Evaluation of cell-based and surrogate SARS-CoV-2 neutralization assays

Running title (54 characters): Evaluation of SARS-CoV-2 neutralizing antibody assays

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Supplementary materials

Supplementary Methods

Protein antigens for the Luminex binding antibody assay. A recombinant form of a synthetic construct (SARS_CoV_2_ectoCSPP (1); GenBank: QJE37812.1) of the spike (S) glycoprotein from SARS-CoV-2 Wuhan-Hu-1 was produced in human HEK293 cells (FreeStyle™ 293-F Cells, ThermoFisher, Waltham, MA) using a lentivirus expression system (2) and purified by nickel affinity and size-exclusion chromatography. Purity and solution monodispersivity were confirmed by comparative reduced/non-reduced PAGE, analytical size-exclusion chromatography, and static/dynamic light scattering on Uncle (Unchained Labs, Pleasanton, CA) and showed uniform trimerization. The recombinant protein was modified by replacing the native leader sequence with a murine Igk leader, removing the polybasic S1/S2 cleavage site (RRAR to A), stabilized with a pair of proline mutations (2P), and incorporating a thrombin cleavage site, a T4 foldon trimerization domain, a hexa-histidine purification tag, and a C-terminal Avi-Tag (3). After purification, the protein was sterile filtered and aliquoted in DPBS, no calcium, no magnesium (ThermoFisher). Alternatively, spike protein was produced as described elsewhere (4). Both spike protein preparations were tested in a binding assay and no difference in recognition by serum and plasma samples from different convalescent subjects was found. Receptor binding domain (RBD) was produced in the same construct, swapping a tobacco etch virus (TEV) protease site (5) for the thrombin cleavage site. SARS-CoV-2 nucleoprotein was purchased from GenScript (Piscataway, NJ) and tetanus toxoid from Lonza (Basel, Switzerland).

In-house Luminex SARS-CoV-2 IgG binding antibody assay. Protein antigens were coupled to the Bio-Plex Pro Magnetic COOH beads in a ratio of 10 µg of antigen per 2.5 x 10^6 beads in a two-step carbodiimide reaction. First, beads were washed and resuspended in Activation Buffer (100 mM MES, pH 6) and then incubated with N-hydroxysulfosuccinimide (Sulfo-NHS, catalog number 24520; ThermoFisher) and 1-ethyl-3-[3-dimethlyaminopropyl]carbodiimide-HCl (EDC, catalog number 77149; ThermoFisher) also dissolved in Activation Buffer for 20 minutes on an end-over-end rotational mixer at room temperature protected from light. Activated beads were washed three times in Activation buffer. For coupling, antigen was mixed with activated beads and reaction was carried out for 2 h on a

rotational mixer at room temperature protected from light. Conjugated beads were washed three times with Wash buffer (PBS, 0.05% Tween-20, 1% BSA, 0.1% NaN_3) and finally resuspended in Wash buffer at 10^7 beads/ml. Beads were stored at 4 ºC for no longer than 30 days.

Antigen-specific IgG was measured using two replicate dilutions. Beads were blocked with phosphate buffered saline (PBS; Gibco) containing 5% Blotto (Bio-Rad) and 0.05% Tween-20 (Sigma) and incubated for 1 hour with serially diluted plasma samples. Next, beads were washed 3 times with 0.05% Tween-20 in PBS and incubated with anti-human IgG Fc-PE (catalog number 2048-09; Sothern Biotech). After incubation with secondary antibody, beads were washed and resuspended in PBS with 1% BSA and 0.05% Tween-20 and binding data were collected on Bio-Plex 200 instrument (Bio-Rad). Median Fluorescence Intensity (MFI) was measured for a minimum of 50 beads per region. Background was established by measuring the MFI of beads conjugated to antigens but incubated in Assay buffer. Background MFI values were subtracted from all readings. We also trialed unconjugated beads and beads conjugated to a decoy antigen with the same plasma samples used in testing and did not detect non-specific binding above the assay background described above.

An IgG standard curve run in duplicate was used to estimate IgG concentration. For that, anti-human IgG Fab-specific (Southern Biotech) was conjugated to MagPlex beads. IgG-coupled beads were blocked, washed and incubated with serially diluted human standard IgG (catalog number I4506; Sigma) for 1 h. Standard beads were washed and incubated with anti-human IgG Fc-PE and MFI was measured as described above. MFI readings and associated IgG concentrations were fitted to a four-parameter logistic curve (4PL) using the R packages *nCal* and *drc*. A standard curve for each experiment was used to obtain the effective concentrations of IgG in serum using the MFI measured with antigen-coated beads. Since plasma samples were also run as a dilution series we used the median of the estimated concentrations from the dilutions that yielded MFIs between 100 and 10,000. Plasma with all values above (below) this range were right (left) censored at the concentration of the minimum (maximum) MFI.

VSV-pseudovirus. The codon-optimized sequence of the SARS-CoV-2 spike protein (YP_009724390.1) with a truncation of the 19 C-terminal amino acids (D19) was cloned into a pcDNA3.1(+) vector (ThermoFisher) under control of the human CMV promoter to generate pcDNA3.1(+)-SARS-CoV-2-D19. The C-terminal truncation leads to a deletion of the ER-retention signal, localizing the spike protein to the cell surface, which enhances pseudovirus packaging (6). VSV(G*ΔG-luciferase) system was purchased from Kerafast (7, 8). Twenty-four hours prior infection with VSV(G*ΔG-luciferase), 293T cells were transfected with pcDNA-WuhanCoV-S-D19. Next day, supernatant was harvest, centrifuged for 5 min at 1,000xg, aliquoted and stored at -80 ºC. $TCID_{50}$ was measured by infecting Vero cells (catalog number CCL-81; ATCC) with serial 2-fold dilutions of the prepared pseudovirus.

LV-pseudovirus. An expression plasmid encoding codon-optimized full-length spike of the Wuhan-1 strain (VRC7480), was provided by Drs. Barney Graham and Kizzmekia Corbett at the Vaccine Research Center, National Institutes of Health (USA). The D614G mutation was introduced into VRC7480 by site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis Kit from (catalog number 210518; Agilent Technologies). The mutation was confirmed by full-length spike gene sequencing. Pseudovirions were produced in HEK 293T/17 cells (catalog number CRL-11268; ATCC) by transfection using Fugene 6 (catalog number E2692; Promega). Pseudovirions for 293T/ACE2 infection were produced by co-transfection with a lentiviral backbone (pCMV-ΔR8.2) and firefly luciferase reporter gene (pHR'-CMV-Luc) (9). Pseudovirions for TZM-bl/ACE2/TMPRSS2 infection were produced by co-transfection with the Env-deficient lentiviral backbone pSG3ΔEnv (kindly provided by Drs Beatrice Hahn and Feng Gao). Culture supernatants from transfections were clarified of cells by low-speed centrifugation and filtration (0.45 µm filter) and stored in 1 ml aliquots at -80ºC.

Live SARS-CoV-2 neutralization assay. All the live virus experiments were performed under BSL-3 conditions at negative pressure, by operators in Tyvek suits wearing personal powered-air purifying respirators. Vero E6 cells were seeded at $2x10^4$ cells/well in a 96-well plate 24 h before the assay. Seventy five pfu of the recombinant SARS-CoV-2-nanoLuc virus (rSARS-CoV-2-nLuc) (10) were mixed

with Ab at 1:1 ratio and incubated at 37ºC for 1h. A 8-points, 3-fold dilution curve was generated for each sample with starting concentration at 1:50. Virus and Ab mix was added to each well and incubated at 37ºC + 5% CO_2 for 48h. Luciferase activities were measured by Nano-Glo Luciferase Assay System (Promega) following manufacturer protocol using SpectraMax M3 luminometer (Molecular Devices). Percent neutralization was calculated by the following equation: [1-(RLU with sample/ RLU with mock treatment)] x 100%.

VSV pseudovirus neutralization assay. Assay was carried out in BSL-2 laboratory. Vero cells (ATCC® CCL-81™) were seeded at $2x10^4$ cells/well in a black-walled 96-well plates 24 hours before the assay. A 7-point, 3-fold dilution curve was generated with starting sample dilution at 1:20 in a separate round-bottom 96-well plate. $3.8x10^2$ TCID50 of rVSV(G*ΔG-luciferase) pseudovirus with SARS-CoV-2-D19 spike protein (PsVSV-Luc-D19) was mixed with the plasma dilutions. Plasma-virus mixture was incubated at 37 ºC in 5% CO_2 for 30 minutes. After incubation, plasma-virus mixture was transferred onto the Vero cells. Cells were then incubated at 37 ºC, 5% CO_2 for 18-20 hours. Luciferase activity was measured by Bio-Glo Luciferase Assay System (catalog number G7940; Promega) following manufacturer protocol using 2030 VICTOR X3 multilabel reader (PerkinElmer). Percent virus neutralization was calculated by the following equation: [1-(luminescence of sample/ luminescence of cells+virus control)] x 100%.

LV-pseudovirus neutralization assays. Assays were carried out in BSL-2 laboratory. Neutralization of SARS-CoV-2 Spike-pseudotyped virus prepared with lentiviral vectors was performed by using infection in either HEK 293T cells expressing human ACE2 (293T/ACE2.MF) or TZM-bl cells expressing both ACE2 and TMPRSS2 (TZM-bl/ACE2/TMPRSS2 cells). Both cell lines kindly provided by Drs. Mike Farzan and Huihui Mu at Scripps). Cells were maintained in DMEM containing 10% FBS, 1% Pen Strep and 3 ug/ml puromycin.

293T/ACE2 cells pseudovirus assay. For the 293T/ACE2 assay, a pre-titrated dose of virus was incubated with serial 3-fold dilutions of test sample in duplicate in a total volume of 150 ul for 1 hr at

37°C in 96-well flat-bottom black/white culture plates. Freshly trypsinized cells (10,000 cells in 100 µl of growth medium) was added to each well. One set of control wells received cells + virus (virus control) and another set received cells only (background control). After 68-72 hours of incubation, 100 ul of cell lysate was transferred to a 96-well black/white plate (catalog number 6005060; Perkin-Elmer) for measurements of luminescence using the Promega Luciferase Assay System (catalog number E1501; Promega). Neutralization titers are the serum dilution at which RLUs were reduced by 50% and 80% compared to virus control wells after subtraction of background RLUs. MPI is the reduction in RLU at the lowest serum dilution tested.

ACE2/TMPRSS2 TZM-bl cells pseudovirus assay. For the TZM-bl/ACE2/TMPRSS2 assay, a pre-titrated dose of virus was incubated with serial 3-fold dilutions of test sample in duplicate in a total volume of 150 µl for 1 hr at 37°C in 96-well flat-bottom culture plates. Freshly trypsinized cells (10,000 cells in 100 ul of growth medium containing 75 µg/ml DEAE dextran) were added to each well. One set of control wells received cells + virus (virus control) and another set received cells only (background control). After 68-72 hours of incubation, 100 µl of cell lysate was transferred to a 96-well black solid plate (Costar) for measurements of luminescence using the BriteLite Luminescence Reporter Gene Assay System (PerkinElmer Life Sciences). Neutralization titers are the serum dilution at which relative luminescence units (RLU) were reduced by 50% and 80% compared to virus control wells after subtraction of background RLUs. Maximum percent inhibition (MPI) is the reduction in RLU at the lowest serum dilution tested.

SARS-CoV-2 Surrogate Virus Neutralization Test (sVNT). Assay was carried out in BSL-1 laboratory and was performed according to manufacturer (GenScript) protocol and recommendations as follows. Capture plate was incubated with plasma samples diluted 1:10, washed and probed with secondary antibody. Assay was developed via TMB (ThermoFisher) and OD at 450 nm was measured using SpectraMax M2 reader (Molecular Devices). Positive and negative controls were provided in the kit. Binding inhibition was determined via the following formula: Inhibition = (1 – (OD of sample / OD of Negative control)) × 100%. Percent binding inhibition was interpreted as a percent neutralization. In

order to determine ND50, plasma samples were serially diluted starting from 1:10 and assay was performed as described above.

Statistical Analysis and Visualization. Neutralization titers were defined as the plasma dilution that reduced relative luminescence units (RLU) by 50% or 80% relative to virus control wells (cells + virus only) after subtraction of background RLU in cells-only control wells. RLU was first transformed to neutralization using the formula neut = 1 – ([RLUsample – bkgd] / [RLUVO – bkgd]). The neutralization vs. dilution curve was then fit with a four-parameter logistic curve (4PL) model that was used to estimate the dilution at which there would be 50% or 80% neutralization. For samples with all dilutions having <50% neutralization the result was right censored at the highest concentration. Fifty and 80 percent neutralization titers (ND50 and ND80) were estimated using the nCal and drc packages in R. Patient demographic information (sex and age) was extracted from a RedEDCap survey database. Abbott assay results (including index value) were extracted from the laboratory information system (Sunquest Laboratory).

Correlations and group differences were estimated using parametric methods and testing (e.g. Pearson correlation and Student's t test). Log-transformed ND50 values and IgG concentrations were approximately normally distributed with few outliers and a low level of censoring, justifying use of these methods. Left censored values were given a value of half the level of detection, which corresponded to the first dilution for each neutralization assay.

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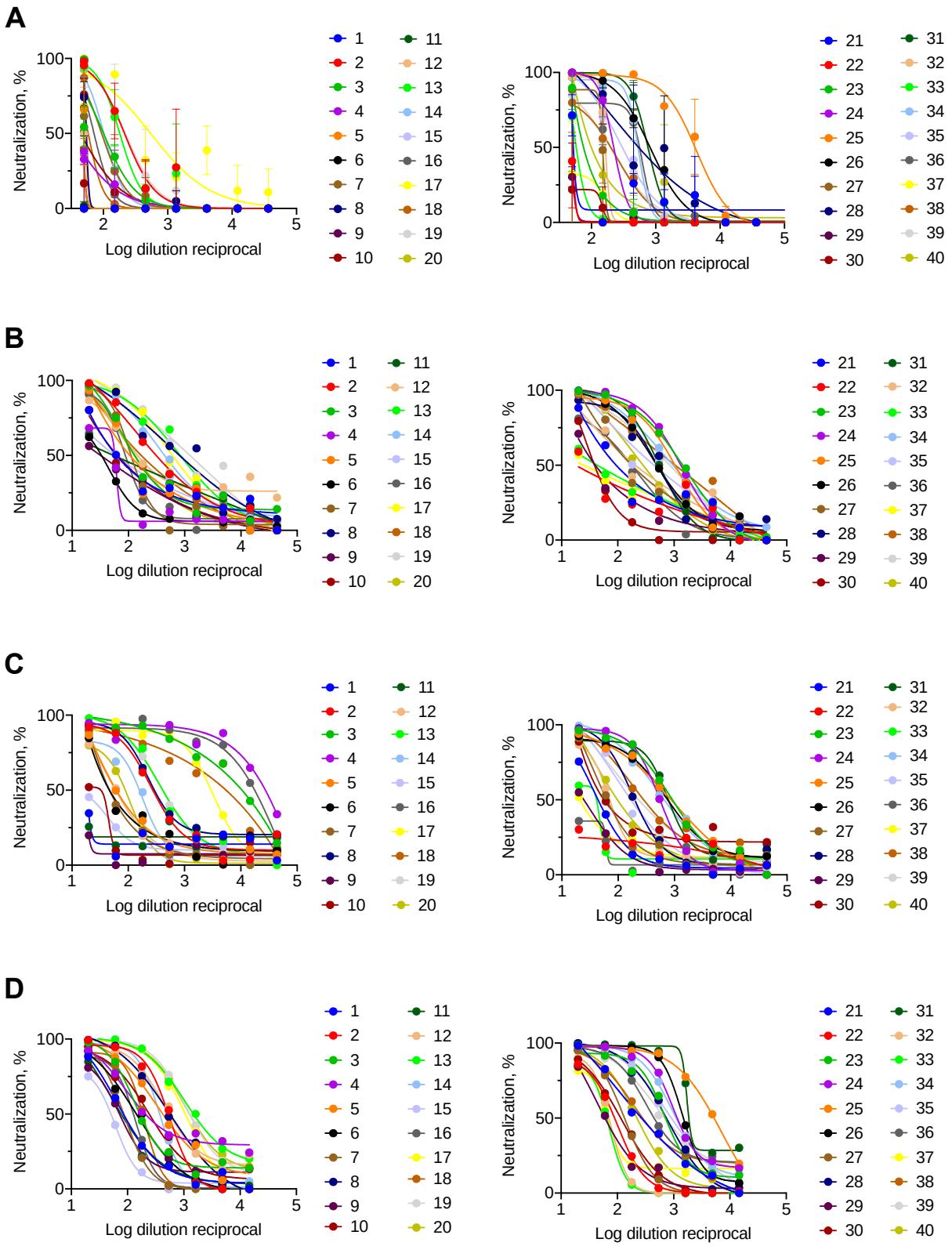
Supplementary Figures and Tables

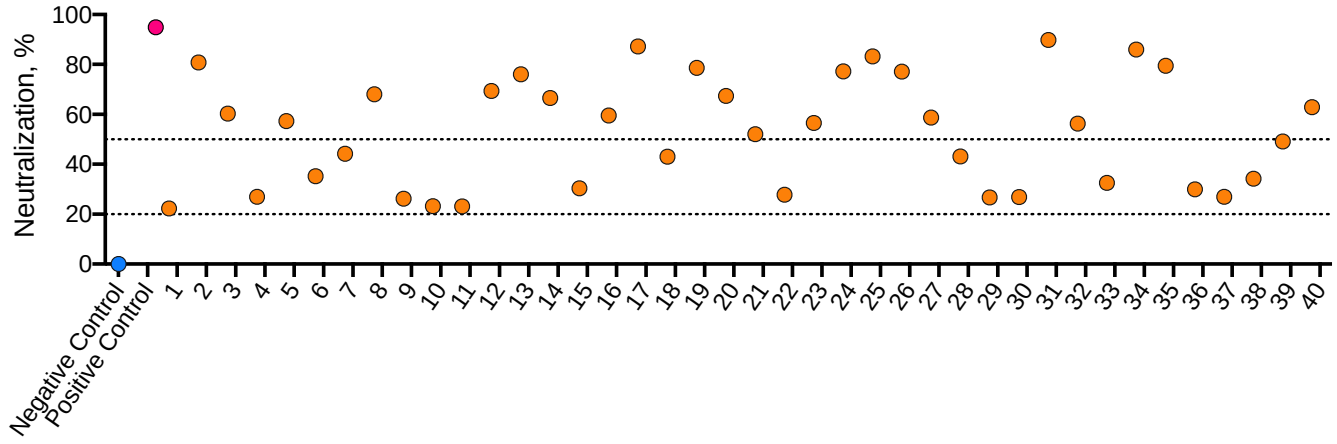
Supplementary Table 1. Symptoms reported by study participants.

Symptom	Yes	No	Missing	Percent reporting symptom
Fever	23	11	6	57.5
Chills	26	10	4	65
Fatigue	35	4	1	87.5
Myalgia	27	9	4	67.5
Sore throat	20	14	6	50
Cough	29	6	5	72.5
Rhinorrhea	24	10	6	60
Dyspnea	22	13	5	55
Wheezing	6	19	15	15
Chest pain	13	16	11	32.5
Other respiratory	8	17	15	20
Headache	27	8	5	67.5
Nausea	9	18	13	22.5
Abdominal pain	6	20	14	15
Diarrhea	14	16	10	35
Loss senses	26	8	6	65
Eye pain	7	19	14	17.5
Rash feet	2	21	17	5
Rash body	4	21	15	10



Supplementary Figure 1. Neutralizing antibody assay. (A) SARS-CoV-2-nLuc in Vero E6 cells. (B) LV-pseudo in 293T/ACE2 cells. (C) LV-pseudo in TZM-bl/ACE2/TMPRSS2 cells. (D) PsVSV-Luc-D19 in Vero cells. Participant samples are as colored lines and circles numbered 1 – 40.

A



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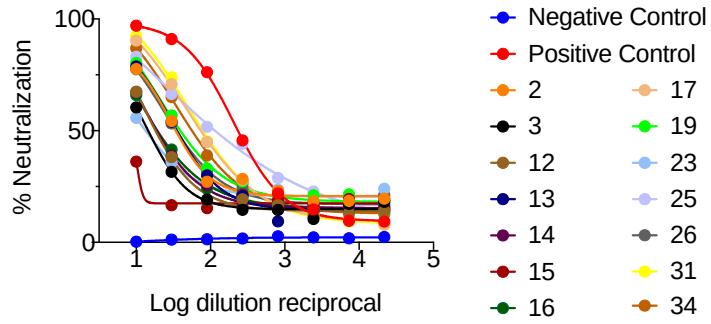
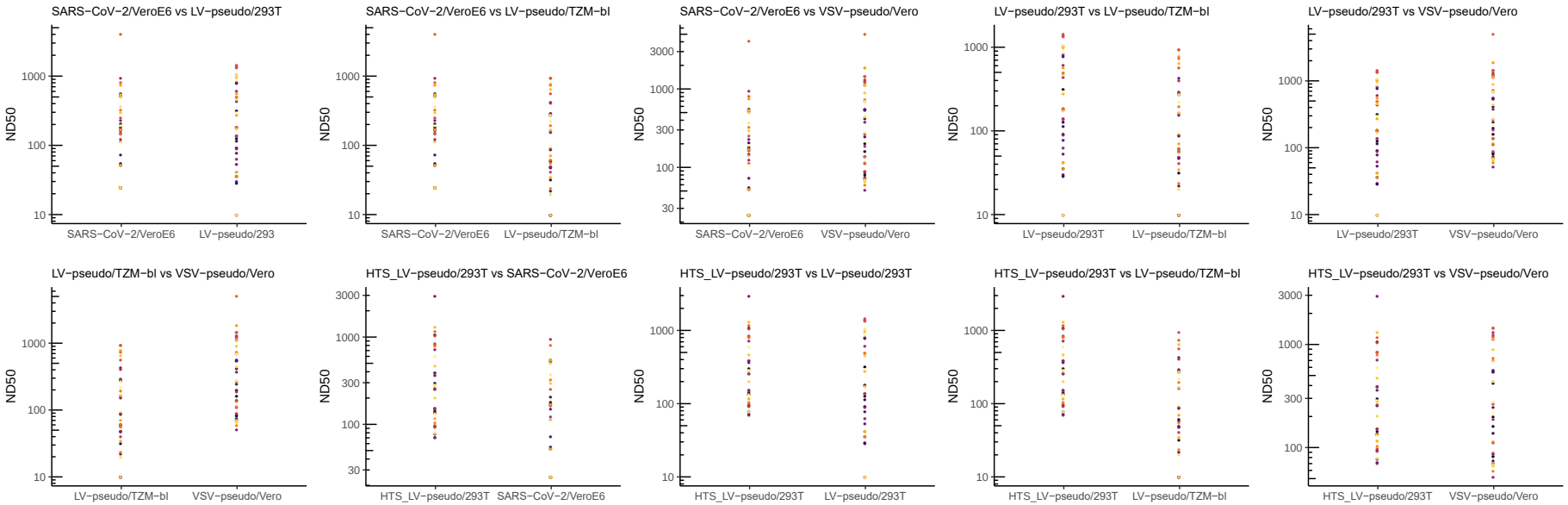


Supplementary Figure 2. Surrogate virus neutralization test from GenScript. A, samples tested in 1:10 dilution as per manufacturer protocol. Dotted lines show 50 and 20% neutralization, respectively. Twenty percent is suggested as positivity cutoff by the manufacturer. B, plasma samples were titrated 2-fold starting at 1:10 to impute ND50 titers.

A


SARS−CoV−2/VeroE6 vs LV−pseudo/293T
SARS−CoV−2/VeroE6 vs LV−pseudo/TZM−bl
SARS−CoV−2/VeroE6 vs VSV−pseudo/Vero
LV−pseudo/293T vs LV−pseudo/TZM−bl
LV−pseudo/293T vs VSV−pseudo/Vero
LV−pseudo/TZM−bl vs VSV−pseudo/Vero
HTS_LV−pseudo/293T vs SARS−CoV−2/VeroE6
HTS_LV−pseudo/293T vs LV−pseudo/293T
HTS_LV−pseudo/293T vs LV−pseudo/TZM−bl
HTS_LV−pseudo/293T vs VSV−pseudo/Vero
ND50
SARS−CoV−2/VeroE6
LV−pseudo/293
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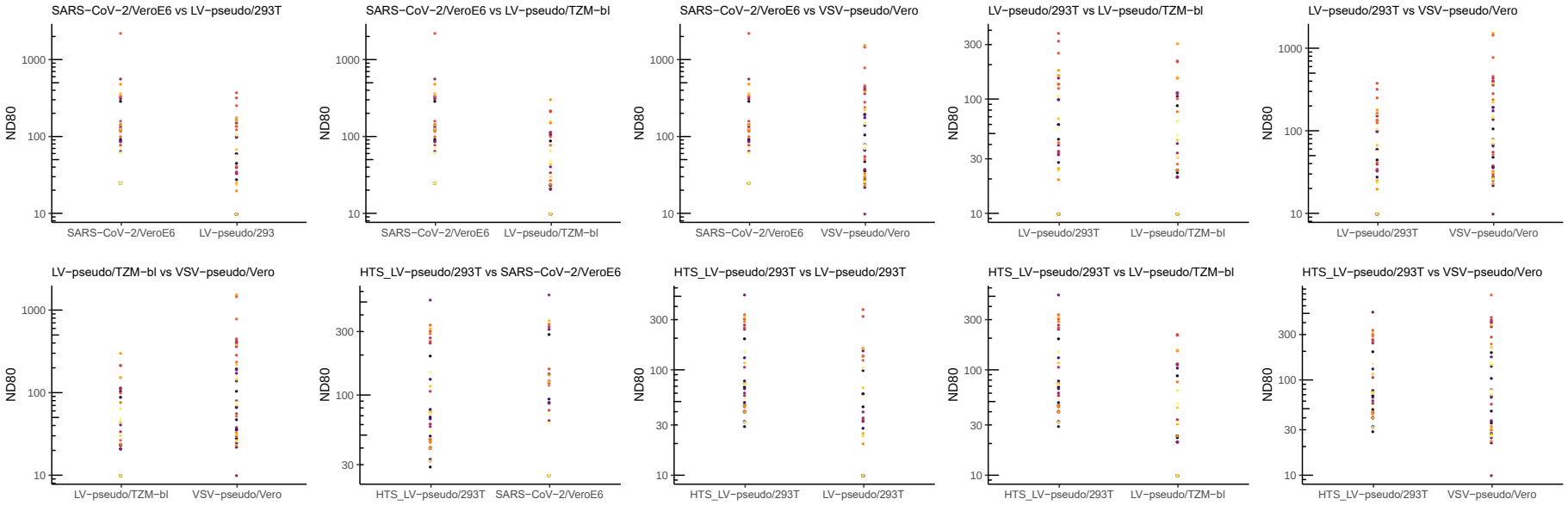
VSV−pseudo/Vero
HTS_LV−pseudo/293T
LV−pseudo/293T

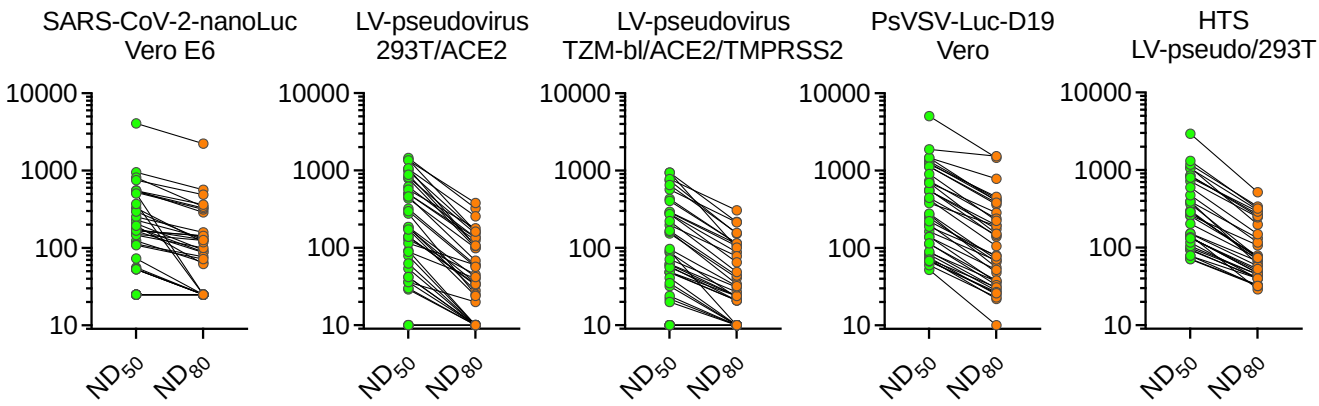
B



Supplementary Figure 3. Comparison of ND50 (A) and ND80 (B) titers measured in cell-based assays. Data used same as represented in Fig. 1 but replotted with lines connecting individual color-coded samples to illustrate the direction of ND50 and ND80 shift between assays.

Supplementary Table 2. GMT of ND50 and ND80 for each neutralization assay represented on Supplementary Figure 3, with fold-differences computed.

	Assay	GMT	95% CI LCL	95% CI UCL	fold-difference	Difference sign	Assay	GMT	95% CI LCL	95% CI UCL	N	p-value
ND50	SARS-CoV-2/VeroE6	141.3	93.7	213.0	12.8	>	sVNT	11.2	7.6	16.4	31	<0.001
	SARS-CoV-2/VeroE6	141.3	93.7	213.0	1.3	<	LV-pseudo/293T	177.9	112.0	282.7	40	0.112
	SARS-CoV-2/VeroE6	141.3	93.7	213.0	1.6	>	LV-pseudo/TZM-bl	89.9	57.0	141.9	40	0.003
	SARS-CoV-2/VeroE6	141.3	93.7	213.0	2.2	<	VSV-pseudo/Vero	309.7	211.3	454.0	40	<0.001
	sVNT	11.2	7.6	16.4	14.8	<	LV-pseudo/293T	177.9	112.0	282.7	31	<0.001
	sVNT	11.2	7.6	16.4	7.6	<	LV-pseudo/TZM-bl	89.9	57.0	141.9	31	<0.001
	sVNT	11.2	7.6	16.4	26.1	<	VSV-pseudo/Vero	309.7	211.3	454.0	31	<0.001
	LV-pseudo/293T	177.9	112.0	282.7	2.0	>	LV-pseudo/TZM-bl	89.9	57.0	141.9	40	<0.001
	LV-pseudo/293T	177.9	112.0	282.7	1.7	<	VSV-pseudo/Vero	309.7	211.3	454.0	40	0.002
	LV-pseudo/TZM-bl	89.9	57.0	141.9	3.4	<	VSV-pseudo/Vero	309.7	211.3	454.0	40	<0.001
	HTS_LV-pseudo/293T	271.7	266.8	643.4	1.92	>	SARS-CoV-2/VeroE6	141.3	93.7	213.0	36	<0.001
	HTS_LV-pseudo/293T	271.7	266.8	643.4	24.3	>	sVNT	11.2	7.6	16.4	31	<0.001
	HTS_LV-pseudo/293T	271.7	266.8	643.4	1.5	>	LV-pseudo/293T	177.9	112.0	282.7	36	<0.001
	HTS_LV-pseudo/293T	271.7	266.8	643.4	3	>	LV-pseudo/TZM-bl	89.9	57.0	141.9	36	<0.001
	HTS_LV-pseudo/293T	271.7	266.8	643.4	1.1	<	VSV-pseudo/Vero	309.7	211.3	454.0	36	0.856
ND80	SARS-CoV-2/VeroE6	79.3	54.8	114.8	13.0	>	sVNT	6.0	5.1	7.0	31	<0.001
	SARS-CoV-2/VeroE6	79.3	54.8	114.8	1.9	>	LV-pseudo/293T	42.0	28.8	61.1	40	<0.001
	SARS-CoV-2/VeroE6	79.3	54.8	114.8	2.4	>	LV-pseudo/TZM-bl	33.0	23.3	46.8	40	<0.001
	SARS-CoV-2/VeroE6	79.3	54.8	114.8	1.3	<	VSV-pseudo/Vero	102.8	69.0	153.2	40	0.027
	sVNT	6.0	5.1	7.0	6.8	<	LV-pseudo/293T	42.0	28.8	61.1	31	<0.001
	sVNT	6.0	5.1	7.0	5.5	<	LV-pseudo/TZM-bl	32.7	21.6	49.4	31	<0.001
	sVNT	6.0	5.1	7.0	16.6	<	VSV-pseudo/Vero	102.8	69.0	153.2	31	<0.001
	LV-pseudo/293T	42.0	28.8	61.1	1.3	>	LV-pseudo/TZM-bl	33.0	23.3	46.8	40	0.001
	LV-pseudo/293T	42.0	28.8	61.1	2.5	<	VSV-pseudo/Vero	102.8	69.0	153.2	40	<0.001
	LV-pseudo/TZM-bl	33.0	23.3	46.8	3.1	<	VSV-pseudo/Vero	102.8	69.0	153.2	40	<0.001
	HTS_LV-pseudo/293T	86.3	83.9	163.4	1.1	>	SARS-CoV-2/VeroE6	79.3	54.8	114.8	36	0.009
	HTS_LV-pseudo/293T	86.3	83.9	163.4	14.4	>	sVNT	6.0	5.1	7.0	31	<0.001
	HTS_LV-pseudo/293T	86.3	83.9	163.4	2.1	>	LV-pseudo/293T	42.0	28.8	61.1	36	<0.001
	HTS_LV-pseudo/293T	86.3	83.9	163.4	2.6	>	LV-pseudo/TZM-bl	33.0	23.3	46.8	36	<0.001
	HTS_LV-pseudo/293T	86.3	83.9	163.4	1.2	<	VSV-pseudo/Vero	102.8	69.0	153.2	36	0.759



ND_{50}	ND_{80}
141.3	79.29

ND_{50}	ND_{80}
177.9	41.96

ND_{50}	ND_{80}
89.95	33.03

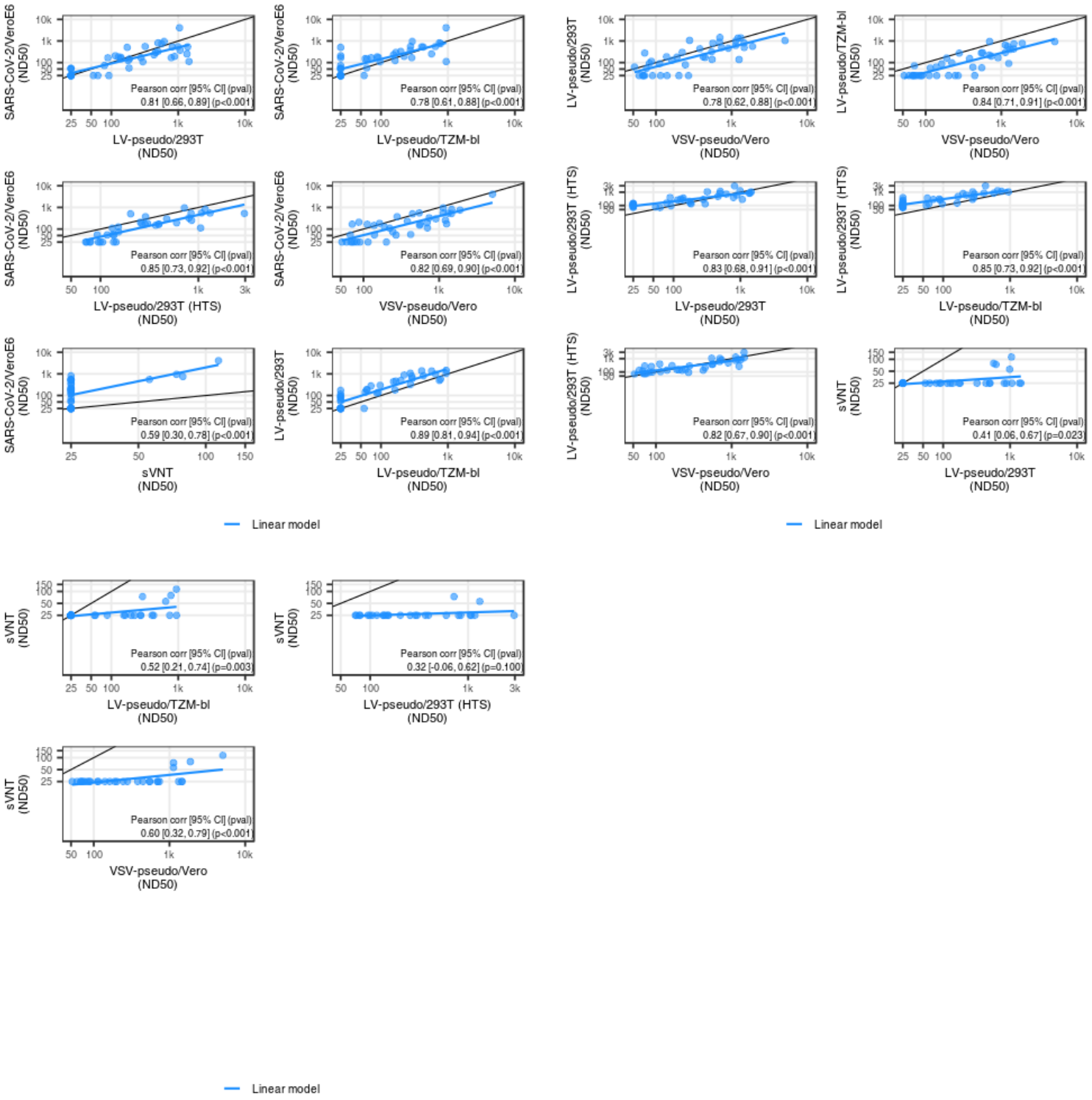
ND_{50}	ND_{80}
309.7	102.8

ND_{50}	ND_{80}
271.7	86.29

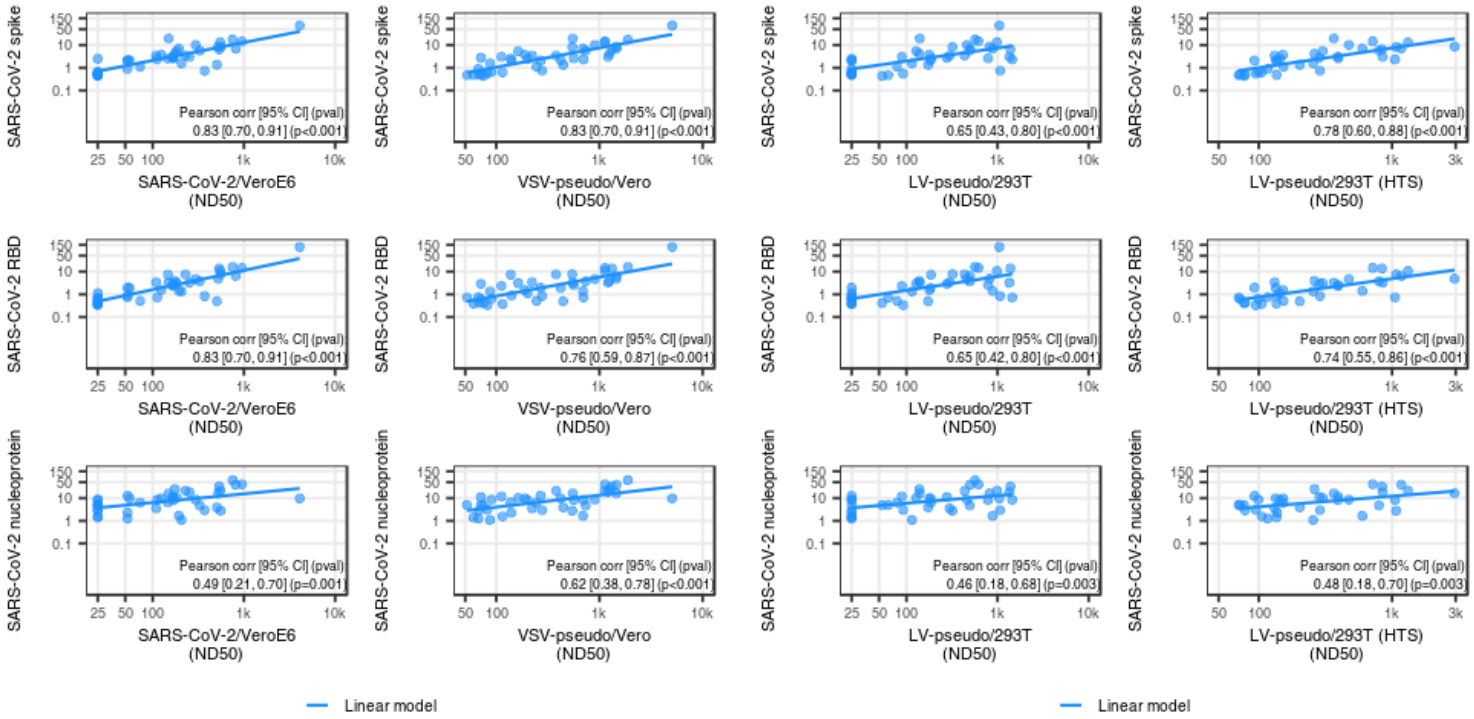
Supplementary Figure 4. Comparison of differences between geometric mean ND50 and ND80 titers for each neutralization assay. GMT ND50 and ND80 for the corresponding assay are shown underneath each graph. Each circle is a participant plasma sample, with lines connecting the same samples analyzed for the two neutralizing dilutions. Green circles are ND50 and orange circles are ND80 values.

Supplementary Table 3. Fold change between ND50 and ND80 values.

Assay	Mean ND fold change [95% CI]	P-value
SARS-CoV-2/Vero E6	1.954 [1.62, 2.29]	<0.0001
LV-pseudo/293T	4.573 [3.65, 5.5]	<0.0001
LV-pseudo/TZM-bl	4.478 [3.48, 5.48]	<0.0001
VSV-pseudo/Vero	2.967 [2.7, 3.24]	<0.0001
HTS_LV-pseudo/293T	3.303 [2.93, 3.68]	<0.0001



Supplementary Figure 5. Pearson correlation model analysis of ND50 titers among neutralization assays.



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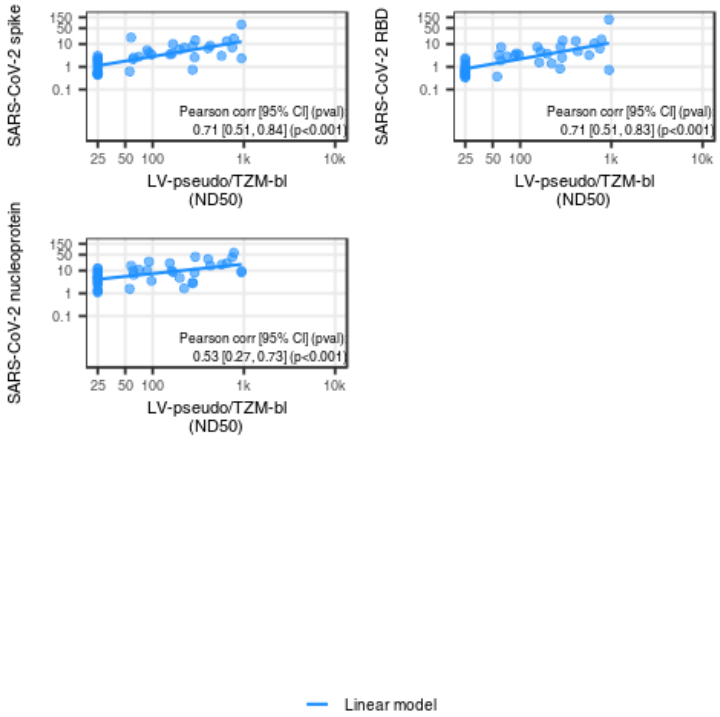

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281 **Supplementary Figure 6. Pearson correlation model analysis of ND50 titers vs SARS-CoV-2**

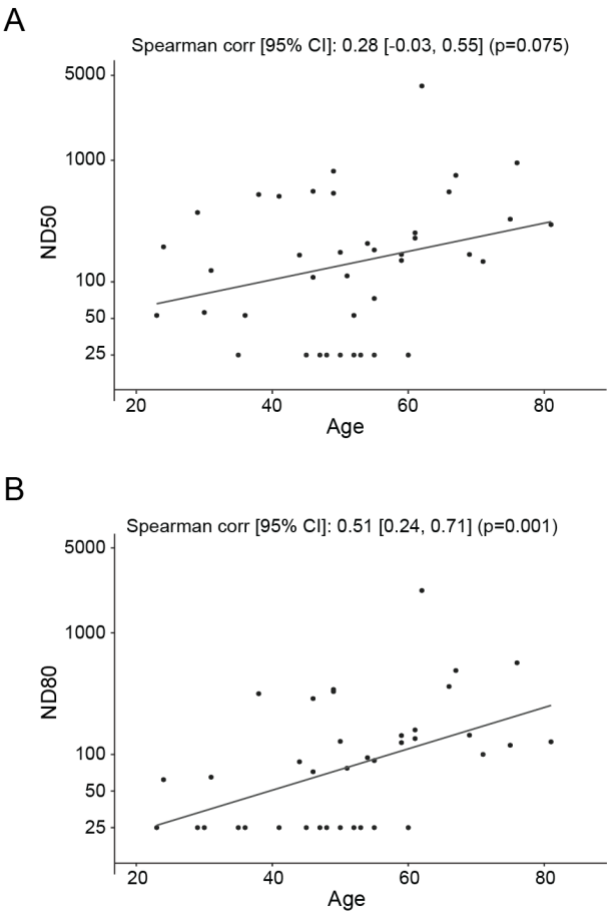
282 **specific IgG concentration in plasma samples.**

283

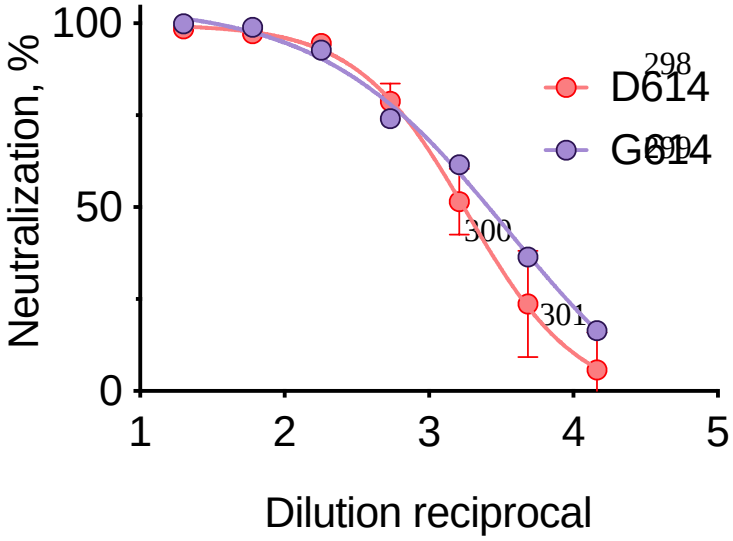
Supplementary Table 4. Tests for association of SARS-CoV-2 antibody neutralization and binding with age of participants.

Assay	Measure	N	Age rho[1] (p-value)
SARS-CoV-2/VeroE6	ND50	40	0.28 (0.0751)
VSV-pseudo/Vero	ND50	40	0.30 (0.0602)
LV-pseudo/293T	ND50	40	0.24 (0.1288)
LV-pseudo/TZM-bl	ND50	40	0.27 (0.0885)
sVNT neutralization	ND50	31	0.43 (0.0160)
SARS-CoV-2/VeroE6	ND80	40	0.51 (0.0007)
VSV-pseudo/Vero	ND80	40	0.32 (0.0466)
LV-pseudo/293T	ND80	40	0.32 (0.0444)
LV-pseudo/TZM-bl	ND80	40	0.29 (0.0738)
sVNT neutralization	ND80	31	0.50 (0.0038)
sVNT neutralization (1:10 dilution)	%	40	0.40 (0.0106)
Abbott nucleoprotein	index	40	0.45 (0.0034)
SARS-CoV-2 spike-specific IgG	µg/mL	40	0.37 (0.0197)
SARS-CoV-2 RBD-specific IgG	µg/mL	40	0.45 (0.0035)
SARS-CoV-2 nucleoprotein-specific IgG	µg/mL	40	0.39 (0.0126)
Tetanus toxoid-specific IgG	µg/mL	40	-0.14 (0.3853)

[1]Spearman's rank correlation coefficient
[2]fold-difference indicates the geometric mean value in females/males, Student's *t* test p-value



Supplementary Figure 7. **Correlation analysis of plasma neutralizing potency and age of participants.** (A) ND50 versus age. (B) ND80 versus age.



	G614	D614
ND50	2584	1741
ND80	465	516

Supplementary Figure 8. **Comparison of neutralization of VSV-pseudovirus bearing D614 and G614 mutations by the WHO standard.** Red, VSV-pseudoviruses with D614; Purple, VSV-pseudoviruses with G614.