

1 Neutralization Assay with SARS-CoV-1 and SARS- 2 CoV-2 Spike Pseudotyped Murine Leukemia Virions

3 Yue Zheng ¹, Erin T. Larragoite ¹, Juan Lama ², Isabel Cisneros ², Julio C. Delgado ^{1,3}, Patricia
4 Slev ^{1,3}, Jenna Rychert ³, Emily A. Innis ¹, Elizabeth S.C.P. Williams ¹, Mayte Coiras ⁴, Matthew T.
5 Rondina ⁵, Adam M. Spivak ⁶ and Vicente Planelles ^{1,*}

6 ¹ Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT.

7 ² RetroVirox, Inc., San Diego, CA.

8 ³ Associated Regional and University Pathologists (ARUP) Laboratories, Salt Lake City, UT.

9 ⁴ AIDS Immunopathology Unit, National Center of Microbiology (CNM), Instituto de Salud Carlos III,
10 Madrid, Spain.

11 ⁵ Department of Human Genetics, University of Utah School of Medicine, Salt Lake City, UT.

12 ⁶ Department of Medicine, University of Utah School of Medicine, Salt Lake City, UT.

13 * Correspondence: vicente.planelles@path.utah.edu

14 **Abstract:** Antibody neutralization is an important prognostic factor in many viral diseases. To easily
15 and rapidly measure titers of neutralizing antibodies in serum or plasma, we developed
16 pseudovirion particles composed of the spike glycoprotein of SARS-CoV-2 incorporated onto
17 murine leukemia virus capsids and a modified minimal MLV genome encoding firefly luciferase.
18 These pseudovirions provide a practical means of assessing immune responses under laboratory
19 conditions consistent with biocontainment level 2.

20 **Keywords:** COVID-19; coronavirus; SARS; SARS-CoV-2; neutralization assay; pseudotyped virus;
21 spike; murine leukemia virus; antibody
22

23 Introduction

24 Coronaviruses are a group of enveloped RNA viruses with a positive-sense single-stranded
25 RNA genome ranging from 26-32 kilobases, which can cause respiratory tract infections. In December
26 2019, a novel coronavirus known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)
27 was identified in China and has caused a global ongoing pandemic of coronavirus disease (COVID-
28 19). To date, SARS-CoV-2 has spread to 188 countries (<https://coronavirus.jhu.edu/>). More than 29
29 million cases and 900,000 deaths have been reported at the time of this writing.

30 Enveloped viruses are known to efficiently package their core elements with heterologous
31 envelope glycoproteins, giving rise to the so called 'pseudotypes' or 'pseudoviruses'. Many
32 laboratories have successfully generated pseudotypes containing the core elements of HIV-1 [1] or
33 MLV [2, 3] and the envelope glycoproteins of vesicular stomatitis virus [4], murine leukemia virus
34 [5], Lassa fever virus, ebola virus, coronavirus spike glycoproteins, and others (reviewed in [6]).

35 In a pseudotype virus, viral attachment [7], entry, and importantly, antibody binding and
36 neutralization sensitivity are dependent on the membrane glycoprotein provided [6]. Using a
37 defective MLV vector genome encoding *firefly* luciferase, and a packaging vector encoding MLV
38 gag/pol, we describe the production of pseudovirus particles containing the spike glycoprotein of
39 SARS-CoV-2. As controls, we also produced similar particles containing SARS-CoV-1, VSV-G or HIV-
40 1 LAI gp160.

41 Materials and Methods

42 Cells

43 HEK293FT cells, Vero E6 cells, SupT1 cells and Huh7 cells were purchased from ATCC.
44 HEK293FT, Vero E6 and Huh7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)
45 (Gibco, US) supplemented with 10% FBS (Gibco, US) and 2mM L-glutamine (Gibco, US) at 37°C with

46 5% CO₂. 293ACE2 cells were cultured in DMEM with 10% FBS, 2mM L-glutamine and 200ug/ml
47 hygromycin B (ThermoFisher, US).

48 *Plasmids*

49 SV-Psi-Env-MLV [8], pHIV-1 LAI gp160 [9], pHCMV-VSV-G [4] and pSIVmac gp130 [10] were
50 previously described. L-LUC-SN was constructed by inserting the *firefly* luciferase gene within the
51 polylinker of pLXSN (Clontech, cat# 631509). pSARS-CoV-1 was purchased from Sino Biologicals.
52 pCAGGS expressing SARS-CoV-2 RBD was obtained from BEI Resources (cat#NR-52309). HEK293T-
53 hACE2 cells were a gift from Adam Bailey and Emma Winkler and were constructed as follows. A
54 DNA fragment containing a codon-optimized version of hACE2 (Genbank NM_021804) was inserted
55 into pLV-EF1a-IRES-Hygro (Addgene Plasmid #85134) using Gibson assembly. 293T cells were then
56 transduced with lentivirus made from this construct. The plasmid pcDNA3.1-SARS-2-S-C9 was a
57 generous gift from Tom Gallagher and expresses a codon-optimized SARS-CoV-2 spike open reading
58 frame with a deletion in the 19 carboxy-terminal deletion amino acids (an endoplasmic reticulum
59 retention signal) and addition of the C9 peptide TETSQVAPA, recognized by antibody 1D4.

60 *Production of pseudotyped MLV*

61 The plasmid SV-Psi-Env-MLV and L-LUC-SN were co-transfected with or without an envelope
62 glycoprotein plasmid (pHCMV-VSV-G/pSARS-CoV-1/pSARS-CoV-2/pHIV-1 LAI gp160) into
63 HEK293FT cells using Lipofectamine™ 3000 (ThermoFisher, US). Cell supernatants containing
64 viruses were collected after 2 days of transfection. Viruses were filtered through a 0.45µm filter (VWR,
65 US) and centrifuged at 4°C, 6500rpm for 18h over a 20% sucrose cushion. Viruses were resuspended
66 in 500µl cell culture medium and stored at -80°C.

67 *Pseudovirus infection*

68 HEK293FT, 293T-ACE2, and Huh7 cells were seeded in 96-well plates (ThermoFisher, US) the
69 day before infection. SupT1 cells were added into a 96-well plate at the time of infection. 5x10⁴ cells
70 were added to each well. Pseudotyped MLV viruses were added to the pre-cultured cells. Cells were
71 cultured at 37°C with 5% CO₂ for 2 days. All cells in each well were lysed and luciferase was measured
72 using ONE-Glo™ Luciferase Assay reagent (Promega, US). RLU are per well of a 96-well plate.

73 *Neutralization assay*

74 293T-ACE2 cells were seeded in 96-well plates at 5x10⁴ cells per well the day prior to infection.
75 Sera from COVID-19-positive patients, negative sera, positive control (RBD) and negative control
76 (SIVgp130) were serially diluted in a volume of 100µL and pre-incubated with 50µL of pseudotyped
77 viruses at 37°C for 1h. For these infections, virus stocks were used at a dilution resulting in 100-200
78 RLU in the absence of serum. Cells were then infected with the serum/pseudovirion mixtures.
79 Luciferase was measured 48 hours post infection using ONE-Glo™ Luciferase Assay reagent.
80 Neutralization titers NT₅₀ and NT₈₀ were calculated using Prism 8 (GraphPad, US).

81

82 **Results**

83 To generate pseudovirion particles, three plasmids were co-transfected into HEK293FT cells. The
84 first plasmid was the packaging construct, SV-Psi-Env-MLV; the second plasmid was L-LUC-SN, a
85 minimal retroviral transfer vector encoding the *firefly* luciferase reporter gene; the third plasmid was
86 an expression construct encoding one of the following membrane viral glycoproteins: SARS-CoV
87 spike (hereafter referred to as SARS-CoV-1), SARS-CoV-2 spike, HIV-1 LAI gp160 and VSV-G. VSV-
88 G pseudotyped virus is used as a positive control because of its high infectivity in most cell types.
89 HIV-1 LAI gp160-pseudotyped virus is used as a negative control as it utilizes CD4 as a primary
90 receptor, which is present in SupT1 cells but absent in HEK293T.

91 Pseudotyped MLV viruses were tested on HEK293FT, HEK293T-ACE2, Huh7 and SupT1 cells.
92 HEK293FT cells were used as a control cell line, which is known to lack of susceptibility of

93 coronavirus and HIV due to the absence of both ACE2 and CD4. As expected, VSV-G pseudotyped
94 viruses infected all cell types and showed the highest infectivity (Figure 1). HIV-1 LAI gp160-
95 pseudotyped viruses only infected SupT1 cells. Both SARS-CoV-1 spike pseudotyped virus and
96 SARS-CoV-2 spike pseudotyped viruses infected 293T-ACE2 and Huh7 cells.

97 Since 293T-ACE2 cells showed the highest susceptibility to both SARS-CoV-1 and SARS-CoV-2
98 pseudotyped MLV viruses, further experiments were all performed in 293T-ACE2 cells. The ultimate
99 goal of our studies was to develop an antibody virus neutralization test based on the above
100 pseudotyped virus. To test for neutralization activity, serum samples from 12 de-identified COVID-
101 19 patients were tested for their ability to neutralize pseudotyped MLV viruses. Samples 1-6 were
102 plasma obtained from patients who had a SARS-CoV-2 positive test for nucleocapsid-specific IgG
103 (Abbot; samples 1 and 12), spike-specific IgG (Euroimmun; samples 2, 4, 5, and 6) or Nucleic Acid
104 Amplification test (ARUP Laboratories; samples 3, and 8-11) either SARS-CoV-2 nucleocapsid ELISA
105 or SARS-CoV-2 PCR.

106 As shown in Figure 2, 11 out of 12 patient serum samples showed neutralizing activity against
107 SARS-CoV-2-spike pseudotyped MLV viruses, with neutralizing titers-50 (NT₅₀) that ranged from
108 1:25 to 1:1,417. Eight out of the 12 samples displayed detectable NT₈₀. We also tested five historical
109 samples from patients who were hospitalized for severe influenza infection in 2016, all of which
110 tested negative in the neutralization assay (NT₅₀ < 25; Figure 3).

111 To test for specificity of neutralization, we asked whether neutralizing antibodies from SARS-
112 CoV-2 patients would exhibit cross-reactivity against a pseudotype expressing SARS-CoV-1 (Figure
113 4). We tested samples #1, 2 and 3, which had the highest NT₅₀ and NT₈₀. None of these sera had
114 detectable neutralizing activity (NT₅₀ <25) against the SARS-CoV-1 pseudotype, which is consistent
115 with previous reports [11-13].

116 As a positive control and also as a standard to monitor variability between neutralization
117 experiments, we used recombinant soluble receptor binding domain from SARS-CoV-2 spike protein.
118 We produced this protein via transient transfection in HEK293FT cells using a mammalian expression
119 vector (pCAGGS) encoding amino acids 319 to 542 of from SARS-CoV-2 S1, encompassing the RBD
120 (BEI Resources, cat.# NR-52309). The apparent NT₅₀ of RBD against SARS-CoV-2 / MLV pseudotype
121 was 1:244. As a negative control for neutralization, the surface glycoprotein from the simian
122 immunodeficiency virus, SIVmac gp130 [10], was similarly produced by transfection.

123

124 Conclusions

125 In summary, we have developed a simple and rapid assay based on pseudovirion particles,
126 which should allow for specific measurement of neutralizing titers in plasma against SARS-CoV-2 in
127 the context of biocontainment level 2 laboratories. Using SARS-CoV-2 RBD as a control in this assay,
128 we observe that, as expected, SARS-CoV-2 RBD was able to block infection with SARS-CoV-2.

129

130 Abbreviations

131 SARS, severe acute respiratory syndrome
132 SARS-CoV-2, severe acute respiratory syndrome coronavirus 2
133 COVID-19, coronavirus disease 2019
134 DMEM, Dulbecco's modified Eagle's medium
135 FBS, fetal bovine serum
136 RBD, receptor binding domain
137 NT, neutralizing titer
138 MLV, murine leukemia virus
139 VSV-G, vesicular stomatitis virus glycoprotein
140 HIV, human immunodeficiency virus
141 ACE2 Angiotensin Converting Enzyme 2

142

143 DECLARATIONS

144 **Ethics approval and consent to participate:** We used de-identified, archived plasma or serum
145 samples throughout the study.
146 **Consent for publication.** All authors have agreed to publication of the manuscript.
147 **Availability of data and materials.** Plasmid constructs and methodology are available upon request.
148 Aliquots of plasma and serum are in limiting quantities and may be available depending on amount
149 requested.
150 **Competing interests.** The authors declare no competing interests.
151 **Funding.** This research was supported by a seed grant from the University of Utah Vice President for
152 Research and the Immunology, Inflammation, and Infectious Disease Initiative. V.P. and M.C. were
153 supported by NIH grant AI143567-01.
154 **Authors contributions.** YZ, ETL, EAI, ESCPW conducted experiments. JL, IC, MC, AMS and VP
155 designed the study. JCD, PS, JR and MTR selected and contributed archived samples.
156 **Acknowledgments:** The construct pcDNA3.1-SARS-2-S-C9 was a generous gift from Tom Gallagher
157 at Loyola University. Human Codon Optimized HEK293T-hACE2 cells were kindly provided by
158 Adam Bailey and Emma Winkler in the laboratory of Michael Diamond at Washington University in
159 St. Louis School of Medicine. We also wish to thank Eloisa Yuste for helpful technical suggestions.
160

161 **References**

- 162 1. Ou X, Liu Y, Lei X, Li P, Mi D, Ren L, Guo L, Guo R, Chen T, Hu J, et al: **Characterization of**
163 **spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with**
164 **SARS-CoV.** *Nat Commun* 2020, **11**:1620.
- 165 2. Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Velesler D: **Structure, Function, and**
166 **Antigenicity of the SARS-CoV-2 Spike Glycoprotein.** *Cell* 2020, **181**:281-292 e286.
- 167 3. Zhang L, Jackson CB, Mou H, Ojha A, Rangarajan ES, Izard T, Farzan M, Choe H: **The**
168 **D614G mutation in the SARS-CoV-2 spike protein reduces S1 shedding and increases**
169 **infectivity.** *bioRxiv* 2020.
- 170 4. Akkina RK, Walton RM, Chen ML, Li QX, Planelles V, Chen IS: **High-efficiency gene**
171 **transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral**
172 **vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G.** *J Virol* 1996,
173 **70**:2581-2585.
- 174 5. Planelles V, Bachelierie F, Jowett JB, Haislip A, Xie Y, Banooni P, Masuda T, Chen IS: **Fate of**
175 **the human immunodeficiency virus type 1 provirus in infected cells: a role for vpr.** *J Virol*
176 1995, **69**:5883-5889.
- 177 6. Steffen I, Simmons G: **Pseudotyping Viral Vectors With Emerging Virus Envelope**
178 **Proteins.** *Curr Gene Ther* 2016, **16**:47-55.
- 179 7. Bosch BJ, van der Zee R, de Haan CA, Rottier PJ: **The coronavirus spike protein is a class I**
180 **virus fusion protein: structural and functional characterization of the fusion core**
181 **complex.** *J Virol* 2003, **77**:8801-8811.
- 182 8. Landau NR, Littman DR: **Packaging system for rapid production of murine leukemia**
183 **virus vectors with variable tropism.** *J Virol* 1992, **66**:5110-5113.
- 184 9. Bosque A, Planelles V: **Induction of HIV-1 latency and reactivation in primary memory**
185 **CD4+ T cells.** *Blood* 2009, **113**:58-65.
- 186 10. Planelles V, Haigwood NL, Marthas ML, Mann KA, Scandella C, Lidster WD, Shuster JR,
187 Van Kuyk R, Marx PA, Gardner MB, et al.: **Functional and immunological characterization**
188 **of SIV envelope glycoprotein produced in genetically engineered mammalian cells.**
189 *AIDS Res Hum Retroviruses* 1991, **7**:889-898.
- 190 11. Yi C, Sun X, Ye J, Ding L, Liu M, Yang Z, Lu X, Zhang Y, Ma L, Gu W, et al: **Key residues of**
191 **the receptor binding motif in the spike protein of SARS-CoV-2 that interact with ACE2**
192 **and neutralizing antibodies.** *Cell Mol Immunol* 2020, **17**:621-630.
- 193 12. Lv H, Wu NC, Tsang OT, Yuan M, Perera R, Leung WS, So RTY, Chan JMC, Yip GK, Chik
194 TSH, et al: **Cross-reactive antibody response between SARS-CoV-2 and SARS-CoV**
195 **infections.** *bioRxiv* 2020.
- 196 13. Ju B, Zhang Q, Ge J, Wang R, Sun J, Ge X, Yu J, Shan S, Zhou B, Song S, et al: **Human**
197 **neutralizing antibodies elicited by SARS-CoV-2 infection.** *Nature* 2020.
- 198
- 199

200 Figure Legends

201 **Figure 1.** Infectivity of pseudotyped MLV Viruses. SARS-CoV-2 spike pseudotyped MLV viruses as
202 well as VSV-G, SARS-CoV-1 spike, and HIV-1 LAIgp160 pseudotyped MLV viruses were tested on
203 HEK293FT, 293T-ACE2, Huh7 and SupT1 cells. 100 μ L of undiluted virus (except for VSV-G
204 pseudotype, which was diluted 1:100) was mixed with 100 μ L of medium and added to cells.
205 Luciferase was measured at 2 days post-infection and values are per well of a 96-well plate. Negative
206 line indicates mean+3SD of luciferase values obtained with virions devoid of glycoprotein.

207 **Figure 2.** Neutralizing activity of COVID-19 patient serum against and SARS-CoV-2 pseudotyped
208 MLV. **A.** Serum of COVID-19 patients were pre-incubated with SARS-CoV-2 spike pseudotyped MLV
209 at 37 °C for 1h. Serum and virus mixture were then incubated with 293T-ACE2 cells for 2 days. SARS-
210 CoV-2 spike RBD was used as a positive control. SIV gp130 was used as a negative control. Luciferase
211 was measured to assess infection. Percentage of neutralization was calculated. **B.** Neutralization titer
212 50 and 80 (NT₅₀, NT₈₀) were calculated as the reciprocal of the dilution resulting in 50 and 80%
213 neutralization, respectively. ELISA tests by Abbot and Euroimmun are not quantitative.

214 **Figure 3. Sera from hospitalized flu patients had no neutralizing activity against SARS-CoV-1**
215 **pseudovirions.** Cryopreserved serum samples from hospitalized flu patients from 2016 were pre-
216 incubated with SARS-CoV-2 spike pseudotyped MLV at 37°C for 1h. Serum and virus mixture were
217 then incubated with 293T-ACE2 cells for 2 days. SARS-CoV-2 spike RBD was used as a positive
218 control. SIV gp130 was used as a negative control.

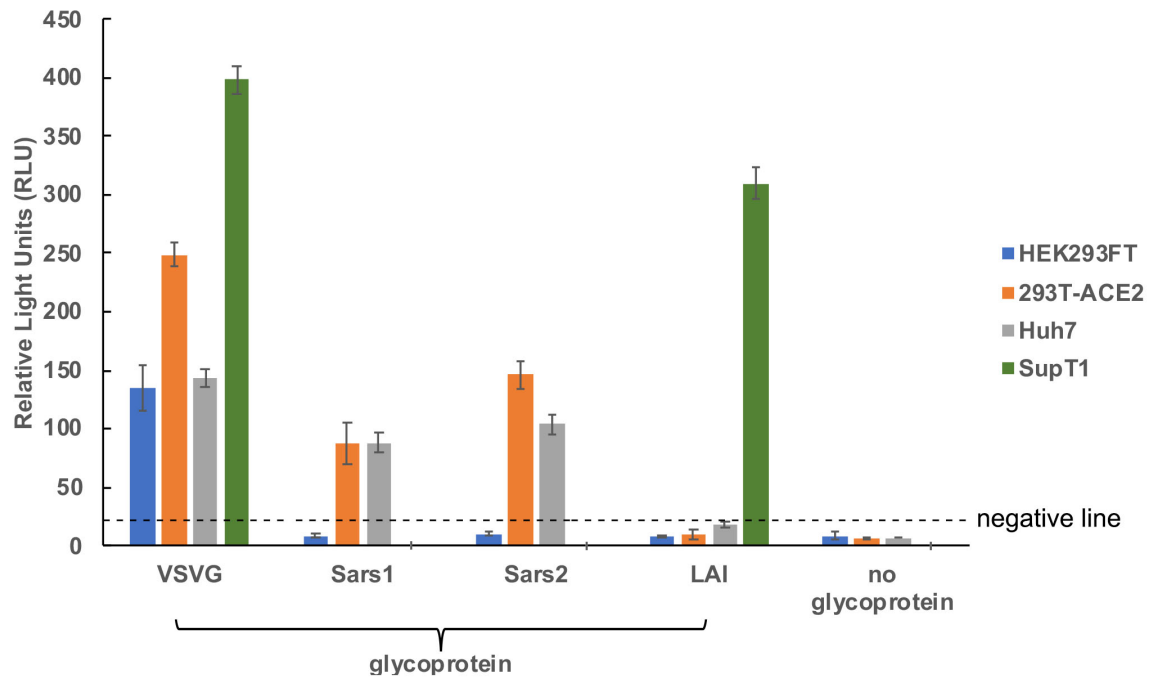
219 **Figure 4.** Neutralizing activity of COVID-19 patient serum against SARS-CoV-1 pseudovirions. **(A)**
220 Serum from COVID-19 patients were pre-incubated with SARS-CoV-1 spike pseudotyped MLV at 37
221 °C for 1h. Serum and virus mixture were then incubated with 293T-ACE2 cells. Percentage of
222 neutralization was calculated. **(B)** NT₅₀ and NT₈₀ were calculated as above. Samples #1, 2 and 3# were
223 tested previously (Figure 2).

224

225

Figures

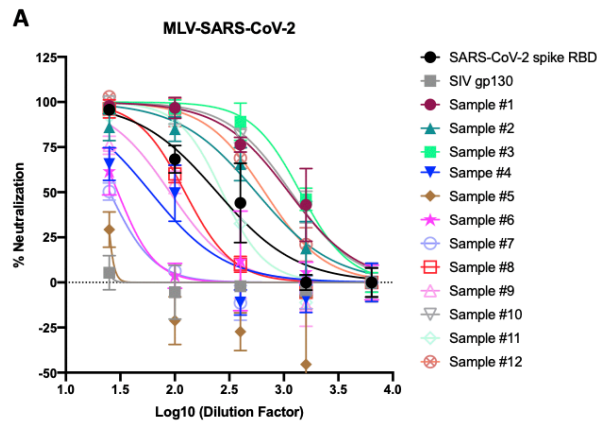
226 **FIGURE 1**



227

228

229 **FIGURE 2**

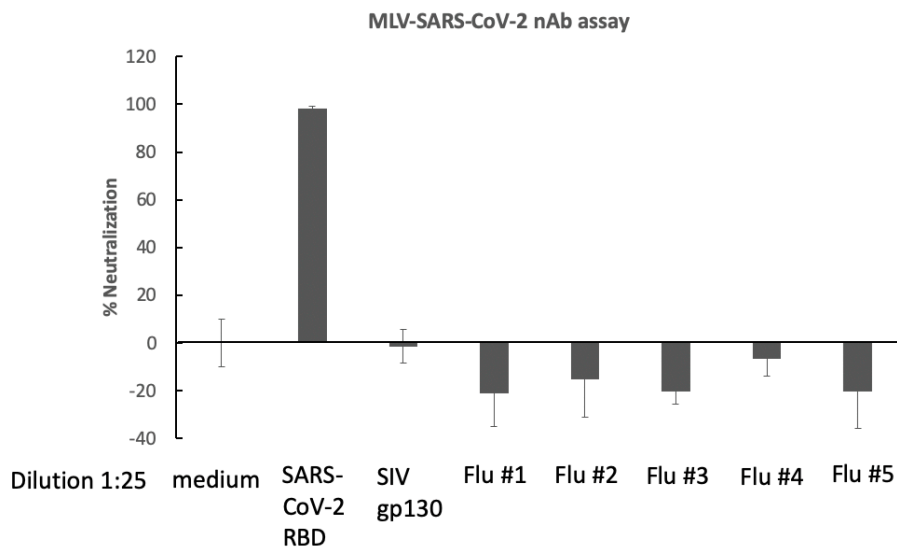


230
231

B

	MLV-Sars-Cov2-C9				
	Serum dilution				
	IC50	IC80	Abbot	Euroimmun	PCR
SARS-CoV-2 spike RBD	244	76			
SIV gp130	<25	<25			
Sample #1	1110	405	5.60	--	--
Sample #2	557	180	--	9.7	--
Sample #3	1417	691	--	--	+
Sample #4	60	<25	--	8.8	--
Sample #5	<25	<25	--	2.8	--
Sample #6	1:30	<25	--	6.6	--
Sample #7	25	<25	--	--	--
Sample #8	124	61	--	--	+
Sample #9	90	36	--	--	+
Sample #10	1162	460	--	--	+
Sample #11	272	144	--	--	+
Sample #12	667	276	<1.4	--	--

232 **FIGURE 3**



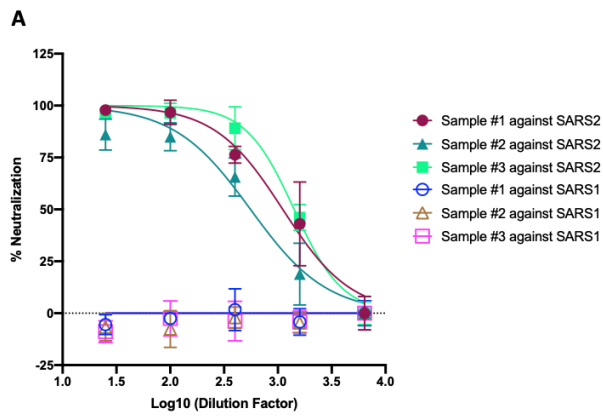
233

234

235

236

237 **FIGURE 4**



B

	Serum dilution			
	MLV-SARS-CoV2		MLV-SARS-CoV1	
	NT50	NT80	NT50	NT80
Sample #1	1110	405	<25	<25
Sample #2	557	180	<25	<25
Sample #3	1417	691	<25	<25

238