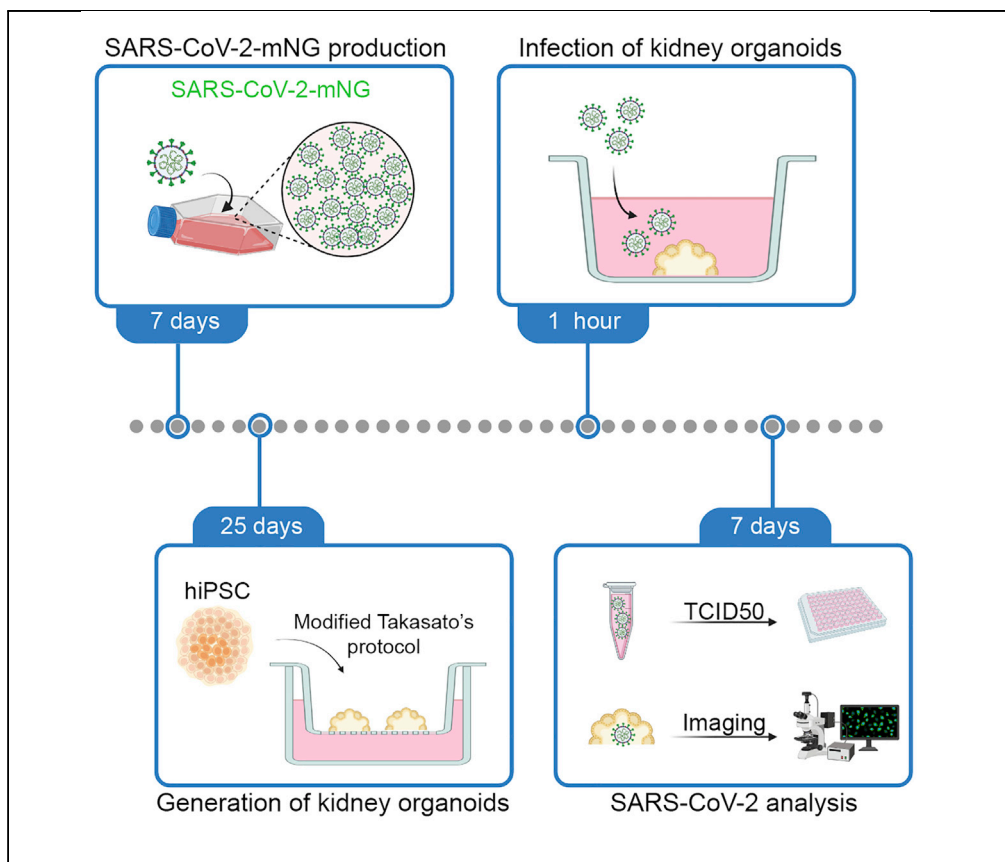


## Protocol

# Infecting kidney organoids with a cDNA reporter clone of SARS-CoV-2



Induced pluripotent stem cell (iPSC)-derived kidney organoids can be used for disease modeling and drug testing. Here, we describe a protocol to prepare stocks of an infectious clone of SARS-CoV-2 expressing a stable mNeonGreen reporter (icSARS-CoV-2-mNG). We demonstrate the infection of kidney organoids, primarily at the proximal tubular cells, with icSARS-CoV-2-mNG. Using a TCID50 (tissue culture infectious dose 50) assay and confocal microscopy, we show the quantification of SARS-CoV-2-mNG signal in proximal tubular cells of the kidney organoids.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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### Highlights

Detailed procedure  
for the production  
and titration of SARS-  
CoV-2-mNG

Efficient SARS-CoV-  
2-mNG infection of  
kidney organoid of  
varying sizes

Quantitative analysis  
of SARS-CoV-2 in  
kidney organoids by  
mNG expression

## Protocol

## Infecting kidney organoids with a cDNA reporter clone of SARS-CoV-2

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## SUMMARY

Induced pluripotent stem cell (iPSC)-derived kidney organoids can be used for disease modeling and drug testing. Here, we describe a protocol to prepare stocks of an infectious clone of SARS-CoV-2 expressing a stable mNeonGreen reporter (icSARS-CoV-2-mNG). We demonstrate the infection of kidney organoids, primarily at the proximal tubular cells, with icSARS-CoV-2-mNG. Using a TCID<sub>50</sub> (tissue culture infectious dose 50) assay and confocal microscopy, we show the quantification of SARS-CoV-2-mNG signal in proximal tubular cells of the kidney organoids.

For complete details on the use and execution of this protocol, please refer to Rahmani et al. (2022).

## BEFORE YOU BEGIN

## Institutional permissions

- △ CRITICAL: Use of human iPSC must conform to all relevant governmental and institutional regulations.
- △ CRITICAL: Handling of icSARS-CoV-2-mNG must be conducted in a contamination level-3 facility and in accordance with your institution's approved biosafety level-3 (BSL-3) biosafety protocols. Work with fixed virus can be performed in a Class II, type A2 biosafety cabinet, herein referred to as a BSC.
- △ CRITICAL: Any biohazardous and/or toxic waste generated must be appropriately disposed of in accordance with local regulations and your institution's BSL-3 biosafety protocols.

Prior to generating kidney organoids, the following steps outline preparations including the coating of plates with Matrigel and routine iPSC culturing techniques.

## Matrigel coating of plates for iPSC culture

- ⌚ Timing: 120 min (coating of 4 × 6 well plates)



### Day 1

1. Thaw human embryonic stem cell (hESC)-qualified Matrigel on ice at 4°C overnight.
2. Place the thawed Matrigel and twenty pre-labeled microcentrifuge tubes on ice in a BSC.
3. Gently mix and aliquot 250 µL Matrigel into the pre-chilled tubes using a pre-chilled P1000 pipet tip.

**△ CRITICAL:** Handle Matrigel on ice as it starts to solidify above 10°C. P1000 and P200 pipet tips used for Matrigel transfer should be pre-chilled by storing them at –20°C.

**Note:** Matrigel aliquots can be stored at –80°C for up to 6 months until ready to use. Thaw the required number of aliquots a day prior to planned coating of plates on ice at 4°C overnight.

4. Dilute 250 µL thawed Matrigel in 25 mL ice-cold DMEM/F-12 on ice.
5. Mix the Matrigel thoroughly with a pre-chilled 10 mL serological pipette.
6. Add 1 mL diluted Matrigel to each well of a 6 well plate.

**Note:** Avoid introducing air bubbles to the Matrigel solution as this can cause uneven coating.

7. Gently swirl the plate/flask to ensure that the entire surface is evenly covered.
8. Incubate for 1.5 h at room temperature in the BSC.
9. Aspirate the Matrigel for iPSC plating.

**▣ Pause point:** Matrigel-coated plates can be sealed with parafilm and stored at 4°C for up to 2 days.

### Thawing of iPSC

**⌚ Timing:** 30 min

10. Prepare a Matrigel-coated 6 well plate and mTeSR1 medium warmed up to room temperature.
11. Obtain a cryovial of high-quality human iPSC from prior culture. Thaw the cryovial in a 37°C water bath for 2 min until a small frozen chunk remains.
12. Transfer thawed iPSC into a 15 mL conical tube using a 5 mL serological pipette in a BSC.
13. Add 5 mL pre-warmed mTeSR1 medium to the cells in a drop-wise manner.
14. Centrifuge the cells at 200 × g for 4 min at room temperature.
15. Aspirate the supernatant and resuspend the cells in 2 mL mTeSR1 medium containing 10 µM ROCK inhibitor (Y-27632).
16. Plate 2 mL cell-suspension into a Matrigel-coated well of the 6 well plate. Gently move the plate a few times back and forth and left to right to ensure the iPSC colonies are evenly dispersed.

**Note:** Our frozen vial contains approximately  $1.0 \times 10^6$  cells (seeding density in a well of 6 well plate should be  $\sim 1.0 \times 10^5$  per  $\text{cm}^3$ ).

17. Incubate the cells for 24 h in a 37°C, 5% CO<sub>2</sub> humidified incubator ([troubleshooting 1](#)).
18. After 24 h, change the iPSC medium to 2 mL fresh mTeSR1 medium without ROCK inhibitor.
19. Change mTeSR1 medium and monitor iPSC confluence daily. iPSC should be passaged when they reach 70%–80% confluency (duration is variable).

**Note:** iPSC should not be cultured in the same well over 7 days ([troubleshooting 2](#)).

**Passaging and maintenance of iPSC**

⌚ Timing: 7 days (30 min for passaging)

*Day 2*

20. Prepare Matrigel-coated plates and mTeSR1 medium warmed to room temperature.
21. Remove the medium and wash the cells with 2 mL PBS.
22. Add 1 mL Gentle Cell Dissociation Reagent (GCDR) to the cells and incubate at room temperature for 5–6 min.
23. Aspirate GCDR and add 2 mL mTeSR1 medium.
24. Gently scrape and detach iPSC colonies using a wide cell scraper. Carefully pipet 4–6 times to break up the floating iPSC colonies using a 10 mL serological pipette.

**Note:** Gentle pipetting is required to preserve uniformly sized iPSC colonies. Heterogeneously sized iPSC colonies can lead to poor iPSC attachment and inconsistent differentiation.

25. Passage iPSC colonies at 1:3 or 1:6 ratio to Matrigel-coated wells of 6 well plates filled with 2 mL mTeSR1 medium.
26. Gently move the plate a few times back and forth and left to right to ensure the iPSC colonies are evenly distributed.
27. Incubate the cells in a 37°C, 5% CO<sub>2</sub> humidified incubator.

**Note:** iPSC should be confluent in 3–4 days. Monitor iPSC proliferation rate daily. Proceed to differentiation when growth of iPSC is in exponential phase.

**KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Sheep polyclonal anti-NPHS1	R&D Systems	Cat. #AF4269 RRID: AB_2154851
Rabbit monoclonal anti-PDGFRβ	Cell Signaling Technology	Cat. #4564 RRID: AB_2236927
Mouse monoclonal anti-CDH1 (clone 36)	BD Transduction Laboratories	Cat. #610181 RRID: AB_397580
Alexa Fluor 647 conjugated anti-sheep IgG	Thermo Fisher Scientific	Cat. #A21448 RRID: AB_1500712
Alexa Fluor 568 conjugated anti-rabbit IgG	Thermo Fisher Scientific	Cat. #A10042 RRID: AB_2534017
Alexa Fluor 405 conjugated anti-mouse IgG	Abcam	Cat. #ab175658; RRID: AB_2687445
Lotus Tetragonolobus Lectin (LTL), Fluorescein conjugated	Vector Laboratories	Cat. #FL-1321-2 RRID: AB_2336559
Lotus Tetragonolobus Lectin (LTL), Cy5-conjugated	GlycoMatrix	Cat. #21761117-1
<b>Bacterial and virus strains</b>		
icSARS-CoV-2-mNG	(Xie et al., 2020)	N/A
<b>Chemicals, peptides, and recombinant proteins</b>		
mTeSR1™1	STEMCELL Technologies	Cat. #85850
Gentle Cell Dissociation Reagent	STEMCELL Technologies	Cat. #7174
STEMdiff™ APEL2™ medium	STEMCELL Technologies	Cat. #05275
Protein-Free Hybridoma Medium (PFHM-II)	Thermo Fisher Scientific	Cat. #12040077
0.05% Trypsin-EDTA	Thermo Fisher Scientific	Cat. #25300-062
Dulbecco's modified Eagle's medium/F12	Thermo Fisher Scientific	Cat. #11330032
DMEM (1×), high glucose, with L-glutamine	Thermo Fisher Scientific	Cat. #11995-065
DMEM (2×), with L-glutamine and sodium pyruvate	Wisent Inc.	Cat. #319-205-CL

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Advanced RPMI 1640 medium	Thermo Fisher Scientific	Cat. #12633012
Embryonic stem cell-qualified fetal bovine serum	Thermo Fisher Scientific	Cat#1614179
Matrigel hESC-Qualified Matrix LDEV-free	Corning	Cat. #354277
Donkey serum	MilliporeSigma	Cat. #S30
Fetal Bovine Serum	MilliporeSigma	Cat. # F1051
Heparin sodium salt	MilliporeSigma	Cat. #H4784
CHIR99021	R&D Systems	Cat. #4423
Y-27632 (ROCK1/2 inhibitor, InSolution™)	MilliporeSigma	Cat. #688001
16% aqueous Paraformaldehyde (PFA)	EMS	Cat. #15710
Recombinant human FGF-9	R&D Systems	Cat. #273-F9-025
GlutaMax (100×)	Thermo Fisher Scientific	Cat. #35050061
Phosphate buffered saline pH 7.4 (1× PBS)	Thermo Fisher Scientific	Cat. #10010-023
Penicillin-Streptomycin-Glutamine (PSQ, 100×)	Thermo Fisher Scientific	Cat. #10378-016
Penicillin-Streptomycin	Gibco	Cat. #15140122
Cellulose – colloidal, microcrystalline	MilliporeSigma	Cat. #435244
Crystal violet	MilliporeSigma	Cat. # C0775
Ethanol	Greenfield Global	Cat. #P006EAAAN
Hoechst 33342	Thermo Fisher Scientific	Cat. #H3570
≥ 36.0% Formaldehyde solution for molecular biology	MilliporeSigma	Cat. #47608
<b>Experimental models: Cell lines</b>		
Human iPSC line (derived from human dermal fibroblast, Cell Applications, Inc. Cat#106-05n, Lot# 1481)	Gift from Dr. Martin Pollak, Harvard Medical School	N/A
Vero C1008 [Vero 76, clone E6, Vero E6]	ATCC	Cat. # CRL-1586
<b>Software and algorithms</b>		
GraphPad Prism 8.3.0	GraphPad	<a href="https://www.graphpad.com/">https://www.graphpad.com/</a>
ImageJ	National Institutes of Health	<a href="https://imagej.nih.gov/ij/">https://imagej.nih.gov/ij/</a> ; RRID: SCR_003070
BioRender	<a href="https://www.biorender.com/">BioRender.com</a>	
<b>Critical commercial assays</b>		
Scepter 2.0 Handheld Automated Cell Counter	Millipore	Cat. #PHCC20040
<b>Other</b>		
μ-slide 8 well glass-bottom chamber	ibidi	Cat. #80826
24 mm Transwell	Corning	Cat. #3450
96-well Clear Round Bottom Ultra-Low Attachment Microplate	Corning	Cat. #7007
Cryogenic Vials	VWR	Cat. #66008-706
Human serum albumin	MilliporeSigma	Cat. #89731
1.5 mL microcentrifuge tubes	VWR	Cat. #82050-504
15 mL conical tubes	VWR	Cat. #89039-664
50 mL conical tubes	VWR	Cat. #89039-656
TC-treated 6-well plate	VWR	Cat. #CA62406-161
TC-treated 12-well plate	VWR	Cat. #82050-930
TC-treated 96-well plate	VWR	Cat. #82050-771
TC-treated T-175 flask	VWR	Cat. #82050-872
CA syringe filter 0.45 μm	VWR	Cat. #CA28145-481
Syringe filter 0.22 μm	VWR	Cat. #CA28143-350
Triton X-100	MilliporeSigma	Cat. #T9284

**MATERIALS AND EQUIPMENT**

**Medium for iPSC differentiation and kidney organoids**

Reagent	Final concentration	Amount
APEL2	N/A	500 mL
PFHM II	~5%	25 mL
<b>Total</b>		<b>525 mL</b>

- Add 25 mL of PFHM II to 500 mL APEL2 and mix thoroughly.
- Aliquot 40 mL in 50 mL conical tubes and store at  $-20^{\circ}\text{C}$  up to 6 months.
- Store APEL2/PFHM II medium (hereafter referred to as APEL2) at  $4^{\circ}\text{C}$  and use it within 2 weeks.
- Warm up APEL2 medium at room temperature.

### Neutralization medium

Reagent	Final concentration	Amount
DMEM/F-12	N/A	450 mL
FBS	10%	50 mL
<b>Total</b>		<b>500 mL</b>

### Kidney organoid medium for SARS-CoV-2 treatment

Reagent	Final concentration	Amount
Advanced RPMI 1640 medium	N/A	490 mL
GlutaMax (100 $\times$ )	1 $\times$	5 mL
Penicillin/Streptomycin (100 $\times$ )	1 $\times$	5 mL
<b>Total</b>		<b>500 mL</b>

### Fixative reagent for control kidney organoids—2% Paraformaldehyde (PFA)

Reagent	Final concentration	Amount
16% PFA	2%	1 mL
1 $\times$ PBS	N/A	7 mL
<b>Total</b>		<b>8 mL</b>

- Store 16% stock PFA at room temperature.
- Prepare fixative solution fresh on day of use. Do not re-use.

### Fixative reagent for kidney organoids uninfected or infected with SARS-CoV-2—4% PFA

Reagent	Final concentration	Amount
16% PFA	4%	10 mL
1 $\times$ PBS	N/A	30 mL
<b>Total</b>		<b>40 mL</b>

- Store 16% stock PFA at room temperature.
- Prepare fixative solution fresh on day of use. Do not re-use.

### Fixative reagent for plaque assay—4% formaldehyde

Reagent	Final concentration	Amount
MilliQ H <sub>2</sub> O	N/A	44.45 mL
Formaldehyde solution for molecular biology, $\geq 36.0\%$ in H <sub>2</sub> O	4%	5.55 mL
<b>Total</b>		<b>50 mL</b>

- Prepare fixative solution fresh on day of use. Do not re-use.

### Vero E6 growth medium—Complete DMEM

Reagent	Final concentration	Amount
DMEM (base)	N/A	495 mL
FBS	10%	50 mL
PSQ (100 $\times$ )	1 $\times$	5 mL
<b>Total</b>		<b>550 mL</b>

- Prepare fresh aliquot. store at 4°C. Warm to 37°C prior to use.
- Use within 4 weeks of preparation.

**Viral inoculum medium—Serum-free DMEM**

Reagent	Final concentration	Amount
DMEM	N/A	50 mL
<b>Total</b>		<b>50 mL</b>

- This is DMEM alone, absent of heat inactivated FBS and PSQ. Warm to 37°C prior to use.

**Virus growth medium—Low-serum DMEM**

Reagent	Final concentration	Amount
DMEM (base)	N/A	48.5 mL
FBS	2%	1 mL
PSQ (100x)	1x	0.5 mL
<b>Total</b>		<b>50 mL</b>

- Prepare fresh then store at 4°C. Warm to 37°C prior to use.
- Use within 4 weeks of preparation.

**2.4% (w/v) colloidal cellulose in H<sub>2</sub>O—2.4% cellulose**

Reagent	Final concentration	Amount
MilliQ H <sub>2</sub> O	N/A	100 mL
Colloidal cellulose	2.4% (w/v)	2.4 g
<b>Total</b>		<b>100 mL</b>

- Gradually add Milli-Q H<sub>2</sub>O to weighed colloidal cellulose while stirring vigorously.
- Continue vigorous stirring for 30 min to avoid forming clumps.
- Autoclave solution on liquid cycle for sterilization then store at room temperature.
- Use within 4 months of preparation and ensure to vigorously mix bottle by hand before each usage.

*Optional:* Initial preparation on a hot plate set to 65°C also helps colloidal cellulose dissolution to avoid clumping.

**Plaque assay overlay medium—Cellulose overlay**

Reagent	Final concentration	Amount
2x DMEM (base)	1x	25 mL
2.4% cellulose (base)	1.2%	25 mL
Heat inactivated FBS	1%	0.5 mL
PSQ (100x)	1x	0.5 mL
<b>Total</b>		<b>51 mL</b>

- Ensure to mix stock 2.4% cellulose well before use in preparing cellulose overlay.
- Prepare cellulose overlay fresh on day of use then incubate at 37°C.
- Any leftover reagent should be disinfected and discarded after use.

### 1.0% (w/v) crystal violet in 20% ethanol—Staining solution

Reagent	Final concentration	Amount
MilliQ H <sub>2</sub> O (base)	80% (v/v)	80 mL
100% ethanol (base)	20% (v/v)	20 mL
Crystal violet	1.0% (w/v)	1.0 g
<b>Total</b>		<b>100 mL</b>

- Add crystal violet powder to 20% (v/v) ethanol solution in MilliQ H<sub>2</sub>O.
- Mix vigorously by shaking for 10 s.
- Wrap bottle completely in aluminum foil since crystal violet is light sensitive.
- Store at room temperature and use within 6 months of preparation.

### Blocking buffer for kidney organoids

Reagent	Final concentration	Amount
Donkey serum	10%	5 mL
PBS	N/A	45 mL
Triton X-100	0.06%	30 $\mu$ L
<b>Total</b>		<b>50.03 mL</b>

- Store at  $-20^{\circ}\text{C}$  and use within 2 months of preparation.

### Wash buffer for kidney organoids

Reagent	Final concentration	Amount
PBS	N/A	49.85 mL
Triton X-100	0.3%	150 $\mu$ L
<b>Total</b>		<b>50 mL</b>

- Store at room temperature and use within 2 weeks of preparation.

### Chemical inhibitors and growth factors

- InSolution™ Y-27632 (ROCK inhibitor, 5 mM stock): Aliquot 10  $\mu$ L and store at  $-20^{\circ}\text{C}$  for up to 6 months. Protect from light.
- CHIR99902 (CHIR): Prepare 10 mM stock solution by adding 2.15 mL DMSO to 10 mg CHIR. Store in 50  $\mu$ L aliquot at  $-20^{\circ}\text{C}$  up to 12 months.
- Human recombinant fibroblast growth factor-9 (FGF-9): Prepare 100  $\mu$ g/mL stock solution. Reconstitute 25  $\mu$ g FGF-9 in 250  $\mu$ L 0.1% human serum albumin (HSA) in PBS. Store at  $4^{\circ}\text{C}$  up to two weeks or  $-80^{\circ}\text{C}$  for up to 6 months.
- Heparin: Prepare a 1 mg/mL stock solution by reconstituting 10 mg heparin in 10 mL ultrapure water. Filter-sterilize the solution using a 0.22  $\mu$ m filter. Store at  $4^{\circ}\text{C}$  for up to 6 months.

## STEP-BY-STEP METHOD DETAILS

### Preparation of icSARS-CoV-2-mNG stock

⌚ Timing: 7 days

This step describes the propagation of an established infectious clone of SARS-CoV-2 which expresses a stable mNeonGreen reporter (herein referred to as icSARS-CoV-2-mNG)



(Xie et al., 2020). While Xie et al. describe how icSARS-CoV-2-mNG was generated, here we describe its propagation in Vero E6 cells.

### Day 1

1. Seed Vero E6 cells in a clearly labeled T-175 flask(s) at a seeding cell density of  $18 \times 10^6$  cells per flask (85%–90% confluency) in 25 mL complete DMEM.
  - a. Incubate flasks overnight in a 37°C, 5% CO<sub>2</sub> humidified incubator.
  - b. Seed at least one additional flask in parallel for each propagation cycle to use for cell counting.

**Note:** Multiple flasks can be seeded if larger quantities of stock are desired. ~80 aliquots can be generated per T-175 flask (see step 16 below).

**Note:** Mock control cells are necessary if different strains of virus are being propagated simultaneously to ensure there is no cross contamination.

### Day 2

2. Determine cell number using the additional flask prepared as follows:
  - a. Aspirate medium from flask and wash once with 12 mL PBS.
  - b. Add 5 mL 0.05% trypsin-EDTA and incubate flask in a 37°C, 5% CO<sub>2</sub> humidified incubator.
  - c. Monitor for cellular detachment using an inverted phase-contrast microscope. Gentle tapping of the flask is encouraged. Detachment should take no longer than 5 min.
  - d. Add 3 mL complete DMEM to the flask to inactivate trypsin. Using a serological pipette, gently wash the cell monolayer several times to create a single cell suspension.
  - e. Collect the cell suspension into a 50 mL conical tube.
  - f. Wash the flask twice with 8 mL complete DMEM to collect residual cells. Transfer washes into same 50 mL conical tube used to collect original cell suspension.
  - g. Centrifuge cell suspension at  $500 \times g$  for 10 min.
  - h. Aspirate the resulting supernatant and resuspend the cells in 8 mL complete DMEM. Perform a cell counting per flask using any desired method (e.g., hemocytometer).

**Note:** We have found that diluting the cell stock 1:8 (dilution factor of 8) in complete DMEM works best for hemocytometer counts using these conditions.

3. Using the total cell number per flask, calculate the required volume of stock icSARS-CoV-2-mNG needed to achieve a multiplicity of infection [MOI; number of plaque forming units (PFU) of virus per cell] of 0.01.
  - a. Prepare viral inoculum to a final volume of 2.5 mL per flask in serum-free DMEM.
  - b. To ensure that your viral inoculum is in excess, prepare for one additional flask than needed in the calculation.
  - c. Example calculation: assume you have  $20 \times 10^6$  cells per flask, and plan to infect 3×T-175 flasks at a MOI of 0.01 with a viral stock that has a titer of  $4 \times 10^6$  PFU/mL. Plan to infect 4 × T-175 flasks; therefore, dilute virus stock in a final volume of 10 mL ( $4 \times 2.5$  mL) in serum-free DMEM.

DEFINE UNITS:  $MOI = \frac{PFU}{cell}$  and stock virus titer =  $\frac{PFU}{mL}$

$$\frac{\# \text{ of cells}}{\text{flask}} \times \# \text{ of flasks} \times \frac{PFU}{\text{cell}} \times \left( \frac{PFU}{mL} \right)^{-1} = \text{mL stock virus}$$

$$\frac{20,000,000 \text{ cells}}{\text{flask}} \times 4 \text{ flasks} \times \frac{0.01 \text{ PFU}}{\text{cell}} \times \frac{mL}{4,000,000 \text{ PFU}} = 0.2 \text{ mL stock virus}$$

In this case, 0.2 mL of stock virus at  $4 \times 10^6$  PFU/mL will be diluted in  $(10 - 0.2 =) 9.8$  mL of serum-free DMEM to infect  $3 \times$  T-175 flasks containing  $20 \times 10^6$  cells/flask at a MOI of 0.01, leaving  $[10 - 3(2.5) =] 2.5$  mL of viral inoculum in excess.

**△ CRITICAL:** While higher MOIs can induce infection and produce infectious virions, the MOI used for virus propagation must be low (i.e., 0.01). Otherwise, infection at relatively higher MOIs can lead to the production and release of defective interfering particles (Thompson and Yin, 2010) which can have downstream impacts in experimental use, particularly for *in vivo* studies (Banerjee et al., 2001; Yang et al., 2019).

4. Remove a stock virus-containing vial from  $-80^{\circ}\text{C}$  storage.
  - a. Thaw virus in a  $37^{\circ}\text{C}$  water bath (2–5 min).
  - b. Transfer tube to BSC and mix the vial by pulse-vortexing for 10 s (within the BSC).
5. Prepare the viral inoculum which was calculated in step 3.
6. Retrieve the T-175 flask(s) of Vero E6 cells from the  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  humidified incubator and bring into the BSC.
  - a. Remove medium from the flask(s) and add 2.5 mL viral inoculum to each flask directly.
  - b. Seal the flask cap using parafilm.
  - c. Rock the flask(s) gently by hand to distribute virus inoculum over cells.
7. Transfer the sealed flask(s) to a  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  humidified incubator for 1 h.
8. Gently rock the flask back-and-forth by hand once every 10 min to redistribute the viral inoculum thus preventing the cells from drying out.
9. After 1 h incubation with regular rocking, transfer the sealed flask(s) from the incubator into the BSC.
10. Add 17.5 mL low-serum DMEM directly to viral inoculated flask(s).

**Note:** Final volume in the flask should be 20 mL (2.5 mL viral inoculum + 17.5 mL low-serum DMEM).

11. Transfer the sealed flask(s) to a  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  humidified incubator and incubate for 40–48 h.

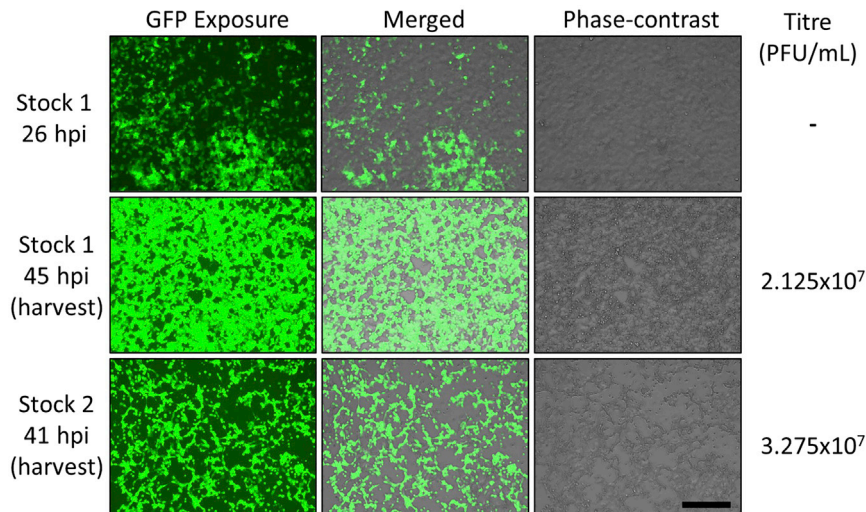
**Note:** Medium does not need to be replenished or spiked with fresh medium during the viral growth incubation period.

### Day 4

12. Monitor for cytopathic effects (CPE) using an inverted phase-contrast microscope after 40 h post infection (hpi). Monitor for viral gene expression by detection of green fluorescence that will be produced in infected cells after expression of the mNeonGreen reporter gene (Figure 1).

**Note:** CPE is evident when cells begin to contract, lose cell-to-cell contact, and lose adherence to the cell culture vessel. Stock 1–26 hpi shows the progression of viral infection (indicated by green fluorescence) but the absence of CPE since all of the cells remain adherent and maintain cell-to-cell contact (Figure 1). Conversely, Stock 1–45 hpi and Stock 2–41 hpi show both the progression of viral infection and presence of CPE with the latter having more severe CPE. Conversely, Stock 1–45 hpi and Stock 2–41 hpi show both the progression of viral infection and presence of CPE with the latter having more severe CPE.

13. Harvest when CPE is visible and remaining adherent cells fluoresce green. Reliance on only green fluorescent signal without CPE will produce a stock of lower titer (Figure 1) (troubleshooting 3).



**Figure 1. Successful propagation of icSARS-CoV-2-mNG in Vero E6 cells**

Vero E6 cells were infected with icSARS-CoV-2-mNG at a MOI of 0.01 for 1 h with intermittent shaking. CPE was monitored at 26 and 45 hpi (Stock 1) or 41 hpi (Stock 2) using an inverted phase-contrast microscope with or without exposure to GFP LED. GFP-positive cells express mNG reporter from icSARS-CoV-2-mNG which indicates successful viral replication within the cells. Titer of each respective stock was performed as described in the “plaque assay for determining icSARS-CoV-2-mNG stock titer” section and is reported as plaque-forming units (PFU)/mL. The CPE observed for stock 2 was more advanced than CPE observed for stock 1, suggesting that titer increases more accurately correlate with CPE stage rather than time of harvest, an effect that can be explained by slight variability between the initial viral inoculum. Scale bar, 300  $\mu$ m.

**△ CRITICAL:** CPE should not progress beyond what is shown in Figure 1. A population of cells which remain adherent to the flask should always remain when harvesting, as late harvest will increase the risk of defective interfering particles (Hein et al., 2021). This decreases the infectious viral stock and can impact experimental procedures, particularly for *in vivo* studies (Banerjee et al., 2001; Yang et al., 2019).

14. When CPE indicates that the virus-containing supernatant is ready to harvest virus, transfer the infected flask(s) to the BSC. Transfer the virus-containing supernatant to 50 mL conical tubes and distribute equal volumes of virus-containing medium into each tube.
15. Centrifuge supernatant at  $500 \times g$  for 10 min to clear cellular debris.
16. Filter the cleared virus-containing supernatant using a 0.45  $\mu$ m CA filter into a new 50 mL conical tube.

**Note:** Try not to disrupt the debris pellet. The filtering step is necessary to clear any residual cellular debris.

17. Mix supernatant thoroughly by pulse-vortexing for 10 s.
  - a. aliquot resulting viral supernatant into externally threaded cryogenic tubes for single-use workable stocks.
  - b. Use 0.25 mL per tube which produces  $\sim 80$  tubes per T-175 flask.

**△ CRITICAL:** It is essential to transfer the resulting centrifuged supernatant into a new tube and mix by pulse-vortexing prior to aliquoting to ensure a consistent titer of virus between aliquots. If a vortex is not available within the BSC, mix well by repeated inversion or pipetting.

△ **CRITICAL:** Viral supernatant from multiple flasks can be combined. However, if you have multiple tubes of collected virus, each tube should be treated as its own stock. The titer may differ between tubes even if collected viral supernatant is from the same propagation cycle.

18. Place the aliquots at  $-80^{\circ}\text{C}$  for long-term storage.

△ **CRITICAL:** Multiple freeze-thaw cycles of virus-containing liquids causes a significant decrease in viral titer (Greiff et al., 1954). Do not thaw and then re-freeze an aliquot for re-use.

### Plaque assay for determining icSARS-CoV-2-mNG stock titer

⌚ Timing: 7 days

This step is necessary for quantifying the titer of each icSARS-CoV-2-mNG stock. Following infection with serially diluted virus samples, utilization of a colloidal cellulose semi-solid overlay restricts spread of virus that would occur in liquid medium. As such, a single infectious virion causes infection and cell lysis in a localized area, resulting in a zone of clearing, or plaque, that can be used to calculate the stock titer.

#### Day 1

19. Seed Vero E6 cells in a 12-well plate at a seeding cell density of  $3.3 \times 10^5$  cells per well in complete DMEM.

a. Incubate plates in a  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  humidified incubator overnight.

**Note:** A minimum of 28 wells (three 12-well plates) are required for each viral stock to be titered (see Figure 2 for plate layout).

#### Day 2

20. Remove an aliquot vial of stock virus (generated as described above) from  $-80^{\circ}\text{C}$  storage.

a. Thaw virus in a  $37^{\circ}\text{C}$  water bath (2–5 min).  
b. transfer tube to BSC and mix the vial by pulse-vortexing for 10 s.

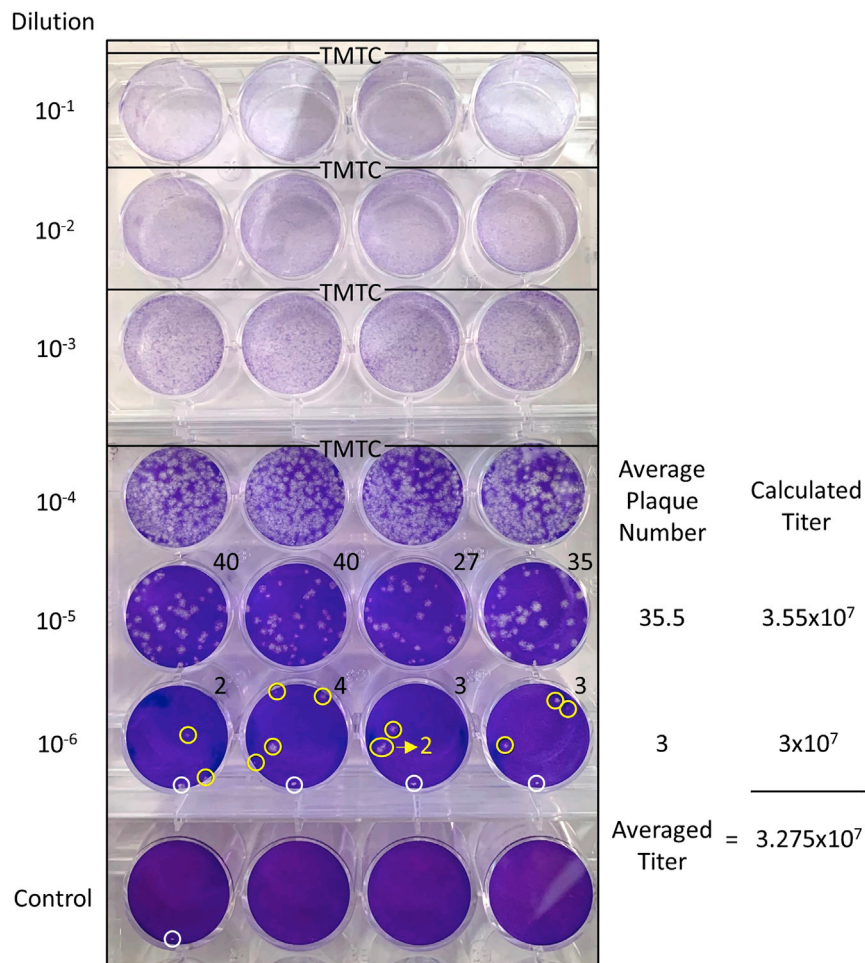
21. Within the BSC, prepare 1:10 serial dilutions by transferring 50  $\mu\text{L}$  of stock into 450  $\mu\text{L}$  of serum-free DMEM up to 6 times (to  $10^{-6}$ ) using sterile 1.5 mL tubes.

**Note:** Each dilution of virus will be used to infect cells in four replicate wells, using 100  $\mu\text{L}$  viral inoculum per well, a total of 400  $\mu\text{L}$ . The dilution series noted above will provide enough excess volume to accommodate this setup.

22. Retrieve the plates of Vero E6 cells from the  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  humidified incubator and bring into the BSC.

23. Aspirate medium and add 100  $\mu\text{L}$  diluted virus stock in 4 replicate wells to each respective well of the 12-well plates (Figure 2).

△ **CRITICAL:** Work with one plate at a time to avoid cells from drying out. Close the plate lid when not actively adding medium/sample to prevent desiccation or contamination of cell monolayers.



**Figure 2. Successful titer determination using plaque assay method in Vero E6 infected with icSARS-CoV-2-mNG**  
Vero E6 cells were infected with serially diluted stock icSARS-CoV-2-mNG for 1 h with intermittent shaking, followed by the addition of cellulose overlay. Cells were fixed and stained 62 hpi, and plaques (black circles at the 10<sup>-6</sup> dilution) were enumerated. Clearings caused by pipetting/tears (white circles at the 10<sup>-6</sup> dilution) were not counted as plaques. Counts for the number of plaques at the 10<sup>-5</sup> and 10<sup>-6</sup> dilution is indicated at the top-right of each respective well with the average plaque number, calculated titer and averaged titer reported to the right. The average titer is used as the stock titer value.

△ **CRITICAL:** Add diluted virus in the following order: most dilute (10<sup>-6</sup>) to least dilute stock (10<sup>-1</sup>). This eliminates the need to change the pipette tip while also avoiding inaccurate titer determination.

24. Seal the plates using parafilm then rock them gently by hand to distribute viral inoculum.
25. Transfer the sealed plates from the BSC to a 37°C, 5% CO<sub>2</sub> humidified incubator for 1 h.

**Note:** Gently rock the plates back-and-forth by hand once every 10 min to redistribute the viral inoculum over the cell monolayer. This step is essential to prevent cells from drying out.

26. During the incubation, freshly prepare the cellulose overlay medium (as described in the [materials and equipment](#) section). Keep the mixture in the 37°C water bath until ready to be used.

**Note:** Store the colloidal cellulose solution at room temperature and use within 4 months.

27. After the incubation, transfer the sealed plates from the incubator into the BSC and remove parafilm.

**Note:** For this assay, the viral inoculum does not need to be removed.

28. Transfer the cellulose overlay mixture to the BSC.
  - a. Add 1 mL cellulose overlay mixture directly to each well of the 12-well plates containing the 100  $\mu$ L viral dilution.
  - b. Re-seal the plates with parafilm then transfer the sealed plates from the BSC into the incubator.
29. After plates are on the shelf in the incubator, gently tilt the plates forward and back, side to side, to evenly distribute the overlay.
30. Incubate the plates for 62–66 h.

**Note:** The incubation time should range from 62–66 hpi for fixation. If the plaque assay is fixed too soon, plaque sizes may be too small to accurately count. If too late, plaque sizes may be too large and not well spaced.

**△ CRITICAL:** Do not touch the plates for the entire incubation period. Colloidal cellulose is a semi-solid overlay and any movement/disruption of the overlay can lead to spread of virus, yielding plaques with comet tails, causing an inaccurate titer determination.

### Day 5

31. Transfer the sealed plates from the incubator into the BSC.
  - a. Remove parafilm.
  - b. Shake the plates to agitate the cellulose overlay.
  - c. Discard overlay according to the appropriate CL3 biosafety protocol.
32. Wash the cells with 1 mL PBS to remove residual cellulose then pipette to remove the PBS.

**△ CRITICAL:** Be careful to limit tears in the monolayer which can appear as plaques to the untrained eye (see white circles in [Figure 2](#)).

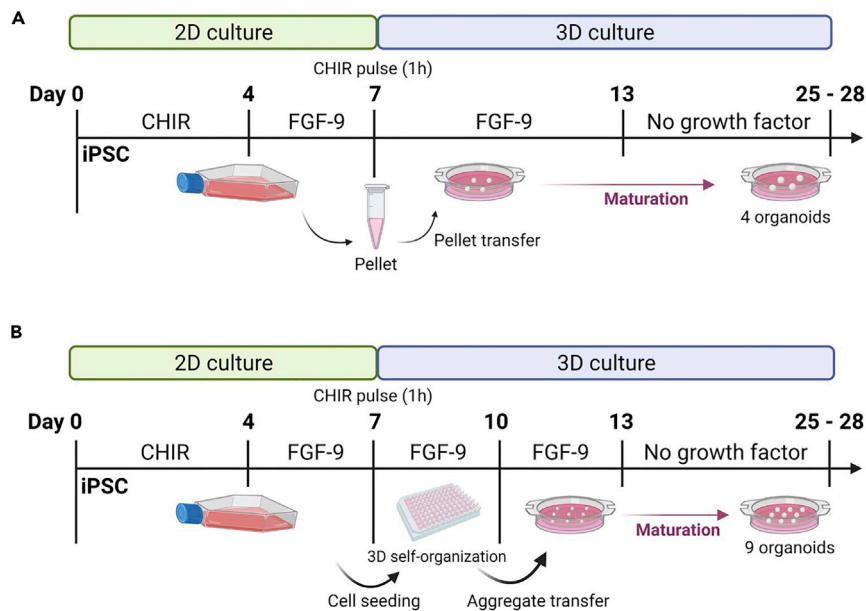
33. Add 1 mL fixative solution (4% formaldehyde) to the cells and incubate for 60 min at room temperature ([troubleshooting 4](#)).

**△ CRITICAL:** The CL3 facility must have a BSC with thimble connection so that toxic fumes from the formaldehyde are not entrapped within the facility. The plates, now containing fixative solution, must remain within this BSC for entire incubation period.

34. In the BSC with thimble connection, remove formaldehyde and wash the cells with 1 mL dH<sub>2</sub>O ([troubleshooting 5](#) and [6](#)).
35. Add 0.5 mL staining solution to each well and incubate at room temperature for 10 min. In the BSC with thimble duct, remove staining solution and wash wells at minimum three times with 0.5 mL dH<sub>2</sub>O.

**Note:** Store the staining solution at room temperature and use within 6 months of preparation.

36. Examine the stained plates for plaques to determine stock titer ([Figure 2](#)). Plaque numbers should decrease with increasing dilution of the viral inoculum ([troubleshooting 7](#)).
37. Count the number of plaques in a minimum of two different viral dilutions.
  - a. Calculate the titer from each well.



**Figure 3. Schematic illustration of protocols for generating kidney organoids using 500K or 250K cells at air-fluid interface**

(A) Standard protocol for 500K kidney organoids.

(B) Alternative protocol for 250K kidney organoids.

- b. Determine the average of two calculated titers to achieve accurate determination of your stock titer (Figure 2).
- c. The following example is based on plaque counts in the  $10^{-5}$  dilution in Figure 2:

$$\text{Titer} \left( \frac{\text{PFU}}{\text{mL}} \right) = \frac{\text{average \# of plaques at observed dilution}}{(\text{dilution factor})(\text{dilution volume})}$$

$$\text{Titer} = \frac{(40 + 40 + 27 + 35)/4}{(10^{-5})(0.1 \text{ mL})}$$

$$\text{Titer} = \frac{35.5 \text{ PFU}}{10^{-6} \text{ mL}}$$

$$\text{Titer} = 3.55 \times 10^7 \frac{\text{PFU}}{\text{mL}}$$

### Generation of kidney organoids from human iPSC lines

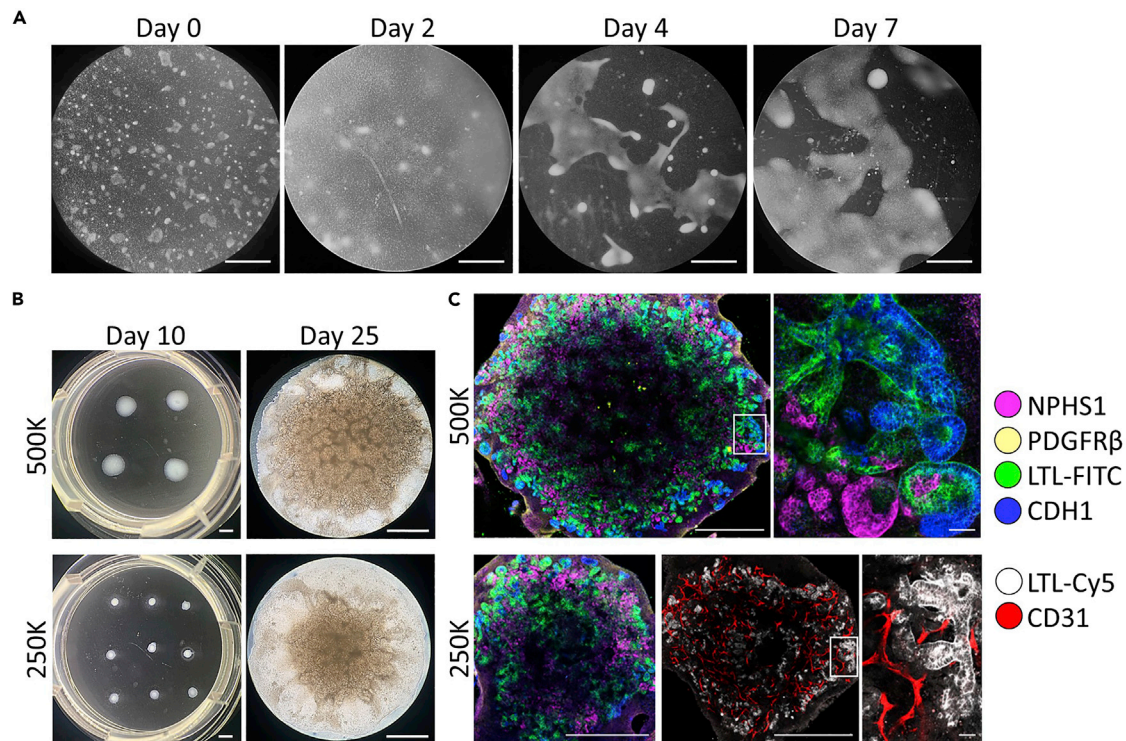
© Timing: 30 days

We have modified an established protocol developed by Takasato (Takasato et al., 2016) to generate kidney organoids more efficiently and economically in our laboratory. The steps below describe the differentiation of kidney organoids from iPSC in 500K and 250K sizes (See Figure 3 for overall steps).

Day – 1

38. Prepare one T-25 flask coated with 2 mL Matrigel on the day of iPSC plating for differentiation.





**Figure 4. Generation of kidney organoids plated using 500K or 250K nephron progenitor cells (NPC) on transwell membranes**  
 (A) Representative bright field images of growth morphological changes of human iPSC colonies during differentiation from day 0 to day 7. Scale bars, 2 mm.  
 (B) Representative bright field images of NPC plated at 500K or 250K at day 25 of differentiation. Scale bars, 1 mm.  
 (C) Immunofluorescence micrographs of whole-mount kidney organoids labeled with NPHS1 (podocytes), PDGFR $\beta$  (stromal cells), LTL-FITC (proximal tubule), CDH1 (distal tubules) at day 30 for 500K and 250K kidney organoids. Lower panels show additional labeling of 250K kidney organoids with CD31 (endothelial cells) and LTL-Cy5 (proximal tubules). Right panels are the magnified views of the white boxes from left panels. Scale bars, 1 mm in whole-mount organoids and 50  $\mu$ m in the magnified views.

39. Passage iPSC at 70%–80% confluence ( $\sim 1.0 \times 10^6$  cells) from a well of 6 well plate to the Matrigel-coated T25 flask with at a seeding density of 50,000 per  $\text{cm}^2$  in 3 mL mTeSR1 medium.
40. Gently agitate the flask to disperse colonies evenly in the flask and incubate in a 37°C, 5%  $\text{CO}_2$  humidified incubator overnight. iPSC at 40%–50% confluence is anticipated on the next day.

**Note:** A cell seeding density for each iPSC line will need to be optimized as the number of attached colonies and their growth rates can vary for each line.

#### Day 0

41. Aspirate the mTeSR1 medium and add 4 mL APEL2 medium containing 8  $\mu$ M CHIR (Figure 4A).

**Note:** Do not start the differentiation if iPSC colonies are at < 20% or > 80% confluence (trou-  
bleshooting 8).

42. Replace the medium with fresh APEL2 containing 8  $\mu$ M CHIR after 2 days.

#### Day 4

43. After 92 h CHIR treatment, replace the medium with 8 mL fresh APEL2 medium supplemented with FGF-9 (200 ng/mL) and heparin (1  $\mu$ g/mL).



44. Replace the medium with fresh APEL2 medium supplemented with FGF-9 and heparin every 2 days until day 7 of differentiation.

#### Day 7

45. Prepare Trypsin-EDTA (0.05%), neutralization medium and APEL2 medium pre-warmed to room temperature.
46. Aspirate the medium and wash the cells with 3 mL PBS.
47. Add 1 mL Trypsin-EDTA and incubate for 2 min in a 37°C, 5% CO<sub>2</sub> humidified incubator.

**Note:** Tap the flask to detach the cells after 2 min of trypsin incubation. Monitor the cells under the microscope to ensure that most of the cells have been detached.

**△ CRITICAL:** Do not expose the cells to trypsin more than 3 min. Over-trypsinization may contribute to breaking of pellet during pellet transfer to transwell membrane (step 54).

48. Add 5 mL neutralization medium to the flask to neutralize trypsin.
49. Gently pipet the cells 6–8 times into single cell suspension using a 10 mL serological pipette.
50. Transfer the cells in suspension to a 15 mL conical tube. Centrifuge at 400 × g for 3 min at room temperature.
51. Aspirate the supernatant and resuspend the cells in 3 mL APEL2 medium.
  - a. Remove 20 μL of cell suspension and perform cell counting using a Scepter 2.0 Cell counter (Millipore) or a hemocytometer.
52. Calculate and transfer the volume required to obtain 500K or 250K cells into 1.5 mL sterile centrifuge tubes.
53. Centrifuge the tubes at 400 × g for 2 min at room temperature to pellet the cells.
54. Prepare wide bore P200 tips by manually cutting off the end of tips with a sterile razor blade. The resulting diameter of open tip should approximately 3–4 mm for 500K pellets and 2–3 mm for 250K pellets ([troubleshooting 9](#)).
55. Collect the pellet using a P200 wide bore tip and transfer up to 4 pellets (500K or 250K cells per pellet) onto a six-well transwell ([Figure 4B](#)).

**△ CRITICAL:** Minimize carry-over volume during pellet transfer. Avoid placing pellets too close to each other or near the corner edge of transwell.

56. Add 1.3 mL APEL2 medium containing 5 μM CHIR to the lower compartment of transwell insert and place the plate in a 37°C, 5% CO<sub>2</sub>, humidified incubator for 1 h.
57. Remove the medium and add 1.3 mL fresh APEL2 medium supplemented with FGF-9 and heparin.
58. Culture the pellets for 6 days in a 37°C, 5% CO<sub>2</sub>, humidified incubator. Refresh APEL2 medium supplemented with FGF-9 and heparin every 2 days for 6 days.

#### Day 13

59. After 6 days of FGF-9 and heparin supplementation, switch the culture medium to APEL2 medium without growth factors every 2 days until the day of experiment (day 28).

### Infecting kidney organoids with SARS-CoV-2

⌚ Timing: 2 h

This step describes the protocol to infect kidney organoids with SARS-CoV-2 and collection of the supernatant from the kidney organoids for a tissue culture infectious dose (TCID<sub>50</sub>) assay.

60. Prepare a sterile fine forceps, razor blade and 10 cm culture plates.
61. Place the transwell plate with kidney organoids in a BSC.
62. Hold the upper plastic region of the transwell insert with one forceps and start cutting the transwell membrane with a razor blade along the edge of the transwell from the bottom side.

**Note:** Leave ~2 mm of the membrane still attached to the transwell insert to avoid a sudden drop of the membrane.

63. Place the transwell insert on the sterile surface of 10 cm culture plate.
64. Detach the membrane from the transwell by pressing the membrane with one forceps while gently pulling the transwell insert in the opposite direction.
65. Cut the membranes around each organoid in approximately 1 cm × 1 cm square using the forceps and razor blade.

**△ CRITICAL:** This process should be completed within 1 min otherwise organoids will start to dry out. Supplementation of small volume of APEL medium (~300 μL) underneath the membrane can prevent organoids from rapid drying out.

66. Transfer each organoid with sterile forceps to a 24-well plate pre-filled with 1 mL APEL2 medium. Place the plate in a 37°C, 5% CO<sub>2</sub>, humidified incubator.
67. Remove an aliquot of stock icSARS-CoV-2-mNeonGreen from -80°C storage.
  - a. Thaw virus in a 37°C water bath (2–5 min).
  - b. Transfer tube to BSC and mix the vial by pulse-vortexing for 10 s.
68. Dilute the stock virus to achieve working stocks of 10<sup>3</sup> PFU/500 μL and 10<sup>5</sup> PFU/500 μL in serum-free, drug-free advanced RPMI medium.
69. Remove medium from the kidney organoids add 500 μL of either 10<sup>3</sup> or 10<sup>5</sup> PFU virus inocula directly into the wells. Seal the plates using parafilm.

**Note:** Kidney organoids are submerged in 500 μL medium containing virus.

70. Transfer the sealed plates to a 37°C, 5% CO<sub>2</sub> humidified incubator and incubate the organoids for 1 h.

**Note:** Plates do not require intermittent shaking as the volume of medium used is enough to cover the organoids. Do not touch the plates during the incubation period.

71. After 1 h incubation, wash the organoids in 0.5 mL PBS three times to remove unbound virus.
72. Replenish 0.5 mL fresh medium and incubate in a 37°C, 5% CO<sub>2</sub> humidified incubator.
73. After 1.5 days post infection (dpi), collect supernatant from each well and replenish fresh medium.
74. Centrifuge the collected supernatant at 500 × g for 10 min to remove cellular debris and particulates.
  - a. Transfer the supernatant to a new centrifuge tube.
  - b. Pulse-vortex to mix, then aliquot into externally threaded cryogenic tubes.
  - c. Store aliquots at -80°C for TCID<sub>50</sub> assay.
75. After 1.5 days (3 dpi), remove the medium and wash the organoids once with 0.5 mL PBS.
76. Fix the organoids in 1 mL 4% PFA for 1 h at room temperature.
77. Remove 4% PFA and wash the organoids in 0.5 mL PBS for three times and keep in PBS at 4°C until further experiment is performed.

## Quantifying the viral titer of collected supernatants from icSARS-CoV-2-mNG-infected kidney organoids

⌚ Timing: 7 days

This step describes the protocol for a TCID assay which was used in our main study to determine the titer of SARS-CoV-2-mNG virus in the supernatant of infected kidney organoid cultures using Kärber method.

### Day 1

78. Seed Vero E6 cells in a 96-well plate at a seeding cell density of  $3 \times 10^4$  cells per well in complete DMEM.
  - a. Incubate plates in a 37°C, 5% CO<sub>2</sub> humidified incubator overnight.

**Note:** Seed a minimum of 14 wells per supernatant sample plus additional control wells which will not be infected. Each plate should have its own set of control wells.

### Day 2

79. Remove the virus-containing supernatant from each kidney organoid culture or retrieve this supernatant from -80°C storage.
  - a. Thaw supernatants in a 37°C water bath (2–5 min).
  - b. Transfer tube to BSC and mix the vial by pulse-vortexing for 10 s.
80. Within the BSC, prepare 1:10 serial dilutions by transferring 50 µL stock into 450 µL low-serum DMEM up to 7 times (to 10<sup>-7</sup>) using sterile 1.5 mL tubes.
81. Retrieve the plates of Vero E6 cells from the incubator and bring into the BSC.
82. Aspirate medium and add 200 µL serially diluted supernatant in duplicate to each respective well of the 96-well plates. Add 200 µL low-serum DMEM to each control well.
83. Seal the plates with parafilm and transfer the plates from the BSC to a 37°C, 5% CO<sub>2</sub> humidified incubator. Incubate for 5 days.

**Note:** Feeding/medium supplementation is not necessary during the entire incubation period.

**Note:** Since the virus expresses an mNG reporter, monolayers can be observed under GFP exposure to check for the progression of CPE during the entire incubation period.

### Day 7

84. Remove plates from the incubator when CPE is observed (5 days post infection in our experience).
  - a. Using an inverted phase-contrast microscope, score each well for presence or absence of CPE by visual inspection.
  - b. Record the number of wells per dilution that are CPE-positive ([troubleshooting 7](#)).

**Optional:** Fix and stain the 96-well plate if you wish to store a physical record of the result. Follow steps 28–33 with the following volume adjustments: use 0.2 mL PBS, 0.2 mL fixative solution (4% formaldehyde), 0.2 mL of dH<sub>2</sub>O, and 0.1 mL staining solution.

**△ CRITICAL:** Control wells should be absent of CPE and absent of fluorescence under GFP exposure. If CPE/fluorescence is present, cross-contamination has occurred, and the assay must be repeated.

85. Based on the CPE scoring, TCID<sub>50</sub>/mL is calculated according to the Kärber method, also referred to as the Spearman-Kärber method (Kärber, 1931; Ramakrishnan, 2016). The formula used an example case is as follows:

$$\log_{10}(\text{TCID}_{50}) = -x_0 - \frac{d}{2} + d * (r_1 + \Sigma r_i + r_0)$$

DEFINE:  $x_0$  = log<sub>10</sub> of last dilution wherein all wells are infected.

$d$  = log<sub>10</sub> of dilution factor; using a dilution 1:10 yields a dilution factor of 10 meaning  $d = 1$ .

$r$  = (number of wells with CPE)/(number of replicate wells).

$r_1$  =  $r$  value of last dilution where all wells have CPE.

$r_0$  =  $r$  value of first dilution where no wells have CPE.

$\Sigma r_i$  = sum of  $r$  values for all dilutions between  $r_1$  and  $r_0$ .

CONDITIONS:  $d$  – using a 1:10 dilution series yield a dilution factor of 10 meaning  $d = 1$ ; if a 1:2.

dilution series is used, the dilution factor is 2 meaning  $d = \log_{10}(2) \approx 0.301$ .

$r_1$  – will always be 1 since number of wells with CPE = number of replicate wells.

$r_0$  – will always be 0 since number of wells with CPE = 0.

$\Sigma r_i$  – if there are no dilutions between  $r_1$  and  $r_0$  then  $\Sigma r_i = 0$ .

**Table 1. Mock data for TCID<sub>50</sub> sample calculation using the Kärber method**

Dilution	# Of wells with CPE	$r$ (# of wells with CPE/# of replicate wells)
10 <sup>-1</sup>	2	1
10 <sup>-2</sup>	2	1
10 <sup>-3</sup>	1	0.5
10 <sup>-4</sup>	1	0.5
10 <sup>-5</sup>	0	0

In this example, we will assume a 1:10 dilution series for a dilution factor of 10, that the total number of replicate wells per dilution is 2, and that 200 μL of dilution was applied to each well.

DEFINE:  $x_0 = \log_{10}(10^{-2}) = -2$

$d = \log_{10}(10) = 1$

$r_1 = r(10^{-2}) = 1$

$r_0 = r(10^{-5}) = 0$

$\Sigma r_i = r(10^{-3}) + r(10^{-4}) = 0.5 + 0.5 = 1$ .

$$\log_{10}(\text{TCID}_{50}) = -x_0 - \frac{d}{2} + d * (r_1 + \Sigma r_i + r_0)$$

$$\log_{10}(TCID_{50}) = -(-2) - \frac{1}{2} + 1 * (1 + 1 + 0)$$

$$\log_{10}(TCID_{50}) = 2 - 0.5 + 2 = 3.5$$

$$TCID_{50} = 10^{3.5}$$

To get  $TCID_{50}/mL$ , divide  $TCID_{50}$  value by the dilution volume applied to each well. In this hypothetical case, the dilution volume is  $200 \mu L = 0.2 mL$  (Table 1).

$$\frac{TCID_{50}}{mL} = \frac{10^{3.5}}{0.2} \cong 1.58 \times 10^4$$

### Quantification of SARS-CoV-2 infected cells in proximal tubular cells by confocal microscopy

⌚ Timing: 2 days

This step describes the protocol for immunostaining of kidney organoids and quantifying SARS-CoV-2 infected proximal tubular cells by confocal microscopy used in our main study.

86. After washing the PFA-fixed kidney organoids once with PBS, place an organoid face-down in each well of an 8 well glass bottom chamber containing  $150 \mu L$  blocking buffer.

**Optional:** Detach the organoid from the membrane. Hold the membrane with one forceps and gently push the organoid off the transwell membrane with another forceps.

87. Block the organoids for 2–3 h at room temperature on a shaker at 400 rpm.

88. Incubate the organoids with primary antibodies (1:200) in  $150 \mu L$  blocking buffer at  $4^{\circ}C$  overnight on a shaker.

89. Wash the organoids in  $150 \mu L$  wash buffer for 15 min each and repeat three times.

90. Incubate the organoids with  $150 \mu L$  secondary antibodies (1:250) and LTL-Cy5 (1:100) in wash buffer at room temperature for 2–3 h.

**Optional:** Incubate Hoechst33342 ( $1 \mu M$ ) for 3 h to stain nuclei.

91. Wash the organoids in  $150 \mu L$  PBS for 10 min each and repeat twice.

92. Remove PBS from the well of uninfected kidney organoid. Locate the organoid using an inverted confocal microscope equipped with a  $20\times$  objective lens (e.g., Zeiss LSM 880 confocal microscope).

93. Use the 633 nm laser to locate proximal tubules labeled with LTL-Cy5 (Figure 5A).

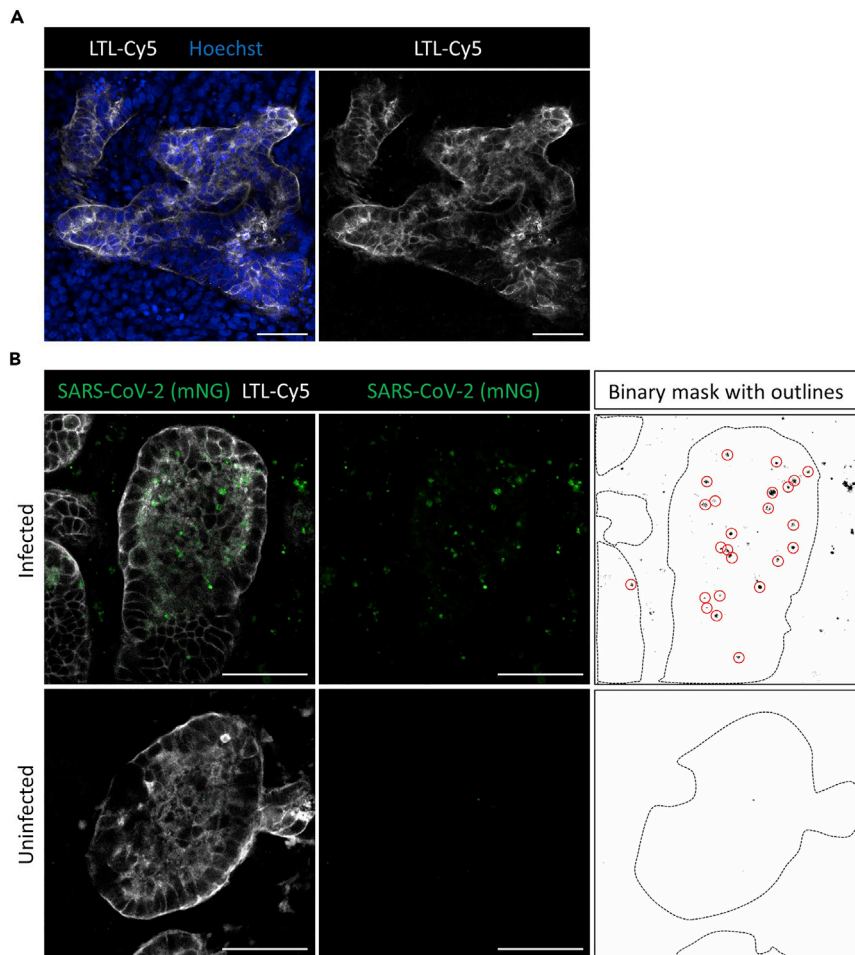
94. Use an optimum 488 nm laser power to determine minimum background signal generated from the organoid. Capture nuclei of the cells in the organoids with 405 nm laser if organoids are stained with Hoechst33342.

**Note:** It is important to eliminate background autofluorescence signal from the organoids to delineate true mNeonGreen signal. Take multiple images from different fields of view to ensure autofluorescence does not produce a false positive signal.

95. Switch to icSARS-CoV-2-mNG infected kidney organoids. Locate proximal tubules using the 633 nm laser with the same setting used in step 93.

96. Use the same 488 nm laser power (step 94) to capture mNeonGreen+ cells in the proximal tubules. Take at least 10 images from multiple fields of view per organoid (Figure 5B).

97. Run ImageJ and open the confocal images of organoids uninfected or infected with SARS-CoV-2-mNG captured by 488 nm laser.



**Figure 5. Quantification of SARS-CoV-2-mNG infected proximal tubular cells using LTL-Cy5 staining in the kidney organoids**

(A) Confocal image of proximal tubular cells labeled with LTL-Cy5 (white) and nuclei (blue) with Hoechst in the kidney organoid. Scale bar, 50  $\mu$ m.

(B) Representative confocal image of proximal tubular cells labeled with LTL-Cy5 (white) and SARS-CoV-2-mNG (green). The far-right panel shows binary masking of SARS-CoV-2-mNG in ImageJ for counting the number of infected cells. Scale bar, 50  $\mu$ m.

98. Determine the empirical threshold level for the intensity of mNeonGreen signal. Apply the same setting to all the images captured under the same condition.
99. Go to "Process" and select "Make Binary". Count total LTL+ cells and LTL+ mNeonGreen + cells per field of view (Figure 5B).

**Note:** Each proximal tubular cell membrane will be labeled with LTL-Cy5 while its nucleus will not be stained and show an empty oval space within the cell. LTL-Cy5 labeled proximal tubular cell has nucleus stained with Hoechst within the cell. We confirmed that Hoechst staining is not necessary for counting LTL+ cells (Figure 5A).

### EXPECTED OUTCOMES

iPSC-derived kidney organoids have emerged as an effective model system for examining SARS-CoV-2 infectivity and response to drugs (Helms et al., 2021; Jansen et al., 2022; Monteil et al., 2020; Wysocki et al., 2021). In this protocol, we report the procedures for generation of kidney

organoids using a modified established protocol by Takasato (Takasato et al., 2016). When kidney organoids are successfully generated, immunostaining of kidney organoids should display the presence of the major kidney structures that include podocytes (NPHS1), proximal tubules (LTL), distal tubules (CDH1, E-Cadherin) and endothelial cells (CD31). The reproducibility of well-developed structures of our modified kidney organoid protocol has been validated in our recent publication (Chun et al., 2022).

The icSARS-CoV-2-mNG reporter virus has been widely used to visually identify infected cells and evaluate viral replication for drug or vaccine development in various model systems including our study (Junqueira et al., 2022; Muruato et al., 2020; Rahmani et al., 2022; Sahin et al., 2021; Xie et al., 2020). In this report, we show methods for production of icSARS-CoV-2-mNG and the direct infection of kidney organoids with icSARS-CoV-2-mNG. Upon incubation, infected cells are expected to show strong cytoplasmic signal observed by confocal microscopy. TCID50 assay using supernatants of infected kidney organoids are expected to show presence of infectious viral particles in a dose-dependent manner. In our main study, we reported that losartan-treated kidney organoids showed lower number of SARS-CoV-2 infected proximal tubular cells and dose-dependent reduction of viral particles (Rahmani et al., 2022). Taken together, our protocols demonstrate the use of SARS-CoV-2 reporter virus in the kidney organoids provides a valuable efficient platform to monitor virus infectivity and test effectiveness of potential drugs.

## LIMITATIONS

The current human kidney organoids model the immature first trimester human fetal kidney that does not fully resemble the adult human kidney and display off-target cell populations. In this respect, interpretation of the data using organoids to humans must be conducted with caution. There may be batch-to-batch variability between organoid generation preps and/or iPSC lines.

## TROUBLESHOOTING

### Problem 1

Only a few small iPSC colonies attached to the plate after thawing.

#### Potential solution

It is expected that not all iPSC colonies following thawing will attach to the plate. More than 50% of iPSC colonies are expected to attach if the iPSC were healthy and properly cryopreserved. We recommended freezing multiple batches of early passage iPSC for reproducibility of organoid quality. iPSC will not also attach and proliferate on the surface that was poorly coated with Matrigel. Ensure that the thawing and coating of plates with Matrigel was properly performed. Addition of 10  $\mu$ M Y-27632 in the mTeSR1 for the first 24 h improves iPSC recovery after cryopreservation.

### Problem 2

iPSC grow slowly and do not reach to  $\sim$ 80% confluence after a week.

#### Potential solution

It will take at least a couple of passaging for iPSC to fully recover from cryopreservation and reach exponential growth phase. If iPSC colonies do not become  $\sim$ 80% confluent in 7 days, passage the cells at 1:1 or 1:2 ratio to a newly prepared well. iPSC should take 3–4 days to reach 70%–80% confluence if they recovered from cryopreservation. If cells do not recover their proliferation rate, test for mycoplasma contamination or thaw a new vial from a different batch of iPSC.

### Problem 3

Progression of CPE is faster or slower than anticipated.

### Potential solution

This is likely due to (1) improper calculation in preparation of the viral inoculum or (2) an inaccurate titer of the stock you are using to propagate infection. To avoid these, (1) you may have to re-titer the original stock.

The benefit of using icSARS-CoV-2-mNG is that CPE can be visualized under GFP exposure. Use an inverted phase-contrast microscope with a GFP LED cube to confirm that the cells are indeed infected then continue to monitor progression of CPE as normal. Even if CPE is faster/slower than anticipated, as long as the cells are infected then you can still harvest for virus given you follow how CPE appears in [Figure 1](#).

### Problem 4

Cells wash away during the plaque assay fixation and staining process.

### Potential solution

This can occur due to one of three reasons: (1) cells were exposed to the air for too long and have dried out rendering them sensitive to manipulation, (2) large perforations in the cell monolayer were created due to pipetting causing the cells to lift during the fixation/staining/washing process, or (3) too rough during the washing process.

Follow these recommendations to avoid this from happening, respectively: (1) work as deep in the BSC as possible so that the airflow does not impact the cell monolayer, and keep the plate lid on as much as possible while adding viral inocula; (2) be gentle when adding and removing medium/reagents to the cells and try not to touch the cell monolayer with the pipette tip; (3) add washes gently and not directly to the cell monolayer, instead, allow washes to run down the side of the wells.

### Problem 5

Plaques are either too small to count or too large and fuse with other plaques.

### Potential solution

Plaque sizes are too small when cells are fixed/stained too early, and too large when cells are fixed/stained too late. Be sure to follow the recommendation of fixing/staining the cells between 62 – 66 hpi. What we usually do is set-up the plaque assay in the afternoon of one day (~16:00) then follow-up with fixation/staining in the morning (~08:00) of the third day post infection.

### Problem 6

Plaques do not have a uniform, circular shape with clear edges.

### Potential solution

This would occur if the plates were moved or agitated during the incubation period. Be sure not to touch/move the plate during the entire incubation period and ensure the plate is in a location (i.e., far back of the incubator) such that no one else will touch it. Also check to make sure that the incubator is stabilized, do not be too rough when opening/closing the incubator and communicate this to others who are accessing the incubator as well.

### Problem 7

Presence of CPE in control wells.

### Potential solution

This indicates that cross-contamination occurred leading to your control wells being infected. In this case, the titer estimate would be inaccurate as the entire plate(s) is unusable; the assay must be repeated. To avoid this, (1) be sure to use a different pipette tip for the addition of control medium



versus the addition of viral inocula, and (2) cover the control wells with the plate while adding viral inocula.

#### **Problem 8**

Low efficiency in kidney organoid formation.

#### **Potential solution**

Low efficiency of kidney organoid formation is likely due to sub-optimal culturing of iPSC. Maintenance of healthy iPSC culture requires regular passaging before reaching their confluence > 80%. It is critical that iPSC are in their exponential growth phase for successful differentiation. If the iPSC confluence on a T-25 flask is < 20%, iPSC can be cultured an additional day to achieve 40%–50% confluence prior to the start of differentiation (day 0). Poor nephrogenesis can result from inadequate intermediate mesoderm induction by CHIR treatment which may occur if the iPSC are seeded at a high (> 80%) confluence. Depending on the iPSC line used, adjustment of CHIR treatment (concentration, duration) may also be required.

#### **Problem 9**

Pellets break apart during transfer to transwell membrane.

#### **Potential solution**

Pellets are fragile and can break during the transfer to the transwell membrane. Over-trypsinization can contribute to a loose pellet following centrifugation. Additionally, a narrow P200 tip bore can result in breakage of the pellets during transfer. To overcome this issue, carefully pre-cut several P200 tips with a sterile razor blade ranging in opening diameters between ~4–6 mm for 500K and ~3–4 mm for 250K pellets. If pellets are not compact enough for pipet transfer, an alternative approach is to use an ultra low binding 96 well plate that enables nephron progenitor cells at day 7 of differentiation to pre-form sphere aggregates. The aggregates can be grown in the liquid phase for 3 days and then transferred to transwell membranes (See [Figure 3B](#)).

## **RESOURCE AVAILABILITY**

### **Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Justin Chun ([chuj@ucalgary.ca](mailto:chuj@ucalgary.ca)).

### **Materials availability**

icSARS-CoV-2-mNG can be shared through a MTA.

### **Data and code availability**

This study did not generate new datasets or codes.

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## AUTHOR CONTRIBUTIONS

H.C. and J.C. cultured iPSC and generated kidney organoids. M.B. performed the SARS-CoV-2 infection experiments. H.C., J.C., and W.R. stained and imaged the kidney organoids. J.A.C. designed and analyzed SARS-CoV-2 infection data. J.A.C. and J.C. conceived experiments and supervised the study. H.C. and M.B. wrote the first draft of the manuscript and prepared the figures. All authors reviewed, edited, and approved the final manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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