

Supplementary Methods

Standard operation procedure (SOP) for mNeonGreen SARS-CoV-2 Neutralization assay

Reagents and equipment

- Greiner Bio-One™ CellStar™ 96-Well, Cell Culture-Treated, Flat-Bottom (Greiner Bio-One™ 655090)
- Corning™ Clear Polystyrene 96-Well Microplates, round bottom (Corning Cat. No: 3799)
- Culture medium: DMEM (Gibco Cat. No: 11965) supplemented with 10% FBS, 1%p/S
- Assay medium: phenol red-free DMEM (Gibco Cat.No: 31053028), supplemented with 2% GlutaMAX (Gibco, Cat. No: 35050079), 2% FBS, 1%P/S.
- Fetal Bovine Serum (Hyclone Cat. No.: SH30071)
- Penicillium-Streptomycin (P/S) (10,000 U/ml) (Gibco Cat. No: 15140122)
- Gibco™ Trysin-EDTA (0.25%), phenol red (Gibco Cat. No.: 25200072)
- Phosphate Buffered Saline (PBS) solution, pH 7.4 (Gibco Cat. No.: 10010049)
- Hoechst 33342 Solution (ThermoFisher Scientific, Cat No: 62249)
- Reagent Reservoirs 25 ml (Gilson Cat. No.: F267660) & 50 ml (Gilson Cat. No.: F267670)
- Cell Counting slides for TC10™/TC20™ Cell Counter, dual-chamber (Bio-Rad Cat. No.: 1450011)
- Corning™ Cell Culture Treated flasks (Corning™ Cat. No.: 431080)
- VACUBOY Hand Operator (INTEGRA Biosciences)
- Eppendorf Xplorer pipette, electronic 12 channel pipette 15-300 µL (Eppendorf Cat. No.: 4861000031)
- TC20™ Automated Cell Counter (Bio-Rad)
- Tissue culture CO₂ incubator
- Cytation™ 7 Cell Imaging Multi-Mode Reader (BioTek)

Prepare cells prior to infection (on day 0)

Seed cells into 96-well plate Solid Black Polystyrene Microplates with clear bottom.

- Cells are grown a T-175 flask. Upon seeing cells, remove the medium from the cells using the VACUBOY.
- Briefly rinse the cell layer with 25 ml PBS to remove all traces of serum. Remove PBS using the VACUBOY. Repeat the PBS wash once.
Note: make sure rinse every corner of the flask to get rid of any trypsin inhibitor.
- Add 3 ml Trypsin-EDTA solution. Observe cells under an inverted microscope until cell layer is dispersed (usually within 2 minutes at room temperature).
- Tap the flask vigorously to detach cells, add 12 ml of complete growth medium and pipet up and down gently to disperse the cell suspension. Transfer the cell suspension into a 50-ml falcon tube.
- Centrifuge at room temperature for 3 min at 1,200×rpm (300g).
- Remove media completely.
- Resuspend cells in 10 ml assay medium. Disperse cells by pipetting up and down.
- Count the cell number using the cell counter (C-Chip DHC-N01-5). Count live cells by mixing 50 µl of trypan blue with 50 µl of cell suspension.
- Dilute cells to a final concentration of 2.4×10^5 cells/ml. Add 50 µl of the diluted cell suspension to each well of a 96-well plate to reach 1.2×10^4 cells/well.
- Incubate the plates at 37°C with 5% CO₂.

Prepare serum dilutions and infection (on day 1)

- Heat inactivate all the sera at 56°C for 30 min.
- Prepare serial dilutions of serum in a round bottom 96-well plate. Prepare 10 serials of 2-fold dilutions. The highest dilution is 10-folds. Prepare dilutions in duplicates. See the diagram below.

D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
10	20	40	80	160	320	640	1280	2156	0

D1: 6 µl serum + 54 µl of assay medium; D2-D9: 30 µl diluted samples + 30 µl assay medium. D10: 30 µl assay medium

- Transfer 30 µl from column #D1 to column #D2 using the electronic pipets with settings of P/M, 30/50 and speed 6/4, 3X. Repeat the step for columns D3 to D9.
- At dilution D9, take 30 µl from after dilution and discard. The total volume of the dilute serum should be 30 µl/well.
- Sera dilution plate set-up. Samples are run in duplicates.

		1	2	3	4	5	6	7	8	9	10	11	12
	A												
Sample 1	B		(+)UN	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	(-)cells	
Sample 1	C		(+)UN	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	(-)cells	
Sample 2	D		(+)UN	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	(-)cells	
Sample 2	E		(+)UN	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	(-)cells	
Sample 3	F		(+)UN	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	(-)cells	
Sample 3	G		(+)UN	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	(-)cells	
	H												

- In BSL-3, add 30 µl of diluted SARS-CoV2-mNG virus (MOI is 0.5) to each well of the serum. Mix the serum with virus solutions thoroughly by gentle pipetting.
- Incubate the plates at 37°C for 1 hour.
- Transfer 50 µl virus-serum complexes to the 96-well plates seeded on day -1 (cell plates containing 50 µl of culture media per well, after adding the diluted reporter virus the total volume of each well will be 100 µl). Mix using an electronic 12-channel pipette (setting, P/M, 50/75, speed 3/3, 2X).
Example: cells+Ab+reporter virus plate setup (black plate)

		1	2	3	4	5	6	7	8	9	10	11	12
	A												
Sample 1	B		+V	+V	+V	+V	+V	+V	+V	+V	+V	+V	No virus
Sample 1	C		+V	+V	+V	+V	+V	+V	+V	+V	+V	+V	No virus
Sample 2	D		+V	+V	+V	+V	+V	+V	+V	+V	+V	+V	No virus
Sample 2	E		+V	+V	+V	+V	+V	+V	+V	+V	+V	+V	No virus
Sample 3	F		+V	+V	+V	+V	+V	+V	+V	+V	+V	+V	No virus
Sample 3	G		+V	+V	+V	+V	+V	+V	+V	+V	+V	+V	No virus
	H												

- Incubate at 37°C for 20 hr.

Data acquisition and analysis (on day 2).

- At 20 h post-infection, in BSL-3 facility, add 25 µl diluted Hoechst 33342 Solution (diluted at 400x in PBS) to each well of the 96-well plate.
- After incubating at 15 min at 37°C, acquire the images with both DAPI staining (in blue) and mNG signals (green) using Cytation™ 7 Cell Imaging Multi-Mode Reader (BioTek) according to the manufacturer's instructions.
 - 1) Count the total cell numbers: mean intensities (in blue) within the primary mask ≥ 5000 .
 - 2) Count the mNG-positive cells: mean intensities (in green) within the secondary mask ≥ 3100 .
The threshold of green intensity was selected to distinguish mNG-positive signals from the background.
 - 3) Determine the infection rate: $(100 \times \text{mNG-positive cell number} / \text{total cell number})\%$.
Optimization may be required to achieve the infection rate in the no-serum controls at 10%-30% for robustness. The cell controls (without viruses) should be $<1\%$.
- Plot the neutralization values in Prism to calculate the NT_{50} s and Hislopes.
 - 1) Normalize the infection rate to the no-serum control wells.

- 2) Plot the relative infection rate versus the dilution (log10 values) in the Prism software 8.3.
- 3) Fit the curve and calculate the Neutralizing titers (NT_{50}) using the nonlinear regression model: log(inhibitor) vs. response-variable slope (four parameters) with constrain of bottom to 0 and top to 100.
- 4) Data interpretation.
 $NT_{50} < 20$: negative; $NT_{50} \geq 20$: Positive

Supplementary Figure 1

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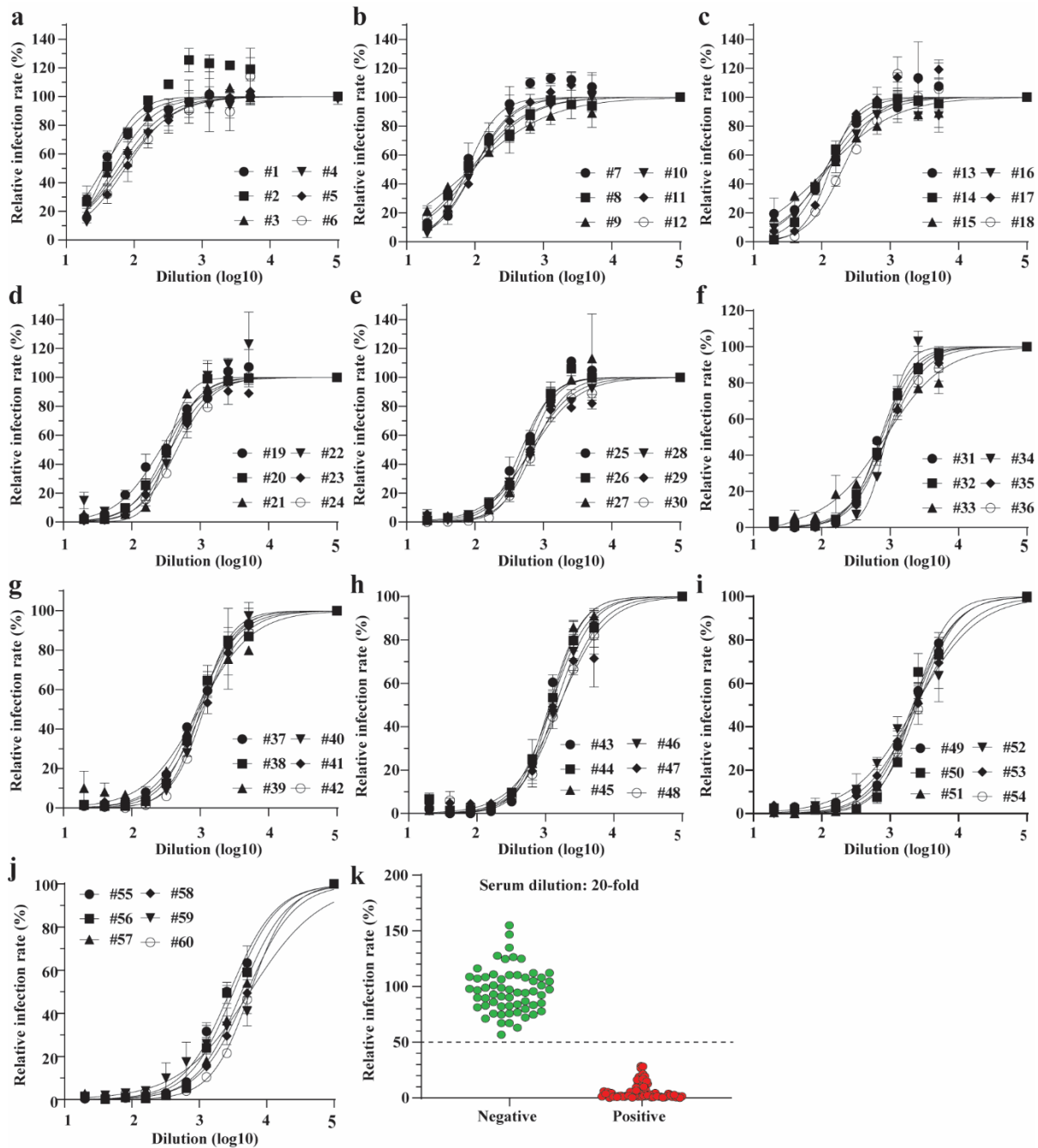
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Supplementary Figure 1. Genome sequence of mNG SARS-CoV-2. The mNG ORF with additional PacI restriction enzyme sites are shown in red.

Supplementary Figure 1



Supplementary Figure 2. Analysis of neutralizing activities of human sera using mNG SARS-CoV-2. (a-j) Neutralization curves for sixty specimens from patients confirmed with RT-PCR test positive. Means and standard deviations from two-independent experiments are shown. The dose-response curves were fitted using a nonlinear regression model in software Prism 8. (k) Relative infection rate of mNG SARS-CoV-2 for sixty COVID-19-positive and sixty COVID-19-negative human sera at dilution of 20 folds.