

1 **Rapid *in vitro* assays for screening neutralizing antibodies and antivirals against**
2 **SARS-CoV-2**

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24 **Abstract**

25 Towards the end of 2019, a novel coronavirus (CoV) named severe acute respiratory
26 syndrome coronavirus-2 (SARS-CoV-2), genetically similar to severe acute respiratory
27 syndrome coronavirus-1 (SARS-CoV-1), emerged in Wuhan, Hubei province of China,
28 and has been responsible of coronavirus disease 2019 (COVID-19) in humans. Since
29 its first report, SARS-CoV-2 has resulted in a global pandemic, with over 10 million
30 human infections and over 560,000 deaths reported worldwide at the end of June 2020.
31 Currently, there are no United States (US) Food and Drug Administration (FDA)-
32 approved vaccines and/or antivirals licensed against SARS-CoV-2, and the high
33 economical and health impact of SARS-CoV-2 has placed global pressure on the
34 scientific community to identify effective prophylactic and therapeutic treatments for the
35 treatment of SARS-CoV-2 infection and associated COVID-19 disease. While some
36 compounds have been already reported to reduce SARS-CoV-2 infection and a handful
37 of monoclonal antibodies (mAbs) have been described that neutralize SARS-CoV-2,
38 there is an urgent need for the development and standardization of assays which can
39 be used in high through-put screening (HTS) settings to identify new antivirals and/or
40 neutralizing mAbs against SARS-CoV-2. Here, we described a rapid, accurate and
41 highly reproducible plaque reduction microneutralization (PRMNT) assay that can be
42 quickly adapted for the identification and characterization of both neutralizing mAbs and
43 antivirals against SARS-CoV-2. Importantly, our MNA is compatible with HTS settings to
44 interrogate large and/or complex libraries of mAbs and/or antivirals to identify those with
45 neutralizing and/or antiviral activity, respectively, against SARS-CoV-2.

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47 **Keywords:** SARS-CoV-2, COVID-19, neutralization, monoclonal antibodies, polyclonal
48 antibodies, neutralizing antibodies, antivirals, plaque reduction microneutralization
49 assay.

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71 **Introduction**

72 A new variant of coronavirus (CoV) emerged around December of 2019 causing
73 multiple hospitalizations in Wuhan, China (Bogoch et al., 2020; Choy et al., 2020). Ever
74 since its first emergence, this novel severe acute respiratory syndrome coronavirus-2
75 (SARS-CoV-2) has been responsible for several cases of pneumonia leading to death
76 among hospitalized patients all around the world. The transboundary spread of this
77 coronavirus-associated acute respiratory disease, designated as coronavirus disease
78 2019 (COVID-19), prompted the World Health Organization (WHO) to declare the
79 disease as a global pandemic on March 11th, 2020 (Organization). Besides the public
80 health significance, COVID-19 has also disrupted the functioning of the global supply
81 chain, bringing to a halt major economic activities all around the world.

82 SARS-CoV-2 is a close relative of severe acute respiratory syndrome coronavirus-1
83 (SARS-CoV-1) in the *Coronaviridae* family and has a single-stranded, non-segmented,
84 positive-sense RNA genome (Schoeman and Fielding, 2019). Like other CoVs, SARS-
85 CoV-2 encodes four conserved structural membrane (M), envelope (E), nucleoprotein
86 (NP), and spike (S) proteins, all of which play an important role in viral replication
87 (Masters, 2006; Mortola and Roy, 2004; Schoeman and Fielding, 2019; Wang et al.,
88 2017). Of the four structural proteins, the S protein determines tissue tropism and host
89 susceptibility to SARS-CoV-2 infection. An early aspect of SARS-CoV-2 replication in a
90 susceptible host is the complex interaction between the viral S protein and the host cell
91 surface receptor, the angiotensin converting enzyme 2 (ACE2). This intricate
92 association enables viral fusion to the host cell membrane facilitating virus entry, an

93 important requisite for successful virus replication. Owing to their importance in
94 facilitating virus entry, the S protein has been a major target of neutralizing antibodies
95 (NAbs) that are currently being produced against SARS-CoV-2.

96 Due to the high impact of COVID-19 and the lack of commercially available vaccines
97 and/or NAbs, many United States (US) Food and Drug Administration (FDA)-approved
98 or repurposed drugs are currently being suggested for the treatment of clinically-
99 infected COVID-19 patients (Elfiky, 2020; Park et al., 2020b; Xu et al., 2020). As
100 scientist and clinicians race to identify drugs for the treatment of SARS-CoV-2 infection
101 and associated COVID-19 disease, some have been used in clinical settings with no
102 supporting evidence of antiviral activity against SARS-CoV-2 (Reihani et al., 2020). The
103 majority of these compounds are being recommended based on their ability to target
104 specific steps of the replication cycle of similar viruses or, as seen in some cases,
105 based on their ability to inhibit protozoan parasites (Yao et al., 2020). Apart from the
106 lack of effective antiviral or immunotherapeutic activity against SARS-CoV-2,
107 administration of certain antiviral compounds in COVID-19 patients has being a subject
108 of safety concern among many clinicians. For instance, at high doses, chloroquine is
109 extremely toxic causing death as a result of cardiac arrhythmias (Juurlink, 2020; Riou et
110 al., 1988), and in the current pandemic, cases of chloroquine poisoning have been
111 reported in Nigeria due to the abuse of the drug for treating COVID-19 patients (Chary
112 et al., 2020). As a result, there is an urgent need for assays that can be used to screen
113 the increasing lists of potential antiviral compounds and/or NAbs for an effective and
114 safe anti-SARS-CoV-2 activity. Herein, we described a rapid approach that can be

115 easily adapted for evaluating the potency and safety of a wide array of antiviral
116 compounds and/or NAbs against SARS-CoV-2 in an *in vitro* setting.

117 **Materials**

118 *Supplies and reagents*

- 119 1. Vero E6 cells (BEI Resources, catalog # NR-596).
- 120 2. SARS-CoV-2 isolate USA-WA1/2020 (BEI Resources, catalog # NR-52281).
- 121 3. Dulbecco's modified Eagle medium, DMEM (Corning, catalog # 15-013-CV).
- 122 4. Penicillin-Streptomycin L-glutamine 100x, PSG (Corning, catalog # 30-009-CI).
- 123 5. Fetal bovine serum, FBS (Avantor Seradigm, catalog # 1500-500).
- 124 6. Agar, 2% (Oxoid, catalog # LP0028).
- 125 7. Bovine serum albumin, 35% (BSA; Sigma-Aldrich, catalog # A7409).
- 126 8. BSA, 2.5% (Sigma-Aldrich, catalog # A9647).
- 127 9. DMEM/F-12 powder (Gibco, catalog # 12400-024).
- 128 10. Cell culture grade water (Corning, catalog # 25-055-CV).
- 129 11. Sodium bicarbonate, 5% (Sigma, catalog # S-5761).
- 130 12. DEAE-Dextran, 1% (MP Biomedicals, catalog # 195133).
- 131 13. Crystal violet, 1% (Fisher Scientific, catalog # C581-100).
- 132 14. Avicel PH-101, 1% (Sigma-Aldrich, catalog # 11365).
- 133 15. Formalin solution, neutral buffered, 10% (Sigma-Aldrich, catalog # HT501128-
134 4L).
- 135 16. Triton X-100, 0.5% (Sigma-Aldrich, catalog # X100-500ML).

- 136 17. Mouse anti-SARS-1 nucleoprotein (NP) mAb 1C7 generated at the Center for
137 Therapeutic Antibody Development at The Icahn School of Medicine at Mount
138 Sinai (ISMMS) (Millipore Sigma, catalog # ZMS1075).
- 139 18. Remdesivir (AOBIOUS, catalog # AOB36496).
- 140 19. Human monoclonal antibody (hmAb) 1207B4 produced in house.
- 141 20. VECTASTAIN® ABC-HRP Kit, Peroxidase (POD) (Mouse IgG) (Vector
142 Laboratory, catalog # P-4002).
- 143 21. DAB Substrate Kit, POD (HRP), with Nickel (Vector Laboratory, catalog # SK-
144 4100).
- 145 22. IRDye 800CW goat anti-mouse IgG secondary antibody (LI-COR, catalog # 926-
146 32210).
- 147 23. DRAQ5™ Fluorescent Probe Solution 5 mM (Thermo scientific, catalog # 62251).
- 148 24. 6-well cell culture plate (Greiner Bio-One, catalog # 657160).
- 149 25. 96-well cell culture plate (Greiner Bio-One, catalog # 655180).
- 150 26. Polystyrene tissue culture flask (Corning, catalog # 431081).
- 151 27. Polypropylene sterile conical tube, 15 mL (Greiner Bio-One, catalog # 188261).
- 152 28. Polypropylene sterile conical tube, 50 mL (Greiner Bio-One, catalog # 227270).
- 153 29. Serological pipette, 5 mL (Greiner Bio-One, catalog # 606 180).
- 154 30. Serological pipette, 10 mL (Greiner Bio-One, catalog # 607 180).
- 155 31. Serological pipette, 25 mL (Greiner Bio-One, catalog # 760 180).
- 156 32. Universal pipette tip, 20 µL (VWR, catalog # 76322-134).
- 157 33. Universal pipette tip, 200 µL (VWR, catalog # 76322-150).
- 158 34. Universal pipette tip, 1000 µL (VWR, catalog # 16466-008).

- 159 35. Microcentrifuge tube, 1.5 mL (VWR, catalog # 89000-028).
160 36. Sterile basin (VWR, catalog # 89094-680).
161 37. CO₂ incubator (PHCbi, model # MCO-170AICUVDL).
162 38. CTL ImmunoSpot plate reader and counting software (Cellular Technology
163 Limited).
164 39. Odyssey Sa Infrared Imaging System (LI-COR, model # 9260).
165 40. GraphPad Prism (GraphPad Software Inc., version 8.0).

166 *Media type and recipes*

- 167 41. Cell-maintenance media (DMEM supplemented with 10% FBS and 1% PSG).
168 42. Post-infection media (DMEM supplemented with 2% FBS and 1% PSG).
169 43. Infection media (DMEM supplemented with 1% PSG).
170 44. DMEM/F-12/Agar mixture (DMEM-F12 with 1% DEAE-Dextran, 2% agar, and 5%
171 NaHCO₃).
172 45. Plaque reduction microneutralization (PRMNT) media (Post-infection media with
173 1% Avicel).

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175 **Biosafety Recommendations:** Although SARS-CoV-2 has been temporarily classified
176 as a category B pathogen, biosafety caution applicable to the category A pathogens are
177 strongly recommended when handling this pathogen. Manipulation of SARS-CoV-2
178 should only be carried out in a biosafety level 3 (BSL3) facility with an appropriate
179 engineering system designed to produce negative air pressure and enhance the safety
180 of laboratory workers. All individuals working with SARS-CoV-2 must have undergone
181 proper biosafety training to work at BSL3 laboratories and be cleared proficient to carry

182 out procedures requiring working with the virus. Cell culture procedures are carried out
183 in BSL2 and moved to BSL3 when ready for viral infection.

184

185 **Cells:** Vero E6 cells, which have been demonstrated to be permissive to SARS-CoV-2,
186 is the preferred cell type for the assays described in this protocol (Imai et al., 2020).

187 Other cells types such as Vero E6 transfected to express transmembrane protease,
188 serine 2 (TMPRSS2), a serine protease which activates SARS-CoV-2 infection

189 (Matsuyama et al., 2020), can also be used. An important consideration when choosing

190 a cell line for this assay is the ability of SARS-CoV-2 to efficiently infect and replicate in

191 the cell line. It is important to seed cells a day before the assay to achieve

192 approximately ~85-95% confluency. Vero E6 cells are maintained in DMEM

193 supplemented with 10% FBS and 1% PSG. We recommend using a low passage of

194 Vero E6 cells as we have found that these cells grow better and are more viable at low

195 passage. It is equally important to make sure the cells are free from any contaminants

196 including mycoplasma.

197

198 **Virus:** For the experiments described in this manuscript we used the SARS-CoV-2

199 isolate USA-WA1/2020 available from BEI Resources. For the proposed experiments,

200 we recommend using ~100-200 plaque forming units (PFU)/well for a 96-well plate.

201 Higher concentrations of the virus may affect the sensitivity of the assay while using

202 lower viral concentrations may result in reduced number of plaques/well. Following the

203 experimental conditions described in this manuscript, you would expect to have ~100-

204 200 positive staining cells/well at 24 h p.i.

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206 **Serum samples:** It is important to heat-inactivate serum or plasma samples from
207 COVID-19 patients or SARS-CoV-2-infected animals at 56°C for 1 h before performing
208 the PRMNT assay to destroy complement proteins or residual SARS-CoV-2 particles.
209 The experimental procedures to inactivate the virus should be confirmed and approved
210 by the institutional biosafety committee (IBC) of the institute. It has been reported that
211 complement deposition on virus envelope may lead to infection-enhancement which
212 may mask the neutralizing effects of Abs contained in serum or plasma samples
213 (Montefiori, 1997). For serum samples or plasma samples, we recommend starting with
214 a 1:100 dilution to avoid potential impurities that may affect the sensitivity of the assay.
215 In the case of mAbs, we recommend starting with 10 µg. However, this will depend on
216 the neutralizing capability of the mAb. We used a hmAb, 1207B4, for the purposes of
217 this study.

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219 **Drugs:** The drug used in this study is remdesivir, a nucleoside analog which interferes
220 with the replication of viral genome by inhibiting viral RNA-dependent RNA polymerase
221 (RdRp) (Gordon et al., 2020). Any other antiviral drug or compound with potential
222 antiviral activity against SARS-CoV-2 can equally be used in the assays described
223 below.

224

225 **Controls:** It is important to validate the described PRMNT assays by including proper
226 positive and negative controls. A mAb with a known neutralizing titer or serum and/or
227 plasma samples from convalescent SARS-CoV-2 patients that contains known NAbs

228 can be used as positive controls. In the case of the antiviral drugs, we recommend to
229 use remdesivir since it has been shown to have antiviral activity against SARS-CoV-2
230 (Choy et al., 2020; Ferner and Aronson, 2020). Negative controls include the use of
231 SARS-CoV-2-infected Vero E6 cells without NABs, sera, or drugs. An additional
232 recommended control to validate the immunostaining with the NP 1C7 mAb is to include
233 cells without viral infection.

234 **General Procedure:** All cell cultures procedures and antibody/antiviral dilutions are
235 generally performed in the BSL2 before transfer to the BSL3 facility on the day of virus
236 infection. For PRMNT assay, the amount of virus per well can be optimized based on
237 the virus titer in the stock. During the inactivation procedure, it is important to ensure
238 that the entire plate and its lid are fully submerged in 10% formalin solution for proper
239 inactivation. After the inactivation procedure plates can be transferred from BSL3 to the
240 BSL2 for immunostaining and development.

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257 **Results**

258 **Preparation of SARS-CoV-2 stock**

259 1. A day before viral infection, seed Vero E6 cells in a T-225 cell culture flask in
260 cell-maintenance media to attain ~85%-95% confluency on the day of infection
261 and place them in a humidified incubator at 37°C with 5% CO₂.

262 2. Thaw the stock virus and prepare an infection media containing the virus
263 inoculum at a MOI of 0.001.

264 3. Remove cell maintenance media and replace with infection media containing the
265 virus in a total volume of 10 mL. Place the tissue culture flasks of infected cells in
266 Ziploc bags and transport them on a flat tray to the humidified incubator and
267 incubate at 37°C in the presence of 5% CO₂. Note: shake the flasks, gently,
268 every 10 minutes to prevent cells from drying out during a 1 h incubation time-
269 point (TP).

270 4. After 1 h of virus adsorption, in a humidified incubator at 37°C with 5% CO₂,
271 remove the virus inoculum and replace with ~40 mL of post-infection media.

272 5. Incubate for 72 h in a humidified incubator at 37°C with 5% CO₂.

273 Note: SARS-CoV-2 behaves differently compared to most other viruses in that
274 the presence of cytopathic effect (CPE) does not correlate with increased virus
275 titer. For our virus stock, we carried out a multistep growth kinetics to determine
276 the best time with the highest virus yield. In our hands, this correlated nicely with
277 low CPE at ~72 h of incubation.

278 6. Freeze-thaw the flask containing the tissue culture supernatant (TCS) once,
279 followed by centrifugation at 2,000 g for 10 min at 4°C.

280 7. Aliquot the TCS in 500 µL volumes, and store at - 80°C until further use.

281

282 **Determining the infectious virus titer by plaque assay**

283 1. The day before infection, prepare Vero E6 cells (6-well plate format, 1×10^6 cells
284 /well) in cell-maintenance media and place them in a humidified incubator at
285 37°C with 5% CO₂.

286 2. Prepare 10-fold serial dilutions of the SARS-CoV-2 stock in infection media.
287 Briefly, pipette 450 µL of infection media and add 50 µL of the virus stock in the
288 first tube. Transfer 50 µL from tube #1 (10^{-1} dilution) to tube #2 (10^{-2} dilution) and
289 continue doing serial dilutions.

290 Note: It is important to change the tips between viral dilutions to prevent the
291 transfer of virus particles from a lower to higher diluted infection medium.

292 3. Infect the 6-well plates of Vero E6 cells with 200 µL of each of the SARS-CoV-2
293 dilutions and incubate for 1 h in a humidified incubator at 37°C with 5% CO₂.

294 Place the plates of infected cells in a Ziploc bag and transport them on a flat tray
295 to the humidified incubator and incubate at 37°C in the presence of 5% CO₂.

296 4. To prevent cells from drying and to also facilitate virus adsorption, shake the
297 plates gently every 10 minutes.

298 5. After 1 h of viral absorption, remove the virus inoculum and overlay the cells with
299 DMEM/F-12/Agar mixture.

300 Note: ensure that at the start of the infection, the 2% agar is melted in a
301 microwave and the temperature brought down to approximately 42°C when
302 mixing with warm DMEM/F-12/Agar mixture.

303 Note: It is important to keep the agar cool enough not to burn the cells, and at the
304 same time warm enough not to solidify in the process of pipetting.

305 6. Allow the agar to solidify and invert the plates to prevent the accumulation of
306 moisture during incubation.

307 7. Incubate the cells for 72 h in a humidified incubator at 37°C in the presence of
308 5% CO₂.

309 8. At 72 h post-infection (h p.i.), fix infected cells in 10% formalin solution for 24 h.

310 Note: After 24 h fixation with 10% formalin solution, plates can be moved from
311 the BSL3 to the BSL2 to complete the viral titration.

312 9. Perform immunostaining using the cross-reactive SARS-CoV-1 NP mAb 1C7, at
313 a working concentration of 1 µg/mL.

314 Note: Alternatively, cells can also be stained with 1% crystal violet for plaque
315 visualization. Count the number of plaques, and calculate the virus titer in plaque
316 forming units per mL (PFU/mL).

317

318 **A plaque reduction microneutralization (PRMNT) assay to identify Abs with**
319 **neutralizing activity against SARS-CoV-2**

320 This assay can be used to evaluate SARS-CoV-2 neutralizing activity by mAbs,
321 polyclonal antibodies (pAbs) or by an Ab-containing sample such as serum plasma from
322 mammalian host species. Since serial dilutions of the Ab containing samples are used,
323 the PRMNT assay measures the neutralizing activity of Abs in a concentration
324 dependent manner. The ability of a NAbs to inhibit virus infection is manifested in the
325 reduced capacity of the virus to produce visible plaques when compared to virus-only
326 infected control cells. The PRMNT assay is similar to a plaque reduction neutralization
327 (PRNT) assay with the only difference that the PRMNT utilizes 96-well plates. Although
328 in this manuscript we describe the use of PRMNT in 96-well plates, similar experimental
329 approaches can be miniaturized and conducted in 384-well cell culture plates for HTS
330 purposes.

331

332 **A PRMNT assay to identify NAbs**

333 The process of entry into a susceptible host cell is an important determinant of infectivity
334 and pathogenesis of viruses, including CoVs (Li, 2016; Perlman and Netland, 2009).
335 SARS-CoV-2 relies on the ability of its S glycoprotein to bind to the ACE2 receptor
336 through its receptor-binding domain (RBD) driving a conformational change that
337 culminates in the fusion of the viral envelope with the host cell membrane, and cell entry
338 (Shang et al., 2020). SARS-CoV-2 S is made up of 2 subunits, S1 and S2. During pre-
339 treatment, mAbs, or Ab containing samples, are pre-incubated with SARS-CoV-2 at
340 37°C for 1h. This enables the Abs to bind to the S protein blocking virus attachment,

341 and subsequently interfering with the process of virus entry. While some SARS-CoV-2-
342 induced NAb can preferentially bind to either the S1 or S2 subunit of the S protein,
343 others can bind, with a high affinity, to the RBD located within the S1 subunit (Wang et
344 al., 2020; Wu et al., 2020). After 1 h of incubation, the Ab-virus mixture is then
345 transferred to a confluent (~85-95%) monolayer of Vero E6 cells and incubated for 24 h
346 at 37°C. During this period, the unbound virus particles are able to attach to cells and
347 initiate virus replication which can be determined by staining for SARS-CoV-2. In the
348 post-treatment condition, virus adsorption is allowed to progress for 1h at 37°C. This
349 gives an opportunity for the virus to initiate viral entry by binding to the cell surface
350 receptor. NAb incubated with the infected cells may interfere with active viral replication
351 partly by blocking later steps of the virus entry into the cell or by inhibiting the cell-to-cell
352 spread of virus progeny. In both pre- and post-treatment conditions, the extent of virus
353 neutralization can be visualized upon staining with a cross-reactive anti-SARS-CoV-1
354 NP mAb (1C7), and the neutralization titer 50 (NT₅₀) is calculated using a sigmoidal
355 dose-response curve. The NT₅₀ of an Ab is the dilution which inhibited viral replication in
356 50% of the infected cells. While the pre-treatment PRMNT assay can be described, to
357 some extent, as a prophylactic assessment of a NAb, the post-treatment PRMNT assay
358 offers a therapeutic evaluation of the neutralizing activity of Abs against SARS-CoV-2.
359 In addition, while the pre-treatment PRMNT assay focuses on identifying NAb targeting
360 the SARS-CoV-2 S RBD, the post-treatment PRMNT assay also allow the identification
361 of Abs targeting other regions on the viral S glycoprotein involved in the fusion event
362 (e.g. S2), or Abs affecting other steps in the replication cycle of the virus (e.g. virus
363 assembly and/or budding). In general, a potent SARS-CoV-2 NAb will show a low NT₅₀

364 titer whereas a weak NAb will give a high NT_{50} value. This protocol precludes the
365 necessity for a cytotoxicity assay since Abs are not known to produce cytotoxic effects
366 in cell cultures. However, a potential toxic effect of the NAbs can be determined when
367 developing the assay with the infrared staining described below.

368

369 **Pre-treatment PRMNT assay**

- 370 1. Seed $\sim 1 \times 10^4$ Vero E6 cells/well the day before virus infection in 96-well plates
371 using cell maintenance media.
- 372 2. On the day of infection, check the confluency of the Vero E6 cells under a light
373 microscope. The optimal cell confluency should be $\sim 85-95\%$.
- 374 3. Prepare a 2-fold serial dilution of the Ab (or Ab containing sample) in an empty,
375 sterile 96-well plate using infection media. Briefly, add 50 μL of infection media to
376 columns 2 to 12, and add 100 μL of the desired starting concentration of each
377 Ab, or Ab containing sample, to column 1. Transfer 50 μL of Ab from column 1 to
378 column 2, and mix ~ 10 times using a multi-channel pipette. Repeat this process
379 from column 2 to column 10, changing the tips between dilutions to prevent
380 transfer of residual Ab. Discard 50 μL from the solution in column 10 after dilution
381 so that each well of the 96-well plate has 50 μL of diluted Ab. Columns 11 and 12
382 are included as internal controls, as virus-only and mock-only, respectively. Each
383 Ab is tested in triplicate.
- 384 4. In the BSL3, prepare $\sim 100-200$ PFU/well of SARS-CoV-2 in infectious media in
385 the biosafety cabinet. From the virus stock, calculate $\sim 1.0-2.0 \times 10^4$ PFU and mix
386 in 5 mL (for one 96-well plate) of infection media.

- 387 Note: The amount of virus per well can be further optimized based on the virus
388 titer in the stock.
- 389 5. Add 50 μ L of SARS-CoV-2 to columns 1 to 11 of the Ab plate and incubate the
390 mixture for 1 h at 37°C. Tap the plate gently to mix the sample with the virus.
- 391 6. After incubation, remove cell maintenance media from the Vero E6 96-well plate.
392 Transfer 50 μ L of the Ab sample-virus mixture from the 96-well plate to the
393 corresponding Vero E6 in 96-well plate using a multi-channel pipette. Incubate
394 for 1 h at 37°C in a 5% CO₂ incubator to allow virus adsorption.
- 395 7. After 1 h, remove the virus inoculum and overlay with post-infection media
396 containing 1% Avicel. Incubate the infected Vero E6 cells for 24 h at 37°C in the
397 5% CO₂ incubator.
- 398 8. At 24 h post-infection (h p.i.), remove infectious media and fix/inactivate the plate
399 in 10% formalin solution for 24 h at 4°C.

400

401 **Post-treatment PRMNT assay**

- 402 1. Vero E6 cells are maintained as described in the previous pre-treatment protocol.
- 403 2. Seed $\sim 1 \times 10^4$ Vero E6 cells/well the day before virus infection in 96-well plates.
- 404 3. On the day of infection, check the confluency of the Vero E6 cells under a light
405 microscope. The optimal cell confluency is between 85-95%.
- 406 4. Prepare a 2-fold serial dilution of the Ab or Ab containing sample in an empty,
407 sterile 96-well cell culture plate using a post-infection media as described in step
408 4 before. Add 50 μ L of 2% Avicel in post-infection media to each well containing

- 409 the diluted Ab or media-only and no-virus control wells (columns 11 and 12,
410 respectively) to give a final concentration of 1% Avicel in each well.
- 411 5. In the BSL3, prepare ~100-200 PFU/well of SARS-CoV-2 in infection media in
412 the biosafety cabinet. From the virus stock, calculate $1.0-2.0 \times 10^4$ PFU and mix
413 in 5 mL (for one 96-well plate) of infection media.
- 414 6. Add 50 μ L of virus inoculum to each well of the cell-cultured Vero E6 96-well
415 plate from column 1 to 11, and incubate for 1 h at 37°C in the CO₂ incubator for
416 virus adsorption.
- 417 7. After 1 h adsorption, remove the virus inoculum and replace with 100 μ L of post-
418 infection media containing the serially-diluted Ab with 1% Avicel.
- 419 8. Incubate the cells for 24 h at 37°C in the CO₂ incubator.
- 420 9. At 24 h p.i., remove media and fix/inactivate the plate in 10% formalin solution for
421 24 h at 4°C.

422

423 **PRMNT assay to identify compounds with antiviral activity against SARS-CoV-2**

424 Several antiviral compounds are currently being evaluated for their effectiveness
425 against SARS-CoV-2 (Buonaguro et al., 2020; Elfiky, 2020; Park et al., 2020b; Xu et al.,
426 2020). Different antiviral compounds target different steps of the viral replication cycle
427 which provides the basis for the use of a PRMNT assay for antiviral screening.
428 Hydroxychloroquine, a potential antiviral compound has been suggested to inhibit
429 SARS-CoV-2 by blocking fusion to the cell membrane, (Fox, 1993; Yao et al., 2020).
430 Other compounds in clinical trials, such as remdesivir, target the viral RdRp which is
431 involved in SARS-CoV-2 replication (Buonaguro et al., 2020). This protocol can be

432 adopted to screen the inhibitory effect and concentration of anti-SARS-CoV-2
433 compounds irrespective of the viral replication step. Although this protocol describes the
434 antiviral activity in post-infection conditions, an alternative assay where the antiviral drug
435 is provided before viral infection will help to provide information on the pre-entry
436 mechanism of antiviral activity of the compound. The antiviral activity is determined by
437 assessing the effective concentration that inhibits virus replication in Vero E6 cells (96-
438 well plates, $\sim 4 \times 10^4$ Vero E6 cells/well at confluency, triplicates) following viral
439 incubation for 24 h. Note that the seeding density of Vero E6 in 96-well is $\sim 1 \times 10^4$
440 cells/well while cells at confluency is $\sim 4 \times 10^4$ cells/well. It is important to include as a
441 positive control in this antiviral PRMNT assay a drug with a known antiviral activity
442 against SARS-CoV-2, such as remdesivir (Elfiky, 2020). Unlike NABs, some compounds
443 could have cytotoxic effects. As a result, it becomes imperative to carry-out a
444 cytotoxicity test (e.g. MTT assay) to evaluate for potential cytotoxicity of a given
445 compound. Vero E6 cells for an MTT assay can be seeded on the same day as the cells
446 for PRMNT assay so that the experiments can be conducted in parallel. Alternatively,
447 our infrared staining technique can be used, concurrently, to evaluate cell viability in the
448 same cells, in 96-well plates, used for PRMNT assay.

449

450 **A PRMNT assay to identify SARS-CoV-2 antivirals**

- 451 1. Vero E6 cells are maintained as described in the previous protocol.
- 452 2. Seed $\sim 1 \times 10^4$ Vero E6 cells/well the day before the virus infection in 96-well
453 plates, using triplicates for each of the drugs that will be tested.

- 454 3. On the day of infection, check the confluency of the Vero E6 cells under a light
455 microscope. The optimal cell confluency is between ~85-95%.
- 456 4. Prepare a 2-fold serial dilution of the antiviral in an empty, sterile 96-well cell
457 culture plates using post-infection media. Briefly, add 50 μ L of infection media to
458 columns 2 to 12, and add 100 μ L of 2X of the desired starting concentration of
459 each antiviral to column 1. The starting concentration of the antiviral will,
460 generally, depend on the type of drug being evaluated. We normally use a
461 starting concentration of 50 μ M for antivirals. Transfer 50 μ L of antiviral from
462 column 1 to column 2, and mix ~ 10 times using a multi-channel pipette. Repeat
463 this process from column 2 to column 10, changing the tips between dilutions to
464 prevent transfer of residual antiviral. Discard 50 μ L from the solution in column 10
465 after dilution so that each well of the 96-well plate has 50 μ L of either diluted
466 antiviral or no antiviral (for columns 11 and 12). Columns 11 and 12 are included
467 as virus-only and mock-only control wells, respectively.
- 468 5. Add 50 μ L of post-infection media containing 2% Avicel to each well in the 96-
469 well plate.
- 470 6. Prepare ~100-200 PFU/well of virus in post-infection media in the biosafety
471 cabinet. From the virus stock, calculate $\sim 1.0\text{-}2.0 \times 10^4$ PFU and mix in 5 mL (for
472 one 96-well plate) of infection media.
- 473 7. Remove media from the cell cultured Vero E6 cells in the 96-well plate and add
474 50 μ L of SARS-CoV-2 to columns 1 to 11. Incubate the plate for 1 h at 37°C in
475 the CO₂ incubator to allow viral adsorption.

- 476 8. After viral adsorption, remove viral inoculum from the 96-well plate. Transfer 100
477 μ L of the serially diluted sample containing 1% Avicel to the corresponding wells
478 in the cell-cultured Vero E6 96-well plate using a multi-channel pipette.
- 479 9. Incubate infected Vero E6 cells in the 96-well plate for 24 h at 37°C in the CO₂
480 incubator.
- 481 10. At 24 h p.i., remove media and wash the cell gently to remove Avicel.
482 Fix/inactivate the plates for 24 h at 4°C with 10% formalin solution.

483

484 **Development of the PRMNT assays**

485 For development of the PRMNT assays described above, we have optimized two
486 different protocols with comparatively similar results: the peroxidase and the infrared
487 staining techniques. The infrared staining technique holds the advantage of its capability
488 to measure cell viability in addition to measuring antiviral activity. In the case of the
489 peroxidase staining, a separate assay is needed to assess cell toxicity of NAbs or
490 antiviral compounds. Both assays rely on the use of the SARS-CoV-1 cross-reactive NP
491 mAb, 1C7 (Amanat et al., 2020) but any other mAb targeting a SARS-CoV-2 viral
492 antigen can be used in the peroxidase and the infrared staining described below.

493

494 **Peroxidase staining**

- 495 1. Once the plates are in the BSL2 after inactivation, remove residual formalin
496 solution by gentle wash with double distilled water (DDW) before staining. Be
497 careful not to touch or dislodge the cells in the 96-well plate with pipette tips.
- 498 2. Gently wash the cells three times with 100 μ L/well of PBS.

- 499 3. Permeabilize the cells with 100 μ L/well of 0.5% Triton X-100 dissolved in PBS,
500 and incubate at room temperature (RT) for 15 min in the biosafety cabinet.
501 Note: If you use an Ab against a viral surface protein (e.g. S), you can skip this
502 permeabilization step.
- 503 4. Wash the cells with 100 μ L/well of PBS, three times, and block with 100 μ L/well
504 of 2.5% BSA in PBS. Incubate cells at 37°C for 1 h.
- 505 5. Prepare primary Ab solution (anti-NP mAb, 1C7, 1 μ g/mL) in 1% BSA, in PBS.
506 Add 50 μ L/well of primary Ab solution and incubate at 37°C for 1 h.
- 507 6. After incubation with the primary Ab, wash each well three times with 100 μ L/well
508 of PBS.
- 509 7. Prepare the biotinylated anti-mouse Ab (VECTASTAIN® ABC-HRP Kit,
510 Peroxidase (Mouse IgG); Vector Laboratory) following the manufacturer's
511 instructions. For one 96-well plate, add 75 μ L of normal blocking serum stock and
512 25 μ L of biotinylated secondary Ab stock to 5 mL of PBS. Add 50 μ L/well of
513 biotinylated Ab solution to each well, and incubate for 30 min at 37°C.
- 514 8. Next, wash each well three times with 100 μ L/well of PBS to remove biotinylated
515 Ab solution thoroughly. Prepare VECTASTAIN ABC Reagent by following
516 manufacturer's instructions (VECTASTAIN® ABC-HRP Kit, Peroxidase (Mouse
517 IgG); Vector Laboratory). For one 96-well plate, add 50 μ L each of Reagent A
518 (Avidin, ABC) and Reagent B (Biotinylated HRP, ABC) to 5 mL of PBS. Then,
519 add 50 μ L/well of VECTASTAIN ABC Reagent and incubate for 30 min at 37°C.
- 520 9. Wash cells three times with 100 μ L/well of PBS. Remove PBS, and dry the plate
521 by gently blotting on paper towels. Prepare developing solution by following

522 manufacturer's instructions (DAB Substrate Kit, Peroxidase (HRP), with Nickel;
523 Vector Laboratory).

524 10. Add 50 μ L of developing solution to each well and wait until visible plaques are
525 observed.

526 11. Stop the reaction by removing the developing solution, and wash with PBS. It is
527 important not to wait for too long once the plaques are discernible to prevent the
528 entire cells from turning black.

529 12. Take images and measure the stained positive cells using a CTL ImmunoSpot
530 plate reader and counting software (Cellular Technology Limited, Cleveland, OH,
531 USA). The formula to calculate percent viral infection for each concentration is
532 given as [(Average # of plaques from each treated wells – average # of plaques
533 from “no virus” wells)/(average # of plaques from “virus only” wells - average # of
534 plaques from “no virus” wells)] x 100. A non-linear regression curve fit analysis
535 over the dilution curve can be performed using GraphPad Prism to calculate NT₅₀
536 or effective concentration 50 (EC₅₀) of the Ab or antiviral (**Figures 2 and 5,**
537 **respectively**).

538

539 **Infrared staining**

540 1. Follow steps 1 to 6 as described for peroxidase staining.

541 2. After 1h of incubation with primary Ab (1C7, 1 μ g/mL), wash cells three times
542 with 100 μ L/well of PBS. Next, prepare secondary Ab (IRDye 800CW goat anti-
543 mouse IgG, 1:1,000 dilution) to stain virus-infected cells, and DRAQ5™

544 Fluorescent Probe Solution (1:4,000 dilution) to stain the nucleus using 1% BSA
545 in PBS, and incubate for 1 h at RT.

546 3. Wash the cells three times with 100 μ L/well of PBS to remove the secondary Ab
547 and DRAQ5 thoroughly. Add 100 μ L/well of PBS.

548 4. Obtain images and measure signal values of the stained positive cells with 700
549 nm (800CW, measuring viral infection) and 800 nm (DRAQ5, measuring cell
550 viability) using Odyssey Sa Infrared Imaging System (LI-COR Biosciences, NE,
551 USA). The formula to calculate percent viral infection (800 nm measurement) or
552 cell viability (700 nm measurement) for each concentration is given as [(Average
553 signal intensity from each treated wells – average signal intensity from “no virus”
554 wells)/(average signal intensity from “virus only” wells - average signal intensity
555 from “no virus” wells)] x 100. A non-linear regression curve fit analysis over the
556 dilution curve can be performed using GraphPad Prism to calculate NT₅₀ or EC₅₀
557 of the Ab or antiviral (**Figures 3 and 6, respectively**).

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578 **Discussion**

579 As COVID-19 cases continue to increase across the globe, there is an urgent need to
580 identify effective and safe prophylactics and/or therapeutics for the treatment of SARS-
581 CoV-2 in infected patients. In this study, we described robust assays and staining
582 techniques which can be employed to evaluate the neutralizing and/or antiviral activity
583 of Abs and/or antivirals, respectively, against SARS-CoV-2 *in vitro*.

584 The PRMNT assay described here measures the neutralization effect of Abs or the
585 antiviral activity of compounds against SARS-CoV-2 in similar manner to what we have
586 previously described with other viruses, including, among others, influenza (Baker et al.,
587 2015; Bauman et al., 2013; Martinez-Sobrido et al., 2010; Nogales et al., 2019; Nogales,
588 Baker, and Martinez-Sobrido, 2015; Nogales et al., 2018; Nogales et al., 2016; Park et
589 al., 2020a; Piepenbrink et al., 2019; Rodriguez, Nogales, and Martinez-Sobrido, 2017;

590 Yang et al., 2016), mammarenaviruses (Brouillette et al., 2018; Ortiz-Riano et al., 2014;
591 Robinson et al., 2016; Rodrigo, de la Torre, and Martinez-Sobrido, 2011), and Zika virus
592 (Park et al., 2019). Although the assay described here was adapted to 96-well plate
593 format, it can be adjusted to 384-well plate format for HTS of SARS-CoV-2 antivirals
594 and/or NAbs. Many factors, including virus isolate, virus titer, cell condition or
595 confluency, nature of samples, and incubation period can affect the outcome of this
596 assay (Amanat et al., 2020). It is important to optimize these conditions so as to be able
597 to generate accurate and reproducible data between different laboratories.

598 To restrict virus spread in infected cells, it is paramount to incubate the infected cells
599 with post-infection media containing 1% Avicel. Incubation without Avicel will enhance a
600 rapid cell-to-cell spread of virus resulting in higher NT_{50} or EC_{50} . Although this assay
601 works best in our hands using 1% Avicel, other reagents, such as 0.75%
602 carboxymethylcellulose (CMC) can also be used (Oladunni et al., 2019).

603 Both the peroxidase and the infrared staining described here measures the level of
604 SARS-CoV-2 infection based on the staining SARS-CoV-2 NP. However, the staining
605 techniques can also be adapted for Abs that recognizes other SARS-CoV-2 proteins.
606 While both staining techniques are comparable with regards to NT_{50} and EC_{50} values,
607 the infrared staining offers the advantage of measuring virus replication and cell viability
608 in a single assay, saving time and reagents needed to separately evaluate cytotoxicity.

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624 **Conflicts of interest**

625 J.G.P., F.S.O., M.P., M.W., J.K., and L.M.S. are listed as inventors on a pending patent
626 application describing the SARS-CoV-2 antibody 1207B4.

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647 **Acknowledgments**

648 We want to thank BEI Resources for providing the SARS-CoV-2 USA-WA1/2020 isolate
649 (NR-52281). We want to thank Maritza Quintero for her technical assistance with the
650 infrared staining with the Odyssey Sa Infrared Imaging System. We would also like to
651 thank members at our institutes for their efforts in keeping them fully operational during
652 the COVID-19 pandemic and the IBC committee for reviewing our protocols in a time
653 efficient manner. We would like to dedicate this manuscript to all COVID-19 victims and
654 to all heroes battling this disease.

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670 **Figure legends**

671 **Figure 1. Schematic representation of the PRMNT assay to identify SARS-CoV-2**

672 **NAbs:** Confluent monolayers of Vero E6 cells (96-well plate format, $\sim 4 \times 10^4$ cells/well,
673 triplicates) are infected with ~ 100 - 200 PFU of SARS-CoV-2 pre-incubated **(A)** or post-
674 treated **(B)** with a 2-fold serially diluted mAbs, pAbs, and/or serum samples. Cells in
675 rows 11 and 12 are incubated as virus only and mock only infected cells, respectively,
676 and are used as internal controls in the assay. After 1 h absorption of the virus-mAb
677 mixture **(A)** or virus alone **(B)**, infected cells are washed 3X with PBS and post-infection
678 media containing 1% Avicel is added in all the wells alone **(A)** or with the 2-fold serially
679 diluted mAbs and/or serum samples **(B)**. At 24 h p.i., cells in the 96-well plate are fixed
680 with 10% formalin solution. After 24 h, fixed cells are washed 3X with PBS and
681 incubated with $1\mu\text{g}/\text{mL}$ of a SARS-CoV-1 cross-reactive NP mAb (1C7, $1\mu\text{g}/\text{mL}$) at

682 37°C. After 1 h incubation with the primary Ab, cells are washed 3X with PBS and
683 incubated with a secondary POD (**Fig. 2**) or IRDye 800CW goat anti-mouse IgG
684 secondary Ab (**Fig. 3**) at 37°C. After 30 minutes incubation with the secondary Ab, cells
685 are washed 3X with PBS and developed with the DAB substrate kit (**Fig. 2**). Cells
686 stained with the IRDye 800CW goat anti-mouse IgG secondary Ab are simultaneously
687 incubated with DRAQ5TM Fluorescent Probe Solution for nuclear staining (**Fig. 3**).
688 Positive staining plaques in each of the wells of the 96-well plate is quantified using an
689 ELISPOT plate reader (**Fig. 2**) or an Odyssey Sa Infrared Imaging System (**Fig. 3**). The
690 neutralizing titer 50 (NT₅₀) is calculated as the highest dilution of the mAb or sera that
691 prevents 50% plaque formation in infected cells, determined by a sigmoidal dose
692 response curve (**Figs. 2 and 3**).

693 **Figure 2. PRMNT assay to identify SARS-CoV-2 NAb, peroxidase staining. A)**

694 **Pre-treatment:** 2-fold serially diluted mAbs, pAbs, or serum samples are pre-incubated
695 with ~100-200 PFU of SARS-CoV-2 for 1 h at RT. After 1 h pre-incubation, confluent
696 monolayers of Vero E6 cells (96-well plate format, ~4 x 10⁴ cells/well, triplicates) are
697 infected with the mAb/pAb/serum-virus mixture for 1 h at RT. Cells in row 11 are
698 incubated with virus only and cells in row are mock-infected and are used as internal
699 controls in each of the plates. After 1 h of virus adsorption, cells are washed 3X with
700 PBS and post-infection media containing 1% Avicel is added in all the wells. **B) Post-**
701 **infection:** confluent monolayers of Vero E6 cells (96-well plate format, ~4 x 10⁴
702 cells/well, triplicates) are infected with ~100-200 PFU/well of SARS-CoV-2. After 1 h of
703 virus adsorption, the infection media is replaced with post-infection media containing 1%
704 Avicel and 2-fold serially diluted mAbs, pAbs or serum samples. Cells in row 11 are

705 incubated with virus only and cells in row are mock-infected and are used as internal
706 controls in each of the plates. **A and B)** At 24 h p.i., cells are fixed with 10% formalin
707 solution. After 24 h fixation, cells are washed 3X with PBS and incubated with 1 μ g/mL of
708 a SARS-CoV-1 cross-reactive NP mAb (1C7) at 37°C. After 1 h incubation with the
709 primary mAb, cells are washed 3X with PBS and incubated with a secondary POD anti-
710 mouse Ab (diluted according to the manufacturer's instruction) at 37°C. After 30 minutes
711 incubation with the secondary Ab, cells are washed 3X with PBS and developed with
712 the DAB substrate kit. Positive staining plaques in each of the wells are quantified using
713 an ELISPOT plate reader. The NT₅₀ is calculated as the highest dilution of the mAb,
714 pAb or sera that prevents 50% plaque formation in infected cells, determined by a
715 sigmoidal dose response curve. Dotted line indicates 50% neutralization. Data were
716 expressed as mean and SD from triplicate wells.

717 **Figure 3. PRMNT assay to identify SARS-CoV-2 NAb, fluorescent staining. A-B)**

718 Confluent monolayers of Vero E6 cells (96-well plate format, $\sim 4 \times 10^4$ cells/well,
719 triplicates) are infected, fixed and staining with the primary NP 1C7 mAb as described in
720 **Figure 2**. After incubation with the primary Ab, cells are incubated, for 1 h, with IRDye
721 800CW goat anti-mouse IgG secondary Ab, and DRAQ5TM Fluorescent Probe Solution
722 for nuclear staining. After 1 h incubation with the secondary Ab and nuclear staining
723 solution, cells are washed 3X with PBS and imaged using an Odyssey Sa infrared
724 imaging system. The neutralizing titer 50 (NT₅₀) is calculated as the highest dilution of
725 the mAb, pAb, or sera that prevents 50% plaque formation in infected cells, determined
726 by a sigmoidal dose response curve. Dotted line indicates 50% neutralization. Data
727 were expressed as mean and SD from triplicate wells.

728 **Figure 4. Schematic representation of the PRMNT assay to identify SARS-CoV-2**
729 **antivirals:** Confluent monolayers of Vero E6 cells (96-well plate format, $\sim 4 \times 10^4$
730 cells/well, triplicates) are infected with ~ 100 - 200 PFU of SARS-CoV-2 for 1 h at 37°C .
731 Cells in rows 11 and 12 are incubated with virus only and mock-infected, respectively,
732 and are used as internal controls in each of the plates. After 1 h viral absorption, cells
733 are incubated with post-infection media containing 2-fold serial dilutions of the antivirals
734 containing 1% Avicel. At 24 h p.i., cells are fixed with 10% formalin solution. After 24 h
735 fixation, cells are washed 3X with PBS and incubated with $1 \mu\text{g/mL}$ of the SARS-CoV-1
736 cross-reactive NP mAb (1C7) at 37°C . After 1 h incubation with the primary mAb, cells
737 are washed 3X with PBS and incubated with a secondary POD (**Fig. 5**) or with IRDye
738 800CW goat anti-mouse IgG secondary Ab, and DRAQ5TM Fluorescent Probe Solution
739 for nuclear staining (**Fig. 6**) at 37°C . After 30 minutes incubation with the secondary
740 POD Ab, cells are washed 3X with PBS and developed with the DAB substrate kit (**Fig.**
741 **5**). Cells stained with the IRDye 800CW goat anti-mouse IgG secondary Ab are
742 simultaneously incubated with DRAQ5TM Fluorescent Probe Solution for nuclear
743 staining (**Fig. 6**). Positive stained cells in each of the wells of the 96-well plate are
744 quantified using an ELISPOT plate reader (**Fig. 5**) or in the Odyssey Sa Infrared
745 Imaging System (**Fig. 6**). The effective concentration 50 (EC_{50}) is calculated as the
746 highest dilution of the drug that prevents 50% plaque formation in infected cells,
747 determined by a sigmoidal dose response curve (**Figs. 5 and 6**).

748 **Figure 5. A PRMNT assay to identify SARS-CoV-2 antivirals, peroxidase staining:**
749 Confluent monolayers of Vero E6 cells (96-well plate format, $\sim 4 \times 10^4$ cells/well,
750 triplicates) are infected with ~ 100 - 200 PFU/well of SARS-CoV-2. After 1 h of virus

751 adsorption, media is replaced with fresh infection media containing 1% Avicel and 2-fold
752 serially diluted drugs. Cells in row 11 are incubated with virus only and cells in row are
753 mock-infected and are used as internal controls in each of the plates. At 24 h p.i., cells
754 are fixed with 10% formalin solution. After 24 h fixation, cells are washed 3X with PBS
755 and incubated with 1µg/mL of a SARS-CoV-1 cross-reactive NP mAb (1C7) at 37°C.
756 After 1 h incubation with the primary mAb, cells are washed 3X with PBS and incubated
757 with a secondary POD anti-mouse Ab (diluted according to the manufacturer's
758 instruction) at 37°C. After 30 minutes incubation with the secondary Ab, cells are
759 washed 3X with PBS and developed with the DAB substrate kit. Positive staining
760 plaques in each of the wells are quantified using an ELISPOT plate reader. The
761 effective concentration 50 (EC₅₀) is calculated as the highest dilution of the mAb, pAb or
762 sera that prevents 50% plaque formation in infected cells, determined by a sigmoidal
763 dose response curve. Dotted line indicates 50% neutralization. Data were expressed as
764 mean and SD from triplicate wells.

765 **Figure 6. A PRMNT assay to identify SARS-CoV-2 antivirals, fluorescent staining:**

766 Confluent monolayers of Vero E6 cells (96-well plate format, ~4 x 10⁴ cells/well,
767 triplicates) are infected, fixed and stained with the primary NP 1C7 mAb as described in

768 **Figure 5.** After 1 h incubation with the primary NP 1C7 mAb, cells are washed 3X with

769 PBS and incubated, at 37°C, with IRDye 800CW goat anti-mouse IgG secondary Ab
770 (1:1000), and DRAQ5TM Fluorescent Probe Solution (1:4000) for nuclear staining. After

771 1 h incubation with the secondary Ab and nuclear stain, cells are washed 3X with PBS

772 imaged using an Odyssey Sa Infrared Imaging System. The effective concentration 50

773 (EC₅₀) is calculated as the highest dilution of the mAb, pAb or sera that prevents 50%

774 plaque formation in infected cells, determined by a sigmoidal dose response curve.
775 Dotted line indicates 50% neutralization. Data were expressed as mean and SD from
776 triplicate wells.

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786 **References**

- 787 Amanat, F., White, K.M., Miorin, L., Strohmeier, S., McMahon, M., Meade, P., Liu, W.C.,
788 Albrecht, R.A., Simon, V., Martinez-Sobrido, L., Moran, T., Garcia-Sastre, A. and
789 Krammer, F., 2020. An In Vitro Microneutralization Assay for SARS-CoV-2
790 Serology and Drug Screening. *Curr Protoc Microbiol* 58, e108.
- 791 Baker, S.F., Nogales, A., Santiago, F.W., Topham, D.J. and Martinez-Sobrido, L., 2015.
792 Competitive detection of influenza neutralizing antibodies using a novel bivalent
793 fluorescence-based microneutralization assay (BiFMA). *Vaccine* 33, 3562-70.
- 794 Bauman, J.D., Patel, D., Baker, S.F., Vijayan, R.S., Xiang, A., Parhi, A.K., Martinez-
795 Sobrido, L., LaVoie, E.J., Das, K. and Arnold, E., 2013. Crystallographic fragment

796 screening and structure-based optimization yields a new class of influenza
797 endonuclease inhibitors. *ACS Chem Biol* 8, 2501-8.

798 Bogoch, Il, Watts, A., Thomas-Bachli, A., Huber, C., Kraemer, M.U.G. and Khan, K.,
799 2020. Potential for global spread of a novel coronavirus from China. *J Travel Med*
800 27.

801 Brouillette, R.B., Phillips, E.K., Patel, R., Mahauad-Fernandez, W., Moller-Tank, S.,
802 Rogers, K.J., Dillard, J.A., Cooney, A.L., Martinez-Sobrido, L., Okeoma, C. and
803 Maury, W., 2018. TIM-1 Mediates Dystroglycan-Independent Entry of Lassa
804 Virus. *J Virol* 92.

805 Buonaguro, L., Tagliamonte, M., Tornesello, M.L. and Buonaguro, F.M., 2020. SARS-
806 CoV-2 RNA polymerase as target for antiviral therapy. *J Transl Med* 18, 185.

807 Chary, M.A., Barbuto, A.F., Izadmehr, S., Hayes, B.D. and Burns, M.M., 2020. COVID-
808 19: Therapeutics and Their Toxicities. *J Med Toxicol* 16, 284-294.

809 Choy, K.T., Wong, A.Y., Kaewpreedee, P., Sia, S.F., Chen, D., Hui, K.P.Y., Chu,
810 D.K.W., Chan, M.C.W., Cheung, P.P., Huang, X., Peiris, M. and Yen, H.L., 2020.
811 Remdesivir, lopinavir, emetine, and homoharringtonine inhibit SARS-CoV-2
812 replication in vitro. *Antiviral Res* 178, 104786.

813 Elfiky, A.A., 2020. Ribavirin, Remdesivir, Sofosbuvir, Galidesivir, and Tenofovir against
814 SARS-CoV-2 RNA dependent RNA polymerase (RdRp): A molecular docking
815 study. *Life Sci* 253, 117592.

816 Ferner, R.E. and Aronson, J.K., 2020. Remdesivir in covid-19. *BMJ* 369, m1610.

817 Fox, R.I., 1993. Mechanism of action of hydroxychloroquine as an antirheumatic drug.
818 *Semin Arthritis Rheum* 23, 82-91.

- 819 Gordon, C.J., Tchesnokov, E.P., Feng, J.Y., Porter, D.P. and Gotte, M., 2020. The
820 antiviral compound remdesivir potently inhibits RNA-dependent RNA polymerase
821 from Middle East respiratory syndrome coronavirus. *J Biol Chem* 295, 4773-
822 4779.
- 823 Imai, M., Iwatsuki-Horimoto, K., Hatta, M., Loeber, S., Halfmann, P.J., Nakajima, N.,
824 Watanabe, T., Ujje, M., Takahashi, K., Ito, M., Yamada, S., Fan, S., Chiba, S.,
825 Kuroda, M., Guan, L., Takada, K., Armbrust, T., Balogh, A., Furusawa, Y.,
826 Okuda, M., Ueki, H., Yasuhara, A., Sakai-Tagawa, Y., Lopes, T.J.S., Kiso, M.,
827 Yamayoshi, S., Kinoshita, N., Ohmagari, N., Hattori, S.I., Takeda, M., Mitsuya,
828 H., Krammer, F., Suzuki, T. and Kawaoka, Y., 2020. Syrian hamsters as a small
829 animal model for SARS-CoV-2 infection and countermeasure development. *Proc*
830 *Natl Acad Sci U S A*.
- 831 Juurlink, D.N., 2020. Safety considerations with chloroquine, hydroxychloroquine and
832 azithromycin in the management of SARS-CoV-2 infection. *CMAJ* 192, E450-
833 E453.
- 834 Li, F., 2016. Structure, Function, and Evolution of Coronavirus Spike Proteins. *Annu*
835 *Rev Virol* 3, 237-261.
- 836 Martinez-Sobrido, L., Cadagan, R., Steel, J., Basler, C.F., Palese, P., Moran, T.M. and
837 Garcia-Sastre, A., 2010. Hemagglutinin-pseudotyped green fluorescent protein-
838 expressing influenza viruses for the detection of influenza virus neutralizing
839 antibodies. *J Virol* 84, 2157-63.
- 840 Masters, P.S., 2006. The molecular biology of coronaviruses. *Adv Virus Res* 66, 193-
841 292.

842 Matsuyama, S., Nao, N., Shirato, K., Kawase, M., Saito, S., Takayama, I., Nagata, N.,
843 Sekizuka, T., Katoh, H. and Kato, F.J.P.o.t.N.A.o.S., 2020. Enhanced isolation of
844 SARS-CoV-2 by TMPRSS2-expressing cells 117, 7001-7003.

845 Montefiori, D.C., 1997. Role of complement and Fc receptors in the pathogenesis of
846 HIV-1 infection. *Springer Semin Immunopathol* 18, 371-90.

847 Mortola, E. and Roy, P., 2004. Efficient assembly and release of SARS coronavirus-like
848 particles by a heterologous expression system. *FEBS Lett* 576, 174-8.

849 Nogales, A., Avila-Perez, G., Rangel-Moreno, J., Chiem, K., DeDiego, M.L. and
850 Martinez-Sobrido, L., 2019. A Novel Fluorescent and Bioluminescent Bireporter
851 Influenza A Virus To Evaluate Viral Infections. *J Virol* 93.

852 Nogales, A., Baker, S.F. and Martinez-Sobrido, L., 2015. Replication-competent
853 influenza A viruses expressing a red fluorescent protein. *Virology* 476, 206-216.

854 Nogales, A., Piepenbrink, M.S., Wang, J., Ortega, S., Basu, M., Fucile, C.F., Treanor,
855 J.J., Rosenberg, A.F., Zand, M.S., Keefer, M.C., Martinez-Sobrido, L. and Kobie,
856 J.J., 2018. A Highly Potent and Broadly Neutralizing H1 Influenza-Specific
857 Human Monoclonal Antibody. *Sci Rep* 8, 4374.

858 Nogales, A., Rodriguez-Sanchez, I., Monte, K., Lenschow, D.J., Perez, D.R. and
859 Martinez-Sobrido, L., 2016. Replication-competent fluorescent-expressing
860 influenza B virus. *Virus Res* 213, 69-81.

861 Oladunni, F.S., Sarkar, S., Reedy, S., Balasuriya, U.B.R., Horohov, D.W. and
862 Chambers, T.M., 2019. Equid Herpesvirus 1 Targets the Sensitization and
863 Induction Steps To Inhibit the Type I Interferon Response in Equine Endothelial
864 Cells. *J Virol* 93.

- 865 Organization, W.H., Coronavirus disease 2019 (COVID-19) Situation Report-51.
866 [https://www.who.int/docs/default-source/coronaviruse/situation-](https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200311-sitrep-51-covid-19.pdf?sfvrsn=1ba62e57_10)
867 [reports/20200311-sitrep-51-covid-19.pdf?sfvrsn=1ba62e57_10](https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200311-sitrep-51-covid-19.pdf?sfvrsn=1ba62e57_10) (Accessed on 5th
868 of July, 2020).
- 869 Ortiz-Riano, E., Ngo, N., Devito, S., Eggink, D., Munger, J., Shaw, M.L., de la Torre,
870 J.C. and Martinez-Sobrido, L., 2014. Inhibition of arenavirus by A3, a pyrimidine
871 biosynthesis inhibitor. *J Virol* 88, 878-89.
- 872 Park, J.G., Avila-Perez, G., Madere, F., Hilimire, T.A., Nogales, A., Almazan, F. and
873 Martinez-Sobrido, L., 2019. Potent Inhibition of Zika Virus Replication by
874 Aurintricarboxylic Acid. *Front Microbiol* 10, 718.
- 875 Park, J.G., Avila-Perez, G., Nogales, A., Blanco-Lobo, P., de la Torre, J.C. and
876 Martinez-Sobrido, L., 2020a. Identification and Characterization of Novel
877 Compounds with Broad-Spectrum Antiviral Activity against Influenza A and B
878 Viruses. *J Virol* 94.
- 879 Park, S.J., Yu, K.M., Kim, Y.I., Kim, S.M., Kim, E.H., Kim, S.G., Kim, E.J., Casel,
880 M.A.B., Rollon, R., Jang, S.G., Lee, M.H., Chang, J.H., Song, M.S., Jeong, H.W.,
881 Choi, Y., Chen, W., Shin, W.J., Jung, J.U. and Choi, Y.K., 2020b. Antiviral
882 Efficacies of FDA-Approved Drugs against SARS-CoV-2 Infection in Ferrets.
883 *mBio* 11.
- 884 Perlman, S. and Netland, J.J.N.r.m., 2009. Coronaviruses post-SARS: update on
885 replication and pathogenesis *7*, 439-450.
- 886 Piepenbrink, M.S., Nogales, A., Basu, M., Fucile, C.F., Liesveld, J.L., Keefer, M.C.,
887 Rosenberg, A.F., Martinez-Sobrido, L. and Kobie, J.J., 2019. Broad and

- 888 Protective Influenza B Virus Neuraminidase Antibodies in Humans after
889 Vaccination and their Clonal Persistence as Plasma Cells. *mBio* 10.
- 890 Reihani, H., Ghassemi, M., Mazer-Amirshahi, M., Aljohani, B. and Pourmand, A., 2020.
891 Non-evidenced based treatment: An unintended cause of morbidity and mortality
892 related to COVID-19. *Am J Emerg Med*.
- 893 Riou, B., Barriot, P., Rimalho, A. and Baud, F.J., 1988. Treatment of severe
894 chloroquine poisoning. *N Engl J Med* 318, 1-6.
- 895 Robinson, J.E., Hastie, K.M., Cross, R.W., Yenni, R.E., Elliott, D.H., Rouelle, J.A.,
896 Kannadka, C.B., Smira, A.A., Garry, C.E., Bradley, B.T., Yu, H., Shaffer, J.G.,
897 Boisen, M.L., Hartnett, J.N., Zandonatti, M.A., Rowland, M.M., Heinrich, M.L.,
898 Martinez-Sobrido, L., Cheng, B., de la Torre, J.C., Andersen, K.G., Goba, A.,
899 Momoh, M., Fullah, M., Gbakie, M., Kanneh, L., Koroma, V.J., Fonnies, R., Jalloh,
900 S.C., Kargbo, B., Vandi, M.A., Gbetuwa, M., Ikponmwosa, O., Asogun, D.A.,
901 Okokhere, P.O., Follarin, O.A., Schieffelin, J.S., Pitts, K.R., Geisbert, J.B.,
902 Kulakoski, P.C., Wilson, R.B., Happi, C.T., Sabeti, P.C., Gevaio, S.M., Khan,
903 S.H., Grant, D.S., Geisbert, T.W., Saphire, E.O., Branco, L.M. and Garry, R.F.,
904 2016. Most neutralizing human monoclonal antibodies target novel epitopes
905 requiring both Lassa virus glycoprotein subunits. *Nat Commun* 7, 11544.
- 906 Rodrigo, W.W., de la Torre, J.C. and Martinez-Sobrido, L., 2011. Use of single-cycle
907 infectious lymphocytic choriomeningitis virus to study hemorrhagic fever
908 arenaviruses. *J Virol* 85, 1684-95.
- 909 Rodriguez, L., Nogales, A. and Martinez-Sobrido, L., 2017. Influenza A Virus Studies in
910 a Mouse Model of Infection. *J Vis Exp*.

- 911 Schoeman, D. and Fielding, B.C., 2019. Coronavirus envelope protein: current
912 knowledge. *Virology* 16, 69.
- 913 Shang, J., Wan, Y., Luo, C., Ye, G., Geng, Q., Auerbach, A. and Li, F.J.P.o.t.N.A.o.S.,
914 2020. Cell entry mechanisms of SARS-CoV-2 117, 11727-11734.
- 915 Wang, C., Li, W., Drabek, D., Okba, N.M.A., van Haperen, R., Osterhaus, A., van
916 Kuppeveld, F.J.M., Haagmans, B.L., Grosveld, F. and Bosch, B.J., 2020. A
917 human monoclonal antibody blocking SARS-CoV-2 infection. *Nature Communications* 11,
918 2251.
- 919 Wang, C., Zheng, X., Gai, W., Zhao, Y., Wang, H., Wang, H., Feng, N., Chi, H., Qiu, B.,
920 Li, N., Wang, T., Gao, Y., Yang, S. and Xia, X., 2017. MERS-CoV virus-like
921 particles produced in insect cells induce specific humoral and cellular immunity
922 in rhesus macaques. *Oncotarget* 8, 12686-12694.
- 923 Wu, F., Wang, A., Liu, M., Wang, Q., Chen, J., Xia, S., Ling, Y., Zhang, Y., Xun, J. and
924 Lu, L., 2020. Neutralizing antibody responses to SARS-CoV-2 in a COVID-19
925 recovered patient cohort and their implications.
- 926 Xu, J., Shi, P.Y., Li, H. and Zhou, J., 2020. Broad Spectrum Antiviral Agent Niclosamide
927 and Its Therapeutic Potential. *ACS Infectious Diseases* 6, 909-915.
- 928 Yang, H., Baker, S.F., Gonzalez, M.E., Topham, D.J., Martinez-Sobrido, L., Zand, M.,
929 Holden-Wiltse, J. and Wu, H., 2016. An improved method for estimating antibody
930 titers in microneutralization assay using green fluorescent protein. *Journal of Biopharmaceutical
931 Statistics* 26, 409-20.
- 932 Yao, X., Ye, F., Zhang, M., Cui, C., Huang, B., Niu, P., Liu, X., Zhao, L., Dong, E., Song,
933 C., Zhan, S., Lu, R., Li, H., Tan, W. and Liu, D., 2020. In Vitro Antiviral Activity

934 and Projection of Optimized Dosing Design of Hydroxychloroquine for the
935 Treatment of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2).
936 Clin Infect Dis.
937

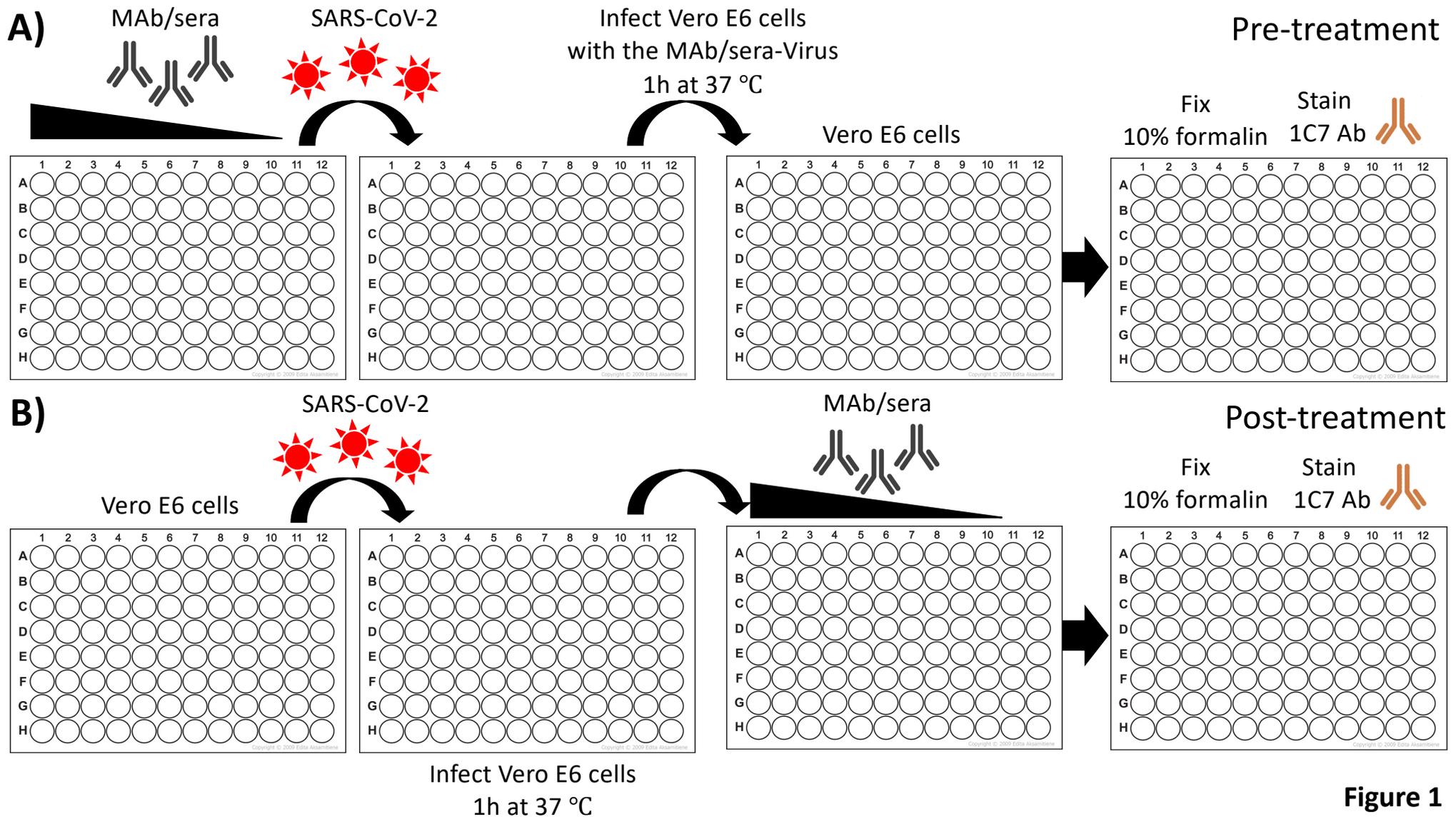


Figure 1

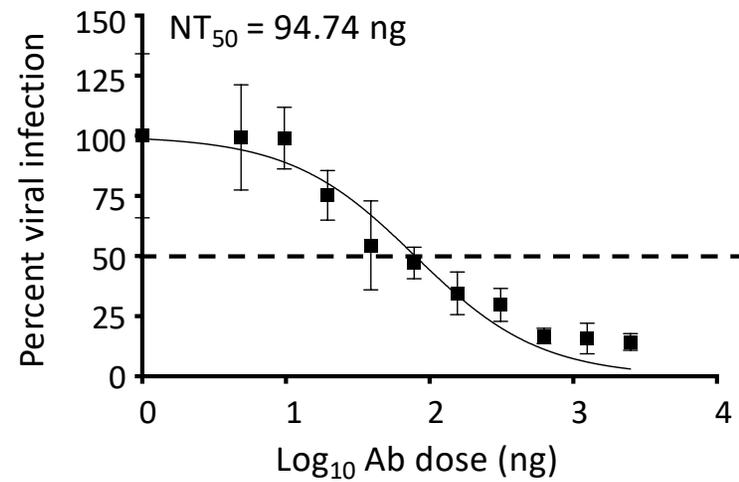
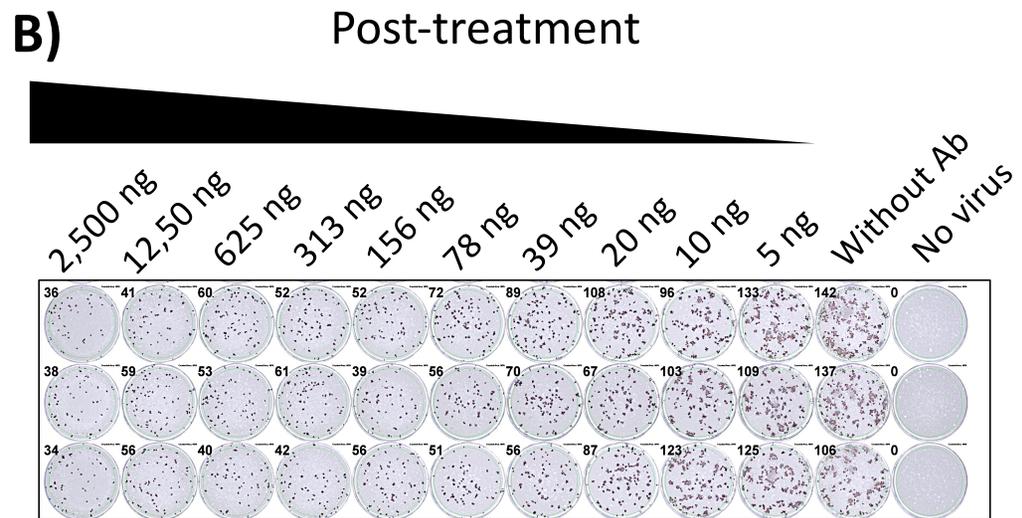
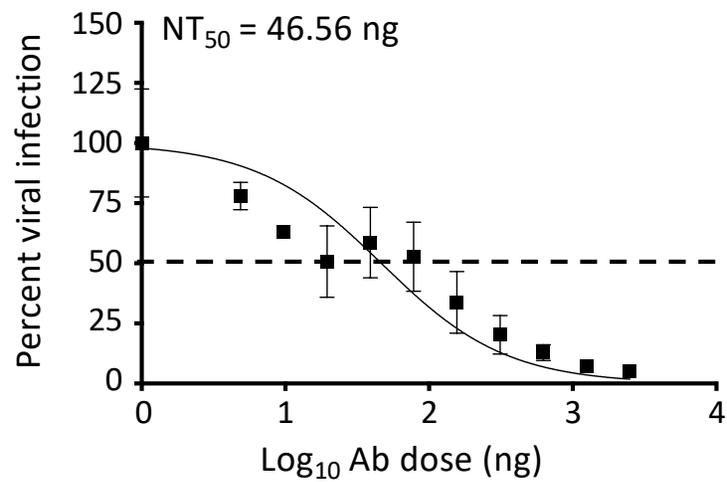
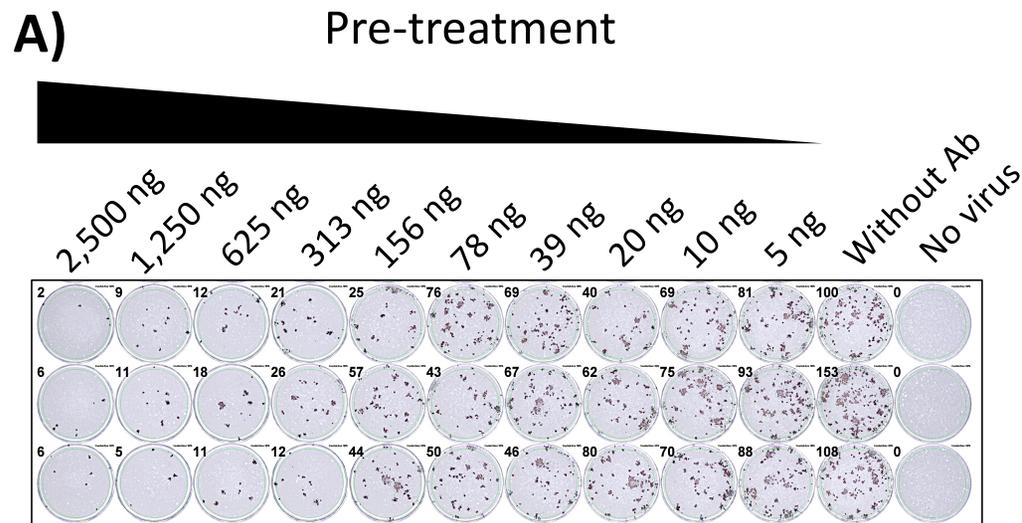


Figure 2

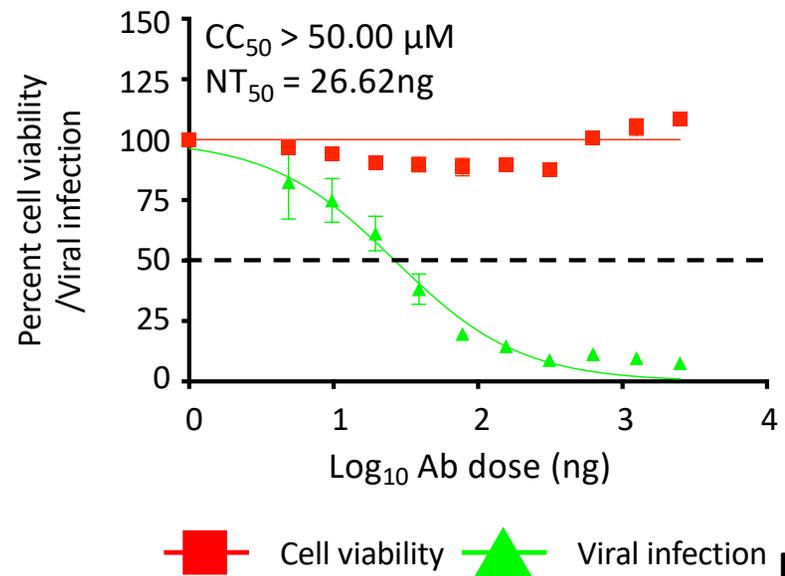
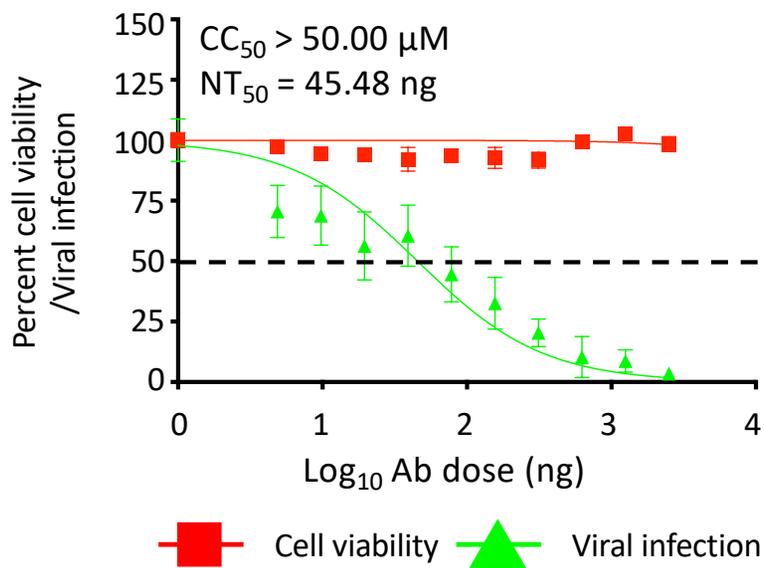
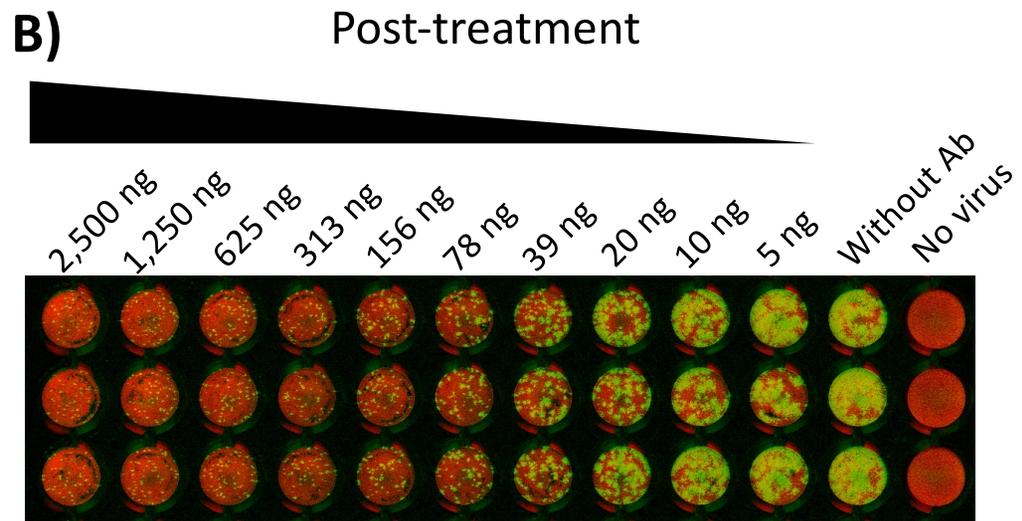
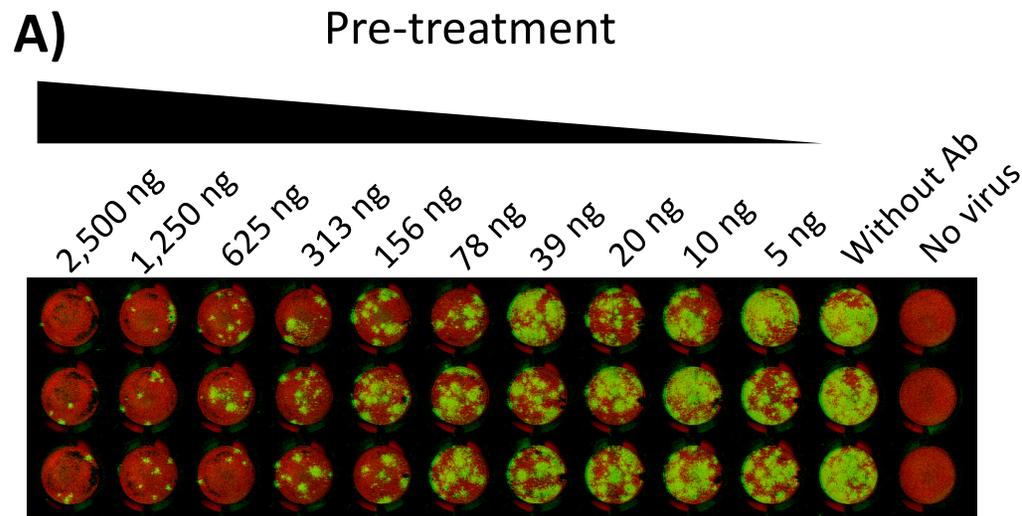


Figure 3

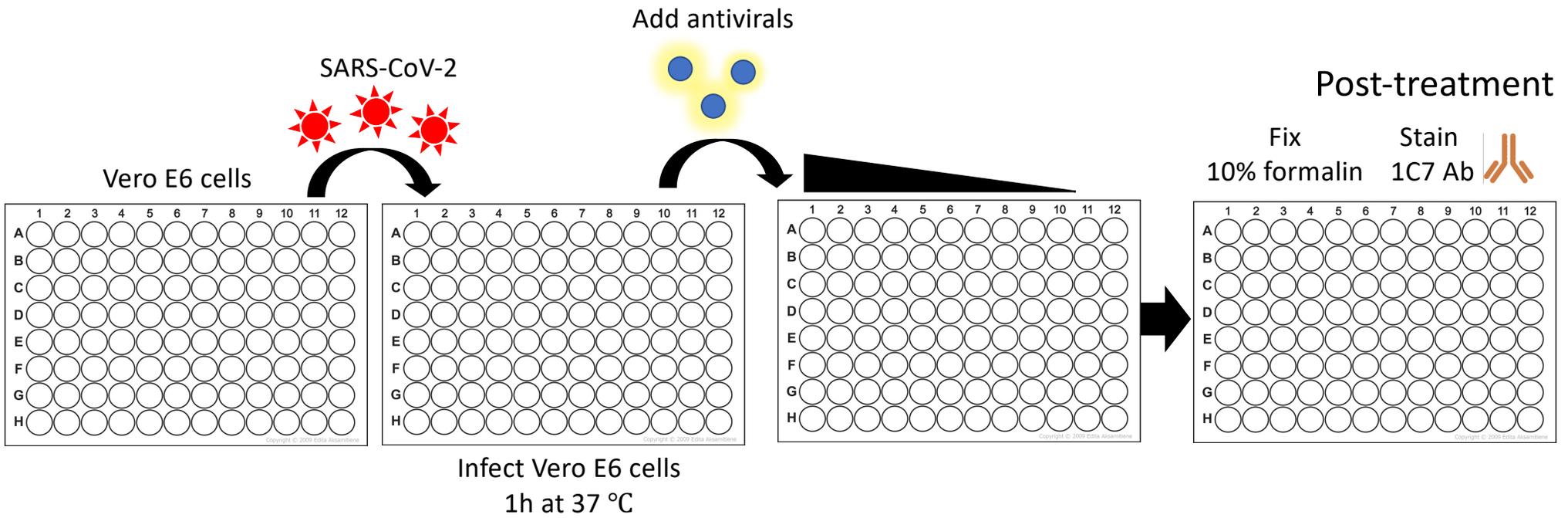


Figure 4

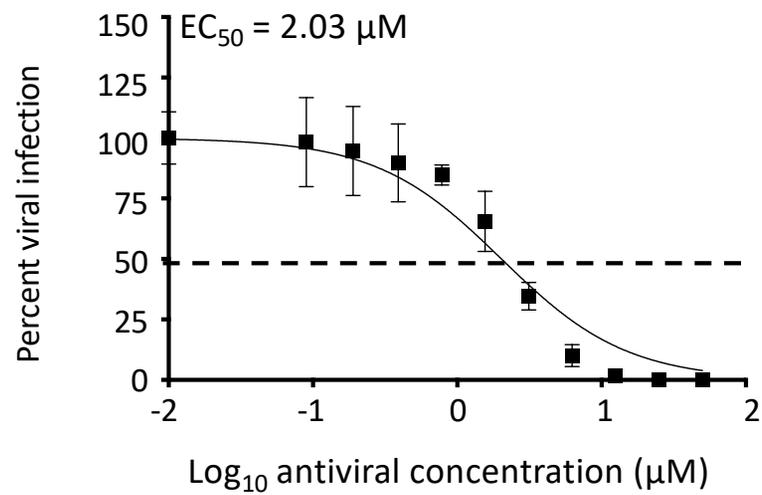
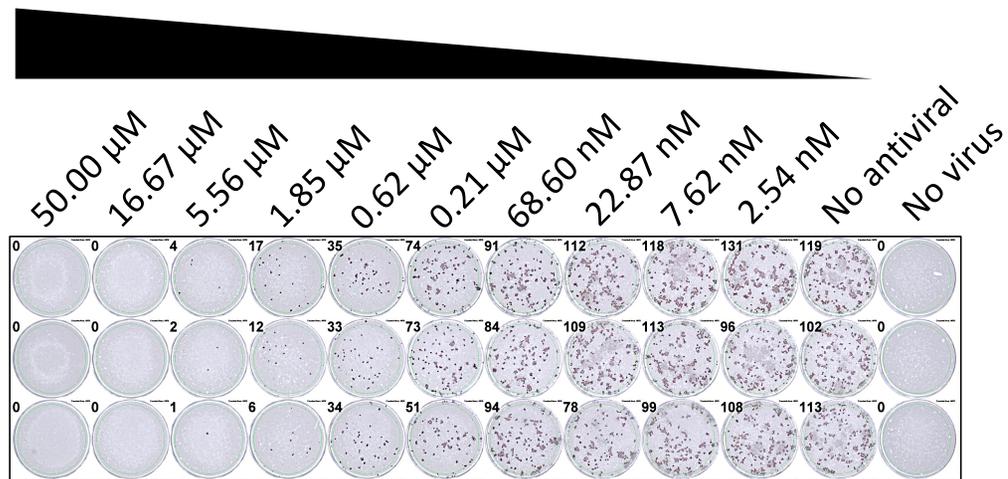


Figure 5

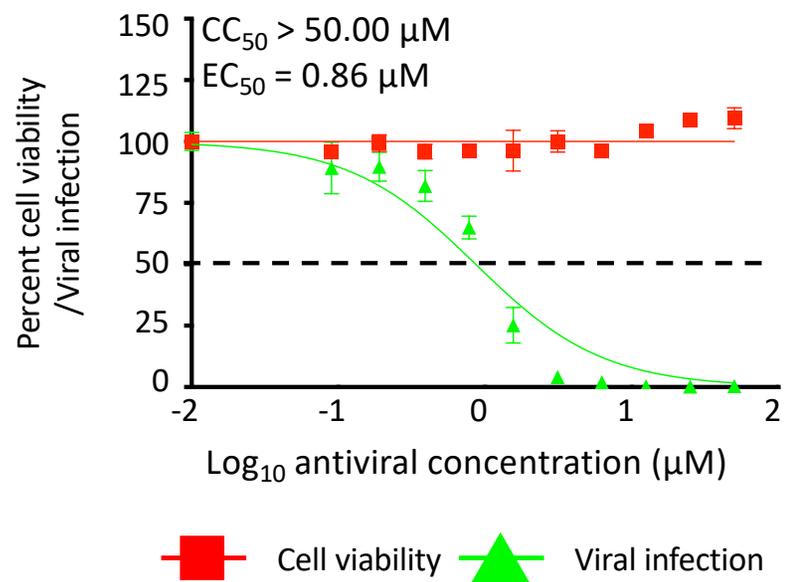
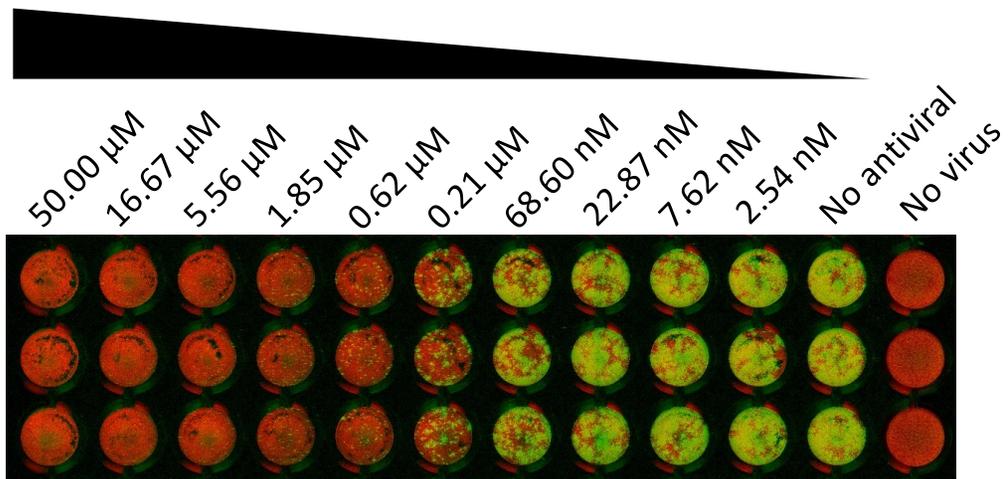


Figure 6