initial U.S. wave of infections between 3/15/2020 and 4/25/2020; and samples were drawn between 6/3/2020-6/4/2020, indicating that all donors were infected with the Washington variant of SARS-CoV-2. The panel consists of 21 female and 9 male donors; ages ranged from 25- to 66-years-old; all donors were symptomatic. A set of 87 COVID-19 negative serum samples collected between 3/24/2017 and 11/9/2018 was also purchased from Access 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). A third set of samples from 30 COVID-19 vaccinated patients was
123 purchased from Access Biologicals. 15 of these samples were selected for evaluation
124 in this study. Participants were vaccinated with the Moderna COVID-19 vaccine
125 between December 2020 and March 2021. Samples were collected 12-17 days after
126 their second dose. This set is referred to as 'Panel H'. Upon receipt, Panels D, E and
127 H serum samples were stored at -80ºC.
128 **2.3 Contrived DBS.** Contrived DBS were created by mixing an equal volume of Panel
129 D, E or H serum with washed human blood cells (BC). Fifty microliters (50 μL) of the
130 BC-serum mixture were spotted onto Whatman 903 filter paper (SigmaAldrich, St.
131 Louis, MO) and allowed to air dry at room temperature for 4 h before being packaged in
132 semi-permeable bags with desiccant and stored at -20ºC. Blood cells (BC) were
133 collected by centrifugation (2200 RCF for 8 min) from commercially obtained fresh
134 human whole blood [Type O EDTA] (ZenBio, Durham, NC).
135
136 **2.4 Plaque reduction neutralization titer (PRNT).** PRNT was determined as described
137 (19, 20). Briefly, 2-fold serially diluted serum (100 µl) was mixed with an equal volume of
138 150-200 plaque forming units (PFUs) of SARS-CoV-2 (isolate USA-WA1/2020, BEI
139 Resources, NR-52281) and incubated for 1 h at 37°C, 5% CO_2. The virus:serum mixture
140 (100 µl) was applied to VeroE6 cells grown to 95%–100% confluency in 6 well plates.
141 Adsorption of the virus:serum mixture was allowed to proceed for 1 h at 37°C, 5% CO_2.
142 Following the adsorption period, the wells were overlaid with 0.6% agar prepared in cell
143 culture medium (Eagle's Minimal Essential Medium, 2% heat inactivated FBS,
144 100 µg/ml penicillin G, 100 U/ml streptomycin). Two days post-infection, a second agar
145 overlay containing 0.2% neutral red prepared in cell culture medium was applied.
146 Plaque determinations were conducted 1-2 days later. Neutralizing titers were defined
147 as the inverse of the highest dilution of serum providing 50% (PRNT50) or 90%
148 (PRNT90) viral plaque reduction relative to a virus only control.
149
150 **2.5 Microsphere Immunoassay (MIA)**. The development and validation of an 8-plex
151 MIA consisting of three different nucleocapsid N antigens, RBD, Spike S1 (S1), full

152 length Spike (FLS), and full length trimer (FLT) is described elsewhere (J. Rock,
153 L.Styer, *manuscript in preparation*). Magplex-C microspheres (Luminex Corp) were
154 coupled with 7 different SARS-CoV-2 antigens and 1 internal control antibody. Each
155 antigen/antibody was coupled to a different bead region allowing the beads to be
156 multiplexed in the same well. A 3.3 mm DBS punch from each sample was combined
157 with 250 µl elution buffer (Tris-buffered saline, 1% casein blocker) (Bio-Rad
158 Laboratories) in a 96 well plate. The DBS punches were eluted for 1 hour at room
159 temperature. Antigen-coated beads (diluted to 1,250 beads/bead set/well with assay
160 buffer (PBS, 2% BSA, pH 7.4)) and 25 µl DBS eluate were combined in a 384 well plate
161 and incubated at 37°C for 30 minutes while shaking (300 RPM) in the dark. Plates were
162 washed for two cycles using the BioTek 405TS microplate washer and 50 µl wash buffer
163 (PBS, 2% BSA, 0.02% Tween-20, 0.05% sodium azide, pH 7.4). 50 µl phycoerythrin-
164 tagged goat anti-human IgG was added to each well. Plates were incubated at 37°C for
165 30 minutes while shaking (300 RPM) in the dark and washed as described above.
166 Antibody-bead complexes were resuspended with 90 µl xMap sheath fluid (Luminex),
167 plates were incubated for 1 minute at room temperature while shaking (300 RPM), and
168 70 µl from each well was read on a Luminex FlexMAP 3D instrument. Results were
169 presented in median fluorescence intensity (MFI) for each well. Positive, negative, and
170 background controls were included with every plate.
171
172 **2.6 Reporter virus particle (RVP) neutralization assays.** Pseudo-typed lentiviral
173 reporter virus particles (RVP) tagged with Renilla luciferase (Luc) and 293T-hsACE2
174 cells were obtained from Integral Molecular, Inc. (Philadelphia, PA). To determine the
175 SARS-CoV-2 neutralizing activity in human serum and paired cDBS eluates, serum and
176 DBS eluates were diluted 1:50 in DMEM containing 10mM HEPES [pH 7.4], 10% fetal
177 bovine serum and 100 U/mL penicillin and 0.1 mg/mL streptomycin (Sigma-Aldrich).
178 Paired sample types were serially diluted in duplicate in cell medium. RVPs were
179 thawed at 37°C and immediately diluted per the manufacturer's specification in cell
180 medium and then 50 µl was added to the assay plate. A total of 50 µl of diluted human
181 serum or DBS eluate were then transferred from the dilution plate and mixed with RVP
182 solution. Serum and DBS eluates were incubated with RVPs for 1 hour at 37°C with 5%

183 CO_2. Positive and negative control wells were included on each assay plate and
184 consisted of RVPs only (100% infectivity control), and no RVPs (0% infectivity control).
185 During RVP-serum incubation 80-90% confluent 293T-hsACE2 cells were harvested
186 and cell density was determined using a hemocytometer. Cells were diluted to a density
187 of 2 x 10^5 cells/mL in cell culture media and 100 µl was added to each well of the RVP +
188 specimen plate and mixed with gentle pipetting. Plates were incubated at 37°C with 5%
189 CO_2 for 72 h. Quantification of SARS-CoV-2 RVP infection was determined using the
190 *Renilla*-Glo® luciferase assay system (Promega Corp., Madison, WI). Microtiter assay
191 plates were centrifuged for 5 min at 2000 rpm to prevent cell loss, then supernatant was
192 removed with a pipet and 30 µl of PBS was added to each well. *Renilla*-Glo® substrate
193 diluted 1:100 (v/v) into buffer was prepared fresh; 30 µl added per well and plates were
194 then incubated for 10 min at room temperature. Relative light units (RLU) were detected
195 on a SpectroMax iD3 (Molecular Devices, LLC, San Jose, CA) and analyzed with
196 SoftMax Pro 7.0 software. A Z' value was calculated for each plate for assay accuracy
197 and comparability, plates with a Z'<0.5 were repeated. The RLU mean and standard
198 deviation (SD) of 100% infected wells was determined, and a minimum limit of detection
199 was calculated for each plate as = $(\text{Mean} - (3\text{xSD}))_{100\%\text{Infected}}$. Samples that failed to
200 reach the LOD cut-off value at the most concentrated dilution were classified as non-
201 neutralizing (<LOD) and assigned a value of 0 for statistical analysis purposes. For each
202 plate the RLU mean value for 0% infected wells was subtracted from the mean of 100%
203 infected wells resulting in a normalized infection value (NIV); % normalized RVP
204 neutralization was calculated as = $100-((S_{AVE}/\text{NIV})*100)$, where S_{AVE}= the average of
205 duplicate sample RLU values.
206
207 **2.7 Human ACE2 inhibition (iACE2) assay.** Recombinant soluble human ACE2 was
208 generated by the Wadsworth Center's Protein Expression core facility. The coding
209 region for human ACE2 (residues 1-615; Genbank #) was cloned into pcDNA3-
210 sACE2(WT)-8his [(https://www.addgene.org/browse/sequence/298420/] and transfected
211 into ExpiCHO-S cells using ExpiFectamine, as per manufacturer's instructions
212 (ThermoFisher (https://www.thermofisher.com/order/catalog/product/A29133). The
213 recombinant protein was affinity purified on 2 x 1ml HisTrap™ Excel (Cytiva) columns

214 connected in series. ACE2 was eluted with a linear gradient of 20 mM Tris-HCl, 500 mM
215 NaCl, 500 mM imidazole pH 8.0 over 20 column volumes. Fractions containing ACE2,
216 as assessed by SDS-PAGE, were pooled and dialyzed twice against PBS [pH 7.4].
217 Protein concentration assessed by Bradford assay with bovine albumin as a standard
218 curve. Biotinylated using EZ-Link NHS Biotin (Thermo Fisher Scientific).
219 Magplex-C microspheres (Luminex) from separate bead regions were coupled
220 with RBD and FLT from SARS-CoV-2 isolate USA-WA1/2020 ("wild type") and
221 resuspended to 2500 beads/region/well. Diluted beads were transferred to wells of a
222 non-binding 96 well plate; 50 µl of serum or DBS eluate (1:138) was then added to each
223 well and incubated for 1 h RT with agitation in the dark. Plates were washed twice
224 manually with wash buffer (190 µl) and then biotinylated-hACE2 (see below; 4 µg/mL)
225 was added to each well, plates were incubated for an additional 45 min at RT, shaking
226 in the dark. Plates were washed, probed with streptavidin-PE (1 µg/mL) and incubated
227 at RT for 30 min. The plates were washed to removed unbound streptavidin-PE then
228 analyzed using FlexMap3D. The MFI for ACE2 binding (in the absence of antibody) was
229 derived from a single column of wells containing diluted beads. Inhibition of ACE2
230 (iACE2) binding calculated as: 190 µl % ACE2 binding-inhibition (iACE2), per sample
231 well, where % iACE2 = 100-[Raw value/(Average of wells with specific VoC beads and
232 no serum or DBS eluate) x 100].
233
234 **2.8 NAB-Sure™**
235 The NAB-Sure™ SARS-CoV-2 Neutralizing Antibody Test (Spear Bio, MA, USA) was
236 performed according to the manufacturer's instructions (www.spear.bio/nabsure). NAB-
237 Sure™ is a competitive binding assay that measures the ability of antibodies in DBS
238 samples to block SARS-CoV-2 Spike Protein (S1) from binding to recombinant hACE2
239 in solution. The assay was validated at the Wadsworth Center using eluates (n=11) from
240 SARS-CoV-2 infected individuals with range of Spike-specific titers provided by
241 SpearBio. Contrived Panel D and H DBS punches (6mm) were incubated in NAB-
242 Sure™ elution buffer at 37°C for 1 h. Afterwards, eluates (15µL) were processed
243 according to the manufacturer's instructions. The test was read using a 7500 Fast Real-
244 Time PCR System (Thermo Fisher Scientific) in the Wadsworth Center's Bloodborne

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

275 We also generated cDBS samples from a commercially available panel of serum
276 samples from a healthy cohort (**Panel E)** as well as a fully vaccinated cohort (**Panel H**).
277 . As detailed in the Materials and Methods, serum was combined with an equal volume
278 of human RBCs then spotted (50 μl) onto Whatman 903 filter paper. The spots were
279 allowed to dry at room temperature for 4 h then stored with desiccant at -20ºC.
280 Recovery of 90% (±0.04%) total IgG was achieved from a single 3 mm punch (1/8'') by
281 incubating the punch in 250 μl in TBS with 1% casein for 1 h at room temperature
282 (**Figure 1**). The cDBS eluates were prepared fresh for each assay described below.
283
284 **3.2 Assessment of SARS-CoV-2 S- and N-specific IgG in DBS eluates by MIA.**
285 Using a recently optimized 8-plex MIA targeting different nucleocapsid (N) and Spike
286 antigens, we examined the relationship between MFIs derived from cDBS eluates and
287 PRNT values. Specifically, the 8-plex MIA includes full length Trimer (FLT), full length
288 spike (FLS), S1, RBD and three N antigens as shown in **Table 1**. As expected, Panel D
289 cDBS eluates had higher median fluorescent intensity (MFI) than Panel E cDBS eluates
290 for all seven SARS-CoV-2 antigens (**Figure S1**). In terms of correlation with PRNT (%
291 neutralization @ 1:100 dilution), the values ranged from 0.76 for FLT (p=0.001) and S1
292 (p=0.001) to 0.82-0.83 for FLS (p =0.0002) and RBD (p=0.0001), respectively (**Figure**
293 **2**). Summing the MFIs for all four spike antigens ("sum Spike") did not greatly increase r
294 values (r = 0.81) (**Figure 2E**). As expected, the correlation between N MFI and PRNT
295 was not significant (r =0.39; p>0.1; **Figure 2F**). We conclude that MFI derived from DBS
296 eluates to a given SARS-CoV-2 spike antigen correlates with virus neutralizing activity
297 as defined by PRNT.
298
299 **3.3. Pseudovirus neutralization activity in cDBS eluates.** Non-replicating, lentiviral-
300 based pseudoviruses expressing SARS-CoV-2 trimeric spike proteins are employed
301 widely to assess SARS and SARS-CoV-2 neutralizing antibody titers in plasma and
302 serum (21-24). However, it is unclear whether DBS eluates are amenable with these
303 cell-based assays. To investigate this, we employed a commercially available non-
304 replicating Reporter Virus Particles (RVP) expressing the SARS-CoV-2 spike protein
305 (D648G) and encoding *Renilla* luciferase (**Figure 3**) (25). We confirmed RVP efficacy

306 using the human monoclonal antibody CC12.3, which neutralizes SARS-CoV-2 with a
307 reported IC_{50} of 0.026 μg/ml (24). In our hands, CC12.3 had an IC_{50} of 0.04 μg/ml
308 (**Figure 3A**). Moreover, the 15 Panel D serum samples had neutralization activities that
309 ranged from 15% to 98% with high concordance with PRNT (r=0.67; p=0.007) (**Figure**
310 **3B**). Thus, RVP serves as reliable measure of SARS-CoV-2 activity in the context of
311 serum.
312 To determine if the RVP assay is compatible with cDBS, we collected eluates
313 from three 1/8" (3 mm) punches from each of the 15 cDBS Panel D spots and 7 Panel E
314 spots as negative controls. Eluates were generated by incubating the punches in
315 DMEM-HEPES at room temperature for 2 h with agitation. Eluates were then diluted
316 1:50 into DMEM-HEPES medium and then mixed with RVPs prior to application to cells
317 293T cells constitutively expressing human ACE2 (see **Materials and Methods**).
318 Panel D eluates had 60-90% neutralization activity [average 68%] that were
319 modestly correlated with PRNT (r value of 0.61; p=0.02) (**Figure 3C**). However, the
320 results were confounded because Panel E eluates (negative controls) had
321 "neutralization" (interfering) activity that was essentially equivalent to Panel D (**Figure**
322 **3D**). Thus, RVP assay was not able to distinguish between Panel D and Panel E
323 eluates. Efforts to improve the sensitivity of the assay through dilution (>1:3200),
324 alternative elution buffers, and removal of hemoglobin using reagents designed for this
325 purpose (e.g., HemogloBind™) were unsuccessful (**Figure 3D**; **data not shown**). We
326 conclude that cDBS eluates from convalescent (unvaccinated) individuals have
327 insufficient virus neutralizing titers to distinguish them from unvaccinated controls in the
328 pseudovirus (RVP) platform.
329
330 **3.4 Relationship between PRNT and iACE2.** We next tested cDBS eluates for the
331 ability to inhibit ACE2-RBD interactions through use of a Luminex-based assay (iACE2).
332 In the assay, RBD- and FLT-coupled microspheres were incubated with soluble
333 biotinylated-ACE2 in the absence or presence of competitor antibodies (e.g.,
334 monoclonal, antisera or DBS eluates), then probed with streptavidin-PE and analyzed
335 by using FlexMap3D (Luminex). A reduction in MFI was interpreted as antibody-
336 mediated inhibition of ACE2 binding to RBD- and FLT. The utility of the in-house iACE2

337 assay was confirmed using the well characterized monoclonal antibody CC12.3, which
338 neutralizes SARS-CoV-2 by interfering with ACE2-RBD interactions (24). In the iACE2
339 assay, CC12.3 had an IC_{50} value for WT FLT and RBD of 0.04 ug/mL and 0.02 ug/mL,
340 respectively (**Figure S2**). These values are consistent with those reported by Rogers
341 and colleagues (24).
342 We examined Panel D DBS eluates in the iACE2 assay, with the expectation that
343 Panel D eluates would have greater inhibition activity than Panel E. Indeed, Panel D
344 had significantly higher iACE2 values (23% average; range 10%-50%) as compared to
345 Panel E (9% average; range 0%-15%) (**Figure 4A**). In terms of the correlation with
346 PRNT, Panel D iACE2 values had an r-value of 0.77 ($p<0.001$) for RBD-coated beads
347 and 0.72 ($p=0.003$) for FLT-coated beads. iACE2 (%) values for FLT-coated beads
348 ranged from 25% to 40% (avg 31%) (**Figure 4; Figure S2**). Panel D DBS eluates
349 performed slightly less well than the matched serum samples from which they were
350 derived, at the same dilution, indicating that a degree of ACE2-RBD inhibitory activity is
351 lost during the collection of cDBS eluates. Panel D serum samples (1:100 dilution) had
352 iACE2 activity that correlated with PRNT with $r= 0.81$ ($p<0.001$; **Figure S2**). These
353 results demonstrate that iACE2 assay affords a reliable estimate of SARS-CoV-2
354 neutralization potential using cDBS eluates.
355
356 **3.5. NAB-Sure™.** NAB-Sure™ is a commercially available cell-free assay based on
357 successive proximity extension amplification reaction (SPEAR) technology with qPCR
358 as a readout. Disruption of a proximity extension amplification reaction primed by
359 recombinant SARS-CoV-2 S1 protein and human ACE2 by neutralizing antibodies in
360 DBS eluates results in increased CT values. To investigate this technology, we first
361 evaluated the NAB-Sure™ assay using a set of 11 previously authenticated DBS
362 eluates provided by the manufacture and which were subjected to five 3-fold serial
363 dilutions (1 to 243) and tested in duplicate using in-house instrumentation. The NAB-
364 Sure™ assay revealed NT_{50} values that ranged from 210 to 9500 (avg. 2333) with 17.7
365 %CV (**Figure 5A**). There was a significant correlation between NT_{50} values from DBS
366 eluates tested by NAB-Sure™ and paired serum samples tested by PRNT [Pearson's r

367 = 0.99; p<0.0001] (**Figure 5A**). Thus, NAB-Sure™ was compatible with DBS eluates
368 and had a high concordance with PRNT.
369 We then used the NAB-Sure™ assay to evaluate cDBS eluates from Panel D
370 (#1-14) and E (#1-7). The samples were tested 3 times in independent reactions under
371 identical assay conditions. Concordance across the different reactions was high, as
372 evidenced by r values between 0.85-0.92 **(Figure 5B-D)**. The average %CV across
373 samples was 20.3%. All reactions reached significance when compared to PRNT. From
374 these results, we conclude that NAB-Sure™ is sufficiently sensitive to detect SARS-
375 CoV-2 neutralizing titers in DBS eluates, based on high degree of concordance with
376 PRNT.
377
378 **3.6 Assessing SARS-CoV-2 neutralizing activity in vaccinated individuals.** Studies
379 prior to this point relied on DBS eluates derived from SARS-CoV-2 infected individuals
380 with relatively low overall spike-specific antibody titers as well as SARS-CoV-2
381 neutralizing levels. With an estimated >90% of the United States population seropositive
382 for SARS-CoV-2 due to vaccination and/or natural infection at this point in time (13), we
383 wished to revisit the utility of DBS eluates in assessing virus-specific antibody titers and
384 neutralizing activity in a vaccinated cohort. For this purpose, we generated cDBS from a
385 commercially available panel (Panel H) of serum samples collected from individuals
386 who had received two doses of the original Moderna (mRNA 1273) vaccine. As shown
387 in **Figure 6**, Panel H DBS eluates (n=15; 15746 ± 1790 MFI) had RBD-specific antibody
388 levels (MFI) what were 1.5 times higher than those in Panel D (10392 ± 3325 MFI) and
389 44 times higher than Panel E (351 ± 313 MFI). Due to the extremely high MFIs
390 associated with Panel H samples, they were diluted 1:10 for all subsequent assays
391 relative to panels D and E.
392 Panel H DBS eluates were evaluated in the iACE2 assay (**Figure 6C, D**), RVP
393 (pseudovirus) neutralization assay (**Figure 6E, F**) and NAB-Sure™ (**Figure 6G, H**). The
394 results demonstrated that all three platforms (iACE2, RVP, NAB-Sure™) were
395 compatible with DBS eluates as evidenced by ACE2 inhibition values approaching
396 100% (**Figure 6C),** RVP NT_{50} values > 5000 (**Figure 6E**), and NAB-Sure™ NT_{50} values
397 approaching 18,000 (**Figure 6G**). There were statistically significant correlations

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

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

460 run on the Luminex platform using the same magnetic microspheres as the MIA. The
461 overall costs of the iACE2 assay are at least an order or magnitude less expensive than
462 cell-based pseudovirus assay with commercial RVPs. Moreover, the in-house iACE2
463 assay is easily multiplexed to include microspheres coated with RBD from circulating
464 VoCs and Omicron subvariants side-by-side with ancestral RBD. Indeed, we have
465 applied a 4-plex RBD assay to several thousand primary DBS samples collected from
466 an elderly cohort (G. Mirabelle, K. Berman, N. Mantis, unpublished results).

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

485 The use of DBS for infectious disease serology and functional antibody testing is
486 not without intrinsic challenges, including variability in blood collection by fingerpick,
487 uniformity of antibody recovery in eluates, and limited sample volumes (11).
488 Nonetheless, the benefits of DBS as a biospecimen type for surveillance of hard-to-
489 reach communities are well recognized. For example, in work spearheaded by the CDC,
490 DBS from ~1,000 children in Bangladesh were sampled for neutralizing antibodies to

polio types 1, 2, and 3 using a modified poliovirus microneutralization assay (26). In the case of polio, a neutralizing antibody titer of 1:8 correlated with protection and was used as the threshold definition of seropositivity. Similarly, in a study examining natural versus vaccine-induced antibody responses to human papillomavirus (HPV), neutralizing activity was detected in 2 mm DBS punches eluted into 0.2 mL saline (27). Another consideration is that for SARS-CoV-2 at least there is a strong congruence between oral fluids and DBS (K. DeRosa, N. Pisanic, K. Kruczynski, L. Styer, M. Parker, C. Heaney, and N. Mantis, *manuscript in preparation*). Thus, DBS and oral fluids might potentially be used interchangeably as means to survey large cohorts for immunity and vaccine responsiveness (32).

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CRediT

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								
JMR			x		x	x					x	x	x	x
RB			x		x	x					x	x	x	x
AKD		x	x								x		x	x
KLD		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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691
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693

694 **FIGURE LEGENDS**

695 **Figure 1. Generation of contrived DBS eluates.** (**Top**) Schematic showing generation
696 of contrived DBS (cDBS), DBS punches, and eluate collection. (**Bottom**) Summary of
697 Panels D, E, and H samples used in this study. RT = room temperature, TBS = tris
698 buffered saline

699

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

707

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

717

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

724 via PRNT. cDBS eluates were evaluated in triplicate. The Pearson r and p-value are
725 shown as a panel inset for (B). The dotted line on (B) represents the average iACE2
726 activity for SARS-CoV-2 negative cohort (Panel E; n=7).
727
728 **Figure 5. NAB-Sure exhibits high concordance with PRNT.** (**Top**) Workflow for the
729 NAB-Sure™ assay. (**Panel A**) Pearson correlation of NAB-Sure ™ NT_{50} from authentic
730 DBS sample eluates compared to PRNT paired serum. DBS samples were evaluated in
731 duplicate. (**Panels B-D**) Pearson correlation comparing NAB-Sure ™ NT_{50} for cDBS
732 eluates and paired serum NT_{50} from PRNT for 14 Panel D samples perfomed in
733 triplicate with each replicate shown. For A-D, Pearson r and p values are in panel
734 insets.
735
736 **Figure 6. Assessment of SAR-CoV-2 neutralizing titers in cDBS eluates from**
737 **vaccinated individuals (Panel H). (A)** Panel H, D and E cDBS eluates were rank
738 ordered from high to low (left to right) based on RBD_{WT} MFI determined in the 8-plex
739 MIA. (**B**) Correlation plot between Panel H RBD_{WT} MFI and Diasorin Liaison values
740 provided from Access Biologics LLC. Panel H cDBS eluate activities in (C) Luminex-
741 based iACE2 assay, (E) RVP neutralization assay and (G) NAB-Sure ™ with
742 corresponding correlation plots shown in right panels (D, F, H). Pearson r and p values
743 are shown as a panel insets. As noted in the text, the Panel H eluates shown in Panel A
744 were diluted 1:10 for the assays descried in panels B-H to avoid saturating MFI values
745 that confound correlation analysis. Moreover, two of the highest Panel H eluates were
746 diluted 1:2 prior to the NAbSure assay to achieve NT_{50} values.
747

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

748

749

750

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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755 **Supplemental Figure Legends**

756

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

761

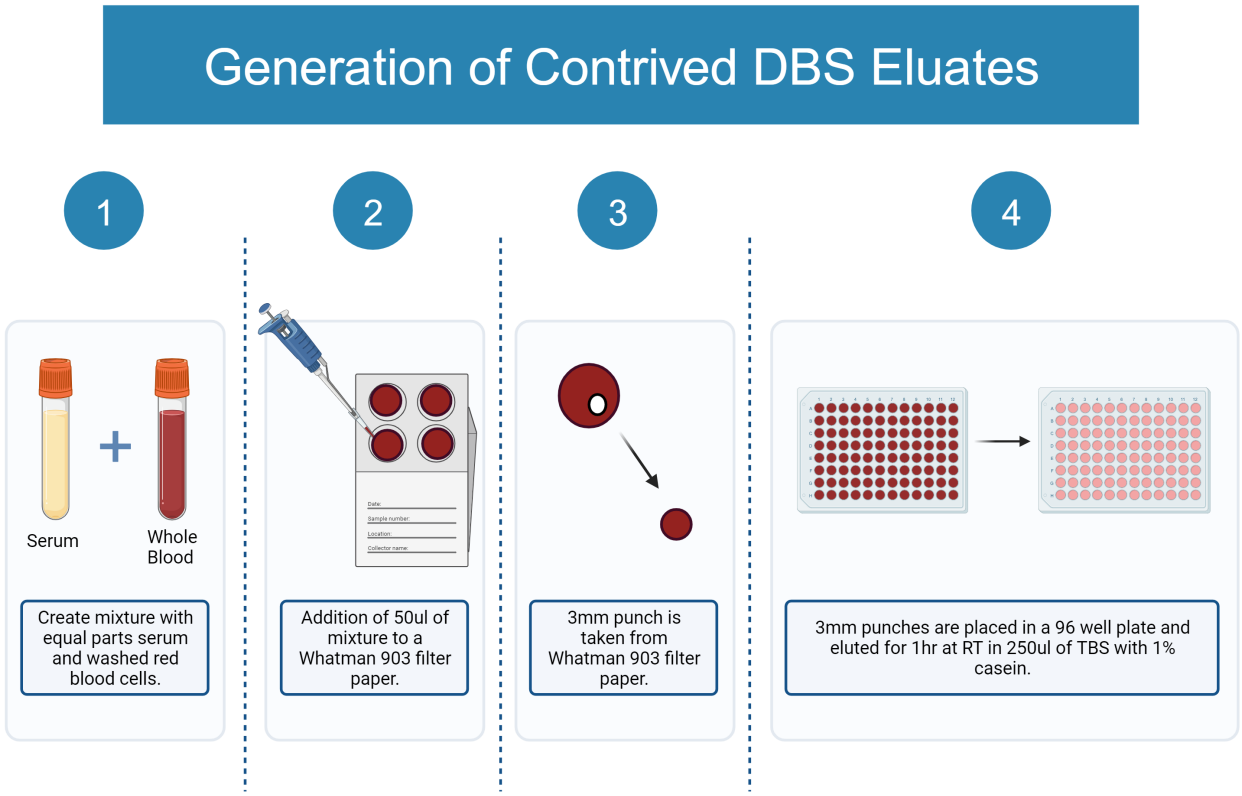
762 **Figure S2. Validation of Luminex-based ACE2 inhibition assay.** (A) MFI values
763 (binding) was assessed in the context of either FLT or RBD with varying concentrations
764 of biotinylated hACE2. (B) ACE2 inhibition titers associated with CC 12.3 in the context
765 of FLT or RBD. IC_{50} is shown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

771

772

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.

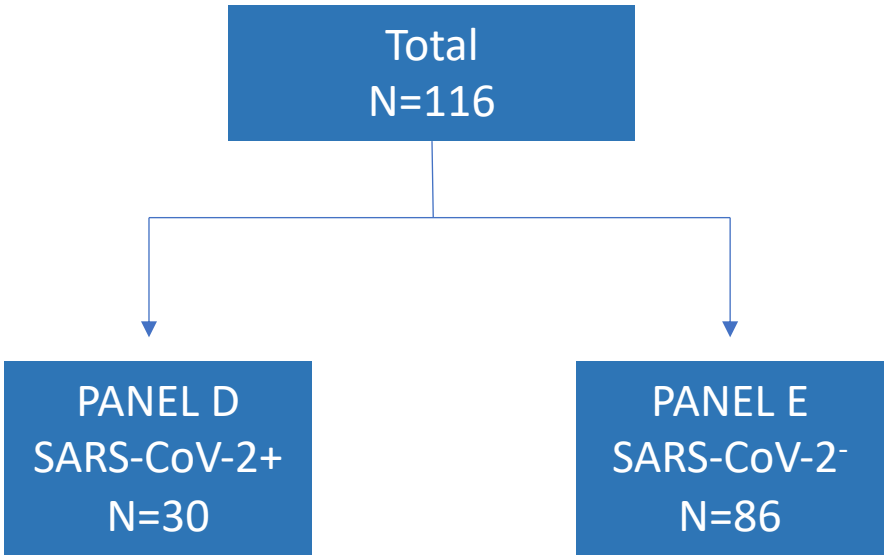
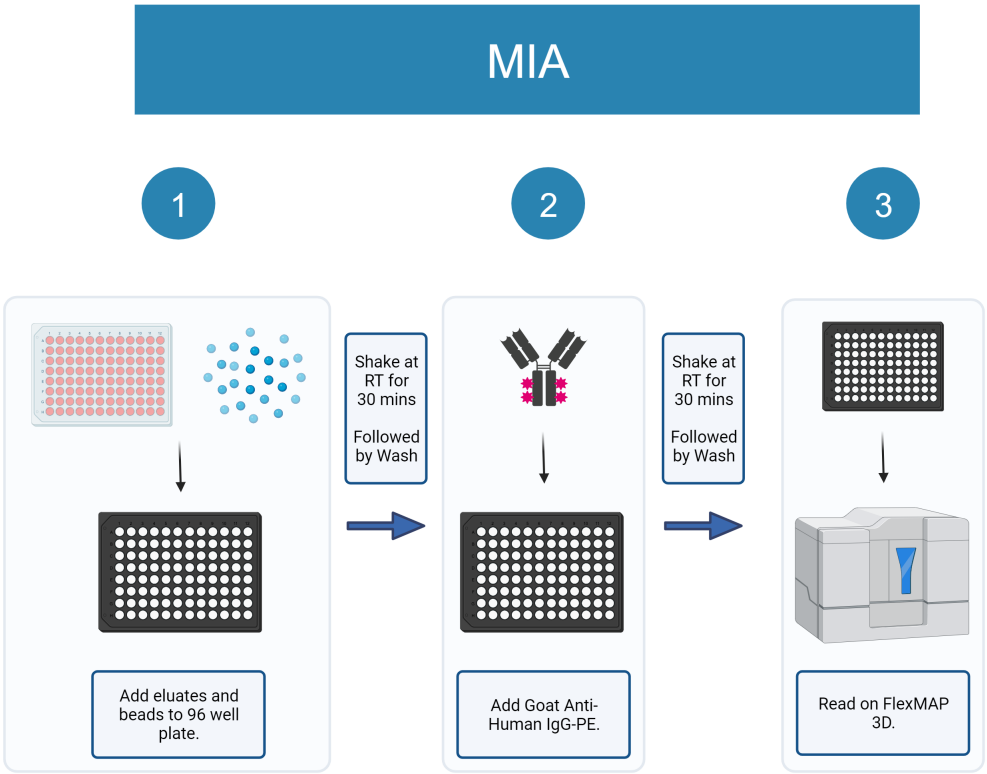

Total
N=116
PANEL D
SARS-CoV-2+
N=30
PANEL E
SARS-CoV-2-
N=86

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.

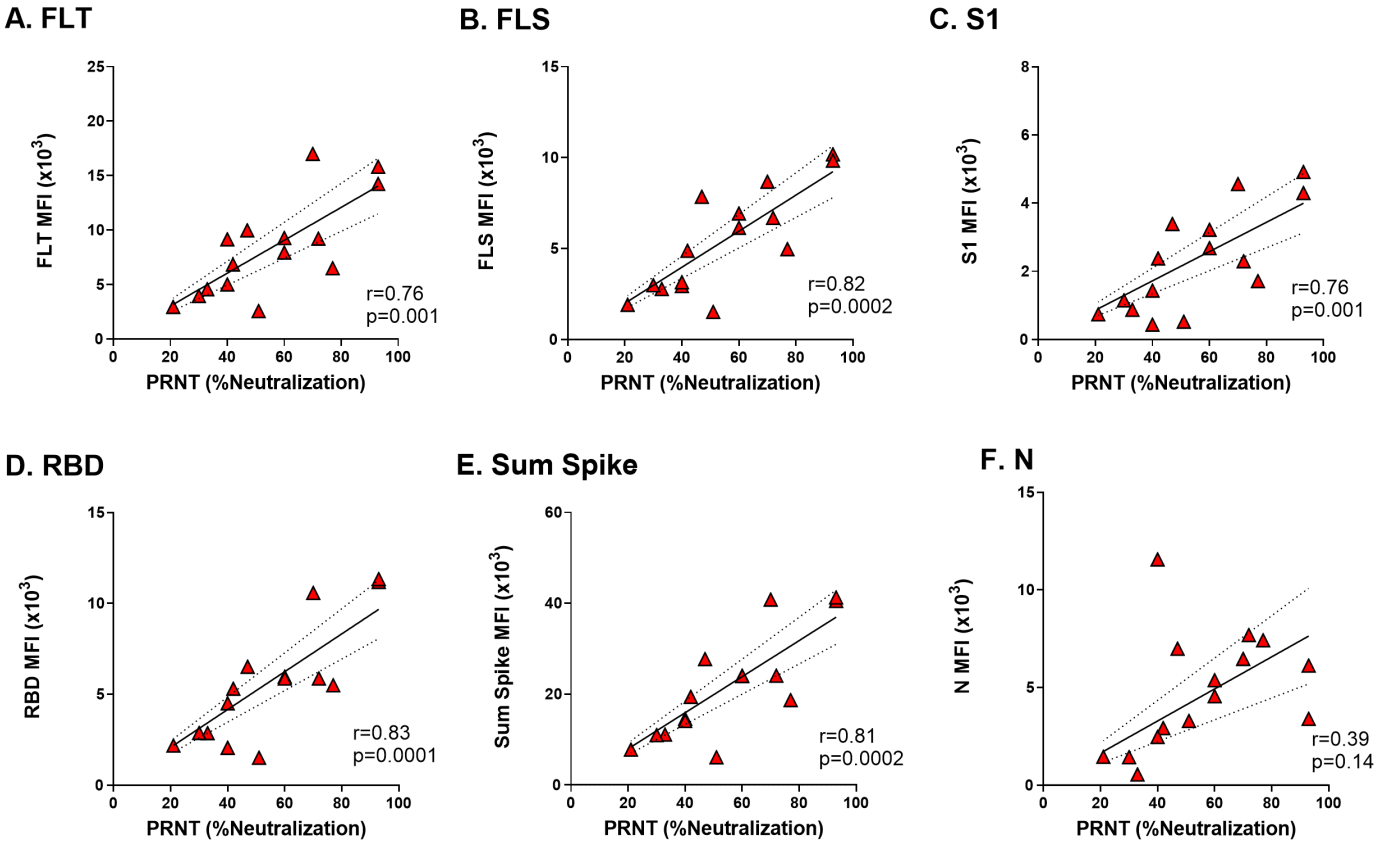
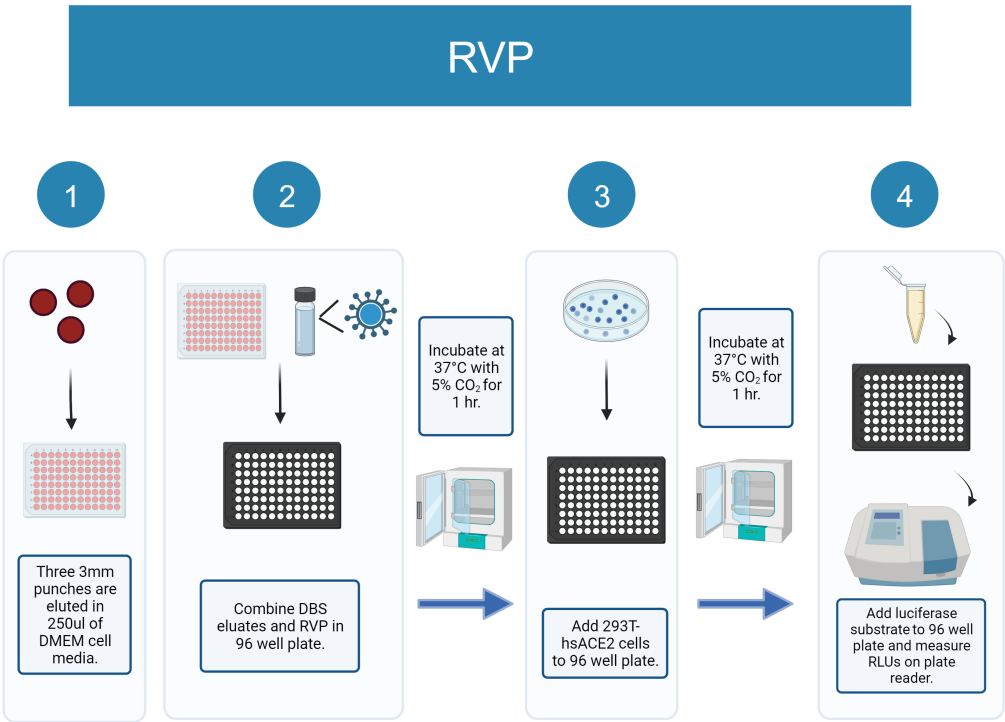
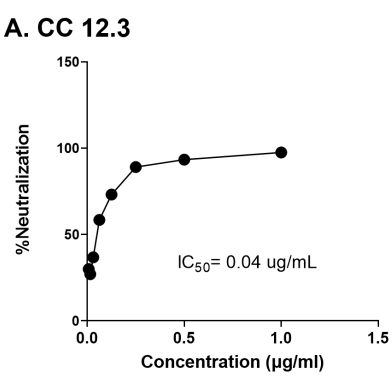
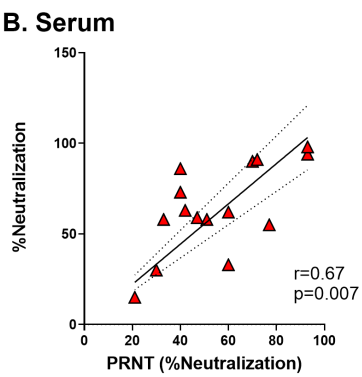
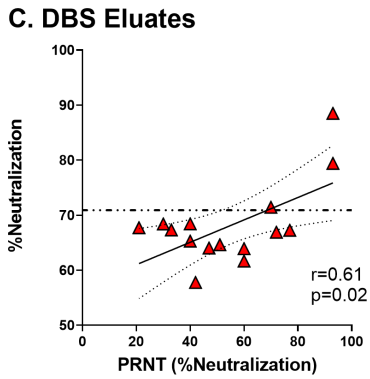

A. FLT
FLT MFI (x10^3)
PRNT (%Neutralization)
r=0.76
p=0.001
B. FLS
FLS MFI (x10^3)
PRNT (%Neutralization)
r=0.82
p=0.0002
C. S1
S1 MFI (x10^3)
PRNT (%Neutralization)
r=0.76
p=0.001
D. RBD
RBD MFI (x10^3)
PRNT (%Neutralization)
r=0.83
p=0.0001
E. Sum Spike
Sum Spike MFI (x10^3)
PRNT (%Neutralization)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

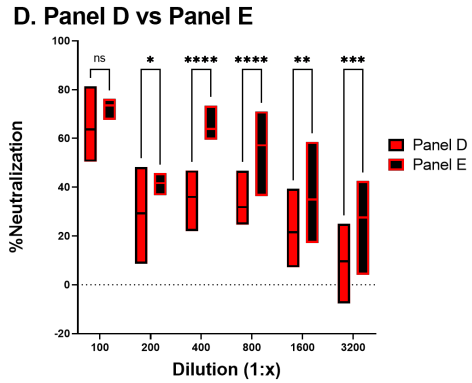
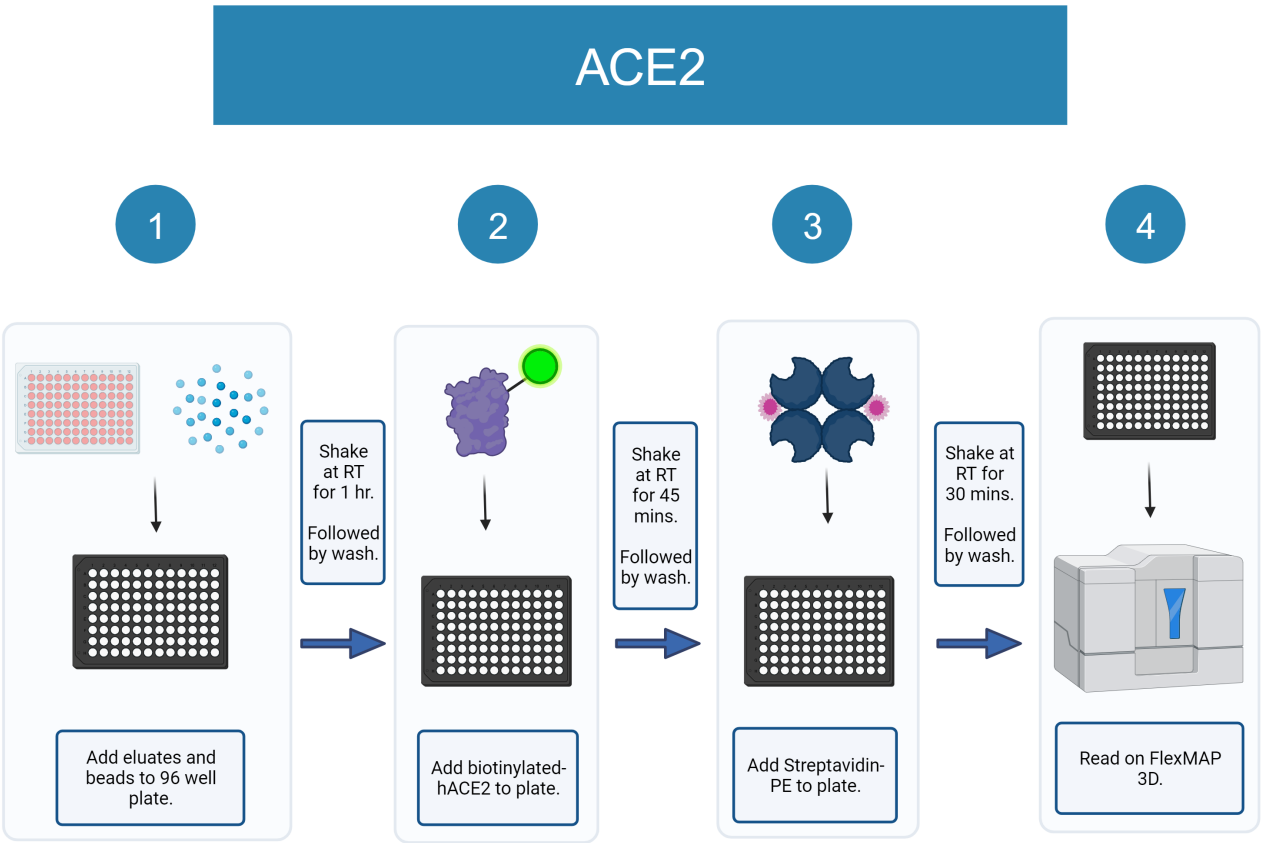

D. Panel D vs Panel E
%Neutralization
Dilution (1:x)
ns
*
****
****
**
***
Panel D
Panel E
-20
0
20
40
60
80
100
100
200
400
800
1600
3200

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-hACE2 to plate.
Shake at RT for 45 mins.
Followed by wash.
Add Streptavidin-PE to plate.
Shake at RT for 30 mins.
Followed by wash.
Read on FlexMAP 3D.

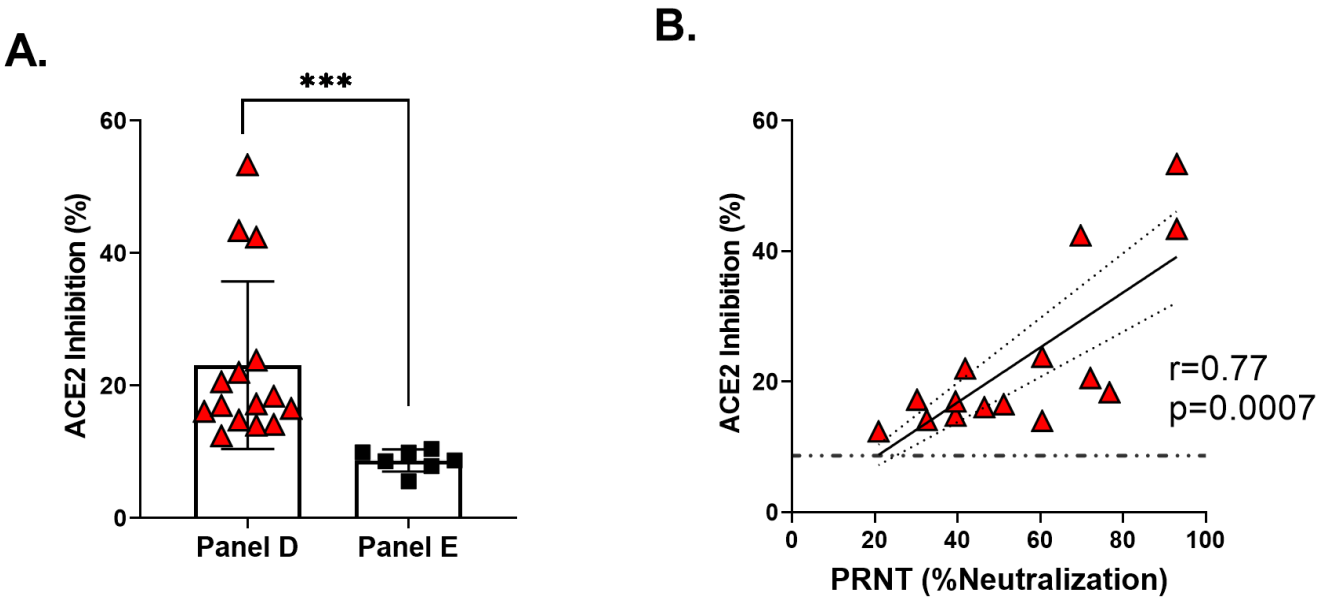
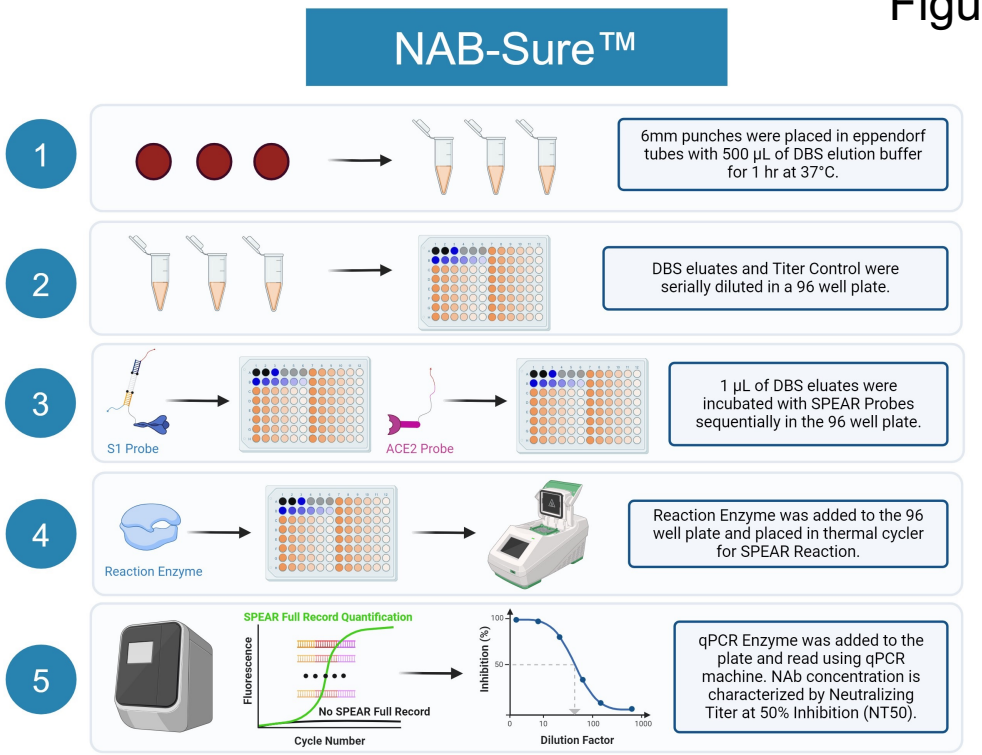

A.
***
ACE2 Inhibition (%)
60
40
20
0
Panel D
Panel E
B.
ACE2 Inhibition (%)
60
40
20
0
0
20
40
60
80
100
PRNT (%Neutralization)
r=0.77
p=0.0007

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).

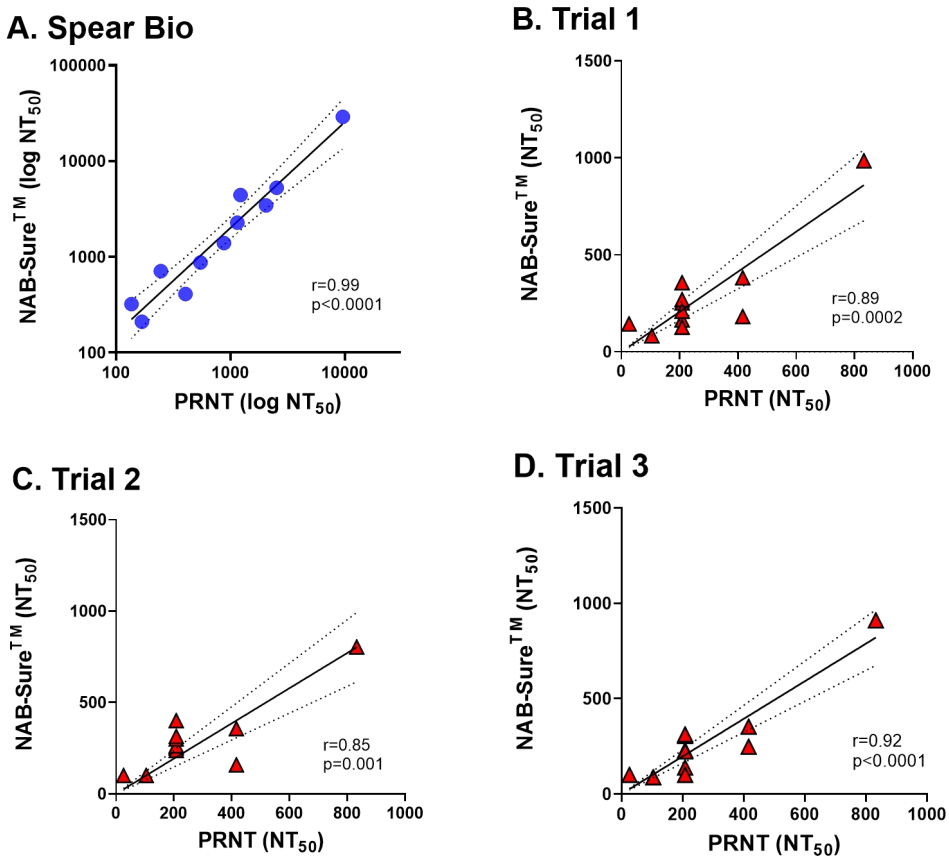

A. Spear Bio
NAB-Sure™ (log NT50)
PRNT (log NT50)
r=0.99
p<0.0001
B. Trial 1
NAB-Sure™ (NT50)
PRNT (NT50)
r=0.89
p=0.0002
C. Trial 2
NAB-Sure™ (NT50)
PRNT (NT50)
r=0.85
p=0.001
D. Trial 3
NAB-Sure™ (NT50)
PRNT (NT50)
r=0.92
p<0.0001

Figure 6

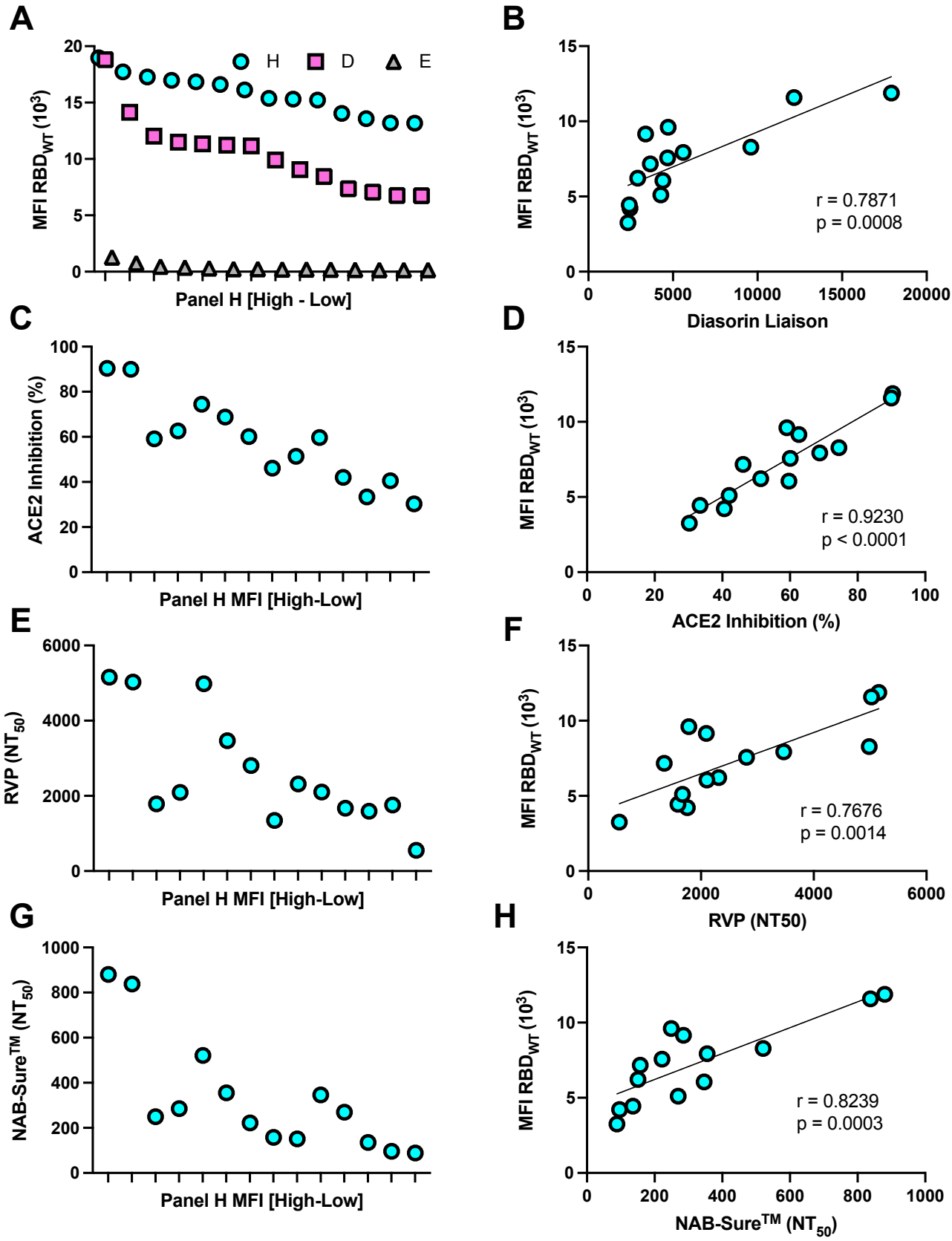

A
H
D
E
MFI RBD$_{WT}$ (10^3)
Panel H [High - Low]
B
MFI RBD$_{WT}$ (10^3)
r = 0.7871
p = 0.0008
Diasorin Liaison
C
ACE2 Inhibition (%)
Panel H MFI [High-Low]
D
MFI RBD$_{WT}$ (10^3)
r = 0.9230
p < 0.0001
ACE2 Inhibition (%)
E
RVP (NT_{50})
Panel H MFI [High-Low]
F
MFI RBD$_{WT}$ (10^3)
r = 0.7676
p = 0.0014
RVP (NT50)
G
NAB-Sure^{TM} (NT_{50})
Panel H MFI [High-Low]
H
MFI RBD$_{WT}$ (10^3)
r = 0.8239
p = 0.0003
NAB-Sure^{TM} (NT_{50})

Figure S1

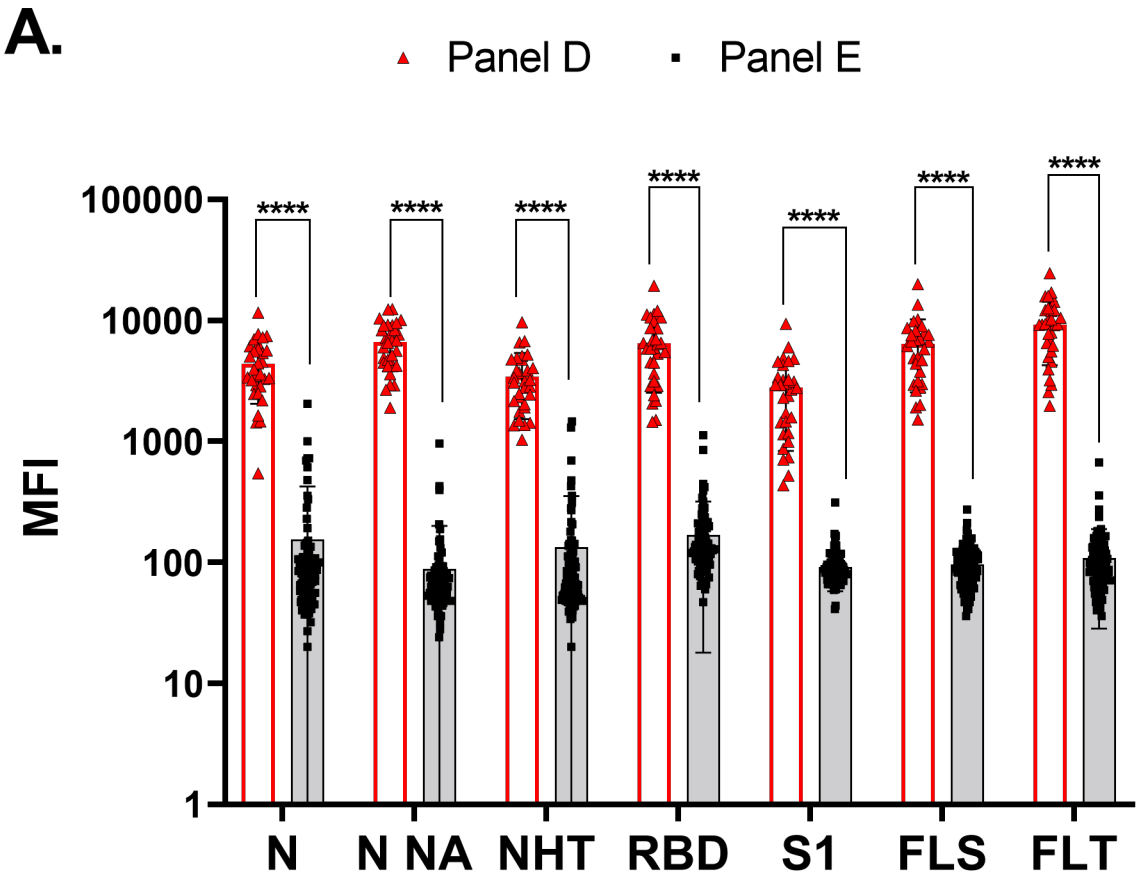
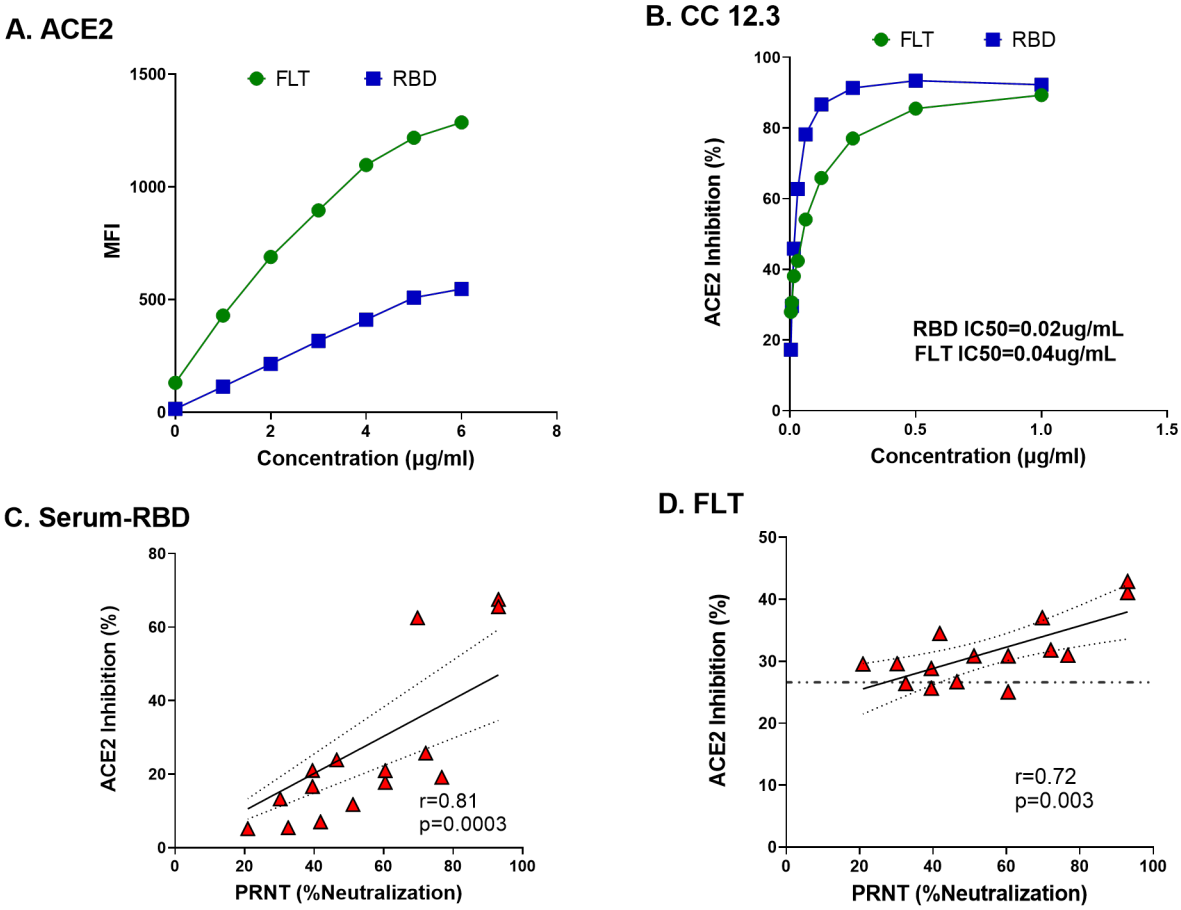

A.
Panel D
Panel E
MFI
100000
10000
1000
100
10
1
****
N
N NA
NHT
RBD
S1
FLS
FLT

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)