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Cytopathic Effect Assay and Plaque Assay to Evaluate *in vitro* Activity of Antiviral Compounds Against Human Coronaviruses 229E, OC43, and NL63

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Abstract

Coronaviruses are important human pathogens, among which the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent for the COVID-19 pandemic. To combat the SARS-CoV-2 pandemic, there is a pressing need for antivirals, especially broad-spectrum antivirals that are active against all seven human coronaviruses (HCoVs). For this reason, we are interested in developing antiviral assays to expedite the drug discovery process. Here, we provide the detailed protocol for the cytopathic effect (CPE) assay and the plaque assay for human coronaviruses 229E (HCoV-229E), HCoV-OC43, and HCoV-NL63, to identify novel antivirals against HCoVs. Neutral red was used in the CPE assay, as it is relatively inexpensive and more sensitive than other reagents. Multiple parameters including multiplicity of infection, incubation time and temperature, and staining conditions have been optimized for CPE and plaque assays for HCoV-229E in MRC-5, Huh-7, and RD cell lines; HCoV-OC43 in RD, MRC-5, and BSC-1 cell lines, and HCoV-NL63 in Vero E6, Huh-7, MRC-5, and RD cell lines. Both CPE and plaque assays have been calibrated with the positive control compounds remdesivir and GC-376. Both CPE and plaque assays have high sensitivity, excellent reproducibility, and are cost-effective. The protocols described herein can be used as surrogate assays in the biosafety level 2 facility to identify entry inhibitors and protease inhibitors for SARS-CoV-2, as HCoV-NL63 also uses ACE2 as the receptor for cell entry, and the main proteases of HCoV-OC43 and SARS-CoV-2 are highly conserved. In addition, these assays can also be used as secondary assays to profile the broad-spectrum antiviral activity of existing SARS-CoV-2 drug candidates.

Keywords: Human Coronavirus, 229E, OC43, NL63, Antiviral

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Background

Humans have been battling viruses throughout history. Viral epidemics and pandemics have caused devastating economic, social, and political unrest, in addition to significant morbidity and mortality (Shang *et al*., 2021). There are seven human coronaviruses: SARS-CoV (Gagneur *et al*., 2002), MERS-CoV (Zumla *et al*., 2015), and SARS-CoV-2 (Hui *et al*., 2020), which cause severe acute respiratory syndrome; and four common human coronaviruses HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1, which cause a significant portion of upper and lower respiratory tract infections in humans worldwide (Mesel-Lemoine *et al*., 2012). Although vaccines are the mainstay for viral prophylaxis, antiviral drugs are essential complements and play a vital role in viral disease containment, especially during the lag period between the initial viral outbreak and the delivery of effective vaccines. Furthermore, vaccines may lose potency and become ineffective when mutations emerge among circulating viruses (Collier *et al*., 2021; Lopez Bernal *et al*., 2021; Williams and Burgers, 2021). As the third coronavirus outbreak in human history, the COVID-19 pandemic is a timely reminder of the urgent need for broad-spectrum antiviral drugs that can be rapidly deployed for the prevention and treatment of emerging and re-emerging viral diseases.

We have routinely used the CPE and plaque assays to test antivirals against many different types of viruses, including influenza, enterovirus, and coronavirus (Hu *et al*., 2017a, 2017b, 2017c, 2017d, 2018, 2021a, 2021b; Li, F. *et al*., 2016, 2017; Ma *et al*., 2019, 2020a, 2020b; Musharrafieh *et al*., 2019a, 2019b, 2020; Smail *et al*., 2021; Wang *et al*., 2018; Xia *et al*., 2021; Zhang *et al*., 2018, 2019, 2020). The CPE and plaque assays have several key advantages over alternative methods. First, these assays measure viral infectivity instead of a given viral protein or gene, as both assays directly report the potency of testing compounds in inhibiting the replication of infectious viruses. Second, CPE and plaque assays are more reproducible than the $TCID_{50}$ assay (Nadgir *et al*., 2013). Third, both CPE and plaque assays are relatively inexpensive compared to RT-qPCR (Corman *et al*., 2020), immunoassays (Liu *et al*., 2005; Payne *et al*., 2006), viral flow cytometry (Brussaard *et al*., 2000), or Transmission Electron Microscope (TEM) (Roingeard, 2008), and do not require expensive reagents or specialized instruments. In this study, we have established two cell-based antiviral assays, the CPE assay and the plaque assay for HCoV-229E, HCoV-NL63, and HCoV-OC43. These assays can be used as alternatives for the primary screening of SARS-CoV-2 antivirals in BSL-2 facilities, and also as secondary assays to characterize the broad-spectrum antiviral activity of potential drug candidates. The CPE assay is appropriate for high-throughput screenings in a 96-well plate format, while the plaque assay is comparatively labor-intense and time-consuming, and is more suitable as a secondary assay to confirm the antiviral activity of initial hits identified from the primary CPE assay (Ratnam *et al*., 1995). Overall, these assays are expected to expedite the discovery of new antivirals against coronaviruses in BSL-2 facilities. In this report, we describe the detailed protocols for utilizing the CPE assay (flowchart illustrated in Figure 1) and the plaque assay (flowchart illustrated in Figure 2) to test antiviral activity of drug candidates against HCoV-229E, HCoV-NL63, and HCoV-OC43. Multiple parameters for the assays have been optimized, including the cell lines, multiplicity of infection, incubation time and temperature, and staining conditions. The optimized CPE and plaque assays conditions for HCoV-229E, HCoV-OC43 and HCoV-NL63 are listed in Table 1. Both assays have been calibrated with the positive control compounds remdesivir (Wang *et al*., 2020) and GC-376 (Ma *et al*., 2020c). Alternatively, CPE and plaque assays protocols for HCoV-229E, HCoV-OC43, and HCoV-NL63 can be found elsewhere (Schmidt *et al*., 1979; Gerna *et al*., 1980; Herzog *et al*., 2008; Bracci *et al*., 2020; Hirose *et al*., 2021; Schirtzinger *et al*., 2021).

Table 1. Cell lines and incubation temperature and time for HCoV-229E, HCoV-OC43, and HCoV-NL63 in the CPE and plaque assays in this protocol.

Virus	Assay	Plate	Cell line	Seeding density; volume per well	Total wells needed for x compounds	Incubation Temperature (°C)	Incubation Time (day)
HCoV-OC43	CPE	96-well	RD	2.5×10^4 cells/mL; 100 µL	6(x+1)*3	33	4.5
	plaque assay	6-well	RD	2.5×10^4 cells/mL; 3 mL	(6x+1)*2	33	4.5
HCoV-229E	CPE	96-well	MRC-5	5×10^4 cells/mL; 100 µL	6(x+1)*3	33	5.5
	plaque assay	6-well	RD	2.5×10^4 cells/ml; 3 mL	(6x+1)*2	33	5.5
HCoV-NL63	CPE	96-well	Vero E6	2×10^4 cells/mL; 100 µL	6(x+1)*3	37	4
	plaque assay	6-well	Vero E6	2×10^4 cells/mL; 3 mL	(6x+1)*2	37	4

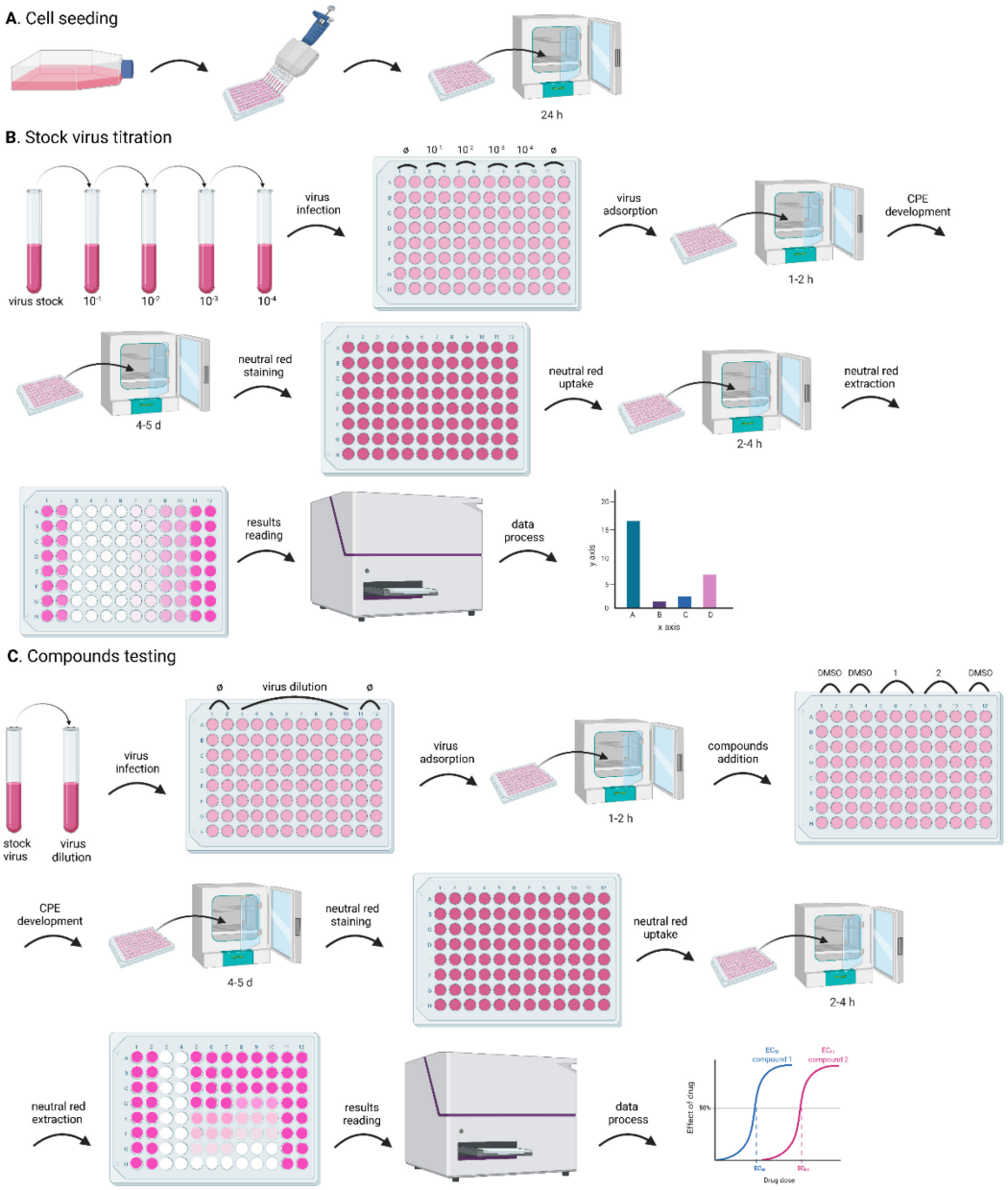

Figure 1. Flowchart for the CPE assay.

The whole process includes: cell seeding, to prepare cells in a 96-well plate for the experiments; stock virus titration, to optimize the assay conditions; and compound testing, for the evaluation of the antiviral activity of test compounds. Both stock virus titration and compound testing comprise the following steps: dilute the stock virus to obtain the desired MOI; infect the cells in the 96-well plate with a small volume (100 µL) of the diluted virus; incubate the 96-well plate in the incubator for 1-2 h, to facilitate viral attachment (virus adsorption); add the test compounds (only for the compound testing experiment); incubate the 96-well plate in the incubator to develop CPE; stain the cells with neutral red; extract neutral red from the cells; quantify neutral red by measuring absorbance; analyze data. A. Preparation of cells in the 96-well plate for the CPE assay; B. Major steps of stock virus titration in the CPE assay; C. Assessment of antiviral activity of test compounds in the CPE assay.

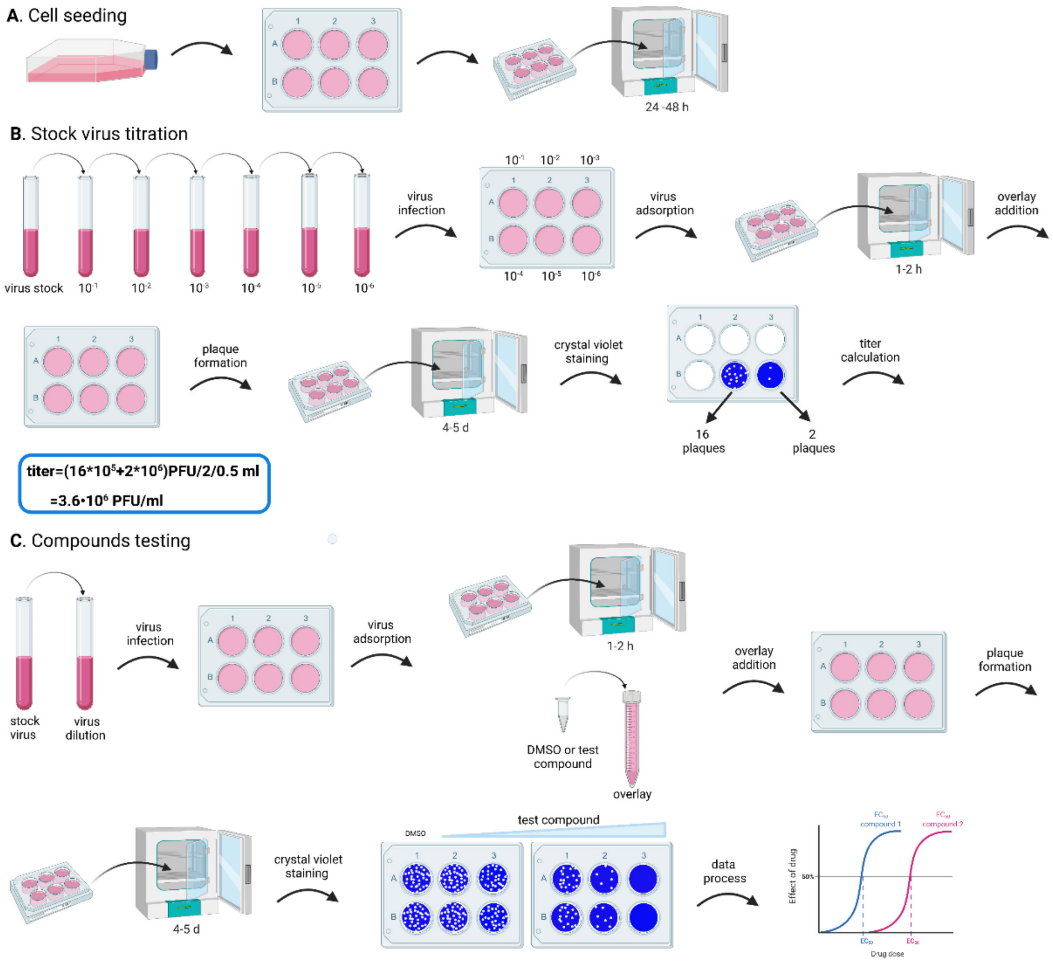

Figure 2. Flowchart for the plaque assay.

The whole process includes: cell seeding, to prepare cells in a 6-well plate for the experiments; stock virus titration, to optimize the assay conditions; and compound testing, for the evaluation of the antiviral activity of test compounds. Both stock virus titration and compound testing comprise the following steps: dilute the stock virus, to obtain the desired MOI; infect the cells in the 6-well plate with a small volume (500 µL) of the diluted virus; incubate the 6-well plate in the incubator for 1-2 h, to facilitate viral attachment (virus adsorption); add an avicel overlay containing the test compounds (only for the compound testing experiment); incubate the 6-well plate in the incubator, to allow plaque formation; stain the cells with crystal violet; analyze data. A. Preparation of cells in the 6-well plate for the plaque assay; B. Major steps of stock virus titration in the plaque assay; C. Assessment of antiviral activity of test compounds in the plaque assay.

Materials and Reagents

1. Flat-bottomed 96-well tissue culture plates (Genesee, catalog number: 25-109)
2. 96-well storage plate (Thermo Scientific, catalog number: AB-1058)
3. 96-well deep well storage plates (ThermoFisher Scientific, catalog number: 260251)
4. 50 mL conical centrifuge tubes, sterile, polypropylene (Genesee, catalog number: 25-108)
5. 15 mL conical centrifuge tubes, sterile, polypropylene (Genesee, catalog number: 25-106)
6. 10 mL serological pipets, sterile (Genesee, catalog number: 25-104)
7. 25 mL serological pipets, sterile (Genesee, catalog number: 25-106)
8. 1.7 mL DNase/RNase-free tubes (Genesee, catalog number: 25-282)
9. 1,000 µL pipette tips, low binding (Genesee, catalog number: 24-160R)

10. 200 µL pipette tips, low binding (Genesee, catalog number: 24-150R)
11. 10 µL pipette tips, low binding (Genesee, catalog number: 24-121R)
12. Tissue culture treated flasks, 600 mL, vent (Genesee, catalog number: 25-211)
13. Tissue culture treated flasks, 250 mL, vent (Genesee, catalog number: 25-209)
14. Sterile syringe filter, 0.2 µm (Fisher Scientific, catalog number: 09-740-100)
15. 6-well cell culture plates (Genesee, catalog number: 25-105)
16. HCoV-OC43 virus (BEI Resources, catalog number: NR-52725)
17. HCoV-229E virus (BEI Resources, catalog number: NR-52726)
18. HCoV-NL63 virus (BEI Resources, catalog number: NR-470)
19. Human rhabdomyosarcoma cell line, RD (ATCC® CCL-136™)
20. Human fibroblast cell line, MRC-5 (ATCC® CCL-171™)
21. HEK-293T-hACE2 cell line (BEI Resources, catalog number: NR-52511)
22. A549-hACE2 cell line (BEI Resources, catalog number: NR-53821)
23. Vero E6 cell line (ATCC® CRL-1586™)
24. HCT-8 cell line (ATCC® CCL-244™)
25. Huh-7 cell line (Millipore Sigma, catalog number: 01042712-1VL, a kind gift from Dr. Tianyi Wang at the University of Pittsburgh)
26. BHK-21 cell line (ATCC® CCL-10™)
27. BSC-1 cell line (ATCC® CCL-26™, a kind gift from Dr. Kui Li at the University of Tennessee Health Science Center)
28. Calu-3 cell line (ATCC® HTB-55™)
29. Caco-2 cell line (ATCC® HTB-37™)
30. Trypsin-EDTA, 0.25% 1×, phenol red (Genesee, catalog number: 25-510)
31. Dulbecco's modified Eagle medium (DMEM) (Genesee, catalog number: 25-501)
32. Eagle's minimum essential medium (EMEM) (ATCC® 30-2003™)
33. FBS (Gibco, catalog number: 26140-095)
34. Penicillin-Streptomycin (P/S) 100× solution (Genesee, catalog number: 25-512)
35. Glacial acetic acid (CAMEO chemicals, catalog number: UN2789)
36. $Na_2HPO_4·2H_2O$ (Sigma, catalog number: 04272-1KG)
37. KH_2PO_4 (Sigma, catalog number: P9791-1KG)
38. NaCl (Fisher Scientific, catalog number: BP358-10)
39. KCl (Sigma, catalog number: P3911-1KG)
40. Ethanol (ThermoFisher, catalog number: A405-20)
41. $MgCl_2·6H_2O$ (Sigma, catalog number: M2670-1KG)
42. $CaCl_2$ (Sigma, catalog number: C1016-2.5KG)
43. DMEM powder (Gibco, catalog number: 12-800-017)
44. HEPES, sodium salt (Gold Biotechnology, catalog number: H-401-2.5)
45. $NaHCO_3$ (Millipore Sigma, catalog number: S5761-5KG)
46. Condensed HCl, 36.5 to 38% (Fisher Scientific, catalog number: A144-212)
47. Avicel microcrystalline cellulose (FMC BioPolymer, Philadelphia, PA)
48. Crystal violet (Fisher Scientific, catalog number: AAB2193236)
49. Nano pure, or distilled and deionized water
50. Phosphate Buffered Saline (PBS) (see Recipes)
51. Neutral red (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride) (Sigma, catalog number: N4638)
52. 10× PBS stock (see Recipes)
53. 100× $MgCl_2$ stock (see Recipes)
54. 100× $CaCl_2$ stock (see Recipes)
55. DPBS, calcium and magnesium free (see Recipes)
56. PBS, with calcium and magnesium (see Recipes)
57. Neutral red stock solution (see Recipes)
58. Complete culture medium (see Recipes)
59. Neutral red working solution (see Recipes)

60. Neutral red de-staining solution (see Recipes)
61. 2× DMEM (see Recipes)
62. 1.2% avicel (see Recipes)
63. 0.2% crystal violet (see Recipes)

Equipment

1. Two cell culture incubators (Eppendorf, Galaxy® 170 R), humidified, 5% CO_2/95% air, with temperatures set up at 33°C and 37°C, respectively
2. Inverted microscope Olympus CKX53 (ThermoFisher Scientific, catalog number: NC1991101)
3. Multiskan™ FC Microplate Photometer (ThermoFisher Scientific, catalog number: 51119000)
4. Countess™ 3 Automated Cell counter (ThermoFisher Scientific, catalog number: AMQAX2000)
5. Fume hood (for hazardous chemicals)
6. Microtiter plate shaker (Fisher Scientific, catalog number: 88-861-023)
7. Hot plate stirrer (Fisher Scientific, catalog number: 11-520-49SH)
8. PIPETMAN Classic P10 (Genesee, catalog number: 37-100P10)
9. PIPETMAN Classic P100 (Genesee, catalog number: 37-100P100)
10. PIPETMAN Classic P1000 (Genesee, catalog number: 37-100P1K)
11. PIPETMAN Classic P20 (Genesee, catalog number: 37-100P20)
12. PIPETMAN Classic P200 (Genesee, catalog number: 37-100P200)
13. Reagent reservoir, sterile (VWR, catalog number: 89094-662)
14. Vortex Mixer (Fisher Scientific, catalog number: 02-215-414)
15. Centrifuge (Beckman, catalog number: A99465)
16. Bottle Top Vacuum Filters (Genesee, catalog number: 25-235)
17. Sartorius™ Biohit™ Picus™ NxT Electronic Pipettes, 50-1,200 µL, 12 Channels (Sartorius™, catalog number: LH745491)
18. Sartorius™ Biohit™ Picus™ NxT Electronic Pipettes, 0.2-10 µL, 12 Channels (Sartorius™, catalog number: LH745421)
19. HandE-Vac Handheld Aspirating System (Argos Technologies™, catalog number: 10-987-042)

Software

1. ImageJ (National Institutes of Health, version 1.50 c, https://imagej.nih.gov/ij/download.html)
2. Prism GraphPad (https://www.graphpad.com)

Procedure

A. Virus propagation

HCoV-OC43 was propagated in HCT-8 or RD cell lines. HCoV-229E was propagated in MRC-5 or Huh-7 cell lines. HCoV-NL63 was initially propagated in the HEK-293T-hACE2 cell line, followed by additional 3-4 passages in Vero E6 or Huh-7 cell lines.

Note: For all cell lines we have tried, only the HEK-293T-hACE2 cell line produces a high titer of HCoV-NL63, and the propagated HCoV-NL63 virus was accommodated in the Vero E6 or Huh-7 cell lines to allow CPE development and plaque formation.

1. Cell seeding (Day 0)

   Note: All procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet.

   a. For a T75 flask of cell culture at 90-95% confluency, aspirate the 20 mL of cell culture medium and rinse the cells with DPBS buffer (10 mL of buffer is used for a 75-cm^2 flask) by gentle agitation to remove any remaining serum, which might inhibit the action of trypsin. Repeat this step once.
   b. Add trypsin-EDTA solution (3 mL is used for a 75-cm^2 flask) to the cell monolayer in the flask, agitate gently, and incubate the flask at 37°C for 5-10 min.
   c. Lightly tap the flask to detach the cells and add prewarmed (to room temperature) complete culture medium (10 mL for a 75-cm^2 flask). Gently disperse the medium by pipetting with a serological pipette over the cell monolayer surface several times to ensure >95% recovery of cells. Pipet up and down for a few times to make sure that a homogeneous suspension is obtained.
   d. Transfer the cell suspension to a 15 mL sterile centrifuge tube and centrifuge at 500 × *g* for 10 min at room temperature (20-30°C) to spin down the cells.
   e. Aspirate the supernatant and add 10 mL of complete culture medium to resuspend the cells.
   f. Count the cell density of the suspension in step e using a cell counter.
   g. Transfer approximately 1 × 10^6 cells into a 175-cm^2 flask and bring the total volume of the cell suspension to 40 mL with complete culture medium. This will allow cells to achieve 70-80% confluency 18-24 h after seeding.
   h. Incubate the flask in a cell culture incubator (humidified, 5% CO_2/95% air, 37°C).
2. Virus infection (Day 1)
   a. Check the cell confluency in the 175-cm^2 flask using a phase-contrast inverted microscope; cells should be at 70-80% confluency.
   b. Warm DMEM medium in a 37°C metallic beads bath (a replacement for water bath to avoid potential contamination due to microbial growth in the water) and thaw the virus stocks (the initial virus was obtained from BEI Resources and stored at -80°C).
   c. Prepare virus infection medium by adding FBS into DMEM to a final concentration of 2%. For 50 mL of DMEM, add 1 mL of FBS.
   d. Dilute 100 µL of the virus stock (from step b) into 10 mL of virus infection medium and mix well by pipetting up and down a few times.
   e. Aspirate the medium in the 175-cm^2 flask and rinse the cells with PBS, calcium and magnesium buffer (50 mL of buffer is used for a 175-cm^2 flask).
   f. Aspirate the PBS, calcium, and magnesium buffer.
   g. Infect the cells in the 175-cm^2 flask by adding 10 mL of virus dilution prepared in step d. Gently swirl the flask to allow the virus dilution to evenly cover the cell monolayer.
   h. Incubate the infected cells in the 175-cm^2 flask in a cell culture incubator (humidified, 5% CO_2/95% air, 33°C for HCoV-OC43 and HCoV-229E; 37°C for HCoV-NL63) for 1-2 h. Swirl the flask every 15 min to allow the virus dilution to evenly cover the cell monolayer to facilitate virus attachment to the infected cells.
   i. Aspirate the virus dilution in the 175-cm^2 flask and add 40 mL of virus infection medium into the flask.
   j. Incubate the 175-cm^2 flask in the cell culture incubator (humidified, 5% CO_2/95% air, 33°C for HCoV-OC43 and HCoV-229E; 37°C for HCoV-NL63) for 3 days.
3. Virus collection (Day 4)

Note: HCoVs achieve the highest viral titer 2-3 days post infection, which is before significant CPE will be observed, and the titer starts to decrease afterwards. Thus, the virus should be collected at no more than 3 days post infection.

b. Transfer supernatant to another 50 mL sterile centrifuge tube using a 10 mL serological pipette. Filter the supernatant through a sterile 0.2 µm filter to remove any remaining cell debris. Aliquot the virus into sterile 1.5 mL DNase/RNase-free tubes at 1 mL per tube. Label each tube with the virus name and the date collected. Flash freeze the tubes (recommended but not necessary) and store all tubes in a -80°C freezer for future use.

B. CPE assay - Titrate the virus stock

We have examined different cell lines for HCoV-OC43, HCoV-229E, and HCoV-NL63 in both the CPE and plaque assays, and the results are listed in Table 2. HCoV-OC43 can develop complete CPE in the RD, MRC-5, and BSC-1 cell lines. HCoV-229E can develop complete CPE in MRC-5, Huh-7, and RD cell lines. HCoV-NL63 can develop complete CPE in Vero E6, Huh-7, MRC-5, and RD cell lines. In this study, the following conditions were shown as representative examples: HCoV-OC43 CPE assay in the RD cell line, HCoV-229E CPE assay in the MRC-5 cell line, and HCoV-NL63 in the Vero E6 cell line.

Table 2. Cell lines tested in the HCoV-OC43, HCoV-229E, and HCoV-NL63 CPE and plaque assays and results.

Virus	Cellular receptor	Cell lines tested in CPE and/or plaque assay	Results (work-Y, not work-N)
HCoV-OC43	9-Oacetylated sialic acid (9-O-Ac-Sia) (Hulswit *et al*., 2019)	RD	CPE assay-Y; plaque assay-Y
		MRC-5	CPE assay-Y; plaque assay-Y
		Huh-7	CPE assay-N; plaque assay-N
		Vero	CPE assay-N; plaque assay-N
		BHK-21	CPE assay-partial CPE observed; Plaque assay-N
		BSC-1	CPE assay-Y; plaque assay-Y
		Calu-3	CPE assay-N; plaque assay-N
HCoV-229E	Human aminopeptidase N (hAPN) (Li, Z. *et al*., 2019; Yeager *et al*., 1992)	RD	CPE assay-Y; plaque assay-Y
		Huh-7	CPE assay-Y; plaque assay-Y
		MRC-5	CPE assay-Y; plaque assay-Y
		Vero	CPE assay-N; plaque assay-N
		BHK-21	CPE assay-N; plaque assay-N
		Calu-3	CPE assay-N; plaque assay-N
HCoV-NL63	Angiotensin-converting enzyme2 (ACE2) (Hofmann *et al*., 2005; Wu *et al*., 2009)	MRC-5	CPE assay-Y; plaque assay-Y
		Huh-7	CPE assay-Y; plaque assay-Y
		Vero	CPE assay-Y; plaque assay-Y
		BHK-21	CPE assay-N; plaque assay-N
		Caco-2	CPE assay-N; plaque assay-N
		RD	CPE assay-Y; plaque assay-Y
		Calu-3	virus can replicate, but no CPE observed, no plaque formation

1. Cell seeding (Day 0)
   Steps a-f is the same as in **A. Virus propagation**.
   a. Dilute the cells with complete culture medium. At least 12 mL of media with a density of approximately 2.5×10^4 cells/mL is needed for one 96-well plate. This will allow the cells to achieve 80-90% confluency at 18-24 h after seeding and be ready for virus infection.
   b. Mix the cell suspension by pipetting up and down a few times and transfer the suspension to a sterile reservoir. Dispense 100 µL of the cell suspension into each well of the 96-well plate using a multichannel pipette.

      Note: Mixing the cell suspension several times to ensure it is homogeneous. Repeat periodically for even distribution.

   c. Cover the plate with a matched lid and incubate the cells in the cell culture incubator (humidified, 5% CO_2/95% air, 37°C) for 18-24 h.
2. Virus infection (Day 1)

   Note: Removing and replacing reagents in the wells needs to be performed gently and carefully to avoid disturbing the cell monolayer. A brief centrifugation step (200 × g for 5 min) can be included if necessary.

   a. Check the cell confluency in the 96-well plate, using a phase-contrast inverted microscope; cells should be 80-90% confluency.
   b. Warm DMEM medium at 37°C in a beads basket and thaw one tube of HCoV virus stock from the -80°C freezer.
   c. Prepare virus infection medium by adding FBS into DMEM to a final concentration of 2%. For 30 mL of DMEM, add 0.6 mL of FBS.
   d. Serially dilute the propagated HCoV virus from 10^1 to 10^4 folds: Label four 15 mL sterile centrifuge tubes with 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}, and add 2.7 mL of virus infection medium into each tube. In the 10^{-1} tube, add 300 µL of propagated HCoV virus stock, and mix well by vortexing at a moderate speed for 10 s; in the 10^{-2} tube, add 300 µL of the virus dilution from the 10^{-1} tube, and mix well by vortexing at moderate speed for 10 s; in the 10^{-3} tube, add 300 µL of the virus dilution from the 10^{-2} tube, and mix well by vortexing at moderate speed for 10 s; in the 10^{-4} tube, add 300 µL of the virus dilution from the 10^{-3} tube, and mix well by vortexing at moderate speed for 10 s.
   e. Aspirate the medium in the 96-well cell culture plate using the HandE-Vac Handheld Aspirating System and rinse the cells by adding 200 µL of PBS, calcium, and magnesium buffer into each well using a multichannel pipette.
   f. On the lid of the 96-well plate, label columns 1-2 and 11-12 (No virus), columns 3-4 (10^{-1}), columns 5-6 (10^{-2}), columns 7-8 (10^{-3}), and columns 9-10 (10^{-4}) as illustrated in Figures 3A, 3F, 3G, 4A, 4F, 4G, 4H, 5A, 5F, and 5G.

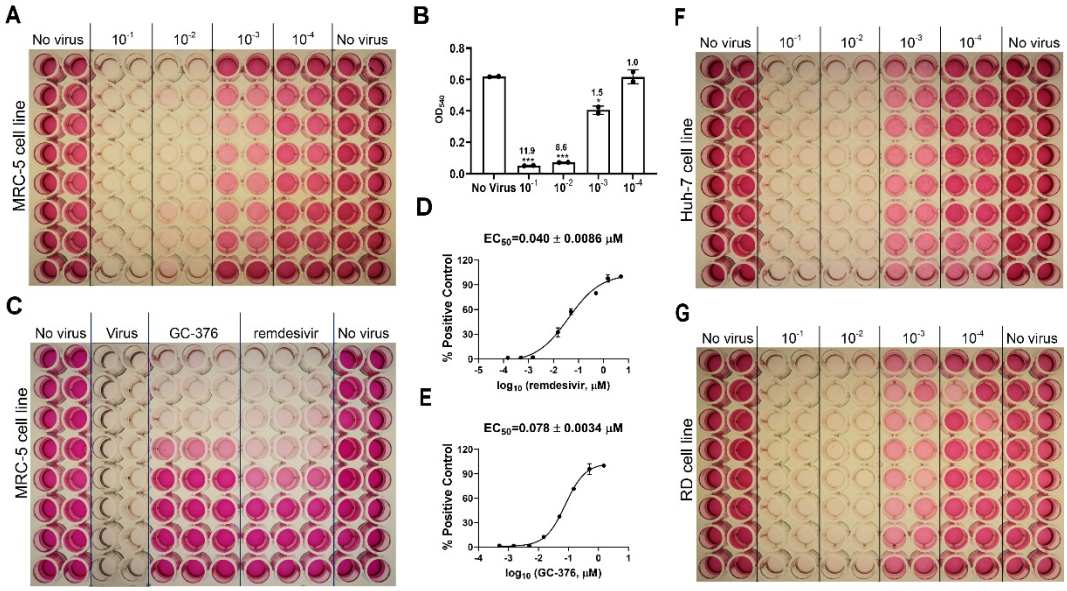

Figure 3. CPE assay for HCoV-229E.

A. Titration of HCoV-229E in the MRC-5 cell line. B. Results of HCoV-229E titration in MRC-5 cell line in CPE assay. The signal to background (S/B) ratios are labeled for each virus dilution. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (Student's *t*-test). C. Determination of EC_{50} values for GC-376 and remdesivir against HCoV-229E in the CPE assay with the MRC-5 cell line. D. EC_{50} curve fitting for remdesivir obtained in GraphPad Prism 8. E. EC_{50} curve fitting for GC-376 obtained in GraphPad Prism 8. F. Titration of HCoV-229E in Huh-7 cell line. G. Titration of HCoV-229E in RD cell line. The images are representatives of three independent repeats.

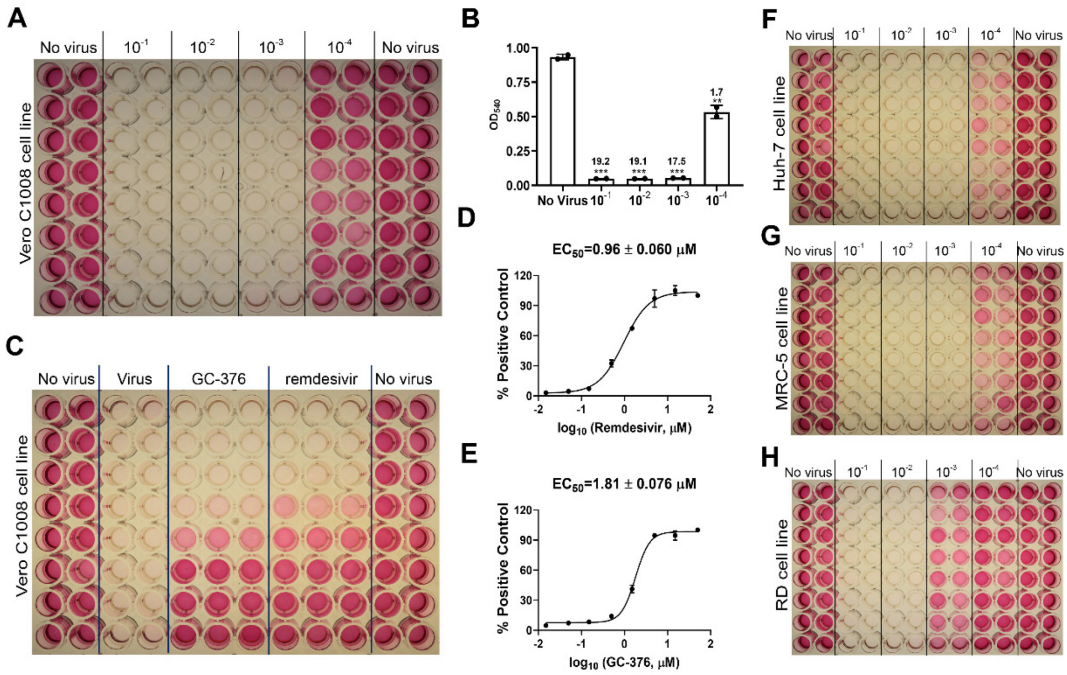

Figure 4. CPE assay for HCoV-NL63.

A. Titration of HCoV-NL63 in the Vero E6 cell line. B. Results of HCoV-NL63 titration in Vero E6 cell line in CPE assay. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (Student's *t*-test). The S/B ratios are labeled for each virus

dilution. C. Determination of EC_{50} values for GC-376 and remdesivir against HCoV-NL63 in the CPE assay. D. EC_{50} curve fitting for remdesivir obtained in GraphPad Prism 8. E. EC_{50} curve fitting for GC-376 obtained in GraphPad Prism 8. F. Titration of the HCoV-NL63 in Huh-7 cell line; G. Titration of HCoV-NL63 in MRC-5 cell line. H. Titration of HCoV-NL63 in RD cell line. The images are representatives of three independent repeats.

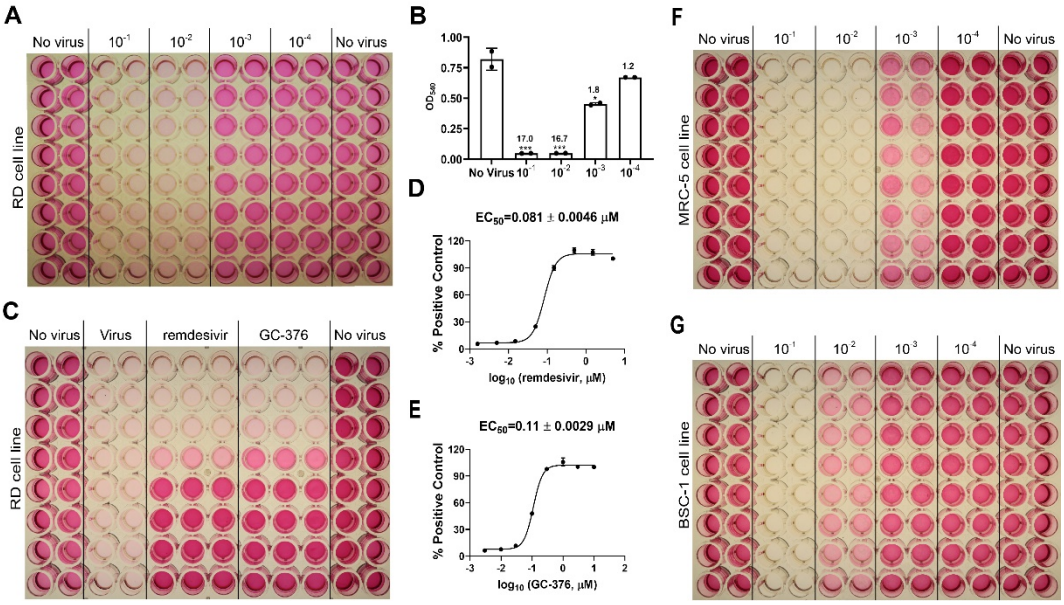

Figure 5. CPE assay for HCoV-OC43.

A. Titration of HCoV-OC43 in the RD cell line. B. Results of HCoV-OC43 titration in RD cell line in CPE assay. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (Student's *t*-test). The S/B ratios are labeled for each virus dilution. C. Determination of EC_{50} values for GC-376 and remdesivir against HCoV-OC43 in CPE assay; D. EC_{50} curve fitting for remdesivir obtained in Prism 8. E. EC_{50} curve fitting for GC-376 obtained in Prism 8. F. Titration of HCoV-OC43 in the MRC-5 cell line. G. Titration of HCoV-OC43 in the BSC-1 cell line. The images are representatives of three independent repeats.

g. Aspirate the PBS, calcium and magnesium buffer using the HandE-Vac Handheld Aspirating System.
h. To the 96-well plate prepared in step e, add 100 µL of virus infection medium into columns 1-2 and 11-12 (No virus), 100 µL of virus dilution from the 10^{-1} tube into columns 3-4 (10^{-1}), 100 µL of virus dilution from the 10^{-2} tube into columns 5-6 (10^{-2}), 100 µL of virus dilution from the 10^{-3} tube into columns 7-8 (10^{-3}), and 100 µL of virus dilution from the 10^{-4} tube into columns 9-10 (10^{-4}).

Note: A small volume of 100 µL of virus dilution was used for infecting to facilitate virus attachment to the cells.

i. Incubate the plate in the cell culture incubator (humidified, 5% CO_2/95% air, 33°C for HCoV-OC43 and HCoV-229E; 37°C for HCoV-NL63) for 1-2 h to ensure viral adsorption.
j. Add 100 µL of virus infection medium into each well in the 96-well plate to bring the total volume in each well to 200 µL, which is suitable for cell maintenance longer than 3 days.
k. Incubate the plate in the cell culture incubator (humidified, 5% CO_2/95% air, 33°C for HCoV-OC43 and HCoV-229E; humidified, 5% CO_2/95% air, 37°C for HCoV-NL63) for 4-5 days.

3. Microscopic evaluation (Days 2, 3, 4, and 5)
Examine the cells using a phase-contrast inverted microscope daily post-infection, and record changes in cell morphology and CPE development.

4. Neutral red uptake assay (Day 5)

Note: Steps a-k do not require sterile conditions or sterilized materials.

a. Centrifuge the neutral red working solution (prepared one day prior to the neutral red uptake assay and incubated in the 37°C cell culture incubator overnight) at 4,000 × *g* for 5 min at room temperature (20-30°C) to remove any precipitations.
b. Aspirate the virus infection medium in the cells and wash the cells by adding 200 µL of PBS, calcium and magnesium buffer into each well.
c. Gently transfer the neutral red working solution (step a) into a reagent reservoir.
d. Add 100 µL of neutral red working solution into each well using the 12-channel Electronic Pipette.
e. Incubate the plate in the 37°C incubator (5% CO_2) for 2-4 h.

Note: For the RD and MRC-5 cell lines, the optimal incubation time is 2 h, and longer incubation times will result in significant loss of sensitivity. For the Vero E6 and A549-hACE2 cell lines, the incubation time can be up to 4 h without affecting assay sensitivity.

f. Aspirate the neutral red working solution.
g. Wash the cells by adding 200 µL of PBS, calcium and magnesium buffer into each well.
h. Aspirate the washing buffer and remove any residual buffer by gently tapping the plate upside down on paper towels or let it air dry.

Note: The wells need to be completely dry before addition of neutral red de-staining solution.

i. Add 100 µL of neutral red de-staining solution into each well.
j. Shake the plate rapidly on a microtiter plate shaker at 200 rpm for 15 min to allow the de-staining solution to completely extract the neutral red from the cells and to form a homogeneous solution.
k. Measure the absorbance of neutral red extract at 540 nm in a spectrophotometer, save the data in .text format for analysis. The data in the .text format file will be transferred into Excel for data processing.
l. Discard the de-staining solution into a proper liquid waste container, and discard the plate.

C. CPE assay - Testing inhibitors

1. Cell seeding (Day 0)
The whole procedure in this part (steps a-i) is exactly the same as in **B. CPE assay - Titrate the virus**.
2. Virus infection (Day 1)
a. Check the confluency of the cells in the 96-well plate using a phase-contrast inverted microscope; cells should be 80-90% confluency.
b. Warm the DMEM medium at 37°C in a beads basket and thaw one tube of propagated HCoV virus stored in -80°C freezer.
c. Prepare dilutions of test compounds (GC-376 and remdesivir) in DMSO by creating eight three-fold dilutions (0.5 log unit), starting from the highest concentration of 1 mM for each compound. The concentrations of the resulting stock solutions are 1, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001, and 0.0003 mM.
d. Make virus infection medium by adding FBS into DMEM to a final concentration of 2%. For 30 mL of DMEM, add 0.6 mL of FBS.
e. Dilute HCoV stock virus for infecting. To obtain a S/B ratio greater than five, the optimal dilution for HCoV-229E is the 10^2 -fold dilution (equal to 2×10^3 PFU/mL) of the stock virus, that is 200 µL of HCoV-229E stock virus in 19.8 mL of virus infection medium. The optimal dilution for HCoV-NL63 is the 10^3 -fold dilution (equal to 7.2×10^3 PFU/mL) of the stock virus, that is 20 µL of HCoV-NL63

stock virus in 19.98 mL of virus infection medium. The optimal dilution for HCoV-OC43 is 10^2 -fold dilution (equal to 1.38×10^4 PFU/mL) of the stock virus, that is 200 µL HCoV-OC43 stock virus in 19.8 mL virus infection medium.

f. Aspirate the medium in the 96-well plate using the HandE-Vac Handheld Aspirating System and rinse the cells by adding 200 µL of PBS, calcium, and magnesium buffer into each well using a multichannel pipette. On the plate lid, label columns 1-2 and 11-12 as 'No virus'; columns 3-4 as 'virus'; columns 5-7 as 'remdesivir'; and columns 8-10 as 'GC-376', as illustrated in Figures 3B, 4B, and 5B.

g. Aspirate the PBS, calcium and magnesium buffer using the HandE-Vac Handheld Aspirating System.

h. Add 100 µL of virus infection medium into columns 1-2 and 11-12 (No virus) in the 96-well cell culture plate, and 100 µL of virus dilution (step e) into columns 3-10.

i. Incubate the 96-well plate in the cell culture incubator (humidified, 5% CO_2/95% air, 33°C for HCoV-OC43 and HCoV-229E, 37°C for HCoV-NL63) for 1-2 h.

j. Add 4 µL of DMSO or each concentration of the test compounds GC-376 and remdesivir (step c) into 400 µL of virus infection medium in a 96-well Deep well storage plate, pipette up and down a few times to mix well.

k. Transfer 100 µL of virus infection medium containing DMSO, remdesivir or GC-376 serial concentrations (in step j) into columns 3-4 (Virus), 5-7 (remdesivir), or 8-10 (GC-376), respectively, in the 96-well plate.

l. Incubate the 96-well plate in the cell culture incubator (humidified, 5% CO_2/95% air, 33°C for HCoV-OC43 and HCoV-229E, 37°C for HCoV-NL63) for 3-5 days.

3. Microscopic evaluation (Days 2, 3, 4, and 5)
Examine the cells using a phase-contrast inverted microscope daily post infection., Complete CPE should develop in columns 3-4 (Virus) by 4 to 5 days post infection.

Note: Finding the proper endpoint of the CPE assay is critical when testing compounds. It is ideal to perform neutral red uptake assay when the virus group gives ~95% CPE.

4. Neutral red uptake assay (Day 5)
Steps a-k are exactly the same as steps a-k in **B. CPE assay - Titrate the virus**.

D. Plaque assay - Titrate the virus

1. Cell seeding (Day 0)
Steps a-f is the same as in **A. Virus propagation**.
   a. Dilute the cells with complete culture medium. At least 20 mL of approximately 2.5×10^4 cells/mL cell stock is needed for one 6-well plate.
   b. Mix the cell suspension to ensure homogeneity by pipetting up and down several times and dispense 3 mL of the cell suspension into each well of the 6-well plate.
   c. Cover the plate with a lid and incubate the cells in the cell culture incubator (humidified, 5% CO_2/95% air, 37°C) for 24 to 48 h.
2. Virus infection (Day 1)
   a. Check confluency of the cells in the 6-well plate using a phase-contrast inverted microscope; cells should be >95% confluency at ~48 h after seeding.
   b. Warm DMEM medium at a 37°C in a beads basket, and thaw one tube of propagated HCoV virus stored in -80°C freezer.
   c. Prepare virus infection medium by adding FBS into DMEM to a final concentration of 2%. For 10 mL of DMEM, add 200 µL of FBS.
   d. Prepare the serial dilution of HCoV virus from 10^1 to 10^6-fold: Label six 15 mL sterile centrifuge tubes with 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. Add 1.8 mL of virus infection medium into each tube. In the 10^{-1} tube, add 200 µL of HCoV virus stock, and mix well by vortexing at moderate speed for 10 s; in the 10^{-2} tube, add 200 µL of virus dilution from the 10^{-1} tube, and mix well by vortexing at

moderate speed for 10 s; in the 10^{-3} tube, add 200 µL of virus dilution from the 10^{-2} tube, and mix well by vortexing at moderate speed for 10 s; in the 10^{-4} tube, add 200 µL of virus dilution from the 10^{-3} tube, and mix well by vortexing at moderate speed for 10 s; in the 10^{-5} tube, add 200 µL of virus dilution from the 10^{-4} tube, mix well by vortexing at moderate speed for 10 s; in the 10^{-6} tube, add 200 µL ofvirus dilution from the 10^{-5} tube, and mix well by vortexing at moderate speed for 10 s.

e. Aspirate the growth medium in the 6-well plate and rinse the cells with 3 mL of PBS, calcium, and magnesium buffer in each well. Gently swirl the plate to wash the cells thoroughly. On the plate cover, label each well with 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}, as illustrated in Figures 6A, 6F, 6G, 7A, 7F, 7G, 7H, 8A, 8F, and 8G.

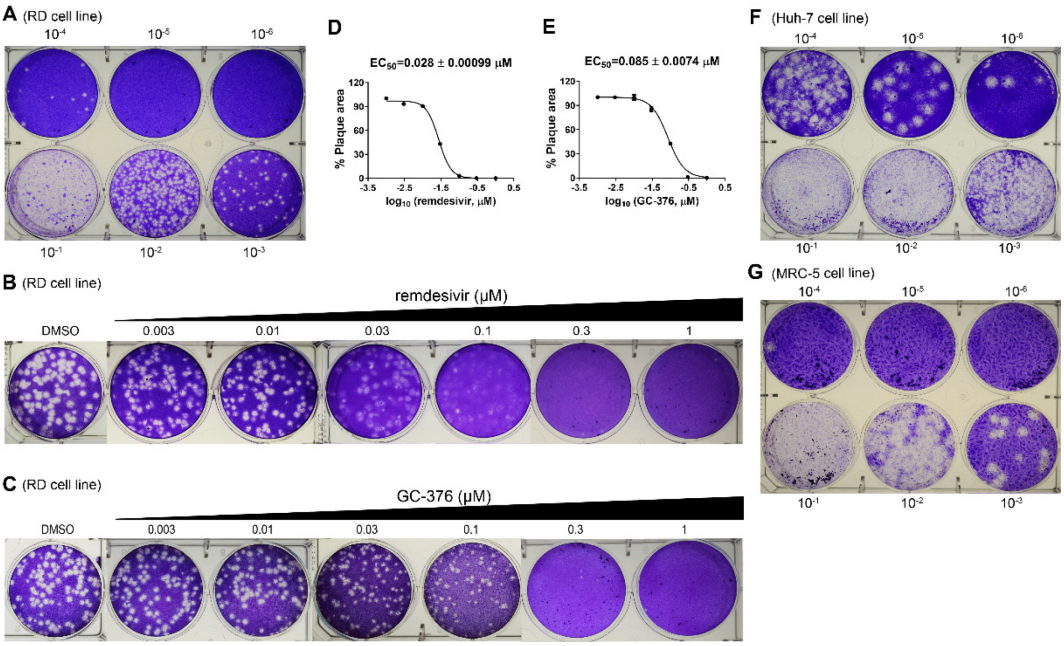

Figure 6. Plaque reduction assay for HCoV-229E.

A. Titration of HCoV-229E in the RD cell line. B. Determination of EC_{50} value for remdesivir against HCoV-229E in plaque assay. C. Determination of EC_{50} value for GC-376 against HcoV-229E in plaque assay. D. EC_{50} curve fitting for remdesivir obtained in GraphPad Prism 8. E. EC_{50} curve fitting for GC-376 obtained in GraphPad Prism 8. F. Titration of HcoV-229E in the Huh-7 cell line. G. Titration of HCoV-229E in the MRC-5 cell line. The images are representatives of three independent repeats.

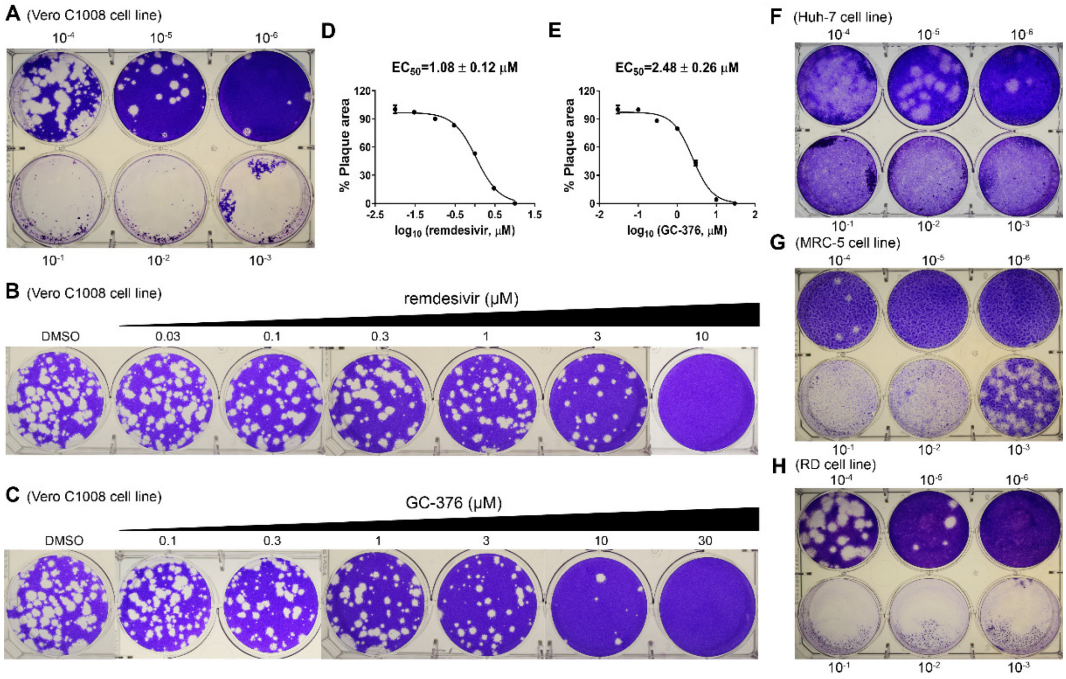

Figure 7. Plaque reduction assay for HCoV-NL63.

A. Titration of HCoV-NL63 in the RD cell line. B. Determination of EC_{50} value for remdesivir against HCoV-NL63 in plaque assay. C. Determination of EC_{50} value for GC-376 against HCoV-NL63 in plaque assay. D. EC_{50} curve fitting for remdesivir obtained in GraphPad Prism 8. E. EC_{50} curve fitting for GC-376 obtained in GraphPad Prism 8. F. Titration of HCoV-NL63 in the Huh-7 cell line. G. Titration of HCoV-NL63 in the MRC-5 cell line. H. Titration of HCoV-NL63 in the RD cell line. The images are representatives of three independent repeats.

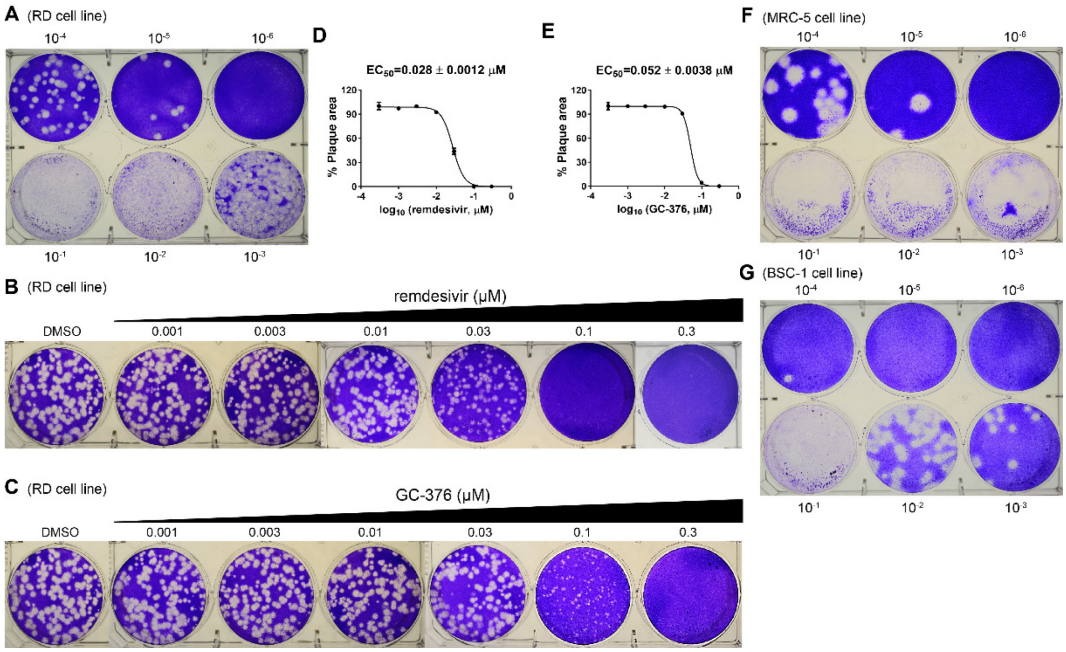

Figure 8. Plaque reduction assay for HCoV-OC43.

A. Titration of HCoV-OC43 in the RD cell line. B. Determination of EC_{50} value for remdesivir against HCoV-OC43 in plaque assay. C. Determination of EC_{50} value for GC-376 against HCoV-OC43 in plaque assay. D. EC_{50} curve fitting for remdesivir obtained in GraphPad Prism 8. E. EC_{50} curve fitting for GC-376 obtained in GraphPad Prism 8. F. Titration of HCoV-OC43 in the MRC-5 cell line. G. Titration of HCoV-OC43 in the BSC-1 cell line. The images are representatives of three independent repeats.

f. Aspirate the PBS, calcium, and magnesium buffer.
g. Add 500 µL of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} virus dilutions from step d into the corresponding wells. Gently swirl the plate to allow the virus inoculum to evenly cover the cell monolayer.

Note: The desired volume of virus inoculum for infecting the cells in a 6-well plate will be 250 µL to 1 mL per well, to allow complete coverage of the cell monolayer and efficient adsorption of viruses.

h. Incubate the infected cells in the 6-well plate in the cell culture incubator (humidified, 5% CO_2/95% air, 33°C for HCoV-OC43 and HCoV-229E, 37°C for HCoV-NL63) for 1-2 h.
i. During the incubation, prepare overlay containing 0.6% Avicel in DMEM plus 2% FBS by mixing equal volumes of 2× DMEM and 1.2% Avicel. For one 6-well plate, mix 12.5 mL of 2× DMEM and 12.5 mL of 1.2% Avicel with 500 µL of FBS.
j. Aspirate viral inoculum from step h and wash the unbound virus in each well with 3 mL of PBS, calcium and magnesium buffer. Gently swirl the plate to wash the cells thoroughly.
k. Aspirate the PBS, calcium and magnesium buffer.
l. Add 4 mL of overlay from step i into each well in the 6-well plate.
m. Incubate the 6-well plate in the cell culture incubator (humidified, 5% CO_2/95% air, 33°C for HCoV-OC43 and HCoV-229E, 37°C for HCoV-NL63) for 4-5 days.

Note: The optimal incubation time for HCoV-OC43 is about 4 days and 15 h. The optimal incubation time for HCoV-229E is about 5 days and 15 h. The optimal incubation time for HCoV-NL63 is about 3 days and 15 h. However, an incubation time that is 6 h shorter or longer than the optimal time will not significantly affect the results.

3. Plate staining (Day 5)
   a. Aspirate the Avicel overlay.
   b. Remove the residual Avicel by washing the cells with 3 mL of PBS, calcium and magnesium buffer each well.
   c. Aspirate the PBS, calcium and magnesium buffer.
   d. Add 1.5 mL of 0.2% crystal violet into each well, incubate at room temperature for 30 min.
   e. Discard the crystal violet into a liquid waste container for biohazard materials, immerse the plate into tap water to remove the extra crystal violet dye.
   f. Leave the plate on a bench to allow it to air dry and take an image of the plate.

E. Plaque assay - Test antiviral activity of compounds

1. Cell seeding (Day 0)
   Steps a-i are exactly the same as in **D. Plaque Assay - Titrate the virus**, except that seeding cells in five 6-well plates for testing two compounds to determine the EC_{50}s.
2. Virus infection (Day 1)
   Steps a-b, follow the same procedure as in **D. Plaque Assay - Titrate the virus**.
   a. Prepare virus dilution for infecting. The optimal condition for testing compounds in the plaque assay is to have 80-150 plaques in each well of a 6-well plate. Based on the titration results in Figure 6A,

the optimal dilution for HCoV-229E is 800-fold dilution (equal to 250 PFU/mL) of stock virus. That is, for five 6-well plates, add 25 µL of stock virus into 20 mL of virus infection medium, and mix well for infecting. Based on the titration results in Figure 7A, the optimal dilution for HCoV-NL63 is 30,000-fold dilution (equal to 240 PFU/mL) of stock virus. That is, for five 6-well plates, add 6.7 × 10^{-4} µL of stock virus into 20 mL of virus infection medium, and mix well for infecting. However, it is preferred to serially dilute the HCoV-NL63 stock virus 10^{-5}-fold, and add 67 µL of the 10^{-5}-fold dilution into 20 mL of virus infection medium, and mix well for infecting. Based on the titration results in Figure 8A, the optimal dilution for HCoV-OC43 is 5,000-fold (equal to 276 PFU/mL) dilution of stock virus. That is, for five 6-well plates, add 4 µL of stock virus into 20 mL of virus infection medium. However, it is preferred to first dilute the HCoV-OC43 stock virus 10^{-1}-fold, and add 40 µL of the 10^{-1}-fold dilution into 20 mL of virus infection medium, to minimize variations.

b. Aspirate the growth medium in the 6-well plate and rinse the cells with 3 mL of PBS, calcium, and magnesium buffer in each well. Gently swirl the plate to wash the cells thoroughly.
c. Aspirate the PBS, calcium, and magnesium buffer.
d. Add 500 µL of diluted virus from step c into each well of the five 6-well plates. Gently swirl the plate to allow the virus inoculum to evenly cover the cell monolayer.
e. Incubate the infected cells in the 6-well plates in the cell culture incubator (humidified, 5% CO_2/95% air, 33°C for HCoV-OC43 and HCoV-229E, 37°C for HCoV-NL63) for 1-2 h.
f. During the incubation, prepare the overlay solution which consists of 0.6% Avicel in DMEM, 2% FBS, and different concentrations of the test compounds or DMSO (control). For five 6-well plates, mix 65 mL of 2× DMEM and 65 mL of 1.2% Avicel with 2.6 mL of FBS. Aliquot 8.5 mL of the overlay to each tube (for two wells in duplicates), label the name and concentration of the test compound on each tube, and add the corresponding concentration of the compound into the overlay in each tube, vortex the tube to mix the compound, and overlay thoroughly.
g. Aspirate the viral inoculum and wash the unbound virus with 3 mL of PBS, calcium and magnesium buffer. Gently swirl the plate to wash the cells thoroughly. On the plate lid, label the name and concentration of the test compounds for each well.
h. Aspirate the PBS, calcium, and magnesium buffer.
i. Transfer 4 mL of overlay containing the test compound from step g into the corresponding wells in the 6-well plate.
j. Incubate the 6-well plate in the cell culture incubator (humidified, 5% CO_2/95% air, 33°C for HCoV-OC43 and HCoV-229E, 37°C for HCoV-NL63) for 4-5 days.

3. Plate staining (Day 5)
   Steps a-f, follow the same procedure as in **D. Plaque Assay - Titrate the virus**.

Note: The optimal incubation time for the three CoVs in the plaque assay was determined in Plaque assay - Titrate the virus. It's recommended to record the time that the incubation of the plates starts, to determine when it is ready for staining.

Data analysis

A. For virus titration in the CPE assay

Take the average reading of each group: No virus, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions. Plot the data in GraphPad Prism 8 and calculate the signal to background ratio (S/B) for each virus dilution group by dividing the average reading of the No virus group by the average reading of each virus dilution group (Figures 3C, 4C, 5C). Choose the virus dilution with the lowest virus amount that gives S/B > 5 for compound testing.

Note: Omit the reading of the outer wells of the plate (wells in row A and row H, columns 1 and 12) and only use data in the inner wells for data processing. The data in the outer wells are not reliable due to the higher rate of evaporation causing inconsistent cell growth compared to the inner wells. An alternative way is to fill the outer wells with PBS or media only when setting up the plates during cell seeding.

B. For testing inhibitors in the CPE assay

In GraphPad Prim 8, create 'New table & graph', select 'XY', and on 'options', enter '3' replicate values for Y. Input log (concentration of testing compound) in column X, and the three replicate values of absorbance at 540 nm corresponding to each concentration of the test compound in column Y. Click on 'Analyze' button, select 'Normalize', enter the average value of absorbance at 540 nm of the 'Virus' group as 0% and the average value of absorbance at 540 nm of the 'No virus' group as 100%. The EC_{50}s of test compounds were determined by fitting the curves with nonlinear regression using log (inhibitor) vs response with variable slopes (Figures 3D, 3E; 4D, 4E; 5D, 5E).

Note: Only use cells in the inner wells for data processing.

C. For virus titration in the plaque assay

Calculation of virus titer: The virus titer will be calculated by taking the average of the last two wells which show countable plaques. For the titration result of HCoV-229E (Figure 6A), the 10^{-4} well had 12 plaques, so the titer is 12×10^4 PFU/0.5 mL, which corresponds to 2.4×10^5 PFU/mL; the 10^{-3} well had 80 plaques, so the titer is 80×10^3 PFU/0.5 mL, which corresponds to 1.6×10^5 PFU/mL. The final titer will be (2.4×10^5 PFU/mL + 1.6×10^5 PFU/mL)/2 = 2×10^5 PFU/mL. For the titration result of HCoV-NL63 (Figure 7A), the 10^{-6} well had 4 plaques, so the titer is 4×10^6 PFU/0.5 mL, which corresponds to 8×10^6 PFU/mL; the 10^{-5} well had 32 plaques, so the titer is 32×10^5 PFU/0.5 mL, which corresponds to 6.4×10^6 PFU/mL. The final titer will be (8×10^6 PFU/mL + 6.4×10^6 PFU/mL)/2 = 7.2×10^6 PFU/mL. For the titration result of HCoV-OC43 (Figure 8A), the 10^{-5} well had 8 plaques, so the titer is 8×10^5 PFU/0.5 mL, which corresponds to 1.6×10^6 PFU/mL; the 10^{-4} well had 58 plaques, so the titer is 58×10^4 PFU/0.5 mL, which corresponds to 1.16×10^6 PFU/mL. The final titer will be (1.6×10^6 PFU/mL + 1.16×10^6 PFU/mL)/2 = 1.38×10^6 PFU/mL. The results of HCoV-229E, HCoV-NL63, and HCoV-OC43 titer calculation are summarized in Table 3.

Table 3. Titer calculation for HCoV-229E, HCoV-NL63, and HCoV-OC43.

Virus	Plaque assay titration results	Plaque counting	Viral titer (PFU/mL)
HCoV-229E	Figure 6A	10^{-4} well: 12	$(12\times10^4/0.5+80\times10^3/0.5)/2=2\times10^5$
		10^{-3} well: 80	
HCoV-NL63	Figure 7A	10^{-6} well: 4	$(4\times10^6/0.5+32\times10^5/0.5)/2=7.2\times10^6$
		10^{-5} well: 32	
HCoV-OC43	Figure 8A	10^{-5} well: 8	$(8\times10^5/0.5+58\times10^4/0.5)/2=1.38\times10^6$
		10^{-4} well: 58	

D. For testing inhibitors in the plaque assay

Quantify the plaque area in each well of the 6-well plate in ImageJ (a step by step detailed instruction for how to measure area percentage using ImageJ can be found at this website:

https://cs.appstate.edu/ret/imageJ/PClabs/imlab/measure/pctarea.html). In GraphPad Prim 8, create 'New table & graph', select 'XY', and on 'options', enter '2' replicate values for Y. Input log (concentration of testing compound) in column X, and the two replicate values of plaque area quantified in ImageJ corresponding to each concentration of the test compound in column Y. Click on 'Analyze' button, select 'Normalize', enter 0 as 0% and the average value of plaque area of the 'DMSO' wells as 100%. The EC_{50}s of test compounds were determined by fitting the curves with nonlinear regression using log (inhibitor) vs response with variable slopes (Figures 3D, 3E; 4D, 4E; 5D, and 5E).

Note: Plaque area was used for quantification because it provides more accurate results, as plaques formed from the same virus infection are not uniform and vary in size. Also, in the plaque assay, some compounds show antiviral activity by reducing the sizes of the plaques at certain lower concentrations and completely inhibiting plaque formation at higher concentrations.

Notes

Troubleshooting

The trouble shooting advice can be found in Table 4.

Table 4. Troubleshooting information for CPE and plaque assay.

Experiment	Problem	Possible reasons	Solutions
CPE	Readings in No virus group are low	Cell density is low	Increase cell density during cell seeding
		Cell detachment during the experiment process	Centrifuge the cells at 200 × *g* for 5 min after each washing step; fix cells before neutral red uptake assay
		Cells in senescence or contaminated	Use a new batch of cells
		Neutral red dye precipitated out	Use freshly made neutral red stock solution
Plaque assay	Cells detached/No crystal violet staining	Cells dried out during the experiment	Always keep the cells in medium or buffer, try to be as fast as possible during solution change
		Cells contaminated	Use a new batch of cells
		Reagents used are not sterile	Make new reagent stocks and sterilize all reagents by autoclave or filtration
		Compounds tested are toxic	Decrease the concentrations of the compounds tested
		MOI of infecting virus is too high	Decrease MOI by increasing the dilution factor of stock virus
	Plaque numbers are low	MOI of infecting virus is too low	Increase MOI by decreasing the dilution factor of stock virus

Recipes

1. **10× PBS stock**
   Dissolve 17.8 g $Na_2HPO_4·2H_2O$, 2.4 g KH_2PO_4, 80 g NaCl and 2 g KCl in 1 L of nano pure H_2O. Autoclave and store at 4°C for up to 2 months.

2. **100× MgCl2 stock**
   Dissolve 10.165 g $MgCl_2·6H_2O$ in 1 L of nano pure H_2O. Filter with 0.2 µm vacuum-driven filter and store at 4°C for up to 2 months.

3. **100× CaCl2 stock**
   Dissolve 9.989 g $CaCl_2$ in 1 L of nano pure H_2O. Filter with 0.2 µm vacuum-driven filter and store at 4°C for up to 2 months.

4. **DPBS, calcium and magnesium-free**
   Add 100 mL of the 10× PBS stock into 900 mL of nano pure water, autoclave and store at room temperature (20-30°C) for up to 2 months.

5. **PBS, with calcium and magnesium**
   For 1 L, add 100 mL of the 10× PBS stock, 10 mL of 100× $MgCl_2$ stock, 10 mL of 100× $CaCl_2$ stock into 880 mL of nano pure water. Filter with 0.2 µm vacuum-driven filter and store at room temperature (20-30°C) for up to 2 months.

6. **Neutral red stock solution (4 mg/mL)**
   Weigh 400 mg neutral red powder, dissolve in 100 mL of DPBS by stirring for 2 h, and filter the solution with 0.2 µm vacuum-driven filter. Protect the solution from light with foil and store at room temperature (20-30°C), for up to 2 months.

7. **Complete culture medium (culture medium plus 10% FBS and 1%P/S, vol/vol)**
   For 500 mL of DMEM, add 50 mL of FBS and 5 mL of P/S. Mix well and store at 4°C for up to 2 weeks.

8. **Neutral red working solution (50 µg/mL)**
   Dilute 1:80 of the neutral red stock solution with DMEM in centrifuge tubes under sterile conditions. For example, mix 150 µL of neutral red stock solution with 11.85 mL of DMEM for one 96-well plate. Incubate overnight in the incubator where cells are cultured.

   Note: Neutral red working solution should be prepared one day before use and incubated in the CO_2 cell culture incubator overnight.

9. **Neutral red de-staining solution (50% ethanol, 49% deionized water, 1% glacial acetic acid, vol/vol)**
   Add 500 mL of ethanol (96%), 490 mL of nano pure water, 10 mL of glacial acetic acid, mix well by stirring for 15 min, and store at room temperature (20-30°C), for up to 2 months.

10. **2× DMEM**
    Dissolve 2 packets of DMEM powder and 7.4 g $NaHCO_3$ into 1 L of nano pure H_2O, add 10 mL 1 M of HEPES buffer (pH7.2), and 20 mL of 100× P/S. Adjust pH to ~7.2 with concentrated HCl. Filter with 0.2 µm vacuum-driven filter and store at 4°C for up to 2 weeks.

11. 1.2% avicel (wt/vol)

Weigh 12 g Avicel powder and add into 1 L of nano pure H_2O, mix by stirring for 1-2 h at room temperature. Autoclave and store at room temperature (20-30°C), for up to 3 months.

12. 0.2% crystal violet (wt/vol)

Weigh 2 g crystal violet, add into 200 mL of methanol and mix to completely dissolve the crystal violet. Add 800 mL of nano pure water, mix well and store at room temperature (20-30°C), for up to 6 months.

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Competing interests

The authors declare no competing interests.

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